Evidence synthesis of probiotic supplementation to dairy calves and its safety: scoping review, meta-analysis, and antimicrobial resistance

By

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ABSTRACT

Probiotics, also known as direct-fed microbials, have been proposed as a strategy to improve growth and enhance health of dairy calves. However, the available literature regarding the efficacy of probiotic supplementation on calf growth and feed intake is inconsistent. Moreover, scarce information exists on the safety of commercially available cattle probiotics, particularly concerning their antibiotic resistance profile. Thus, the objectives of this dissertation were to: (i) identify, describe, and characterize the literature on probiotic supplementation in dairy calves, (ii) quantify the effect of probiotic supplementation, considering different probiotics combined and categorized by probiotic genus, on ADG, feed intake, and feed efficiency of dairy calves and (iii) evaluate the phenotypic antimicrobial susceptibility of Enterococcus and Bacillus ssp. isolated from commercially available cattle probiotics. For the first objective, a comprehensive scoping review was performed, compromising studies with non-randomized, quasi- randomized, and randomized controlled trials in English, Spanish, or Portuguese that evaluated the effect of probiotic supplementation on the growth and health of dairy calves. Searches were conducted in Biosis, CAB Abstracts, Medline, Scopus, and the Dissertations and Theses Database. In total, 4,467 records were retrieved, of which 103 studies (110 controlled trials) met the inclusion criteria. The studies were published between 1980 and 2021 and originated from 28 different countries. Eighty percent of the trials were randomized, with most using Holstein calves (74.5%) which were under 15 d old at the start of probiotic supplementation (71.8%). The most studied probiotics were *Lactobacillus acidophilus* and *Enterococcus faecium*. On average, supplementation lasted 50 d, and probiotics were mostly mixed into feed (88.5%). Most trials measured weight gain (88.2%) and fecal consistency (64.5%) to assess growth and health, respectively. For the second objective a scoping review and meta-analysis were conducted. It

included quasi-randomized and randomized controlled trials that assessed the effects of probiotic supplementation on the growth and feed intake of dairy calves. After applying the inclusion criteria, 48 studies (49 controlled trials) were included in the study. Meta-analyses indicated that probiotics did not significantly affect total dry matter intake (DMI) or feed efficiency (FE) in dairy calves compared to controls. However, probiotic supplementation improved starter intake ($P = 0.02$) and average daily gain (ADG, $P = 0.001$) and showed a trend toward reduced milk intake ($P = 0.09$). Upon examining specific probiotic types, only Bacillus ssp. supplementation significantly increased ADG ($P = 0.03$). High and significant heterogeneity was observed for all outcomes. Meta-regression demonstrated significant associations between total DMI and probiotic type ($P = 0.001$) as well as the duration of supplementation ($P <0.001$). Additionally, meta-regression results indicated a significant association between starter intake and probiotic type ($P = 0.006$) and the duration of probiotic supplementation ($P = 0.003$). For the last objective, the antimicrobial susceptibility of 35 cattle probiotic products that claimed to contain Enterococcus spp. or Bacillus ssp. was determined. All 16 Enterococcus isolates were susceptible to chloramphenicol, streptomycin, tetracycline, tigecycline, and vancomycin. Nine Enterococcus isolates were resistant to one antibiotic (ciprofloxacin, erythromycin, penicillin, and daptomycin) and two isolates were resistant to two antibiotics. One Enterococcus isolate was multidrug-resistant to ciprofloxacin, daptomycin, and quinupristin/dalfopristin. All 15 Bacillus isolates showed susceptibility to tetracycline and vancomycin. However, one Bacillus isolate displayed resistance to chloramphenicol, and the other to erythromycin. In conclusion, the incomplete reporting across trials suggests a need for adhering to standardized guidelines in future research. While probiotic supplementation may increase ADG and starter intake in dairy calves, the existing evidence is limited due to substantial heterogeneity, and more research is

needed. The results of antimicrobial susceptibility profiles emphasize the need for safety assessment in commercially available probiotics.

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Chapter 1: Characterization of controlled trials on probiotic supplementation to dairy calves: a scoping review

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ABSTRACT

The objective of this scoping review was to identify, describe, and characterize the literature on probiotic supplementation in dairy calves. Eligible studies were non-randomized, quasi-randomized and randomized controlled trials in English, Spanish, or Portuguese that evaluated the effect of probiotic supplementation on growth and health of dairy calves. The search strategies were based on a modification of **PICO** (Population, Intervention, Comparator, Outcome) framework and used synonyms and related words to "dairy calves" (population), "probiotics" (intervention), and "growth and health measurements" (outcomes). No restrictions for publication year or language were applied. Searches were conducted in Biosis, CAB Abstracts, Medline, Scopus, and the Dissertations and Theses Database. In total, the search identified 4,467 records, of which 103 studies (110 controlled trials) met the inclusion criteria. The studies were published between 1980 and 2021 and originated from 28 different countries. Trials were randomized (80.0%), non-randomized (16.4%), and quasi-randomized (3.6%), ranging in sample size from 5 to 1,801 dairy calves (mode = 24; average = 64). Enrolled calves were frequently Holstein (74.5%), males (43.6%), and younger than 15 d at the beginning of probiotic supplementation (71.8%). Often trials were conducted in research facilities (47.3%). Trials evaluated probiotics with single or multiple species of the same genus: Lactobacillus (26.4%), Saccharomyces (15.4%), Bacillus (10.0%), Enterococcus (3.6%) or multiple species of various genera (31.8%). Eight trials did not report the probiotic species used. *Lactobacillus*

acidophilus and *Enterococcus faecium* were the species most supplemented to calves. The duration of probiotic supplementation ranged from 1 to 462 d (mode = 56; average = 50). In trials with a constant dose, it ranged from 4.0×10^{6} to 3.7×10^{11} cfu/calf/d. Most probiotics were administered mixed solely into feed (88.5%; whole milk, milk replacer, starter or TMR) and less frequently orally as a drench or oral paste (7.9%). Most trials evaluated weight gain (88.2%) as a growth indicator, and fecal consistency score (64.5%) as a health indicator. Our scoping review summarizes the breadth of controlled trials evaluating probiotic supplementation in dairy calves. Differences in intervention design (mode of probiotic administration, dose, and duration of probiotic supplementation) and outcomes evaluation (type and methods) justify future efforts towards standardized guidelines in clinical trials.

Keywords: cattle, direct-fed microbial, feed additive, review.

INTRODUCTION

Probiotics, also known as direct-fed microbials, have been proposed as a strategy to improve growth and enhance health of dairy calves (Cangiano et al., 2020). The International Scientific Association for Probiotics and Prebiotics defines probiotics as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill et al., 2014). According to the US National Animal Health Monitoring System, 23% of dairy heifer operations supplement probiotics, and this management strategy is more common in large operations (38%; >500 cows; USDA, 2016).

Traditionally, lactic acid-producing bacteria (e.g., Lactobacillus ssp.; Uyeno et al., 2015) and yeast-based products (e.g., Saccharomyces ssp.; Alugongo et al., 2017) have been the most studied dairy calves probiotics. Additionally, according to the 2019 Direct-fed-Microbial, Enzyme and Forage Additive Compendium (Feedstuffs, 2019), most probiotics commercialized

for cattle contain *Lactobacillus acidophilus* and *Enterococcus faecium*. In dairy calves, the proposed benefits associated with probiotic supplementation include improving average daily gain and feed efficiency (Sun et al., 2010; Le et al., 2017), reducing antibiotic use, maintaining growth rate in diarrheic calves (Villot et al., 2019), and decreasing the incidence of scours (Timmerman et al., 2005). Probiotics provide health or productivity benefits to the host by different mechanisms, such as competing for adhesion to the epithelium and for nutrients, producing antibacterial substances (e.g., bacteriocins and hydrogen peroxide), changing the gastrointestinal microenvironment (e.g., pH lowering), and modulating the immune system (Bermudez-Brito et al., 2012).

Evidence synthesis methods have been used to evaluate the efficacy of probiotics on calves (Frizzo et al., 2011; Signorini et al., 2012; Alawneh et al., 2020). However, none of these previous reviews has mapped current literature trends, or addressed differences in study characteristics (e.g., funding source and facility where experiment was carried out), study design (e.g., sample size justification and randomization process) and outcomes characteristics (e.g., description of evaluators and their training) across controlled studies. This information is key to interpreting results from existing studies and informing future research during the process of study design. A scoping review (**ScR**) is the appropriate evidence synthesis method to examine how research on a certain topic is conducted and to identify and analyze knowledge gaps (Munn et al., 2018). A scoping review addresses broader review questions than systematic reviews and meta-analyses, which are frequently focused on effectiveness (Peters et al., 2020). The results of ScR are not used for recommendations; these reviews describe rather than analyze the available information (Lockwood et al., 2019). Therefore, the objective of this study was to identify, describe, and characterize the literature on probiotic supplementation in dairy calves.

METHODS

The protocol for this ScR was adapted from a protocol developed for a systematic review on probiotic use for dairy calves which is deposited on UC Davis eScholarship (https://escholarship.org/uc/item/2r93v26f) and is available via Systematic Reviews for Animals and Food (http:// https://www.syreaf.org/protocol/). A summarized version of the protocol is presented in Supplemental material 1. This manuscript is reported following the Preferred Reporting Items for Systematic Review and Meta-Analysis (**PRISMA**) extension for scoping reviews (**PRISMA-ScR**; Tricco et al., 2018).

Eligibility Criteria

Eligible studies were primary research studies, including non-randomized, quasi-randomized and randomized controlled trials, written in English, Spanish, or Portuguese (at least one reviewer was proficient in these languages). Published studies as peer-reviewed and non-peer-reviewed articles (thesis and dissertations) were included in the ScR. Eligibility criteria were defined based on the PICO (Population, Intervention, Comparator, Outcome) framework (Cooper et al., 2018). The target population was dairy calves (up to 7 mo of age at enrolment), with no restrictions on calves' breed or sex. The intervention of interest was probiotic supplementation, regardless of probiotic type, dose, or supplementation duration. Studies that named probiotics as direct-fed microbials were also included. Studies using probiotics as treatment therapy were not considered. The eligible comparator was control group consisted of calves that were untreated or received placebo. Eligible studies included publications that measured a growth performance outcome (e.g., ADG, body traits) or a health outcome (e.g., serum metabolites, immunological parameters).

Information Sources

Identification of the electronic databases was completed with the assistance of a UC Davis research librarian specialized in veterinary science. The following databases were searched to identify relevant literature: Biosis (Web of Science, 1926 to 2021), CAB Abstracts (CAB Direct, 1973 to 2021), Medline (PubMed, 1966 to 2021), and Scopus (Scopus, 1996 to 2021). The Dissertations and Theses Database (ProQuest, 1861 to 2021) was searched to retrieve grey literature. In addition, the bibliography of relevant studies was hand-searched.

Search Strategy

The search strategy was developed based on the PICO framework and in collaboration with a UC Davis librarian. Key words were selected for each PICO concept from relevant literature. Posteriorly, the librarian performed a keyword mining in PubMed and CAB Direct. The Yale MeSH analyzer was also used to compare common Medical Subject Headings across studies for PubMed. No restrictions for publication year, study design, or language were applied during the searches. The literature search was conducted on February 27th and March 3rd of 2020 and updated on 19th August 2021. The search strategy used in Medline (PubMed) is described in Table 1.1, search strings were adjusted accordingly to each database to fit its specific formats (Supplemental material 1; escholarship.org/uc/item/4js544c0). Search results were uploaded to the reference manager F1000 (Faculty of 1000 Limited, London, UK), and duplicates were removed. The de-duplicated results were then exported to the Covidence systematic review management software (Veritas Health Innovation, Melbourne, AU) for screening.

Selection of Sources of Evidence

All records were screened for eligibility twice by two reviewers independently. Both reviewers were trained on the methodology, and none of them were blinded to journal or author names. First, the titles were screened, using the following questions to identify eligible studies:

1) Does the title describe a study involving dairy calves?
2) Does the title describe a study with probiotic(s) supplementation?

Second, the abstracts were screened, using the following questions:

3) Does the abstract describe a primary research study?
4) Does the abstract describe a study involving dairy calves supplemented with probiotic(s)?
5) Does the abstract describe one or more measurements of performance (e.g., ADG, feed efficiency) or health (e.g., fecal consistency score, diarrhea incidence)?

For title and abstract screening questions, the possible answers were ‘no’, ‘maybe’, and ‘yes’. Studies were excluded if both reviewers answered ‘no’ to any question. In both screenings (title and abstract), conflicts between the two reviewers were discussed until a consensus was reached.

Full-text screening was conducted by one reviewer. This screening included questions 3, 4, and 5, which were adapted for full-text screening (the word “study” was used instead of “abstract”), plus:

6) Is the study a trial with a control group?
7) Is the study written in English, Spanish, or Portuguese?
8) Is the probiotic a supplementation strategy (not treatment for sick animals)?
9) Is the study population (dairy calves) equal or less than 7 mo old at enrollment?

During the full-text manuscript screening the available answers were ‘no’ and ‘yes’. Studies were excluded if the answer was ‘no’ for at least one of the questions. The exclusion reason was recorded at this screening level. All screening questions were pilot tested. For this, the questions were developed according to the eligibility criteria and tested on the first 30 studies listed in the systematic review management software (Covidence). Primary screening questions (1 to 5) were tested by two reviewers and secondary questions (6 to 9) by one reviewer, subsequently clarity and objectivity of questions were discussed with a third reviewer.

Data Charting Process and Data Items

Data from studies that met eligibility criteria were extracted into a pre-designed form built on Microsoft Excel (Excel 2022 v. 2206, Microsoft Corp.). Data extraction form was pilot tested on 5 studies randomly selected and the relevance of the data extracted was discussed with a second reviewer. Study-level data consisted of authors’ name, journal’s name, language, trial’s country (when not available, the country was based on first author’s affiliation), year of publication, funding source, randomization process, sample size, sample size justification and facility type (commercial vs. research). Population characteristics consisted of calf breed, sex, age at the start of probiotic supplementation. Intervention and comparator data consisted of description of comparator, commercial name of probiotic, scientific name, dose, mode of probiotic administration (e.g., whole milk, milk replacer), and duration of probiotic supplementation. Outcomes data consisted of all outcomes evaluated, method of measurement, evaluation frequency, evaluators, and their training and blinding. To guarantee the accuracy of data extracted from selected studies, the extraction process was performed twice, and discrepancies were corrected.

Synthesis of Results

The screening process was described using the PRISMA flowchart (Page et al., 2021). Microsoft Excel was used to summarize eligible studies in tables and graphs and to generate numerical outcomes' metrics such as average, mode and range. The Choropleth map was developed using MapChart (MapChart, 2021). Based on the reported probiotic genus, trials were classified into six categories: Bacillus (**BC**, including monospecies and multispecies), Enterococcus (**ENT**, including monospecies), Lactobacillus (**LB**, including monospecies and multispecies), Saccharomyces (**SC**, including monospecies), multiple genera (**Multi**, including monospecies and multispecies**)**, or undefined species and less frequent genera (**Other**, including monospecies). Throughout the manuscript the data is presented at 3 different levels: study, trial, and arm (or treatment group).

RESULTS

Selection of Sources of Evidence

Results of the databases search, screening process, and exclusion reasons are summarized in Fig. 1.1. In total, the search identified 4,467 records, of which 3,426 remained after de-duplication. After all screening levels, 3,323 records were considered irrelevant and excluded. During the full-text manuscript screening, 46 manuscripts were excluded, mainly due to lack of clarity on breed purpose of enrolled animals (41.3%; 19/46 studies with inconclusive dairy or beef breed). Overall, 103 studies met the eligibility criteria and were included in the ScR, reflecting 110 controlled trials and 322 arms (165 arms evaluated probiotics). The complete list of included papers is presented in Supplemental material 1 (escholarship.org/uc/item/4js544c0).

General Study Characteristics

Study-level characteristics. The included studies were published between 1980 and 2021 as peer-reviewed articles (n = 100; 97.1%) or as dissertations or theses (n = 3; 2.9%). Most of the studies were published within the last decade (n = 61; 59.2%) indicating increased research interest in probiotic supplementation to dairy calves over the years. The number of studies published per year stratified by probiotic type is represented in Fig. 1.2. The geographical distribution of studies is shown in Supplemental material 1. Studies were conducted in 28 different countries, mainly in the US (n = 22; 21.3%), Brazil (n = 13; 12.6%), and China (n = 9; 8.7%). Most studies were written in English (n = 88; 85.4%), followed by Portuguese (n = 11; 10.7%), Spanish (n = 2; 1.9%), or Spanish and English (n = 2; 1.9%).

Study design and randomization. The 103 studies included 110 trials (control vs. treatment: n = 52; control vs. multiple treatments: n = 58), of which 88 were randomized, 18 non-randomized, and 4 quasi-randomized trials. Randomized trials were designed as randomized controlled (n = 50; 56.8%), randomized block (n = 21; 23.9%), factorial (n = 16; 18.2%), or incomplete block designs (n = 1; 1.1%). Among randomized and quasi-randomized trials (n = 92), only nine trials described the randomization process (i.e., preassigned ballots “in a hat” strategy, random list, ear tag, location, alternation), while the remaining trials solely noted that calves were randomly allocated to treatments (n = 83). Randomized block trials (n = 21) used the following variables alone or in combination as blocking factors: body weight or birth body weight (n = 10), sex (n = 7), birth date (n = 4), age (n = 2), serum total protein (n = 2), breed (n =1) and farm of origin (n =1).

Sample size. Trial sample size ranged from 5 to 1,801 dairy calves (mode = 24; average = 64). Half of trials (n = 56; 50.9%) enrolled 30 or fewer calves (Table 1.2). The sample size was

justified in nine trials based on power analysis (n = 8), and facilities capacity (n = 1). Also, one trial reported a sample size justification, but the outcome used for it was unclear. The outcomes used for power analysis calculations were growth performance (n = 3), gut total bacteria concentration (n = 1), antimicrobial resistance of fecal coliforms counts (n = 1), diarrhea incidence (n = 1), *Mannheimia haemolytica* incidence (n = 1), and both rectal temperature and total peripheral blood neutrophil counts (n = 1).

Funding. Studies were funded by public institutions (n = 39; 37.9%), private companies (n = 13; 12.6%), both public and private institutions (n =10; 9.7%), did not receive financial support (n = 2; 1.9%), or did not disclose funding (n = 39; 37.9%). Some studies that did not provide funding source had probiotic, feed, seed, or pharmaceutical-industry authorship (n = 10; 25.6%).

Facility type. Trials were conducted in research facilities (n = 52; 47.3%), commercial dairy facilities (n = 28; 25.4%), or did not provide the facility type (n = 30; 27.3%; Table 2). Among trials performed in commercial settings, 78.6% were conducted in farms (n = 17; dairy farm and n = 5; unspecified type of farm), and less frequently in calf raising operations (n = 3; 10.7% calf ranch and n = 3; 10.7% veal facility).

Population

Breed. Enrolled calves were mainly Holstein (n = 82; 74.5%); others were Holstein crossbreed (n = 7; 6.4%), Jersey (n = 3; 2.7%), at least two different breeds (n = 7; 6.4%), and other breeds (n = 9; 8.2%). Calves' breed was not reported in two trials, although the authors stated that calves were from dairy herds (Table 2).

Sex. Often trials included only males (castrated or not; n = 48; 43.6%; Table 2), followed by trials that enrolled both males and females (n = 31; 28.2%), and those including only females (n = 21; 19.1%); sex of calves was not provided in 10 trials (9.1%).

Age. The calves' age at start of supplementation ranged from birth to 192 d (mode = 1; average = 21; Table 2). Most trials (n = 79; 71.8%) started probiotic supplementation when calves were younger than 15 d. The calf age was unclear or not reported in nine trials.

Intervention and Comparator

Probiotics. A full description of the species investigated by the studies is provided in Supplemental material 1. The individual probiotic genus most investigated was Lactobacillus (n = 29 trials; 26.4%), followed by Saccharomyces (n = 17 trials; 15.4%), Bacillus (n = 11 trials; 10.0%); however, most of the studies investigated multispecies probiotics which contained diverse genera (n = 35 trials; 31.8%). Among trials, *L. acidophilus* was the species most studied (n = 58 arms); 11 different strains of *L. acidophilus* were evaluated. *Enterococcus faecium* was the second most evaluated strain (n = 33 arms) and 6 different *E. faecium* strains were assessed. Eight trials did not report the probiotic species used. Forty-eight different commercial products were assessed; but only few of those were evaluated three or more times [Levucell SC (n = 6 trials; Lallemand Biochem International, Milwaukee, WI, USA), Levucell SB20 (n = 6 trials; Lallemand Biochem International, Milwaukee, WI, USA), Protexin (n = 4 trials; Probiotics International Ltd., South Petherton, UK), Biomate FG (n = 3 trials; Chr Hansen, Milwaukee, WI, USA), and BioPLus 2B (n = 3 trials; Chr Hansen, Milwaukee, WI, USA)].

Duration of supplementation. The duration of probiotic supplementation is represented in Fig. 1.3. Overall, the duration of probiotic supplementation ranged from 1 to 462 d (mode = 56; average = 50). Probiotics were often supplemented for 60 d or less (n = 109 arms; 66.1%),

and less frequently more than 76 d (n = 17 arms; 10.3%). The duration of supplementation was unclear or not reported in 16.4% of arms (n = 27).

Mode of administration. The mode of probiotic administration is represented in Fig. 1.4. Probiotics were administered mixed solely into feed (n = 146 arms; 88.5%; colostrum, milk, milk replacer, starter, concentrate, or TMR) and less frequently orally as drench, gel, or paste (n = 13 arms; 7.9%), ruminally (n = 1 arm; 0.6%), or nasally (n = 1 arm; 0.6%). For all six probiotic categories, milk (colostrum, skimmed, whole or waste milk, and milk replacer) was the most adopted mode of probiotic administration (BC: 53.3%; ENT: 50.0%; LB: 76.6%; Multi: 67.3%; SC: 43.3%; Other: 64.7%).

Dose. The probiotic dose administered to dairy calves in each trial is presented in Supplemental material 1. In trials with a constant dose throughout the experiment, the probiotic dose ranged from 4.0×10^{6} to 3.7×10^{11} cfu/calf/d. The constant dose was $\geq10^{9}$ cfu/calf/d in 100% of ENT and SC, 85.7% of BC and Other, 63.1% of LB, and 57.8% of Multi trials. The dose was not reported in 7 trials (6.4%).

Comparator. Few trials used a placebo (n = 19; 17.3%) as the comparator group, whereas most of the trials adopted a non-supplemented group as control (n = 91; 82.7%).

Outcomes

Growth. The most common growth performance outcomes were weight gain (n = 97; 88.2%), feed intake (n = 75; 68.2%), and feed efficiency (n = 53; 48.2%; Fig. 1.5). The method used to measure or estimate weight was specified in 16.5% of the trials (scale: n = 15; tape: n = 1). Calves' weight was determined weekly (n = 43; 44.3%), every other week (n = 10; 10.3%), or in a different pattern (e.g., at enrollment and at the end of the experimental period, monthly, 3

times during experiment; n = 35; 36.1%). The frequency of weight measurement was not reported in 9 trials (9.3%).

