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A systematic review for evidence-based studies of probiotics supporting a health claim for microbial dysbiosis.

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ABSTRACT

Objective: To quantify and analyse the quality of evidence for the claim that probiotics can correct dysbiosis of the normal microbiota from randomised controlled trials.

Setting: Systematic review of current published clinical trials of adult or pediatric patients receiving a probiotic intervention for the prevention or treatment of various diseases.

Data sources: Sources searched: PubMed (1985-2013), EMBASE (1985-2013), Cochrane Database of Systematic Reviews (1990-2013), CINAHL (1985-2013), AMED (1985-2013), and ISI Web of Science (2000-2013). Three on-line clinical trial registries were searched: Cochrane Central Register of Controlled trials, MetaRegister of Controlled Trials, and National Institutes of Health.

Review methods: Included trials were randomised evaluations of host normal microbiota embedded in trials of probiotic interventions. Studies were evaluated following PRISMA guidelines for specific probiotic strains. A standard data extraction form was used to collect the raw data. The degree of dysbiosis correction is grouped into three different outcomes (restoration, alteration or no effect) depending upon the type of study design used (Models A-C).

Outcome measures: The primary outcome is the degree of microbiota correction by specific probiotic strains. Secondary outcome was the association of the degree of dysbiosis correction and the strength of efficacy found in randomized controlled trials.

Results: 63 trials (with 69 treatment arms) are included. Complete restoration of the microbiota was found in 83% of 12 probiotic products, altered microbiota was documented in 56% of 18 probiotics and no change in microbiota was found in 79% of 19 probiotics. Clinical efficacy was associated with strains capable of restoration of the normal microbiota.

Conclusions: Only five (10%) of the 49 probiotic strains have evidence for normal microbiota restoration or alteration with supportive clinical efficacy trial results. The health claim for correcting dysbiosis is poorly supported for most probiotic strains and requires further research.

Systematic review registration: PROSPERO (CRD42014007224)

Strengths and Limitations

Strengths include:

- A comprehensive review of the published literature from 1985-2013
- Literature search unrestricted by language or country
- Analysis of study designs resulted in novel strategy to limit bias and classify outcomes
- Three types of outcomes of dysbiosis applied to evidence-based studies of specific probiotic strains
- Author has over 40 years of research experience in the probiotic field

Limitations include:

- Pooled clinical trials using different study populations
- Pooled probiotic doses and regimens
- Indirect evidence linking probiotic strains and dysbiosis
- Review done by sole author

INTRODUCTION

The popularity of probiotics has expanded exponentially recently, but along with their increased use, debate rages on how probiotics should be regulated and whether probiotics should be considered as a drug or a food supplement. In the U.S., unlike approved prescription drugs, which are regulated by the Food and Drug Administration (FDA), probiotics are typically available as over-the-counter medications or as dietary supplements and thus are limited to 'structure or function health claims' and are not permitted to claim to 'treat' or 'cure' disease. In Europe and the United Kingdom, probiotics are allowed to have 'disease or symptom claims'. These claims are required to be supported by well-conducted human trials in the targeted population or in healthy volunteers, but the European Food Safety Authority (EFSA) has rejected 80% of claims submitted to them.[1-3] In many cases, scientific substantiation of a specific health claim was judged insufficient or based on an indirect effect.[4] One such health claim made for probiotic products is that they correct dysbiosis (or the disruption of bacterial and fungal species after antibiotics or other disruptive exposures) and thus may be beneficial to maintain health. Probiotics are uniquely qualified act as temporary surrogate normal microbiota or act to protect the disrupted niche until dysbiosis is corrected. A wide variety of mechanisms-of-action have been documented for probiotics (ranging from blocking pathogen attachment sites, destruction of the pathogen by bacteriocins or proteases that degrade toxins to regulation of the immune system),[5,6] and while clinical evidence supports efficacy of some probiotic strains, the evidence linking these mechanisms-of-action to health claims is not as clear.

A classic example of the consequence of dysbiosis is antibiotic-associated diarrhea (AAD).[7,8] While antibiotics may be effective in the elimination of pathogenic organisms, a common, unintended effect is the killing or inhibition of beneficial microbes due to shared susceptibility to the antibiotic. One of the many functions for normal microbiota is the ability to resist infection by pathogenic organisms, termed 'colonization resistance'.[9,10] The loss of a sub-population of the normal microbiota, for example, can lead to the loss of the ability to break down fibers and starches into absorbable short chain fatty acids, resulting in high level of undigested carbohydrates, which can trigger diarrhea.[11] Disruption of the normal microbiota has been

shown to lead to higher rates of infections in other body systems other than the intestinal tract including the skin,[12,13] vagina,[14,15] respiratory tract,[16,17] and in the buccal cavity.[18-20]

The challenge to proving the microbiota has been restored after a disrupting event is that it is comprised of $>10^{13}$-10^{14} organisms and standard microbial culturing methods miss 75-95% of these organisms. [21,22] The development of metagenomics (cataloguing individual and disease-specific bacterial gene profiles) and the creation of the international Human Microbiome Project ushered in a new era for our understanding of the complexity of these interactions within the body.[23,24] This paradigm shift from culturing to metagenomic analysis has expanded our ability to document shifts in microbial populations to an unparalleled degree, but the interpretation of these shifts continues to be under debate.[25-28] With the advent of these newer metagenomic tools, the role of probiotics in the restoration of normal microbiota is being re-visited.[29]

In light of new guidance documents and recommendations, the goal of this systematic review is to determine if health claims for the restoration of the normal microbiota and the correction of dysbiosis have been studied with well-designed trials and which probiotic strains have evidence-based data to support these claims.

METHODS

Study Objective

To systematically review the literature to analyse the quality of evidence for the claim that probiotics can correct dysbiosis of the normal microbiota from randomised controlled trials.

Search Strategy

Search terms included: health claims for restoring normal microbiota, dysbiosis, normal microbiota, pharmacokinetics, metagenomics, probiotics, dietary supplements, randomized controlled trials and specific probiotic strains or products. Search strategies were broad-based initially, then narrowed to clinical trials with probiotics.

Data Sources

PubMed (1985-2013), EMBASE (1985-2013), Cochrane Database of Systematic Reviews (1990-2013), CINAHL (1985-2013), AMED (1985-2013), and ISI Web of Science (2000-2013). Three on-line clinical trial registries were searched: Cochrane Central Register of Controlled trials (http://www.cochrane.org), MetaRegister of Controlled Trials (http:www.controlled-trials.com/mrct) and National Institutes of Health (http://www.clinicaltrials.gov).

Criteria for study selection and data extraction

Abstracts of all citations were reviewed and rated for inclusion for randomized controlled trials of probiotic treatments. Full articles were retrieved if normal microbiota assays were mentioned. Non-English language trials were translated and included whenever possible. Exclusion criteria included pre-clinical studies (animal models or *in vitro* assays), safety or phase 2 studies, reviews, efficacy trials with no assays for normal microbiota species, metagenomic methods only, mechanism of action of normal microbiota or probiotic, cross-sectional surveys, case reports or case series, duplicate reports, or trials of unspecified types of probiotics. All pharmacokinetic studies in humans were reviewed, as abstracts often did not include normal microbiota assay data. Data extraction and the review process followed the PRISMA statement guidelines using a 27-item checklist and flow diagram.[30] A standardized data extraction form was used to collect data on the probiotic (strain type, daily dose, duration), type of controls (placebo, active or no treatment), study design (status of microbiota at baseline and follow-up times), type of microbiota assay (microbial culturing, molecular biomarkers, etc.), enrolled study population (adult vs. pediatric, healthy volunteers, disease condition), type and timing of disruptive agent (antibiotics, chemotherapy, etc.), study size and attrition, outcome assessment (efficacy and/or microbiota status at end of study, adverse events) and type of health claim.

Outcomes and definitions

The primary outcome is the degree of microbiota correction or improvement by the probiotic strain. The secondary outcome is the association of the degree of dysbiosis correction and the strength of efficacy found in randomized controlled trials of probiotic interventions. Dysbiosis is defined as an alteration or disruption of the normal microbiota (bacterial or fungal species) due

to exposure of an inciting factor (such as antibiotics, chronic disease, stress, medical procedures or medications, etc.). Included studies were required to have at least a pre-probiotic treatment assay and a post-probiotic treatment assay for normal microbiota determination. As a variety of microbial assays were available during the search period (1985-2013), we included documentation of the microbiota by either microbial cultures, or metagenomic methods [16s rRNA-targeted probes using fluorescent *in situ* hybridization (FISH) or other polymerase chain reaction (PCR) technique][8,21,28,31] or by indirect methods (Nugent scores).[15] Nugent scores (ranged 0-10) are used to diagnose bacterial vaginosis (scores $\geq$7) or normal vaginal microbiota (scores 0-3) based on the quantitated morphotypes of small gram negative rods (*G. vaginalis/Bacteroides* spp.) and curved gram negative rods (*Mobiluncus* spp.) from gram stains of vaginal discharge smears. Microbial assays of the strain(s) contained in the probiotic product are considered as pharmacokinetic studies and were not included in the normal microbiota profiles.

To determine the impact on normal microbiota, only direct evidence of microbiota change (species, profiles, diversity indices, or diagnostic criteria) were included and indirect effects were excluded (changes in intestinal enzymes, short chain fatty acids, immune system parameters or disease symptoms). The degree to which dysbiosis was improved is categorized into three levels: (1) recovery of the normal microbiota back to baseline levels; (2) alteration or improvement of the normal microbiota; and (3) no change in normal microbiota. 'Recovery' of the normal microbiota is defined as a restoration of the microbiota back to a normal baseline. To determine this outcome, only those studies that enrolled subjects with undisrupted microflora at baseline and assayed subjects before a disruptive event occurred (such as antibiotic exposure or chronic disease) and compared the microbiota using another assay taken after the study intervention was completed were used, as shown in Figure 1. These types of clinical types are typically preventive probiotic interventions. This type of study design is termed 'Model A'. Recovery may be complete recovery (all assayed microbial levels returned to baseline) or incomplete recovery (partial recovery of some microbial strains, but not all returned to baseline levels). In studies enrolling subjects with dysbiosis at baseline (typically due to chronic diseases), it is not possible to show a restoration to normal microbiota levels because a normal, undisturbed microbiota was not present in these types of study subjects at the time of enrollment.

Therefore, the strongest claim possible is for an 'alteration or improvement' of the normal microbiota. To determine this outcome, studies in which the first assay occurred after a disruptive exposure (e.g., post-antibiotic exposure or during active disease) and the second assay occurred post-probiotic intervention were grouped (Model B in Figure 1). These types of studies are typically for the treatment of existing diseases by probiotics. In studies enrolling healthy subjects who had a normal microbiota at baseline and were not exposed to a disruptive factor at any time during the study intervention trial, dysbiosis did not occur. These types of clinical trials enrolled healthy volunteers and were assayed before and after the probiotic intervention was given (Model C in Figure 1). Only data from the probiotic-exposed subjects were analysed in this paper. Data from the control groups were used to confirm dysbiosis for subjects with chronic diseases or after a disruptive exposure, such as antibiotics or chemotherapy, unaffected by probiotic exposure.[32-34]

Assessment of methodological quality

The GRADE (Grading of Recommendations, Assessment, Development and Evaluation) system for rating overall study quality will be used for each probiotic strain or type (single strains and mixtures of strains).[35] Recommendation for the support of the claim of each probiotic strain or mixture can be assessed by the overall strength of the evidence [“strong”, many randomized controlled trials show significant recovery of the microbiota, or "moderate" only one randomized controlled trial; or “weak”, only case series or reports, limited number of small trials, *etc.*]. Quality of the evidence is graded as “high quality” (well-defined study design for determining restoration with normal microbiota at baseline), or “moderate quality” (disrupted microbiota at baseline), or “low quality” (no disruptive event occurred). Measurement of publication bias was not assessed for this review, as pooled outcome estimates of efficacy were not done, as typical in meta-analysis, but all studies with assays of microbiota were included to limit bias.

Net efficacy rating

To determine if the ability to correct dysbiosis is associated with clinical efficacy, we reviewed the published literature for randomized controlled trials (RCTs) or meta-analyses of probiotics for various disease indications, including antibiotic associated diarrhea (AAD),[5,36,37] *Clostridium difficile* infection (CDI), [5,38] inflammatory bowel disease (IBD),[39] irritable bowel syndrome

(IBS), [40] traveler's diarrhea (TD), [41] eradication of *Heliobacter pylori* (Hp),[36,37] bacterial vaginosis (BV) or vaginitis,[42] and treatment of acute pediatric diarrhea. [43-45] The net rank was calculated by subtracting the number of RCTs showing non-significant or equivalent efficacy from the number of RCTs having significant efficacies. The ranks were categorized as follows: ++, ≥2 net RCTs showing significant efficacy; +, net of one RCT showing significant efficacy; 0, equal number of RCTs showing significant and non-significant efficacy results and -, ≥1 net negative or non-significant RCTs. Probiotics with no RCTs were not ranked.

RESULTS

A review of the literature from 1985-2013 found 353 articles that dealt with probiotic treatments and their potential effect on normal microbiota.

Excluded studies

As shown in Figure 2, a total of 272 articles were excluded for the following reasons: reviews (n=116), probiotic efficacy studies with no data on normal microbiota assays (n=54), animal models of probiotics and changes in microbiota (n=38), metagenomic or microbiota methods only (n=17), studies on normal microbiota but with no use of probiotics (n=14), *in vitro* assays of microbiota (n=10), or miscellaneous (n=23), which included probiotic mechanism of action studies, safety studies, duplicative reports, cross-sectional surveys and two with poorly described probiotic interventions.[46,47] We reviewed 81 full articles which mentioned changes in normal microbiota or indicated a health claim for probiotics and effects on normal microbiota.

Probiotic pharmacokinetic studies. . We found 18 studies reporting concentrations of probiotic strains before and post-treatment, but did not assay for other species of normal microbiota. Studies that assay for only the probiotic strain(s) that are given, and do not assay for other normal microbiota bacteria or fungi, cannot determine what impact the probiotics have on normal microbiota. While several studies using this study design claim probiotics had an impact on normal microbiota, type of data generated is pharmacokinetic behavior of the probiotics themselves and not the normal microbiota. Several studies stated that the normal microbiota was altered because an increase in various bacterial species was observed after the probiotics were

given, but the species assayed were those contained in the probiotic product, so an increase is not unexpected. Pharmacokinetic studies have documented that probiotic strains taken orally can survive transit through the intestinal tract with recovery rates in feces ranging from <1% to 22%.[48,49] These pharmacokinetic studies were excluded from this analysis, as they did not assay other types of normal microbiota not found in the probiotic product.

Included studies

Of the 63 included clinical trials, five trials had multiple treatment arms, which resulted in a total of 68 treatment arms for analysis. Engelbrektson et al. tested a mixture of 5 probiotic strains in volunteers exposed to antibiotics and also tested a mixture of 4 probiotic strains in healthy volunteers with no antibiotic exposure.[50] Zoppi et al. had eight different treatment arms in his study, and we included four treatment arms in our analysis [*Saccharomyces boulardii* (*S. boulardii*) alone and *Lactobacillus (L.) rhamnosus GG* alone], a mixture of two probiotics (*L. acidophilus* and *Bifido. bifidum*) and a mixture of three probiotic strains (*L. acidophilus, L. rhamnosus* and *Bifido. bifidum*).[51] Orrhage et al. had two treatment arms (*Bifido. longum* alone and a mixture of *Bifido. longum* and *L. acidophilus*).[52] Larsen et al. tested two single probiotics (*Bifido. lactis* and *L. acidophilus*) in separate treatment arms.[53] Lidbeck et al. gave either enoxacin or clindamycin and randomized patients to either *L. acidophilus* or placebo. [54]

Normal microbiota assay methods. Of the 69 treatment arms that did normal microbiota assays, diverse methods were used to profile the microbiota. Many studies used only standard microbiological culture assays (37, 54%), while others (28, 40%) used techniques to detect non-cultivatable bacterial strains, which included metagenomic assays (FISH, TRFLP, 16s rRNA sequencing) or other PCR techniques. Some studies (4, 6%) used an indirect measure of normal microbiota, using the Nugent score to diagnose bacterial vaginosis, which relies upon gram stain of the vaginal secretions, vaginal pH and symptoms to characterize if normal microbiota is present or absent.[15]

Probiotic strains. In the 69 treatment arms, most (36, 52%) used a single strain of probiotic, while 14 (20%) tested a mix of two probiotic strains and 19 (28%) tested a mix of three or more probiotic strains. The distribution of single versus multiple strain probiotics did not significant vary by the model of study design (X^2_2=2.3, P=0.32). Of the 15 restorative (Model A) study arms, 47% used a single strain of probiotic and 53% used multiple strains. Of the 25 treatment arms with disrupted microbiota at baseline (Model B), 44% used a single strain and 56% used multiple strains. Of the 29 study arms with undisrupted microbiota (Model C), 62% used a single strain and 38% used multiple strains.

Normal microbiota restoration model (Model A)

The study design with the highest quality capable of documenting the recovery of normal microbiota due to a probiotic treatment requires that subjects be enrolled with a normal, undisrupted microbiota, then be exposed to a disruptive factor (e.g. antibiotics, chemotherapy, disease onset, etc.), followed by probiotic treatment and a follow-up period post-probiotic to document recovery of the microbiota back to the baseline profiles. We found only 10 studies (with 15 treatment arms) that fit these criteria (Table 1).[32,34,50-52,54-58] The type of enrolled subjects varied from healthy volunteers to children with untreated respiratory infections, to pediatric cancer patients. For subjects with acute infections or cancer, baseline assays were done prior to the disrupting agent (antibiotics or chemotherapy). The number of subjects given probiotics averaged 20/study and ranged from 5 to 83. In 93%, the disruptive factor was antibiotic exposure and in one study, chemotherapy caused the microbiota disruption. Only 8 (53%) of the study arms did an assay during a 1-8 week follow-up period after the probiotic was discontinued.

Of the 15 probiotic treatment arms, 7 (47%) showed restoration of the assayed normal microbiota, 5 (33%) showed partial recovery and 3 (20%) showed no change in the microbiota. Analysis of the probiotic strain(s) separately found only two probiotic products with more than one randomized controlled trial. The probiotic mix of *L. acidophilus* and *Bifido. bifidum* showed a complete restoration in one study, but only a partial recovery in the other. (Strength: strong,

Quality: high). The probiotic mix of *L. acidophilus* (2 strains) with *Bifido. bifidum* and *Bifido. animalis* showed complete restoration in one study, but only a partial recovery in the other. (Strength: strong, Quality: high). Five other probiotic products with only one supporting clinical trial showed microbiota restoration (*Bifido. longum*, *Clostridium butyricum*, *L. acidophilus*, mix of *L. acidophilus* with *L. paracasei* and *Bifido. lactis*, and the mix of *L. acidophilus* with *L. paracasei* and *Bifido. bifidum* and two strains of *Bifido. lactis*). (Strength: moderate, Quality: high). Three probiotic products with one supporting clinical trial showed partial restoration (*S. boulardii*, *L. rhamnosus* GG, mix of *L. rhamnosus* with *L. bifidus* and *L. acidophilus*), (Strength: moderate, Quality: high). Only two probiotic products using Model A showed no change in the microbiota (*Bifido. breve* and a mix of *L. acidophilus* and *Bifido. longum*). (Strength: moderate, Quality: high).

Of the 11 probiotic products with claims of 'restores or improves normal microbiota', 10 (91%) were supported by our analysis, but only seven showed complete restoration and five had partial restoration of the microbiota (Table 1). We confirmed that the mixture of *L. acidophilus* and *Bifido. longum* did not show any changes in the microbiota. We disagreed with the conclusions of one of the studies. Wada et al. claimed *Bifido. breve* 'enhanced intestinal anaerobes', but this was only compared to the placebo group.[32] Their data showed chemotherapy is a disruptive event, resulting in more Enterobacteria in the intestine in the placebo group, but there were no significant differences seen by the end of the 8 week follow-up in either the probiotic or the placebo group compared to baseline microbiota levels.

Disrupted normal microbiota at baseline studies (Model B)

Model B is a study design enrolling subjects with a pre-existing disrupted microbiota related to ongoing disease or conditions. In 25 treatment arms, patients with acute or chronic disease were enrolled and randomized to probiotics or controls and normal microbiota was assayed at enrollment, during treatment and/or post-treatment (Table 2).[33,53,59-80] The number of subjects given probiotics averaged 23 $\pm$ 16/study and ranged from 7-83 participants. The types of pre-existing factors that disrupted the microbiota included atopic dermatitis patients, allergies,

cirrhosis, bacterial vaginosis, irritable bowel syndrome, inflammatory bowel disease (ulcerative colitis and pouchitis), idiopathic diarrhea, enteral feeding, short-bowel syndrome and colon cancer. Only 10 (40%) of the study arms did an assay during the post-probiotic follow-up period.

Our analysis found 12 (48%) of the study arms supported an alteration or modification of the microbiome, while 13 (52%) found no significant change in the microbiota. Three of the probiotics had multiple clinical trials to support the claim of an improvement in the microbiota due to the probiotic. *S. boulardii* was used in two trials either with enteral fed patients or patients with active diarrhea and found an improvement in the habitual microbiota in the patients with active diarrhea[66], but only showed indirect evidence of short-chain fatty acid changes in the other study.[65] (Strength: strong, Quality: moderate) A mix of four probiotic strains (2 strains of *L. rhamnosus*, *P. freudenreichii* + *Bifido. breve*) showed improved microbiota in two clinical trials.[74,75] (Strength: strong, Quality: moderate) Of four clinical trials testing a mixture of seven probiotic strains, two showed no significant change in microbiota [77,78], one showed more anaerobes post-probiotic treatment[79] and one found a reduction in Bacteroides species.[80] (Strength: strong, Quality: moderate) Three clinical trials determined there were no significant changes due to *L. plantarum* 299v.[62-64] (Strength: strong, Quality: moderate). Of those probiotics with only one supporting clinical trial (Strength: moderate, Quality: moderate), two single probiotic strains (*E. coli* Nissle and *L. casei rhamnosus*) and five different mixtures of probiotic strains support the claim that the probiotic alters the microbiota (Table 2).

Of the 25 treatment arms, we confirmed the paper's claim in 14 (56%) of the studies. We agreed that there was no significant change in the microbiota due to the probiotic in nine treatment arms only an alteration of the microbiota in five others (Table 2). We disagree with the claimed outcomes in 11 (46%) of the other treatment arms. In seven treatment arms, it was claimed the tested probiotic 'restored normal microbiota', but it is uncertain how this conclusion was reached, since there was no time when a normal undisrupted microbiota was present. Of the seven studies that claimed their probiotic 'restored' normal microbiota, our analysis determined none were

capable of documenting restoration, but we do confirm that probiotics improved or altered the microbiota. Four studies claimed the probiotic 'altered or improved' normal microbiota, but we found no significant differences when post-probiotic and baseline assays were compared for the probiotic groups. Girard-Pipau et al. concluded that *S. boulardii* 'altered normal flora' because more gram positive anaerobes were seen in the probiotic group compared to the controls and an increase in three short-chain fatty acids were observed in the *S. boulardii* group.[65] However, when the analysis is restricted to trends observed in the probiotic group only, no significant differences were observed in pre-probiotic versus post-probiotic microbiota profiles. Venturi et al. concluded that the mix of seven probiotic strains enhanced the concentration of some beneficial strains in the intestines. [77] However, the only strains having a significant increase were those contained in the probiotic mix, and not specifically normal microbiota of the host. As this study did not have an undisturbed microbiota baseline, the increased numbers of Lactobacilli and Bifidobacteria may not have reflected their normal levels. Van der Aa et al. claimed that *Bifido. breve* 'successfully modulates the intestinal flora', but no significant changes were observed in the probiotic group when comparing the baseline to the post-probiotic levels. [59] Odamaki et al. did show an increase in Faecalibacterium ssp. and *Bacteroides fragilis* ssp. at the end of *Bifido. longum* BB536 treatment, but the same increase was also observed in the placebo group.[33]

Undisrupted normal microbiota studies (Model C)

Twenty nine trials enrolled healthy adults who had no disruptive factor present during the study (either no antibiotic or no medication exposure or presence of acute or chronic disease) that might impact normal microbiota, as shown in Table 3.[14,49,50,81-106] The average number of subjects given probiotics was 23/study and ranged from 7 to 160/study. Of the 29 study arms, assays were taken during a follow-up period in only 52%. Not surprisingly, if the normal microbiota was not disturbed, most probiotic treatment arms (25, 86%) did not show a significant change of the microbiota and only 4 (14%) indicated some alteration in the microbiota. Fujiwara et al. cultured seven healthy volunteers and found Enterobacteriaceae and Clostridial species post-*Bifido. longum* was reduced by 10^1/g compared to baseline ($P<0.03$), but no other changes in the microbiota were detected.[84] Karlsson et al. found a significant increase in intestinal diversity in nine male volunteers with atherosclerosis given *L. plantarum* 299v, but because

terminal restriction fragment length polymorphism assays were used instead of cultures for bacterial species, the specific changes in the microbiota species could not be determined.[94] Yang and Sheu cultured 63 children (55% with *H. pylori*) given a yogurt with *L. acidophilus* and *Bifido. lactis* but only found a decrease in *E. coli* counts in the *H.pylori* negative children sub-group, no significant changes in normal microbiota was found in the *H. pylori* positive children.[100] Kubota et al. assayed 29 subjects with Japanese cedar pollen allergy and found milk fermented with *L. rhamnosus* GG and *L. gasseri* TMC0356 suppressed microbiota changes (less intestinal profile changes), but could not determine specific bacterial species changes due to the type of assay used (FISH and TRFLP).[103]

Of the seven studies that claimed their probiotic(s) 'restored or altered' the normal microbiota, we confirmed only four claims, but disagree with three studies. Sierra et al. claimed *L. salivarius* given to 20 healthy adults 'improved gut microbiota', but only increased levels of Lactobacilli were found and no other changes in normal microbiota species were detected. The only other evidence was indirect from changes observed in immune parameters.[96] He et al. claimed a mixture of *Bifido. longum* and *Bifido. animalis* 'modified' microbiota, but changes were seen only during the yogurt administration and not after the one week follow-up period.[99] Vitali et al. claimed that the mixture of four Lactobacilli strains and three Bifidobacteria strains 'modulated vaginal microbiota', but the only significant changes were due to an increase in the bacterial species contained in the probiotic mixture.[14]

Of the probiotics supported by multiple clinical trials (*Bifido. animalis*, *Bifido. longum*, *L. casei*, *L. plantarum* 299v, the mixture of *Bifido. animalis and Bifido. lactis*), 13 (87%) support there is no significant change in normal microbiota if the microbiota is not disrupted. [Strength: strong, Quality: low)

Association of clinical efficacy and normal microbiota restoration

Few studies concurrently compared clinical efficacy and the ability to restore or improve normal microbiota after dysbiosis. A synthesis of the literature of RCT for eight common disease indications was performed and the overall net strength was ranked. Probiotics with the ability to restore normal microbiota were frequently supported by RCTs for efficacy, as shown in Table 4. Of the 10 probiotics with evidence for restoration, 7 (70%) also had at least one RCT testing for at least one of the eight diseases, while 30% did not have any supportive RCTs for efficacy. Of the 7 probiotics with associated RCTs, only two probiotics (*S. boulardii* and *L. acidophilus*) have strong evidence for efficacy across most of the disease indications, while five probiotics with the ability to restore the microbiota had weak or no evidence of efficacy. For example, *S. boulardii*, which has studies supporting restoration, has strong evidence for clinical efficacy for AAD (ranked ++: 11 RCTs had significant results and 6 had non-significant results), CDI (ranked ++: had two RCTs with significant results), IBD (ranked ++: had two RCTs with significant results), IBS (ranked 0: had one RCT with significant efficacy and one RCT with non-significant results), TD (ranked +: 3 RCTs with significant efficacy and 2 with non-significant efficacy), *H. pylori* eradication (ranked -: 2 RCTs with significant results and 4 with non-significant results) and no studies for BV. *L. acidophilus*, which partially restored the microbiota in a study, is associated with clinical efficacy for AAD, IBS and BV, but not for TD or eradication of *H. pylori* and treatment of acute pediatric diarrhea (ranked ++: had 19 RCTs with significant protection and five with non-significant results). In contrast, *L. rhamnosus GG,* another probiotic capable of restoring microbiota, is often cited in meta-analysis as having significant efficacy for AAD. Our results of an updated review of the literature indicate a net weak evidence rating for clinical efficacy across all disease indications: AAD (ranked -: 3 RCTs had significant results and 6 had non-significant results), CDI (ranked -: two RCTs with non-significant results), IBD (ranked -: one RCT with non-significant results), IBS (ranked 0: 2 RCTs with significant efficacy and two RCTs with non-significant results), TD (ranked 0: one RCT with significant efficacy and one with non-significant efficacy), *H. pylori* eradication (ranked -: 3 RCTs with non-significant results), no RCTs for BV and treatment of acute pediatric diarrhea (ranked ++: 10 RCTs with significant efficacy and one with non-significant findings).

Efficacy trials were not done as frequently for probiotics shown to only have the ability to alter or improve, but not restore, the microbiota after dysbiosis. Of nine probiotics that can alter the microbiota, 6 (67%) have supporting RCTs for at least one disease, but the diversity of investigated diseases was more limited. *L. casei* had moderate net strength for AAD and bacterial vaginosis, but was neutral for the ability to eradicate *H. pylori* and other disease indications were not tested in RCTs with *L. casei*. The probiotic mixture of *L. reuteri* and *L. fermentum* has strong evidence for bacterial vaginosis, but not for any other disease indications listed in Table 4.

Of the eight probiotics not capable of altering or restoring normal microbiota, only *L. plantarum* 299v had RCTs for AAD and IBS, both with net negative or weak strength of clinical efficacy. *Bifido. lactis* and the mixture of *L. rhamnosus* and *L. reuteri* had net neutral rankings for efficacy for the treatment of acute pediatric diarrhea. The other four probiotic products with no effect on normal microbiota lacked any RCTs for clinical efficacy. Studies with *B. clausii* did not assay for normal microbiota and had non-significant trial results for *H. pylori* eradication and the treatment of pediatric diarrhea.

Of the six probiotics with only pharmacokinetic data on the probiotic itself and no other investigation of other normal microbiota strains, five had RCTs showing varying net efficacies for different disease indications, as shown in Table 4.

Six popular probiotics (*Bacillus clausii*, *Bifido. infantis*, *L. reuteri*, *L. acidophilus* + *L. helveticus*, *L. acidophilus* + *L. casei* and *L. acidophilus* + *Bifido. animalis*) have only clinical efficacy RCTs, but have not published studies investigating their role in restoring or improving the normal microbiota.

