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Impact of probiotic administration on the health and fecal microbiota of young calves: A meta-analysis of randomized controlled trials of lactic acid bacteria

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ABSTRACT

Before weaning, dairy calves are susceptible to many pathogens which can affect their subsequent performance. The use of lactic acid bacteria (LAB) has been identified as a tool to maintain the intestinal microbial balance and to prevent the establishment of opportunistic pathogenic bacterial populations. However, a consensus has not been reached as to whether probiotics may be effective in reducing the prevalence of gastrointestinal diseases in young calves. The aim of this meta-analysis was to assess the effect of probiotics on diarrhea incidence and the intestinal microbial balance. LAB supplementation has been shown to exert a protective effect and to reduce the incidence of diarrhea (relative risk, RR = 0.437, 95% confidence interval (CI) 0.251–0.761). In the subanalysis, this protective effect of the probiotics against diarrhea was observed only in trials that used whole milk (RR = 0.154, 95% CI 0.079–0.301) and trials that used multistrain inocula (RR = 0.415, 95% CI 0.227–0.759). Probiotics did not improve the fecal characteristics (standardized mean difference, SMD = −0.4904, 95% CI −1.011–0.035) and were unable to change the LAB:coliforms ratio (SMD = 0.016, 95% CI −0.701–0.733). Probiotics showed a beneficial impact on the LAB:coliforms ratio in the subanalysis that included trials that used whole milk (SMD = 0.780, 95% CI 0.141–1.418) and monostrain inocula (SMD = 0.990, 95% CI 0.340–1.641). The probability of significant effects (probiotic positive effect) in a new study was >0.70 for diarrhea and fecal consistency. Whole milk feeding improved the action of the probiotic effect on the incidence of diarrhea and LAB:coliforms ratio. The probability to find significant effects in the diarrhea frequency and LAB:coliforms ratio was higher ($P > 0.85$) if the new studies were conducted using whole milk to feed calves. This paper defines the guidelines to standardize the experimental designs of future trials. LAB can be used as growth promoters in calves instead of antibiotics to counteract the negative effects of their widespread use.
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1. Introduction

Neonatal calf diarrhea, which affects mostly animals under 6 weeks of ages, is easy to diagnose, and is characterized by frequent removal of soft feces (less than 10% dry content) (Millemann, 2009). Many factors, including the calf's exposure to pathogens, the weather conditions, the production systems and the nutritional and immunological condition of young calves, impact on the occurrence of diarrhea (Barrington et al., 2002). Both, the number of calves with diarrhea and the severity of the disease increase during the winter (Millemann, 2009).

Several pathogens, alone or most often in combination with other pathogens, are etiologic agents of diarrhea in young calves. Most of these agents are predominantly transmitted by the fecal-oral route from the feces of infected animals to the mouths of susceptible animals (Barrington et al., 2002). A precise diagnosis is often not necessary because it does not affect the treatment (usually rehydration and antibiotic treatment). However, at farm level, diagnosis is important and influences on the selection of management measures (Millemann, 2009).

Lactic acid bacteria (LAB) are natural components of the normal intestinal microbiota in both humans and animals (Schneider et al., 2004) and have been used to control the effects of pathogens such as *Salmonella* spp. (Gill et al., 2001) and *Escherichia coli* (Shu and Gill, 2002). These two pathogens are the most frequent bacterial etiologic agents in calf scours during the first week of life (Barrington et al., 2002; Millemann, 2009).

It is very important to reduce the prevalence of gastrointestinal infections in young calves because when animals are sick at this

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stage their subsequent growth is delayed, thus affecting their productivity (Rosmini et al., 2004). The incidence of intestinal disease is especially high in intensive rearing systems, where exposure to pathogens is increased due to the confinement of large numbers of animals in small spaces (Callaway et al., 2002).

The use of probiotics has increased as an alternative therapy that prevents the use of antibiotics and thus, reduces the emergence and spread of antibiotic-resistant bacteria and residual antibiotics in dairy foods, meat and milk (Abu-Tarboush et al., 1996). However, a consensus has not been reached as to whether probiotics may be effective in reducing the prevalence of gastrointestinal diseases in young calves.

A systematic review consists of a scientific technique that involves reviewing the available literature, using explicit methods to identify, select and critically evaluate the relevant studies (Faria Filho et al., 2006). In the present work, a consistent review of the studies about probiotics applied to calf rearing published up to date was performed using meta-analysis.

The objective of this meta-analysis was to assess the effect of probiotics on the incidence of diarrhea and the intestinal microbial balance (LAB:coliforms ratio, fecal consistency) of young calves.

2. Materials and methods

2.1. Criteria for study selection

The studies included in the meta-analysis were selected based on the following criteria: randomized and controlled trials and studies published in peer-reviewed journals, which used young calves (younger than 10 days old) without diseases and with good passive immunity and which used LAB added in whole milk, pasteurized whole milk or milk replacer, and starter as a solid diet. The studies considered reported data on incidence of diarrhea, fecal consistency and LAB:coliforms ratio with deviation around the mean values.

2.2. Outcomes and definitions

The impact of LAB supplementation on the incidence of diarrhea, fecal consistency and LAB:coliforms ratio, was analyzed. The data obtained from each study corresponded to the whole trial. In studies that included more than one LAB group or the same LAB but applied in different diets (e.g. whole milk and milk replacer), each LAB group was compared with the control group separately.

2.3. Data sources

PubMed and Scopus databases were searched for articles unrestricted by language published from 1980 to 2010. Search terms included probiotic* and calves*. The abstracts were assessed and the articles that met the *a priori* inclusion criteria were selected.