Health. Common health outcomes evaluated included fecal consistency score (n = 71; 64.5%), fecal, intestinal, or ruminal microbiota (n = 52; 47.3%), and blood parameters (n = 51; 46.4%; Fig. 5). Most trials (n = 42; 59.1%) provided a reference for the fecal consistency scoring system used; 19 different references were used across trials. Larson et al. (1977) was the most commonly adopted scoring system (n = 13; 30.9%). The frequency of fecal consistency score assessments varied across trials from daily (n = 51; 71.8%) or 2 to 3 times/d (n = 5; 7.0%) to less frequently (e.g., every other day, twice a week, or weekly; n = 7; 9.8%). Eleven percent of the trials did not report the frequency of assessment (n = 8).

Evaluator. The evaluator of the outcomes was reported in 12 trials (10.9%), and in 7 trials (6.4%) evaluators were blinded while assessing at least one outcome. Measurement of at least one outcome was performed by veterinarians (n = 5), farm workers (n = 3), researchers (n = 3), and a specialist in veterinary medicine (n = 1). The training of the evaluators was reported in only 3 trials (2.7%); however, one trial reported training for outcomes that posteriorly were not statistically analyzed or described in the results.

DISCUSSION

This scoping review aimed to identify, describe, and characterize controlled trials evaluating probiotic supplementation in dairy calves. The large number of studies included in this review demonstrates extensive research on probiotics supplementation to dairy calves, which has spanned four decades and has been performed worldwide. This substantial literature supports future systematic reviews and meta-analyses, which may be more comprehensive than previous reviews (Frizzo et al., 2011; Signorini et al., 2012; Alawneh et al., 2020). However, the

variability of the experimental design and the incompleteness of reporting among studies may make meta-analyses challenging. Given the observed trend for increased research interest in probiotic supplementation to dairy calves, it is of utmost importance that researchers exercise best practices when designing future experiments so the aforementioned powerful synthesis methods can help the research community and additional stakeholders reach conclusions on a demonstrated area of research interest.

General Characteristics of Selected Studies

Randomization. In our ScR, description of the randomization process was uncommon among the randomized trials (9.7%). Adequate reporting of the allocation method is essential in systematic reviews, which evaluate the risk of bias (Page et al., 2021). The assessment of risk of bias considers biases arising from different domains (different stages of a trial). There are different tools to evaluate the risk of bias, for example, the Cochrane tool includes five bias domains and one is related to randomization (Sterne et al., 2019). An inadequate reporting of a randomization process does not mean that the methods were inappropriate or that the study had flaws (details could be omitted due to limited word counts); however, it will increase the risk of bias when summarized by synthesis methods such as systematic reviews (Drucker et al., 2016).

Randomization may not always result in comparable groups and pre-treatment assessment may be important to inspect baseline differences. Jarett et al. (2021) proposed best practices for microbiome study design in companion animals and recommended pre-screening of the gut microbiome of all animals before enrollment. Previous research in humans suggests that probiotic efficacy might depend on the initial characteristics of the microbial ecosystem (e.g., butyrate concentration; Ferrario et al., 2014). There are different study designs to tackle interindividual microbiome variability, for example, one study included in our ScR adopted a

cross-over design with a 20 d washout period (Watanabe et al., 2019). However, determining the length of the washout period may be challenging and the probiotic may persist after the washout (Gibson et al., 2011). Although the consideration of initial microbiome composition in the study design (e.g., treatment allocation) may not be always feasible, future studies should assess and report microbiome composition prior to probiotic treatment as it may be a key determinant of probiotic effectiveness.

Sample size. Fifty percent of trials enrolled 30 or less calves and only 10% justified the sample size. Consequently, some of the trials included in the present ScR might have underpowered statistical tests, which are prone to incur a Type II error (Christley, 2010), and fail to reject a null hypothesis that is actually false. A power analysis estimates the minimum sample size needed to statistically detect the difference between treatment groups (Cohen, 1992). It should be calculated during the experimental design, as post hoc analyses are considered conceptually flawed and analytically misleading (Zhang et al., 2019). If power analysis is used to justify the sample size for a primary outcome as shown in this ScR (e.g., based on diarrhea incidence or *Mannheimia haemolytica* incidence), the remaining outcomes of that study should be interpreted with caution.

Funding. In our ScR, 37.9% of the reviewed studies failed to disclose sponsorship; this is a larger proportion than previously reported in probiotics human studies (23.9 to 32.0%; Mugambi et al., 2013; Saa et al., 2019). One review by Mugambi et al. (2013) reported that industry-funded studies were more likely to report at least one favorable outcome than non-industry-funded trials, but Saa et al. (2019) did not find that association. Disclosing the funding source would help readers evaluate publication bias, which occurs when studies with positive results are more likely to be published (Song et al., 2010).

Facility type. Our ScR revealed that trials were often conducted at research facilities (47.3%). It is generally assumed that research facilities are managed following industry best practices, which may decrease the challenges that animals are exposed to. Moreover, personnel of research facilities might be better trained to implement treatments and handle samples. In contrast, using commercial facilities in research studies may require undesired adjustments to the study protocol based on their husbandry practices, and farm personnel may have little experience or time to comply with the study protocol. However, Johnston et al. (2003) pointed out that experiments conducted in commercial farms may provide valuable information on the efficacy of new technologies. Additionally, large commercial operations may offer the opportunity to enroll larger number of animals (Engstrom et al., 2010). Thus, study methods should clearly describe the trial facilities, as well as implemented husbandry practices (Nevalainen, 2014) and the role of farm personnel in study implementation regardless if studies are conducted in commercial or research facilities.

Population

Based on the PICO framework, our eligibility criteria for population (dairy calves up to 7 mo at enrollment) were established to include studies supplementing probiotics in preweaned and weaned dairy calves. Traditionally, probiotics have been studied as a strategy to improve gut health and decrease diarrhea (Cangiano et al., 2020), which is more likely in preweaned calves, especially during the first three weeks of life (Klein-Jöbstl et al., 2014; Cruvinel et al., 2020). The high interest on probiotic supplementation in early life is seen in this ScR with most of the trials starting probiotic supplementation at <15 d of life. At early ages, the gastrointestinal microbiota is highly variable and prone to change (Jami et al., 2013; Yáñez-Ruiz et al., 2015) potentially making microbial modulation by probiotics more effective. Probiotics have also been

explored as growth promoters in calves and in other livestock animals (Barba-Vidal et al., 2019; Jha et al., 2020). Thus, a different reasoning may explain the use of probiotics at older age, as shown in the range of age at start of supplementation summarized in this ScR.

Our ScR revealed that frequently probiotic research in dairy calves was undertaken with Holstein and male calves. We hypothesized that the lower purchase price of males compared to females dairy calves (Marquou et al., 2019) may have influenced the higher use of males, especially in experiments that euthanized animals. It has been suggested that the composition of gastrointestinal microbiota may differ by breed (Gonzalez-Recio et al., 2018) and sex (Li et al., 2019). Thus, since probiotic effectiveness may be influenced by the initial microbiome, it is important to conduct probiotic research with different sexes and breeds, and these need to be taken into consideration when evaluating probiotic research.

Intervention

Our ScR found that most studied genus was Lactobacillus. Additionally, it is noteworthy that eight studies did not state the species evaluated. *L. acidophilus* followed by *E. faecium* were the most evaluated strains among trials. Both species are highly used in commercial probiotics designed for cattle use (Feedstuffs, 2019) and are constituents of calves' gut microbiota (Rodriguez-Palacios et al., 2009; Kumar et al., 2022). Autochthonous strains seem to establish more efficiently in the gastrointestinal tract compared to allochthonous strains (Frese et al., 2012). In our ScR, recent studies reported two species (*Bacillus megaterium* and *Candida tropicalis*) that had not been studied in previous years. *B. megaterium* was isolated from chicken manure (Deng et al., 2021), while *C. tropicalis* source was not specified (Bi et al., 2017; Kong et al., 2019). However, it has been reported that Candida spp. can be found in the gastrointestinal tracts of different animals (Sidrim et al., 2010; Marrero et al., 2011; Mandal and Ghosh, 2013).

The screening of autochthonous strains is becoming more feasible with the accessibility of genetic sequencing tools and is contributing to the emergence of a new kind of probiotics termed Next-Generation Probiotics (microorganisms identified based on comparative microbiota analyses; Martín and Langella, 2019). Future researchers have the opportunity to incorporate the new technologies into their screening for new probiotics strains.

Our ScR revealed that the probiotic dose supplemented to calves varied among studies. The probiotic dose is fundamental for its effectiveness, as shown by Renaud et al. (2019a) in a study where only the highest evaluated dose of *Saccharomyces cerevisiae boulardii* CNCM I-1079 had an effect on calf growth performance. The probiotic definition states that "probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill et al., 2014). However, "adequate amounts" is subjective and Hill et. al. (2014) did not provide a dose recommendation for humans or animals. The effective dose for each probiotic might depend on the strain, desired health outcome, vehicle, and mode of probiotic administration (Ouwehand, 2017). However, shipment and storage conditions (e.g., temperature, humidity, pressure) may decrease probiotic viability (Gurram et al., 2021). Thus, an overage is commonly included in the commercial probiotics to ensure that cfu are equal or greater than the label dose, and to take into account potential losses in viability (Weitzel et al., 2021).

In line with young calves' feeding management, most of the studies included in the ScR provided the probiotic mixed into the liquid feed. Some included studies that evaluated probiotics with expected rumen effects, mixed them into milk (Kong et al., 2019; Takemura et al., 2020). This likely implies probiotics bypassing the rumen, reticulum, and omasum through the esophageal groove (Ørskov, 1972), and reaching directly into the abomasum. Calf probiotics aiming to promote rumen development may be more effective if incorporated into starter (Diao

et al., 2019), although Stefańska et al. (2021) hypothesized that probiotics that by-pass rumen may contribute to ruminal development by alteration of small intestine metabolism.

Outcomes

Although the definition of "probiotics" is linked to health benefits (Hill et al., 2014), weight gain was the most evaluated outcome across trials. According to the USDA (2021), calf growth performance may be an indirect assessment of the overall calf health status, supported by the reported negative relationship between health status and growth performance (Shivley et al., 2018). Feed efficiency (calculated based on feed intake and calf weight), which potentially holds more information than the ADG and feed intake, was the third most reported outcome. Feed efficiency is an economically important outcome, as feed represents the main cost to raise a heifer from birth to calving (Heinrichs et al., 2013; Boulton et al., 2017). In dairy calves, the gut microbiota may play a role in feed efficiency by increasing the availability of energy substrates (volatile fatty acids) and essential nutrients (amino acids and vitamins) from the diet (Elolimy et al., 2020).

The most assessed health outcome across studies was fecal consistency score alone or as a tool to identify diarrhea. However, there was a disagreement across studies on references used for fecal consistency scoring and a variation on the assessment frequency. These unstandardized measurements make the comparison among trials challenging. Training for evaluation of fecal consistency was reported in only two trials included in our ScR. In a small study, Steen et al. (2011) reported inter-evaluator discrepancies in fecal consistency score across swine veterinarians (kappa = 0.24), partially attributed to the previous experience and knowledge of evaluators. The variation between evaluators has been reported even for objective measurements, such as rectal temperature (Naylor et al., 2012). Therefore, training the evaluators, independently

of their previous knowledge, will increase the measurements' repeatability. Fecal consistency score was validated to assess fecal dry matter in dairy calves by Renaud and Wilms (2020), and the authors recommended that research using this metric should assess internal and external reliability to guarantee repeatability. Furthermore, agreement on a fecal consistency scoring system would facilitate studies comparisons and summarization using evidence synthesis methods.

Our ScR identified some limitations in the selected studies, these include inconsistency of outcomes measured (e.g., fecal consistency score with different scoring systems) and incomplete data reporting (e.g., missing breed or age). Efforts have been made to decrease the inconsistency in methods and data reporting. In 1977, a standardized guideline was published to orientate researchers designing calf experiments (Larson et al., 1977), and the Reporting Guidelines for Randomized Controlled Trials for Livestock and Food Safety (REFLECT) was launched in 2010 to provide a checklist of essential items that authors should report when publishing their study (O'Connor et al., 2010). Additionally, McNamara et al. (2016) reviewed key aspects of experimental design and data reporting, and encouraged data sharing in support of animal systems modeling research. More recently, in human health research, the Core Outcome Measures in Effectiveness Trials (COMET) initiative has been developed, aiming to diminish the heterogeneity in outcome reporting (Williamson et al., 2017). The COMET initiative proposes a Core Outcome Set (COS), a minimum set of outcomes that should be measured and reported in experiments evaluating a specific condition.

This ScR has several strengths; in order to identify the highest number of available studies the search strategy was designed with librarian support, and language was not restricted to English (12.6% of the studies were not written in English). Also, multiple databases were

searched to maximize coverage, and grey literature was included. To minimize bias, the title and abstracts screenings were performed independently by two reviewers using pre-tested forms, and to decrease errors the data extraction was performed twice. This study had some limitations. It focuses only on controlled trials; observational studies were not included. There were 19 reports excluded due to the unclarity of cattle production system (dairy or beef). Moreover, the last literature search was performed on 19th August 2021. Thus, relevant recent literature might be missing. Additionally, only one person screened full-text manuscripts and extracted the data, even though data was extracted twice to minimize error.

CONCLUSIONS

This scoping review reveals the breadth of controlled trials evaluating probiotic supplementation in dairy calves. Future research should describe the randomization process, provide sample size justification, identify the evaluators, and follow the available guidelines for evaluating and reporting data.

TABLES AND FIGURES

Table 1.1. Results of the search strategy used to identify records

Search ID	Terms[1]	Results
#1 population	"calf" OR "calves" [tiab] OR "veal" [tiab] OR "preweaned dairy heifers" [tiab]	64,421
#2 intervention	"direct fed microbial" [tiab] OR "DFM" [tiab] OR "probiotic" [tiab] OR "probiotics" [tiab] OR "probiotics"[Mesh] OR "Faecalibacterium" [tiab] OR "Lactobacilli" [tiab] OR "LAB" [tiab] OR "Lactobacillus" [tiab] OR "Propionibacterium" [tiab] OR "Bacillus" [tiab] OR "Pediococcus" [tiab] OR "Enterococcus" [tiab] OR "Saccharomyces" [tiab] OR "Lactococcus" [tiab] OR "Megasphaera" [tiab] OR "Bifidobacterium" [tiab] OR "Faecalibacterium" [tiab] OR "digestive system diseases/prevention and control" [Mesh] OR "dietary Supplements" [Mesh] OR "dietary Supplements/administration and dosage" [Mesh] OR "Dietary Supplements/therapeutic use" [Mesh] OR "dietary dupplements/therapy" [Mesh] OR "Lactobacillus/therapeutic use" [Mesh] OR "Propionibacterium" [Mesh] OR "Bacillus" [Mesh] OR "Pediococcus" [Mesh] OR "Enterococcus" [Mesh] OR "Saccharomyces" [Mesh] OR "Lactococcus"[Mesh] OR "Megasphaera" [Mesh] OR "Bifidobacterium" [Mesh] OR "Faecalibacterium" [Mesh]	553,003
#3 outcomes	"Fecal score" [tiab] OR "faecal score" [tiab] OR "weight gain" [tiab] OR "feed efficiency" [tiab] OR "diarrhea" [tiab] OR "diarrhoea" [tiab] OR "diarrheal" [tiab] OR "diarrhoeal" [tiab] OR "scours" [tiab] OR "scouring" [tiab] OR "intestinal development" [tiab] OR "intestinal bacterial community" [tiab] OR "microbiome" [tiab] OR "microbiota" [tiab] OR "microbial community" [tiab] OR "gut flora" [tiab] OR "intestinal flora"[tiab] OR "microbial flora"[tiab] OR "growth"[tiab] OR "health" [tiab] OR "mortality" [tiab] OR "diarrhea/microbiology" [Mesh] OR "diarrhea/mortality" [Mesh] OR "diarrhea/veterinary" [Mesh] OR "cattle/growth and development"[Mesh] OR "microbiota" [Mesh] OR "gut health" [tiab] OR "weight gain" [Mesh]	3,987,048
#4	#1 AND #2 AND #3	661
#5	#4 AND (2020:2021[pdat])	121

[1]tiab = Title/Abstract; words included in the title or abstract of a citation. MeSH = Medical Subject Headings; controlled vocabulary of terms from the US National Library of Medicine. Pdat = Publication Date.

Table 1.2. Description of calves' characteristics and sample size from 110 trials included in the scoping review

		Probiotic type (n = trials)[1]					
Item	Categories[2]	BC (n = 11)	ENT (n = 4)	LB (n = 29)	SC (n = 17)	Multi (n = 35)	Other (n =14)
Sample size (calves)	<15	1	-	5	1	4	1
	15 to 30	4	3	13	5	15	4
	31 to 45	3	-	6	3	6	3
	46 to 60	-	1	3	3	3	3
	61 to 75	2	-	-	1	2	-
	76 to 100	1	-	1	2	1	-
	>100	-	-	1	2	4	3
Age supplementation start (d)	<7	5	4	14	8	20	5
	7 to 15	2	-	8	1	6	6
	16 to 30	2	-	-	3	1	2
	31 to 60	-	-	2	-	1	-
	61 to 120	1	-	2	1	2	-
	>120	-	-	2	2	1	-
	NR or unclear	1	-	1	2	4	1
Breed	Holstein	10	2	21	13	26	10
	Holstein cross	-	-	2	1	3	1
	Jersey	-	-	1	-	2	-
	Other	-	2	3	1	2	1
	NR	-	-	-	1	1	-
	Two breeds	1	-	2	1	1	2
Sex	Male	4	-	15	12	14	3
	Female	1	-	4	1	9	6
	Female and Male	5	4	5	4	9	4
	NR	1	-	5	0	3	1
Facility	Research	6	2	14	8	16	6
	Commercial	2	-	5	5	10	6
	NR	3	2	10	4	9	2

[1] BC: Bacillus, ENT: Enterococcus, LB: Lactobacillus, SC: Saccharomyces, Multi: multiple genera, Other [less frequent genera (n = 6) and not reported species (n = 8)].

[2] NR = not reported

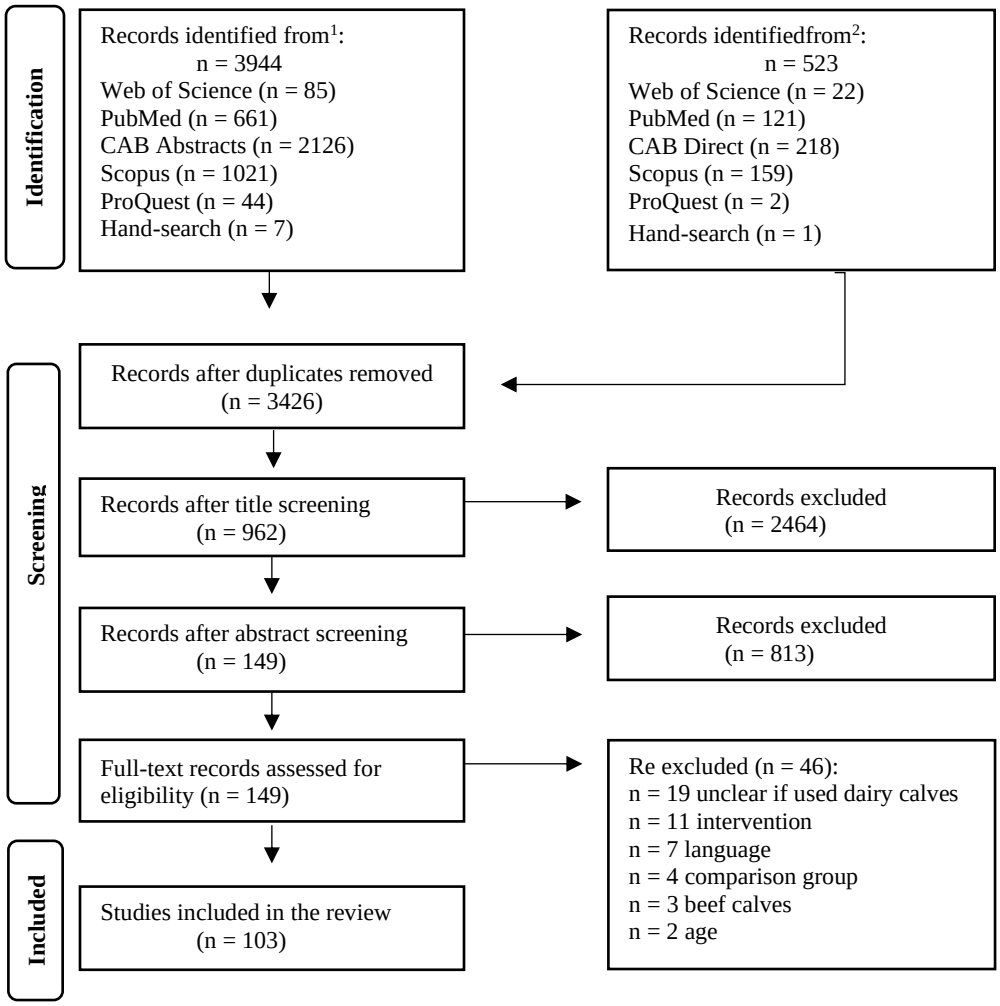

Fig. 1.1. Flow chart describing the number of records identified, included, and excluded, and the reasons for exclusions through the screening process of the scoping review ([1]searched on 27th February to 3rd March 2020; [2]search was updated on 19th August 2021; chart adapted from Page et al., 2021).

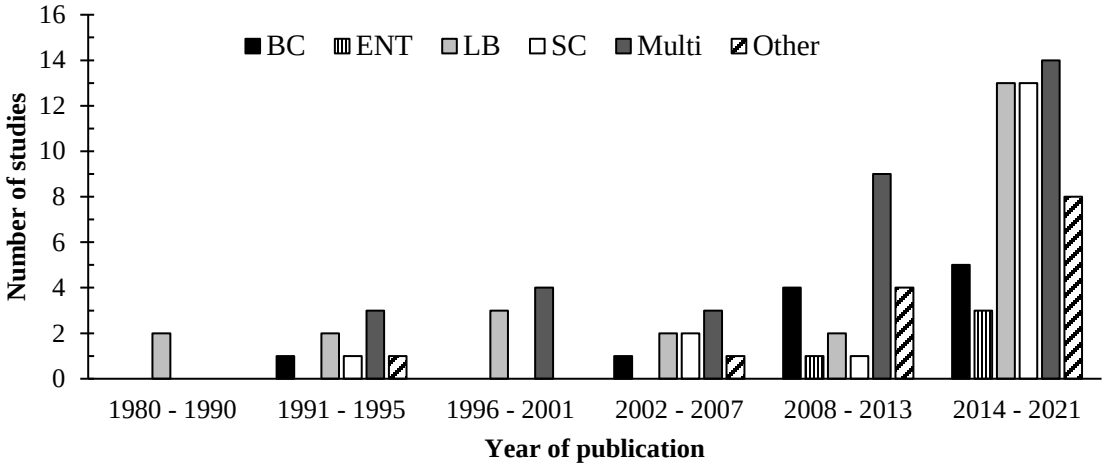

Fig. 1.2. Distribution of the 103 studies included in the scoping review by publication year and probiotic type. BC: Bacillus (n = 11), ENT: Enterococcus (n = 4), LB: Lactobacillus (n = 24), SC: Saccharomyces (n = 17), Multi: multiple genera (n = 33), Other [n = 14; less frequent genera (n = 6) and not reported species (n = 8)].