Discussion

Developing and evaluating health claims for probiotics is an important issue and is now identified as a priority for research by several international organizations, including the World

Gastroenterology Organization [107] and the American Society for Nutrition.[2] The U.S. Food and Drug Administration has struggled with appropriate evidence-based health claims for probiotic products and recommends the use of structure/function claims, such as "maintains bowel regularity", but the claim for restoring normal microbiota is under debate.[108] The European Food Safety Authority (EFSA) provides guidance materials that recommend health claims for probiotics should have beneficial physiological effects and have appropriate scientific trials to substantiate the health claims.[3] Acceptable claims for intestinal health include functional claims (improved transit time, softer stool consistency, reduction in gastrointestinal discomfort, defense against pathogens) and changes related to gastrointestinal microbiota. As it is currently not possible to define a standard normal microbiota profile, the EFSA recommends functional claims for the restoration of normal microbiota should be accompanied by a beneficial physiological or clinical outcome.[3] In addition, because the efficacy and mechanisms are strain-specific and may vary by probiotic strain, the evidence must be analyzed for each probiotic product individually.[5,6,9,109, 110]

When the literature on probiotics and its impact on the normal microbiota was reviewed from 69 different treatment arms, the evidence shows probiotics restored normal microbiota in only 10% of the trials, probiotics altered normal microbiota in 30% and no change in the normal microbiota was found (60%).

An underappreciated finding was the influence that study design and study populations have on the interpretation of study results. In this review, five different types of study designs were found in the literature relating to probiotics and dysbiosis. The most common study type was a randomized controlled trial testing the efficacy and safety outcomes in patients, but these trials did not typically document the impact of the probiotic on the normal microbiota of the patient. The second type of study design was for pharmacokinetic studies (documenting recovery of oral dose of probiotic or increase in probiotic strains post-treatment compared to pre-treatment or clearance of the probiotic). Even though these kinetic studies did not assay for non-probiotic strains, some extrapolated their results and concluded some effect or improvement of the normal microbiota was observed by their probiotic.[19,111] These two first types of study designs do not support evidence-based conclusions for the restoration or alteration of the normal microbiota and

were excluded from this review. The third type of study design started with inappropriate baselines (baseline samples taken after normal microbiota had been disrupted by chronic disease). For patients with established chronic diseases, there is no "normal microbiota" baseline in either the probiotic or the control group. Even if baselines are taken during remission, the microbiota may still be impacted by chronic disease or acute diarrhea. Studies of probiotics in chronic diseases or acute disease typically report on 'pre-probiotic treatment' and 'post-probiotic treatment' and may show significant shifts in microbial species, but it is uncertain if this reflects a true re-establishment of normal microbiota profiles. The fourth type of study design enrolled healthy volunteers, who were not challenged with antibiotics (so no normal microbiota disruption occurred), and show only the effect of probiotics on a healthy microbiota (typically mild or no effects). The fifth type of study design had normal microbiota assayed at least twice (at baseline, which was before exposure to a disruptive event or probiotics and then again during or post-probiotic treatment) to show actual recovery of assayed normal microbiota back to baseline levels. Control groups were not required for our assessment of the impact of probiotics on microbiota, but control groups can document the degree normal microbiota is disrupted by inciting agents (antibiotic, disease onset, etc.).

Five single strain probiotics (*Bifido. longum*, *Clost. butyricum*, *L. acidophilus, L. rhamnosus* and *S. boulardii*) and four probiotic mixtures (*L. acidophilus* + *Bifido. bifidum*, *L. rhamnosus* + *L. bifidus* + *L. acidophilus*, *L. acidophilus* + *L. paracasei* + *Bifido. lactis, L. acidophilus*, 2 strains, *Bifido. bifidum, Bifido. animalis*) documented either complete or partial recovery of normal microbiota (Model A). Only two probiotic mixtures [(2 strain mixture: *L. acidophilus* + *Bifido. bifidum*) and (4 strain mixture: *L. acidophilus*, 2 strains, *Bifido. bifidum, Bifido. animalis*)] were supported by a confirmatory study. Evidence that probiotics may alter or improve normal microbiota (Model B) was found for three single strain probiotics (*E. coli* Nissle, *S. boulardii* and *L. casei rhamnosus*) and seven mixtures of 2-7 probiotic strains. Of these ten probiotics finding alteration of the microbiota, only three had multiple trials [*S. boulardii*, and a four strain mixture (2 strains of *L. rhamnosus* + *P. freudenreichii* + *Bifido. breve*), and a seven strain mixture (4 Lactobacilli and 3 Bifidobacteria strains)], but only one had consistent results

showing improvements in the microbiota. [74,75] Clearly, more than one study is needed to confirm the impact of a probiotic on the normal microbiota.

Of the 19 probiotic strains (or mixtures) studied in healthy volunteers who were not exposed to disruptive factors, no change in the normal microbiota was observed for 79%, indicating the robustness of the microbiota.

Improvement in the normal microbiota by specific probiotic strains seemed to be associated with better clinical endpoints. Within eight common diseases typically treated with probiotics, more trials with significant efficacy were associated with probiotic strains shown to restore the normal microbiota, and only one trial with significant efficacy was found for probiotics that did not alter the microbiota. However, few probiotics had efficacy trials for all eight diseases and many did not have any efficacy trials.

Some probiotics which have published efficacy trials for various diseases did not have studies investigating the effect of the probiotic on normal microbiota: *Bacillus clausii*, *Bifido. infantis*, *L. brevis*, *L. reuteri*, mix of 2 strains (*L. acidophilus* + *L. helveticus*), mix of 2 strains (*L. acidophilus* + *L. casei*) or (*L. acidophilus* + *Bifido. animalis*), mix of 4 strains [*L. rhamnosus* (2 strains), *Propionibacterium freudenreichii* + *Bifido. animalis*)] and mix of 7 strains (*L. sporogens, L. bifidum, L. bulgaricus, L. thermophilus, L. acidophilus, L. casei, L. rhamnosus).*

Comparison of results with other studies

Other reviews in the literature of health claims for probiotics relating to changes in the normal microbiota have focused on the broad issues of regulatory standardization of health claims, the use of proper study designs and the challenge of defining biomarkers for a 'healthy microbiota'.[3,29,112] Donovan et al. recommends that health claims for probiotics be supported by well-conducted human trials in the targeted population.[2] These reviews also recommend that gut

biomarkers need to be correlated with clinical endpoints, however none of these reviews attempted to do so.[29,112] No prior review has attempted to analyze the association between probiotic strains and their impact on normal microbiota by stratifying on the quality of study design. [111] This review addressed these concerns by analyzing probiotic strains by the quality of the study design and only including trials that assessed the normal microbiota (either by microbial culturing or molecular strain biomarkers) and assessed the degree of dysbiosis improvement with clinical outcomes for each probiotic strain.

Opportunities for future research

Most of the studies (80%) using Model A to document restoration of the normal microbiota only used microbiologic culturing techniques, which can only detect those organisms that grow in culture. Use of the more advanced molecular metagenomic techniques have found that culturing alone misses up to 95% of these organisms.[21,22] The use of the metagenomic techniques was more common in the studies using Model B (48%) and Model C (45%) study designs, which only addresses potential alteration of the microbiota. Characterization of the microbiota is a complex issue and a comprehensive accounting of all the bacterial and fungal strains in the body is beyond our current capabilities. Therefore, any studies of changes to the microbiota are incomplete at best, but general trends in bacterial phylotypes can be documented using DNA probes and metagenomic techniques. Differential detection bias may be present due to the variety of assays used in these studies and should be accounted for in future studies.

Another suggestion for future studies is to include an appropriate follow-up time period post-probiotic administration. Fewer than half of the reviewed trials did assays for normal microbiota during an appropriate follow-up period. As it has been shown that recovery from a disrupting factor can be prolonged (typically eight weeks),[7,8] and studies that failed to find microbiota recovery might have detected a return to normal baseline levels if a sufficiently long time was given for the recovery to have occurred. Future studies should strive to allow time for the restoration of the normal microbiota to occur.

Strengths and weaknesses

The strengths of this review included the completeness of the search strategy, which reviewed multiple citation databases, trial registries and author searches, use of established PRISMA protocols for reviews and the use of an outcome classification scheme for different degrees of assessment for microbial recovery. This analysis controlled the confounding effects of different study populations and study designs present in the literature. Pharmacokinetic studies of just the probiotic strain(s) itself were excluded and only trials that assayed other species found in the microbiota were included. By applying a standard definition for 'restoring' versus 'improving' normal microbiota, it is possible to distinguish significant differences by the type of study designs used and differential effects of the different probiotic strains. Limitations of this review include pooling trials from different populations (adult versus pediatric) and different probiotic doses and regimens used. Incomplete retrieval of all studies assessing the effect that probiotics have on human microbiota is also a potential limitation of any literature search. Another limitation is that dysbiosis improvement and clinical efficacy for probiotic strains is also indirectly associated, no direct cause and effect relationship was possible with the types of studies done.

Conclusion

The challenges in recommending a specific probiotic to patients who need to restore or improve their normal microbiota after a disrupting event occurs is two-fold: one is the diversity of probiotic products available and second is the varying strength of evidence provided by clinical trials using different outcome measures and study designs. By grouping studies into three groups that result in three different degrees of probiotic effect (restoration, improvement or no change), an overview of the body of evidence is possible. By comparing the strength of the clinical evidence for common diseases by the degree to which the probiotics could impact the restoration of the normal microbiota, it became obvious that those probiotics with a greater ability to restore the microbiota are associated with the strongest strength of clinical efficacy. While this evidence only indirectly links clinical efficacy with the ability to restore the microbiota, the overall review of the evidence shows this is an important mechanism of action for probiotics. What becomes

obvious is that more studies are required to conclude which probiotic strains have a beneficial impact on the normal microbiota, as most strains have only a single clinical trial and many probiotic products overstate the strength of their claim to restore normal microbiota.

Figure 1. Time sequence of events and three models of study designs determining three different degrees of dysbiosis correction by probiotics.

Figure 2. Flow-chart of literature review results (1985-2013) of included and excluded studies for the restoration or improvement of normal microbiota by probiotics.

Abbreviations: RCT, randomized-controlled trials; MOA, mechanism of action; NM, normal microbiota

Table 1. Model A: Evidence-based data for restoration of normal microbiota (NM) for 12 probiotics from 10 studies (15 treatment arms).

Probiotic	Reference	No. treated with probiotic	Type(s) of assay for NM	Enrolled population	Type of disrupting factor	Follow-up post-treatment (wks)	Claims stated in papers	Evidence-based claim
Bifido. breve	Wada 2010[32]	19	FISH	pediatric cancer patients	chemotherapy	8	enhances anaerobes	no change
Bifido. longum	Orrhage 1994[52]	10	culture	healthy volunteers	clindamycin	0	restores	restores
Clost. butyricum	Seki 2003[34]	83	culture	pediatric respiratory or GI infections	antibiotics	0	restores	restores
L. acidophilus	Lidbeck 1988[54]	5 5	culture culture	healthy volunteers volunteers	enoxacin or clindamycin	1 1	restores only in enoxacin no change	restores only in enoxacin, no change in clindamycin
L. rhamnosus GG	Zoppi 2001[51]	7	culture	pediatric respiratory infections	ceftriaxone	0	partially corrects	partially restores
S. boulardii	Zoppi 2001[51]	6	culture	pediatric respiratory infections	ceftriaxone	0	improves	partially restores
L. acidophilus + *Bifido. bifidum*	Black 1991, [55] Zoppi 2001[51]	10, 7	culture, culture	healthy volunteers, pediatric respiratory	ampicillin, ceftriaxone	2, 0	recovers more rapidly, less change	restores, partially restores
L. acidophilus + *Bifido. longum*	Orrhage 1994[52]	10	culture	healthy volunteers	clindamycin	0	no change	no change
L. rhamnosus + *L. bifidus* + *L. acidophilus*	Zoppi 2001[51]	7	culture	pediatric respiratory infections	ceftriaxone	0	partially corrects	partially restores
L. acidophilus + *L. paracasei* + *Bifido lactis*	Jernberg 2005[56]	4	culture PCR TRFLP	healthy volunteers	clindamycin	2	restores	restores
L. acidophilus + *L. acidophilus* + *Bifido. bifidum* + *Bifido animalis*	Madden 2005, [57] Plummer 2005[58]	15, 76	culture, culture	*H. pylori* +, *H. pylori* +	amoxicillin + metronidazole, amoxicillin + clarithromycin	2, 2	restores, restores more rapidly	restores, partially restores
L. acidophilus + *L. paracasei* + *Bifido. bifidum* + *Bifido. lactis* + *Bifido. lactis*	Engelbrek-tson 2006[50]	20	culture PCR TRFLP	healthy volunteers	augmentin	2	restores	restores

Table 2. Model B: Evidence-based data for improvement or alteration of normal microbiota (NM) in 18 probiotics from 24 studies (25 treatment arms) with disturbed microbiota at baseline.

Probiotic	Reference	No. treated with probiotic	Type(s) of assay for NM	Pre-existing disrupting factor	Follow-up time	Claims stated in papers	Evidence-based claim	Type of change found in NM
Bifido. breve	Van der Aa 2010[59]	46	FISH	Atopic dermatitis	0	modulates NF	no change	--
Bifido. lactis	Larsen 2011[53]	17	PCR	Atopic dermatitis	0	no change	no change	--
Bifido. longum	Odamaki 2007[33]	22	TRFLP PCR	Cedar pollen allergy	4 wk	maintains NF	no change	--
E. coli	Lata 2007[60]	22	culture	liver cirrhosis	0	restores	improves	more Bifido. & Lacto.
L. acidophilus	Larsen 2011[53]	17	PCR	atopic dermatitis	0	no change	no change	--
L. casei rhamnosus	Petricevic 2008[61]	83	Nugent scores	bacterial vaginosis	4 wk	restores	improves	improved Nugent scores
L. plantarum 299v	Nobaek 2000,[62] Klarin 2005,[63] Klarin 2008[64]	25, 17, 22	culture, culture, culture	IBS, enterally-fed, antibiotics	4 wk, 0, 0	no change, no change, no change	no change, no change, no change	--
S. boulardii	Girard 2002,[65] Swidsinski 2008[66]	10, 20	culture, FISH	enterally-fed, active diarrhea	9 d, 3 wk	alters NF, improves	no change, improves	-- more 'habitual microbiota'
L. reuteri + *L. fermentum*	Reid 2001[67]	33	Nugent scores	bacterial vaginosis	2 wk	restores	improves	improved Nugent scores
L. rhamnosus + *L. fermentum*	Reid 2003[68]	31	Nugent scores and culture	bacterial vaginosis	30 d	restores	improves	improved Nugent scores
L. plantarum + *Bifido bifidum*	Kirpich 2008[69]	32	culture	colon cancer	0	restores	improves	more *E. coli* and *Enterococci*
L. rhamnosus + *L. reuteri*	Hummelen 2010[70]	23	Nugent score	bacterial vaginosis	0	no change	no change	--
L. casei + *Bifido breve*	Uchida 2007[71]	4	culture	short bowel syndrome	0	no change	no change	--
L. brevis + *L. salivaris* + *L. plantarum*	Mastromarini 2009[72]	19	Nugent score	bacterial vaginosis	2 wk	restores	improves	improved Nugent scores
L. paracasei + *L. acidophilus* + *Bifido. animalis*	Roessler 2012[73]	30	PCR	atopic dermatitis	0	no change	no change	--

L. rhamnosus + *L. rhamnosus* + *P. freudenreichii* + *Bifido. breve*	Kajander 2005, [74] Lyra 2010[75]	41, 22	PCR, PCR	IBS, IBS	0, 0	restores, alters	improves, alters	Improved similarity index More Clostridia and Rumino-coccus
L. acidophilus + *L. plantarum* + *L. rhamnosus* + *Bifido. bifidum*	Wong 2013[76]	7	PCR	liver disease	0	improves	alters	Less Firmicutes, more Bacterio-detes
L. acidophilus + *L. paracasei* + *L. delbrueckii ssp. bulgaricus* + *L. plantarum* + *Bifido. longum* + *Bifido. infantis* + *Bifido. breve*	Venturi 1999, [77] Brigidi 2001, [78] Kuhbacher 2006[79] Ng 2013[80]	20, 10, 10 10	culture culture & PCR FISH PCR	ulcerative colitis, IBS, pouchitis IBS	15 d, 10 d, 0 0	enhances, no change, altered richness modulates	no change, no change, altered altered	-- -- More anaerobes Less Bacteroides

*disruption of normal microbiota at baseline shown by significant differences compared to control (non-diseased) population.

Table 3. Model C: Evidence-based data for improvement or alteration of normal microbiota (NM) in 19 probiotics in healthy volunteers enrolled in 29 studies (29 treatment arms) in studies with no disruptive exposures.

Probiotic	Reference	No. treated with probiotic	Type of assay for NM	Enrolled population	Type of disrupt-ing factor	Follow-up post-treat-ment	Claims stated in papers	Evidence-based claim
Bifido. animalis (lactis)	Rochet 2008,[49] Oswari 2013[81]	12, 160	FISH PCR	healthy volunteers	none, none	10 d, 6 mon	no change, no change	no change, no change
Bifido. bifidum	Langhendries 1995[82]	20	culture	healthy volunteers	none	0	no change	no change
Bifido. longum	Benno 1992,[83] Fujiwara 2001,[84] Harmsen 2002[85]	5, 7, 14	culture, culture, FISH	healthy volunteers	none, none, none	0, 30 d, 0	no change, alters, no change	no change, alters, no change
L. casei	Guerin 1998,[86] Rochet 2006,[87] Rochet 2008[88]	12, 12, 7	culture, FISH, FISH	healthy volunteers	none, none, none	1 wk, 10 d, 0	no change, no change, no change	no change, no change, no change
L. johnsonii	Brunser 2006[89]	32	culture & FISH	healthy volunteers	none	2 wk	no claim	no change
L. plantarum 299v	Goossens 2003,[90] Goossens 2005,[91] Goossens 2006,[92] Berggren 2003,[93] Karlsson 2010[94]	11, 32, 15, 33, 9	culture, culture, culture, culture, TRFLP	healthy, healthy, colonic polyps, healthy, atherosclerosis	none, none, none, none, none	3 wk, 4 wk, 0, 0, 0	no change, no change, no change, no change, alters	no change, no change, no change, no change, alters
L. rhamnosus GG	Gueimonde 2006[95]	29	PCR	healthy volunteers	none	0	no change	no change
L. salivarius	Sierra 2010[96]	20	culture	healthy volunteers	none	0	improves	no change
S. boulardii	Vanhoutte 2006[97]	30	PCR	healthy volunteers	none	0	no change	no change
Bifido. animalis + Bifido. longum	Zhong 2006,[98] He 2008[99]	11, 11	FISH, FISH	healthy volunteers	none	7 d, 7d	no change, modifies	no change, no change
L. acidophilic + Bifido. lactis	Yang 2012[100]	63	culture	healthy but 55% *H. pylori* +	none	0	restores	alters
L. rhamnosus GG + Bifido. longum	Mah 2007[101]	20	FISH	healthy neonates	none	6 mon	no change	no change
L. rhamnosus GG + Bifido. lactis	Rafter 2007[102]	38	culture	colon cancer patients or at risk	none	0	no change	no change

L. rhamnosus GG + *L. gasseri*	Kubota 2009[103]	14	culture FISH TRFLP	healthy, allergy patients	none	0	suppressed changes	alters
L. paracasei + *L. paracasei* + *L. gasseri*	Morelli 2003[104]	12	culture	healthy volunteers	none	3 d	no claims	no change
L. acidophilus + *L. paracasei* + *Bifido. lactis*	Sullivan 2009[105]	15	culture	chronic fatigue patients	none	4 wk	no change	no change
L. rhamnosus + *L. acidophilus* + *L. paracasei* + *Bifido. animalis*	Engelbrektson 2006[50]	22	culture TRFLP PCR	healthy volunteers	none	2 wk	no change	no change
Bifido. animalis + *L. delbrueckii* + *L. delbrueckii* + *L. lactis*	McNulty 2011[106]	7	PCR	healthy twins volunteers	none	4 wk	no change	no change
L. acidophilus + *L. paracasei* + *L. delbrueckii ssp. bulgaricus* + *L. plantarum* + *Bifido. longum* + *Bifido. infantis* + *Bifido. breve*	Vitali 2012[14]	15	PCR	healthy pregnant volunteers	none	0	modulates	no change

Abbreviations: FISH, fluorescence *in situ* hybridization analysis; TRFLP, terminal restriction fragment length polymorphism analysis; PCR, polymerase chain reaction

Table 4. Comparison of the ability of probiotic to restore or improve dysbiosis with ranked clinical efficacy for various disease indications.

Probiotic	Restored normal microbiota*	Altered normal microbiota*	Ranked net evidence for efficacy*							
			AAD	CDI	IBD	IBS	TD	H pylori	Vaginitis/ BV	Acute Ped diar
Restores microbiota										
Clost. butyricum	yes	nd	-					-		
L. acidophilus + *Bifido. bifidum*	yes	nd	**0**	-						
L. acidophilus + *L. paracasei* + *Bifido. lactis*	yes	nd				-				
Bifido. longum	yes	no			-	+				
L. acidophilus + *L. acidophilus* + *Bifido. bifidum* + *Bifido. animalis*	yes	nd								
L. acidophilus + *L. paracasei* + *Bifido. lactis (2)*	yes	no								
S. boulardii	partial	yes	++	++	++	**0**	+	-		++
L. rhamnosus GG	partial	nd	-	-	-	**0**	**0**	-	**0**	++
L. acidophilus	partial	no	++			++	-	-	+	**0**
L. acidophilus + *L. bifidus* + *L. rhamnosus*	partial	nd								
Alters microbiota										
E. coli	nd	yes			-					+
L. casei (DN114001 or Lcr35)	nd	yes	+					**0**	+	++
L. reuteri + *L. fermentum*	nd	yes							++	
L. rhamnosus + *L. fermentum*	nd	yes								

L. plantarum + Bifido. bifidum	nd	yes								
L. rhamnosus + L. rhamnosus + P. freudenreichii + Bifido. breve	nd	yes				++				
L. acidophilus + L. plantarum + L. rhamnosus + Bifido bifidum	nd-	yes								
L. brevis + L. salivarus + L. plantarum	nd	yes							+	
L. acidophilus + L. paracasei + L. delbrueckii ssp. bulgaricus + L. plantarum, Bifido. longum, Bifido. infantis, Bifido. breve	nd	yes	-		++	+				++
No effect on microbiota										
B. clausii	nd	nd						-		-
L. plantarum 299v	nd	no	-	-		-				
Bifido. lactis	nd	no	+							**0**
Bifido. breve	no	no								
L. acidophilus + Bifido. longum	no	--								
L. rhamnosus + L. reuteri	nd	no								**0**
L. casei + Bifido. breve	nd	no								
L. paracasei + L. acidophilus + Bifido animalis	nd	no								
Pharmacokinetic only										
L. reuteri	nd	nd								+
L. johnsonii La1	nd	nd			-			+		
L. salivarius	nd	nd				-				
Bifido. animalis	nd	nd				**0**				
Bifido. bifidum	nd	nd				+				
L. rhamnosus + Bifido. longum	nd	nd								

*** Rank:** ++, ≥2 net RCTs (randomized controlled trials) with significant protective efficacy; +, only one net protective RCT; 0, equal number of significant and non-significant RCTs; -, ≥1 net non-significant RCT. Blank indicates no RCT done for the disease indication.

Abbreviations: nd, not determined; AAD, antibiotic associated diarrhea; CDI, Clostridium difficile infections; IBD, inflammatory bowel disease; IBS, irritable bowel syndrome; TD, traveler's diarrhea; BV, bacterial vaginosis; Acute Ped Diar, treatment of acute pediatric diarrhea.

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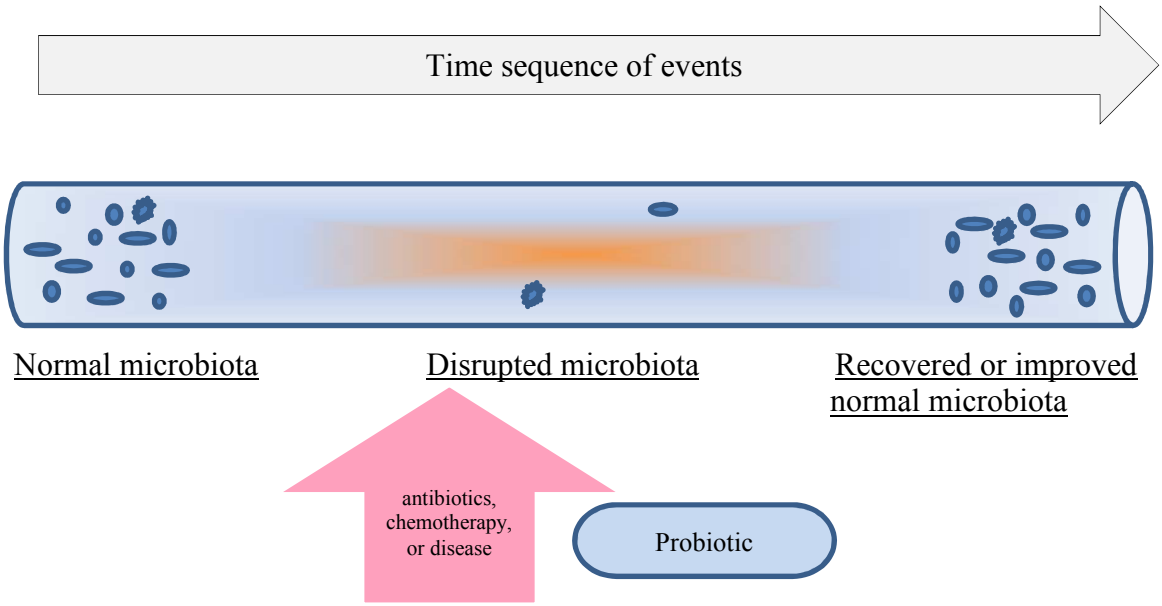
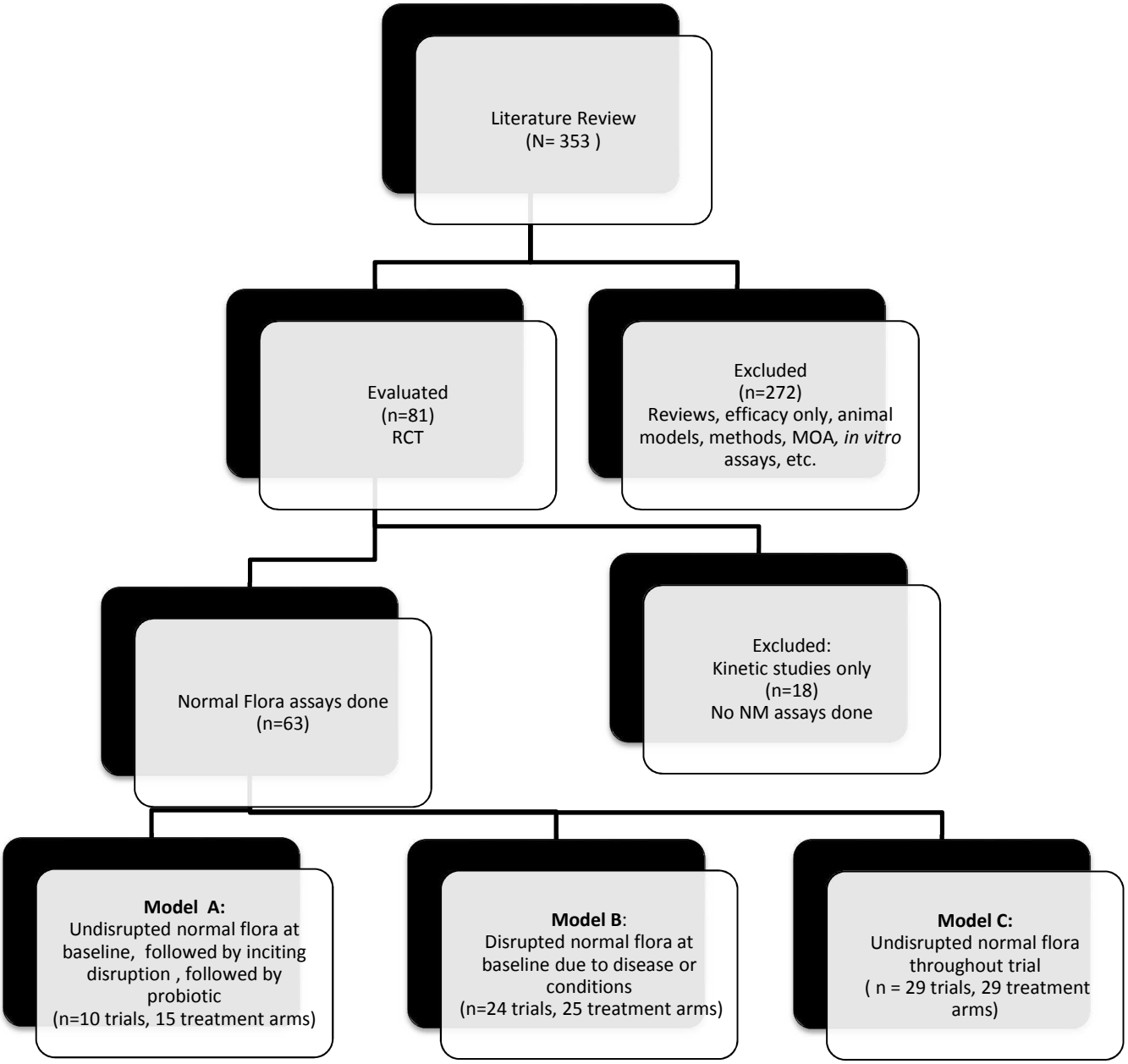


Model	Type of population enrolled	Dysbiosis	Time microbiota disrupted	Probiotic or control intervention	Potential outcomes
A	Healthy volunteers or at-risk patients	no	post-baseline	preventive	restoration
B	Patients with active disease at enrollment	yes	pre-baseline	treatment	altered or improved
C	Healthy volunteers	no	not disrupted	preventive	no change


Literature Review
(N= 353)
Evaluated
(n=81)
RCT
Excluded
(n=272)
Reviews, efficacy only, animal models, methods, MOA, *in vitro* assays, etc.
Normal Flora assays done
(n=63)
Excluded:
Kinetic studies only
(n=18)
No NM assays done
Model A:
Undisrupted normal flora at baseline, followed by inciting disruption , followed by probiotic
(n=10 trials, 15 treatment arms)
Model B:
Disrupted normal flora at baseline due to disease or conditions
(n=24 trials, 25 treatment arms)
Model C:
Undisrupted normal flora throughout trial
(n = 29 trials, 29 treatment arms)

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BMJ Open

A systematic review for evidence-based studies of probiotics supporting a claim for microbial dysbiosis.

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A systematic review for evidence-based studies of probiotics supporting a claim for microbial dysbiosis.

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ABSTRACT

Objective: To assess the evidence for the claim probiotics can correct dysbiosis of the normal microbiota resulting from disease or disruptive events.

Setting: Systematic review of published clinical trials of patients receiving a probiotic intervention for the prevention or treatment of various diseases.