2.4. Data extraction

Information on the study design, methods (diets), treatments (LAB strains, treatment dose and duration), number of animals, calves' sex and breed, and outcomes, were extracted from each article. Regarding the outcomes from each study, the methodology applied, were evaluated. Fecal consistency was used as an indicator of the intensity and duration of deposition, using a four-point scale (1 = normal, 2 = soft, 3 = liquid and 4 = watery).

2.5. Statistical analysis

Statistical analysis was performed using Epidat software version 3.1 (Information Service on Public Health, Province of Galicia (Spain) in collaboration with the PanAmerican Health Organization PAHO-WHO) (EPIDAT, 2006). Results of continuous variables (fecal consistency and LAB:coliforms ratio) were analyzed as standardized mean differences (SMD) between the probiotic treatment and controls with 95% confidence intervals (CI). Incidence of diarrhea was analyzed using the relative risk (RR) estimation.

Heterogeneity among studies was evaluated using DerSimonian and Laird test (Q-statistic) and Inconsistency index (I^2-statistic) (Higgins and Thompson, 2002). A classification of the I^2 values was used to interpret the heterogeneity magnitude: values around 25%, 50% and 75% were considered as low, medium and high heterogeneity, respectively (Higgins and Thompson, 2002). A random effect model was used.

A Sensitivity analysis was completed to assess the robustness of the results of the meta-analysis. Sensitivity analyses have also been previously used to examine the effects of studies identified as being aberrant or being highly influential in the analysis (Lean et al., 2009). These analyses consist in completing the same analysis (SMD or RR), but dropping one study each time. The Influence graph shows the global effect without each study.

Two approaches were used to investigate the causes of heterogeneity were conducted: (1) stratified analyses and (2) meta-regression. In stratified analyses, the data were stratified according to a factor thought to influence the treatment effect (e.g. number of animals per trial, duration of the trial, type of inocula, feed, etc.), and a separate meta-analysis carried out in each of the strata. Stratified analysis was not conducted when individual strata contained relatively few studies (<2). In the meta-regression, the same factors used in the stratified analysis were included as predictors in a weighted regression (weight equal to the inverse variance of the results of each study) (Dohoo et al., 2003).

An adjusted rank correlation test using the Egger method (which examines the correlation between study size and effect) (Egger et al., 1997) and the Begg test (which examines the association between study size and effect) (Begg and Mazumdar, 1994) was used to assess publication bias.

Separate prediction intervals (PIs) were calculated for incidence of diarrhea, fecal consistency and LAB:coliforms ratio, by using the following formula from Higgins et al. (2009):

$$\mu \pm t^{\alpha}_{k-2}\sqrt{\mathrm{SE}(\mu)^2 + \tau^2}$$

where μ is the average weighted estimate across studies, t^{α}_{k-2} is the $100(1-\alpha)\%$ percentile of the t-distribution with $k-2$ degrees of freedom, $\mathrm{SE}(\mu)^2$ is the estimated squared standard error of μ, and τ^2 is the estimated between study variance. The probability of significant effect of new individual trials was obtained from the T-student distribution.

3. Results

3.1. Excluded studies

A total of 34 out of the 66 papers identified at the beginning of the meta-analysis failed to meet one or more of the inclusion criteria. Review articles, trials conducted to assess the impact of probiotics and prebiotics, and studies to isolate and select strains with potential probiotic activity without *in vivo* tests to study their effects on the incidence of diarrhea, fecal consistency or LAB:coliforms ratio were excluded from the meta-analysis. A total of 18 out of the 32 trials that met the inclusion criteria were excluded due to lack of statistical information to conduct a meta-analysis and non-additional information was requested from the authors. Trials that analyzed only the effect of probiotics on growth performance ($n = 6$), trials that included ill calves or calves without a

good passive immunization ($n = 2$) and trials that used non-viable LAB ($n = 2$) were also excluded from the meta-analysis.

3.2. Overview of included studies

The literature search yielded 66 scientific papers on probiotics and calves. Nine of these 66 articles met all the inclusion criteria previously established to assess the probiotic effect on the incidence of diarrhea (with 15 trials), while six (with seven trials) met the inclusion criteria to assess the probiotic effect on fecal consistency. Finally, eight studies (with 14 trials) were included to assess the effect of probiotic on the LAB:coliforms ratio.

Among the studies included to assess the probiotic effect on incidence of diarrhea in young calves ($n = 9$), three were conducted before 2000, and the rest after 2001 ($n = 6$). Most part of the experiments ($n = 8$) used Holstein calves. The number of animals included in the trials was variable: two trials included less than 20 animals, six trials included between 21 and 50 animals, and one trial included more than 50 animals. Five of these studies were conducted using multistrain probiotics, whereas the other four used monostrain probiotics. Calves were fed whole milk ($n = 2$), milk replacer ($n = 5$), or both types of feed in the same trial ($n = 2$). Studies were conducted for less than 45 days ($n = 3$), between 45 and 60 days ($n = 5$) or more than 60 days ($n = 1$) (Table 1). None of these trials reported the treatments applied to the calves which suffered diarrhea.

Among the studies included to assess the impact of probiotics on fecal consistency ($n = 6$), three were conducted before 2000. All the trials used Holstein calves. The number of animals included in the trials was variable: four included less than 50 animals and two included more than 50 animals. Only one trial used monostrain probiotics, whereas four trials were conducted using multistrain probiotics, and the remaining study was carried out with both monostrain and multistrain probiotics in the same experiment. Calves were fed with milk replacer in all cases. Studies were conducted for either less than 45 days ($n = 4$) or more than 60 days ($n = 2$) (Table 1).

Among the studies included to assess the probiotic effect on the LAB:coliforms ratio in young calves, two were conducted before 1990 and five between 1991 and 2000. Most of the experiments

Table 1
Randomized, controlled trials to study the effect of supplementation with probiotics on diarrheal incidence, fecal consistency or LAB:coliforms ratio in young calves.