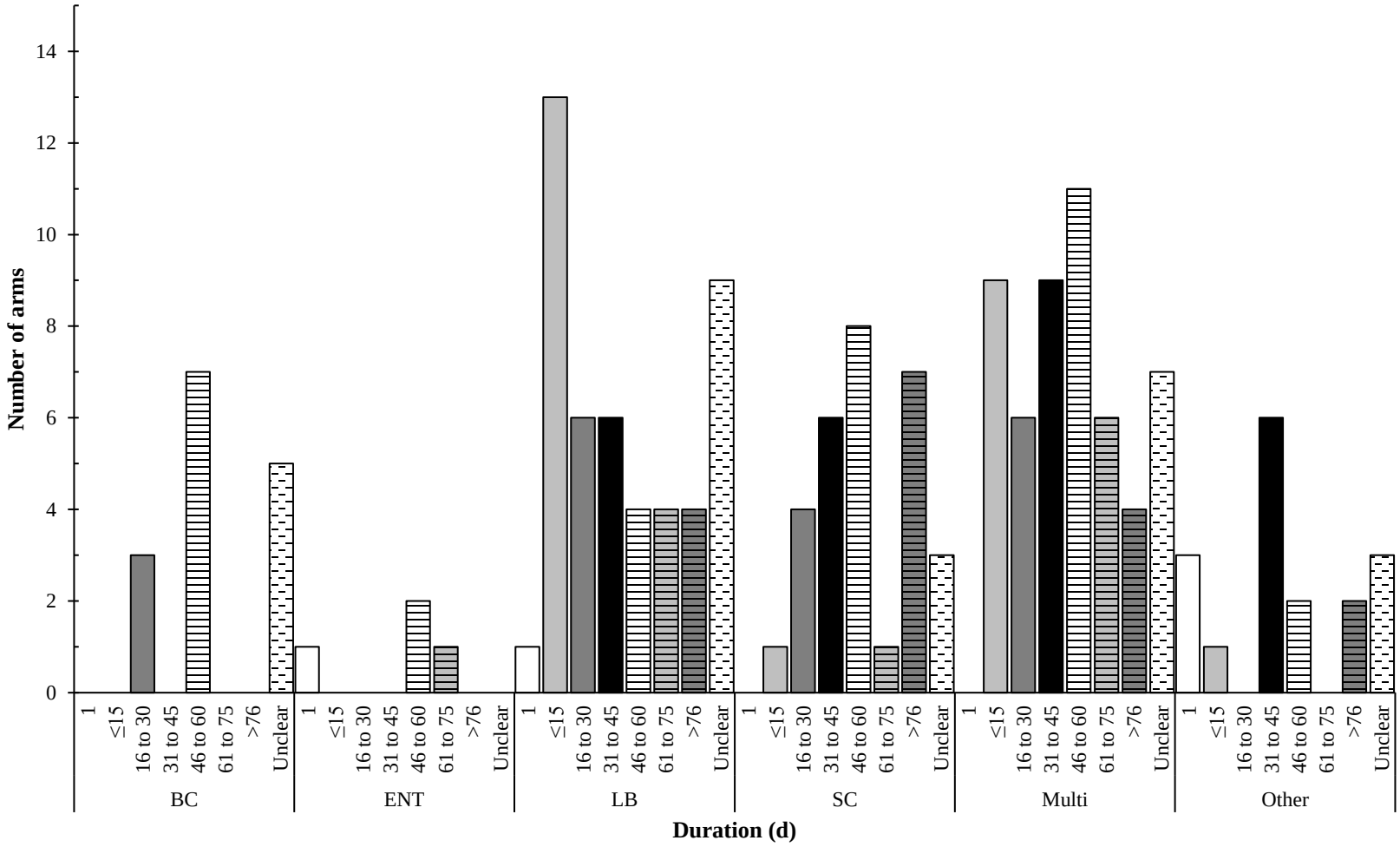

Fig. 1.3. Duration (d) of the probiotic supplementation across probiotic arms included in the scoping review (n = 165 arms). BC: Bacillus (n = 15), ENT: Enterococcus (n = 4), LB: Lactobacillus (n = 47), SC: Saccharomyces (n = 30), Multi: multiple genera (n = 52), Other [n = 17; less frequent genera (n = 6) and not reported species (n = 11)].

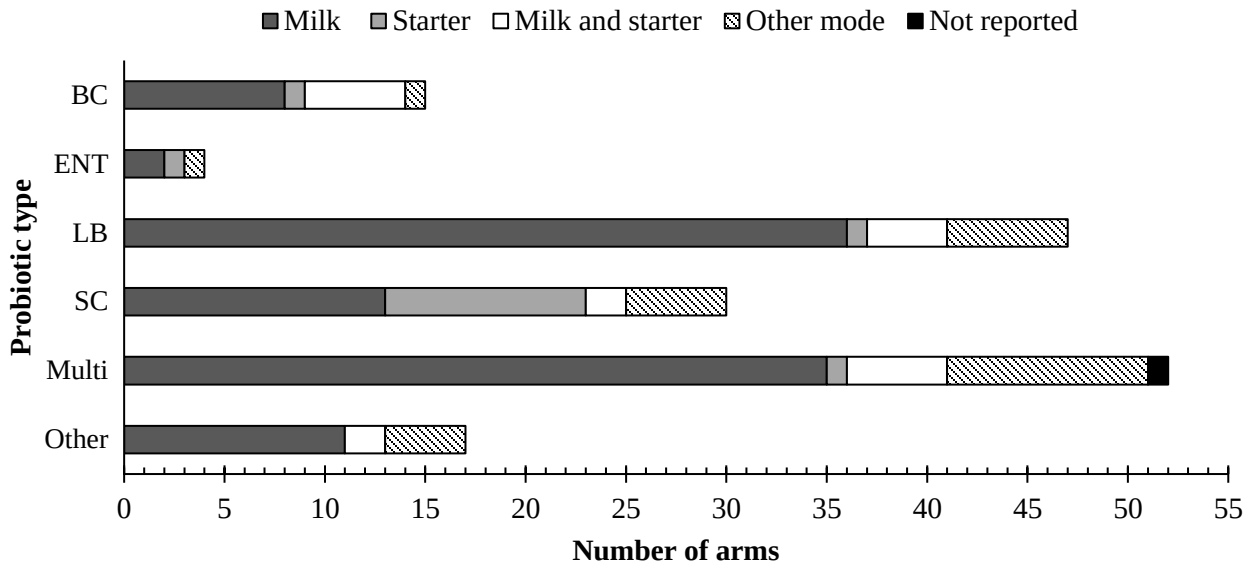

Fig. 1.4. Mode of probiotic administration across probiotic arms included in the scoping review (n = 165 arms). BC: Bacillus (n = 15), ENT: Enterococcus (n = 4), LB: Lactobacillus (n = 47), SC: Saccharomyces (n = 30), Multi: multiple genera (n = 52), Other [n = 17; less frequent genera (n = 6) and not reported species (n = 11)]. The milk category included trials that mixed probiotics into: colostrum, whole, skimmed or waste milk, a mixture of whole milk and milk replacer and milk replacer solely. The stater category included trials that administered the probiotic into starter or concentrate. The milk and starter category included trials that mixed the probiotics into: milk (replacer or whole) and starter during the whole experiment or separately. The other mode category included trials that administered the probiotic via oral (syringe, drench, paste, gel), ruminal (fistula), feed (milk replacer combined with oral application, milk replacer combined with total mixed ration, total mixed ration), nasal. The not reported category included trials that did not reported mode of probiotic administration.

		Probiotic type (n = trials)						
	Outcome	Total (n = 110)	BC (n = 11)	ENT (n = 4)	LB (n = 29)	SC (n = 17)	Multi (n = 35)	Other (n =14)
Growth	Weight gain[1]	97	11	3	22	15	33	13
	Feed intake	75	11	3	12	13	26	10
	Feed efficiency	53	10	3	8	9	17	6
	Body traits	24	5	1	3	0	12	3
	Rumen parameters[2]	18	2	0	2	4	5	5
	Digestibility	9	1	1	4	1	2	0
	Weaning	12	3	0	2	2	5	0
Health	Fecal consistency score[3]	71	8	3	16	12	25	7
	Microbiota[4]	52	6	4	18	7	14	3
	Blood parameters[5]	51	8	0	13	5	16	9
	Clinical examination[7]	41	4	0	7	10	14	6
	Mortality	16	1	2	1	3	8	1
	Other[7]	34	4	0	11	6	13	0

[1]Average daily gain and body gain.
[2]Volatile fatty acids, ruminal histomorphology, ruminal pH, enzymatic activity, ruminal ammonia.
[3] Fecal consistency score and diarrhea.
[4] Fecal, intestinal, or ruminal microbiota assessments.
[5] Glucose, blood urea nitrogen, cholesterol, triglycerides, cortisol, plasma total protein, albumin, globulin, beta hydroxybutyrate, non-esterified fatty acids, insulin, ghrelin, glucagon, creatinine kinase, aspartate aminotransferase, alkaline phosphatase, glutamate dehydrogenase, gamma-glutamyl transferase, lactate dehydrogenase, catalase, inorganic phosphorus, inorganic calcium, inorganic iron, hematocrit, red and white blood cells, IgA, IgE, IgM, IgG, IL-1β, IL-4, IL-6, IL-10, IFN-γ, TNF-α.
[6] Rectal temperature, heart rate, respiration rate, ear score, eye score, navel score, attitude score, nasal discharge, dehydration score, joint score, bloating, umbilical palpation, clinical examination to identify bovine respiratory disease.
[7] Duration diarrhea treatment, days with diarrhea, antibiotic use, colon histomorphology, intestine pH, fecal pH, fecal bacteria antibiotic susceptibility test, Johne's disease, meat and carcass traits, nasal microbiota, fecal dry matter, gene expression.

Fig. 1.5. Heat map representing the frequencies of growth and health outcomes evaluated in the included trials (n = 110). Frequencies of 0 to 25% are white, 26 to 50% light grey, 51 to 75% dark grey and 76 to 100% black. BC: Bacillus, ENT: Enterococcus, LB: Lactobacillus, SC: Saccharomyces, Multi: multiple genera, Other [n = 17; less frequent genera (n = 6) and not reported species (n = 11)].

Chapter 2: Effects of probiotic supplementation on growth performance and feed intake of dairy calves: a meta-analysis

ABSTRACT

The objective of this systematic review (**SR**) and meta-analysis (**MA**) was to quantify the effect of probiotic supplementation, considering different probiotics combined and categorized by probiotic genus, on ADG, feed intake, and feed efficiency of dairy calves. Our study included quasi-randomized and randomized controlled trials written in English, Spanish, or Portuguese that assessed the effects of probiotic supplementation on the growth of dairy calves. No restrictions were placed on the publication year. A total of 4,467 records were initially identified after conducting searches in Biosis, CAB Abstracts, Medline, Scopus, and the Dissertations and Theses Database. After applying inclusion criteria, 48 studies (49 controlled trials) were included in the analysis. Multi-level random-effects models were fitted for a single data set combining all trials and for four data sets stratified by probiotic types (Bacillus, Lactobacillus, Saccharomyces, and multiple strains with diverse genera). Meta-analyses showed that calf supplementation with probiotics did not result in significant differences in total dry matter intake (**DMI**) and feed efficiency (**FE**) compared to control groups (no treatment or placebo). Probiotic supplementation improved starter intake and ADG and tended to decrease milk intake. Analyses by probiotic type revealed that supplementation with Bacillus ssp., Lactobacillus ssp., Saccharomyces ssp., and multi-genera probiotic supplementation did not yield significant differences in DMI, FE, and starter intake. However, supplementation with Bacillus ssp. significantly increased the ADG of calves, while Lactobacillus ssp., Saccharomyces ssp., and multi-genera probiotic supplementation did not yield significant differences. High and significant heterogeneity was observed for all outcomes; thus, results must be interpreted carefully. A meta-regression analysis

demonstrated significant associations between total DMI and probiotic type as well as the duration of supplementation. Moreover, meta-regression results indicated a significant association between starter intake and probiotic type and the duration of probiotic supplementation. Probiotics may be beneficial for enhancing ADG and starter intake in dairy calves; however, current evidence remains limited due to study heterogeneity. To establish appropriate recommendations, additional studies are required to address the heterogeneity observed in the existing research.

Keywords: direct fed-microbials, weight gain, feed additive, efficiency, dairy calf

INTRODUCTION

The growth performance of dairy cattle calves influences their future reproductive and productive performance as parous cows. Age at first calving and milk yield of primiparous cows has been associated with a calf's growth rate during both preweaning and postweaning periods (Moallem et al., 2010; Soberon et al., 2012; Krpálková et al., 2014). The research estimated that milk production of future dams is optimized when ADG is at least 0.5 kg/d during preweaning (Gelsinger et al., 2016) and at least 0.8 kg/d after weaning (Zanton and Heinrichs, 2005). Feed additives, such as probiotics, have been proposed as a strategy to improve growth in dairy calves (Cangiano et al., 2020). Probiotics have become a popular feeding management strategy in dairy calf rearing, with 38% of large dairy operations in the US using probiotics in heifers (USDA, 2016) and 57% of Ohio dairy producers using probiotics in newborn calves (Habing et al., 2016).

According to the International Scientific Association for Probiotics and Prebiotics, probiotics are "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill et al., 2014). Products containing live microorganisms that are administered to animals are categorized as direct-fed-microbials (**DFM**) by the U. S. Food and

Drug Administration (FDA, 1995). Despite slight differences in their definitions, the terms DFM and probiotics are often used interchangeably. In dairy calves, the efficacy of probiotic supplementation on growth performance is inconsistent across studies. Some studies reported that supplementing dairy calves' diets with probiotics improves average daily gain and feed efficiency (Le et al., 2017; Wu et al., 2021), while others have found no effect (He et al., 2017) or even negative effects (Corbett et al., 2015) on calf performance.

Previous systematic reviews and meta-analyses (MAs) assessed the effect of probiotics supplementation on the performance of dairy calves (Alawneh et al., 2020; Frizzo et al., 2011; Wang et al., 2023). However, the findings of Frizzo et al. (2011) are outdated as a substantial amount of literature has been published in the past decade (Branco-Lopes et al., 2023). In recent years, two MAs have been conducted on this topic. One MA included dairy, beef, and buffalo calves from birth to one year of age (Alawneh et al., 2020); the other MA focused specifically on dairy calves but was limited to the preweaning period (Wang et al., 2023). Although both MAs provided a risk of bias assessment and were well-reported (e.g., followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses Protocols [**PRISMA-P**]), neither took into consideration potential dependency in effect sizes. Dependency in effect sizes can occur when studies are related in some way, such as studies conducted by the same researchers or in the same laboratory. Ignoring this dependency could lead to underestimated standard errors of the overall effect, and potentially biasing the conclusions drawn from the analysis (López-López et al., 2018).

Currently, there is no MA assessing the effect of probiotic supplementation during preweaning and postweaning on dairy calves' growth performance. This information is crucial to guide the decisions and recommendations of veterinarians, dairy nutritionists, and industry and

dairy producers. Furthermore, considering that not all probiotics share the same mechanisms of actions (Hill et al., 2014), a genus-level analysis for probiotic type is required. Therefore, the objective of this study was to quantify the effect of probiotic supplementation, considering different probiotics combined and categorized by probiotic genus, on ADG, feed intake, and feed efficiency of dairy calves.

METHODS

A protocol was designed *a priori* and reported according to PRISMA-P (Moher et al., 2015); it also followed the guidelines for systematic reviews in animal agriculture and veterinary medicine (O'Connor et al., 2014). The protocol was deposited with UC Davis eScholarship (https://escholarship.org/uc/item/2r93v26f) and is available in the Systematic Reviews for Animals and Food (https://syreaf.org/protocols/). A summarized version of the protocol is presented in Supplemental Material 1. Additionally, this SR and MA is reported according to PRISMA (Page et al., 2021).

Eligibility Criteria

This SR included primary research studies, including randomized and quasi-randomized controlled trials, that were written in English, Spanish, or Portuguese. Observational and non-randomized studies were excluded as well as studies that were not randomized. Peer-reviewed and non-peer-reviewed studies (specifically theses and dissertations) were included in the SR. Eligibility criteria were defined based on the population, intervention, comparator, and outcome (**PICO**) framework (Cooper et al., 2018). Eligible studies were those that evaluated preweaned or weaned dairy calves (up to 7 mo of age at enrollment); no restrictions for breed, sex, or raising facilities were applied. Additionally, studies were required to have investigated supplementation with probiotics (no restriction regarding probiotic species, dose, mode of administration and

duration of supplementation). Studies that named probiotics as direct-fed microbials were also included. Research studies assessing probiotics as therapy to control or treat diseases were excluded. The comparator group must have been either no intervention, a placebo, or a negative control. Eligible studies included a growth performance outcome (e.g., ADG) or a health outcome (e.g., immunological parameters). In this manuscript, we report findings for growth measurements, with average daily gain as the primary outcome, and total dry matter intake, milk intake, starter intake and feed efficiency as secondary outcomes.

Information Sources

To conduct a comprehensive literature search, databases were identified with the assistance of a UC Davis research librarian specialized in veterinary science. The selected databases were Biosis (Web of Science, 1926 to present), CAB Abstracts (CAB Direct, 1973 to present), Medline (PubMed, 1966 to present), and Scopus (Scopus, 1996 to present), along with the Dissertations and Theses Database (ProQuest, 1861 to present) to search for unpublished records. The databases were selected based on their high coverage of veterinary journals and other journals with significant veterinary or animal science content. The bibliographies of all relevant studies were hand-searched to identify additional manuscripts.

Search Strategy

In collaboration with a UC Davis librarian, the search strategies were developed using the PICO framework and relevant literature to select keywords for each PICO concept. The search strings were adjusted accordingly to each database to fit its specific formats. No restrictions were applied for publication year, study design, or language. The searches were performed on February 27, 2020 and March 3, 2020 and were updated on August 19, 2021. The search results were uploaded to the reference manager F1000 (Faculty of 1000 Limited, London, UK),

duplicates were removed, and the de-duplicated results were exported to the Covidence systematic review management software (Veritas Health Innovation, Melbourne, AU) for screening.

Study Records

Selection Process

Two independent reviewers trained on the methodology screened all records twice for eligibility. The reviewers were not blinded to journals or author names. All screening questions were pilot tested. The first screening involved assessing the studies' titles for eligibility based on two questions:

1) Does the title describe a study involving dairy calves?

2) Does the title describe a study with probiotic(s) supplementation?

The second screening involved assessing the abstracts using three questions:

1) Does the abstract describe a primary research study?

2) Does the abstract describe a study involving dairy calves supplemented with probiotic(s)?

3) Does the abstract describe one or more measurements of performance (e.g., ADG, feed efficiency) or health (e.g., fecal consistency score, diarrhea incidence)?

During title and abstract screenings, the possible answers were 'no,' 'maybe,' and 'yes.' If both reviewers selected 'no' for any question, the study was excluded. During both screenings (title and abstract), conflicts between the two reviewers were discussed until a consensus was reached.

Full-text screening was conducted by one reviewer and included questions 1 to 3 from the abstract screening, adapted to the full-text manuscript, as well as four additional questions:

1) Is the study a trial with a control group?

2) Is the study written in English, Spanish, or Portuguese?

3) Is the probiotic a supplementation strategy (not treatment for sick animals)?

4) Is the study population (dairy calves) equal or less than 7 mo old at enrollment?

In the screening process, two post hoc modifications from the original protocol were made: (1) to exclude non-randomized trials and (2) to exclude studies with poor reporting regarding probiotic type, or data needed for meta-analysis (i.e., SD, SE, sample size). For this, the following questions were included:

1) Is the study quasi-randomized or randomized trial?

2) Did the study report SD or SE for at least one of the outcomes of interest, group size and probiotic type?

The available answers for the full-text screening were 'no' and 'yes,' and studies were excluded if the answer was 'no' for at least one of the questions. The reason for exclusion was recorded at this stage.

Data Collection Process

Study-level data that were extracted consisted of author(s)'s name(s), journal's name, language, trial country (when not available, the country was based on first author's affiliation), year of publication, and funding information. Population characteristics consisted of calf breed, sex, age at the start of probiotic supplementation (when not specified, the enrollment age was used or an estimation was done based on the description of calf management), and herd type (commercial vs. research). Intervention and comparator data consisted of description of comparator, commercial name of probiotic, scientific name, dose, dose adjustment, administration method (e.g., whole milk, milk replacer), and duration of supplementation. If the

probiotic dose was unclear, we calculated it based on the information provided in the manuscript. When more than one dose was tested, we extracted the information for the highest dose. If the duration of supplementation was unclear, we assumed it was the entire experimental period. In trials in which calves were fed probiotic via milk, we considered the weaning date as the last day of probiotic supplementation. Outcomes-level data consisted of number of experimental units for each treatment group, means for each treatment group, unit of results, standard deviation for each treatment group, and time point of each measurement. If SD was not reported, the standard error was extracted, and SD was back-calculated according to Higgins et al. (2019).

Data extraction forms were designed based on previous studies, and pre-tested using five studies. Data regarding study-level, population, and intervention from selected studies were input into a Microsoft Excel spreadsheet by one reviewer twice. The outcome data (data used for MA) were extrapolated into a Microsoft Excel spreadsheet by two reviewers independently and then checked for discrepancies.

Risk of Bias in Individual Studies

The risk of bias was assessed at the outcome level (considering ADG) by two reviewers independently using the Cochrane Risk of Bias 2.0 tool (Sterne et al., 2019), with adaptations in the signaling questions as described by Lopes et al. (2021). For each domain, the risk of bias was be classified as “high,” “some concerns,” or “low.”

Data Synthesis and Meta-Bias

All analyses were performed in R 4.0.3 (R Foundation for Statistical Computing, Vienna, Austria) using RStudio version 1.3.1093 (RStudio Inc., Boston, MA). Based on a previous meta-analysis (effect size for average daily gain = 0.2, sample size of studies = 43, degree of heterogeneity = 60; Frizzo et al. 2011), a statistical power analysis was calculated using the

package metapower (Griffin, 2021). To achieve an 80% statistical power for detecting difference in ADG, the MA needed to include 47 trials. The meta-analyses were performed with the metafor package (Viechtbauer, 2010). Five data sets were employed for the calculations. One data set included all trials, which combined all different probiotics and was used for fitting the multilevel models and meta-regressions, performing sensitivity analysis, and assessing influential trials and publication bias. The remaining four datasets were specific to a type of probiotic (i.e., Bacillus, Lactobacillus, Saccharomyces, and probiotics multiple strains with diverse genera) and used solely for fitting the multilevel models. The association of probiotic type and the outcomes of interest were also investigated through meta-regressions.

The 'escalc' function was used to compute standard mean difference (milk intake and feed efficiency), mean differences (DMI, starter intake, and ADG), and their corresponding sampling variances for all datasets. Most studies yielded complex data structures; trials were nested within studies, themselves clustered within laboratories. Therefore, to account for the dependency of effect sizes, multilevel models were used with considerations given to the random variation of observation, trial, study, and laboratory. All models were fitted with the rma.mv function. Between studies variance was estimated by the restricted maximum likelihood (REML). To assess heterogeneity, the I^2 statistic was calculated for each model. The I^2 values of 25%, 50%, and 75% were interpreted as representing small, moderate, and substantial levels of heterogeneity (Higgins et al., 2003). Cochran's *Q* test was used to evaluate heterogeneity significance (Higgins et al., 2003).