Data sources: Sources searched (1985-2013): PubMed, EMBASE, Cochrane Database of Systematic Reviews, CINAHL, AMED, and ISI Web of Science. Three on-line clinical trial registries were searched: Cochrane Central Register of Controlled trials, MetaRegister of Controlled Trials, and National Institutes of Health.

Review methods: Included studies were randomized clinical trials of probiotic interventions having microbiologic assays. Studies were evaluated following PRISMA guidelines for specific probiotic strains. A standard data extraction form was used to collect the raw data.

Outcome measures: The primary outcome is the degree of microbiota correction by specific probiotic strains. Secondary outcome was the association between the degree of dysbiosis correction and clinical efficacy.

Results: The review of the literature found three distinct study designs: Model A (restoration) assayed patients enrolled with a healthy, undisturbed microbiota and then assayed post-disruptive event and probiotic therapy; Model B (alteration) assayed patients with pre-existing disrupted microbiota and then post-probiotic therapy; Model C (no dysbiosis) assayed volunteers with no disruptive event pre and post-probiotic. From a total of 63 trials, 83% of the probiotic products using Model A restored the microbiota, 56% using Model B improved the microbiota and only 21% using Model C had any effect on microbiota. Clinical efficacy was more commonly associated with strains capable of restoration of the normal microbiota.

Conclusions: The ability to assess the degree of dysbiosis improvement is dependent upon the enrolled population and the timing of microbiologic assays. The functional claim for correcting dysbiosis is poorly supported for most probiotic strains and requires further research.

Systematic review registration: PROSPERO (CRD42014007224)

Strengths and Limitations

Strengths include:

- A comprehensive review of the published literature from 1985-2013
- Literature search unrestricted by language or country
- Analysis of study designs resulted in novel strategy to limit bias and classify outcomes
- Three types of outcomes of dysbiosis applied to evidence-based studies of specific probiotic strains
- Author has over 40 years of research experience in the probiotic field

Limitations include:

- Pooled clinical trials using different study populations
- Pooled probiotic doses and regimens
- Indirect evidence linking probiotic strains and dysbiosis
- Review done by sole author

INTRODUCTION

The popularity of probiotics has expanded exponentially recently, but along with their increased use, debate rages on how probiotics should be regulated and whether probiotics should be considered as a medical food, drug or a food supplement. In the U.S., probiotics are typically available as dietary supplements and thus are limited to 'structure or function' health claims and, unlike prescription drugs, are not permitted to claim to 'treat' or 'cure' disease. In Europe and the United Kingdom, probiotics are allowed to have health or function claims. These claims are required to be supported by well-conducted human trials in the targeted population or in healthy volunteers, but the European Food Safety Authority (EFSA) has rejected >80% of claims submitted to them. [1-3] In many cases, scientific substantiation of a specific health claim was judged insufficient or based on an indirect effect.[4] One such functional claim made for probiotic products is they correct dysbiosis (or the disruption of bacterial and fungal species after antibiotics or other disruptive exposures) and thus may be beneficial to maintain health. Probiotics are active during this susceptible window from the time of the disruptive event to the time when normal microbiota is restored. A wide variety of mechanisms-of-action have been documented for probiotics (ranging from blocking pathogen attachment sites, destruction of the pathogen by bacteriocins or proteases that degrade toxins, to regulation of the immune system),[5,6] and while clinical evidence supports efficacy of some probiotic strains, the evidence linking these mechanisms-of-action to a specific health or function claims is not as clear.

A classic example of the consequence of dysbiosis is antibiotic-associated diarrhea (AAD).[7,8] While antibiotics may be effective in the elimination of pathogenic organisms, a common, unintended effect is the killing or inhibition of beneficial microbes due to shared susceptibility to the antibiotic. One of the many functions for normal microbiota is the ability to resist infection by pathogenic organisms, termed 'colonization resistance'.[9,10] The loss of a sub-population of the normal microbiota, for example, can lead to the loss of the ability to break down fibers and starches into absorbable short chain fatty acids, resulting in high level of undigested carbohydrates, which can trigger diarrhea.[11] Disruption of the normal microbiota has been shown to lead to higher rates of infections in other body systems other than the intestinal tract including the skin,[12,13] vagina,[14,15] respiratory tract,[16,17] and in the buccal cavity.[18-20]

The major challenge to establishing a cause and effect for the improvement of dysbiosis by probiotics is a lack of a standard definition of 'normal' microbiota. There is substantial inter-individual variation of the species of microbes present at different body niches, which also varies by age, geographic area and health status of the host. In addition, a complete accounting of the microbiota is currently impossible, as there are no assays to detect all of $>10^{13}$-10^{14} organisms in the intestines and standard microbial culturing methods miss 75-95% of these organisms. [21,22] The development of metagenomics (cataloguing individual and disease-specific bacterial gene profiles) and the creation of the international Human Microbiome Project ushered in a new era for our understanding of the complexity of these interactions within the body.[23,24] This paradigm shift from culturing to metagenomic analysis has expanded our ability to document shifts in microbial populations to an unparalleled degree, but the interpretation of these shifts continues to be under debate.[25-28] With the advent of these newer metagenomic tools, the role of probiotics in the restoration of normal microbiota is being re-visited.[29]

In light of new guidance documents and recommendations, the goal of this systematic review is to determine how claims for the restoration of the normal microbiota and the correction of dysbiosis have been studied using well-designed trials and which probiotic strains have evidence-based data to support these claims.

METHODS

Study Objective

To systematically review the literature to analyse the evidence for the claim probiotics can correct dysbiosis of the normal microbiota from randomised controlled trials.

Search Strategy

Search terms included: probiotics + health claims, restoring normal microbiota, dysbiosis, normal microbiota, pharmacokinetics, metagenomics, probiotics, dietary supplements,

randomized controlled trials, antibiotic associated diarrhea (AAD), *Clostridium difficile* infection (CDI), inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), traveler's diarrhea (TD), eradication of *Heliobacter pylori,* bacterial vaginosis or vaginitis, treatment of acute pediatric diarrhea, and specific probiotic strains or products. Search strategies were broad-based initially, then narrowed to clinical trials with probiotics.

Data Sources

PubMed (1985-2013), EMBASE (1985-2013), Cochrane Database of Systematic Reviews (1990-2013), CINAHL (1985-2013), AMED (1985-2013), and ISI Web of Science (2000-2013). Three on-line clinical trial registries were searched: Cochrane Central Register of Controlled trials (http://www.cochrane.org), MetaRegister of Controlled Trials (http:www.controlled-trials.com/mrct) and National Institutes of Health (http://www.clinicaltrials.gov).

Criteria for study selection and data extraction

Abstracts of all citations were reviewed and rated for inclusion for randomized controlled trials of probiotic treatments. Full articles were retrieved if normal microbiota assays were mentioned. Non-English language trials were translated and included whenever possible. Exclusion criteria included pre-clinical studies (animal models or *in vitro* assays), safety or phase 2 studies, reviews, efficacy trials with no assays for normal microbiota species, metagenomic methods only, mechanism of action of normal microbiota or probiotic, cross-sectional surveys, case reports or case series, duplicate reports, or trials of unspecified types of probiotics. All pharmacokinetic studies in humans were reviewed, as abstracts often did not include normal microbiota assay data. Data extraction and the review process followed the PRISMA statement guidelines using a 27-item checklist and flow diagram.[30] A standardized data extraction form was used to collect data on the probiotic (strain type, daily dose, duration), type of controls (placebo, active or no treatment), study design (status of microbiota at baseline and follow-up times), type of microbiota assay (microbial culturing, molecular biomarkers, etc.), enrolled study population (adult vs. pediatric, healthy volunteers, disease condition), type and timing of disruptive agent (antibiotics, chemotherapy, etc.), study size and attrition, outcome assessment (efficacy and/or microbiota status at end of study, adverse events) and type of health claim.

Outcomes and definitions

The primary outcome is the degree of microbiota correction or improvement by specific probiotic strain(s). The secondary outcome is the association between the degree of dysbiosis correction and the net efficacy found from randomized controlled trials of probiotic interventions. Dysbiosis is defined as an alteration or disruption of the normal microbiota (bacterial or fungal species) due to exposure of an disruptive factor (such as antibiotics, chronic disease, stress, medical procedures or medications, etc.). As there is no current standard definition of 'normal' microbiota, for this review, restoration of normal microbiota is defined as a return to the assayed microbial species or profile taken from a healthy individual (before a disruptive event has occurred). Included studies are required to have at least a pre-probiotic treatment assay and a post-probiotic treatment assay. A variety of microbial assays were available during the search period (1985-2013), including documentation of the microbiota by either microbial cultures, or metagenomic methods [16s rRNA-targeted probes using fluorescent *in situ* hybridization (FISH) or other polymerase chain reaction (PCR) technique][8,21,28,31] or by indirect methods (Nugent scores).[15] Nugent scores (ranged 0-10) are used to diagnose bacterial vaginosis (scores ≥7) or normal vaginal microbiota (scores 0-3) based on the quantitated morphotypes of small gram negative rods (*G. vaginalis/Bacteroides* spp.) and curved gram negative rods (*Mobiluncus* spp.) from gram stains of vaginal discharge smears. Microbial assays of only the strain(s) contained in the probiotic product are considered as pharmacokinetic studies and were not included in the normal microbiota profiles.

Models of dysbiosis. To determine the impact on normal microbiota, only direct evidence of microbiota change (species, profiles, diversity indices, or diagnostic criteria) were included and indirect effects were excluded (changes in intestinal enzymes, immune system parameters or disease symptoms). The degree to which dysbiosis was improved is categorized into three levels: (1) recovery of the normal microbiota back to baseline levels; (2) alteration or improvement of the normal microbiota; and (3) no change in normal microbiota.
The literature contained three dysbiosis models: Model A (restoration of the normal microbiota), which assayed patients enrolled with a healthy, undisturbed microbiota and then assayed again after a disruptive event (such as antibiotic exposure) and probiotic therapy occurred; Model B (alteration of the microbiota) assayed patients with pre-existing disrupted microbiota (for

example, pre-existing chronic disease or active disease) and then post-probiotic therapy; Model C (no dysbiosis) assayed volunteers with no disruptive event (before or during the clinical trial) at both pre-probiotic and post-probiotic times, as shown in Figure 1. 'Recovery' of the normal microbiota is defined as a restoration of the microbiota back to a normal healthy baseline. Recovery may be complete recovery (all assayed microbial levels returned to baseline) or incomplete recovery (partial recovery of some microbial strains, but not all returned to baseline levels). In studies enrolling subjects with dysbiosis at baseline (typically due to chronic diseases), it is not possible to show a restoration to normal microbiota levels because a normal, undisturbed microbiota was not present in these types of study subjects at the time of enrollment. Therefore, the strongest claim possible for Model B designs is for an 'alteration or improvement' of the microbiota. Only data from the probiotic-exposed subjects were analysed in this paper. Data from the control groups were used to confirm dysbiosis for subjects with chronic diseases or after a disruptive exposure, such as antibiotics or chemotherapy, unaffected by probiotic exposure.[32-34]

Assessment of methodological strength and quality

The GRADE (Grading of Recommendations, Assessment, Development and Evaluation) system for rating overall study quality will be used for each probiotic strain or type (single strains and mixtures of strains).[35] Recommendation for the support of the claim of each probiotic strain or mixture can be assessed by the overall strength of the evidence [“strong”, many randomized controlled trials show significant recovery of the microbiota, or "moderate" only one randomized controlled trial; or “weak”, only case series or reports, limited number of small trials, *etc.*].

Quality of the evidence is based on study design and graded as “high quality” (well-defined study design for determining restoration with normal microbiota, Model A), or “moderate quality” (disrupted microbiota at baseline, Model B), or “low quality” (no disruptive event occurred, Model C). Measurement of publication bias was not assessed for this review, as pooled outcome estimates of efficacy were not done, as typical in meta-analysis, but all studies with assays of microbiota were included to limit bias.

Net efficacy rating

To determine if the ability to correct dysbiosis is associated with clinical efficacy, the published literature for randomized controlled trials (RCTs) or meta-analyses of probiotics for various disease indications, including antibiotic associated diarrhea (AAD),[5,36,37] *Clostridium difficile* infection (CDI), [5,38] inflammatory bowel disease (IBD),[39] irritable bowel syndrome (IBS), [40] traveler's diarrhea (TD), [41] eradication of *Heliobacter pylori* (Hp),[36,37] bacterial vaginosis (BV) or vaginitis,[42] and treatment of acute pediatric diarrhea was reviewed. [43-45] The net rank was calculated by subtracting the number of RCTs showing non-significant or equivalent efficacy from the number of RCTs having significant efficacies. The ranks were categorized as follows: ++, ≥2 net RCTs showing significant efficacy; +, net of one RCT showing significant efficacy; 0, equal number of RCTs showing significant and non-significant efficacy results and -, ≥1 net negative or non-significant RCTs. Probiotics with no RCTs were not ranked.

RESULTS

A review of the literature from 1985-2013 found 353 articles that dealt with probiotic treatments and their potential effect on normal microbiota.

Excluded studies

As shown in Figure 2, a total of 272 articles were excluded for the following reasons: reviews (n=116), probiotic efficacy studies with no data on normal microbiota assays (n=54), animal models of probiotics and changes in microbiota (n=38), metagenomic or microbiota methods only (n=17), studies on normal microbiota but with no use of probiotics (n=14), *in vitro* assays of microbiota (n=10), duplicative reports (n=2) or miscellaneous (n=21), which included probiotic mechanism of action studies, safety studies, duplicative reports, cross-sectional surveys and two with poorly described probiotic interventions.[46,47] A total of 81 full articles were reviewed which mentioned changes in normal microbiota or indicated a health claim for probiotics and effects on normal microbiota.

Probiotic pharmacokinetic studies (n=18) reporting concentrations of probiotic strains before and post-treatment, but did not assaying for other species of normal microbiota were excluded. While several studies using this study design claim probiotics had an impact on normal

microbiota, type of data generated is pharmacokinetic behavior of the probiotics themselves and not the normal microbiota. Several studies stated that the normal microbiota was altered because an increase in various bacterial species was observed after the probiotics were given, but the species assayed were those contained in the probiotic product, so an increase is not unexpected. Pharmacokinetic studies have documented that probiotic strains taken orally can survive transit through the intestinal tract with recovery rates in feces ranging from <1% to 22%.[48,49] These pharmacokinetic studies were excluded from this analysis, as they did not assay other types of normal microbiota not found in the probiotic product.

Included studies

Of the 63 included clinical trials, five trials had multiple treatment arms, which resulted in a total of 68 treatment arms for analysis. Engelbrektson et al. tested a mixture of 5 probiotic strains in volunteers exposed to antibiotics and also tested a mixture of 4 probiotic strains in healthy volunteers with no antibiotic exposure.[50] Zoppi et al. had eight different treatment arms in his study, and probiotic arms were included in our analysis [*Saccharomyces boulardii* (*S. boulardii*) alone and *Lactobacillus (L.) rhamnosus GG* alone], a mixture of two probiotics (*L. acidophilus* and *Bifido. bifidum*) and a mixture of three probiotic strains (*L. acidophilus, L. rhamnosus* and *Bifido. bifidum*).[51] Orrhage et al. had two treatment arms (*Bifido. longum* alone and a mixture of *Bifido. longum* and *L. acidophilus*).[52] Larsen et al. tested two single probiotics (*Bifido. lactis* and *L. acidophilus*) in separate treatment arms.[53] Lidbeck et al. gave either enoxacin or clindamycin and randomized patients to either *L. acidophilus* or placebo. [54]

Normal microbiota assay methods. Of the 69 treatment arms that did normal microbiota assays, diverse methods were used to profile the microbiota. Many studies used only standard microbiological culture assays (37, 54%), while others (28, 40%) used techniques to detect non-cultivatable bacterial strains, which included metagenomic assays (FISH, TRFLP, 16s rRNA sequencing) or other PCR techniques. Some studies (4, 6%) used an indirect measure of normal microbiota, using the Nugent score to diagnose bacterial vaginosis, which relies upon gram stain of the vaginal secretions, vaginal pH and symptoms to characterize if normal microbiota is present or absent.[15]

Probiotic strains. In the 69 treatment arms, most (36, 52%) used a single strain of probiotic, while 14 (20%) tested a mix of two probiotic strains and 19 (28%) tested a mix of three or more probiotic strains. The distribution of single versus multiple strain probiotics did not significant vary by the model of study design (X^2_2=2.3, P=0.32). Of the 15 restorative (Model A) study arms, 47% used a single strain of probiotic and 53% used multiple strains. Of the 25 treatment arms with disrupted microbiota at baseline (Model B), 44% used a single strain and 56% used multiple strains. Of the 29 study arms with undisrupted microbiota (Model C), 62% used a single strain and 38% used multiple strains.

Normal microbiota restoration model (Model A)

Only 10 studies (with 15 treatment arms) using Model A to determine restoration of the microbiota were found (Table 1).[32,34,50-52,54-58] The type of enrolled subjects varied from healthy volunteers to children with untreated respiratory infections, to pediatric cancer patients. For subjects with acute infections or cancer, baseline assays were done prior to the disrupting agent (antibiotics or chemotherapy). The number of subjects given probiotics averaged 20/study and ranged from 5 to 83. In 93%, the disruptive factor was antibiotic exposure and in one study, chemotherapy caused the microbiota disruption. Only 8 (53%) of the study arms did an assay during a 1-8 week follow-up period after the probiotic was discontinued.

Analysis of the probiotic strain(s) separately found only two probiotic products with more than one randomized controlled trial. The probiotic mix of *L. acidophilus* and *Bifido. bifidum* showed a complete restoration in one study, but only a partial recovery in the other. (Strength: strong, Quality: high). The probiotic mix of *L. acidophilus* (2 strains) with *Bifido. bifidum* and *Bifido. animalis* showed complete restoration in one study, but only a partial recovery in the other. (Strength: strong, Quality: high). Five other probiotic products with only one supporting clinical trial showed microbiota restoration (*Bifido. longum*, *Clostridium butyricum*, *L. acidophilus*, mix of *L. acidophilus* with *L. paracasei* and *Bifido. lactis*, and the mix of *L. acidophilus* with *L. paracasei* and *Bifido. bifidum* and two strains of *Bifido. lactis*). (Strength: moderate, Quality:

high). Three probiotic products with one supporting clinical trial showed partial restoration (*S. boulardii*, *L. rhamnosus* GG, mix of *L. rhamnosus* with *L. bifidus* and *L. acidophilus*), (Strength: moderate, Quality: high). Only two probiotic products using Model A showed no change in the microbiota (*Bifido. breve* and a mix of *L. acidophilus* and *Bifido. longum*). (Strength: moderate, Quality: high). In summary, 10 of 12 (83%) of the probiotic products showed complete or partial restoration of the normal microbiota.

Of the 11 probiotic products with claims of 'restores or improves normal microbiota', 10 (91%) were supported by this review, but only seven showed complete restoration and five had partial restoration of the microbiota (Table 1). The mixture of *L. acidophilus* and *Bifido. longum* did not show any changes in the microbiota. Wada et al. claimed *Bifido. breve* 'enhanced intestinal anaerobes', but this was only compared to the placebo group.[32] Their data showed chemotherapy is a disruptive event, resulting in more Enterobacteria in the intestine in the placebo group, but there were no significant differences seen by the end of the 8 week follow-up in either the probiotic or the placebo group compared to baseline microbiota levels.

Disrupted normal microbiota at baseline studies (Model B)

Twenty-four studies (with 25 treatment arms) used Model B that enrolled subjects with a pre-existing disrupted microbiota related to ongoing disease or conditions (Table 2).[33,53,59-80] The number of subjects given probiotics averaged 23 $\pm$ 16/study and ranged from 7-83 participants. The types of pre-existing factors that disrupted the microbiota included atopic dermatitis patients, allergies, cirrhosis, bacterial vaginosis, irritable bowel syndrome, inflammatory bowel disease (ulcerative colitis and pouchitis), idiopathic diarrhea, enteral feeding, short-bowel syndrome and colon cancer. Only 10 (40%) of the study arms did an assay during the post-probiotic follow-up period.

Three of the probiotics had multiple clinical trials to support the claim of an improvement in the microbiota due to the probiotic. *S. boulardii* was used in two trials either with enteral fed

patients or patients with active diarrhea and found an improvement in the habitual microbiota in the patients with active diarrhea[66], but only showed indirect evidence of short-chain fatty acid changes in the other study.[65] (Strength: strong, Quality: moderate) A mix of four probiotic strains (2 strains of *L. rhamnosus*, *P. freudenreichii* + *Bifido. breve*) showed improved microbiota in two clinical trials.[74,75] (Strength: strong, Quality: moderate) Of four clinical trials testing a mixture of seven probiotic strains, two showed no significant change in microbiota [77,78], one showed more anaerobes post-probiotic treatment[79] and one found a reduction in Bacteroides species.[80] (Strength: strong, Quality: moderate) Three clinical trials determined there were no significant changes due to *L. plantarum* 299v.[62-64] (Strength: strong, Quality: moderate). Of those probiotics with only one supporting clinical trial (Strength: moderate, Quality: moderate), two single probiotic strains (*E. coli* Nissle and *L. casei rhamnosus*) and five different mixtures of probiotic strains support the claim that the probiotic alters the microbiota (Table 2). In summary, 10 of 18 (56%) probiotic products altered or improved microbiota in individuals with pre-existing disease.

Of the 25 treatment arms, the paper's claim was confirmed in 14 (56%) of the studies. There was no significant change in the microbiota due to the probiotic in nine treatment arms and only an alteration of the microbiota in five others (Table 2). Our review disagreed with the claimed outcomes in 11 (46%) of the other treatment arms. In seven treatment arms, it was claimed the tested probiotic 'restored normal microbiota', but it is uncertain how this conclusion was reached, since there was no time when a normal undisrupted microbiota was present. Of the seven studies that claimed their probiotic 'restored' normal microbiota, our analysis determined none were capable of documenting restoration, but it is confirmed probiotics improved or altered the microbiota in these studies. Four studies claimed the probiotic 'altered or improved' normal microbiota, but this review found no significant differences when post-probiotic and baseline assays were compared for the probiotic groups. Girard-Pipau et al. concluded that *S. boulardii* 'altered normal flora' because more gram positive anaerobes were seen in the probiotic group compared to the controls and an increase in three short-chain fatty acids were observed in the *S. boulardii* group.[65] However, when the analysis is restricted to trends observed in the probiotic group only, no significant differences were observed in pre-probiotic versus post-probiotic

microbiota profiles. Venturi et al. concluded that the mix of seven probiotic strains enhanced the concentration of some beneficial strains in the intestines. [77] However, the only strains having a significant increase were those contained in the probiotic mix, and not specifically normal microbiota of the host. As this study did not have an undisturbed microbiota baseline, the increased numbers of Lactobacilli and Bifidobacteria may not have reflected their normal levels. Van der Aa et al. claimed that *Bifido. breve* 'successfully modulates the intestinal flora', but no significant changes were observed in the probiotic group when comparing the baseline to the post-probiotic levels. [59] Odamaki et al. did show an increase in Faecalibacterium ssp. and *Bacteroides fragilis* ssp. at the end of *Bifido. longum* BB536 treatment, but the same increase was also observed in the placebo group.[33]

Undisrupted normal microbiota studies (Model C)

Twenty nine trials enrolled healthy adults who had no disruptive factor present during the study (either no antibiotic or no medication exposure or presence of acute or chronic disease) that might impact normal microbiota, as shown in Table 3.[14,49,50,81-106] The average number of subjects given probiotics was 23/study and ranged from 7 to 160/study. Of the 29 study arms, assays were taken during a follow-up period in only 52%. Fujiwara et al. cultured seven healthy volunteers and found Enterobacteriaceae and Clostridial species post-*Bifido. longum* was reduced by 10^1/g compared to baseline ($P<0.03$), but no other changes in the microbiota were detected.[84] Karlsson et al. found a significant increase in intestinal diversity in nine male volunteers with atherosclerosis given *L. plantarum* 299v, but because terminal restriction fragment length polymorphism assays were used instead of cultures for bacterial species, the specific changes in the microbiota species could not be determined.[94] Yang and Sheu cultured 63 children (55% with *H. pylori*) given a yogurt with *L. acidophilus* and *Bifido. lactis* but only found a decrease in *E. coli* counts in the *H.pylori* negative children sub-group, no significant changes in normal microbiota was found in the *H. pylori* positive children.[100] Kubota et al. assayed 29 subjects with Japanese cedar pollen allergy and found milk fermented with *L. rhamnosus* GG and *L. gasseri* TMC0356 suppressed microbiota changes (less intestinal profile changes), but could not determine specific bacterial species changes due to the type of assay used

(FISH and TRFLP).[103] In summary, only 4 of 19 (21%) probiotic products altered microbiota in healthy individuals who had no disruptive event.

Of the seven studies that claimed their probiotic(s) 'restored or altered' the normal microbiota, only four claims were confirmed. Sierra et al. claimed *L. salivarius* given to 20 healthy adults 'improved gut microbiota', but only increased levels of Lactobacilli were found and no other changes in normal microbiota species were detected. The only other evidence was indirect from changes observed in immune parameters.[96] He et al. claimed a mixture of *Bifido. longum* and *Bifido. animalis* 'modified' microbiota, but changes were seen only during the yogurt administration and not after the one week follow-up period.[99] Vitali et al. claimed that the mixture of four Lactobacilli strains and three Bifidobacteria strains 'modulated vaginal microbiota', but the only significant changes were due to an increase in the bacterial species contained in the probiotic mixture.[14]

Of the probiotics supported by multiple clinical trials (*Bifido. animalis*, *Bifido. longum*, *L. casei*, *L. plantarum* 299v, the mixture of *Bifido. animalis and Bifido. lactis*), 13 of the trials (87%) support there is no significant change in normal microbiota if the microbiota is not disrupted. [Strength: strong, Quality: low)

Association of clinical efficacy and normal microbiota restoration

Few studies concurrently compared clinical efficacy and the ability to restore or improve normal microbiota after dysbiosis. A synthesis of the literature of RCT for eight common disease indications was performed and the overall net strength was ranked. Probiotics with the ability to restore normal microbiota were frequently supported by RCTs for efficacy, as shown in Table 4. Of the 10 probiotics with evidence for restoration, 7 (70%) also had at least one RCT testing for at least one of the eight diseases, while 30% did not have any supportive RCTs for efficacy. Of the 7 probiotics with associated RCTs, only two probiotics (*S. boulardii* and *L. acidophilus*) have strong evidence for efficacy across most of the disease indications, while five probiotics with the

ability to restore the microbiota had weak or no evidence of efficacy. For example, *S. boulardii*, which has studies supporting restoration, has strong evidence for clinical efficacy for AAD (ranked ++: 11 RCTs had significant results and 6 had non-significant results), CDI (ranked ++: had two RCTs with significant results), IBD (ranked ++: had two RCTs with significant results), IBS (ranked 0: had one RCT with significant efficacy and one RCT with non-significant results), TD (ranked +: 3 RCTs with significant efficacy and 2 with non-significant efficacy), *H. pylori* eradication (ranked -: 2 RCTs with significant results and 4 with non-significant results) and no studies for BV. *L. acidophilus*, which partially restored the microbiota in a study, is associated with clinical efficacy for AAD, IBS and BV, but not for TD or eradication of *H. pylori* and treatment of acute pediatric diarrhea (ranked ++: had 19 RCTs with significant protection and five with non-significant results). In contrast, *L. rhamnosus GG,* another probiotic capable of restoring microbiota, is often cited in meta-analysis as having significant efficacy for AAD. Our results of an updated review of the literature indicate a net weak evidence rating for clinical efficacy across all disease indications: AAD (ranked -: 3 RCTs had significant results and 6 had non-significant results), CDI (ranked -: two RCTs with non-significant results), IBD (ranked -: one RCT with non-significant results), IBS (ranked 0: 2 RCTs with significant efficacy and two RCTs with non-significant results), TD (ranked 0: one RCT with significant efficacy and one with non-significant efficacy), *H. pylori* eradication (ranked -: 3 RCTs with non-significant results), no RCTs for BV and treatment of acute pediatric diarrhea (ranked ++: 10 RCTs with significant efficacy and one with non-significant findings).

Efficacy trials were not done as frequently for probiotics shown to only have the ability to alter or improve, but not restore, the microbiota after dysbiosis. Of nine probiotics that can alter the microbiota, 6 (67%) have supporting RCTs for at least one disease, but the diversity of investigated diseases was more limited. *L. casei* had moderate net strength for AAD and bacterial vaginosis, but was neutral for the ability to eradicate *H. pylori* and other disease indications were not tested in RCTs with *L. casei*. The probiotic mixture of *L. reuteri* and *L. fermentum* has strong evidence for bacterial vaginosis, but not for any other disease indications listed in Table 4.

Of the eight probiotics not capable of altering or restoring normal microbiota, only *L. plantarum* 299v had RCTs for AAD and IBS, both with net negative or weak strength of clinical efficacy. *Bifido. lactis* and the mixture of *L. rhamnosus* and *L. reuteri* had net neutral rankings for efficacy for the treatment of acute pediatric diarrhea. The other four probiotic products with no effect on normal microbiota lacked any RCTs for clinical efficacy. Studies with *B. clausii* did not assay for normal microbiota and had non-significant trial results for *H. pylori* eradication and the treatment of pediatric diarrhea.

Of the six probiotics with only pharmacokinetic data on the probiotic itself and no other investigation of other normal microbiota strains, five had RCTs showing varying net efficacies for different disease indications, as shown in Table 4.

Six popular probiotics (*Bacillus clausii*, *Bifido. infantis*, *L. reuteri*, *L. acidophilus* + *L. helveticus*, *L. acidophilus* + *L. casei* and *L. acidophilus* + *Bifido. animalis*) have only clinical efficacy RCTs, but have not published studies investigating their role in restoring or improving the normal microbiota.

Discussion

Developing and evaluating health or function claims for probiotics is an important issue and is now identified as a priority for research by several international organizations, including the World Gastroenterology Organization [107] and the American Society for Nutrition.[2] The U.S. Food and Drug Administration has struggled with appropriate evidence-based health claims for probiotic products and currently recommends the use of structure/function claims, such as "maintains bowel regularity", but the claim for restoring normal microbiota is still under debate.[108] The European Food Safety Authority (EFSA) provides guidance materials that recommend health or function claims for probiotics should have beneficial physiological effects and have appropriate scientific trials to substantiate the health claims.[3] Acceptable claims for intestinal health may include functional claims (improved transit time, softer stool consistency, reduction in gastrointestinal discomfort, defense against pathogens). As it is currently not possible to define a standard normal microbiota profile, the EFSA recommends functional claims

for the restoration of normal microbiota should document a recovery of healthy microbiota and be accompanied by a beneficial physiological or clinical outcome.[3] In addition, because the efficacy and mechanisms are strain-specific and may vary by probiotic strain, the evidence must be analyzed for each probiotic product individually.[5,6,9,109, 110-112]

An underappreciated finding was the influence that study design and study populations have on the interpretation of study outcomes. In the literature, five different types of study designs are commonly found relating to probiotics. The most common study type is a randomized controlled trial testing the efficacy and safety outcomes in patients, but these trials did not typically document the impact of the probiotic on the normal microbiota. The second most common type of study design is pharmacokinetic studies (documenting recovery of oral dose of probiotic or increase in probiotic strains post-treatment compared to pre-treatment or clearance of the probiotic). Even though these kinetic studies did not assay for non-probiotic strains, some extrapolated their results and concluded some effect or improvement of the normal microbiota was observed by their probiotic.[19,111] These two first types of study designs do not support evidence-based conclusions for the restoration or alteration of the normal microbiota and were excluded from this review.