Year	N	Breed	Probiotic strains	Duration of the study (days)	Treatment period (days)	Feed	Outcomes analyzed	Reference
1980	10	Holstein × Ayrshire	*L. acidophilus* (from human)	14	14	Pasteurized whole milk	LCR	Gilliland et al., 1980
1980	10	Holstein × Ayrshire	*L. acidophilus* (from calves)	14	14			
1985	45	Swedish Red and Withe	*Lactobacillus* spp.	50	50	Milk replacer	DI LCR	Jonsson and Olsson, 1985
1991	56	Holstein	*L. acidophilus* and *L. lactis*	42	42	Milk replacer	FC	Jenny et al., 1991
1991	53	Holstein	*Bacillus subtilis*	42	42		LCR	
1993	15	Holstein	*L. acidophilus* and *Streptococcus faecium*	36	36	Milk replacer	FC LCR	Higginbotham and Bath, 1993
1995	19	Holstein	*Lactobacillus acidophilus*, *Bacillus thermophilum* and *Enterococcus faecium*	56	56	Milk replacer	DI	Abe et al., 1995
1996	16	Holstein	L. acidophilus and *L. plantarum*	84	84	Pasteurized whole milk and milk replacer	LCR	Abu-Tarboush et al., 1996
1996	16	Holstein	*L. acidophilus* 27SC	84	84			
1998	28	Holstein	*S. faecium*, *L. acidophilus*, *Saccharomyces cerevisae*, *Bacillus subtilis* and *Aspergillus oryzae*	56	56	Milk replacer	FC LCR	Higginbotham et al., 1998
1999	24	Holstein × Cebú	*Lactobacillus acidophilus*	56	56	Whole milk	DI LCR	Chaves et al., 1999
2002	51	Holstein	*Lactobacillus acidophilus*	42	42	Whole milk	DI	Abdala et al., 2002
2002	52	Holstein	*Lactobacillus acidophilus*	42	42	Milk replacer		
2003	22	Holstein	*Lactobacillus plantarum*, *L. bulgaricus*, *L. acidophilus*, *L. mamnsus*, *Bifidobacterium bifidum*, *Streptococcus thermophilus*, *Enterococcus faecium*, *Aspergillus oryza* and *Candida pintolopessi*	60	60	Whole milk	DI	Gorgulu et al., 2003
2005	360	Holstein	*L. acidophilus*, *L. salivarius*, *L. paracasei spp. paracasei*, *L. plantarum*, *L. lactis* and *Enterococcus faecium*	53	14			
2005	62	Holstein	*L. acidophilus*, *L. salivarius*, *L. paracasei spp. paracasei*, *L. plantarum*, *L. lactis* and *Enterococcus faecium*	56	14			
2005	48	Holstein	*L. acidophilus*, *L. salivarius*, *L. paracasei spp. paracasei*, *L. plantarum*, *L. lactis* and *Enterococcus faecium*	187	56	Milk replacer	DI LCR	Timmerman et al., 2005
2005	48	Holstein	*Lactobacillus* spp.	187	56			
2005	41	Holstein	*L. acidophilus*, *L. salivarius*, *L. paracasei spp. paracasei*, *L. plantarum*, *L. lactis* and *Enterococcus faecium*	56	56			
2005	41	Holstein	*Lactobacillus* spp.	56	56			
2007	112	Not specified	*Bifidobacterium bifidum*, *Enterococcus faecium*, *Streptococcus thermophilus*, *Aspergillus oryzae* and *Candida pinotopesti*	90	90	Whole milk	DI	Mokhber-Dezfouli et al., 2007
2008	24	Holstein	*Lactobacillus casei*, *L. salivarius* and *Pediococcus acidilactici*	35	35	Milk replacer	DI FC	Frizzo et al., 2008
2010	40	Holstein	*S. faecium*	52	52	Milk replacer	FC	Morrison et al., 2010
2010	16	Holstein	*Lactobacillus casei*, *L. salivarius* and *Pediococcus acidilactici*	35	35	Milk replacer	DI FC	Frizzo et al., 2010a,b

References: DI = diarrhea incidence; FC = fecal consistency; LCR = LAB:coliforms ratio.

($n = 7$) used Holstein calves. The number of animals included in the trials was variable: two included less than 20 animals and six included between 21 and 50 animals. Two studies used multistrain probiotics, three used monostrain probiotics, and the remaining three studies used monostrain and multistrain probiotics in the same experiment. Calves were fed whole milk ($n = 2$), milk replacer ($n = 5$), or both types of feed in the same trial ($n = 1$). Studies were conducted for less than 45 days ($n = 3$), between 45 and 60 days ($n = 3$) or more than 60 days ($n = 2$) (Table 1).