The possible sources of heterogeneity were investigated through meta-regressions and the multilevel models were extended with moderators ("mod" argument of the "rma.mv" function). Probiotic type, maximum dose, duration of supplementation and period of supplementation were

tested as moderators. A minimum of three trials in each class of the different moderators were included in the meta-regressions. Probiotic type was categorized as Bacillus, Lactobacillus, Saccharomyces, and probiotics with multiple strains of diverse genera. Maximum dose was categorized as $< 10^{10}$ and $\geq 10^{10}$ cfu/d. Supplementation period was dichotomized as preweaning and other (preweaning and postweaning as well as postweaning). Only two studies evaluated postweaning; thus, it was combined with those studies measuring both preweaning and postweaning periods. Initially, univariate analyses were performed to evaluate the influence of each moderator separately ($P \leq 0.10$). Next, multivariate meta-regressions were fitted, including all significant moderators and their interaction terms. Variables were removed from the models using backward elimination with significance criteria of $P \leq 0.05$. Additionally, both Cochran Q and I^2 statistics were performed for each meta-regression.

Cook's distances was used to examine whether trials were influential in the context of the models (Viechtbauer and Cheung, 2010). To assess the impact of influential trials identified by Cook's distances, sensitivity analyses were carried out by removing the influential trials from the dataset and re-fitting the multilevel models. An additional sensitivity analysis was performed to examine the effect of removing the trials with high risk of bias on estimates for ADG (the outcome for which risk of bias was assessed). Publication bias was investigated both graphically with contour-enhanced funnel plots (Peters et al., 2008) and statistically using Egger's test (Egger et al., 1997). In the manuscript, $P \leq 0.05$ was considered significant, while $0.05 < P \leq 0.10$ was referred to as tendencies.

RESULTS

Study Selection

The initial search, conducted in the electronic databases, yielded a total of 4,459 papers; a manual search of references turned up eight additional studies (Fig. 2.1). After duplicates were removed and titles and abstracts were screened, 4,318 studies were excluded. Following the full-text screening of the remaining 149 studies, 101 studies were excluded. The primary exclusion reason was the lack of clarity regarding the productive orientation of the enrolled calves (18.8%; 19/101 studies were inconclusive if calves were dairy or beef breeds). Overall, 48 studies (comprising 49 trials with 69 arms evaluating probiotics) met the inclusion criteria and were included in the SR and MA. A comprehensive list of the included studies is presented in Supplemental Material 2.

Study Characteristics

The characteristics of the included trials are described in Table 2.1. The years of publication ranged from 1991 to 2021, with 68.7% (n = 33) published in the last decade. Most studies were in English (n = 43; 89.6%), with Portuguese accounting for a smaller portion (n = 4; 8.3%), and a single study was written in both Spanish and English (n = 1; 2.1%). Studies were funded by public institutions (n = 23; 47.9%), private companies (n = 5; 10.4%), both public and private institutions (n = 3; 6.2%), did not receive financial support (n = 1; 2.1%), or did not disclose funding (n = 16; 33.3%). Out of the 49 trials included, 48 were randomized controlled trials and one was a quasi-randomized controlled trial. Trials often included male calves (n = 21; 42.8%) that were predominantly pure or crossbred Holstein (n = 44; 89.7%). The calves' age at the start of supplementation ranged from birth to 120 d (mode = 1; average = 12; interquartile = 2 to 10). Most trials (n = 39; 79.5%) started probiotic supplementation when calves were

younger than 15 d. The calves received probiotics during the preweaning period (n = 44; 89.8%), postweaning period (n = 2; 4.1%), or both preweaning and postweaning periods (n = 2; 4.1%). In one trial, the supplementation period was not clearly stated (2.0%). The interventions included 42 monostrains probiotics (Saccharomyces ssp., n = 14; Lactobacillus ssp., n = 12; Bacillus ssp., n = 8; Enterococcus ssp., n = 3; Megasphaera ssp., n = 2; Candida ssp., n = 2; Bifidobacterium ssp., n = 1), five multistrains probiotics from the same genus (Bacillus ssp. n = 4; Lactobacillus ssp., n = 1), and 22 multistrains probiotics from diverse genera. Overall, the duration of probiotic supplementation ranged from 1 to 175 d (mode = 56; average = 48; interquartile = 36 to 56), and the probiotic maximum dose ranged from 3.7×10^{7} to 1.2×10^{11} cfu/calf/d.

Feed Intake Results

Dry Matter Intake

All probiotics. Twenty-one trials evaluated the total DMI over the study period. According to the four-level random-effects model, the total DMI for calves supplemented with probiotics and control calves did not differ ($P = 0.12$; Table 2.2) and a significant heterogeneity was observed ($P < 0.001$; $I^2 = 91.3\%$). An analysis using Cook's distance measure identified one influential trial (Deng et al., 2021). After excluding this trial from the dataset, the total DMI remained unaffected by probiotic supplementation ($P = 0.29$), and the heterogeneity was significant and substantial ($P < 0.001$; $I^2 = 78.4\%$). Meta-regression revealed a significant association of total DMI with probiotic type ($P = 0.001$) and period of supplementation ($P < 0.001$; Table 2.4).

By probiotic type. The total DMI was measured in trials evaluating Bacillus (n = 6), Lactobacillus (n =4), Saccharomyces (n = 3), and multiple genera (n =5). No differences in total DMI were detected for any of the probiotic types studied (Table 2.3). The heterogeneity was not

significant for the analysis of Lactobacillus ($P = 0.60$; $I^2 = 26.1\%$), while it was significant for the remaining probiotic types ($P < 0.001$; $I^2 = 93.3\%$ Bacillus; $P = 0.04$; $I^2 = 59.9\%$ Saccharomyces and multi genera probiotics $P = 0.001$; $I^2 = 90.7\%$ multi genera probiotics).

Starter Intake

All probiotics. Out of the 49 included trials, 23 assessed starter intake. Utilizing the four-level random-effects model, the analysis revealed that probiotic supplementation significantly improved the starter intake of calves compared to the control group ($P = 0.02$; Table 2.2). Additionally, significant and substantial heterogeneity was observed ($P < 0.001$; $I^2 = 98.9\%$). The Cook's distance measure analysis identified one influential study (Muya et al., 2015). After removal of Muya et al. (2015), starter intake remained unaffected by probiotic treatment ($P = 0.01$), and heterogeneity was significant and substantial ($P < 0.001$; $I^2 = 96.0\%$). Additionally, meta-regression indicated that starter intake was associated with the probiotic type ($P = 0.006$) and the duration of probiotic supplementation ($P = 0.003$, Table 2.4).

By probiotic type. The starter intake was evaluated in five trials for Bacillus, three trials for Lactobacillus, five trials for Saccharomyces, and eight trials for multiple genera. No effect of starter intake of calves was detected for any of the four probiotic types considered in this study (Table 2.3). Additionally, heterogeneity was significant only for Saccharomyces ($P = 0.01$) with I^2 values varying: 41.7% for Bacillus, 0% for Lactobacillus, 68.6% for Saccharomyces and 93.6% for multi genera probiotics.

Milk Intake

All probiotics. Six trials evaluated milk intake. The four-level random-effects model showed that calves receiving probiotic supplementation tended to have a lower milk intake compared to those in the control group ($P = 0.09$; Table 2.2). Additionally, heterogeneity

between trials was not significant ($P = 0.34$; $I^2 = 28.9\%$). Due to the low number of trials, sensitivity analysis and meta-regression were not performed for this outcome.

By probiotic type. The analysis by probiotic type was not conducted due to the insufficient number of trials. Two studies reported either a reduction (*Megasphaera elsdenii* NCIMB 41125, single dose 5×10^9 cfu; Muya et al., 2017) or a tendency for a reduction (*Bacillus subtills*, 1.24×10^{10} cfu/d for 27 d; Jenny et al., 1991) or multiple strains probiotic (*Lactobacillus acidophilus*, *Lactobacillus lactis*, and *Bacillus subtilis*, 3.3×10^9 cfu/d for 27 d). In contrast, no effect was observed in the milk intake of calves supplemented with *Bacillus amyloliquefaciens* H57 (3.16×10^8 cfu/kg of dry matter for 56 d; Le et al., 2017), *Bacillus subtilis* natto (1×10^{10} cfu/d for 44 d; Sun et al., 2010), *Candida tropicalis* (5×10^9 cfu/d for 52d; Kong et al., 2019), *Saccharomyces cerevisiae* boulardii CNCMI-1079 (daily 7.5×10^8 cfu/L of milk and 3×10^9 cfu/kg of starter for 96 d; Fomenky et al., 2017), and *Lactobacillus acidophilus* (daily 2.5×10^8 cfu/L of milk and 1×10^9 cfu/kg of starter; Fomenky et al., 2017).

ADG Results

All probiotics. Of the 49 trials, 45 measured ADG. Based on the four-level random-effects model, probiotic supplementation significantly increased ADG of calves ($P = 0.001$; Table 2.2) relative to comparator; although, substantial heterogeneity was observed ($P < 0.001$, $I^2 = 99.5\%$). Analysis of Cook's distance measure revealed one influential study (Deng et al., 2021). The finding that probiotic supplementation increased ADG was robust after removing Deng et al. (2021) from the dataset ($P = 0.002$). Sensitivity analysis, assessing the robustness of the results by excluding studies with a high risk of bias, showed that the probiotic effect on ADG remained significant (mean difference = 0.04 kg/d, 95% CI = 0.02 to 0.07, $P = 0.0002$) as well as had significant and substantial heterogeneity ($P < 0.0001$; $I^2 = 99.4\%$). The meta-regression

indicated that moderators were not significantly associated with the estimated probiotic effect on ADG.

By probiotic type. The ADG was assessed in 10 trials for Bacillus, eight trials for Lactobacillus, nine trials for Saccharomyces, and 14 trials for multiple genera. Analyses by probiotic type revealed that supplementation with Bacillus ssp. significantly increased the ADG of calves ($P = 0.03$; Table 2.3). However, supplementation with Lactobacillus ssp., Saccharomyces ssp., and multi-genera probiotics did not result in significant differences in ADG. Additionally, heterogeneity varied for each probiotic type; the I^2 values were 99.0, 50.3, 26.4, and 99.6% for Bacillus, Lactobacillus, Saccharomyces, and multi-genera, respectively.

Feed efficiency results

All probiotics. Thirty-three trials reported feed efficiency. Based on the four-level random-effects model, no significant difference was observed in feed efficiency between the calves' receiving probiotics and those in the control group ($P = 0.23$; Table 2.2). A significant heterogeneity was detected across the trials ($P< 0.001$; $I^2 = 70.8\%$). The Cook's distance measure analysis identified two significant studies (Abe et al., 1995; Yao et al., 2020). After removing these studies from the analysis, the feed efficiency remained unaffected by probiotic treatment ($P = 0.81$), but heterogeneity decreased ($P = 0.0001$; $I^2 = 54.0\%$).

By probiotic type. Feed efficiency was assessed in eight trials for Bacillus, eight trials for Lactobacillus, five trials for Saccharomyces, and 11 trials for multiple genera. Bacillus supplementation showed a tendency to improve feed efficiency ($P = 0.07$; Table 2.3), but heterogeneity was significant and substantial ($P = 0.002$; $I^2 = 74.8\%$). No differences in feed efficiency were detected for Lactobacillus, Saccharomyces, and multi-genera probiotics. Heterogeneity was significant for the analyses of all probiotic types.

Risk of Bias

The results of the risk of bias assessments are presented in Fig. 2.2. In the first domain (randomization process), three trials were classified as high risk of bias due to the alternative allocation (Cruywagen et al., 1996), significant difference in IgG baseline (Riddell et al., 2010), and substantial differences between treatment group sizes (Timmerman et al., 2005). No trials reported if the allocation sequence was concealed, which contributed to most trials being classified as "some concerns" in the first domain. In the second domain (deviations from intended interventions), all trials were classified as low risk even though most trials did not report if blinding was used, and only one trial disclosed that study personnel were not blinded (Corbett et al., 2015). In the third domain (missing outcome data), all trials were classified as low risk of bias; however, 23 trials did not report the number of calves used for the analysis of ADG. In the fourth domain (measurement of the outcome), one trial was classified as high risk due to the use of tape to estimate body weight (Corbett et al., 2015). The remaining trials were considered as low risk, but 13 trials failed to specify the method used to measure ADG in calves. In the fifth domain (selection of the reported result), all trials raised some concerns due to lack of clarity regarding a pre-specified analysis plan. Two trials measured body weight using multiple time points and reported selected results without justification (Fomenky et al., 2018; Górka et al., 2021). However, the selected results were not statistically significant; thus, they were not considered as high risk.

Publication Bias

The counter-enhanced funnel plots for DMI, starter intake, ADG and feed efficiency are shown in Fig. 2.3. Funnel plots appeared symmetrical for feed efficiency, while the funnel plots for DMI, starter intake, and ADG seemingly suggested potential asymmetry. To further assess

the presence of publication bias, Egger's test was performed, and the results suggested no evidence of publication bias (DMI: $P = 0.13$; starter intake: $P = 0.38$; ADG: $P = 0.45$; feed efficiency: $P = 0.87$).

DISCUSSION

This SR and MA estimated the overall effect of probiotic supplementation on feed intake and growth performance of preweaned and weaned dairy calves and examined whether the type of probiotic, maximum dose, duration of probiotic supplementation and period of supplementation (i.e., preweaning, postweaning, or both) was associated with the intervention effect. This study was the first quantitative summary that took the hierarchical structure of the data into account. A substantial body of evidence was found that aimed to evaluate the effect of probiotic supplementation on calf performance. However, data were not extracted from 26 studies due to incomplete reporting. Specifically, these studies did not report a variance measure, sample size, or probiotic type. Overall, the present study systematically reviewed and analyzed 48 studies.

Our MA included 33 probiotic species compromising 14 probiotic genera. Monostrain probiotics were the most common intervention, and Saccharomyces ssp., Lactobacillus ssp., Bacillus ssp. were the most commonly evaluated species. These species are considered "traditional probiotics," which, in general, have been isolated from many sources (Martín and Langella, 2019). Although not entirely understood, probiotics may confer benefits to the host through several proposed mechanisms, including competitive exclusion, synthesis of various compounds, enzymatic activity, and immunomodulation (Plaza-Diaz et al., 2019). Further, different probiotics may present different mechanisms of action. However, the International Scientific Association for Probiotics and Prebiotics considers that some mechanisms (e.g., acid

and short-chain fatty acids production) are widespread among a diversity of probiotics, whereas other mechanisms are species- or strain-specific (Hill et al., 2014). According to Sanders et al. (2018), these commonalities are due to shared metabolic pathways or molecular mechanisms. Thus, pooling results from trials evaluating different strains is supported by the existence of shared mechanisms of action.

Feed Intake

Based on our findings, probiotic supplementation did not impact total DMI, but it enhanced starter intake and tended to decrease milk intake. The MA by Wang et al. (2023) reported an increase in DMI among preweaned calves but only when probiotics were administered via milk and milk replacer. Alawneh et al. (2020) found no difference in DMI between calves and buffalos receiving probiotics compared to the control group; however, their MA included studies with calves from birth to 1 year. In contrast to our study, previous MAs did not assess the impact of probiotics on starter and milk intake. Considering the significance of these two outcomes, particularly for preweaned dairy calves, further evaluation can provide more meaningful insights than solely focusing on total DMI.

According to a survey conducted in the UK, dairy producers may use age, weight, starter intake, or a combination of these factors to determine when to wean calves (Mahendran et al., 2022). Successful preweaning calf rearing programs aim to maximize early starter intake, as this can accelerate the transition from preruminant to a ruminant calf and reduce rearing costs (Khan et al., 2016). At birth, calves' rumen is physically underdeveloped and metabolically nonfunctional. Consequently, calves depend on milk feeding to sustain their growth, especially during their first weeks of life (Baldwin et al., 2004). Although high milk allowance enhances preweaning growth and feed efficiency (Jafari et al., 2020), improves calf welfare (reduces

behavioral signs of hunger), and increases mammary development (Geiger et al., 2016), it also delays starter intake (Jafari et al., 2020) and therefore rumen development. Several factors may influence starter intake, such as physical form of solid feed (i.e., pelleted, ground, textured; Ghaffari and Kertz, 2021), forage intake (Imani et al., 2017), weaning process (Sweeney et al., 2010), incidence of respiratory disease (Cantor et al., 2022), and personality traits (Neave et al., 2018). Based on our results and those from Wang et al. (2023), inclusion of probiotic supplementation in preweaning calf feeding programs may enhance starter intake. The consumption of solid feed, particularly readily fermentable carbohydrates, aids in rumen epithelium development (Chai et al., 2021). This development is likely explained by short-chain fatty acid production via ruminal fermentation, especially butyrate, which can induce morphological and functional changes in ruminal papillae (Sander et al., 1959).

ADG

Our MA indicated that probiotic supplementation increases ADG in dairy calves, and this result was not modified after removing studies with a high risk of bias from the analysis. Previous MAs also documented higher weight gain following the administration of probiotics in preweaned dairy calves (Wang et al., 2023), calves and buffalos (Alawneh, et al., 2020), piglets (Zimmermann et al., 2016), and preterm infants (Panchal et al., 2023). However, other evidence syntheses concluded that probiotics have an anti-obesity effect in mice and humans (Zhang et al., 2016a). Although evaluating different hosts, those studies evaluated similar probiotics species (e.g., *Lactobacillus acidophilus*). Moreover, a comparative MA evaluating the weight gain effect of Lactobacillus probiotics on animals and humans revealed that probiotic effect on weight was species-specific (Million et al., 2012). In our MA, we were able to evaluate probiotic only at the genera level. Our results indicated that Bacillus supplementation increased ADG. Bacillus ssp.

are known for their capacity to produce extracellular enzymes (e.g., amylase, protease, and lipase; Hmani et al., 2017), which could enhance nutrient digestion and consequently increase weight gain.

In our analysis for ADG, the results were robust when an influential study (Deng et al., 2021) was removed. Deng et al. (2021) supplemented preweaned calves (n = 24) with *Bacillus megaterium* 1259 (1.2×10^{11} cfu/d) for 56 d. Among the included trials, Deng et al. (2021) had the second biggest difference in ADG between intervention and comparison groups (0.200 kg/d). Similarly, Wu et al. (2021) reported important differences (0.210 kd/d), but only at the intermediate dose tested (7×10^{9} cfu/d) and not at different doses (3.5×10^{9} and 1.4×10^{10} cfu/d).

Feed Efficiency

According to our findings, probiotic supplementation did not affect feed efficiency. Enhancing the feed efficiency of dairy calves is crucial for the economic performance of dairy enterprises. Since feeding costs represent a significant expense in heifer rearing, even small improvements in feed efficiency can result in substantial savings (Bach et al., 2021). Previous MAs reported varying feed efficiency results regarding the effect of probiotics supplementation in different livestock animals. The supplementation of probiotics enhanced feed efficiency in calves (Wang et al., 2023), calves and buffalos (Alawaneh et al., 2020), broilers (Blajman et al., 2014), and during growing and finishing periods of pigs (Zimmermann et al., 2016). However, in swine, no effect on feed efficiency was observed after probiotic supplementation during the lactation period (Zimmermann et al., 2016). In a sub-group analysis, Frizzo et al. (2011) found that probiotic supplementation for calves had no effect on feed efficiency when probiotics were administered via milk but improved feed efficiency when given through a milk replacer. According to a review of biological determinants of feed efficiency in beef cattle, several factors,

such as animals' eating behaviors, physical activity levels, feed digestibility, rumen microbiome, energy metabolism, protein turnover, body composition, and endocrine system play a role in the inter-animal variation of feed efficiency (Cantalapiedra-Hijar et al., 2018).

Heterogeneity

Substantial heterogeneity was detected in our MA for all outcomes; therefore, results must be interpreted carefully. Although Cochran's test is commonly adopted to assess heterogeneity, it is sensitive to the number of studies included in an MA. The test has poor power with few studies, and excessive power to detect unimportant heterogeneity when there are many studies (Higgins et al., 2003). Therefore, we also used the I^2 to assess heterogeneity, which indicates the amount of variability in the observed effects that are due to real differences between studies, rather than chance (Higgins et al., 2003). The observed heterogeneity suggests a lack of consistency in the effects observed across trials, which could be attributed to various factors, such as study design differences and methodological variations. The results of meta-regression indicated that the heterogeneity observed can be partially attributed to probiotic type, duration of supplementation, and period of supplementation. However, other factors, such as the mode of probiotic administration, diets, age, and housing adopted by the trials included in our MA, likely contribute to the high heterogeneity.

Risk of Bias

In our MA, we assessed the risk of bias at the outcome level (ADG) and identified six trials with a high risk of bias. The exclusion of those trials from the analysis did not affect the ADG results. Two trials were classified as high risk of bias due to concerns with the randomization process. One trial had a significant imbalance in sample size between the treatment and control groups. In unbalanced studies, Type I error rates could be an issue,

especially when sample sizes do not reach 200 (Alamolhoda et al., 2017). In another trial, a significant difference in baseline IgG levels was observed between treatment groups. While random imbalances do not introduce systematic bias, the randomization process might have been affected due to the importance of IgG in the future performance of calves (Robison et al., 1988). One trial was considered as high risk due to the method used to estimate body weight gain (i.e., tape). Heinrichs et al. (1992) developed an equation to calculate body weight from heart girth using tape for Holstein animals. However, poor agreement between tape and weight scale have been observed in calves younger than 3 mo (Dingwell et al., 2006).

Some concerns arise from the lack of reporting regarding allocation concealment and pre-specified analysis plans. In an evaluation of the completeness of reporting in dairy cattle studies published over a one year period, allocation concealment was described in only 3 of 104 randomized trials (Winder et al., 2019). In randomized controlled trials, adequate allocation concealment prevents people enrolling animals from having prior knowledge of the upcoming treatment assignments (O'Connor et al., 2010). Without concealment, selection bias can occur as animals may be selectively assigned to treatment groups. In human research, failure to report allocation concealment has been associated with exaggerated treatment effects (Schulz et al., 1995). However, in livestock research allocation concealment may be less critical when all eligible animals are enrolled and there is no preference for treatment group (Sargeant et al., 2023).

Pre-specification of the planned statistical analysis minimizes bias in trials stemming from selective reporting (Kahan et al., 2020). Such bias can occur if a finding is reported based on its direction, magnitude, or statistical significance. Protocols for human research studies are typically registered and made publicly accessible before a trial begins. However, this practice is

not widespread in dairy science, as evident in the American Veterinary Medical Association's animal health studies database, which does not feature any dairy protocols. Additionally, in our MA, two trials failed to inform the results for all time points that body weight was measured. To prevent this incomplete reporting, comprehensive detailed reporting of results could be made available in supplementary materials.

The present MA had several strengths. We developed a search strategy with the support of a research librarian. Multiple electronic databases and grey literature were searched, and not only studies written in English were included. The data extraction process involved two individuals to enhance reliability. Furthermore, the MA models accounted for data dependency, which was not considered in previous studies. Some studies were excluded due to unclear reporting, potentially leading to the omission of relevant findings. Additionally, the interventions involving probiotics varied significantly, including differences in the type of probiotics, dosage, mode of administration, and duration of supplementation, which contributed to high heterogeneity and was not fully explained by meta-regressions.

CONCLUSIONS

Probiotics have the potential to enhance ADG and starter intake in dairy calves. However, the current evidence is limited due to the heterogeneity of current studies. To establish more accurate recommendations, it is necessary to conduct further studies that specifically address variations in methodology. Additionally, future research should adhere to reporting guidelines, which ensure adequate study selection, data extraction, and risk of bias assessment in evidence synthesis studies.