Three types of study designs are appropriate for the study of dysbiosis. The first type of study design had normal microbiota assayed at least twice (at baseline, which was before exposure to a disruptive event or probiotics and then again during or post-probiotic treatment) to show actual recovery of assayed normal microbiota back to healthy baseline levels. The second type of study design started with inappropriate baselines (baseline samples taken after normal microbiota had been disrupted by chronic disease). For patients with established chronic diseases, there is no "normal microbiota" baseline in either the probiotic or the control group. Even if baselines are taken during remission, the microbiota may still be impacted by chronic disease or acute diarrhea. Studies of probiotics in chronic diseases or acute disease typically report on 'pre-probiotic treatment' and 'post-probiotic treatment' and may show significant shifts in microbial species, but it is uncertain if this reflects a true re-establishment of normal microbiota profiles. The third type of study design enrolled healthy volunteers, who were not challenged with antibiotics (so no normal microbiota disruption occurred), and show only the effect of probiotics on a healthy microbiota (typically mild or no effects). Control groups were not required for our

assessment of the impact of probiotics on microbiota, but control groups can document the degree normal microbiota is disrupted by inciting agents (antibiotic, disease onset, etc.).

Five single strain probiotics (*Bifido. longum*, *Clost. butyricum*, *L. acidophilus, L. rhamnosus* and *S. boulardii*) and five probiotic mixtures [(*L. acidophilus* + *Bifido. bifidum)*, (*L. rhamnosus* + *L. bifidus* + *L. acidophilus)*, (*L. acidophilus* + *L. paracasei* + *Bifido. lactis),(L. acidophilus*, 2 strains, *Bifido. bifidum, Bifido. animalis)* and *(L. acidophilus* + *L. paracasei* + *Bifido. bifidum* +*2 strains of Bifido. lactis*)] documented either complete or partial recovery of normal microbiota (Model A). Only two probiotic mixtures [(2 strain mixture: *L. acidophilus* + *Bifido. bifidum*) and (4 strain mixture: *L. acidophilus*, 2 strains, *Bifido. bifidum, Bifido. animalis*)] were supported by a confirmatory study. Evidence that probiotics may alter or improve normal microbiota (Model B) was found for three single strain probiotics (*E. coli* Nissle, *S. boulardii* and *L. casei rhamnosus*) and seven mixtures of 2-7 probiotic strains. Of these ten probiotics finding alteration of the microbiota, only three had multiple trials [*S. boulardii*, and a four strain mixture (2 strains of *L. rhamnosus* + *P. freudenreichii* + *Bifido. breve*), and a seven strain mixture (4 Lactobacilli and 3 Bifidobacteria strains)], but only one had consistent results showing improvements in the microbiota. [74,75] Clearly, more than one study is needed to confirm the impact of a probiotic on the normal microbiota. Of the 19 probiotic strains (or mixtures) studied in healthy volunteers who were not exposed to disruptive factors (Model C), no change in the normal microbiota was observed for 79%, indicating the robustness of the microbiota.

Improvement in the normal microbiota by specific probiotic strains seemed to be associated with better clinical endpoints. Within eight common diseases typically treated with probiotics, more trials with significant efficacy were associated with probiotic strains shown to restore the normal microbiota, and only one trial with significant efficacy was found for probiotics that did not alter the microbiota. However, few probiotics had efficacy trials for all eight diseases and many did not have any efficacy trials.

Some probiotics which have published efficacy trials for various diseases did not have studies investigating the effect of the probiotic on normal microbiota: *Bacillus clausii*, *Bifido. infantis*, *L. brevis*, *L. reuteri*, mix of 2 strains (*L. acidophilus* + *L. helveticus*), mix of 2 strains (*L. acidophilus* + *L. casei*) or (*L. acidophilus* + *Bifido. animalis*), mix of 4 strains [*L. rhamnosus* (2 strains), *Propionibacterium freudenreichii* + *Bifido. animalis*)] and mix of 7 strains (*L. sporogens, L. bifidum, L. bulgaricus, L. thermophilus, L. acidophilus, L. casei, L. rhamnosus).*

Comparison of results with other studies

Other reviews in the literature of claims for probiotics relating to changes in the normal microbiota have focused on the broad issues of regulatory standardization of health or function claims, the use of proper study designs and the challenge of defining biomarkers for a 'healthy microbiota'.[3,29,112] Donovan et al. recommends that health claims for probiotics be supported by well-conducted human trials in the targeted population.[2] These reviews also recommend that gut biomarkers need to be correlated with clinical endpoints, however none of these reviews attempted to do so.[29,112] No prior review has attempted to analyze the association between probiotic strains and their impact on normal microbiota by stratifying on the quality of study design. [111] This review addressed these concerns by analyzing probiotic strains by the quality of the study design and only including trials that assessed the normal microbiota (either by microbial culturing or molecular strain biomarkers) and assessed the degree of dysbiosis improvement with clinical outcomes for each probiotic strain.

Opportunities for future research

Most of the studies (80%) using Model A to document restoration of the normal microbiota only used microbiologic culturing techniques, which can only detect those organisms that grow in culture. Use of the more advanced molecular metagenomic techniques have found that culturing alone misses up to 95% of these organisms.[21,22] The use of the metagenomic techniques was more common in the studies using Model B (48%) and Model C (45%) study designs, which only addresses potential alteration of the microbiota. Characterization of the microbiota is a

complex issue and a comprehensive accounting of all the bacterial and fungal strains in the body is beyond our current capabilities. Therefore, any studies of changes to the microbiota are incomplete at best, but general trends in bacterial phylotypes can be documented using DNA probes and metagenomic techniques. Differential detection bias may be present due to the variety of assays used in these studies and should be accounted for in future studies.

Another suggestion for future studies is to include an appropriate follow-up time period post-probiotic administration. Fewer than half of the reviewed trials did assays for normal microbiota during an appropriate follow-up period. As it has been shown that recovery from a disrupting factor can be prolonged (typically eight weeks),[7,8] and studies that failed to find microbiota recovery might have detected a return to normal baseline levels if a sufficiently long time was given for the recovery to have occurred. Future studies should strive to allow time for the restoration of the normal microbiota to occur.

As the effects of probiotics are strain-specific, and many studies typically only report the genus and species of the tested probiotic, future reports should include a complete description of the probiotic to the stain level. [5,112]

Strengths and weaknesses

The strengths of this review included the completeness of the search strategy, which reviewed multiple citation databases, trial registries and author searches, use of established PRISMA protocols for reviews and the use of an outcome classification scheme for different degrees of assessment for microbial recovery. This analysis controlled the confounding effects of different study populations and study designs present in the literature. Pharmacokinetic studies of just the probiotic strain(s) itself were excluded and only trials that assayed other species found in the microbiota were included. By applying a standard definition for 'restoring' versus 'improving' normal microbiota, it is possible to distinguish significant differences by the type of study designs used and differential effects of the different probiotic strains. Limitations of this review

include pooling trials from different populations (adult versus pediatric) and different probiotic doses and regimens used. Incomplete retrieval of all studies assessing the effect that probiotics have on human microbiota is also a potential limitation of any literature search. Another limitation is that dysbiosis improvement and clinical efficacy for probiotic strains is also indirectly associated, no direct cause and effect relationship was possible with the types of studies done. Another limitation is the current lack of a standard definition of what comprises a 'normal microbiota'. The constituents of the microbiota vary by individual, by age, geographic location and health status of the host. Current microbiologic techniques are improving, but can not detect all species present in the host.

Conclusion

The challenges in recommending a specific probiotic to patients who need to restore or improve their normal microbiota after a disrupting event occurs is two-fold: one is the diversity of probiotic products available and second is the varying strength of evidence provided by clinical trials using different outcome measures and study designs. By grouping studies into three groups that result in three different degrees of probiotic effect (restoration, improvement or no change), an overview of the body of evidence is possible. By comparing the strength of the clinical evidence for common diseases by the degree to which the probiotics could impact the restoration of the normal microbiota, it became obvious that those probiotics with a greater ability to restore the microbiota are associated with the strongest strength of clinical efficacy. While this evidence only indirectly links clinical efficacy with the ability to restore the microbiota, the overall review of the evidence shows this is an important mechanism of action for probiotics. What becomes obvious is that more studies are required to conclude which probiotic strains have a beneficial impact on the normal microbiota, as most strains have only a single clinical trial and many probiotic products overstate the strength of their claim to restore normal microbiota. These types of issues should be considered for health care policy makers and researchers for future studies and for creating guidelines for health/function claims.

Table 1. Model A: Evidence-based data for restoration of normal microbiota (NM) for 12 probiotics from 10 studies (15 treatment arms).

Probiotic*	Reference	No. treated with probiotic	Typeof assay for NM	Enrolled population	Type of disrupting factor	Follow-up post-treatment (wks)	Claims stated in papers	Evidence-based claim
Bifido. breve	Wada 2010[32]	19	FISH	pediatric cancer patients	chemotherapy	8	enhances anaerobes	no change
Bifido. longum BB536	Orrhage 1994[52]	10	culture	healthy volunteers	clindamycin	0	restores	restores
Clost. butyricum MIYAIRI	Seki 2003[34]	83	culture	pediatric respiratory or GI infections	antibiotics	0	restores	restores
L. acidophilus NCFB1748	Lidbeck 1988[54]	5 5	culture culture	healthy volunteers volunteers	enoxacin or clindamycin	1 1	restores only in enoxacin no change	restores only in enoxacin, no change in clindamycin
L. rhamnosus GG	Zoppi 2001[51]	7	culture	pediatric respiratory infections	ceftriaxone	0	partially corrects	partially restores
S. boulardii lyo	Zoppi 2001[51]	6	culture	pediatric respiratory infections	ceftriaxone	0	improves	partially restores
L. acidophilus + *Bifido. bifidum*	Black 1991, [55] Zoppi 2001[51]	10, 7	culture, culture	healthy volunteers, pediatric respiratory	ampicillin, ceftriaxone	2, 0	recovers more rapidly, less change	restores, partially restores
L. acidophilus 1748 + *Bifido. longum* BB536	Orrhage 1994[52]	10	culture	healthy volunteers	clindamycin	0	no change	no change
L. rhamnosus + *L. bifidus* + *L. acidophilus*	Zoppi 2001[51]	7	culture	pediatric respiratory infections	ceftriaxone	0	partially corrects	partially restores
L. acidophilus 1748 + *L. paracasei* F19 + *Bifido lactis* Bb12	Jernberg 2005[56]	4	culture PCR TRFLP	healthy volunteers	clindamycin	2	restores	restores
L. acidophilus CUL60+ *L. acidophilus* CUL21 + *Bifido. bifidum* CUL17 + *Bifido animalis lactis*	Madden 2005, [57] Plummer 2005[58]	15, 76	culture, culture	*H. pylori* +, *H. pylori* +	amoxicillin + metronidazole, amoxicillin + clarithromycin	2, 2	restores, restores more rapidly	restores, partially restores
L. acidophilus NCFM + *L. paracasei* Lpc-37 + *Bifido. bifidum* Bb02+ *Bifido. lactis* Bi-04 + *Bifido. lactis* Bi-07	Engel-brektson 2006[50]	20	culture PCR TRFLP	healthy volunteers	augmentin	2	restores	restores

*including strain (when reported)

Table 2. Model B: Evidence-based data for improvement or alteration of normal microbiota (NM) in 18 probiotics from 24 studies (25 treatment arms) with disturbed microbiota at baseline.

Probiotic*	Reference	No. treated with probiotic	Type(s) of assay for NM	Pre-existing disrupting factor**	Follow-up time	Claims stated in papers	Evidence-based claim	Type of change found in NM
Bifido. breve M-16V	Van der Aa 2010[59]	46	FISH	Atopic dermatitis	0	modulates NF	no change	--
Bifido. lactis Bi-07	Larsen 2011[53]	17	PCR	Atopic dermatitis	0	no change	no change	--
Bifido. longum BB536	Odamaki 2007[33]	22	TRFLP PCR	Cedar pollen allergy	4 wk	maintains NF	no change	--
E. coli Nissle	Lata 2007[60]	22	culture	liver cirrhosis	0	restores	improves	more Bifido. & Lacto.
L. acidophilus 700396	Larsen 2011[53]	17	PCR	atopic dermatitis	0	no change	no change	--
L. casei rhamnosus Lcr35	Petricevic 2008[61]	83	Nugent scores	bacterial vaginosis	4 wk	restores	improves	improved Nugent scores
L. plantarum 299v	Nobaek 2000, [62] Klarin 2005, [63] Klarin 2008[64]	25, 17, 22	culture, culture, culture	IBS, enterally-fed, antibiotics	4 wk, 0, 0	no change, no change, no change	no change, no change, no change	--
S. boulardii lyo	Girard 2002, [65] Swidsinski 2008[66]	10, 20	culture, FISH	enterally-fed, active diarrhea	9 d, 3 wk	alters NF, improves	no change, improves	-- more 'habitual microbiota'
L. rhamnosus GR-1 + *L. fermentum* RC14	Reid 2001[67]	33	Nugent scores	bacterial vaginosis	2 wk	restores	improves	improved Nugent scores
L. rhamnosus GR-1 + *L. fermentum* RC14	Reid 2003[68]	31	Nugent scores and culture	bacterial vaginosis	30 d	restores	improves	improved Nugent scores
L. plantarum 8PA3 + *Bifido bifidum*	Kirpich 2008[69]	32	culture	colon cancer	0	restores	improves	more *E. coli* and *Enterococci*
L. rhamnosus GR1 + *L. reuteri* RC14	Hummelen 2010[70]	23	Nugent score	bacterial vaginosis	0	no change	no change	--
L. casei Shirota+ *Bifido breve* BBG01	Uchida 2007[71]	4	culture	short bowel syndrome	0	no change	no change	--
L. brevis CD2 + *L. salivaris* FV2 + *L. plantarum* FV9	Mastromarino 2009[72]	19	Nugent score	bacterial vaginosis	2 wk	restores	improves	improved Nugent scores

L. paracasei Lpc37+ *L. acidophilus* 74-2 + *Bifido. animalis* DGCC420	Roessler 2012[73]	30	PCR	atopic dermatitis	0	no change	no change	--
L. rhamnosus GG + *L. rhamnosus* Lc705 + *P. freudenreichii shermanii* JS + *Bifido. breve* Bb99	Kajander 2005,[74] Lyra 2010[75]	41, 22	PCR, PCR	IBS, IBS	0, 0	restores, alters	improves, alters	Improved similarity index More Clostridia and Rumino-coccus
L. acidophilus 4356 + *L. plantarum* 14917 + *L. rhamnosus* 7469 + *Bifido. bifidum* 2952	Wong 2013[76]	7	PCR	liver disease	0	improves	alters	Less Firmicutes, more Bacterio-detes
L. acidophilus + *L. paracasei* + *L. delbrueckii ssp. bulgaricus* + *L. plantarum* + *Bifido. longum* + *Bifido. infantis* + *Bifido. breve*	Venturi 1999, [77] Brigidi 2001, [78] Kuhbacher 2006[79] Ng 2013[80]	20, 10, 10 10	culture culture & PCR FISH PCR	ulcerative colitis, IBS, pouchitis IBS	15 d, 10 d, 0 0	enhances, no change, altered richness modulates	no change, no change, altered altered	-- -- More anaerobes Less Bacteroides

*including strain (when reported)

**disruption of normal microbiota at baseline shown by significant differences compared to control (non-diseased) population.

Table 3. Model C: Evidence-based data for improvement or alteration of normal microbiota (NM) in 19 probiotics in healthy volunteers enrolled in 29 studies (29 treatment arms) in studies with no disruptive exposures.

Probiotic	Reference	No. treated with probiotic	Type of assay for NM	Enrolled population	Type of disrupt-ing factor	Follow-up post-treat-ment	Claims stated in papers	Evidence-based claim
Bifido. animalis lactis DN173010	Rochet 2008,[49] Oswari 2013[81]	12, 160	FISH PCR	healthy volunteers	none, none	10 d, 6 mon	no change, no change	no change, no change
Bifido. bifidum	Langhendries 1995[82]	20	culture	healthy volunteers	none	0	no change	no change
Bifido. longum	Benno 1992,[83] Fujiwara 2001,[84] Harmsen 2002[85]	5, 7, 14	culture, culture, FISH	healthy volunteers	none, none, none	0, 30 d, 0	no change, alters, no change	no change, alters, no change
L. casei ND114001	Guerin 1998,[86] Rochet 2006,[87] Rochet 2008[88]	12, 12, 7	culture, FISH, FISH	healthy volunteers	none, none, none	1 wk, 10 d, 0	no change, no change, no change	no change, no change, no change
L. johnsonii La1	Brunser 2006[89]	32	culture & FISH	healthy volunteers	none	2 wk	no claim	no change
L. plantarum 299v	Goossens 2003,[90] Goossens 2005,[91] Goossens 2006,[92] Berggren 2003,[93] Karlsson 2010[94]	11, 32, 15, 33, 9	culture, culture, culture, culture, TRFLP	healthy, healthy, colonic polyps, healthy, atherosclerosis	none, none, none, none, none	3 wk, 4 wk, 0, 0, 0	no change, no change, no change, no change, alters	no change, no change, no change, no change, alters
L. rhamnosus GG	Gueimonde 2006[95]	29	PCR	healthy volunteers	none	0	no change	no change
L. salivarius CECT5713	Sierra 2010[96]	20	culture	healthy volunteers	none	0	improves	no change
S. boulardii lyo	Vanhoutte 2006[97]	30	PCR	healthy volunteers	none	0	no change	no change
Bifido. animalis + *Bifido. longum*	Zhong 2006,[98] He 2008[99]	11, 11	FISH, FISH	healthy volunteers	none	7 d, 7d	no change, modifies	no change, no change
L. acidophilic + *Bifido. lactis*	Yang 2012[100]	63	culture	healthy but 55% *H. pylori* +	none	0	restores	alters
L. rhamnosus GG + *Bifido. longum* Bb536	Mah 2007[101]	20	FISH	healthy neonates	none	6 mon	no change	no change
L. rhamnosus GG + *Bifido. lactis* Bb12	Rafter 2007[102]	38	culture	colon cancer patients or at risk	none	0	no change	no change

L. rhamnosus GG + *L. gasseri* TMC0356	Kubota 2009[103]	14	culture FISH TRFLP	healthy, allergy patients	none	0	suppressed changes	alters
L. paracasei B21060 + *L. paracasei* B21070 + *L. gasseri* B21090	Morelli 2003[104]	12	culture	healthy volunteers	none	3 d	no claims	no change
L. acidophilus 1748 + *L. paracasei* F19 + *Bifido. lactis* Bb12	Sullivan 2009[105]	15	culture	chronic fatigue patients	none	4 wk	no change	no change
L. rhamnosus 271 + *L. acidophilus* NCFM + *L. paracasei* 114001 + *Bifido. animalis* 1017	Engelbrektson 2006[50]	22	culture TRFLP PCR	healthy volunteers	none	2 wk	no change	no change
Bifido. animalis lactis + *L. delbrueckii* I-1632 + *L. delbrueckii I-*1519 + *L. lactis cremoris*	McNulty 2011[106]	7	PCR	healthy twins volunteers	none	4 wk	no change	no change
L. acidophilus + *L. paracasei* + *L. delbrueckii ssp. bulgaricus* + *L. plantarum* + *Bifido. longum* + *Bifido. infantis* + *Bifido. breve*	Vitali 2012[14]	15	PCR	healthy pregnant volunteers	none	0	modulates	no change

*including strain (when reported)

Abbreviations: FISH, fluorescence *in situ* hybridization analysis; TRFLP, terminal restriction fragment length polymorphism analysis; PCR, polymerase chain reaction

Table 4. Comparison of the ability of probiotic to restore or improve dysbiosis with ranked clinical efficacy for various disease indications.

Probiotic*	**Restored normal microbiota***	**Altered normal microbiota***	**Ranked net evidence for efficacy****							
			AAD	**CDI**	**IBD**	**IBS**	**TD**	**H pylori**	**Vaginitis/ BV**	**Acute Ped diar**
Restores microbiota										
Clost. butyricum MIYAIRI	yes	nd	-					-		
L. acidophilus + *Bifido. bifidum*	yes	nd	0	-						
L. acidophilus 1748 + *L. paracasei* F19 + *Bifido. lactis* Bb12	yes	nd				-				
Bifido. longum	yes	no			-	+				
L. acidophilus + *L. acidophilus* + *Bifido. bifidum* + *Bifido. animalis*	yes	nd								
L. acidophilus + *L. paracasei* + *Bifido. lactis (2)*	yes	no								
S. boulardii lyo	partial	yes	++	++	++	0	+	-		++
L. rhamnosus GG	partial	nd	-	-	-	0	0	-	0	++
L. acidophilus	partial	no	++		++	-	-	+	0	
L. acidophilus + *L. bifidus* + *L. rhamnosus*	partial	nd								
Alters microbiota										
E. coli Nissle	nd	yes			-					+
L. casei (DN114001 or Lcr35)	nd	yes	+					0	+	++
L. rhamnosus GR1 + *L. fermentum* RC14	nd	yes							++	
L. plantarum 8PA3 + *Bifido. bifidum*	nd	yes								
L. rhamnosus GG + *L. rhamnosus* Lc705 + *P. freudenreichii shermanii* JS + *Bifido. breve* Bb99	nd	yes				++				
L. acidophilus + *L. plantarum* + *L. rhamnosus* + *Bifido bifidum*	nd-	yes								

L. brevis CD2 + *L. salivarus* FV2 + *L. plantarum* FV9	nd	yes							+	
L. acidophilus + *L. paracasei* + *L. delbrueckii ssp. bulgaricus* + *L. plantarum, Bifido. longum, Bifido. infantis, Bifido. breve*	nd	yes	-		++	+				++
No effect on microbiota										
B. clausii	nd	nd						-		-
L. plantarum 299v	nd	no	-	-		-				
Bifido. lactis	nd	no	+							0
Bifido. breve	no	no								
L. acidophilus + *Bifido. longum*	no	--								
L. rhamnosus 19070-2 + *L. reuteri* DSM	nd	no								0
L. casei + *Bifido. breve*	nd	no								
L. paracasei + *L. acidophilus* + *Bifido animalis*	nd	no								
Pharmacokinetic only										
L. reuteri 55730	nd	nd								+
L. johnsonii La1	nd	nd			-			+		
L. salivarius UCC4331	nd	nd				-				
Bifido. infantis 35624	nd	nd				0				
Bifido. bifidum MIMBb75	nd	nd				+				
L. rhamnosus + *Bifido. longum*	nd	nd								

*including strain (when reported)

** **Rank:** **++**, ≥2 net RCTs (randomized controlled trials) with significant protective efficacy; +, only one net protective RCT; 0, equal number of significant and non-significant RCTs; -, ≥1 net non-significant RCT. Blank indicates no RCT done for the disease indication.

Abbreviations: nd, not determined; AAD, antibiotic associated diarrhea; CDI, Clostridium difficile infections; IBD, inflammatory bowel disease; IBS, irritable bowel syndrome; TD, traveler's diarrhea; BV, bacterial vaginosis; Acute Ped Diar, treatment of acute pediatric diarrhea.

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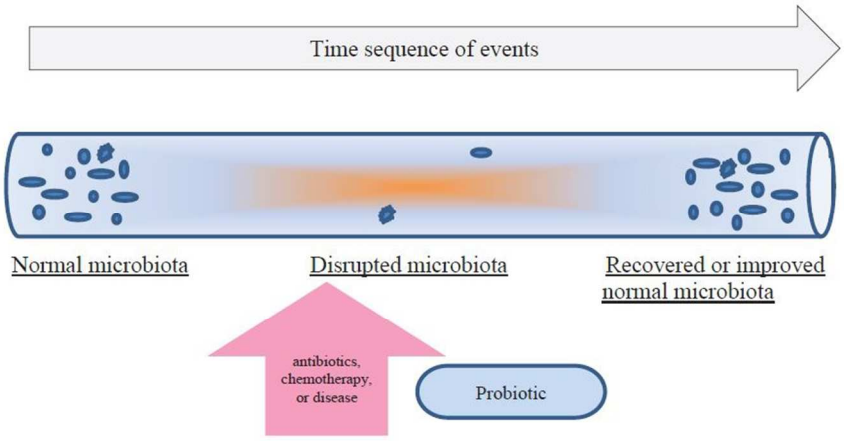
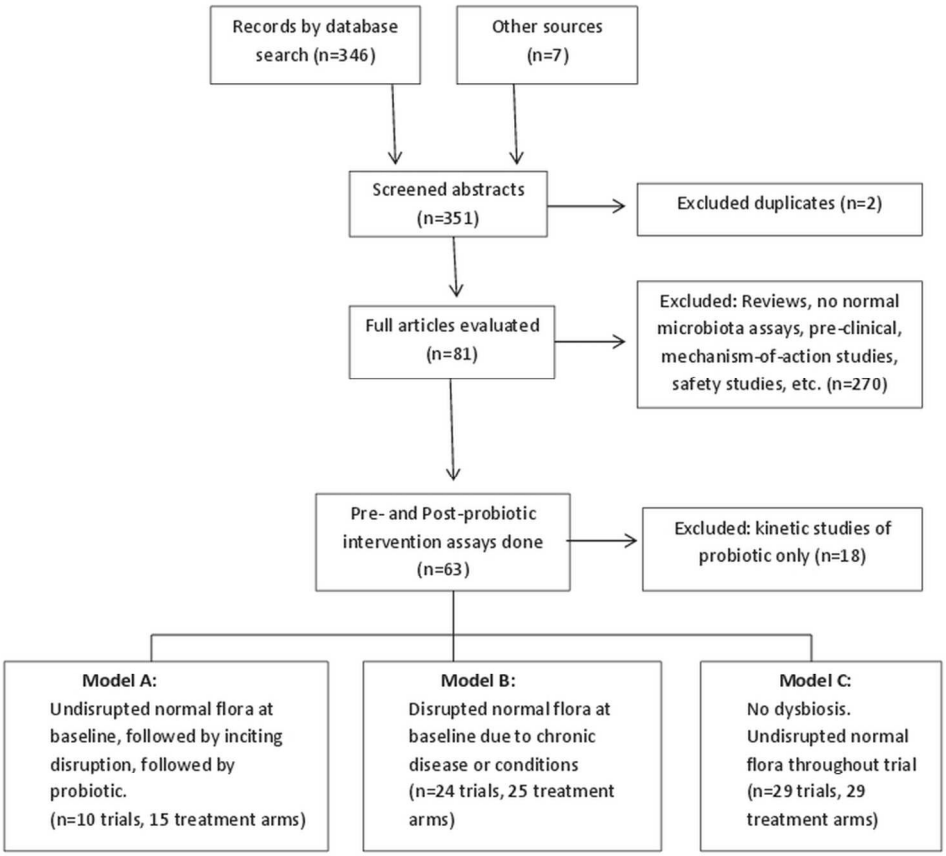
Figure 1. Time sequence of events and three models of study designs determining three different degrees of dysbiosis correction by probiotics.

Figure 2. Flow-chart of literature review results (1985-2013) of included and excluded studies for the restoration or improvement of normal microbiota by probiotics. Abbreviations: RCT, randomized-controlled trials; MOA, mechanism of action; NM, normal microbiota



Model	Type of population enrolled	Dysbiosis at baseline	Time microbiota disrupted	Probiotic or control intervention	Potential outcomes
A	Healthy volunteers or at-risk patients	no	post-baseline	preventive	restoration
B	Patients with active disease at enrollment	yes	pre-baseline	treatment	altered or improved
C	Healthy volunteers	no	not disrupted	preventive	altered

Time sequence of events and three models of study designs determining three different degrees of dysbiosis correction by probiotics.
272x231mm (96 x 96 DPI)



Flow-chart of literature review results (1985-2013) of included and excluded studies for the restoration or improvement of normal microbiota by probiotics. Abbreviations: RCT, randomized-controlled trials; MOA, mechanism of action; NM, normal microbiota.
101x90mm (300 x 300 DPI)

PRISMA 2009 Checklist

Section/topic	#	Checklist item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both. **This is a systematic review**	1
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	2
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known. **Probiotics are promising candidates to prevent or treat disease, but are typically supported by structure/function claims in most countries. The function claim for the restoration of normal microbiota is commonly cited in efficacy trials, but the evidence for this claim has not been examined systematically for all probiotic strains. Differences in study populations and study design effect the type of conclusions that can be drawn. This is the first systematic review and proof of principle of this type of analysis for the function claim of dysbiosis.**	4-5
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS). **A comprehensive literature review of the evidence from randomized controlled trials will discuss the strength of the evidence for the restoration or improvement of dysbiosis by specific probiotic strain. The interventions include probiotic or control (typically placebo) given for a specific time enrolled in clinical trials for either the prevention or treatment of disease. All trials which stated some impact on the normal microbiota will be reviewed and analyzed for the ability to document changes in the normal microbiota. The outcomes are the degree of dysbiosis restoration depending upon the study design and type of enrolled participants**.	5
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number. **Review protocol is described in Methods section of paper.** **Prospero registration number is: CRD42014007224**	5-9
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale. **Participants: without restriction, any enrolled in clinical trial (adults and pediatrics)**	6

PRISMA 2009 Checklist

		Interventions: all probiotics **Comparisons: controlled (typically placebo)** **Outcomes: microbiologic assays of the intestinal flora or microbiota** **Study design: required to have pre-intervention (baseline) and post-intervention microbiological assays** **Length of follow-up: unrestricted** **Language: unrestricted** **Publication and years considered: peer-reviewed publications from PubMed (1985-2013, unless otherwise noted), EMBASE, Cochrane Database (1990-2013), CINAHL, AMED, ISI Web of Science (2000-2013). On-line trial registries: (Cochrane Central Register of Controlled trials, MetaRegister of Controlled Trials, and National Institutes of Health.**	
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched. **See item above**	6
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated. **All probiotics + health claims, structure/function claims, normal microbiota, normal intestinal flora, dysbiosis, pharmacokinetics, metagenomics, dietary supplements, randomized controlled trials, antibiotic-associated diarrhea, Clostridium difficile infections, H. pylori treatments, inflammatory bowel disease, irritable bowel disease, travelers diarrhea, bacterial vaginosis or vaginitis, treatment of pediatric acute diarrhea, healthy volunteer trials and specific probiotic strains. (PubMed)**	5-6
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis). **Screening:** **Eligibility: Must have at least one pre-intervention (baseline) microbiological assay of normal flora or metagenomic analysis and one post-intervention (post-probiotic) assay. Genus and species of probiotic strain(s) provided. Normal microbiota assayed during a randomized, controlled trial.** **Excluded: pre-clinical studies, safety studies, reviews, mechanism of action studies, case reports or case series, duplicate reports, unspecified type of probiotics.**	6-7
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators. **Pilot data extraction form used modified from standard meta-analysis data extraction form (McFarland and Goh 2013, World J Gastroenterol). Questionable results were queried from original authors of papers.**	6
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made. **The timing and type of microbiologic assays of intestinal or vaginal microbiota were collected. As the literature review has a length inclusion period (1985-2013), the types of microbiologic assays have evolved, but all types were included from basic microbiologic assays to metagenomic profiling. The type of probiotic intervention was collected by genus, species and strain (if stated in paper). Types of normal microbiota assays varied by technique. The patient population (healthy volunteers, acute disease or chronic disease) was also collected. Also collected: study size, type of disruptive factor, follow-up duration, stated claims in**	7-8

PRISMA 2009 Checklist

		paper.	
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis. **Quality of study design (restoration, improvement or no dysbiosis) was used when assessing quality of individual studies. These were then analyzed by stratification on the quality of study design.**	8
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means). **NA, No pooled RR or DWMs used in this systematic review.**	na
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis. **NA, No pooled RR or DWMs used in this systematic review.**	na

Section/topic	#	Checklist item	Reported on page #
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies). **All studies with microbiota were included to limit bias, but no measurement for publication bias was done for this systematic review.**	8
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified. **association of degree of dysbiosis correction with clinical efficacy by probiotic strain(s)**	8-9
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram. **See flow diagram, Figure 2.**	25
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations. **Extracted data were cited in tables. See paper-Tables 1-4.**	26-32
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12). **Presented in Discussion section.**	11-15
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot. **Cited in Tables 1-4.**	26-32
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency. **NA, this is a systematic review, not a meta-analysis.**	na
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see item 15).	18-19

PRISMA 2009 Checklist

		Presented in Discussion section (bias due to study design).	
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	15-17
DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers). **Strength of the evidence is provided in the Results section.** **Relevance to key groups is in the Discussion section.**	19, 22
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias). **Provided in Discussion section and "Opportunities for Future Research" section and 'Strengths and Weaknesses" section.**	21-22
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research. **The weight of the evidence for the function claim that probiotics can improve or restore normal microbiota is strong for a few probiotic strains, but in general, more confirmatory studies that are properly timed and designed are required for the majority of probiotic strains.**	20-21
FUNDING			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review. **This review was unfunded.**	23

From: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLoS Med 6(6): e1000097. doi:10.1371/journal.pmed1000097

For more information, visit: www.prisma-statement.org.