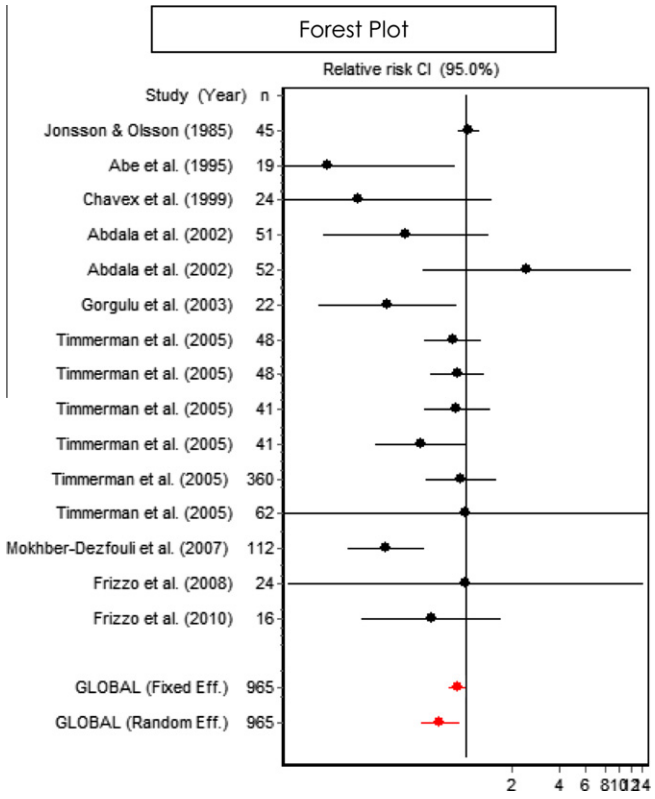

Fig. 1. Forest plot of 15 randomized controlled trials to study the effect of supplementation with probiotics on diarrhea incidence in young calves.

3.3. Incidence of diarrhea

Of the nine studies that met with the inclusion criteria, 15 trials (965 calves) that combined calves fed with probiotics and control groups were identified. In the pooled estimate, the relative risk (RR) to present diarrhea was lower in the animals fed with probiotics than in controls (RR = 0.437, 95% CI 0.251–0.761) (Fig. 1). Significant heterogeneity was observed across the 15 trials ($Q = 28.244$; $I^2 = 50.43$; $P < 0.0013$). The prediction interval (95% confidence interval) for an expected treatment effect in a new trial was 0–1.91 (Table 2).

Two subgroup analyses restricted to the type of feed provided to the calves (whole milk and milk replacer) were conducted. Four trials using whole milk were identified, and probiotics caused a protective effect against diarrhea (RR = 0.154, 95% CI 0.079–0.301). Another subgroup analysis restricted to 11 trials that used milk replacer to fed calves found no effect on the risk of diarrhea as a consequence of the supplementation with LAB (RR = 0.674, 95% CI 0.414–1.098). The type of feed seems to account for some of the heterogeneity between studies. There was no longer any evidence of heterogeneity within the two groups of feed: whole milk ($Q = 1.506$, $I^2 = 0$, $P = 0.681$) and milk replacer ($Q = 12.189$, $I^2 = 17.96$, $P = 0.273$).

Another subgroup analysis restricted to the type of inocula provided to the calves (monostrain *versus* multistrain) was conducted. Three trials using monostrain inocula were identified, and the protective effect of the probiotics against diarrhea was non-statistically different (RR = 0.543, 95% CI 0.097–3.048). Another subgroup analysis restricted to 12 trials that used multistrain inocula found a positive effect on the risk of diarrhea (RR = 0.415, 95% CI 0.227–0.759). Within the groups that used monostrain inocula, there was no longer any evidence of heterogeneity, but the Inconsistence Index was high ($Q = 5.347$, $I^2 = 62.59$, $P = 0.069$). However, there was still heterogeneity among the studies based on multistrain inocula ($Q = 22.706$, $I^2 = 51.55$, $P = 0.019$). However, since the number of studies that used monostrain inocula was relatively small, the summary effects must be interpreted with caution.

Two meta-regressions were conducted to analyze the effect of the number of animals used in the trials and the duration of the study as prediction variables. The duration of the study was not a significant predictor of the study effects observed ($P = 0.1752$). The number of animals had an overall significance ($P = 0.0402$), but with a low regression coefficient ($R = 0.14$). There was a trend

Table 2
Mean of probiotic effect and prediction intervals for diarrhea incidence, fecal consistency and LAB:coliforms ratio in young calves.

Variable	Analysis	n[a]	μ (95% CI for overall mean effect)[b]	95% Prediction Interval	Probability[c]
Diarrhea incidence	Global effect	15	0.437 (0.251–0.761)	0–1.91	0.730
	Whole milk	4	0.154 (0.079–0.301)	0–1.61	0.883
	Milk replacer	11	0.674 (0.414–1.098)	0–1.50	0.755
	Monostrain inocula	3	0.543 (0.097–3.048)	0–17.12	0.554
	Multistrain inocula	12	0.415 (0.227–0.759)	0–1.93	0.749
Fecal consistency		7	−0.490 (−1.011–0.030)	−2.54–1.28	0.731
BAL:Coliforms ratio	Global effect	14	0.016 (−0.701–0.733	−2.68–2.71	0.504
	Whole milk	5	0.780 (0.141–1.418)	−0.62–2.18	0.859
	Milk replacer	9	−0.401 (−1.334–0.532)	−3.69–2.89	0.412
	Monostrain inocula	4	0.990 (0.340–1.641)	−0.47–2.45	0.907
	Multistrain inocula	10	−0.360 (−1.220–0.500)	−3.40–2.68	0.416

References:
[a] n = number of trials;
[b] Relative risk for diarrhea incidence or SMD for fecal consistency and BAL:coliforms ratio';
[c] Probability for an expected benefic treatment effect (supplementation with probiotics) in a new trial on diarrhea incidence, fecal consistency or BAL:coliforms ratio.

towards greater treatment effects as the number of animals increased.

No significant publication bias was observed for these 15 trials as shown by the funnel plot in Fig. 2, confirmed by Begg's test ($P = 0.5526$) and Egger's test ($P = 0.30$). The funnel plot shows that the points are symmetrically arranged with the exception of one trial (Mokhber-Dezfouli et al., 2007).