TABLES AND FIGURES

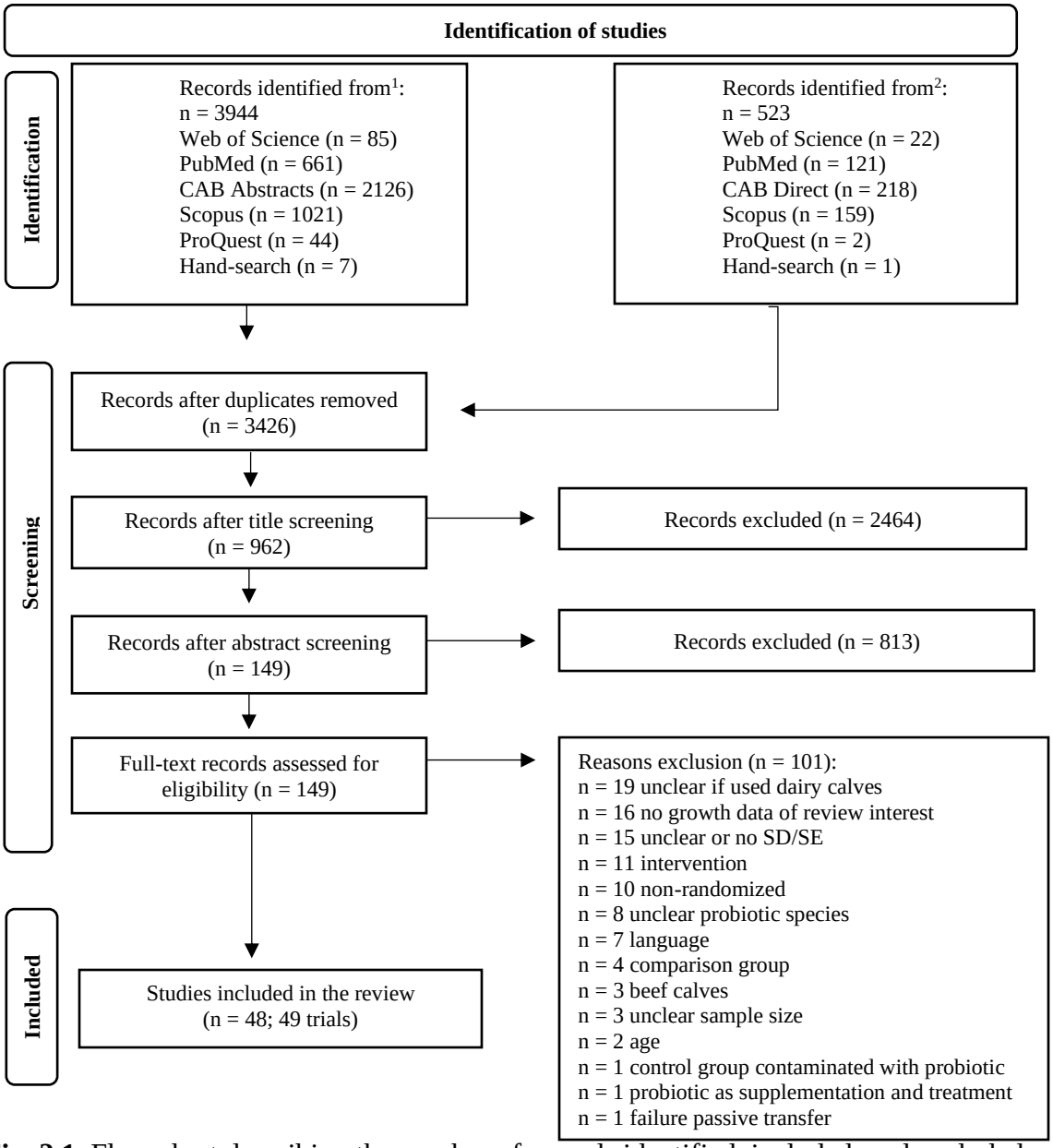

Fig. 2.1. Flow chart describing the number of records identified, included, and excluded, and the reasons for exclusions through the screening process of the systematic review and meta-analysis ([1]searched on 27th February to 3rd March 2020; [2]search was updated on 19th August 2021; chart adapted from Page et al., 2021).

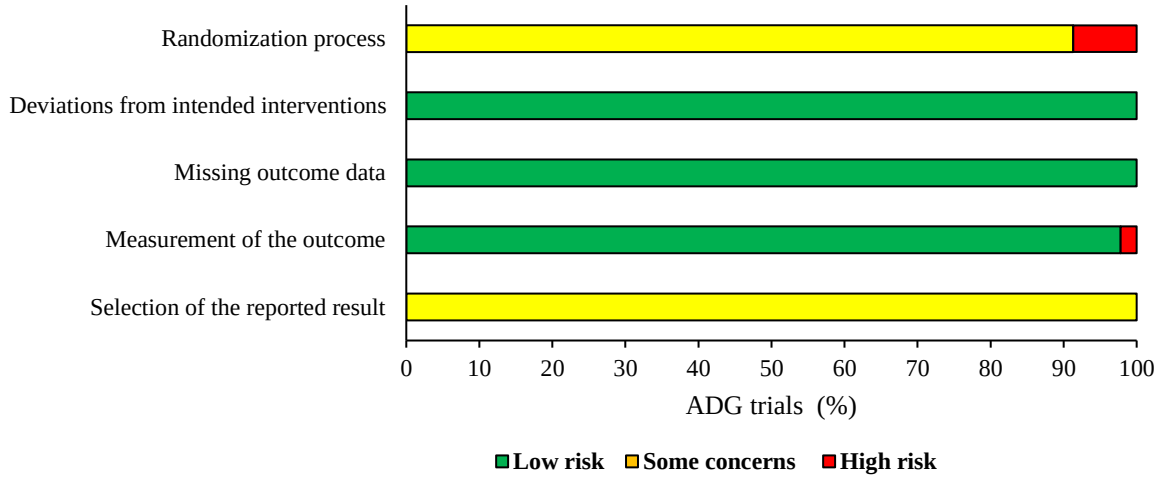

Fig. 2.2. Overall risk of bias for 45 trials that evaluated average daily gain. Risk of bias was evaluated based on Revised Cochrane risk-of-bias tool for randomized trials version 2 (RoB 2; Sterne et al., 2019). In the color-coded ranking, green, yellow, and red stand for low risk of bias, some concerns, and high risk of bias, respectively.

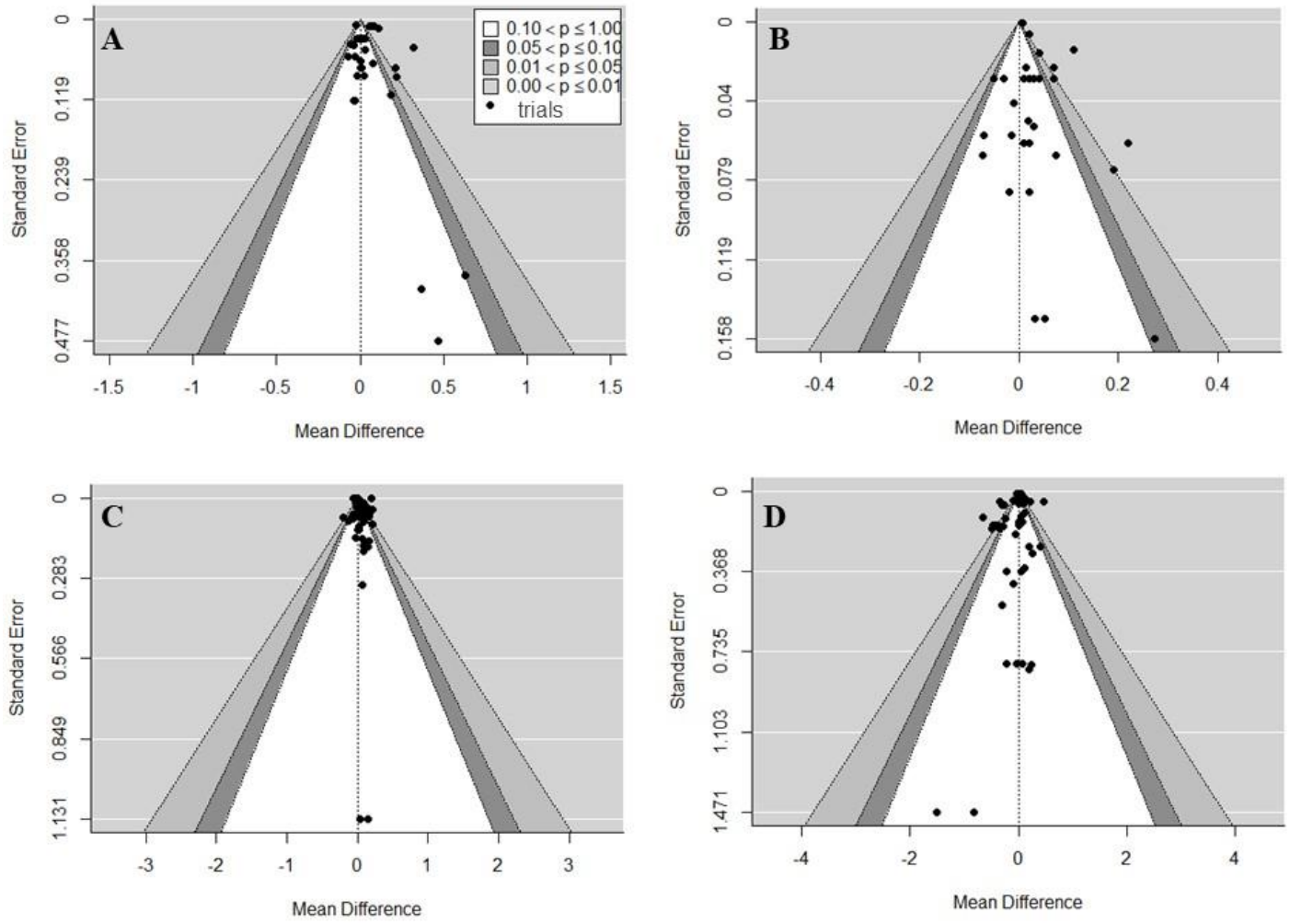

Fig. 2.3. Contour-enhanced funnel plots for DMI (A), starter intake (B), ADG (C), and feed efficiency (D) in trials that supplemented calves with probiotic. The shaded region indicates areas of levels of statistical significance for trials (•) and non-statistical significance is represented in white.

Table 2.1. Descriptive characteristics of the 48 studies reporting 49 trials included in the meta-analysis

Variable	Categories	Number of trials (arms)
Breed	Holstein	41 (57)
	Holstein cross[1]	3 (4)
	other	4 (5)
	not reported	1 (3)
Sex	male	21 (28)
	female	6 (11)
	male and female	19 (26)
	not reporting	3 (4)
Age	≤ 7d	29 (42)
	≤ 20d	12 (15)
	>21 – 120d	7 (10)
	not reporting[2]	1 (2)
Herd type	commercial	15 (21)
	research	22 (30)
	not reporting	12 (18)
Probiotic[3]	Bacillus	10 (12)
	Enterococcus	3 (3)
	Lactobacillus	12 (15)
	Saccharomyces	9 (14)
	Multistrains multi genera[4]	16 (20)
	other[5]	5 (5)
Maximum dose (cfu/d)	10^7 - 10^8	8 (8)
	$\geq 10^9$	18 (23)
	$\geq 10^{10}$	22 (27)
	$\geq 10^{11}$	2 (2)
	not reporting	5 (9)
Mode of administration[3]	milk replacer	16 (19)
	whole milk	13 (16)
	starter	5 (7)
	other[6]	21 (27)
Duration (d)[3]	≤ 21d	8 (10)
	≤ 42d	15 (18)
	≤ 56d	21 (26)
	>57 - 175d	11 (15)

[1]This includes one trial with both Holstein and Holstein x Jersey.

[2] This includes one trial described calves as newborn (not age specified).

[3]Six trials evaluated more than one probiotic type and were included in more than one category.

[4]Multistrains multi genera included more than one genus of the following, Aspergillus, Bacillus, Bifidobacterium, Candida, Enterococcus, Lactobacillus, Lactococcus, Pediococcus, Propionibacterium, Ruminobacter, Saccharomyces, Streptococcus, and Succinovibrio.

[5]other = included genera were Bifidobacterium, Candida, and Megasphaera.

[6]other = TMR, drench, feed, and the following combinations: colostrum and milk replacer, milk replacer and starter, milk replacer and waste milk, whole milk and milk replacer, whole and skimmed milk.

Table 2.2. Summary of meta-analyses on the effects of probiotics on the growth performance and feed intake of dairy calves

Outcome	Included trials	No. of trials	Effect size		Heterogeneity	
			MD or SMD (95% CI)	*P*-value	*P*-value	I^2 (%)
DMI (kg/d)	all	21	0.03 (-0.01, 0.08)	0.12	<0.001	91.3
	without influential	20	0.01 (-0.01, 0.04)	0.29	<0.001	78.4
Starter intake (kg/d)	all	23	0.02 (0.00, 0.04)	0.02	<0.001	98.9
	without influential	22	0.02 (0.00, 0.03)	0.01	<0.001	96.0
Milk intake (kg/d)	all	6	-0.35 (-0.80, 0.08)	0.09	0.34	28.9
ADG (kg/d)	all	45	0.03 (0.01, 0.06)	0.001	<0.001	99.5
	without influential	44	0.03 (0.01, 0.05)	0.002	<0.001	99.2
Feed efficiency	all	33	-0.14 (-0.39, 0.10)	0.23	<0.001	70.8
	without influential	31	-0.02 (-0.21, 0.16)	0.81	<0.001	54.0

Table 2.3. Summary of meta-analyses on the effects of different probiotic types on the growth performance and feed intake of dairy calves

			Effect size		Heterogeneity	
Probiotic type	Outcome	n (trials)	SMD or MD (95% CI)[1]	*P*-value	*P*-value	I^2 (%)
Bacillus	DMI (kg/d)	6	0.11 (-0.07, 0.29)	0.18	<0.001	93.3
	Starter intake (kg/d)	5	0.00 (-0.06, 0.07)	0.88	0.08	41.7
	ADG (kg/d)	10	0.06 (0.00, 0.12)	0.03	<0.001	99.0
	Feed efficiency	8	-0.59 (-1.26, 0.06)	0.07	0.002	74.8
Lactobacillus	DMI (kg/d)	4	-0.01 (-0.11, 0.08)	0.65	0.60	26.1
	Starter intake (kg/d)	3	0.05 (-0.04, 0.16)	0.13	0.73	0
	ADG (kg/d)	8	0.04 (-0.02, 0.11)	0.15	0.05	50.3
	Feed efficiency	8	-0.33 (-0.94, 0.26)	0.22	0.008	64.4
Saccharomyces	DMI (kg/d)	3	0.03 (-0.15, 0.22)	0.55	0.04	59.9
	Starter intake (kg/d)	5	0.03 (-0.04, 0.10)	0.34	0.01	68.6
	ADG (kg/d)	9	0.00 (-0.02, 0.04)	0.54	0.23	26.4
	Feed efficiency	5	0.48 (-1.08, 2.77)	0.59	<0.001	96.1
Multi genera	DMI (kg/d)	5	0.03 (-0.05, 0.12)	0.36	0.001	90.7
	Starter intake (kg/d)	8	0.01 (-0.00, 0.02)	0.11	0.09	93.6
	ADG (kg/d)	14	0.02 (-0.02, 0.06)	0.29	<0.001	99.6
	Feed efficiency	11	-0.16 (-0.45, 0.12)	0.22	0.03	39.6

Table 2.4. Summary of meta-regressions for dry matter intake and starter intake

Outcomes	Moderator	Coefficient (95% CI)	*P*-value
DMI (kg/d)	probiotic	0.11 (0.00, 0.04)	0.001
	period	0.35 (0.18, 0.51)	<0.001
Starter intake (kg/d)	probiotic	0.01 (0.00, 0.03)	0.006
	duration	-0.001 (-0.0017, -0.0003)	0.003

Chapter 3: Antimicrobial susceptibility of Enterococcus and Bacillus species isolated from commercial probiotic products used in cattle

ABSTRACT

The objective of this study was to evaluate the phenotypic antimicrobial susceptibility of Enterococcus ssp. and Bacillus ssp. isolated from commercially available cattle probiotics. Cattle probiotic products claiming to contain at least one type of Enterococcus spp. or Bacillus ssp. and marketed in North America and Europe were identified through different sources. A total of 35 products were included in the final list. Phenotypic antimicrobial susceptibility was evaluated by determining minimum inhibitory concentrations of 16 antimicrobials using broth microdilution. The Enterococcus ssp. were categorized as susceptible, intermediate, or resistant according to the breakpoints of the Clinical Laboratory and Standards Institute and the National Antimicrobial Resistance Monitoring System. The Bacillus ssp. was classified as susceptible or resistant using cut-off from the European Food Safety Authority Panel on Additives and Products or Substances used in Animal Feed. Among Enterococcus-based probiotics, 17 isolates were identified as *Enterococcus faecium*, and 1 as *Enterococcus hirae*. For Bacillus-based probiotics, 2 isolates were identified as *Bacillus licheniformis*, 4 as *Bacillus amyloliquefaciens*, 6 as *Bacillus subtilis*, and 3 were only identified at the genus level as Bacillus. All Enterococcus isolates exhibited susceptibility to chloramphenicol, streptomycin, tetracycline, tigecycline, and vancomycin. Nine Enterococcus isolates were resistant to one antibiotic (ciprofloxacin, erythromycin, penicillin, and daptomycin), and two isolates were resistant to two antibiotics. One Enterococcus isolate was multidrug-resistant to ciprofloxacin, daptomycin, and quinupristin/dalfopristin. Out of 15 Bacillus isolates, 13 isolates were susceptible to all investigated antibiotics with thresholds available. All Bacillus isolates showed susceptibility to both tetracycline and vancomycin.

However, one Bacillus isolate displayed resistance to chloramphenicol, and the other to erythromycin. In conclusion, Enterococcus isolates exhibited varying susceptibilities to a range of antimicrobials. Bacillus isolates displayed a higher degree of antibiotic susceptibility, with the majority of isolates exhibiting susceptibility to the antibiotics under investigation. The diversity in antimicrobial susceptibility patterns among Enterococcus and Bacillus isolates highlights the need for safety assessments of commercially available probiotics.

Keywords: antibiotic resistance, antibiogram, feed additives

INTRODUCTION

Misuse and overuse of antimicrobials contribute to rises in antimicrobial resistance (**AMR**), which is a global threat to human and animal health (Davies and Davies, 2010). In the US and Europe, mastitis and diarrhea have been reported as the most prevalent reasons for using antimicrobials in dairy cows and calves, respectively (De Briyne et al., 2014; USDA, 2018). Further, misdiagnosis of diseases by farm personnel can lead to inappropriate treatment decisions (Olson et al., 2019), which may contribute to AMR. Besides antimicrobial treatment for diseased animals, management practices (e.g., feeding waste milk or medicated milk replacer and culling frequency) have been associated with AMR in dairy cattle (Springer et al., 2019; Pandit et al., 2021).

Probiotics have emerged as an alternative treatment option to minimize antimicrobials use. They are defined as “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” (Hill et al., 2014). For instance, probiotics have reduced the need of therapeutic treatments and duration of diarrhea in calves (Timmerman et al., 2005; Renaud et al., 2019b), and showed a comparable cure rate to antibiotics in mastitic cows (Kitching et al., 2019). Furthermore, probiotic supplementation is widely adopted by dairy

producers in the US, with 38% of large dairy operations using it for heifers and 42% for cows (USDA, 2014).

Although some microorganisms used as probiotics are generally recognized as safe (GRAS) or have qualified presumption of safety (QPS) status, there is a growing research interest in assessing their safety (Merenstein et al., 2023). One of the concerns associated with probiotic use is the presence of AMR genes and the potential transmission of these genes to pathogenic bacteria (Li et al., 2020). Strains of lactic acid bacteria selected as potential probiotics for cattle carried tetracycline-resistant *tet(S)* gene and erythromycin-resistant *erm(B)* gene (Ficoseco et al., 2018). Additionally, a in vivo study suggested that antimicrobial resistant fecal coliform counts were higher after probiotic supplementation in calves (Bacillus and Lactobacillus spp.; Corbett et al., 2015).

Enterococcus and Bacillus ssp. are commonly investigated probiotics for dairy cattle use (Branco-Lopes et al., 2023). According to the 2019 Direct-fed-Microbial, Enzyme, and Forage Additive Compendium (Feedstuffs, 2019), probiotics commercialized for cattle commonly contain *E. faecium*. However, scarce information exists on the antibiotic resistance or susceptibility of Enterococcus- or Bacillus-based probiotics marketed to cattle. Amachawadi et al. (2018) reported that commercial strains of *E. faecium* for cattle and swine are resistant to a variety of antimicrobials (e.g., chloramphenicol, ciprofloxacin, tetracycline). Given the possible role of probiotics in antimicrobial stewardship programs and their extensive use as feed additives by dairy producers, it is crucial to assess their safety. The objective of this study was to evaluate the phenotypic antimicrobial susceptibility of Enterococcus and Bacillus ssp. isolated from commercially available cattle probiotics.

MATERIAL AND METHODS

Identification of probiotics products with Enterococcus ssp. and Bacillus ssp.

Cattle probiotics products marketed in North America and Europe that claimed to contain at least one Enterococcus spp. or Bacillus ssp. were identified through various sources. Products available in the US market were identified based on the 2019-Direct-fed Microbial, Enzyme, and Forage Additive Compendium (Feedstuffs, 2019) and the 12th Edition of Compendium of Veterinary Products (Bayer HealthCare, 2010). The European Union Register of Feed Additives (EFSA, 2022a) and Health Canada's Notification Program for Veterinary Health Products (VHP, 2022) were consulted to identify probiotics in Europe and Canada, respectively. Additionally, researchers, dairy consultants, and common web search engines were used to identify probiotic products available in the market. The final list included a total of 35 products. To keep the confidentiality of the commercial products, letters were assigned to each product.

Isolation and Identification of probiotics

For both Enterococcus and Bacillus probiotics, each probiotic product (0.5 g or ml) was reactivated in 5 mL of Tryptic Soy Broth at 35°C for 2h. For Enterococcus, 0.5 ml of the activated cultures were plated onto bile esculin agar (BEA) and incubated at 35°C for 24h. To ensure purification, the putative colonies were streaked onto BEA and subsequently onto blood agar (Remel, Lenexa, KS), with both plates being incubated at 35°C for 24h each time. As for Bacillus, once reactivated, the cultures were plated onto blood agar and incubated at 37°C for 18h. For purification, the putative colonies were streaked onto blood agar plates and incubated at 37°C for 18h each time. Presumptive isolates were maintained at −80°C in the presence of glycerol 20% (v/v), as a cryoprotective agent. Bacteria from frozen stock cultures were transferred to blood agar plates and incubated (at 35°C for 24h for Enterococcus and 37°C for

18h for Bacillus) prior to identification. For all isolates, species identification was performed using matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS) at California Animal Health and Food Safety Laboratory System (Tulare, CA, Clark et al., 2013). In brief, a single colony was directly spotted on the MALDI plate and treated with 1 µL of formic acid and 1 µL Bruker matrix. The plate, once loaded, was inserted into the instrument per manufacturer instructions (Bruker Daltonics, Germany) and was run in duplicate. Identifications were classified using the following values: a score ≥ 2.00 denoted species level identification, a score from 1.99 to 1.70 indicated at the genus level identification, and a score <1.70 meant no significant similarity of the obtained spectrum with any database entry.