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Use of probiotics to correct dysbiosis of normal microbiota following disease or disruptive events: a systematic review.

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Use of probiotics to correct dysbiosis of normal microbiota following disease or disruptive events: a systematic review.

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ABSTRACT

Objective: To assess the evidence for the claim probiotics can correct dysbiosis of the normal microbiota resulting from disease or disruptive events.

Setting: Systematic review of published clinical trials of patients receiving a probiotic intervention for the prevention or treatment of various diseases.

Data sources: Sources searched (1985-2013): PubMed, EMBASE, Cochrane Database of Systematic Reviews, CINAHL, AMED, and ISI Web of Science. Three on-line clinical trial registries were searched: Cochrane Central Register of Controlled trials, MetaRegister of Controlled Trials, and National Institutes of Health.

Review methods: Included studies were randomized clinical trials of probiotic interventions having microbiologic assays. Studies were evaluated following PRISMA guidelines for specific probiotic strains. A standard data extraction form was used to collect the raw data.

Outcome measures: The primary outcome is the degree of microbiota correction by specific probiotic strains. Secondary outcome was the association between the degree of dysbiosis correction and clinical efficacy.

Results: The review of the literature found three distinct study designs: Model A (restoration) assayed patients enrolled with a healthy, undisturbed microbiota and then assayed post-disruptive event and probiotic therapy; Model B (alteration) assayed patients with pre-existing disrupted microbiota and then post-probiotic therapy; Model C (no dysbiosis) assayed volunteers with no disruptive event pre and post-probiotic. From a total of 63 trials, 83% of the probiotic products using Model A restored the microbiota, 56% using Model B improved the microbiota and only 21% using Model C had any effect on microbiota. Clinical efficacy was more commonly associated with strains capable of restoration of the normal microbiota.

Conclusions: The ability to assess the degree of dysbiosis improvement is dependent upon the enrolled population and the timing of microbiologic assays. The functional claim for correcting dysbiosis is poorly supported for most probiotic strains and requires further research.

Systematic review registration: PROSPERO (CRD42014007224)

Strengths and Limitations

Strengths include:

- A comprehensive review of the published literature from 1985-2013
- Literature search unrestricted by language or country
- Analysis of study designs resulted in novel strategy to limit bias and classify outcomes
- Three types of outcomes of dysbiosis applied to evidence-based studies of specific probiotic strains
- Author has over 40 years of research experience in the probiotic field

Limitations include:

- Pooled clinical trials using different study populations
- Pooled probiotic doses and regimens
- Indirect evidence linking probiotic strains and dysbiosis
- Review done by sole author

INTRODUCTION

The popularity of probiotics has expanded exponentially recently, but along with their increased use, debate rages on how probiotics should be regulated and whether probiotics should be considered as a medical food, drug or a food supplement. In the U.S., probiotics are typically available as dietary supplements and thus are limited to 'structure or function' health claims and, unlike prescription drugs, are not permitted to claim to 'treat' or 'cure' disease. In Europe and the United Kingdom, probiotics are allowed to have health or function claims. These claims are required to be supported by well-conducted human trials in the targeted population or in healthy volunteers, but the European Food Safety Authority (EFSA) has rejected >80% of claims submitted to them. [1-3] In many cases, scientific substantiation of a specific health claim was judged insufficient or based on an indirect effect.[4] One such functional claim made for probiotic products is they correct dysbiosis (or the disruption of bacterial and fungal species after antibiotics or other disruptive exposures) and thus may be beneficial to maintain health. Probiotics are active during this susceptible window from the time of the disruptive event to the time when normal microbiota is restored. A wide variety of mechanisms-of-action have been documented for probiotics (ranging from blocking pathogen attachment sites, destruction of the pathogen by bacteriocins or proteases that degrade toxins, to regulation of the immune system),[5,6] and while clinical evidence supports efficacy of some probiotic strains, the evidence linking these mechanisms-of-action to a specific health or function claims is not as clear.

A classic example of the consequence of dysbiosis is antibiotic-associated diarrhea (AAD).[7,8] While antibiotics may be effective in the elimination of pathogenic organisms, a common, unintended effect is the killing or inhibition of beneficial microbes due to shared susceptibility to the antibiotic. One of the many functions for normal microbiota is the ability to resist infection by pathogenic organisms, termed 'colonization resistance'.[9,10] The loss of a sub-population of the normal microbiota, for example, can lead to the loss of the ability to break down fibers and starches into absorbable short chain fatty acids, resulting in high level of undigested carbohydrates, which can trigger diarrhea.[11] Disruption of the normal microbiota has been shown to lead to higher rates of infections in other body systems other than the intestinal tract including the skin,[12,13] vagina,[14,15] respiratory tract,[16,17] and in the buccal cavity.[18-20]

The major challenge to establishing a cause and effect for the improvement of dysbiosis by probiotics is a lack of a standard definition of 'normal' microbiota. There is substantial inter-individual variation of the species of microbes present at different body niches, which also varies by age, geographic area and health status of the host. In addition, a complete accounting of the microbiota is currently impossible, as there are no assays to detect all of $>10^{13}$-10^{14} organisms in the intestines and standard microbial culturing methods miss 75-95% of these organisms. [21,22] The development of metagenomics (cataloguing individual and disease-specific bacterial gene profiles) and the creation of the international Human Microbiome Project ushered in a new era for our understanding of the complexity of these interactions within the body.[23,24] This paradigm shift from culturing to metagenomic analysis has expanded our ability to document shifts in microbial populations to an unparalleled degree, but the interpretation of these shifts continues to be under debate.[25-28] With the advent of these newer metagenomic tools, the role of probiotics in the restoration of normal microbiota is being re-visited.[29]

In light of new guidance documents and recommendations, the goal of this systematic review is to determine how claims for the restoration of the normal microbiota and the correction of dysbiosis have been studied using well-designed trials and which probiotic strains have evidence-based data to support these claims.

METHODS

Study Objective

To systematically review the literature to analyse the evidence for the claim probiotics can correct dysbiosis of the normal microbiota from randomised controlled trials.

Search Strategy

Search terms included: probiotics + health claims, restoring normal microbiota, dysbiosis, normal microbiota, pharmacokinetics, metagenomics, probiotics, dietary supplements,

randomized controlled trials, antibiotic associated diarrhea (AAD), *Clostridium difficile* infection (CDI), inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), traveler's diarrhea (TD), eradication of *Heliobacter pylori,* bacterial vaginosis or vaginitis, treatment of acute pediatric diarrhea, and specific probiotic strains or products. Search strategies were broad-based initially, then narrowed to clinical trials with probiotics.

Data Sources

PubMed (1985-2013), EMBASE (1985-2013), Cochrane Database of Systematic Reviews (1990-2013), CINAHL (1985-2013), AMED (1985-2013), and ISI Web of Science (2000-2013). Three on-line clinical trial registries were searched: Cochrane Central Register of Controlled trials (http://www.cochrane.org), MetaRegister of Controlled Trials (http:www.controlled-trials.com/mrct) and National Institutes of Health (http://www.clinicaltrials.gov).

Criteria for study selection and data extraction

Abstracts of all citations were reviewed by a single author and rated for inclusion for randomized controlled trials of probiotic treatments. Full articles were retrieved if normal microbiota assays were mentioned. Non-English language trials were translated and included whenever possible. Exclusion criteria included pre-clinical studies (animal models or *in vitro* assays), safety or phase 2 studies, reviews, efficacy trials with no assays for normal microbiota species, metagenomic methods only, mechanism of action of normal microbiota or probiotic, cross-sectional surveys, case reports or case series, duplicate reports, or trials of unspecified types of probiotics. All pharmacokinetic studies in humans were reviewed, as abstracts often did not include normal microbiota assay data. Data extraction and the review process followed the PRISMA statement guidelines using a 27-item checklist and flow diagram.[30] A standardized data extraction form was used to collect data on the probiotic (strain type, daily dose, duration), type of controls (placebo, active or no treatment), study design (status of microbiota at baseline and follow-up times), type of microbiota assay (microbial culturing, molecular biomarkers, etc.), enrolled study population (adult vs. pediatric, healthy volunteers, disease condition), type and timing of disruptive agent (antibiotics, chemotherapy, etc.), study size and attrition, outcome assessment (efficacy and/or microbiota status at end of study, adverse events) and type of health claim.

Outcomes and definitions

The primary outcome is the degree of microbiota correction or improvement by specific probiotic strain(s). The secondary outcome is the association between the degree of dysbiosis correction and the net efficacy found from randomized controlled trials of probiotic interventions. Dysbiosis is defined as an alteration or disruption of the normal microbiota (bacterial or fungal species) due to exposure of an disruptive factor (such as antibiotics, chronic disease, stress, medical procedures or medications, etc.). As there is no current standard definition of 'normal' microbiota, for this review, restoration of normal microbiota is defined as a return to the assayed microbial species or profile taken from a healthy individual (before a disruptive event has occurred). Included studies are required to have at least a pre-probiotic treatment assay and a post-probiotic treatment assay. A variety of microbial assays were available during the search period (1985-2013), including documentation of the microbiota by either microbial cultures, or metagenomic methods [16s rRNA-targeted probes using fluorescent *in situ* hybridization (FISH) or other polymerase chain reaction (PCR) technique][8,21,28,31] or by indirect methods (Nugent scores).[15] Nugent scores (ranged 0-10) are used to diagnose bacterial vaginosis (scores ≥7) or normal vaginal microbiota (scores 0-3) based on the quantitated morphotypes of small gram negative rods (*G. vaginalis/Bacteroides* spp.) and curved gram negative rods (*Mobiluncus* spp.) from gram stains of vaginal discharge smears. Microbial assays of only the strain(s) contained in the probiotic product are considered as pharmacokinetic studies and were not included in the normal microbiota profiles.

Models of dysbiosis. To determine the impact on normal microbiota, only direct evidence of microbiota change (species, profiles, diversity indices, or diagnostic criteria) were included and indirect effects were excluded (changes in intestinal enzymes, immune system parameters or disease symptoms). The degree to which dysbiosis was improved is categorized into three levels: (1) recovery of the normal microbiota back to baseline levels; (2) alteration or improvement of the normal microbiota; and (3) no change in normal microbiota.
The literature contained three dysbiosis models: Model A (restoration of the normal microbiota), which assayed patients enrolled with a healthy, undisturbed microbiota and then assayed again after a disruptive event (such as antibiotic exposure) and probiotic therapy occurred; Model B (alteration of the microbiota) assayed patients with pre-existing disrupted microbiota (for

example, pre-existing chronic disease or active disease) and then post-probiotic therapy; Model C (no dysbiosis) assayed volunteers with no disruptive event (before or during the clinical trial) at both pre-probiotic and post-probiotic times, as shown in Figure 1. 'Recovery' of the normal microbiota is defined as a restoration of the microbiota back to a normal healthy baseline. Recovery may be complete recovery (all assayed microbial levels returned to baseline) or incomplete recovery (partial recovery of some microbial strains, but not all returned to baseline levels). In studies enrolling subjects with dysbiosis at baseline (typically due to chronic diseases), it is not possible to show a restoration to normal microbiota levels because a normal, undisturbed microbiota was not present in these types of study subjects at the time of enrollment. Therefore, the strongest claim possible for Model B designs is for an 'alteration or improvement' of the microbiota. Only data from the probiotic-exposed subjects were analysed in this paper. Data from the control groups were used to confirm dysbiosis for subjects with chronic diseases or after a disruptive exposure, such as antibiotics or chemotherapy, unaffected by probiotic exposure.[32-34]

Assessment of methodological strength and quality

The GRADE (Grading of Recommendations, Assessment, Development and Evaluation) system for rating overall study quality will be used for each probiotic strain or type (single strains and mixtures of strains).[35] Recommendation for the support of the claim of each probiotic strain or mixture can be assessed by the overall strength of the evidence [“strong”, many randomized controlled trials show significant recovery of the microbiota, or "moderate" only one randomized controlled trial; or “weak”, only case series or reports, limited number of small trials, *etc.*].

Quality of the evidence is based on study design and graded as “high quality” (well-defined study design for determining restoration with normal microbiota, Model A), or “moderate quality” (disrupted microbiota at baseline, Model B), or “low quality” (no disruptive event occurred, Model C). Measurement of publication bias was not assessed for this review, as pooled outcome estimates of efficacy were not done, as typical in meta-analysis, but all studies with assays of microbiota were included to limit bias.

Net efficacy rating

To determine if the ability to correct dysbiosis is associated with clinical efficacy, the published literature for randomized controlled trials (RCTs) or meta-analyses of probiotics for various disease indications, including antibiotic associated diarrhea (AAD),[5,36,37] *Clostridium difficile* infection (CDI), [5,38] inflammatory bowel disease (IBD),[39] irritable bowel syndrome (IBS), [40] traveler's diarrhea (TD), [41] eradication of *Heliobacter pylori* (Hp),[36,37] bacterial vaginosis (BV) or vaginitis,[42] and treatment of acute pediatric diarrhea was reviewed. [43-45] The net rank was calculated by subtracting the number of RCTs showing non-significant or equivalent efficacy from the number of RCTs having significant efficacies. The ranks were categorized as follows: ++, ≥2 net RCTs showing significant efficacy; +, net of one RCT showing significant efficacy; 0, equal number of RCTs showing significant and non-significant efficacy results and -, ≥1 net negative or non-significant RCTs. Probiotics with no RCTs were not ranked.

RESULTS

A review of the literature from 1985-2013 found 353 articles that dealt with probiotic treatments and their potential effect on normal microbiota.

Excluded studies

As shown in Figure 2, a total of 272 articles were excluded for the following reasons: reviews (n=116), probiotic efficacy studies with no data on normal microbiota assays (n=54), animal models of probiotics and changes in microbiota (n=38), metagenomic or microbiota methods only (n=17), studies on normal microbiota but with no use of probiotics (n=14), *in vitro* assays of microbiota (n=10), duplicative reports (n=2) or miscellaneous (n=21), which included probiotic mechanism of action studies, safety studies, duplicative reports, cross-sectional surveys and two with poorly described probiotic interventions.[46,47] A total of 81 full articles were reviewed which mentioned changes in normal microbiota or indicated a health claim for probiotics and effects on normal microbiota.

Probiotic pharmacokinetic studies (n=18) reporting concentrations of probiotic strains before and post-treatment, but did not assaying for other species of normal microbiota were excluded. While several studies using this study design claim probiotics had an impact on normal

microbiota, type of data generated is pharmacokinetic behavior of the probiotics themselves and not the normal microbiota. Several studies stated that the normal microbiota was altered because an increase in various bacterial species was observed after the probiotics were given, but the species assayed were those contained in the probiotic product, so an increase is not unexpected. Pharmacokinetic studies have documented that probiotic strains taken orally can survive transit through the intestinal tract with recovery rates in feces ranging from <1% to 22%.[48,49] These pharmacokinetic studies were excluded from this analysis, as they did not assay other types of normal microbiota not found in the probiotic product.

Included studies

Of the 63 included clinical trials, five trials had multiple treatment arms, which resulted in a total of 68 treatment arms for analysis. Engelbrektson et al. tested a mixture of 5 probiotic strains in volunteers exposed to antibiotics and also tested a mixture of 4 probiotic strains in healthy volunteers with no antibiotic exposure.[50] Zoppi et al. had eight different treatment arms in his study, and probiotic arms were included in our analysis [*Saccharomyces boulardii* (*S. boulardii*) alone and *Lactobacillus (L.) rhamnosus GG* alone], a mixture of two probiotics (*L. acidophilus* and *Bifido. bifidum*) and a mixture of three probiotic strains (*L. acidophilus, L. rhamnosus* and *Bifido. bifidum*).[51] Orrhage et al. had two treatment arms (*Bifido. longum* alone and a mixture of *Bifido. longum* and *L. acidophilus*).[52] Larsen et al. tested two single probiotics (*Bifido. lactis* and *L. acidophilus*) in separate treatment arms.[53] Lidbeck et al. gave either enoxacin or clindamycin and randomized patients to either *L. acidophilus* or placebo. [54]

Normal microbiota assay methods. Of the 69 treatment arms that did normal microbiota assays, diverse methods were used to profile the microbiota. Many studies used only standard microbiological culture assays (37, 54%), while others (28, 40%) used techniques to detect non-cultivatable bacterial strains, which included metagenomic assays (FISH, TRFLP, 16s rRNA sequencing) or other PCR techniques. Some studies (4, 6%) used an indirect measure of normal microbiota, using the Nugent score to diagnose bacterial vaginosis, which relies upon gram stain of the vaginal secretions, vaginal pH and symptoms to characterize if normal microbiota is present or absent.[15]

Probiotic strains. In the 69 treatment arms, most (36, 52%) used a single strain of probiotic, while 14 (20%) tested a mix of two probiotic strains and 19 (28%) tested a mix of three or more probiotic strains. The distribution of single versus multiple strain probiotics did not significant vary by the model of study design (X^2_2=2.3, P=0.32). Of the 15 restorative (Model A) study arms, 47% used a single strain of probiotic and 53% used multiple strains. Of the 25 treatment arms with disrupted microbiota at baseline (Model B), 44% used a single strain and 56% used multiple strains. Of the 29 study arms with undisrupted microbiota (Model C), 62% used a single strain and 38% used multiple strains.

Normal microbiota restoration model (Model A)

Only 10 studies (with 15 treatment arms) using Model A to determine restoration of the microbiota were found (Table 1).[32,34,50-52,54-58] The type of enrolled subjects varied from healthy volunteers to children with untreated respiratory infections, to pediatric cancer patients. For subjects with acute infections or cancer, baseline assays were done prior to the disrupting agent (antibiotics or chemotherapy). The number of subjects given probiotics averaged 20/study and ranged from 5 to 83. In 93%, the disruptive factor was antibiotic exposure and in one study, chemotherapy caused the microbiota disruption. Only 8 (53%) of the study arms did an assay during a 1-8 week follow-up period after the probiotic was discontinued.

Analysis of the probiotic strain(s) separately found only two probiotic products with more than one randomized controlled trial. The probiotic mix of *L. acidophilus* and *Bifido. bifidum* showed a complete restoration in one study, but only a partial recovery in the other. (Strength: strong, Quality: high). The probiotic mix of *L. acidophilus* (2 strains) with *Bifido. bifidum* and *Bifido. animalis* showed complete restoration in one study, but only a partial recovery in the other. (Strength: strong, Quality: high). Five other probiotic products with only one supporting clinical trial showed microbiota restoration (*Bifido. longum*, *Clostridium butyricum*, *L. acidophilus*, mix of *L. acidophilus* with *L. paracasei* and *Bifido. lactis*, and the mix of *L. acidophilus* with *L. paracasei* and *Bifido. bifidum* and two strains of *Bifido. lactis*). (Strength: moderate, Quality:

high). Three probiotic products with one supporting clinical trial showed partial restoration (*S. boulardii*, *L. rhamnosus* GG, mix of *L. rhamnosus* with *L. bifidus* and *L. acidophilus*), (Strength: moderate, Quality: high). Only two probiotic products using Model A showed no change in the microbiota (*Bifido. breve* and a mix of *L. acidophilus* and *Bifido. longum*). (Strength: moderate, Quality: high). In summary, 10 of 12 (83%) of the probiotic products showed complete or partial restoration of the normal microbiota.

Of the 11 probiotic products with claims of 'restores or improves normal microbiota', 10 (91%) were supported by this review, but only seven showed complete restoration and five had partial restoration of the microbiota (Table 1). The mixture of *L. acidophilus* and *Bifido. longum* did not show any changes in the microbiota. Wada et al. claimed *Bifido. breve* 'enhanced intestinal anaerobes', but this was only compared to the placebo group.[32] Their data showed chemotherapy is a disruptive event, resulting in more Enterobacteria in the intestine in the placebo group, but there were no significant differences seen by the end of the 8 week follow-up in either the probiotic or the placebo group compared to baseline microbiota levels.

Disrupted normal microbiota at baseline studies (Model B)

Twenty-four studies (with 25 treatment arms) used Model B that enrolled subjects with a pre-existing disrupted microbiota related to ongoing disease or conditions (Table 2).[33,53,59-80] The number of subjects given probiotics averaged 23 $\pm$ 16/study and ranged from 7-83 participants. The types of pre-existing factors that disrupted the microbiota included atopic dermatitis patients, allergies, cirrhosis, bacterial vaginosis, irritable bowel syndrome, inflammatory bowel disease (ulcerative colitis and pouchitis), idiopathic diarrhea, enteral feeding, short-bowel syndrome and colon cancer. Only 10 (40%) of the study arms did an assay during the post-probiotic follow-up period.

Three of the probiotics had multiple clinical trials to support the claim of an improvement in the microbiota due to the probiotic. *S. boulardii* was used in two trials either with enteral fed

patients or patients with active diarrhea and found an improvement in the habitual microbiota in the patients with active diarrhea[66], but only showed indirect evidence of short-chain fatty acid changes in the other study.[65] (Strength: strong, Quality: moderate) A mix of four probiotic strains (2 strains of *L. rhamnosus*, *P. freudenreichii* + *Bifido. breve*) showed improved microbiota in two clinical trials.[74,75] (Strength: strong, Quality: moderate) Of four clinical trials testing a mixture of seven probiotic strains, two showed no significant change in microbiota [77,78], one showed more anaerobes post-probiotic treatment[79] and one found a reduction in Bacteroides species.[80] (Strength: strong, Quality: moderate) Three clinical trials determined there were no significant changes due to *L. plantarum* 299v.[62-64] (Strength: strong, Quality: moderate). Of those probiotics with only one supporting clinical trial (Strength: moderate, Quality: moderate), two single probiotic strains (*E. coli* Nissle and *L. casei rhamnosus*) and five different mixtures of probiotic strains support the claim that the probiotic alters the microbiota (Table 2). In summary, 10 of 18 (56%) probiotic products altered or improved microbiota in individuals with pre-existing disease.

Of the 25 treatment arms, the paper's claim was confirmed in 14 (56%) of the studies. There was no significant change in the microbiota due to the probiotic in nine treatment arms and only an alteration of the microbiota in five others (Table 2). Our review disagreed with the claimed outcomes in 11 (46%) of the other treatment arms. In seven treatment arms, it was claimed the tested probiotic 'restored normal microbiota', but it is uncertain how this conclusion was reached, since there was no time when a normal undisrupted microbiota was present. Of the seven studies that claimed their probiotic 'restored' normal microbiota, our analysis determined none were capable of documenting restoration, but it is confirmed probiotics improved or altered the microbiota in these studies. Four studies claimed the probiotic 'altered or improved' normal microbiota, but this review found no significant differences when post-probiotic and baseline assays were compared for the probiotic groups. Girard-Pipau et al. concluded that *S. boulardii* 'altered normal flora' because more gram positive anaerobes were seen in the probiotic group compared to the controls and an increase in three short-chain fatty acids were observed in the *S. boulardii* group.[65] However, when the analysis is restricted to trends observed in the probiotic group only, no significant differences were observed in pre-probiotic versus post-probiotic

microbiota profiles. Venturi et al. concluded that the mix of seven probiotic strains enhanced the concentration of some beneficial strains in the intestines. [77] However, the only strains having a significant increase were those contained in the probiotic mix, and not specifically normal microbiota of the host. As this study did not have an undisturbed microbiota baseline, the increased numbers of Lactobacilli and Bifidobacteria may not have reflected their normal levels. Van der Aa et al. claimed that *Bifido. breve* 'successfully modulates the intestinal flora', but no significant changes were observed in the probiotic group when comparing the baseline to the post-probiotic levels. [59] Odamaki et al. did show an increase in Faecalibacterium ssp. and *Bacteroides fragilis* ssp. at the end of *Bifido. longum* BB536 treatment, but the same increase was also observed in the placebo group.[33]

Undisrupted normal microbiota studies (Model C)

Twenty nine trials enrolled healthy adults who had no disruptive factor present during the study (either no antibiotic or no medication exposure or presence of acute or chronic disease) that might impact normal microbiota, as shown in Table 3.[14,49,50,81-106] The average number of subjects given probiotics was 23/study and ranged from 7 to 160/study. Of the 29 study arms, assays were taken during a follow-up period in only 52%. Fujiwara et al. cultured seven healthy volunteers and found Enterobacteriaceae and Clostridial species post-*Bifido. longum* was reduced by 10^1/g compared to baseline ($P<0.03$), but no other changes in the microbiota were detected.[84] Karlsson et al. found a significant increase in intestinal diversity in nine male volunteers with atherosclerosis given *L. plantarum* 299v, but because terminal restriction fragment length polymorphism assays were used instead of cultures for bacterial species, the specific changes in the microbiota species could not be determined.[94] Yang and Sheu cultured 63 children (55% with *H. pylori*) given a yogurt with *L. acidophilus* and *Bifido. lactis* but only found a decrease in *E. coli* counts in the *H.pylori* negative children sub-group, no significant changes in normal microbiota was found in the *H. pylori* positive children.[100] Kubota et al. assayed 29 subjects with Japanese cedar pollen allergy and found milk fermented with *L. rhamnosus* GG and *L. gasseri* TMC0356 suppressed microbiota changes (less intestinal profile changes), but could not determine specific bacterial species changes due to the type of assay used

(FISH and TRFLP).[103] In summary, only 4 of 19 (21%) probiotic products altered microbiota in healthy individuals who had no disruptive event.

Of the seven studies that claimed their probiotic(s) 'restored or altered' the normal microbiota, only four claims were confirmed. Sierra et al. claimed *L. salivarius* given to 20 healthy adults 'improved gut microbiota', but only increased levels of Lactobacilli were found and no other changes in normal microbiota species were detected. The only other evidence was indirect from changes observed in immune parameters.[96] He et al. claimed a mixture of *Bifido. longum* and *Bifido. animalis* 'modified' microbiota, but changes were seen only during the yogurt administration and not after the one week follow-up period.[99] Vitali et al. claimed that the mixture of four Lactobacilli strains and three Bifidobacteria strains 'modulated vaginal microbiota', but the only significant changes were due to an increase in the bacterial species contained in the probiotic mixture.[14]

Of the probiotics supported by multiple clinical trials (*Bifido. animalis*, *Bifido. longum*, *L. casei*, *L. plantarum* 299v, the mixture of *Bifido. animalis and Bifido. lactis*), 13 of the trials (87%) support there is no significant change in normal microbiota if the microbiota is not disrupted. [Strength: strong, Quality: low)

Association of clinical efficacy and normal microbiota restoration

Few studies concurrently compared clinical efficacy and the ability to restore or improve normal microbiota after dysbiosis. A synthesis of the literature of RCT for eight common disease indications was performed and the overall net strength was ranked. Probiotics with the ability to restore normal microbiota were frequently supported by RCTs for efficacy, as shown in Table 4. Of the 10 probiotics with evidence for restoration, 7 (70%) also had at least one RCT testing for at least one of the eight diseases, while 30% did not have any supportive RCTs for efficacy. Of the 7 probiotics with associated RCTs, only two probiotics (*S. boulardii* and *L. acidophilus*) have strong evidence for efficacy across most of the disease indications, while five probiotics with the

ability to restore the microbiota had weak or no evidence of efficacy. For example, *S. boulardii*, which has studies supporting restoration, has strong evidence for clinical efficacy for AAD (ranked ++: 11 RCTs had significant results and 6 had non-significant results), CDI (ranked ++: had two RCTs with significant results), IBD (ranked ++: had two RCTs with significant results), IBS (ranked 0: had one RCT with significant efficacy and one RCT with non-significant results), TD (ranked +: 3 RCTs with significant efficacy and 2 with non-significant efficacy), *H. pylori* eradication (ranked -: 2 RCTs with significant results and 4 with non-significant results) and no studies for BV. *L. acidophilus*, which partially restored the microbiota in a study, is associated with clinical efficacy for AAD, IBS and BV, but not for TD or eradication of *H. pylori* and treatment of acute pediatric diarrhea (ranked ++: had 19 RCTs with significant protection and five with non-significant results). In contrast, *L. rhamnosus GG,* another probiotic capable of restoring microbiota, is often cited in meta-analysis as having significant efficacy for AAD. Our results of an updated review of the literature indicate a net weak evidence rating for clinical efficacy across all disease indications: AAD (ranked -: 3 RCTs had significant results and 6 had non-significant results), CDI (ranked -: two RCTs with non-significant results), IBD (ranked -: one RCT with non-significant results), IBS (ranked 0: 2 RCTs with significant efficacy and two RCTs with non-significant results), TD (ranked 0: one RCT with significant efficacy and one with non-significant efficacy), *H. pylori* eradication (ranked -: 3 RCTs with non-significant results), no RCTs for BV and treatment of acute pediatric diarrhea (ranked ++: 10 RCTs with significant efficacy and one with non-significant findings).