3.4. Fecal consistency

From six studies that met with the inclusion criteria, seven trials (232 calves) that evaluated the effect of probiotics on the fecal consistency were identified. The meta-analysis showed that the animals fed with probiotics did not improve the consistency of feces (low level of fecal consistency) in comparison with animals without probiotics supplementation (SMD = −0.4904, 95% CI −1.011–0.035) (Fig. 3). The prediction interval (95% CI) for an expected treatment effect in a new trial was −2.54–1.28 (Table 2).

Significant heterogeneity was observed across the seven trials ($Q = 20.887$; $I^2 = 72.26$; $P < 0.0019$).

Significant publication bias was observed for these seven trials as shown by the funnel plot (Fig. 4) and confirmed by Begg's test ($P = 0.0069$) and Egger's test ($P = 0.0019$). Two studies appeared outside the funnel plot (Higginbotham and Bath, 1993; Frizzo et al., 2010a,b).

3.5. LAB:coliforms ratio

Of the eight studies that met with the inclusion criteria, 14 trials (451 calves) that combined calves fed with probiotics and control groups were identified. In the pooled estimate, there was no effect on the LAB:coliforms ratio due to the supplementation with probiotics (SMD = 0.016, 95% CI −0.701–0.733) (Fig. 5). Prediction interval (95% CI) for an expected treatment effect in a new trial was −2.68–2.71 (Table 2).

Significant heterogeneity was observed across the 14 trials ($Q = 154.408$; $I^2 = 91.581$; $P < 0.0001$).

Two subgroup analyses restricted to the type of feed provided to the calves (whole milk and milk replacer), were conducted. Five trials using whole milk were identified, and probiotics showed a beneficial impact on the LAB:coliforms ratio (SMD = 0.780, 95% CI

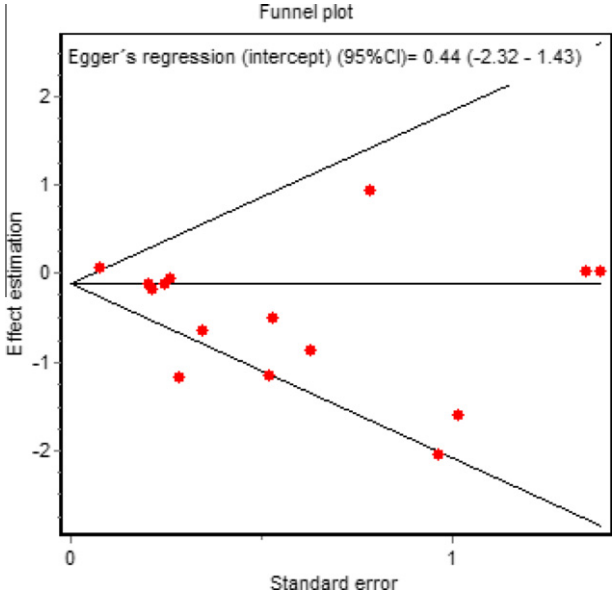

Fig. 2. Funnel plot of 15 randomized controlled trials to study the effect of supplementation with probiotics on diarrhea incidence in young calves.

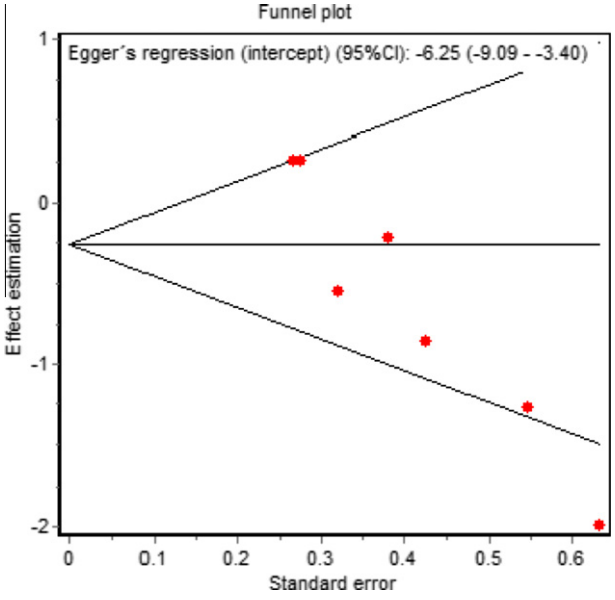

Fig. 4. Funnel plots of seven randomized, controlled trials to study the effect of supplementation with probiotics on fecal consistency in young calves.

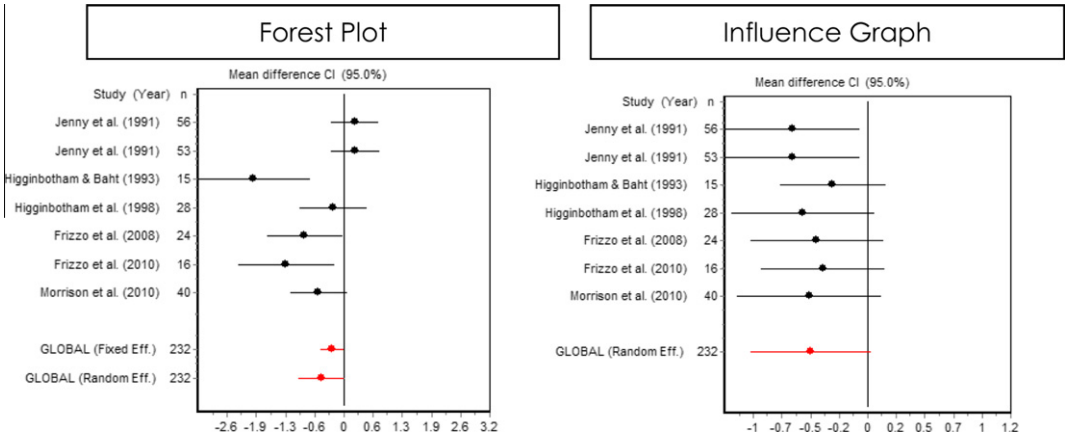

Fig. 3. Forest plot and influence graph of seven randomized, controlled trials to study the effect of supplementation with probiotics on fecal consistency in young calves.