Phenotypic Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing (AST) of the probiotic isolates was performed by the microdilution method in accordance with the Clinical Laboratory and Standards Institute (CLSI, 2019) to determine minimum inhibitory concentrations (MIC) of antimicrobials. Bacterial suspensions were prepared to an equivalent 0.5 McFarland turbidity standard by mixing individual colonies with demineralized water (Trek Diagnostics Systems, Cleveland, OH). A 10 µl aliquot of the standardized bacterial suspension was added to Mueller-Hinton broth (Trek Diagnostics Systems, Cleveland, OH) and vortexed. Fifty microliters of the final inoculum suspension were dispensed into each well of the Gram-positive NARMS panel plates (CMV3AGPF, Trek Diagnostics Systems, Cleveland, OH) using the Sensititre automated inoculation delivery system (Trek Diagnostics Systems, Cleveland, OH). Plates were incubated for 24 h at 35°C. To determine the antimicrobial susceptibility phenotype, interpretive criteria (clinical breakpoints or epidemiological cut-off) were applied to interpret MIC values. In our study, clinical breakpoints were used for Enterococcus ssp. due to their wider availability. For

Enterococcus ssp., isolates were recorded as resistant, intermediate, or sensitive based on the breakpoints established by CLSI (2020) if available; otherwise, breakpoints from National Antimicrobial Resistance Monitoring System (NARMS, 2021) were used (Supplemental material 3). Currently, CLSI or NARMS breakpoints for Bacillus ssp. are not available. Thus, Bacillus isolates were determined as resistant or susceptible using microbiological cut-off values defined by the European Food Safety Authority (EFSA) Panel on Additives and Products or Substances used in Animal Feed (FEEDAP, 2018; Supplemental Material 3). Quality assurance was performed by concurrently testing *E. faecalis* ATCC 29212, which is a reference strain recommended by the CLSI (2019). The MIC_{50} and MIC_{90} were calculated according to (Schwarz et al., 2010). The MIC_{50} and MIC_{90} are defined as the antimicrobial concentration that inhibited ≥50 and ≥ 90% of the isolates within a population, respectively.

RESULTS

Characterization of the products and identification of Enterococcus and Bacillus isolates

The characteristics of the 35 probiotic products are shown in Table 3.1. For Enterococcus-based probiotics, no visible bacterial growth was observed for 6 products (A, J, K, M, W and Y, all presumptive *E. faecium*). From 20 products, 17 isolates were identified as E. faecium, 1 as *E. hirae* and 2 as not Enterococcus ssp. (Pantoea ssp. and *Acinetobacter baumannii*). For Bacillus-based probiotics, visible growth was observed for all products. From 20 products, 2 isolates were identified as *B. licheniformis*, 4 as *B. amyloliquefaciens*, 6 as *B. subtilis*, and 3 were only identified at the genus level as belonging to Bacillus. Additionally, 7 were not identified by MALDI-TOF MS.

Antimicrobial susceptibility test for Enterococcus and Bacillus isolates

Enterococcus ssp. In total, 17 Enterococcus ssp. isolates were subjected to MIC determination for 16 antimicrobials, but one isolate was subjected to MIC determination due to a lack of supplies. The MICs of antimicrobials for Enterococcus ssp. are presented in Table 3.2. The MIC values for chloramphenicol (8 µg/mL), nitrofurantoin (64 µg/mL), streptomycin (512 µg/mL) and tetracycline (1 µg/mL) were the same against all isolates. The MIC for the remaining antibiotics varied from 0.06 (tigecycline) to 1024 µg/mL (gentamicin). Tigecycline exhibited the lowest MIC90 value (0.12 µg/mL), whereas streptomycin and kanamycin had the highest MIC90 values (512 µg/mL) against Enterococcus ssp.

Out of 16 isolates, 4 isolates had susceptible or intermediate phenotypes to all investigated antibiotics with thresholds available. All isolates were susceptible to chloramphenicol, nitrofurantoin, streptomycin, tetracycline, tigecycline, and vancomycin. Nine isolates were resistant to one antibiotic (4 isolates to ciprofloxacin, 2 isolates to erythromycin, 2 isolates to penicillin and 1 isolate to daptomycin). Two isolates were resistant to two antibiotics (1 isolate was resistant to erythromycin and gentamicin and 1 isolate was resistant to daptomycin and erythromycin). One isolate was multidrug-resistant (concurrent resistance to three or more agents from different antimicrobial classes) to ciprofloxacin, daptomycin and quinupristin/dalfopristin. Five isolates were resistant to ciprofloxacin (31.2%), 4 to erythromycin (25.0%), 3 to daptomycin (18.7%), 2 to penicillin (12.5%), 1 to gentamycin and quinupristin/dalfopristin (6.2%).

Bacillus ssp. The MIC determination for 16 antimicrobials was performed on 15 Bacillus ssp. isolates (Table 3.3). All isolates exhibited identical MIC values for ciprofloxacin (0.12 µg/mL), gentamicin (µg/mL), kanamycin (µg/mL), lincomycin (µg/mL), and streptomycin (µg/mL).

The MIC for the other 15 antibiotics varied from 0.03 (tigecycline) to 512 µg/mL (streptomycin). Against Bacillus ssp., ciprofloxacin and tigecycline displayed the lowest MIC90 values (0.12 µg/mL), while streptomycin exhibited the highest MIC90 values (512 µg/mL). Out of 15 isolates, 13 isolates were susceptible to all investigated antibiotics with thresholds available. All isolates showed susceptibility to both tetracycline and vancomycin. However, there was antibiotic resistance in two isolates; one displayed resistance to chloramphenicol, and the other to erythromycin.

DISCUSSION

In the dairy industry, the effects of probiotics on cattle performance and health have been largely studied and their application in dairy operations is widespread. However, their safety, specifically in relation to AMR, has not been as thoroughly examined. In this study, a total of 31 bacterial species isolated from cattle probiotics were tested for their susceptibility to 16 antimicrobials (NARMS panel). Our study provides valuable information into the antibiotic susceptibility of Enterococcus and Bacillus ssp. present in commercially available cattle probiotics, which is a critical step towards incorporating these feed additives into antimicrobial stewardship programs.

Our results indicated a mismatch between the declared content on probiotic labels and their actual composition. Although it is not the gold standard method for bacterial identification, MALDI-TOF MS, which was used in our study, can accurately identify Bacillus ssp. and Enterococcus ssp. (Celandroni et al., 2019; Kim et al., 2023). Thus, the disagreement within the same genus may be due to the discriminatory capabilities of MALDI-TOF MS (Santos et al., 2016). However, the identification of contaminants is unlikely to be a lack of accuracy in the bacterial identification method. Previous research on veterinary and human probiotics also

identified additional bacteria that were not listed in the products label (Berreta et al., 2021; Syromyatnikov et al., 2022). In our study, one product contained Pantoea ssp., a gram-negative bacterial genus that belongs to Enterobacteriaceae family, which have demonstrated some probiotic properties but have also been linked to infections (Van Rostenberghe et al., 2006; Amenyogbe et al., 2021) Another product contained *A. baumannii*, which is an opportunistic nosocomial pathogen responsible for a vast array of infections with a high mortality rate in humans (Antunes et al., 2014). In probiotics, bacterial contaminants are a concern, especially due to their potential to translocate from gut to bloodstream. Indeed, probiotic-associated bacteremia has been observed in infants (Bertelli et al., 2015). These results raise some concern about quality control in the manufacturing of these products.

In our study, we observed no visible bacterial growth in 6 Enterococcus-based probiotics, despite modifications to our isolation protocol, which included the use of blood agar media and variations in incubation times and temperatures. The definition of a probiotic is linked to microbial viability (Hill et al., 2014). To ensure that a probiotic product has the necessary number of live organisms, regulatory agencies require data on viability, which is typically assessed through plate count enumeration (Wendel, 2022). However, the inability to culture probiotic bacteria in vitro does not necessarily equate to a loss of microbial viability (Wendel, 2022). During manufacturing, transportation or storage, these bacteria can shift into a viable but not culturable state, which is a defensive adaptation that helps them withstand stressful conditions (Davis, 2014). Thus, despite being unculturable, the bacteria in this state remain metabolically active.

Antimicrobial susceptibility testing is utilized as a tool to assess the safety of probiotic products in different guidelines. The FAO/WHO developed a guideline for the evaluation of

probiotics, which highlighted the need for developing a standardized AST for microorganisms used as probiotics (FAO/WHO, 2002). Particularly important is the establishment of breakpoints to differentiate resistant from susceptible strains in beneficial bacteria, as these values are well-established for pathogenic bacteria. More recently, the EFSA-FEEDAP developed a guidance document to assist applications for marketing probiotics and defined microbiological cut-off for 13 relevant antimicrobials (EFSA-FEEDAP, 2018). Additionally, AMR testing is required as part of the safety assessments of probiotics by regulatory agencies in several countries, such as Brazil, Canada, China, India, the US, and the European Union (Roe et al., 2022). The EFSA established the QSP approach for live microorganisms used in foods and feeds (EFSA, 2022b). *E. faecium* lacks a QPS status due to the presence of potential harmful traits, requiring individual strain-level assessments (EFSA, 2022b). On the other hand, all the Bacillus species examined in our study (*B. subtilis*, *B. licheniformis*, *B. amyloliquefaciens*) possess a QPS status (EFSA, 2022b). The US Food and Drug Administration's Center for Veterinary Medicine has a GRAS notification program for ingredients in animal food. The FDA has not issued a list of Animal Food GRAS probiotic species; however, they provide information on GRAS notices, detailing the safety assessments conducted. Currently, the GRAS notices inventory did not include any notifications for the bacterial species that were investigated in our study (GRAS Notices, 2023).

In our study, some Enterococcus ssp. isolates demonstrated resistance to ciprofloxacin, erythromycin, penicillin, daptomycin, and gentamicin. Additionally, one isolate was multidrug-resistant to ciprofloxacin, daptomycin, and quinupristin/dalfopristin. For Bacillus ssp., one isolate was resistant to chloramphenicol and another to erythromycin. Within the Bacillus genus, intrinsic resistance to erythromycin, streptomycin, and chloramphenicol has been observed (Agersø et al., 2019). Enterococcus spp. are intrinsically resistant to cephalosporins,

lincosamides, low levels of aminoglycosides, fluoroquinolones, and folate pathway antagonists (CLSI, 2020). Research indicated that acquired AMR exists for high levels of ciprofloxacin (MIC >16 mg/L; Werner et al., 2010). However, in our study, high levels of resistance to ciprofloxacin could not be identified due to the restricted testing range on the NARMS panel, with the highest MIC on the panel being 4μg/mL. In line with our results, Shridhar et al. (2022) observed resistance to erythromycin in *E. faecium* isolated from cattle probiotics (MIC = 8 μg/mL) and identified that erythromycin-resistant strains harbored the *msrC* gene. However, the *msrC* gene, commonly found in *E. faecium*, is thought to be a result of acquired resistance dating back to the early stages of the species (Werner et al., 2001). Additionally, *E. faecium* LBB.E81 carrying the *msrC* gene was considered as safe and not able to confer acquired AMR (Urshev and Yungareva, 2021). Previously, resistance to daptomycin (MIC = 8 μg/mL) and penicillin (MIC = 16 μg/mL) in Enterococcus-based probiotics was observed; however, the strains did not carry any relevant AMR gene (Shridhar et al., 2022). Contrary to ours results, *E. faecium* from swine and cattle probiotics exhibited susceptibility to high-level gentamicin in one previous study (MIC ≥500 μg/mL; Amachawadi et al., 2018). In line with our findings, multidrug resistance was also detected in Enterococcus isolates from commercial probiotics (Amachawadi et al., 2018), and it has been associated with acquired resistance through mobile genetic elements (Partridge et al., 2018).

All 16 Enterococcus ssp. isolates were susceptible to chloramphenicol, streptomycin, tetracycline, tigecycline, and vancomycin. Additionally, all 15 Bacillus ssp. isolates showed susceptibility to both tetracycline and vancomycin. As with our results, 22 *E. faecium* strains isolated from cattle and swine probiotics were susceptible to high-level of streptomycin in one study (MIC = 512 μg/mL; Amachawadi et al., 2018). Despite being widely recognized for their

intrinsic low-level resistance to aminoglycosides, Enterococcus spp. may acquire high-level aminoglycoside resistance (streptomycin MIC ≥1000 µg/mL; Chow, 2000). Vancomycin susceptibility holds crucial importance since *E. faecium* is a major species involved in vancomycin-resistant enterococci infections (O'Toole et al., 2023). In the US, about 30% of all healthcare-associated enterococcal infections are resistant to vancomycin (CDC, 2019). Our findings for vancomycin susceptibility align with prior studies investigating safety of a potential probiotic strain (*E. lactis* JDM1, MIC ≤0.5 µg/mL; Fu et al., 2022) and commercial probiotics (MIC = 0.5 to 2.0 µg/mL; Amachawadi et al., 2018). However, Berreta et al. (2020) identified a transferrable vancomycin resistance gene (*vanA*) in 2 of 36 commercial veterinary probiotics. Similar to our results, vancomycin susceptibility was observed in all 114 Bacillus ssp. isolated from milk (MIC susceptible breakpoint ≤4 µg/mL; Zhai et al., 2023). In the US, the use of vancomycin and chloramphenicol as extra-label drug in food animals is prohibited (FARAD, 2023). Amachawadi et al. (2018) reported that 23% of *E. faecium* strains isolated from cattle probiotics were resistant to chloramphenicol (MIC = 32 µg/mL), while all strains isolated from swine probiotics were susceptible to this antimicrobial (MIC = 2 to 8 µg/mL). Zhang et al. (2016b) screened 108 enterococci isolated from Chinese infants as probiotic candidates and observed all strains were susceptible to chloramphenicol (disk diffusion content = 30 µg but diameter of inhibition zone and interpretative breakpoints not reported). Susceptibility to tetracycline is important as it is frequently used with dairy cattle. A survey on antimicrobial use in California dairies found tetracycline was the first-choice antimicrobial for hoof treatment, and it was employed for treating mastitis, metritis, and pneumonia (Abdelfattah et al., 2021). Intermediate sensitivity to tetracycline has been observed in candidate probiotic strains *E. faecium* NM213 and *E. faecium* NM113, which were isolated from infant feces (disk diffusion

content = 30 mg/mL but diameter of inhibition zone and interpretative breakpoints not reported; Mansour et al., 2014). However, probiotic candidate *E. faecium* MK-SQ-1 isolated from chicken bile have shown resistance to tetracycline (diameter of inhibition zone = 9 mm; Shi et al., 2020). Tetracycline susceptibility was observed in most (n =113/114) Bacillus ssp. (including *B. subtilis* and *B. licheniformis*) isolated from milk (MIC resistant breakpoint ≥16 μg/mL; Zhai et al., 2023). However, Zhai et al. (2023) identified that the only tetracycline-resistant strain (*B. cereus*) carried the gene *tetL*, which can be transferred across Bacillus stains.

Some limitations of this study should be noted. Our difficulty in culturing specific probiotic microorganisms could be due to their transition into a viable but non-culturable state. Despite attempting various isolation protocols, these microorganisms seemed to require growth conditions different from those utilized in our study. Only the phenotypic AMR was accessed in our study; given that susceptible species can carry AMR genes, further investigation is warranted to detect AMR determinants. Despite these limitations, the results presented here provide valuable information on the antimicrobial susceptibility profiles of Bacillus and Enterococcus isolates collected from cattle probiotic market in North America and Europe.

CONCLUSIONS

Resistance to multiple antimicrobials was observed in 11 Enterococcus ssp. isolates. Furthermore, one Enterococcus isolate showed multidrug resistance. In contrast, only two Bacillus ssp. isolates exhibited antimicrobial resistance. Notably, all the tested Enterococcus ssp. isolates were susceptible to important antimicrobials, such as chloramphenicol, streptomycin, tetracycline, tigecycline, and vancomycin. All Bacillus ssp. isolates displayed susceptibility to both tetracycline and vancomycin. This research underscores the vital importance of evaluating commercial probiotics for potential antimicrobial resistance.

TABLES

Table 3.1. Characteristics of the 35 probiotic products and identification of bacterial isolates

ID code	Label relevant microorganisms	Place of origin	Identification	
			Enterococcus ssp.	Bacillus ssp.
A	*Bacillus subtills* and *Enterococcus faecium*	North America	No growth	*B. amyloliquefaciens*
B	*Enterococcus faecium*	North America	*E. faecium*	
C	*Enterococcus faecium*	North America	*E. faecium*	
D	*Enterococcus faecium*	North America	*E. faecium*	
E	*Enterococcus faecium*	North America	*E. faecium*	
F	*Enterococcus faecium*	North America	*E. faecium*	
G	*Bacillus subtilis* and *Enterococcus faecium*	North America	*E. faecium*	Not significant match
H	*Enterococcus faecium*	North America	*E. faecium*	
I	*Enterococcus faecium* and *Enterococcus lactis*	Europe	*E. faecium*	
J	*Bacillus subtills, Bacillus licheniformis and Enterococcus faecium*	North America	No growth	*B. subtilis*
K	*Enterococcus faecium*	Europe	No growth	
L	*Enterococcus faecium, Bacillus subtilis* and *Bacillus licheniformis*	North America	*E. hirae*	*B. licheniformis*
M	*Enterococcus faecium* and *Bacillus subtilis*	North America	No growth	*B. amyloliquefaciens, B. subtilis*
N	*Enterococcus faecium*	North America	Pantoea ssp.	
O	*Enterococcus faecium*	North America	*Acinetobacter baumannii*	
P	*Enterococcus faecium*	North America	*E. faecium*	
Q	*Enterococcus faecium*	North America	*E. faecium*	
R	*Enterococcus faecium* and *Bacillus subtilis*	North America	*E. faecium*	Not significant match
S	*Bacillus licheniformis* and *Bacillus subtilis*	North America		*B. subtilis*
T	*Bacillus subtilis*	North America		*B. amyloliquefaciens*
U	*Bacillus subtilis*	North America		Bacillus ssp.
V	*Bacillus amyloliquefaciens*	Europe		Not significant match

W	*Bacillus subtilis* and *Enterococcus faecium*	North America	No growth	Not significant match
X	*Enterococcus faecium*	North America	*E. faecium*	
Y	*Bacillus subtills, Bacillus licheniformis* and *Enterococcus faecium*	North America	No growth	*B. subtilis, B. licheniforms*
Z	*Bacillus subtilis* and *Enterococcus faecium*	North America	*E. faecium*	Not significant match
AA	*Enterococcus faecium, Bacillus subtilis*	North America	*E. faecium*	Not significant match
BB	*Enterococcus faecium, Bacillus subtilis, Bacillus licheniformis* and *Bacillus coagulans*	North America	*E. faecium*	Bacillus ssp.
CC	*Enterococcus faecium*	North America	*E. faecium*	
DD	*Bacillus subtilis*	North America		*B. subtilis*
EE	*Bacillus ssp.*	Europe		Bacillus ssp.
FF	*Bacillus subtilis*	North America		Not significant match
GG	*Bacillus subtilis*	North America		B. amyloliquefaciens
HH	*Enterococcus faecium*	North America/Europe	*E. faecium*	
II	*Bacillus licheniformis* and *Bacillus subtilis*	North America/Europe		*B. subtilis*

Table 3.2. Distribution of the MIC, MIC50, and MIC90 values of 16 antimicrobials among Enterococcus ssp. isolates, the colors indicate the antimicrobial susceptibility phenotype; green represents susceptible, yellow represents intermediate, and red indicates resistant

	MIC values (µg/mL)																				
Antimicrobial	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	2048	R[1] (%)	MIC_{50}	MIC_{90}
Chloramphenicol										16									0	8	8
Ciprofloxacin							9	2	5										31.2	1	4
Daptomycin								1	12	3									18.7	4	8
Erythromycin									12	4									25.0	4	8
Gentamicin														15			1		6.2	128	128
Kanamycin														7	6	3			-	256	512
Lincomycin							5			11									-	8	8
Linezolid								15	1										0	2	2
Nitrofurantoin													16						0	64	64
Penicillin								5	8	1	2								12.5	4	16
Quinupristin/ dalfopristin						4		11	1										6.2	2	2
Streptomycin																16			0	512	512
Tetracycline							16												0	1	1
Tigecycline			2	14															0	0.12	0.12
Tylosin								8	6	2									-	2	8
Vancomycin						12	4												0	0.5	1

[1] R = % of resistant isolates

Table 3.3. Distribution of the MIC, MIC50, and MIC90 values of 16 antimicrobials among Bacillus ssp. isolates, the colors indicate the antimicrobial susceptibility phenotype; green represents susceptible and red indicates resistant

	MIC values (µg/mL)																				
Antimicrobial Drug	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	2048	R[1](%)	MIC_{50}	MIC_{90}
Chloramphenicol								7	5	2	1								6.7	4	8
Ciprofloxacin				15															-	0.12	0.12
Daptomycin					3	9	1		2										-	0.5	4
Erythromycin					13	1				1									6.7	0.25	0.5
Gentamicin														15					-	128	128
Kanamycin														15					-	128	128
Lincomycin										15									-	8	8
Linezolid						12	3												-	0.5	1
Nitrofurantoin										4	11								-	16	16
Penicillin					14		1												-	0.25	0.25
Quinupristin/ dalfopristin							1	11	3										-	2	4
Streptomycin																15			0	512	512
Tetracycline							8	3	2	2									-	1	8
Tigecycline		1		2	11	1													-	0.12	0.12
Tylosin					3	10	1	1											-	0.5	1
Vancomycin					14	1													0	0.25	0.25

[1] R = % of resistant isolates

GENERAL CONCLUSIONS

Chapter 1

The scoping review provided a comprehensive synthesis of controlled trials that evaluated probiotic supplementation in dairy calves. Over the years, there has been a consistent interest in probiotics for calves as evidenced by the widespread global research spanning four decades. One important finding of the scoping review was the lack of comprehensive reporting, which potentially poses challenges for future meta-analyses. The limited description of randomization methods, small sample sizes without justifiable rationale, and failure of funding disclosures reveal some areas that should be addressed in future research. Attending to these problems in future research is crucial as biases in experimental design and incomplete reporting may affect the interpretation and replicability of research.

The focus of most included studies was on early probiotic supplementation, which could be attributed to the higher susceptibility of younger calves to gut health issues. While Holstein and male calves were commonly studied, there is a need to diversify research to account for potential breed and sex-specific microbiota differences that could affect probiotic effectiveness. Lactobacillus was the most studied genus, and the dose and mode of probiotic administration differed widely among studies. The review underscored the importance of adopting standardized measurements, particularly for health evaluation.

In conclusion, while there is a wealth of research on probiotic supplementation in dairy calves, there is an overarching need for standardization and best practices in experimental design and outcome measurements. Adopting these standardizations will facilitate the scientific community's ability to derive meaningful conclusions from future primary and secondary research.

Chapter 2

The systematic review and meta-analysis undertaken provided a comprehensive evaluation of the effects of probiotic supplementation on feed intake and growth performance of preweaned and weaned dairy calves. Although probiotics did not alter total dry matter intake, they seem to enhance starter intake and showed a tendency to decrease milk intake. Starter intake is pivotal for preweaned dairy calves. Probiotic supplementation may increase starter intake, aiding in the efficient transition from the preruminant to ruminant state. Furthermore, probiotic supplementation positively impacted average daily gain, particularly with Bacillus ssp. Our results did not reveal any significant impact of probiotics on feed efficiency.