Efficacy trials were not done as frequently for probiotics shown to only have the ability to alter or improve, but not restore, the microbiota after dysbiosis. Of nine probiotics that can alter the microbiota, 6 (67%) have supporting RCTs for at least one disease, but the diversity of investigated diseases was more limited. *L. casei* had moderate net strength for AAD and bacterial vaginosis, but was neutral for the ability to eradicate *H. pylori* and other disease indications were not tested in RCTs with *L. casei*. The probiotic mixture of *L. reuteri* and *L. fermentum* has strong evidence for bacterial vaginosis, but not for any other disease indications listed in Table 4.

Of the eight probiotics not capable of altering or restoring normal microbiota, only *L. plantarum* 299v had RCTs for AAD and IBS, both with net negative or weak strength of clinical efficacy. *Bifido. lactis* and the mixture of *L. rhamnosus* and *L. reuteri* had net neutral rankings for efficacy for the treatment of acute pediatric diarrhea. The other four probiotic products with no effect on normal microbiota lacked any RCTs for clinical efficacy. Studies with *B. clausii* did not assay for normal microbiota and had non-significant trial results for *H. pylori* eradication and the treatment of pediatric diarrhea.

Of the six probiotics with only pharmacokinetic data on the probiotic itself and no other investigation of other normal microbiota strains, five had RCTs showing varying net efficacies for different disease indications, as shown in Table 4.

Six popular probiotics (*Bacillus clausii*, *Bifido. infantis*, *L. reuteri*, *L. acidophilus* + *L. helveticus*, *L. acidophilus* + *L. casei* and *L. acidophilus* + *Bifido. animalis*) have only clinical efficacy RCTs, but have not published studies investigating their role in restoring or improving the normal microbiota.

Discussion

Developing and evaluating health or function claims for probiotics is an important issue and is now identified as a priority for research by several international organizations, including the World Gastroenterology Organization [107] and the American Society for Nutrition.[2] The U.S. Food and Drug Administration has struggled with appropriate evidence-based health claims for probiotic products and currently recommends the use of structure/function claims, such as "maintains bowel regularity", but the claim for restoring normal microbiota is still under debate.[108] The European Food Safety Authority (EFSA) provides guidance materials that recommend health or function claims for probiotics should have beneficial physiological effects and have appropriate scientific trials to substantiate the health claims.[3] Acceptable claims for intestinal health may include functional claims (improved transit time, softer stool consistency, reduction in gastrointestinal discomfort, defense against pathogens). As it is currently not possible to define a standard normal microbiota profile, the EFSA recommends functional claims

for the restoration of normal microbiota should document a recovery of healthy microbiota and be accompanied by a beneficial physiological or clinical outcome.[3] In addition, because the efficacy and mechanisms are strain-specific and may vary by probiotic strain, the evidence must be analyzed for each probiotic product individually.[5,6,9,109, 110-112]

An underappreciated finding was the influence that study design and study populations have on the interpretation of study outcomes. In the literature, five different types of study designs are commonly found relating to probiotics. The most common study type is a randomized controlled trial testing the efficacy and safety outcomes in patients, but these trials did not typically document the impact of the probiotic on the normal microbiota. The second most common type of study design is pharmacokinetic studies (documenting recovery of oral dose of probiotic or increase in probiotic strains post-treatment compared to pre-treatment or clearance of the probiotic). Even though these kinetic studies did not assay for non-probiotic strains, some extrapolated their results and concluded some effect or improvement of the normal microbiota was observed by their probiotic.[19,111] These two first types of study designs do not support evidence-based conclusions for the restoration or alteration of the normal microbiota and were excluded from this review.

Three types of study designs are appropriate for the study of dysbiosis. The first type of study design had normal microbiota assayed at least twice (at baseline, which was before exposure to a disruptive event or probiotics and then again during or post-probiotic treatment) to show actual recovery of assayed normal microbiota back to healthy baseline levels. The second type of study design started with inappropriate baselines (baseline samples taken after normal microbiota had been disrupted by chronic disease). For patients with established chronic diseases, there is no "normal microbiota" baseline in either the probiotic or the control group. Even if baselines are taken during remission, the microbiota may still be impacted by chronic disease or acute diarrhea. Studies of probiotics in chronic diseases or acute disease typically report on 'pre-probiotic treatment' and 'post-probiotic treatment' and may show significant shifts in microbial species, but it is uncertain if this reflects a true re-establishment of normal microbiota profiles. The third type of study design enrolled healthy volunteers, who were not challenged with antibiotics (so no normal microbiota disruption occurred), and show only the effect of probiotics on a healthy microbiota (typically mild or no effects). Control groups were not required for our

assessment of the impact of probiotics on microbiota, but control groups can document the degree normal microbiota is disrupted by inciting agents (antibiotic, disease onset, etc.).

Five single strain probiotics (*Bifido. longum*, *Clost. butyricum*, *L. acidophilus, L. rhamnosus* and *S. boulardii*) and five probiotic mixtures [(*L. acidophilus* + *Bifido. bifidum)*, (*L. rhamnosus* + *L. bifidus* + *L. acidophilus)*, (*L. acidophilus* + *L. paracasei* + *Bifido. lactis),(L. acidophilus*, 2 strains, *Bifido. bifidum, Bifido. animalis)* and *(L. acidophilus* + *L. paracasei* + *Bifido. bifidum* +*2 strains of Bifido. lactis*)] documented either complete or partial recovery of normal microbiota (Model A). Only two probiotic mixtures [(2 strain mixture: *L. acidophilus* + *Bifido. bifidum*) and (4 strain mixture: *L. acidophilus*, 2 strains, *Bifido. bifidum, Bifido. animalis*)] were supported by a confirmatory study. Evidence that probiotics may alter or improve normal microbiota (Model B) was found for three single strain probiotics (*E. coli* Nissle, *S. boulardii* and *L. casei rhamnosus*) and seven mixtures of 2-7 probiotic strains. Of these ten probiotics finding alteration of the microbiota, only three had multiple trials [*S. boulardii*, and a four strain mixture (2 strains of *L. rhamnosus* + *P. freudenreichii* + *Bifido. breve*), and a seven strain mixture (4 Lactobacilli and 3 Bifidobacteria strains)], but only one had consistent results showing improvements in the microbiota. [74,75] Clearly, more than one study is needed to confirm the impact of a probiotic on the normal microbiota. Of the 19 probiotic strains (or mixtures) studied in healthy volunteers who were not exposed to disruptive factors (Model C), no change in the normal microbiota was observed for 79%, indicating the robustness of the microbiota.

Improvement in the normal microbiota by specific probiotic strains seemed to be associated with better clinical endpoints. Within eight common diseases typically treated with probiotics, more trials with significant efficacy were associated with probiotic strains shown to restore the normal microbiota, and only one trial with significant efficacy was found for probiotics that did not alter the microbiota. However, few probiotics had efficacy trials for all eight diseases and many did not have any efficacy trials.

Some probiotics which have published efficacy trials for various diseases did not have studies investigating the effect of the probiotic on normal microbiota: *Bacillus clausii*, *Bifido. infantis*, *L. brevis*, *L. reuteri*, mix of 2 strains (*L. acidophilus* + *L. helveticus*), mix of 2 strains (*L. acidophilus* + *L. casei*) or (*L. acidophilus* + *Bifido. animalis*), mix of 4 strains [*L. rhamnosus* (2 strains), *Propionibacterium freudenreichii* + *Bifido. animalis*)] and mix of 7 strains (*L. sporogens, L. bifidum, L. bulgaricus, L. thermophilus, L. acidophilus, L. casei, L. rhamnosus).*

Comparison of results with other studies

Other reviews in the literature of claims for probiotics relating to changes in the normal microbiota have focused on the broad issues of regulatory standardization of health or function claims, the use of proper study designs and the challenge of defining biomarkers for a 'healthy microbiota'.[3,29,112] Donovan et al. recommends that health claims for probiotics be supported by well-conducted human trials in the targeted population.[2] These reviews also recommend that gut biomarkers need to be correlated with clinical endpoints, however none of these reviews attempted to do so.[29,112] No prior review has attempted to analyze the association between probiotic strains and their impact on normal microbiota by stratifying on the quality of study design. [111] This review addressed these concerns by analyzing probiotic strains by the quality of the study design and only including trials that assessed the normal microbiota (either by microbial culturing or molecular strain biomarkers) and assessed the degree of dysbiosis improvement with clinical outcomes for each probiotic strain.

Opportunities for future research

Most of the studies (80%) using Model A to document restoration of the normal microbiota only used microbiologic culturing techniques, which can only detect those organisms that grow in culture. Use of the more advanced molecular metagenomic techniques have found that culturing alone misses up to 95% of these organisms.[21,22] The use of the metagenomic techniques was more common in the studies using Model B (48%) and Model C (45%) study designs, which only addresses potential alteration of the microbiota. Characterization of the microbiota is a

complex issue and a comprehensive accounting of all the bacterial and fungal strains in the body is beyond our current capabilities. Therefore, any studies of changes to the microbiota are incomplete at best, but general trends in bacterial phylotypes can be documented using DNA probes and metagenomic techniques. Differential detection bias may be present due to the variety of assays used in these studies and should be accounted for in future studies.

Another suggestion for future studies is to include an appropriate follow-up time period post-probiotic administration. Fewer than half of the reviewed trials did assays for normal microbiota during an appropriate follow-up period. As it has been shown that recovery from a disrupting factor can be prolonged (typically eight weeks),[7,8] and studies that failed to find microbiota recovery might have detected a return to normal baseline levels if a sufficiently long time was given for the recovery to have occurred. Future studies should strive to allow time for the restoration of the normal microbiota to occur.

As the effects of probiotics are strain specific, and many studies typically only report the genus and species of the tested probiotic, future reports should include a complete description of the probiotic to the stain level. [5,112]

Strengths and weaknesses

The strengths of this review included the completeness of the search strategy, which reviewed multiple citation databases, trial registries and author searches, use of established PRISMA protocols for reviews and the use of an outcome classification scheme for different degrees of assessment for microbial recovery. This analysis controlled the confounding effects of different study populations and study designs present in the literature. Pharmacokinetic studies of just the probiotic strain(s) itself were excluded and only trials that assayed other species found in the microbiota were included. By applying a standard definition for 'restoring' versus 'improving' normal microbiota, it is possible to distinguish significant differences by the type of study designs used and differential effects of the different probiotic strains. Limitations of this review

include: a single author reviewed and extracted the literature, pooling trials from different populations (adult versus pediatric) and different probiotic doses and regimens used. Incomplete retrieval of all studies assessing the effect that probiotics have on human microbiota is also a potential limitation of any literature search. Another limitation is that dysbiosis improvement and clinical efficacy for probiotic strains is also indirectly associated, no direct cause and effect relationship was possible with the types of studies done. Another limitation is the current lack of a standard definition of what comprises a 'normal microbiota'. The constituents of the microbiota vary by individual, by age, geographic location and health status of the host. Current microbiologic techniques are improving, but can not detect all species present in the host.

Conclusion

The challenges in recommending a specific probiotic to patients who need to restore or improve their normal microbiota after a disrupting event occurs is two-fold: one is the diversity of probiotic products available and second is the varying strength of evidence provided by clinical trials using different outcome measures and study designs. By grouping studies into three groups that result in three different degrees of probiotic effect (restoration, improvement or no change), an overview of the body of evidence is possible. By comparing the strength of the clinical evidence for common diseases by the degree to which the probiotics could impact the restoration of the normal microbiota, it became obvious that those probiotics with a greater ability to restore the microbiota are associated with the strongest strength of clinical efficacy. While this evidence only indirectly links clinical efficacy with the ability to restore the microbiota, the overall review of the evidence shows this is an important mechanism of action for probiotics. What becomes obvious is that more studies are required to conclude which probiotic strains have a beneficial impact on the normal microbiota, as most strains have only a single clinical trial and many probiotic products overstate the strength of their claim to restore normal microbiota. These types of issues should be considered for health care policy makers and researchers for future studies and for creating guidelines for health/function claims.

Table 1. Model A: Evidence-based data for restoration of normal microbiota (NM) for 12 probiotics from 10 studies (15 treatment arms).

Probiotic*	Reference	No. treated with probiotic	Typeof assay for NM	Enrolled population	Type of disrupting factor	Follow-up post-treatment (wks)	Claims stated in papers	Evidence-based claim
Bifido. breve	Wada 2010[32]	19	FISH	pediatric cancer patients	chemotherapy	8	enhances anaerobes	no change
Bifido. longum BB536	Orrhage 1994[52]	10	culture	healthy volunteers	clindamycin	0	restores	restores
Clost. butyricum MIYAIRI	Seki 2003[34]	83	culture	pediatric respiratory or GI infections	antibiotics	0	restores	restores
L. acidophilus NCFB1748	Lidbeck 1988[54]	5 5	culture culture	healthy volunteers volunteers	enoxacin or clindamycin	1 1	restores only in enoxacin no change	restores only in enoxacin, no change in clindamycin
L. rhamnosus GG	Zoppi 2001[51]	7	culture	pediatric respiratory infections	ceftriaxone	0	partially corrects	partially restores
S. boulardii lyo	Zoppi 2001[51]	6	culture	pediatric respiratory infections	ceftriaxone	0	improves	partially restores
L. acidophilus + *Bifido. bifidum*	Black 1991, [55] Zoppi 2001[51]	10, 7	culture, culture culture	healthy volunteers, pediatric respiratory	ampicillin, ceftriaxone	2, 0	recovers more rapidly, less change	restores, partially restores
L. acidophilus 1748 + *Bifido. longum* BB536	Orrhage 1994[52]	10	culture	healthy volunteers	clindamycin	0	no change	no change
L. rhamnosus + *L. bifidus* + *L. acidophilus*	Zoppi 2001[51]	7	culture	pediatric respiratory infections	ceftriaxone	0	partially corrects	partially restores
L. acidophilus 1748 + *L. paracasei* F19 + *Bifido lactis* Bb12	Jernberg 2005[56]	4	culture PCR TRFLP	healthy volunteers	clindamycin	2	restores	restores
L. acidophilus CUL60+ *L. acidophilus* CUL21 + *Bifido. bifidum* CUL17 + *Bifido animalis lactis*	Madden 2005, [57] Plummer 2005[58]	15, 76	culture, culture	*H. pylori* +, *H. pylori* +	amoxicillin + metronidazole, amoxicillin + clarithromycin	2, 2	restores, restores more rapidly	restores, partially restores
L. acidophilus NCFM + *L. paracasei* Lpc-37 + *Bifido. bifidum* Bb02+ *Bifido. lactis* Bi-04 + *Bifido. lactis* Bi-07	Engel-brektson 2006[50]	20	culture PCR TRFLP	healthy volunteers	augmentin	2	restores	restores

*including strain (when reported)

Table 2. Model B: Evidence-based data for improvement or alteration of normal microbiota (NM) in 18 probiotics from 24 studies (25 treatment arms) with disturbed microbiota at baseline.

Probiotic*	Reference	No. treated with probiotic	Type(s) of assay for NM	Pre-existing disrupting factor**	Follow-up time	Claims stated in papers	Evidence-based claim	Type of change found in NM
Bifido. breve M-16V	Van der Aa 2010[59]	46	FISH	Atopic dermatitis	0	modulates NF	no change	--
Bifido. lactis Bi-07	Larsen 2011[53]	17	PCR	Atopic dermatitis	0	no change	no change	--
Bifido. longum BB536	Odamaki 2007[33]	22	TRFLP PCR	Cedar pollen allergy	4 wk	maintains NF	no change	--
E. coli Nissle	Lata 2007[60]	22	culture	liver cirrhosis	0	restores	improves	more Bifido. & Lacto.
L. acidophilus 700396	Larsen 2011[53]	17	PCR	atopic dermatitis	0	no change	no change	--
L. casei rhamnosus Lcr35	Petricevic 2008[61]	83	Nugent scores	bacterial vaginosis	4 wk	restores	improves	improved Nugent scores
L. plantarum 299v	Nobaek 2000, [62] Klarin 2005, [63] Klarin 2008[64]	25, 17, 22	culture, culture, culture	IBS, enterally-fed, antibiotics	4 wk, 0, 0	no change, no change, no change	no change, no change, no change	--
S. boulardii lyo	Girard 2002, [65] Swidsinski 2008[66]	10, 20	culture, FISH	enterally-fed, active diarrhea	9 d, 3 wk	alters NF, improves	no change, improves	-- more 'habitual microbiota'
L. rhamnosus GR-1 + *L. fermentum* RC14	Reid 2001[67]	33	Nugent scores	bacterial vaginosis	2 wk	restores	improves	improved Nugent scores
L. rhamnosus GR-1 + *L. fermentum* RC14	Reid 2003[68]	31	Nugent scores and culture	bacterial vaginosis	30 d	restores	improves	improved Nugent scores
L. plantarum 8PA3 + *Bifido bifidum*	Kirpich 2008[69]	32	culture	colon cancer	0	restores	improves	more *E. coli* and *Enterococci*
L. rhamnosus GR1 + *L. reuteri* RC14	Hummelen 2010[70]	23	Nugent score	bacterial vaginosis	0	no change	no change	--
L. casei Shirota+ *Bifido breve* BBG01	Uchida 2007[71]	4	culture	short bowel syndrome	0	no change	no change	--
L. brevis CD2 + *L. salivaris* FV2 + *L. plantarum* FV9	Mastromarino 2009[72]	19	Nugent score	bacterial vaginosis	2 wk	restores	improves	improved Nugent scores

L. paracasei Lpc37+ *L. acidophilus* 74-2 + *Bifido. animalis* DGCC420	Roessler 2012[73]	30	PCR	atopic dermatitis	0	no change	no change	--
L. rhamnosus GG + *L. rhamnosus* Lc705 + *P. freudenreichii shermanii* JS + *Bifido. breve* Bb99	Kajander 2005,[74] Lyra 2010[75]	41, 22	PCR, PCR	IBS, IBS	0, 0	restores, alters	improves, alters	Improved similarity index More Clostridia and Rumino-coccus
L. acidophilus 4356 + *L. plantarum* 14917 + *L. rhamnosus* 7469 + *Bifido. bifidum* 2952	Wong 2013[76]	7	PCR	liver disease	0	improves	alters	Less Firmicutes, more Bacterio-detes
L. acidophilus + *L. paracasei* + *L. delbrueckii ssp. bulgaricus* + *L. plantarum* + *Bifido. longum* + *Bifido. infantis* + *Bifido. breve*	Venturi 1999,[77] Brigidi 2001,[78] Kuhbacher 2006[79] Ng 2013[80]	20, 10, 10 10	culture culture & PCR FISH PCR	ulcerative colitis, IBS, pouchitis IBS	15 d, 10 d, 0 0	enhances, no change, altered richness modulates	no change, no change, altered altered	-- -- More anaerobes Less Bacteroides

*including strain (when reported)

**disruption of normal microbiota at baseline shown by significant differences compared to control (non-diseased) population.

Table 3. Model C: Evidence-based data for improvement or alteration of normal microbiota (NM) in 19 probiotics in healthy volunteers enrolled in 29 studies (29 treatment arms) in studies with no disruptive exposures.

Probiotic	Reference	No. treated with probiotic	Type of assay for NM	Enrolled population	Type of disrupt-ing factor	Follow-up post-treat-ment	Claims stated in papers	Evidence-based claim
Bifido. animalis lactis DN173010	Rochet 2008,[49] Oswari 2013[81]	12, 160	FISH PCR	healthy volunteers	none, none	10 d, 6 mon	no change, no change	no change, no change
Bifido. bifidum	Langhendries 1995[82]	20	culture	healthy volunteers	none	0	no change	no change
Bifido. longum	Benno 1992,[83] Fujiwara 2001,[84] Harmsen 2002[85]	5, 7, 14	culture, culture, FISH	healthy volunteers	none, none, none	0, 30 d, 0	no change, alters, no change	no change, alters, no change
L. casei ND114001	Guerin 1998,[86] Rochet 2006,[87] Rochet 2008[88]	12, 12, 7	culture, FISH, FISH	healthy volunteers	none, none, none	1 wk, 10 d, 0	no change, no change, no change	no change, no change, no change
L. johnsonii La1	Brunser 2006[89]	32	culture & FISH	healthy volunteers	none	2 wk	no claim	no change
L. plantarum 299v	Goossens 2003,[90] Goossens 2005,[91] Goossens 2006,[92] Berggren 2003,[93] Karlsson 2010[94]	11, 32, 15, 33, 9	culture, culture, culture, culture, TRFLP	healthy, healthy, colonic polyps, healthy, atherosclerosis	none, none, none, none, none	3 wk, 4 wk, 0, 0, 0	no change, no change, no change, no change, alters	no change, no change, no change, no change, alters
L. rhamnosus GG	Gueimonde 2006[95]	29	PCR	healthy volunteers	none	0	no change	no change
L. salivarius CECT5713	Sierra 2010[96]	20	culture	healthy volunteers	none	0	improves	no change
S. boulardii lyo	Vanhoutte 2006[97]	30	PCR	healthy volunteers	none	0	no change	no change
Bifido. animalis + *Bifido. longum*	Zhong 2006,[98] He 2008[99]	11, 11	FISH, FISH	healthy volunteers	none	7 d, 7d	no change, modifies	no change, no change
L. acidophilic + *Bifido. lactis*	Yang 2012[100]	63	culture	healthy but 55% *H. pylori* +	none	0	restores	alters
L. rhamnosus GG + *Bifido. longum* Bb536	Mah 2007[101]	20	FISH	healthy neonates	none	6 mon	no change	no change
L. rhamnosus GG + *Bifido. lactis* Bb12	Rafter 2007[102]	38	culture	colon cancer patients or at risk	none	0	no change	no change

L. rhamnosus GG + *L. gasseri* TMC0356	Kubota 2009[103]	14	culture FISH TRFLP	healthy, allergy patients	none	0	suppressed changes	alters
L. paracasei B21060 + *L. paracasei* B21070 + *L. gasseri* B21090	Morelli 2003[104]	12	culture	healthy volunteers	none	3 d	no claims	no change
L. acidophilus 1748 + *L. paracasei* F19 + *Bifido. lactis* Bb12	Sullivan 2009[105]	15	culture	chronic fatigue patients	none	4 wk	no change	no change
L. rhamnosus 271 + *L. acidophilus* NCFM + *L. paracasei* 114001 + *Bifido. animalis* 1017	Engelbrektson 2006[50]	22	culture TRFLP PCR	healthy volunteers	none	2 wk	no change	no change
Bifido. animalis lactis + *L. delbrueckii* I-1632 + *L. delbrueckii I-*1519 + *L. lactis cremoris*	McNulty 2011[106]	7	PCR	healthy twins volunteers	none	4 wk	no change	no change
L. acidophilus + *L. paracasei* + *L. delbrueckii ssp. bulgaricus* + *L. plantarum* + *Bifido. longum* + *Bifido. infantis* + *Bifido. breve*	Vitali 2012[14]	15	PCR	healthy pregnant volunteers	none	0	modulates	no change

*including strain (when reported)

Abbreviations: FISH, fluorescence *in situ* hybridization analysis; TRFLP, terminal restriction fragment length polymorphism analysis; PCR, polymerase chain reaction

Table 4. Comparison of the ability of probiotic to restore or improve dysbiosis with ranked clinical efficacy for various disease indications.

Probiotic*	**Restored normal microbiota***	**Altered normal microbiota***	**Ranked net evidence for efficacy****							
			AAD	**CDI**	**IBD**	**IBS**	**TD**	**H pylori**	**Vaginitis/ BV**	**Acute Ped diar**
Restores microbiota										
Clost. butyricum MIYAIRI	yes	nd	**-**					**-**		
L. acidophilus + *Bifido. bifidum*	yes	nd	**0**	**-**						
L. acidophilus 1748 + *L. paracasei* F19 + *Bifido. lactis* Bb12	yes	nd				**-**				
Bifido. longum	yes	no			**-**	+				
L. acidophilus + *L. acidophilus* + *Bifido. bifidum* + *Bifido. animalis*	yes	nd								
L. acidophilus + *L. paracasei* + *Bifido. lactis (2)*	yes	no								
S. boulardii lyo	partial	yes	++	++	++	**0**	+	**-**		++
L. rhamnosus GG	partial	nd	**-**	**-**	**-**	**0**	**0**	**-**	**0**	++
L. acidophilus	partial	no	++		++	-	**-**	+	**0**	
L. acidophilus + *L. bifidus* + *L. rhamnosus*	partial	nd								
Alters microbiota										
E. coli Nissle	nd	yes			**-**					+
L. casei (DN114001 or Lcr35)	nd	yes	+					**0**	+	++
L. rhamnosus GR1 + *L. fermentum* RC14	nd	yes							++	
L. plantarum 8PA3 + *Bifido. bifidum*	nd	yes								
L. rhamnosus GG + *L. rhamnosus* Lc705 + *P. freudenreichii shermanii* JS + *Bifido. breve* Bb99	nd	yes				++				
L. acidophilus + *L. plantarum* + *L. rhamnosus* + *Bifido bifidum*	nd-	yes								

L. brevis CD2 + *L. salivarus* FV2 + *L. plantarum* FV9	nd	yes							+	
L. acidophilus + *L. paracasei* + *L. delbrueckii ssp. bulgaricus* + *L. plantarum, Bifido. longum, Bifido. infantis, Bifido. breve*	nd	yes	-		++	+				++
No effect on microbiota										
B. clausii	nd	nd						-		-
L. plantarum 299v	nd	no	-	-		-				
Bifido. lactis	nd	no	+							**0**
Bifido. breve	no	no								
L. acidophilus + *Bifido. longum*	no	--								
L. rhamnosus 19070-2 + *L. reuteri* DSM	nd	no								**0**
L. casei + *Bifido. breve*	nd	no								
L. paracasei + *L. acidophilus* + *Bifido animalis*	nd	no								
Pharmacokinetic only										
L. reuteri 55730	nd	nd								+
L. johnsonii La1	nd	nd			-			+		
L. salivarius UCC4331	nd	nd				-				
Bifido. infantis 35624	nd	nd				**0**				
Bifido. bifidum MIMBb75	nd	nd				+				
L. rhamnosus + *Bifido. longum*	nd	nd								

*including strain (when reported)

** **Rank:** ++, ≥2 net RCTs (randomized controlled trials) with significant protective efficacy; +, only one net protective RCT; 0, equal number of significant and non-significant RCTs; -, ≥1 net non-significant RCT. Blank indicates no RCT done for the disease indication.

Abbreviations: nd, not determined; AAD, antibiotic associated diarrhea; CDI, Clostridium difficile infections; IBD, inflammatory bowel disease; IBS, irritable bowel syndrome; TD, traveler's diarrhea; BV, bacterial vaginosis; Acute Ped Diar, treatment of acute pediatric diarrhea.

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Figure 1. Time sequence of events and three models of study designs determining three different degrees of dysbiosis correction by probiotics.

Figure 2. Flow-chart of literature review results (1985-2013) of included and excluded studies for the restoration or improvement of normal microbiota by probiotics. Abbreviations: RCT, randomized-controlled trials; MOA, mechanism of action; NM, normal microbiota.

Use of probiotics to correct dysbiosis of normal microbiota following disease or disruptive events: a systematic review.

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ABSTRACT

Objective: To assess the evidence for the claim probiotics can correct dysbiosis of the normal microbiota resulting from disease or disruptive events.

Setting: Systematic review of published clinical trials of patients receiving a probiotic intervention for the prevention or treatment of various diseases.

Data sources: Sources searched (1985-2013): PubMed, EMBASE, Cochrane Database of Systematic Reviews, CINAHL, AMED, and ISI Web of Science. Three on-line clinical trial registries were searched: Cochrane Central Register of Controlled trials, MetaRegister of Controlled Trials, and National Institutes of Health.

Review methods: Included studies were randomized clinical trials of probiotic interventions having microbiologic assays. Studies were evaluated following PRISMA guidelines for specific probiotic strains. A standard data extraction form was used to collect the raw data.

Outcome measures: The primary outcome is the degree of microbiota correction by specific probiotic strains. Secondary outcome was the association between the degree of dysbiosis correction and clinical efficacy.

Results: The review of the literature found three distinct study designs: Model A (restoration) assayed patients enrolled with a healthy, undisturbed microbiota and then assayed post-disruptive event and probiotic therapy; Model B (alteration) assayed patients with pre-existing disrupted microbiota and then post-probiotic therapy; Model C (no dysbiosis) assayed volunteers with no disruptive event pre and post-probiotic. From a total of 63 trials, 83% of the probiotic products using Model A restored the microbiota, 56% using Model B improved the microbiota and only 21% using Model C had any effect on microbiota. Clinical efficacy was more commonly associated with strains capable of restoration of the normal microbiota.

Conclusions: The ability to assess the degree of dysbiosis improvement is dependent upon the enrolled population and the timing of microbiologic assays. The functional claim for correcting dysbiosis is poorly supported for most probiotic strains and requires further research.

Systematic review registration: PROSPERO (CRD42014007224)

Strengths and Limitations

Strengths include:

- A comprehensive review of the published literature from 1985-2013
- Literature search unrestricted by language or country
- Analysis of study designs resulted in novel strategy to limit bias and classify outcomes
- Three types of outcomes of dysbiosis applied to evidence-based studies of specific probiotic strains
- Author has over 40 years of research experience in the probiotic field

Limitations include:

- Pooled clinical trials using different study populations
- Pooled probiotic doses and regimens
- Indirect evidence linking probiotic strains and dysbiosis
- Review done by sole author

INTRODUCTION

The popularity of probiotics has expanded exponentially recently, but along with their increased use, debate rages on how probiotics should be regulated and whether probiotics should be considered as a medical food, drug or a food supplement. In the U.S., probiotics are typically available as dietary supplements and thus are limited to 'structure or function' health claims and, unlike prescription drugs, are not permitted to claim to 'treat' or 'cure' disease. In Europe and the United Kingdom, probiotics are allowed to have health or function claims. These claims are required to be supported by well-conducted human trials in the targeted population or in healthy volunteers, but the European Food Safety Authority (EFSA) has rejected >80% of claims submitted to them. [1-3] In many cases, scientific substantiation of a specific health claim was judged insufficient or based on an indirect effect.[4] One such functional claim made for probiotic products is they correct dysbiosis (or the disruption of bacterial and fungal species after antibiotics or other disruptive exposures) and thus may be beneficial to maintain health. Probiotics are active during this susceptible window from the time of the disruptive event to the time when normal microbiota is restored. A wide variety of mechanisms-of-action have been documented for probiotics (ranging from blocking pathogen attachment sites, destruction of the pathogen by bacteriocins or proteases that degrade toxins, to regulation of the immune system),[5,6] and while clinical evidence supports efficacy of some probiotic strains, the evidence linking these mechanisms-of-action to a specific health or function claims is not as clear.