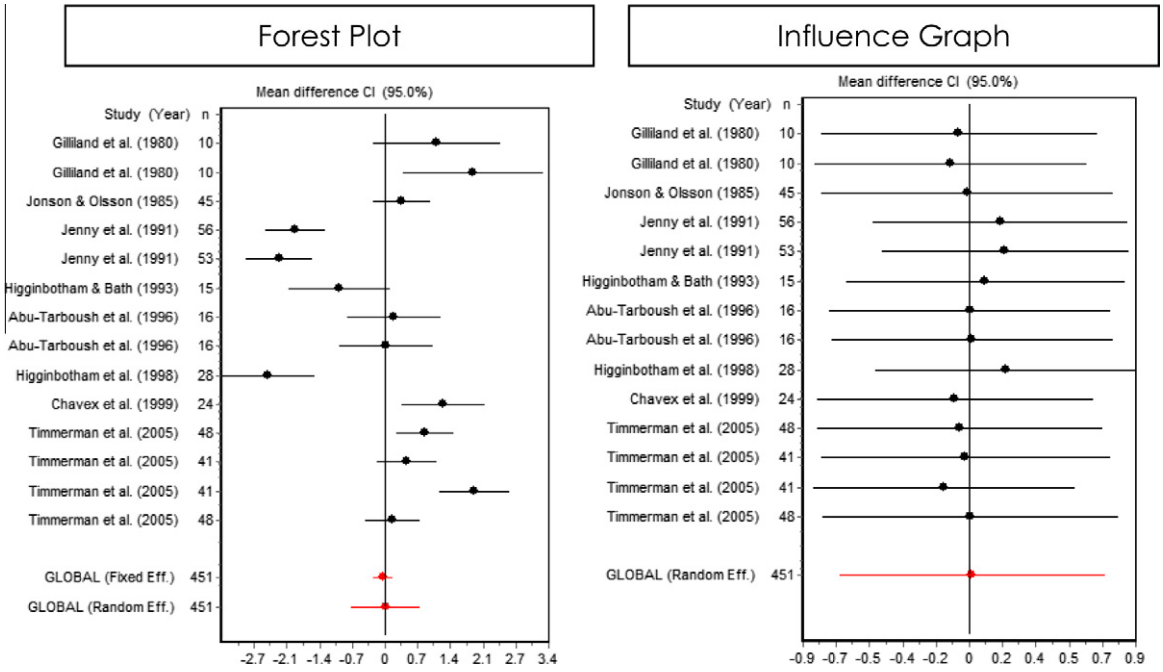

Fig. 5. Forest plot and influence graph of 14 randomized controlled trials to study the effect of supplementation with probiotics on the LAB:coliforms ratio in young calves.

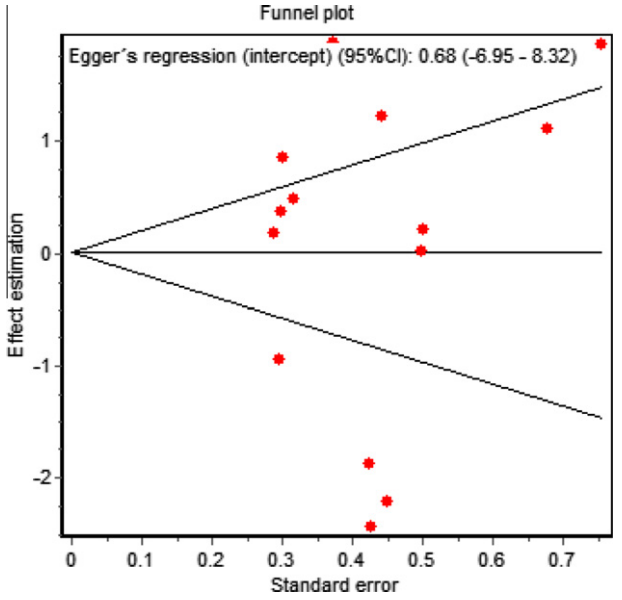

Fig. 6. Funnel plots of 14 randomized controlled trials to study the effect of supplementation with probiotics on the LAB:coliforms ratio in young calves.

0.141–1.418, $n = 5$). However, when the trials that used milk replacer to feed the calves were analyzed, this beneficial effect was not observed (SMD = -0.401, 95% CI −1.334–0.532, $n = 9$). Among the groups of whole milk there was no longer any evidence of heterogeneity ($Q = 6.855$, $I^2 = 41.649$, $P = 0.144$). However, there was still heterogeneity among the studies based on milk replacer ($Q = 134.912$, $I^2 = 94.07$, $P < 0.001$).

Another subgroup analysis restricted to the type of inocula provided to the calves (monostrain *versus* multistrain) was conducted. Four trials using monostrain inocula were identified, and the LAB:coliforms ratio was statistically different (SMD = 0.990, 95% CI 0.340–1.641). Another subgroup analysis restricted to 10 trials that used multistrain inocula could not find a positive effect on LAB:coliforms ratio (SMD = -0.360, 95% CI −1.220–0.500). Among the groups of monostrain inocula, there was no longer any evidence of heterogeneity ($Q = 4.075$, $I^2 = 26.38$, $P = 0.253$). However, there was still heterogeneity between the studies based on multistrain inocula ($Q = 135.105$, $I^2 = 93.34$, $P < 0.001$).