Some concerns were identified regarding the risk of bias in the included studies. Specifically, concerns were raised in areas like randomization, missing outcome data, and reporting methods. Such potential biases stress the importance of reducing allocation concealment and improving complete reporting to avoid potential biases that can affect outcomes. It is noteworthy that despite the exclusion of some trials due to high risk of bias, the findings remained consistent. In summary, these insights highlight the potential benefits of probiotic supplementation in dairy calves but also emphasize the need for further research with standardized methods to derive robust conclusions on their efficacy.

Chapter 3

The safety implications concerning antimicrobial resistance of probiotics have been under-investigated. Our study sought to fill this knowledge gap by examining antimicrobial susceptibility in bacterial species present in cattle probiotics. The absence of bacterial growth in several probiotic samples brings the product's viability into question. Though non-culturable bacteria might still be metabolically active, future research should evaluate the product's

viability. Discrepancies between the labeled and actual contents of the probiotics were identified, raising concerns about the manufacturing process. Surprisingly, contaminants, including an opportunistic pathogen, were present in some products, emphasizing the importance of rigorous quality control.

Notably in our investigation, while certain Enterococcus ssp. isolates displayed resistance to various antimicrobials, all 16 isolates of Enterococcus ssp. and all 15 of Bacillus ssp. demonstrated susceptibility to key antimicrobials like vancomycin and tetracycline. In conclusion, this study underscored the need for rigorous safety evaluations, particularly concerning antimicrobial resistance. The antibiotic susceptibility profile of these isolates suggests that more comprehensive safety assessments are necessary to ensure that probiotic supplements do not inadvertently contribute to the global challenge of antimicrobial resistance.

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APPENDIX

SUPPLEMENTAL MATERIAL 1

Effect of probiotics on performance and health of dairy calves: protocol for a systematic review and meta-analysis

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Amendments: Any amendments to this protocol will be documented and justified in the final systematic review.

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INTRODUCTION

Rationale: For over 60 years, antimicrobials have been used to both prevent and treat diseases in food animals (Xiong et al., 2018). However, the global concern with antimicrobial resistance has been increased the interest in alternative products, such as probiotics, that might reduce the use of antimicrobials. The International Scientific Association for Probiotics and Prebiotics defined probiotics as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill et al., 2014). The mechanisms used by probiotics to promote health benefits to their host are not fully elucidated. However, it seems that, in general, probiotics modulate the host's gut microbiota and immune system (Ma et al., 2018). Some studies have shown the supplementation of probiotics dairy calves to reduce incidence of diarrhea and promote growth (Foditsch et al., 2015; Fomenky et al., 2017). On the other hand, results from other studies have indicated that prebiotic supplementation has no effect (He et al., 2017) or even negative (Corbett et al., 2015) effect on health or performance of calves.

Previous systematic reviews (SR)evaluated the effect of probiotics on performance (Frizzo et al., 2011) and health (Signorini et al., 2012) of dairy calves. However, both SRs addressed only lactic acid bacteria, excluding other important probiotics, such as yeasts. The latest SR was published 8years ago, and since then several new studies have been published. Moreover, according to O'Connor et al. (2014) the median survival time of systematic reviews 5.5 years for human research.

Objectives: The first objective of this review is to identify, summarize, appraise, and discuss the current literature on probiotic supplementation for dairy calves. The second objective is to evaluate the effect of probiotic supplementation on performance and health of dairy calves. The research question addressed in this protocol and in the future systematic review is: does the probiotic supplementation effect performance or health of dairy calves?

a) Population: dairy calves (up to 7 months of age) of both sexes

b) Intervention: probiotic supplementation (only as prophylactic, not therapeutic use) c) Comparator: placebo or no probiotic supplementation

d) Outcomes: any performance measurement [e.g. body weight, average daily gain, body traits(heart girth, wither height, hip width, or body length), feed efficiency, dry matter intake, gastrointestinal tract measurements (volatile fatty acid concentration, rumen pH, papilla length and papilla width)] or any health measurement[e.g. serum metabolites (glucose and beta-hydroxybutyrate), immunoglobulins, cytokines, fecal score, diarrhea incidence, pneumonia incidence, mortality, days on treatment, microbiota and microbiome].

METHODS

Eligibility criteria: Besides the PICO elements described above, the systematic review will include only primary research studies, and of these, randomized and non-randomized controlled trials which are available in English, Spanish and Portuguese. No restriction for date will be imposed other than that of the databases searched. The studies can be published and non-published since the primary data is reported.

Information sources: Electronic searches were conducted using the following electronic databases: Biosis (Web of Science, 1926 to present), CAB Abstracts (CAB Direct, 1973 to present), Medline (PubMed, 1966 to present), and Scopus (Scopus, 1996 to present). Grey literature was searched to find unpublished data using Dissertations and Theses Database (ProQuest, 1861 to present). The bibliography of relevant studies was hand searched. The search was conducted between February 27th and March 3rd of 2020.

Data management: The studies identified in the searches were uploaded to the reference manager Sciwheel formerly known asF1000(Faculty of 1000 Limited, London, UK) and duplicates were

removed. The de-duplicated results were exported to the Covidence systematic review management software (Veritas Health Innovation, Melbourne, AU).

Selection process: Two screenings were conducted by two independent reviewers (RBL and another reviewer), first assessing manuscript title and secondly abstracts. The title screening used the following questions: 1) Does the title describe a study involving dairy calves?2) Does the title describe a study with probiotic supplementation? The abstract screening used the following questions:1) Does the abstract describe a primary intervention study? 2) Does the abstract describe a study involving dairy calves supplemented with probiotic? 3) Does the abstract describe one or more of the measurements in performance (e.g., average daily gain, feed efficiency) or health (e.g., fecal score, diarrhea incidence,)? Studies were excluded if both reviewers answer "no" for one of the questions. Only studies with "maybe "or "yes "answers were selected for following step. Conflicts between inclusion and exclusion by the two reviewers were discussed until a consensus was reached. A pilot test was conducted in 30 abstracts and the reviewers were trained on systematic review methodology. A full manuscript screening was performed by RBL on the remnant studies. This screening included the 3 previous abstract questions plus: 4) Is the study a controlled trial? 5) Is the study written in English, Spanish or Portuguese? 6) Is the probiotic supplementation strategy (prophylactic treatment for sick animals)? 7) Is the study population (dairy calves) equal or less than 7 months old? Studies were excluded if RBL answer "no" for one or more of the questions. The exclusion reason was recorded at this screening level.

Data collection process: Data from eligible studies is being extracted by RBL into an electronic spreadsheet and it will be reviewed by another reviewer. Data extraction forms, adapted from previous studies, were tested on 5 studies randomly selected by RBL.

General information data consist of: 1) journal name, 2) language, 3) country, 4) author affiliation, 5) year of publication, 6) year study was performed, 7) month study was performed, 8) funding information. Population characteristics consist of: 1) breed, 2) sex, 3) age, 4) housing system, 5) type production system (conventional vs organic), 6) assessment of passive transfer, 7) commercial or research herd. Intervention and comparator data consist of:1) description of comparator,2) commercial name of probiotic, 3) single or multistrain, 4) genera, 5) scientific name, 6) concentration, 7) dose, 8) via of administration (e.g. whole milk, milk replacer), 9) duration of supplementation. Outcomes: For continuous outcomes (e.g., average daily gain) the following information will be extracted: 1) number of experimental units for each treatment level, 2) least square or contrast means for each treatment level, 3) mean differences from control, 4) unit of results, 5) lower/upper 95% CI, 6) standard error, 7) P-value, and 8) timepoint of each measurement. For dichotomous outcomes (e.g., occurrence of diarrhea) information:1) number of positive experimental units per treatment group, 2) proportion of positive experimental units per treatment group, 3) total number of experimental units per treatment group, 4) unit of results, 5) odd ratio, 6) relative risk, 7) lower/ upper 95% CI,8) P-value, and 9) timepoint of each measurement.

Data items Outcomes and prioritization: The main performance outcomes are average daily gain and feed efficiency, and the secondary performance outcomes are body weight, body traits (heart girth, wither height, hip width, or body length), dry matter intake and rumen development indicators(volatile fatty acid concentration, ruminal pH, papilla length and papilla width).The main health outcomes are fecal score and diarrhea incidence and the secondary health outcomes are serum metabolites (glucose, beta-hydroxybutyrate), immunoglobulins, cytokines, pneumonia incidence, mortality, days on treatment, and rumen and gut microbiota and microbiome. The

prioritization of the performance outcomes was based on their impact on animal growth, weaning age, and economic results. The health outcomes were prioritized based on their easiness to evaluate gut health and also, they are frequently used. Moreover, fecal score is a feasible indicator for farm use. Risk of bias assessment: Risk of bias of randomized studies will be assessed for each outcome by RBL, using the Cochrane risk of bias 2.0 tool with the necessary adaptations to fit the specific review question.

Data synthesis: If more than 3studies investigated similar treatments with the same outcome a meta-analysis will be conducted. A random effects meta-analysis will be conducted. Studies will be weighted using the inverse variance method. Heterogeneity between studies will be assessed using Cochran's Q statistic and I^{2s}tatistic. Heterogeneity will be explored via sub-group analysis and/or meta-regression, if enough studies are found for a single outcome. A sub-group analysis will be performed categorizing the studies in pre-and postweaning and according with probiotics. If there are more than 10 studies, publication bias will be investigated using funnel plots, Begg's adjusted rank correlation, and Egger's test.

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Table 1.1.a. Results of the search strategy used to identify records; search strategy used in CAB Direct (CAB Abstracts)

Search ID	Terms	Results
#1 Population	title: (“calf” OR “calves” OR “veal” OR “preweaned dairy heifers”) OR ab: (“calf” OR “calves” OR “veal” OR “preweaned dairy heifers”) OR de: (“calf feeding” OR “calves” OR “veal calves”)	102,176
#2 Intervention	(de: ("probiotics" OR "yeasts" OR "feed supplements") OR od: ("Lactobacillus" OR "Faecalibacterium" OR "*Lactobacillus acidophilus*" OR "Propionibacterium" OR "Bacillus" OR "Enterococcus") OR od: ("Pediococcus" OR "Saccharomyces" OR "Lactococcus" OR "Megasphaera" OR "Enterobacteriaceae" OR "Bifidobacterium" OR "*Pediococcus acidilactici*" or "*Bifidobacterium bifidum*" or "*Enterococcus faecium*") OR od: ("*Lactobacillus casei* subsp. casei" OR "Actinobacteria" OR "*Faecalibacterium prausnitzii*" OR "Proteobacteria" OR "Firmicutes")) OR (ab: ("Direct fed microbial" OR "DFM" OR "probiotic" OR "probiotics" OR "Faecalibacterium" OR "Lactobacilli") OR ab: ("LAB" OR "Lactobacillus" OR "Propionibacterium" OR "Bacillus" OR "Pediococcus" OR "Enterococcus" OR "enterococcus" OR "Saccharomyces" OR "Lactococcus") OR ab: ("Megasphaera" OR "Bifidobacterium" OR "Faecalibacterium")) OR (title: ("Direct fed microbial" OR "DFM" OR "probiotic" OR "probiotics" OR "Faecalibacterium" OR "Lactobacilli" OR "Dietary Supplements") OR title: ("LAB" OR "Lactobacillus" OR "Propionibacterium" OR "Bacillus" OR "Pediococcus" OR "Enterococcus" OR "Saccharomyces" OR "Lactococcus") OR title: ("Megasphaera" OR "Bifidobacterium" OR "Faecalibacterium"))	305,648
#3 Outcome	(id: (“diarrhea” OR “fecal coliforms” OR “fecal flora” OR “feces” OR “gut flora” OR “intestinal microorganisms” OR “microflora” OR “scouring” OR “death rate” OR “liveweight gains” OR “digestive tract contents”)) OR (de: (“coliform count” OR “diarrhoea” OR “ faecal coliforms” OR "faecal flora" OR "intestinal microorganisms") OR de: ("microbial flora" OR "microorganisms" OR “growth rate” OR "liveweight gain" OR “animal health” OR “microbial flora” OR “liveweight” OR “weight gain” OR “faecal flora” OR “faeces”)) OR (ab: (“fecal score” OR “faecal score” OR “feces score” OR “weight gain” OR “feed efficiency” OR “diarrheal” OR “diarrhea” OR “diarrhoea” OR “diarrhoeal”) OR ab: (“intestinal development” OR “intestinal bacterial community” OR “microbiom*” OR “microbiota” OR “microbial community” OR “gut flora” OR “intestinal flora” OR “growth” OR “health” OR “mortality” OR “gut health”) OR ab: (“average daily gain” OR “ADG”)) OR (title: (“fecal score” OR “faecal score” OR “feces score”OR “weight gain” OR “feed efficiency” OR “diarrheal” OR “diarrhea” OR “diarrhoea”OR “diarrhoeal”) OR title: (“intestinal development” OR “intestinal bacterial community” OR “microbiom*” OR “microbiota” OR “microbial community” OR “gut flora” OR “intestinal flora” OR “growth” OR “health” OR “mortality” OR “gut health”) OR title: (“average daily gain” OR “ADG”))	2,297,396
#4	#1 AND #2 AND #3	2,126
#5	#4 AND yr: [2020 TO 2021]	218

Table 1.2.a. Results of the search strategy used to identify records; search strategy used in Scopus (Scopus)

Search ID	Terms	Results
#1 Population	TITLE-ABS-KEY (“calf” OR "calves” OR “veal" OR "preweaned dairy heifers")	95,378
#2 Intervention	TITLE-ABS-KEY(probiotic* OR "yeasts" OR "feed supplements" OR "Lactobacillus" OR "Faecalibacterium" OR {*Lactobacillus acidophilus*} OR “Propionibacterium” OR "Bacillus" OR "Enterococcus" OR "Pediococcus" OR "Saccharomyces" OR "Lactococcus" OR "Megasphaera" OR "Enterobacteriaceae" OR "Bifidobacterium" OR {*Pediococcus acidilactici*} OR {*Bifidobacterium bifidum*} OR {*Enterococcus faecium*} OR {*Lactobacillus casei*} OR "Actinobacteria" OR {*Faecalibacterium prausnitzii*} OR "Proteobacteria" OR "Firmicutes" OR "Direct fed microbial" OR "DFM" OR "Lactobacilli" OR "LAB")	968,182
#3 Outcomes	(TITLE-ABS-KEY (diarrh* OR "fecal coliforms" OR "fecal flora" OR "feces" OR "gut flora" OR "intestinal microorganisms" OR "microflora" OR scour* OR "death rate" OR "liveweight gains") OR TITLE-ABS-KEY ("digestive tract contents" OR "coliform count" OR "faecal flora" OR "intestinal microorganisms" OR "microbial flora" OR "microorganisms") OR TITLE-ABS-KEY ("growth rate" OR "animal health" OR "microbial flora" OR "liveweight" OR "weight gain" OR "faeces" OR "fecal score" OR "faecal score" OR "feces score" OR "weight gain" OR "feed efficiency") OR TITLE-ABS-KEY ("intestinal development" OR "intestinal bacterial community" OR microbiom* OR "microbiota") OR TITLE-ABS-KEY ("microbial community" OR "gut flora" OR "intestinal flora" OR "growth" OR "health" OR "mortality" OR "gut health" OR "intestinal development" OR "average daily gain" OR "ADG"))	10,221,498
#4	#1 AND #2 AND #3	1,021
#5	#4 AND (LIMIT-TO (PUBYEAR, 2021) OR LIMIT-TO (PUBYEAR, 2020))	159

Table 1.3.a. Results of the search strategy used to identify records; search strategy used in Web of Science (Biosis)

Search ID	Terms	Results
#1 Population	TI= ("calf" OR "calves" OR "veal" OR "preweaned dairy heifers")	34,452
#2 Intervention	TI= (probiotic* OR "yeasts" OR "feed supplements" OR "Lactobacillus" OR "Faecalibacterium" OR "*Lactobacillus acidophilus*" OR "Propionibacterium" OR "Bacillus" OR "Enterococcus" OR "Pediococcus" OR "Saccharomyces" OR "Lactococcus" OR "Megasphaera" OR "Enterobacteriaceae" OR "Bifidobacterium" OR "*Pediococcus acidilactici*" OR "*Bifidobacterium bifidum*" OR "*Enterococcus faecium*" OR "*Lactobacillus cas*ei" OR "Actinobacteria" OR "*Faecalibacterium prausnitzii*" OR "Proteobacteria" OR "Firmicutes" OR "Direct fed microbial" OR "DFM" OR "Lactobacilli" OR "LAB")	171,887
#3 Outcome	TI= (diarrh* OR "fecal coliforms" OR "fecal flora" OR "feces" OR "gut flora" OR "intestinal microorganisms" OR "microflora" OR scour* OR "death rate" OR "liveweight gains" OR "digestive tract contents" OR "coliform count" OR "faecal flora" OR "intestinal microorganisms" OR "microbial flora" OR "microorganisms" OR "growth rate" OR "animal health" OR "microbial flora" OR "liveweight" OR "weight gain" OR "faeces" "fecal score" OR "faecal score" OR "feces score" OR "weight gain" OR "feed efficiency" OR "intestinal development" OR "intestinal bacterial community" OR microbiom* OR "microbiota" OR "microbial community" OR "gut flora" OR "intestinal flora" OR "growth" OR "health" OR "mortality" OR "gut health" OR "intestinal development" OR "average daily gain" OR "ADG")	984,443
#4	#1 AND #2 AND #3	85
#5	#4 AND 2021 or 2020 (Publication Years)	22

Table 1.4.a. Results of the search strategy used to identify records; search strategy used in ProQuest (Dissertation and Theses database)

Search ID	Terms	Results
#1 Population	ab ("calf" OR "calves" OR "veal" OR "preweaned dairy heifers") OR ti ("calf" OR "calves" OR "veal" OR "preweaned dairy heifers")	5,602
#2 Intervention	ab ("Bifidobacterium" OR "*Pediococcus acidilactici*" OR "*Bifidobacterium bifidum*" OR "*Enterococcus faecium*" OR "*Lactobacillus casei*") OR ti("*bifidobacterium*" OR "*Pediococcus acidilactici*" OR "*Bifidobacterium bifidum*" OR "*Enterococcus faecium*" OR "*Lactobacillus casei*") OR ab (probiotic* OR "yeasts" OR "feed supplements" OR "Lactobacillus" OR "Faecalibacterium" OR "*Lactobacillus acidophilus*" OR "Propionibacterium" OR "Bacillus" OR "Enterococcus" OR "Pediococcus" OR "Saccharomyces" OR "Lactococcus" OR "Megasphaera" OR "Enterobacteriaceae") OR ti ("Actinobacteria" OR "*Faecalibacterium prausnitzii*" OR "Proteobacteria" OR "Firmicutes" OR "Direct fed microbial" OR DFM OR "Lactobacilli" OR "LAB") OR ab("Actinobacteria" OR "*Faecalibacterium prausnitzii*" OR "Proteobacteria" OR "Firmicutes" OR "Direct fed microbial" OR "DFM" OR "Lactobacilli" OR "LAB") OR ti(probiotic* OR "yeasts" OR "feed supplements" OR "Lactobacillus" OR "Faecalibacterium" OR "*Lactobacillus acidophilus*" OR "Propionibacterium" OR "Bacillus" OR "Enterococcus" OR "Pediococcus" OR "Saccharomyces" OR "Lactococcus" OR "Megasphaera" OR "Enterobacteriaceae")	44,356
#3 Outcome	ab (“gut flora” OR “intestinal flora” OR “growth” OR “health” OR “mortality” OR “gut health” OR “intestinal development” OR “average daily gain” OR “ADG”) OR ti (“gut flora” OR “intestinal flora” OR “growth” OR “health” OR “mortality” OR “gut health” OR “intestinal development” OR “average daily gain” OR “ADG”) OR ab (“feed efficiency” OR “intestinal development” OR “intestinal bacterial community” OR microbiom* OR “microbiota” OR “microbial community”) OR ti (“feed efficiency” OR “intestinal development” OR “intestinal bacterial community” OR microbiom* OR “microbiota” OR “microbial community”) OR ab (“growth rate” OR “animal health” OR “microbial flora” OR “liveweight” OR “weight gain” OR “faeces” “fecal score” OR “faecal score” OR “feces score” OR “weight gain”) OR ti (“growth rate” OR “animal health” OR “microbial flora” OR “liveweight” OR “weight gain” OR “faeces” “fecal score” OR “faecal score” OR “feces score” OR “weight gain”) OR ab (diarrh* OR “fecal coliforms” OR “fecal flora” OR “feces” OR “gut flora” OR “intestinal micro-organisms” OR “microflora” OR scour* OR “death rate” OR “liveweight gains” OR “digestive tract contents” OR “coliform count” OR "faecal flora" OR "intestinal microorganisms" OR "microbial flora" OR "microorganisms") OR ti (diarrh* OR “fecal coliforms” OR “fecal flora” OR “feces” OR “gut flora” OR “intestinal micro-organisms” OR “microflora” OR scour* OR “death rate” OR “liveweight gains” OR “digestive tract contents” OR “coliform count” OR "faecal flora" OR "intestinal microorganisms" OR "microbial flora" OR "microorganisms")	519,527
#4	#1 AND #2 AND #3	44

#5	#4 AND Limits applied 2020 - 2021	2

Fig. 1.1.a. World choropleth map indicating the geographic distribution of the 103 studies included in the scoping review (USA: n = 22; Brazil: n = 13; China: n = 9; Canada/Iran: n = 7; Argentina/Japan/Poland: n = 6; Lithuania/South Africa: n = 3; Cuba/India/Uruguay: n = 2; Australia/Bulgaria/Chile/Colombia/Czech Republic/Egypt/Finland/Italy/Netherlands/New Zealand/Mexico/Saudi Arabia/Spain/South Korea/Venezuela: n = 1).

Table 1.5.a. Description of trials that supplemented Bacillus to calves (BC category)

Study	Scientific name	Dose[1]	Unit
Riddell et al., 2010[2]	*Bacillus subtilis* and *Bacillus lichenformis*	10^9 and 10^6	cfu/d and cfu/g starter
Torrezan et al., 2016	*B. subtilis* and *B. lichenformis*	2	g/d
Bakhshi et al., 2006	*B. subtilis* and *B. lichenformis*	3.2×10^9	cfu/d
Le et al., 2017[3]	*B. amyloliquefaciens* H57	3.16×10^8	cfu/kg starter
Sun et al., 2010	*B. subtilis* natto	10^{10}	cfu/d
Jenny et al., 1991[4]	*Lactobacillus acidophilus*, *L. lactis*, and *B. subtilis*	2.2×10^9, 2.2×10^6, and 1.1×10^9	cfu/d per strain
	B. subtilis	1.24×10^{10}	cfu/d
Kowalski et al., 2009[5]	*B. licheniformis* and *B. subtilis*	1.32×10^9 and 1.13×10^9	cfu/d milk replacer and cfu/d starter
Garcia, 2008	*B. subtilis*	10^9	cfu/d
	B. subtilis	2×10^9	cfu/d
	B. subtilis	4×10^9	cfu/d
Deng et al., 2021	*B. megatherium* 1259	1.2×10^{11}	cfu/d
Górka et al., 2021	*B. licheniformis* and *B. subtilis*	5.85×10^8	cfu/d
Yao et al., 2020	*B. megaterium*	5×10^9	cfu/d

[1]Multistrains probiotics = dose is presented as the total bacterial count, when concentration is stratified by strain the amounts are indicated.
[2]10^9 cfu/d in the milk replacer and 10^6 cfu/g in the starter (unclear starter intake).
[3] Starter intake = 700g/d at weaning.
[4]2.2×10^9 cfu/d of *Lactobacillus acidophilus*, 2.2×10^6 cfu/d of *L. lactis*, and 1.1×10^9 cfu/d of *B. subtilis*.
[5]1.32×10^9 cfu/d in the milk replacer and 1.13×10^9 cfu/d in the starter.