A classic example of the consequence of dysbiosis is antibiotic-associated diarrhea (AAD).[7,8] While antibiotics may be effective in the elimination of pathogenic organisms, a common, unintended effect is the killing or inhibition of beneficial microbes due to shared susceptibility to the antibiotic. One of the many functions for normal microbiota is the ability to resist infection by pathogenic organisms, termed 'colonization resistance'.[9,10] The loss of a sub-population of the normal microbiota, for example, can lead to the loss of the ability to break down fibers and starches into absorbable short chain fatty acids, resulting in high level of undigested carbohydrates, which can trigger diarrhea.[11] Disruption of the normal microbiota has been shown to lead to higher rates of infections in other body systems other than the intestinal tract including the skin,[12,13] vagina,[14,15] respiratory tract,[16,17] and in the buccal cavity.[18-20]

The major challenge to establishing a cause and effect for the improvement of dysbiosis by probiotics is a lack of a standard definition of 'normal' microbiota. There is substantial inter-individual variation of the species of microbes present at different body niches, which also varies by age, geographic area and health status of the host. In addition, a complete accounting of the microbiota is currently impossible, as there are no assays to detect all of $>10^{13}$-10^{14} organisms in the intestines and standard microbial culturing methods miss 75-95% of these organisms. [21,22] The development of metagenomics (cataloguing individual and disease-specific bacterial gene profiles) and the creation of the international Human Microbiome Project ushered in a new era for our understanding of the complexity of these interactions within the body.[23,24] This paradigm shift from culturing to metagenomic analysis has expanded our ability to document shifts in microbial populations to an unparalleled degree, but the interpretation of these shifts continues to be under debate.[25-28] With the advent of these newer metagenomic tools, the role of probiotics in the restoration of normal microbiota is being re-visited.[29]

In light of new guidance documents and recommendations, the goal of this systematic review is to determine how claims for the restoration of the normal microbiota and the correction of dysbiosis have been studied using well-designed trials and which probiotic strains have evidence-based data to support these claims.

METHODS

Study Objective

To systematically review the literature to analyse the evidence for the claim probiotics can correct dysbiosis of the normal microbiota from randomised controlled trials.

Search Strategy

Search terms included: probiotics + health claims, restoring normal microbiota, dysbiosis, normal microbiota, pharmacokinetics, metagenomics, probiotics, dietary supplements,

randomized controlled trials, antibiotic associated diarrhea (AAD), *Clostridium difficile* infection (CDI), inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), traveler's diarrhea (TD), eradication of *Heliobacter pylori,* bacterial vaginosis or vaginitis, treatment of acute pediatric diarrhea, and specific probiotic strains or products. Search strategies were broad-based initially, then narrowed to clinical trials with probiotics.

Data Sources

PubMed (1985-2013), EMBASE (1985-2013), Cochrane Database of Systematic Reviews (1990-2013), CINAHL (1985-2013), AMED (1985-2013), and ISI Web of Science (2000-2013). Three on-line clinical trial registries were searched: Cochrane Central Register of Controlled trials (http://www.cochrane.org), MetaRegister of Controlled Trials (http:www.controlled-trials.com/mrct) and National Institutes of Health (http://www.clinicaltrials.gov).

Criteria for study selection and data extraction

Abstracts of all citations were reviewed by a single author and rated for inclusion for randomized controlled trials of probiotic treatments. Full articles were retrieved if normal microbiota assays were mentioned. Non-English language trials were translated and included whenever possible. Exclusion criteria included pre-clinical studies (animal models or *in vitro* assays), safety or phase 2 studies, reviews, efficacy trials with no assays for normal microbiota species, metagenomic methods only, mechanism of action of normal microbiota or probiotic, cross-sectional surveys, case reports or case series, duplicate reports, or trials of unspecified types of probiotics. All pharmacokinetic studies in humans were reviewed, as abstracts often did not include normal microbiota assay data. Data extraction and the review process followed the PRISMA statement guidelines using a 27-item checklist and flow diagram.[30] A standardized data extraction form was used to collect data on the probiotic (strain type, daily dose, duration), type of controls (placebo, active or no treatment), study design (status of microbiota at baseline and follow-up times), type of microbiota assay (microbial culturing, molecular biomarkers, etc.), enrolled study population (adult vs. pediatric, healthy volunteers, disease condition), type and timing of disruptive agent (antibiotics, chemotherapy, etc.), study size and attrition, outcome assessment (efficacy and/or microbiota status at end of study, adverse events) and type of health claim.

Outcomes and definitions

The primary outcome is the degree of microbiota correction or improvement by specific probiotic strain(s). The secondary outcome is the association between the degree of dysbiosis correction and the net efficacy found from randomized controlled trials of probiotic interventions. Dysbiosis is defined as an alteration or disruption of the normal microbiota (bacterial or fungal species) due to exposure of an disruptive factor (such as antibiotics, chronic disease, stress, medical procedures or medications, etc.). As there is no current standard definition of 'normal' microbiota, for this review, restoration of normal microbiota is defined as a return to the assayed microbial species or profile taken from a healthy individual (before a disruptive event has occurred). Included studies are required to have at least a pre-probiotic treatment assay and a post-probiotic treatment assay. A variety of microbial assays were available during the search period (1985-2013), including documentation of the microbiota by either microbial cultures, or metagenomic methods [16s rRNA-targeted probes using fluorescent *in situ* hybridization (FISH) or other polymerase chain reaction (PCR) technique][8,21,28,31] or by indirect methods (Nugent scores).[15] Nugent scores (ranged 0-10) are used to diagnose bacterial vaginosis (scores ≥7) or normal vaginal microbiota (scores 0-3) based on the quantitated morphotypes of small gram negative rods (*G. vaginalis/Bacteroides* spp.) and curved gram negative rods (*Mobiluncus* spp.) from gram stains of vaginal discharge smears. Microbial assays of only the strain(s) contained in the probiotic product are considered as pharmacokinetic studies and were not included in the normal microbiota profiles.

Models of dysbiosis. To determine the impact on normal microbiota, only direct evidence of microbiota change (species, profiles, diversity indices, or diagnostic criteria) were included and indirect effects were excluded (changes in intestinal enzymes, immune system parameters or disease symptoms). The degree to which dysbiosis was improved is categorized into three levels: (1) recovery of the normal microbiota back to baseline levels; (2) alteration or improvement of the normal microbiota; and (3) no change in normal microbiota.
The literature contained three dysbiosis models: Model A (restoration of the normal microbiota), which assayed patients enrolled with a healthy, undisturbed microbiota and then assayed again after a disruptive event (such as antibiotic exposure) and probiotic therapy occurred; Model B (alteration of the microbiota) assayed patients with pre-existing disrupted microbiota (for

example, pre-existing chronic disease or active disease) and then post-probiotic therapy; Model C (no dysbiosis) assayed volunteers with no disruptive event (before or during the clinical trial) at both pre-probiotic and post-probiotic times, as shown in Figure 1. 'Recovery' of the normal microbiota is defined as a restoration of the microbiota back to a normal healthy baseline. Recovery may be complete recovery (all assayed microbial levels returned to baseline) or incomplete recovery (partial recovery of some microbial strains, but not all returned to baseline levels). In studies enrolling subjects with dysbiosis at baseline (typically due to chronic diseases), it is not possible to show a restoration to normal microbiota levels because a normal, undisturbed microbiota was not present in these types of study subjects at the time of enrollment. Therefore, the strongest claim possible for Model B designs is for an 'alteration or improvement' of the microbiota. Only data from the probiotic-exposed subjects were analysed in this paper. Data from the control groups were used to confirm dysbiosis for subjects with chronic diseases or after a disruptive exposure, such as antibiotics or chemotherapy, unaffected by probiotic exposure.[32-34]

Assessment of methodological strength and quality

The GRADE (Grading of Recommendations, Assessment, Development and Evaluation) system for rating overall study quality will be used for each probiotic strain or type (single strains and mixtures of strains).[35] Recommendation for the support of the claim of each probiotic strain or mixture can be assessed by the overall strength of the evidence [“strong”, many randomized controlled trials show significant recovery of the microbiota, or "moderate" only one randomized controlled trial; or “weak”, only case series or reports, limited number of small trials, *etc.*].

Quality of the evidence is based on study design and graded as “high quality” (well-defined study design for determining restoration with normal microbiota, Model A), or “moderate quality” (disrupted microbiota at baseline, Model B), or “low quality” (no disruptive event occurred, Model C). Measurement of publication bias was not assessed for this review, as pooled outcome estimates of efficacy were not done, as typical in meta-analysis, but all studies with assays of microbiota were included to limit bias.

Net efficacy rating

To determine if the ability to correct dysbiosis is associated with clinical efficacy, the published literature for randomized controlled trials (RCTs) or meta-analyses of probiotics for various disease indications, including antibiotic associated diarrhea (AAD),[5,36,37] *Clostridium difficile* infection (CDI), [5,38] inflammatory bowel disease (IBD),[39] irritable bowel syndrome (IBS), [40] traveler's diarrhea (TD), [41] eradication of *Heliobacter pylori* (Hp),[36,37] bacterial vaginosis (BV) or vaginitis,[42] and treatment of acute pediatric diarrhea was reviewed. [43-45] The net rank was calculated by subtracting the number of RCTs showing non-significant or equivalent efficacy from the number of RCTs having significant efficacies. The ranks were categorized as follows: ++, ≥2 net RCTs showing significant efficacy; +, net of one RCT showing significant efficacy; 0, equal number of RCTs showing significant and non-significant efficacy results and -, ≥1 net negative or non-significant RCTs. Probiotics with no RCTs were not ranked.

RESULTS

A review of the literature from 1985-2013 found 353 articles that dealt with probiotic treatments and their potential effect on normal microbiota.

Excluded studies

As shown in Figure 2, a total of 272 articles were excluded for the following reasons: reviews (n=116), probiotic efficacy studies with no data on normal microbiota assays (n=54), animal models of probiotics and changes in microbiota (n=38), metagenomic or microbiota methods only (n=17), studies on normal microbiota but with no use of probiotics (n=14), *in vitro* assays of microbiota (n=10), duplicative reports (n=2) or miscellaneous (n=21), which included probiotic mechanism of action studies, safety studies, duplicative reports, cross-sectional surveys and two with poorly described probiotic interventions.[46,47] A total of 81 full articles were reviewed which mentioned changes in normal microbiota or indicated a health claim for probiotics and effects on normal microbiota.

Probiotic pharmacokinetic studies (n=18) reporting concentrations of probiotic strains before and post-treatment, but did not assaying for other species of normal microbiota were excluded. While several studies using this study design claim probiotics had an impact on normal

microbiota, type of data generated is pharmacokinetic behavior of the probiotics themselves and not the normal microbiota. Several studies stated that the normal microbiota was altered because an increase in various bacterial species was observed after the probiotics were given, but the species assayed were those contained in the probiotic product, so an increase is not unexpected. Pharmacokinetic studies have documented that probiotic strains taken orally can survive transit through the intestinal tract with recovery rates in feces ranging from <1% to 22%.[48,49] These pharmacokinetic studies were excluded from this analysis, as they did not assay other types of normal microbiota not found in the probiotic product.

Included studies

Of the 63 included clinical trials, five trials had multiple treatment arms, which resulted in a total of 68 treatment arms for analysis. Engelbrektson et al. tested a mixture of 5 probiotic strains in volunteers exposed to antibiotics and also tested a mixture of 4 probiotic strains in healthy volunteers with no antibiotic exposure.[50] Zoppi et al. had eight different treatment arms in his study, and probiotic arms were included in our analysis [*Saccharomyces boulardii* (*S. boulardii*) alone and *Lactobacillus (L.) rhamnosus GG* alone], a mixture of two probiotics (*L. acidophilus* and *Bifido. bifidum*) and a mixture of three probiotic strains (*L. acidophilus, L. rhamnosus* and *Bifido. bifidum*).[51] Orrhage et al. had two treatment arms (*Bifido. longum* alone and a mixture of *Bifido. longum* and *L. acidophilus*).[52] Larsen et al. tested two single probiotics (*Bifido. lactis* and *L. acidophilus*) in separate treatment arms.[53] Lidbeck et al. gave either enoxacin or clindamycin and randomized patients to either *L. acidophilus* or placebo. [54]

Normal microbiota assay methods. Of the 69 treatment arms that did normal microbiota assays, diverse methods were used to profile the microbiota. Many studies used only standard microbiological culture assays (37, 54%), while others (28, 40%) used techniques to detect non-cultivatable bacterial strains, which included metagenomic assays (FISH, TRFLP, 16s rRNA sequencing) or other PCR techniques. Some studies (4, 6%) used an indirect measure of normal microbiota, using the Nugent score to diagnose bacterial vaginosis, which relies upon gram stain of the vaginal secretions, vaginal pH and symptoms to characterize if normal microbiota is present or absent.[15]

Probiotic strains. In the 69 treatment arms, most (36, 52%) used a single strain of probiotic, while 14 (20%) tested a mix of two probiotic strains and 19 (28%) tested a mix of three or more probiotic strains. The distribution of single versus multiple strain probiotics did not significant vary by the model of study design (X^2_2=2.3, P=0.32). Of the 15 restorative (Model A) study arms, 47% used a single strain of probiotic and 53% used multiple strains. Of the 25 treatment arms with disrupted microbiota at baseline (Model B), 44% used a single strain and 56% used multiple strains. Of the 29 study arms with undisrupted microbiota (Model C), 62% used a single strain and 38% used multiple strains.

Normal microbiota restoration model (Model A)

Only 10 studies (with 15 treatment arms) using Model A to determine restoration of the microbiota were found (Table 1).[32,34,50-52,54-58] The type of enrolled subjects varied from healthy volunteers to children with untreated respiratory infections, to pediatric cancer patients. For subjects with acute infections or cancer, baseline assays were done prior to the disrupting agent (antibiotics or chemotherapy). The number of subjects given probiotics averaged 20/study and ranged from 5 to 83. In 93%, the disruptive factor was antibiotic exposure and in one study, chemotherapy caused the microbiota disruption. Only 8 (53%) of the study arms did an assay during a 1-8 week follow-up period after the probiotic was discontinued.

Analysis of the probiotic strain(s) separately found only two probiotic products with more than one randomized controlled trial. The probiotic mix of *L. acidophilus* and *Bifido. bifidum* showed a complete restoration in one study, but only a partial recovery in the other. (Strength: strong, Quality: high). The probiotic mix of *L. acidophilus* (2 strains) with *Bifido. bifidum* and *Bifido. animalis* showed complete restoration in one study, but only a partial recovery in the other. (Strength: strong, Quality: high). Five other probiotic products with only one supporting clinical trial showed microbiota restoration (*Bifido. longum*, *Clostridium butyricum*, *L. acidophilus*, mix of *L. acidophilus* with *L. paracasei* and *Bifido. lactis*, and the mix of *L. acidophilus* with *L. paracasei* and *Bifido. bifidum* and two strains of *Bifido. lactis*). (Strength: moderate, Quality:

high). Three probiotic products with one supporting clinical trial showed partial restoration (*S. boulardii*, *L. rhamnosus* GG, mix of *L. rhamnosus* with *L. bifidus* and *L. acidophilus*), (Strength: moderate, Quality: high). Only two probiotic products using Model A showed no change in the microbiota (*Bifido. breve* and a mix of *L. acidophilus* and *Bifido. longum*). (Strength: moderate, Quality: high). In summary, 10 of 12 (83%) of the probiotic products showed complete or partial restoration of the normal microbiota.

Of the 11 probiotic products with claims of 'restores or improves normal microbiota', 10 (91%) were supported by this review, but only seven showed complete restoration and five had partial restoration of the microbiota (Table 1). The mixture of *L. acidophilus* and *Bifido. longum* did not show any changes in the microbiota. Wada et al. claimed *Bifido. breve* 'enhanced intestinal anaerobes', but this was only compared to the placebo group.[32] Their data showed chemotherapy is a disruptive event, resulting in more Enterobacteria in the intestine in the placebo group, but there were no significant differences seen by the end of the 8 week follow-up in either the probiotic or the placebo group compared to baseline microbiota levels.

Disrupted normal microbiota at baseline studies (Model B)

Twenty-four studies (with 25 treatment arms) used Model B that enrolled subjects with a pre-existing disrupted microbiota related to ongoing disease or conditions (Table 2).[33,53,59-80] The number of subjects given probiotics averaged 23 $\pm$ 16/study and ranged from 7-83 participants. The types of pre-existing factors that disrupted the microbiota included atopic dermatitis patients, allergies, cirrhosis, bacterial vaginosis, irritable bowel syndrome, inflammatory bowel disease (ulcerative colitis and pouchitis), idiopathic diarrhea, enteral feeding, short-bowel syndrome and colon cancer. Only 10 (40%) of the study arms did an assay during the post-probiotic follow-up period.

Three of the probiotics had multiple clinical trials to support the claim of an improvement in the microbiota due to the probiotic. *S. boulardii* was used in two trials either with enteral fed

patients or patients with active diarrhea and found an improvement in the habitual microbiota in the patients with active diarrhea[66], but only showed indirect evidence of short-chain fatty acid changes in the other study.[65] (Strength: strong, Quality: moderate) A mix of four probiotic strains (2 strains of *L. rhamnosus*, *P. freudenreichii* + *Bifido. breve*) showed improved microbiota in two clinical trials.[74,75] (Strength: strong, Quality: moderate) Of four clinical trials testing a mixture of seven probiotic strains, two showed no significant change in microbiota [77,78], one showed more anaerobes post-probiotic treatment[79] and one found a reduction in Bacteroides species.[80] (Strength: strong, Quality: moderate) Three clinical trials determined there were no significant changes due to *L. plantarum* 299v.[62-64] (Strength: strong, Quality: moderate). Of those probiotics with only one supporting clinical trial (Strength: moderate, Quality: moderate), two single probiotic strains (*E. coli* Nissle and *L. casei rhamnosus*) and five different mixtures of probiotic strains support the claim that the probiotic alters the microbiota (Table 2). In summary, 10 of 18 (56%) probiotic products altered or improved microbiota in individuals with pre-existing disease.

Of the 25 treatment arms, the paper's claim was confirmed in 14 (56%) of the studies. There was no significant change in the microbiota due to the probiotic in nine treatment arms and only an alteration of the microbiota in five others (Table 2). Our review disagreed with the claimed outcomes in 11 (46%) of the other treatment arms. In seven treatment arms, it was claimed the tested probiotic 'restored normal microbiota', but it is uncertain how this conclusion was reached, since there was no time when a normal undisrupted microbiota was present. Of the seven studies that claimed their probiotic 'restored' normal microbiota, our analysis determined none were capable of documenting restoration, but it is confirmed probiotics improved or altered the microbiota in these studies. Four studies claimed the probiotic 'altered or improved' normal microbiota, but this review found no significant differences when post-probiotic and baseline assays were compared for the probiotic groups. Girard-Pipau et al. concluded that *S. boulardii* 'altered normal flora' because more gram positive anaerobes were seen in the probiotic group compared to the controls and an increase in three short-chain fatty acids were observed in the *S. boulardii* group.[65] However, when the analysis is restricted to trends observed in the probiotic group only, no significant differences were observed in pre-probiotic versus post-probiotic

microbiota profiles. Venturi et al. concluded that the mix of seven probiotic strains enhanced the concentration of some beneficial strains in the intestines. [77] However, the only strains having a significant increase were those contained in the probiotic mix, and not specifically normal microbiota of the host. As this study did not have an undisturbed microbiota baseline, the increased numbers of Lactobacilli and Bifidobacteria may not have reflected their normal levels. Van der Aa et al. claimed that *Bifido. breve* 'successfully modulates the intestinal flora', but no significant changes were observed in the probiotic group when comparing the baseline to the post-probiotic levels. [59] Odamaki et al. did show an increase in Faecalibacterium ssp. and *Bacteroides fragilis* ssp. at the end of *Bifido. longum* BB536 treatment, but the same increase was also observed in the placebo group.[33]

Undisrupted normal microbiota studies (Model C)

Twenty nine trials enrolled healthy adults who had no disruptive factor present during the study (either no antibiotic or no medication exposure or presence of acute or chronic disease) that might impact normal microbiota, as shown in Table 3.[14,49,50,81-106] The average number of subjects given probiotics was 23/study and ranged from 7 to 160/study. Of the 29 study arms, assays were taken during a follow-up period in only 52%. Fujiwara et al. cultured seven healthy volunteers and found Enterobacteriaceae and Clostridial species post-*Bifido. longum* was reduced by 10^1/g compared to baseline ($P<0.03$), but no other changes in the microbiota were detected.[84] Karlsson et al. found a significant increase in intestinal diversity in nine male volunteers with atherosclerosis given *L. plantarum* 299v, but because terminal restriction fragment length polymorphism assays were used instead of cultures for bacterial species, the specific changes in the microbiota species could not be determined.[94] Yang and Sheu cultured 63 children (55% with *H. pylori*) given a yogurt with *L. acidophilus* and *Bifido. lactis* but only found a decrease in *E. coli* counts in the *H.pylori* negative children sub-group, no significant changes in normal microbiota was found in the *H. pylori* positive children.[100] Kubota et al. assayed 29 subjects with Japanese cedar pollen allergy and found milk fermented with *L. rhamnosus* GG and *L. gasseri* TMC0356 suppressed microbiota changes (less intestinal profile changes), but could not determine specific bacterial species changes due to the type of assay used

(FISH and TRFLP).[103] In summary, only 4 of 19 (21%) probiotic products altered microbiota in healthy individuals who had no disruptive event.

Of the seven studies that claimed their probiotic(s) 'restored or altered' the normal microbiota, only four claims were confirmed. Sierra et al. claimed *L. salivarius* given to 20 healthy adults 'improved gut microbiota', but only increased levels of Lactobacilli were found and no other changes in normal microbiota species were detected. The only other evidence was indirect from changes observed in immune parameters.[96] He et al. claimed a mixture of *Bifido. longum* and *Bifido. animalis* 'modified' microbiota, but changes were seen only during the yogurt administration and not after the one week follow-up period.[99] Vitali et al. claimed that the mixture of four Lactobacilli strains and three Bifidobacteria strains 'modulated vaginal microbiota', but the only significant changes were due to an increase in the bacterial species contained in the probiotic mixture.[14]

Of the probiotics supported by multiple clinical trials (*Bifido. animalis*, *Bifido. longum*, *L. casei*, *L. plantarum* 299v, the mixture of *Bifido. animalis and Bifido. lactis*), 13 of the trials (87%) support there is no significant change in normal microbiota if the microbiota is not disrupted. [Strength: strong, Quality: low)

Association of clinical efficacy and normal microbiota restoration

Few studies concurrently compared clinical efficacy and the ability to restore or improve normal microbiota after dysbiosis. A synthesis of the literature of RCT for eight common disease indications was performed and the overall net strength was ranked. Probiotics with the ability to restore normal microbiota were frequently supported by RCTs for efficacy, as shown in Table 4. Of the 10 probiotics with evidence for restoration, 7 (70%) also had at least one RCT testing for at least one of the eight diseases, while 30% did not have any supportive RCTs for efficacy. Of the 7 probiotics with associated RCTs, only two probiotics (*S. boulardii* and *L. acidophilus*) have strong evidence for efficacy across most of the disease indications, while five probiotics with the

ability to restore the microbiota had weak or no evidence of efficacy. For example, *S. boulardii*, which has studies supporting restoration, has strong evidence for clinical efficacy for AAD (ranked ++: 11 RCTs had significant results and 6 had non-significant results), CDI (ranked ++: had two RCTs with significant results), IBD (ranked ++: had two RCTs with significant results), IBS (ranked 0: had one RCT with significant efficacy and one RCT with non-significant results), TD (ranked +: 3 RCTs with significant efficacy and 2 with non-significant efficacy), *H. pylori* eradication (ranked -: 2 RCTs with significant results and 4 with non-significant results) and no studies for BV. *L. acidophilus*, which partially restored the microbiota in a study, is associated with clinical efficacy for AAD, IBS and BV, but not for TD or eradication of *H. pylori* and treatment of acute pediatric diarrhea (ranked ++: had 19 RCTs with significant protection and five with non-significant results). In contrast, *L. rhamnosus GG,* another probiotic capable of restoring microbiota, is often cited in meta-analysis as having significant efficacy for AAD. Our results of an updated review of the literature indicate a net weak evidence rating for clinical efficacy across all disease indications: AAD (ranked -: 3 RCTs had significant results and 6 had non-significant results), CDI (ranked -: two RCTs with non-significant results), IBD (ranked -: one RCT with non-significant results), IBS (ranked 0: 2 RCTs with significant efficacy and two RCTs with non-significant results), TD (ranked 0: one RCT with significant efficacy and one with non-significant efficacy), *H. pylori* eradication (ranked -: 3 RCTs with non-significant results), no RCTs for BV and treatment of acute pediatric diarrhea (ranked ++: 10 RCTs with significant efficacy and one with non-significant findings).

Efficacy trials were not done as frequently for probiotics shown to only have the ability to alter or improve, but not restore, the microbiota after dysbiosis. Of nine probiotics that can alter the microbiota, 6 (67%) have supporting RCTs for at least one disease, but the diversity of investigated diseases was more limited. *L. casei* had moderate net strength for AAD and bacterial vaginosis, but was neutral for the ability to eradicate *H. pylori* and other disease indications were not tested in RCTs with *L. casei*. The probiotic mixture of *L. reuteri* and *L. fermentum* has strong evidence for bacterial vaginosis, but not for any other disease indications listed in Table 4.

Of the eight probiotics not capable of altering or restoring normal microbiota, only *L. plantarum* 299v had RCTs for AAD and IBS, both with net negative or weak strength of clinical efficacy. *Bifido. lactis* and the mixture of *L. rhamnosus* and *L. reuteri* had net neutral rankings for efficacy for the treatment of acute pediatric diarrhea. The other four probiotic products with no effect on normal microbiota lacked any RCTs for clinical efficacy. Studies with *B. clausii* did not assay for normal microbiota and had non-significant trial results for *H. pylori* eradication and the treatment of pediatric diarrhea.

Of the six probiotics with only pharmacokinetic data on the probiotic itself and no other investigation of other normal microbiota strains, five had RCTs showing varying net efficacies for different disease indications, as shown in Table 4.

Six popular probiotics (*Bacillus clausii*, *Bifido. infantis*, *L. reuteri*, *L. acidophilus* + *L. helveticus*, *L. acidophilus* + *L. casei* and *L. acidophilus* + *Bifido. animalis*) have only clinical efficacy RCTs, but have not published studies investigating their role in restoring or improving the normal microbiota.

Discussion

Developing and evaluating health or function claims for probiotics is an important issue and is now identified as a priority for research by several international organizations, including the World Gastroenterology Organization [107] and the American Society for Nutrition.[2] The U.S. Food and Drug Administration has struggled with appropriate evidence-based health claims for probiotic products and currently recommends the use of structure/function claims, such as "maintains bowel regularity", but the claim for restoring normal microbiota is still under debate.[108] The European Food Safety Authority (EFSA) provides guidance materials that recommend health or function claims for probiotics should have beneficial physiological effects and have appropriate scientific trials to substantiate the health claims.[3] Acceptable claims for intestinal health may include functional claims (improved transit time, softer stool consistency, reduction in gastrointestinal discomfort, defense against pathogens). As it is currently not possible to define a standard normal microbiota profile, the EFSA recommends functional claims

for the restoration of normal microbiota should document a recovery of healthy microbiota and be accompanied by a beneficial physiological or clinical outcome.[3] In addition, because the efficacy and mechanisms are strain-specific and may vary by probiotic strain, the evidence must be analyzed for each probiotic product individually.[5,6,9,109, 110-112]

An underappreciated finding was the influence that study design and study populations have on the interpretation of study outcomes. In the literature, five different types of study designs are commonly found relating to probiotics. The most common study type is a randomized controlled trial testing the efficacy and safety outcomes in patients, but these trials did not typically document the impact of the probiotic on the normal microbiota. The second most common type of study design is pharmacokinetic studies (documenting recovery of oral dose of probiotic or increase in probiotic strains post-treatment compared to pre-treatment or clearance of the probiotic). Even though these kinetic studies did not assay for non-probiotic strains, some extrapolated their results and concluded some effect or improvement of the normal microbiota was observed by their probiotic.[19,111] These two first types of study designs do not support evidence-based conclusions for the restoration or alteration of the normal microbiota and were excluded from this review.

Three types of study designs are appropriate for the study of dysbiosis. The first type of study design had normal microbiota assayed at least twice (at baseline, which was before exposure to a disruptive event or probiotics and then again during or post-probiotic treatment) to show actual recovery of assayed normal microbiota back to healthy baseline levels. The second type of study design started with inappropriate baselines (baseline samples taken after normal microbiota had been disrupted by chronic disease). For patients with established chronic diseases, there is no "normal microbiota" baseline in either the probiotic or the control group. Even if baselines are taken during remission, the microbiota may still be impacted by chronic disease or acute diarrhea. Studies of probiotics in chronic diseases or acute disease typically report on 'pre-probiotic treatment' and 'post-probiotic treatment' and may show significant shifts in microbial species, but it is uncertain if this reflects a true re-establishment of normal microbiota profiles. The third type of study design enrolled healthy volunteers, who were not challenged with antibiotics (so no normal microbiota disruption occurred), and show only the effect of probiotics on a healthy microbiota (typically mild or no effects). Control groups were not required for our

assessment of the impact of probiotics on microbiota, but control groups can document the degree normal microbiota is disrupted by inciting agents (antibiotic, disease onset, etc.).

Five single strain probiotics (*Bifido. longum*, *Clost. butyricum*, *L. acidophilus, L. rhamnosus* and *S. boulardii*) and five probiotic mixtures [(*L. acidophilus* + *Bifido. bifidum)*, (*L. rhamnosus* + *L. bifidus* + *L. acidophilus)*, (*L. acidophilus* + *L. paracasei* + *Bifido. lactis),(L. acidophilus*, 2 strains, *Bifido. bifidum, Bifido. animalis)* and *(L. acidophilus* + *L. paracasei* + *Bifido. bifidum* +*2 strains of Bifido. lactis*)] documented either complete or partial recovery of normal microbiota (Model A). Only two probiotic mixtures [(2 strain mixture: *L. acidophilus* + *Bifido. bifidum*) and (4 strain mixture: *L. acidophilus*, 2 strains, *Bifido. bifidum, Bifido. animalis*)] were supported by a confirmatory study. Evidence that probiotics may alter or improve normal microbiota (Model B) was found for three single strain probiotics (*E. coli* Nissle, *S. boulardii* and *L. casei rhamnosus*) and seven mixtures of 2-7 probiotic strains. Of these ten probiotics finding alteration of the microbiota, only three had multiple trials [*S. boulardii*, and a four strain mixture (2 strains of *L. rhamnosus* + *P. freudenreichii* + *Bifido. breve*), and a seven strain mixture (4 Lactobacilli and 3 Bifidobacteria strains)], but only one had consistent results showing improvements in the microbiota. [74,75] Clearly, more than one study is needed to confirm the impact of a probiotic on the normal microbiota. Of the 19 probiotic strains (or mixtures) studied in healthy volunteers who were not exposed to disruptive factors (Model C), no change in the normal microbiota was observed for 79%, indicating the robustness of the microbiota.