Two meta-regression analyses were conducted to analyze the effect of the number of animals used in the trials and the duration of the study as prediction variables. Neither variable was a significant predictor of the effects observed. The regression coefficients for the number of animals and duration of the study were 0.08 ($P > 0.05$) and 0.039 ($P > 0.05$), respectively.

No significant publication bias was observed for these 14 trials, as shown by the funnel plot in Fig. 6 and confirmed by Begg's test ($P = 0.434$) and Egger's test ($P = 0.682$). Four studies appeared outside the funnel plot (Higginbotham and Bath, 1993; Higginbotham et al., 1998; Jenny et al., 1991; Timmerman et al., 2005). No individual study had a particularly large influence on the summary BAL:coliforms ratio estimate (Fig. 5).

4. Discussion

The composition of the intestinal microbiota is complex and varies due to environmental conditions and host factors (Vlková et al., 2006). In young calves, *Lactobacillus,* the most important genus found in the gastrointestinal tract and faeces, adapts and develops beneficial symbiosis with the host, reaching levels of 10^7–10^8 CFU/g during the first week of life (Karney et al., 1986; Rada et al., 2006). At this moment, the intestinal microbiota is extremely unstable (Lukás et al., 2007). Healthy animals have a balanced intestinal microbiota that allows them to growth properly. However, when calves are under stress conditions (especially due to intensive rearing systems), a microbiota imbalance occurs, and Lactobacilli and Bifidobacteria populations may diminish and pathogen microorganisms may increase. The use of probiotics may either prevent pathogen colonization of the digestive tract (Fuller, 1989) or significantly reduce the prevalence of diarrhea in young calves (Abe et al., 1995). Many of the problems that affect the growth performance of young calves are related to low digestion

and reduced absorption of nutrients due to colonization of pathogenic bacteria. However, nutritional diarrhea often precedes and predisposes the calf diarrhea syndrome caused by pathogenic microorganisms. In these cases, the use of probiotics aims to prevent the diarrhea. The results of this meta-analysis show that probiotics induce a beneficial effect on the incidence of diarrhea. Moreover, in the stratified analyses, a beneficial effect due to probiotic supplementation was observed in the studies that used whole milk to feed the young calves. The main factors that account for the differences between the studies are related to the health status, stress level of animals and degree of exposure to pathogens during rearing. The supplementation with LAB may be useful in young calves, animals treated with antibiotics or other animals with a temporarily disturbed intestinal microbiota (Jonsson, 1985). A beneficial effect due to probiotic supplementation was observed in the studies that used multistrain inocula. The probiotic microorganism performance may vary from one animal to another of the same species, and for that reason, some authors (Gardiner et al., 2004; Timmerman et al., 2004) have recommended the administration of an inoculum formed by a mixture of different strains. Therefore, we recommend further research to test a limited number of strains and their association in specific management conditions to evaluate the additive or synergistic effects on the calves' health (especially the antagonist effects on intestinal pathogen microorganisms).

The intestinal homeostasis is based on the balance between absorption (nutrients, ions), secretion (ions, IgA) and the ability of the digestive epithelium to act as a barrier (against pathogens and macromolecules). These functions are controlled by multiple interactions between the endocrine, neurocrine, stromal and immune cells or the natural intestinal microbiota, which controls the epithelial functions (Heyman and Ménard, 2002). The biological activity of LAB has a direct influence on the metabolic processes, the dynamics of the gut microbiota and host resistance (Novik et al., 2006). The beneficial action of probiotics should be always aimed at keeping indigenous intestinal microbiota in balance so that the animal is ready to respond successfully to eventual pathogen colonization (Morrill et al., 1995). Different stressing situations (e.g. intake of high-density feed, drastic weather changes,—particularly temperature—changes in the feed components and conditions after transport) are some of the main factors causing diarrhea (Ishihara et al., 2001). In our meta-analysis, we found that supplementation with probiotics in the calves' diet did not improve the fecal characteristics. This could be because this parameter is not very sensitive and more time may be necessary for the probiotic to generate its beneficial effect. A good consistency of the feces is due to better digestion and absorption of feed and an adequate state of the intestinal mucosa.

Probiotic microorganisms can shift part of the normal microbiota and significantly increase the number of total LAB, a situation that is evidenced by recovering a greater number of microorganisms from different parts of the intestine and from the feces of treated animals. Probiotics found in a viable form in the gastrointestinal tract are the result of: (a) the number of LAB inoculated that were capable of surviving the biological barriers, (b) their growth in the intestinal lumen and saturation of intestinal niche, and (c) evacuation due to the difficulty to adhere to the intestinal epithelium (Frizzo et al., 2010a). The effect of the probiotic is related to the ability of LAB to ensure implantation in the gastrointestinal tract, improving the microbial balance and stimulating the immune system. The persistence of LAB in the gastrointestinal tract should be maintained at least for a few days to generate the beneficial effects (Heyman and Ménard, 2002). Fecal counts of *Lactobacillus* are normally higher than counts of coliforms in healthy calves (LAB:coliforms ratio >1) but, in calves suffering diarrhea, this relationship can change dramatically (Abu-Tarboush et al., 1996). The beneficial effects of LAB are, probably, due to their growth in the intestinal tract, which creates a microbiological barrier against the development of pathogenic bacteria (Heyman and Ménard, 2002). The ability of LAB to compete against pathogenic bacteria depends on the diet offered to calves (Jonsson, 1985). Increases in fecal *Lactobacilli* have been reported by many researchers (Ellinger et al., 1980; Gilliland et al., 1980; Jenny et al., 1991; Abu-Tarboush et al., 1996). The results of this meta-analysis show that the supplementation with probiotics did not modify the LAB:coliforms ratio. However, the stratified analysis showed a probiotic beneficial effect on the LAB:coliforms ratio in young calves fed with whole milk. Moreover, a beneficial effect due to probiotic supplementation was observed in the studies that used monostrain inocula. It is possible that the components of the whole milk and the probiotic inoculum cause synergistic effects to reduce the incidence of diarrhea and improve the LAB:coliforms ratio.