Table 1.6.a. Description of trials that supplemented Enterococcus to calves (ENT category)

Study	Scientific name	Dose	Unit
Salazar et al., 2019[1]	*Enterococcus faecium* NCIMB 10415	1.4×10^{9}	cfu/kg starter
Jatkauskas and Vrotniakiene, 2010	*E. faecium* M74	1.2×10^{11}	cfu/d
Jatkauskas and Vrotniakiene, 2014[2]	*E. faecium* M74 NCIMB 11181	9×10^{10} then 1.5×10^{10}	cfu/d
Šmídková and Čížek, 2017	*E. faecium* M74 NCIMB 11181	NR	NR

[1]Stater intake over the experiment = 843.9 g/d and 1590.8 g/d for calves supplemented with probiotic pre- and post-weaning, respectively.
[2]9×10^{10} cfu/d from 4 to day 24 of age and 1.5×10^{10} cfu/d from day 25 to day 67 of age.
NR = not reported.

Table 1.7.a. Description of trials that supplemented Saccharomyces to calves (SC category)

Study	Scientific name	Dose	Unit
He et al., 2017	*Saccharomyces boulardii* CNCM I-1079	10^{10}	cfu/d
Huuskonen and Pesonen, 2015[1]	*S. cerevisiae* Sc 47	10^{10}	cfu/g starter
Lee et al., 2019	*S. boulardii* CNCM I-1079	10^{10}	cfu/d
	S. boulardii CNCM I-1079	2×10^{10}	cfu/d
	S. boulardii CNCM I-1079	4×10^{10}	cfu/d
Melendez et al., 2018	*S. cerevisiae*	10^{10}	cfu/d
Terré et al., 2015[2]	*S. cerevisiae* CNCM I-1077	1.5×10^{6}	cfu/g stater
Renaud et al., 2019	*S. boulardii* CNCM I-1079	10^{10}	cfu/d
	S. boulardii CNCM I-1079	2×10^{10}	cfu/d
Villot et al., 2019	*S. boulardii* CNCM I-1079	1×10^{10}	cfu/d
Rokde et al., 2007	*S. cerevisiae* NCDC 47	5×10^{9}	cfu/d
Neumann et al., 2014	*S. cerevisiae* KA500	2×10^{11}	cfu/d
Neumann et al., 2015	*S. cerevisiae* NCYC 996	3×10^{10}	cfu/d
Turney et al., 2017[3]	*S. cerevisiae* CNCM I-1077	10^{10}	cfu/g starter
Galvão et al., 2005[4]	*S. cerevisiae* CNCM I-1077	10^{10}	cfu/d
	S. boulardii CNCM I-1079	10^{10}	cfu/d
	S. cerevisiae CNCM I-1077 / *S. boulardii* CNCM I-1079	10^{10} / 10^{10}	cfu/d
Seymour et al., 1995	*S. cerevisae*	1	% starter
Watanabe et al., 2019	*S. cerevisae* CNCM I-1077	2×10^{10}	cfu/d
Pinos-Rodríguez et al., 2008	*S. cerevisae* CNCM I-1077	2×10^{10}	cfu/d
	S. boulardii CNCM I-1079	2×10^{10}	cfu/d
Takemura et al., 2020	*S. cerevisae* CNCM I-1077	2×10^{9}	cfu/d
Villot et al., 2020	*S. boulardii* CNCM I-1079	10^{10}	cfu/d

[1]Stater intake over the experiment = 0.67 kg/d and 2.46 kg/d for calves supplemented with probiotic during pre- and post-weaning, respectively.
[2]Stater intake over the experiment = 0.57 kg/d and 2.34 kg/d for calves supplemented with probiotic during pre- and post-weaning, respectively.
[3]Stater intake over the experiment = 0.607 kg/d/pen (4 -11 d old) and 60.770 kg/d/pen (40 - 46 d old) for calves supplemented with probiotic (10 calves/pen).
[4]*S. cerevisiae* CNCM I-1077 = 10^{10} cfu/d (in milk) and *S. boulardii* CNCM I-1079 (in starter) = 10^{10} cfu/d

Table 1.8.a. Description of trials that supplemented Lactobacillus to calves (LB category)

Study	Scientific name	Dose[1]	Unit
Ávila et al., 1995	*L. acidophilus*	2×10^{8}	cfu/d
Gilliland et al., 1980[2]	Trial 1 and 2: *L. acidophilus* NCFM	2×10^{6} to 10^{7}	cfu/ml milk
	Trial 1 and 2: *L. acidophilus* C-28	2.8×10^{6} to 2×10^{7}	cfu/ml milk
Fomenky et al., 2017[3]	*S. boulardii* CNCMI-1079	7.5×10^{8} and 3×10^{9}	cfu/L milk replacer and cfu/kg starter
	L. acidophilus BT1386	2.5×10^{8} and 10^{9}	cfu/L milk replacer and cfu/kg starter
Nagashima et al., 2010[4]	Trial 1: *L. plantarum* HOKKAIDO	10^{9}	cfu/d
	Trial 2: *L. plantarum* HOKKAIDO	2×10^{9}	cfu/d
	Trial 2: *L. plantarum* 220, *E. faecium*, and *Clostridium butyricum* Miyari	2×10^{8}, 2×10^{7}, and 2×10^{6}	cfu/d per strain
Abe et al., 1995[5]	Trial 1: *Bifidobacterium pseudolongum* M-602	3×10^{9}	cfu/d
	Trial 1: *L. acidophilus* LAC-300	3×10^{9}	cfu/d
	Trial 2: *B. thermophilum* S-501, *E. faecium* FA-5, and *L. acidophilus* LAC-300	10^{10}, 10^{10}, and 10^{9}	cfu/d per strain
Cruywagen et al., 1996	*L. acidophilus*	5×10^{7}	cfu/d
Luyai, 2004	Trial 1 and 2: *L. acidophilus* 381 IL-28	10^{9}	cfu/d
Chaves et al., 1999	*L. acidophilus* LT 516	1.9×10^{10}	cfu/d
Soca et al., 2011	*L. rhamnosus* and *L. acidophilus*	10^{11}	cfu/d
Abdala et al., 2001	*L. acidophilus*	4×10^{8}	cfu/d
Abu-Tarboush et al., 1996[6]	*L. acidophilus* and *L. plantarum*	1.25	g/100 kg milk
	L. acidophilus 27SC	1.85×10^{7}	cfu/L milk
Rodriguez-Palacios et al., 2017	*L. plantarum* B80	$10^{7\text{-}8}$	cfu/d
	L. plantarum B80	$10^{10\text{-}11}$	cfu/d
Ellinger et al., 1980	*L. acidophilus*	4.24×10^{6}	cfu/d
Gupta et al., 2016[7]	*L. acidophilus*	6.8×10^{8}	cfu/L milk
	L. plantarum	6.8×10^{8}	cfu/L milk
Zhang et al., 2019	*L. rhamnosus* GG	10^{10}	cfu/d
Zhang et al., 2016[8]	*L. plantarum* GF103	1.7×10^{10}	cfu/d
	L. plantarum GF103 and *B. subtilis* B27	1.7×10^{10} and 1.7×10^{8}	cfu/d per strain

Fernández et al., 2020	Trial 1 and 2: *L. reuteri* TP1.3B	2×10^{10}	cfu/d
	Trial 1 and 2: *L. johnsonii* TP1.6	2×10^{10}	cfu/d
Rondón et al., 2020	*L. salivarius* C-65	10	mL/kg DM
	L. salivarius C-65	20	mL/kg DM
Stefańska et al., 2021[9]	*L. casei* PKM B/00103, *L. salivarius* PKM B/00102, and *L. sakei* PKM B/00101	2.5×10^{10}	cfu/d per strain
Casper et al., 2021	*L. plantarum* GB LP 1	4.8×10^{9}	cfu/d
	L. plantarum GB LP 1	9.6×10^{9}	cfu/d
Fernández-Ciganda et al., 2021	*L. reuteri* TP1.3B	2×10^{10}	cfu/d
	L. plantarum TP1.6	2×10^{10}	cfu/d
Jiang et al., 2020	*L. plantarum* 299v	10^{10}	cfu/d
Zavistanaviciute et al., 2020	*L. uvarum* LUHS245	$5\times10^{8.5}$	cfu/d
Amat et al., 2020	*L. amylovorus* 72B, *L. buchneri* 63A and 86D, *L. curvatus* 103C, and *L. paracasei* 3E and 57A	3×10^{9}	cfu/d per strain

[1]Multistrains probiotics = dose is presented as the total bacterial count, when concentration is stratified by strain the amounts are indicated.
[2]*L. acidophilus* NCFM ranged from 2×10^{6} to 10^{7} cfu/ml of milk and *L. acidophilus* C-28 ranged from 2.8×10^{6} to 2×10^{7} cfu/ml of milk. Milk intake = calves were fed daily a milk amount of 12.5% of their metabolic size (body weight not informed).
[3]*S. boulardii* CNCMI-1079 (7.5×10^{8} cfu/L in the milk replacer and 3×10^{9} cfu/kg in the starter), *L. acidophilus* BT1386 (2.5×10^{8} cfu/L in the milk replacer and 10^{9} cfu/kg in the starter). Milk intake = 6 L/d (first 4 d) and 9 L (5 - 53 d) for both arms. Stater intake (53 - 88 d) = 3.60 and 3.58 kg/d for calves supplemented with *S. boulardii* CNCMI-1079 and *L. acidophilus* BT1386, respectively.
[4]2×10^{8} cfu/d of *L. plantarum*, 2×10^{7} cfu/d of *E. faecium*, and 2×10^{6} cfu/d of *Clostridium butyricum* Miyari.
[5]10^{10} cfu/d of *B. thermophilum* S-501, 10^{10} cfu/d of *E. faecium* FA-5, and 10^{9} cfu/d of *L. acidophilus* LAC-300
[6]Milk intake = a maximum of 4 kg/d for 9 wk, and restricted to 2.5 kg/d at 10 wk.
[7]Milk intake = 2 L/d (9 – 18 d) and 1 L/d (31 – 90 d).
[8]1.7×10^{10} cfu/d of *L. plantarum* GF103 and 1.7×10^{8} cfu/d of *B. subtilis* B27.
[9]2.5×10^{10} cfu/d for each of the following strains: *L. casei* PKM B/00103, *L. salivarius* PKM B/00102, *L. sakei* PKM B/00101.
NR = not reported.

Table 1.9.a. Description of trials that supplemented calves with multiple strains probiotics from different genera (Multi category)

Study	Scientific name	Dose[1]	Unit
Batista et al., 2008[2]	*L. acidophilus*, *B. bifidum*, and *E. faecium*	6.66×10^{6}	cfu/d per strain
Bayatkouhsar et al., 2013	*L. acidophilus*, *L. casei*, *B. bifidium*, and *E. faecium*	2×10^{8}	cfu/d
	L. acidophilus PTCC 1643, *L. rhamnosus* PTCC 1637, *L. casei* PTCC 1608, and *L. delbrueckii* PTCC 1333	2×10^{8}	cfu/d
Corbett et al., 2015[3]	*B. licheniformis*, *B. subtilis*, *L. acidophilus*, *L. lactis*, *B. animalis* lactis, and *E. faecium*	7.2×10^{9} all and 10^{5} *E. faecium*	cfu/d
	B. licheniformis, *B. subtilis*, *L. acidophilus*, *L. lactis*, *B. animalis* lactis, and *E. faecium*	3.6×10^{9} all and 10^{5} *E. faecium*	cfu/d
Görgülü et al., 2003	*L. plantarum*, *L. bulgaricus*, *L. acidophilus*, *L. rhamnosus*, *B. bifidum*, *Streptococcus thermophilus*, *E. faecium*, *Aspergillus oryza*, and *Candida pintolopesii*	6.16×10^{8}	cfu/d
Cantor et al., 2019[4]	*E. faecium* M74, *L. acidophilus*, *L. casei*, *S. cerevisiae*, *B. bifidum*, and *L. lactis*	10/5/1.75	cc/cc/g
Frizzo et al., 2011[5]	*L. casei* DSPV 318 T, *L. salivarius* DSPV 315 T, and *Pediococcus acidilactici* DSPV 006 T	10^{9}	cfu/kg BW
Windschitl et al., 1991	*S. cerevisiae*, *E. faecium*, *L. acidophilus*, *A. oryzae*, and *B. subtilis*	28	g/d
Kawakami et al., 2010[6]	*L. plantarum* chikuso-1 and *Candida* sp. CO119	3.7×10^{11} and 2.6×10^{9}	cfu/d per strain
Flores et al., 2019[7]	*E. faecium* and *S. cerevisiae*	5×10^{9} and 2×10^{9}	cfu/d per strain
Strzetelski et al., 1998	*L. acidophilus*	4×10^{6}	cfu/d
	L. acidophilus, *L. casei*, *L. plantarum*, and *E. faecium*	4×10^{7}	cfu/d
	E. faecium	4×10^{10}	cfu/d
	B. bifidum	4×10^{10}	cfu/d
Qadis et al., 2014[8]	*L. plantarum* 220, *E. faecium* 26, and *C. butyricum* Miyari	9×10^{6}, 9×10^{5}, and 9×10^{4}	cfu/g per strain
Dick et al., 2013[9]	Trial 1: *L. acidophilus* and *Propionibacterium freudenreichii*	5×10^{8}	cfu/d
	Trial 2: *L. acidophilus* and *P. freudenreichii*	10^{5} and 10^{9}	cfu/d per strain

	Trial 2: *L. acidophilus* and *P. freudenreichii*	10^6 and 10^9	cfu/d per strain
Soto et al., 2014	*L. casei* DSPV 318T, *L. salivarius* DSPV 315T and *P. acidilactici* DSPV 006T	10^{10}	cfu/d
	L. plantarum DSPV 354T	10^{10}	cfu/d
Quintero-Gonzalez et al., 2003	*L. acidophilus, B. subtilis, B. licheniformis*, and *L. lactis*	3.3×10^8	cfu/d
	L. acidophilus	2×10^{10}	cfu/d
Agazzi et al., 2014	*L. animalis* SB310, *L. paracasei paracasei* SB137, and *B. coagulans* SB117	1.8×10^{10}	cfu/d
Rodriguez, 1994	*L. acidophilus* and *E. faecium*	10^9	cfu/d
Frizzo et al., 2010a[10]	*L. casei* DSPV 318T, *L. salivarius* DSPV 315T, and *P. acidilactici* DSPV 006T	10^9	cfu/kg BW
Bittar et al., 2016	*B. cereus, E. faecium, L. acidophilus, Ruminobacter amylophilum, R. succinogenes*, and *S. dextrinosolvens*	2	g/d
Frizzo et al., 2010b[11]	*L. casei* DSPV 318T, *L. salivarius* DSPV 315T, and *P. acidilactici* DSPV 006T	10^9	cfu/kg BW
Badiei et al., 2013	*L. plantarum*, *L. delbrueckii* bulgaricus, *L. acidophilus, L. rhamnosus*, *B. bifidum*, *S. salivarius, C. pintolopesii*. thermophilus, *E. faecium*, and *A. oryzae*	2	g/d
Frizzo et al., 2008[12]	*L. casei* DSPV 318T, *L. salivarius* DSPV 315T, and *P. acidilactici* DSPV 006T	10^9	cfu/kg BW
Seifzadeh et al., 2017	*L. plantarum*, *L. delbrueckii* bulgaricus, *L. acidophilus*, *L. rhamnosus* thermophilus, *E. faecium*, *A. oryzae*, and *C. pintolopesii*	2	g/d
Gonçalves et al., 2000[13]	*L. acidophilus, B. subtilis, L. lactis*	5	g/d
	L. acidophilus, B. subtilis, B. lifidem, L. lactis and *L. acidophilus, B. subtilis*, and *L. lactis*	10 and 5	ml/d and g/d
Alves et al., 2000[14]	*L. acidophilus*, *E. faecium*, and *S. cerevisae*	3×10^7	cfu/g probiotic
Quintero-Moreno et al., 1998	*L. acidophilus, L. lactis*, *B. bifidum* and *B. subtilis*	3.25×10^8	cfu/d
Higginbotham et al., 1993	*L. acidophilus* and *E. faecium*	10^9	cfu/d
Moghadam et al., 2020	*L. plantarum*, *L. delbrueckii* bulgaricus, *L. acidophilus, L. rhamnosus, B.*	2	g/d

	bifidum, *S. salivarius* thermophilus, *E. faecium*, *A. oryzae*, and *C. pintolopesii*		
Karamzadeh-Dehaghani et al., 2021	*E. faecium*, *P. acidilactici*, S. *thermophilus*, *L. bulgaricus*, *L. acidophilus*, *L. rhamnosus*, and *B. bifidum*	10^8	cfu/d
Lucey et al., 2021[15]	*B. subtilis* and *L. plantarum*	10^9 and 2.5×10^8	cfu/d per strain
Liang et al., 2020[16]	*L. casei* and *E. faecium*	2×10^{10} then 2×10^9	cfu/d
Mandouh et al., 2020[17]	*B. subtilis* DSMZ 5750, *B. licheniformis* DSMZ 5749, and *E. faecium*	6.4×10^8, 6.4×10^8, and 10^9	cfu/d per strain
Wu et al., 2021[18]	*L. acidophilus*, *B. subtilis*, and *S. cerevisiae*	1.5×10^9, 1.5×10^9, and 5×10^8	cfu/d per strain
	L. acidophilus, *B. subtilis*, and *S. cerevisiae*	3×10^9, 3×10^9, and 10^9	cfu/d per strain
	L. acidophilus, *B. subtilis*, and *S. cerevisiae*	6×10^9, 6×10^9, 2×10^9	cfu/d per strain
Timmerman et al., 2005[19]	Trial 1 and 2: *L. acidophilus* W55, *L. salivarius* W57, L. paracasei spp. W56, *L. plantarum* W59, *L. lactis* W58, and *E. faecium* W54	10^9	cfu/kg BW

[1]Dose is presented as the total bacterial count, when concentration is stratified by strain the amounts are indicated.
[2]6.66×10^6 cfu/d for each of the following strains: *L. acidophilus, B. bifidum, and E. faecium.*
[3]7.2×10^9 cfu/d of total bacteria count (*B. licheniformis*, *B. subtilis*, *L. acidophilus*, *L. lactis*, *B. animalis* lactis) and 10^5 cfu/d for *E. faecium.* 3.6×10^9 cfu/d of total bacteria count (*B. licheniformis*, *B. subtilis*, *L. acidophilus*, *L. lactis*, *B. animalis* lactis) and 10^5 cfu/d for *E. faecium.*
[4]10 cc/d from birth to 7 ± 2 d of age and 3.5 g/d from 7±2 until 53 d of age.
[5] BW at end of experiment = 65.5 kg for calves supplemented with probiotics.
[6]3.7×10^{11} cfu/d of *L. plantarum* chikuso-1 and 2.6×10^9 cfu/d of *Candida* sp. CO119.
[7]5×10^9 cfu/d of *E. faecium* and 2×10^9 cfu/d of *S. cerevisiae*.
[8]9×10^6 cfu/g of *L. plantarum* 220, 9×10^5 cfu/g of *E. faecium* 26 9×10^4 cfu/g of *C. butyricum* Miyari [1.5 or 3.0 g/100 kg BW (initial BW = 95 ± 2 kg)].
[9]10^5 or 10^6 cfu/d of *L. acidophilus* and 10^9 cfu/d of *P. freudenreichii*.
[10]Initial BW = not reported, final BW = 58.3 kg for calves supplemented with probiotic.
[11]Initial BW = 41.1±3.5 kg.
[12]Initial BW = 42.8±3.07 kg.
[13]5 g/d of *L. acidophilus*, *B. subtilis*, *L. lactis.* 10 ml/d *L. acidophilus*, *B. subtilis*, *B. lifidem*, *L. lactis* (at birth and at 30 d of age) + 5g/d *L. acidophilus*, *B. subtilis*, *L. lactis*.
[14]4g/d of probiotic for 4d then 2g/d for the rest of the experiment.
[15]10^9 cfu/d of *B. subtilis* and 2.5×10^8 cfu/d of *L. plantarum*.
[16]2×10^{10} for 3 d and 2×10^9 for the rest of the experiment.
[17]6.4×10^8 cfu/d of *B. subtilis* DSMZ 5750, 6.4×10^8 cfu/d of *B. licheniformis* DSMZ 5749 and 10^9 cfu/d of *E. faecium*.
[18]1.5×10^9, 3×10^9 or 6×10^9 cfu/d of *L. acidophilus*, 1.5×10^9, 3×10^9 or 6×10^9 cfu/d of *B. subtilis* and 5×10^8, 10^9 or 2×10^9 cfu/d of *S. cerevisiae*.
[19]Initial BW = 44.6 kg (trial 1) and 39.7kg (trial 2).

Table 1.10.a. Description of trials that supplemented calves with *Candida tropicalis*, *Megasphaera elsdenii*, and *Faecalibacterium prausnitzii* and the trials that did not inform the probiotic species (Other category)

Study	Scientific name	Dose	Unit
Bi et al., 2017	*Candida tropicalis*	5×10^9	cfu/d
Kong et al., 2019	*C. tropicalis*	5×10^9	cfu/d
Muya et al., 2015	*Megasphaera elsdenii* NCIMB 41125	5×10^9	cfu/d
Muya et al., 2017	*M. elsdenii* NCIMB 41125	5×10^9	cfu/d
Yohe et al., 2018	*M. elsdenii* NCIMB 41125	5×10^9	cfu/d
Foditsch et al., 2015	*Faecalibacterium prausnitzii*	5.72×10^8	cfu/d
Aldana et al., 2009	NR	NR	NR
Dimova et al., 2013	NR	NR	NR
Szewczuk et al., 2013	NR	NR	NR
Lima et al., 2006	NR	NR	NR
Geiger et al., 2014	NR	NR	NR
Hill et al., 2009[1]	NR (live yeast)	NR	NR
Morril et al., 1995	NR	NR	NR
Roodposhti et al., 2012	NR	2×10^9	cfu/d

[1]Probiotic species not reported but informed that it was a live yeast.
NR = not reported.

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List of included studies in the Systematic Review and Meta-analysis

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SUPPLEMENTAL MATERIAL 3

Table 3.1.a Breakpoints (µg/mL) adopted by CLSI

Antimicrobial	S	SDD	I	R
Chloramphenicol	<=8		16	>=32
Ciprofloxacin	<=1		2	>=4
Daptomycin		<=4		>=8
Erythromycin	<=0.5		1 to 4	>=8
Linezolid	<=2		4	>=8
Nitrofurantoin	<=32		64	>=128
Penicillin	<=8			>=16
Quinupristin/dalfopristin	<=1		2	>=4
Tetracycline	<=4		8	>=16
Vancomycin	<=4		8 to 16	>=32

S = susceptible, SDD = susceptible dose dependent, I = intermediate, R = resistant

Table 3.2.a Breakpoints (µg/mL) adopted by NARMS

Antimicrobial	S	I	R
Gentamicin	<=500	NA	>500
Streptomycin	<=512	NA	>=1024
Tigecycline	<=0.25	NA	>=0.5

S = susceptible, I = intermediate, R = resistant

Table 3.3.a. Cut-off values (µg/mL) adopted by FEEDAP

Antimicrobial	R
Chloramphenicol	>8
Erythromycin	>4
Tetracycline	>8
Vancomycin	>4

R = resistant