Improvement in the normal microbiota by specific probiotic strains seemed to be associated with better clinical endpoints. Within eight common diseases typically treated with probiotics, more trials with significant efficacy were associated with probiotic strains shown to restore the normal microbiota, and only one trial with significant efficacy was found for probiotics that did not alter the microbiota. However, few probiotics had efficacy trials for all eight diseases and many did not have any efficacy trials.

Some probiotics which have published efficacy trials for various diseases did not have studies investigating the effect of the probiotic on normal microbiota: *Bacillus clausii*, *Bifido. infantis*, *L. brevis*, *L. reuteri*, mix of 2 strains (*L. acidophilus* + *L. helveticus*), mix of 2 strains (*L. acidophilus* + *L. casei*) or (*L. acidophilus* + *Bifido. animalis*), mix of 4 strains [*L. rhamnosus* (2 strains), *Propionibacterium freudenreichii* + *Bifido. animalis*)] and mix of 7 strains (*L. sporogens, L. bifidum, L. bulgaricus, L. thermophilus, L. acidophilus, L. casei, L. rhamnosus).*

Comparison of results with other studies

Other reviews in the literature of claims for probiotics relating to changes in the normal microbiota have focused on the broad issues of regulatory standardization of health or function claims, the use of proper study designs and the challenge of defining biomarkers for a 'healthy microbiota'.[3,29,112] Donovan et al. recommends that health claims for probiotics be supported by well-conducted human trials in the targeted population.[2] These reviews also recommend that gut biomarkers need to be correlated with clinical endpoints, however none of these reviews attempted to do so.[29,112] No prior review has attempted to analyze the association between probiotic strains and their impact on normal microbiota by stratifying on the quality of study design. [111] This review addressed these concerns by analyzing probiotic strains by the quality of the study design and only including trials that assessed the normal microbiota (either by microbial culturing or molecular strain biomarkers) and assessed the degree of dysbiosis improvement with clinical outcomes for each probiotic strain.

Opportunities for future research

Most of the studies (80%) using Model A to document restoration of the normal microbiota only used microbiologic culturing techniques, which can only detect those organisms that grow in culture. Use of the more advanced molecular metagenomic techniques have found that culturing alone misses up to 95% of these organisms.[21,22] The use of the metagenomic techniques was more common in the studies using Model B (48%) and Model C (45%) study designs, which only addresses potential alteration of the microbiota. Characterization of the microbiota is a

complex issue and a comprehensive accounting of all the bacterial and fungal strains in the body is beyond our current capabilities. Therefore, any studies of changes to the microbiota are incomplete at best, but general trends in bacterial phylotypes can be documented using DNA probes and metagenomic techniques. Differential detection bias may be present due to the variety of assays used in these studies and should be accounted for in future studies.

Another suggestion for future studies is to include an appropriate follow-up time period post-probiotic administration. Fewer than half of the reviewed trials did assays for normal microbiota during an appropriate follow-up period. As it has been shown that recovery from a disrupting factor can be prolonged (typically eight weeks),[7,8] and studies that failed to find microbiota recovery might have detected a return to normal baseline levels if a sufficiently long time was given for the recovery to have occurred. Future studies should strive to allow time for the restoration of the normal microbiota to occur.

As the effects of probiotics are strain specific, and many studies typically only report the genus and species of the tested probiotic, future reports should include a complete description of the probiotic to the stain level. [5,112]

Strengths and weaknesses

The strengths of this review included the completeness of the search strategy, which reviewed multiple citation databases, trial registries and author searches, use of established PRISMA protocols for reviews and the use of an outcome classification scheme for different degrees of assessment for microbial recovery. This analysis controlled the confounding effects of different study populations and study designs present in the literature. Pharmacokinetic studies of just the probiotic strain(s) itself were excluded and only trials that assayed other species found in the microbiota were included. By applying a standard definition for 'restoring' versus 'improving' normal microbiota, it is possible to distinguish significant differences by the type of study designs used and differential effects of the different probiotic strains. Limitations of this review

include: a single author reviewed and extracted the literature, pooling trials from different populations (adult versus pediatric) and different probiotic doses and regimens used. Incomplete retrieval of all studies assessing the effect that probiotics have on human microbiota is also a potential limitation of any literature search. Another limitation is that dysbiosis improvement and clinical efficacy for probiotic strains is also indirectly associated, no direct cause and effect relationship was possible with the types of studies done. Another limitation is the current lack of a standard definition of what comprises a 'normal microbiota'. The constituents of the microbiota vary by individual, by age, geographic location and health status of the host. Current microbiologic techniques are improving, but can not detect all species present in the host.

Conclusion

The challenges in recommending a specific probiotic to patients who need to restore or improve their normal microbiota after a disrupting event occurs is two-fold: one is the diversity of probiotic products available and second is the varying strength of evidence provided by clinical trials using different outcome measures and study designs. By grouping studies into three groups that result in three different degrees of probiotic effect (restoration, improvement or no change), an overview of the body of evidence is possible. By comparing the strength of the clinical evidence for common diseases by the degree to which the probiotics could impact the restoration of the normal microbiota, it became obvious that those probiotics with a greater ability to restore the microbiota are associated with the strongest strength of clinical efficacy. While this evidence only indirectly links clinical efficacy with the ability to restore the microbiota, the overall review of the evidence shows this is an important mechanism of action for probiotics. What becomes obvious is that more studies are required to conclude which probiotic strains have a beneficial impact on the normal microbiota, as most strains have only a single clinical trial and many probiotic products overstate the strength of their claim to restore normal microbiota. These types of issues should be considered for health care policy makers and researchers for future studies and for creating guidelines for health/function claims.

Table 1. Model A: Evidence-based data for restoration of normal microbiota (NM) for 12 probiotics from 10 studies (15 treatment arms).

Probiotic*	Reference	No. treated with probiotic	Typeof assay for NM	Enrolled population	Type of disrupting factor	Follow-up post-treatment (wks)	Claims stated in papers	Evidence-based claim
Bifido. breve	Wada 2010[32]	19	FISH	pediatric cancer patients	chemotherapy	8	enhances anaerobes	no change
Bifido. longum BB536	Orrhage 1994[52]	10	culture	healthy volunteers	clindamycin	0	restores	restores
Clost. butyricum MIYAIRI	Seki 2003[34]	83	culture	pediatric respiratory or GI infections	antibiotics	0	restores	restores
L. acidophilus NCFB1748	Lidbeck 1988[54]	5 5	culture culture	healthy volunteers volunteers	enoxacin or clindamycin	1 1	restores only in enoxacin no change	restores only in enoxacin, no change in clindamycin
L. rhamnosus GG	Zoppi 2001[51]	7	culture	pediatric respiratory infections	ceftriaxone	0	partially corrects	partially restores
S. boulardii lyo	Zoppi 2001[51]	6	culture	pediatric respiratory infections	ceftriaxone	0	improves	partially restores
L. acidophilus + *Bifido. bifidum*	Black 1991, [55] Zoppi 2001[51]	10, 7	culture, culture culture	healthy volunteers, pediatric respiratory	ampicillin, ceftriaxone	2, 0	recovers more rapidly, less change	restores, partially restores
L. acidophilus 1748 + *Bifido. longum* BB536	Orrhage 1994[52]	10	culture	healthy volunteers	clindamycin	0	no change	no change
L. rhamnosus + *L. bifidus* + *L. acidophilus*	Zoppi 2001[51]	7	culture	pediatric respiratory infections	ceftriaxone	0	partially corrects	partially restores
L. acidophilus 1748 + *L. paracasei* F19 + *Bifido lactis* Bb12	Jernberg 2005[56]	4	culture PCR TRFLP	healthy volunteers	clindamycin	2	restores	restores
L. acidophilus CUL60+ *L. acidophilus* CUL21 + *Bifido. bifidum* CUL17 + *Bifido animalis lactis*	Madden 2005, [57] Plummer 2005[58]	15, 76	culture, culture	*H. pylori* +, *H. pylori* +	amoxicillin + metronidazole, amoxicillin + clarithromycin	2, 2	restores, restores more rapidly	restores, partially restores
L. acidophilus NCFM + *L. paracasei* Lpc-37 + *Bifido. bifidum* Bb02+ *Bifido. lactis* Bi-04 + *Bifido. lactis* Bi-07	Engel-brektson 2006[50]	20	culture PCR TRFLP	healthy volunteers	augmentin	2	restores	restores

*including strain (when reported)

Table 2. Model B: Evidence-based data for improvement or alteration of normal microbiota (NM) in 18 probiotics from 24 studies (25 treatment arms) with disturbed microbiota at baseline.

Probiotic*	Reference	No. treated with probiotic	Type(s) of assay for NM	Pre-existing disrupting factor**	Follow-up time	Claims stated in papers	Evidence-based claim	Type of change found in NM
Bifido. breve M-16V	Van der Aa 2010[59]	46	FISH	Atopic dermatitis	0	modulates NF	no change	--
Bifido. lactis Bi-07	Larsen 2011[53]	17	PCR	Atopic dermatitis	0	no change	no change	--
Bifido. longum BB536	Odamaki 2007[33]	22	TRFLP PCR	Cedar pollen allergy	4 wk	maintains NF	no change	--
E. coli Nissle	Lata 2007[60]	22	culture	liver cirrhosis	0	restores	improves	more Bifido. & Lacto.
L. acidophilus 700396	Larsen 2011[53]	17	PCR	atopic dermatitis	0	no change	no change	--
L. casei rhamnosus Lcr35	Petricevic 2008[61]	83	Nugent scores	bacterial vaginosis	4 wk	restores	improves	improved Nugent scores
L. plantarum 299v	Nobaek 2000, [62] Klarin 2005, [63] Klarin 2008[64]	25, 17, 22	culture, culture, culture	IBS, enterally-fed, antibiotics	4 wk, 0, 0	no change, no change, no change	no change, no change, no change	--
S. boulardii lyo	Girard 2002, [65] Swidsinski 2008[66]	10, 20	culture, FISH	enterally-fed, active diarrhea	9 d, 3 wk	alters NF, improves	no change, improves	-- more 'habitual microbiota'
L. rhamnosus GR-1 + *L. fermentum* RC14	Reid 2001[67]	33	Nugent scores	bacterial vaginosis	2 wk	restores	improves	improved Nugent scores
L. rhamnosus GR-1 + *L. fermentum* RC14	Reid 2003[68]	31	Nugent scores and culture	bacterial vaginosis	30 d	restores	improves	improved Nugent scores
L. plantarum 8PA3 + *Bifido bifidum*	Kirpich 2008[69]	32	culture	colon cancer	0	restores	improves	more *E. coli* and *Enterococci*
L. rhamnosus GR1 + *L. reuteri* RC14	Hummelen 2010[70]	23	Nugent score	bacterial vaginosis	0	no change	no change	--
L. casei Shirota+ *Bifido breve* BBG01	Uchida 2007[71]	4	culture	short bowel syndrome	0	no change	no change	--
L. brevis CD2 + *L. salivaris* FV2 + *L. plantarum* FV9	Mastromarino 2009[72]	19	Nugent score	bacterial vaginosis	2 wk	restores	improves	improved Nugent scores

L. paracasei Lpc37+ *L. acidophilus* 74-2 + *Bifido. animalis* DGCC420	Roessler 2012[73]	30	PCR	atopic dermatitis	0	no change	no change	--
L. rhamnosus GG + *L. rhamnosus* Lc705 + *P. freudenreichii shermanii* JS + *Bifido. breve* Bb99	Kajander 2005,[74] Lyra 2010[75]	41, 22	PCR, PCR	IBS, IBS	0, 0	restores, alters	improves, alters	Improved similarity index More Clostridia and Rumino-coccus
L. acidophilus 4356 + *L. plantarum* 14917 + *L. rhamnosus* 7469 + *Bifido. bifidum* 2952	Wong 2013[76]	7	PCR	liver disease	0	improves	alters	Less Firmicutes, more Bacterio-detes
L. acidophilus + *L. paracasei* + *L. delbrueckii ssp. bulgaricus* + *L. plantarum* + *Bifido. longum* + *Bifido. infantis* + *Bifido. breve*	Venturi 1999,[77] Brigidi 2001,[78] Kuhbacher 2006[79] Ng 2013[80]	20, 10, 10 10	culture culture & PCR FISH PCR	ulcerative colitis, IBS, pouchitis IBS	15 d, 10 d, 0 0	enhances, no change, altered richness modulates	no change, no change, altered altered	-- -- More anaerobes Less Bacteroides

*including strain (when reported)

**disruption of normal microbiota at baseline shown by significant differences compared to control (non-diseased) population.

Table 3. Model C: Evidence-based data for improvement or alteration of normal microbiota (NM) in 19 probiotics in healthy volunteers enrolled in 29 studies (29 treatment arms) in studies with no disruptive exposures.

Probiotic	Reference	No. treated with probiotic	Type of assay for NM	Enrolled population	Type of disrupt-ing factor	Follow-up post-treat-ment	Claims stated in papers	Evidence-based claim
Bifido. animalis lactis DN173010	Rochet 2008,[49] Oswari 2013[81]	12, 160	FISH PCR	healthy volunteers	none, none	10 d, 6 mon	no change, no change	no change, no change
Bifido. bifidum	Langhendries 1995[82]	20	culture	healthy volunteers	none	0	no change	no change
Bifido. longum	Benno 1992,[83] Fujiwara 2001,[84] Harmsen 2002[85]	5, 7, 14	culture, culture, FISH	healthy volunteers	none, none, none	0, 30 d, 0	no change, alters, no change	no change, alters, no change
L. casei ND114001	Guerin 1998,[86] Rochet 2006,[87] Rochet 2008[88]	12, 12, 7	culture, FISH, FISH	healthy volunteers	none, none, none	1 wk, 10 d, 0	no change, no change, no change	no change, no change, no change
L. johnsonii La1	Brunser 2006[89]	32	culture & FISH	healthy volunteers	none	2 wk	no claim	no change
L. plantarum 299v	Goossens 2003,[90] Goossens 2005,[91] Goossens 2006,[92] Berggren 2003,[93] Karlsson 2010[94]	11, 32, 15, 33, 9	culture, culture, culture, culture, TRFLP	healthy, healthy, colonic polyps, healthy, atherosclerosis	none, none, none, none, none	3 wk, 4 wk, 0, 0, 0	no change, no change, no change, no change, alters	no change, no change, no change, no change, alters
L. rhamnosus GG	Gueimonde 2006[95]	29	PCR	healthy volunteers	none	0	no change	no change
L. salivarius CECT5713	Sierra 2010[96]	20	culture	healthy volunteers	none	0	improves	no change
S. boulardii lyo	Vanhoutte 2006[97]	30	PCR	healthy volunteers	none	0	no change	no change
Bifido. animalis + *Bifido. longum*	Zhong 2006,[98] He 2008[99]	11, 11	FISH, FISH	healthy volunteers	none	7 d, 7d	no change, modifies	no change, no change
L. acidophilic + *Bifido. lactis*	Yang 2012[100]	63	culture	healthy but 55% *H. pylori* +	none	0	restores	alters
L. rhamnosus GG + *Bifido. longum* Bb536	Mah 2007[101]	20	FISH	healthy neonates	none	6 mon	no change	no change
L. rhamnosus GG + *Bifido. lactis* Bb12	Rafter 2007[102]	38	culture	colon cancer patients or at risk	none	0	no change	no change

L. rhamnosus GG + *L. gasseri* TMC0356	Kubota 2009[103]	14	culture FISH TRFLP	healthy, allergy patients	none	0	suppressed changes	alters
L. paracasei B21060 + *L. paracasei* B21070 + *L. gasseri* B21090	Morelli 2003[104]	12	culture	healthy volunteers	none	3 d	no claims	no change
L. acidophilus 1748 + *L. paracasei* F19 + *Bifido. lactis* Bb12	Sullivan 2009[105]	15	culture	chronic fatigue patients	none	4 wk	no change	no change
L. rhamnosus 271 + *L. acidophilus* NCFM + *L. paracasei* 114001 + *Bifido. animalis* 1017	Engelbrektson 2006[50]	22	culture TRFLP PCR	healthy volunteers	none	2 wk	no change	no change
Bifido. animalis lactis + *L. delbrueckii* I-1632 + *L. delbrueckii I*-1519 + *L. lactis cremoris*	McNulty 2011[106]	7	PCR	healthy twins volunteers	none	4 wk	no change	no change
L. acidophilus + *L. paracasei* + *L. delbrueckii ssp. bulgaricus* + *L. plantarum* + *Bifido. longum* + *Bifido. infantis* + *Bifido. breve*	Vitali 2012[14]	15	PCR	healthy pregnant volunteers	none	0	modulates	no change

*including strain (when reported)

Abbreviations: FISH, fluorescence *in situ* hybridization analysis; TRFLP, terminal restriction fragment length polymorphism analysis; PCR, polymerase chain reaction

Table 4. Comparison of the ability of probiotic to restore or improve dysbiosis with ranked clinical efficacy for various disease indications.

Probiotic*	Restored normal microbiota *	Altered normal microbiota*	Ranked net evidence for efficacy**							
			AAD	CDI	IBD	IBS	TD	H pylori	Vaginitis/ BV	Acute Ped diar
Restores microbiota										
Clost. butyricum MIYAIRI	yes	nd	-					-		
L. acidophilus + *Bifido. bifidum*	yes	nd	**0**	-						
L. acidophilus 1748 + *L. paracasei* F19 + *Bifido. lactis* Bb12	yes	nd				-				
Bifido. longum	yes	no			-	+				
L. acidophilus + *L. acidophilus* + *Bifido. bifidum* + *Bifido. animalis*	yes	nd								
L. acidophilus + *L. paracasei* + *Bifido. lactis (2)*	yes	no								
S. boulardii lyo	partial	yes	++	++	++	**0**	+	-		++
L. rhamnosus GG	partial	nd	-	-	-	**0**	**0**	-	**0**	++
L. acidophilus	partial	no	++		++	-	-	+	**0**	
L. acidophilus + *L. bifidus* + *L. rhamnosus*	partial	nd								
Alters microbiota										
E. coli Nissle	nd	yes			-					+
L. casei (DN114001 or Lcr35)	nd	yes	+					**0**	+	++
L. rhamnosus GR1 + *L. fermentum* RC14	nd	yes							++	
L. plantarum 8PA3 + *Bifido. bifidum*	nd	yes								
L. rhamnosus GG + *L. rhamnosus* Lc705 + *P. freudenreichii shermanii* JS + *Bifido. breve* Bb99	nd	yes				++				
L. acidophilus + *L. plantarum* + *L. rhamnosus* + *Bifido bifidum*	nd-	yes								

L. brevis CD2 + *L. salivarus* FV2 + *L. plantarum* FV9	nd	yes							+	
L. acidophilus + *L. paracasei* + *L. delbrueckii ssp. bulgaricus* + *L. plantarum, Bifido. longum, Bifido. infantis, Bifido. breve*	nd	yes	-		++	+				++
No effect on microbiota										
B. clausii	nd	nd						-		-
L. plantarum 299v	nd	no	-	-		-				
Bifido. lactis	nd	no	+							**0**
Bifido. breve	no	no								
L. acidophilus + *Bifido. longum*	no	--								
L. rhamnosus 19070-2 + *L. reuteri* DSM	nd	no								**0**
L. casei + *Bifido. breve*	nd	no								
L. paracasei + *L. acidophilus* + *Bifido animalis*	nd	no								
Pharmacokinetic only										
L. reuteri 55730	nd	nd								+
L. johnsonii La1	nd	nd			-			+		
L. salivarius UCC4331	nd	nd				-				
Bifido. infantis 35624	nd	nd				**0**				
Bifido. bifidum MIMBb75	nd	nd				+				
L. rhamnosus + *Bifido. longum*	nd	nd								

*including strain (when reported)

** **Rank:** ++, ≥2 net RCTs (randomized controlled trials) with significant protective efficacy; +, only one net protective RCT; 0, equal number of significant and non-significant RCTs; -, ≥1 net non-significant RCT. Blank indicates no RCT done for the disease indication.

Abbreviations: nd, not determined; AAD, antibiotic associated diarrhea; CDI, Clostridium difficile infections; IBD, inflammatory bowel disease; IBS, irritable bowel syndrome; TD, traveler's diarrhea; BV, bacterial vaginosis; Acute Ped Diar, treatment of acute pediatric diarrhea.

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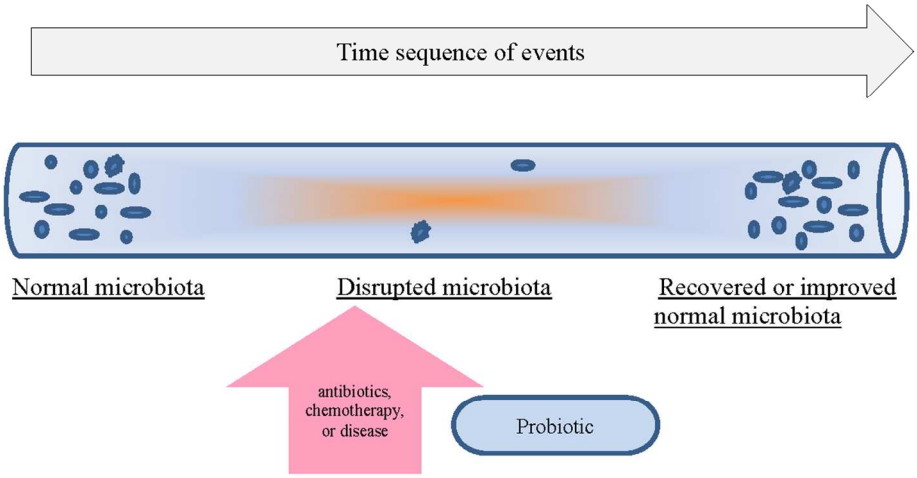
Figure 1. Time sequence of events and three models of study designs determining three different degrees of dysbiosis correction by probiotics.

Figure 2. Flow-chart of literature review results (1985-2013) of included and excluded studies for the restoration or improvement of normal microbiota by probiotics. Abbreviations: RCT, randomized-controlled trials; MOA, mechanism of action; NM, normal microbiota.



Model	Type of population enrolled	Dysbiosis at baseline	Time microbiota disrupted	Probiotic or control intervention	Potential outcomes
A	Healthy volunteers or at-risk patients	no	post-baseline	preventive	restoration
B	Patients with active disease at enrollment	yes	pre-baseline	treatment	altered or improved
C	Healthy volunteers	no	not disrupted	preventive	altered

Figure 1. Time sequence of events and three models of study designs determining three different degrees of dysbiosis correction by probiotics
112x102mm (300 x 300 DPI)

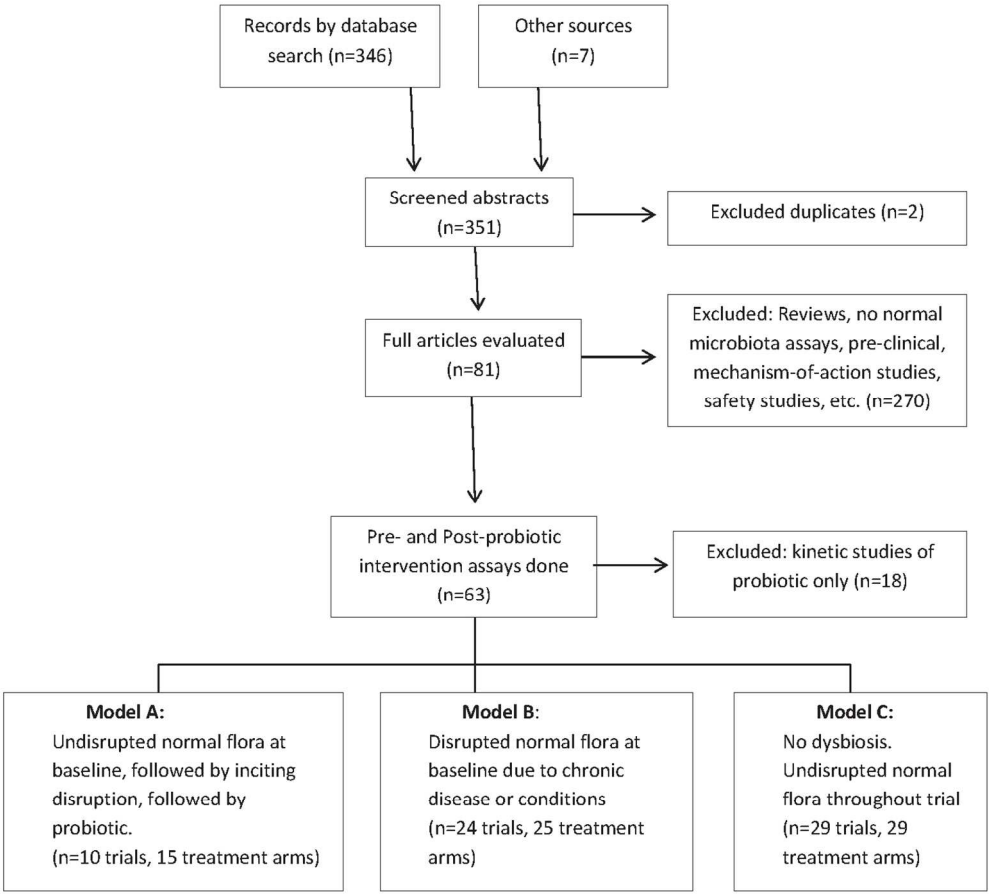


Figure 2. Flow-chart of literature review results (1985-2013) of included and excluded studies for the restoration or improvement of normal microbiota by probiotics. Abbreviations: RCT, randomized-controlled trials; MOA, mechanism of action; NM, normal microbiota.
125x113mm (300 x 300 DPI)

PRISMA 2009 Checklist

Section/topic	#	Checklist item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both. **This is a systematic review**	1
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	2
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known. **Probiotics are promising candidates to prevent or treat disease, but are typically supported by structure/function claims in most countries. The function claim for the restoration of normal microbiota is commonly cited in efficacy trials, but the evidence for this claim has not been examined systematically for all probiotic strains. Differences in study populations and study design effect the type of conclusions that can be drawn. This is the first systematic review and proof of principle of this type of analysis for the function claim of dysbiosis.**	4-5
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS). **A comprehensive literature review of the evidence from randomized controlled trials will discuss the strength of the evidence for the restoration or improvement of dysbiosis by specific probiotic strain. The interventions include probiotic or control (typically placebo) given for a specific time enrolled in clinical trials for either the prevention or treatment of disease. All trials which stated some impact on the normal microbiota will be reviewed and analyzed for the ability to document changes in the normal microbiota. The outcomes are the degree of dysbiosis restoration depending upon the study design and type of enrolled participants**.	5
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number. **Review protocol is described in Methods section of paper.** **Prospero registration number is: CRD42014007224**	5-9
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale. **Participants: without restriction, any enrolled in clinical trial (adults and pediatrics)**	6

PRISMA 2009 Checklist

		Interventions: all probiotics **Comparisons: controlled (typically placebo)** **Outcomes: microbiologic assays of the intestinal flora or microbiota** **Study design: required to have pre-intervention (baseline) and post-intervention microbiological assays** **Length of follow-up: unrestricted** **Language: unrestricted** **Publication and years considered: peer-reviewed publications from PubMed (1985-2013, unless otherwise noted), EMBASE, Cochrane Database (1990-2013), CINAHL, AMED, ISI Web of Science (2000-2013). On-line trial registries: (Cochrane Central Register of Controlled trials, MetaRegister of Controlled Trials, and National Institutes of Health.**	
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched. **See item above**	6
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated. **All probiotics + health claims, structure/function claims, normal microbiota, normal intestinal flora, dysbiosis, pharmacokinetics, metagenomics, dietary supplements, randomized controlled trials, antibiotic-associated diarrhea, Clostridium difficile infections, H. pylori treatments, inflammatory bowel disease, irritable bowel disease, travelers diarrhea, bacterial vaginosis or vaginitis, treatment of pediatric acute diarrhea, healthy volunteer trials and specific probiotic strains. (PubMed)**	5-6
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis). **Screening:** **Eligibility: Must have at least one pre-intervention (baseline) microbiological assay of normal flora or metagenomic analysis and one post-intervention (post-probiotic) assay. Genus and species of probiotic strain(s) provided. Normal microbiota assayed during a randomized, controlled trial.** **Excluded: pre-clinical studies, safety studies, reviews, mechanism of action studies, case reports or case series, duplicate reports, unspecified type of probiotics.**	6-7
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators. **Pilot data extraction form used modified from standard meta-analysis data extraction form (McFarland and Goh 2013, World J Gastroenterol). Questionable results were queried from original authors of papers.**	6
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made. **The timing and type of microbiologic assays of intestinal or vaginal microbiota were collected. As the literature review has a length inclusion period (1985-2013), the types of microbiologic assays have evolved, but all types were included from basic microbiologic assays to metagenomic profiling. The type of probiotic intervention was collected by genus, species and strain (if stated in paper). Types of normal microbiota assays varied by technique. The patient population (healthy volunteers, acute disease or chronic disease) was also collected. Also collected: study size, type of disruptive factor, follow-up duration, stated claims in**	7-8

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		paper.	
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis. **Quality of study design (restoration, improvement or no dysbiosis) was used when assessing quality of individual studies. These were then analyzed by stratification on the quality of study design.**	8
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means). **NA, No pooled RR or DWMs used in this systematic review.**	na
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis. **NA, No pooled RR or DWMs used in this systematic review.**	na

Section/topic	#	Checklist item	Reported on page #
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies). **All studies with microbiota were included to limit bias, but no measurement for publication bias was done for this systematic review.**	8
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified. **association of degree of dysbiosis correction with clinical efficacy by probiotic strain(s)**	8-9
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram. **See flow diagram, Figure 2.**	25
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations. **Extracted data were cited in tables. See paper-Tables 1-4.**	26-32
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12). **Presented in Discussion section.**	11-15
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot. **Cited in Tables 1-4.**	26-32
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency. **NA, this is a systematic review, not a meta-analysis.**	na
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see item 15).	18-19

PRISMA 2009 Checklist

		Presented in Discussion section (bias due to study design).	
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	15-17
DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers). **Strength of the evidence is provided in the Results section.** **Relevance to key groups is in the Discussion section.**	19, 22
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias). **Provided in Discussion section and "Opportunities for Future Research" section and 'Strengths and Weaknesses" section.**	21-22
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research. **The weight of the evidence for the function claim that probiotics can improve or restore normal microbiota is strong for a few probiotic strains, but in general, more confirmatory studies that are properly timed and designed are required for the majority of probiotic strains.**	20-21
FUNDING			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review. **This review was unfunded.**	23

From: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLoS Med 6(6): e1000097. doi:10.1371/journal.pmed1000097

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