The viability of probiotics, a source of heterogeneity, is an important factor that affects their efficiency, and may be related to the loss of viability and stability of the probiotics used. In most of the studies reviewed, there were no data regarding the storage conditions and the viability of the strain. Another source of heterogeneity is the type of inoculum, the doses and dosage used in the studies. To assess the influence of these variables, it would be necessary to conduct specific trials.

The season of the year in which the trial was conducted is another source of heterogeneity in this type of studies with calves. Although this variable may have a great impact on the calves' health status and therefore on the results obtained, in the studies analyzed reported no information on this issue. Another source of heterogeneity is related to the type of diarrhea and its etiology. Although these data are usually not described in scientific articles, they are important to assess the impact of different probiotics preparations against specific pathogens.

Another factor to consider in these trials is the safety of the probiotic strain used. In order to test the harmlessness of potentially beneficial bacterial strains when raising young calves, their safety level should be verified before they are included into a probiotic formulation (Frizzo et al., 2010a). This problem is not sufficiently documented in the studies considered in this meta-analysis, and in many cases data on clinical response of the supplemented animal are not reported. The gastrointestinal tract of a young animal at birth is physiologically immature when compared with that of an adult animal (Lee et al., 2000). Young ruminants might be more vulnerable to the translocation of microorganisms because their monogastric digestive systems undergo a physiological transition to become polygastric (Frizzo et al., 2010a). All sources of heterogeneity were related to methodological differences between the studies. Although many of these data have been collected, it was not possible to establish a statistical model that includes all the variables.

The main factors that can explain the differences observed between the studies, regardless the LAB inoculum, are related to the health status, the level of stress suffered by calves and the exposure to intestinal pathogens during rearing. The beneficial effect due to LAB supplementation (e.g. growth performance, health and fecal microflora) can be detected more easily in farms that present high morbidity and mortality rates caused mainly by intestinal pathogens. Growth performance parameters are more sensitive than the health status parameters to assess the beneficial effect of probiotics applied to the calves' diet. A possible explanation might be the incidence of sub-clinical gastrointestinal diseases that can be detected only by a reduction in growth performance (Frizzo et al., 2010b). To improve the detection of probiotics effects on the calves' health status, trials using experimental models with pathogens should be conducted. These types of trials may improve the sensitivity of the health indicators. Trials should be designed with the aim to assess the probiotics effects as a prophylactic tool

to protect young calves against pathogen colonization of the digestive tract, stimulating the development of the immune system and counteracting the negative effects of such pathogens.

The wide variety of experimental designs detected in this meta-analysis, is a source of heterogeneity that affects the results and reduces the consistency of the findings. However, this meta-analysis allowed us to identify certain components of the experimental designs that could affect the probiotic effect on animal health. This, in turn allowed us to define guidelines to standardize the experimental designs of future trials, which should be added to the basic rules reported by other authors to the general use of probiotics (Fuller, 1989, 2006; FAO/WHO, 2001). Some of these guidelines are that: (1) there are more chances of finding beneficial effects on health indicators by designing experimental models that induce nutritional diarrhea (e.g. introduce some stressful substance to the diet such as lactose); (2) probiotic viability should be maintained during all the trial; (3) trials should focus on the first week of life (6–9 weeks), because the highest incidence of diarrhea usually occurs during this period and (4) trials designed as experimental disease models can be useful to assess the effectiveness of the probiotic against specific pathogens.

The publication bias is an important issue to discuss in this type of studies. In this meta-analysis, publication bias was minimized by conducting extensive searches through multiple databases and including studies conducted in different countries.

The sensitivity analysis shows the influence of each study on the overall estimation and, therefore, the robustness or stability of the final measure obtained. The results of the sensitivity analysis performed in this meta-analysis show that the global estimations were not stable along the different studies, indicating that in some cases, a particular trial could change the overall results. Considering these analyses, the results can not be conclusive, and different trials with appropriate experimental designs should be conducted to determine the probiotic effect on the young calves' health.

The prediction intervals (PIs) approach has been developed and recommended determines how treatment effects of a new individual trial are distributed around the mean in a random-effects meta-analysis (Kelley and Kelley, 2009). Prediction intervals applied to the meta-analysis results (global effects and the different stratified analyses) overlapping one for relative risk of diarrhea and zero for fecal consistency and LAB:coliforms ratio. However, the probability of significant effects (probiotic positive effect) in a new study was >0.70 for diarrhea and fecal consistency, but it was approximately 0.5 for the LAB:coliforms ratio (i.e. this probability is not higher than the probability only for chance). Additionally, the probability to find significant effects in the diarrhea frequency and LAB:coliforms ratio is higher ($P > 0.85$) if the new study is conducted using whole milk to feed calves.

5. Conclusions

This meta-analysis included a large number of trials assessing health issues during the growth of calves and showed that the addition of probiotics in the calves' diet causes a reduction in the incidence of diarrhea of young calves. Whole milk feeding improved the action of the probiotic effect on the incidence of diarrhea and LAB:coliforms ratio. Probiotics may be an alternative to the use of antibiotics as a growth promoter in calves. Although the use of meta-analysis as a statistical tool is powerful, all issues discussed show that the results should be considered with caution, since they are highly dependent on the data set and methodology used. However, this meta-analysis shows that both the data quality and the approach used were relevant. To increase comparability, efforts should be made to standardize the doses, the duration of treatments and the populations studied. This work defines guidelines to standardize the experimental designs of future trials.

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