Research Article

A Carrier Emulsion, Based on Anaerobic Probiotic Bacteria, for Dosing Newborn Calves against Diarrhea and Improving Body Weight Gain

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Abstract | Probiotic additives are used to prevent diarrhea episodes in weanling dairy calves and to improve animal well-being, growth, and weight gain. In this study, we developed an emulsion that offered protection over time to its four constituent anaerobic probiotic bacteria (*Butyrivibrio fibrisolvens* B9, *Streptococcus bovis* C2, *Fibrobacter succinogenes* Fs, and *Ruminococcus flavefaciens* Rf). When it was stored over 6 months at 4ºC, a significant reduction ($P \leq 0.05$) of viable cell counts, from 1.95×10^9 CFU/mL at time zero to 3.02×10^8 CFU/mL at 6 months, was noted; despite this, viable cell counts were never less than 1×10^8 CFU/mL; the minimum suggested for probiotic effectiveness. During *in vivo* trials, weanling dairy calves were given either a no-bacteria control, a freshly prepared, or stored probiotic emulsion. After 2 and 3 months, as compared to control calves, greater weight gains (up to 15.5%), lower weaning time (15 d), and lower frequency of diarrhea (up to 67.7%) were seen in calves given the freshly prepared and stored probiotic emulsions ($P \leq 0.05$). The carrier emulsion protected the viability of the cells and thus their efficacy.

Keywords | Anaerobic bacteria, Rumen, Probiotic, Emulsion, Newborn calves

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INTRODUCTION

The incidence of diarrhea is high in the calves, especially in the first 4 weeks of their life, and it is closely associated with other major diseases and mortality (Togzhan et al., 2020). Calf diarrhea can have an effect both economically and in terms of animal welfare. Losses are due to death, treatment costs, and time spent on care, as well as subsequent chronic ill thrift and poor growth (Bazeley, 2003). Although calf diarrhea is frequently treated with antibiotics, their effectiveness is debatable (Kim et al., 2021). Additionally, the use of antibiotics has been shown to have a deleterious effect on the microbial makeup of the calf gut (Ianiro et al., 2016; Ramirez et al., 2020). Also, the excessive use of antibiotics in the food animal sector is contributing to the spread of antimicrobial-resistant bacteria in humans and food animals.

The search for non-antibiotic alternatives is one of the aims of sustainable dairy farming: producing healthy newborn calves in the face of the previously mentioned threat, under safe and high-quality standards. Non-ruminal microorganisms, as well as plant and microbial-derived feed additives, have been reported to prevent the occurrence of diarrhea episodes in weanling calves and to improve animal well-being, growth, and weight gain (Roodposthi and Dabiri, 2012; Foditsch et al., 2015; Cantor et al., 2019; Lucey et al., 2021; Szlufman and Shemesh, 2021).

The majority of microbial additives in those commercial biologicals are based on bacterial genera, such as: *Bacillus*, *Bifidobacterium*, *Lactobacillus*, *Enterococcus*, *Pediococcus*, *Leuconostoc, Megasphaera, Propionibacterium*, and *Streptococcus;* and yeasts, such as: *Saccharomyces boulardii* and *S. cerevisiae* (Agarwal et al., 2002; Stella et al., 2007; Desnoyers et al., 2009; Yadav et al., 2016; Ricci et al., 2017; Adjei-Fremah et al., 2018; Markowiak and Śliżewska, 2018; Zommiti et al., 2019). These amendments are generally formulated as liquids or in solid form as powders, granules, or microcapsules (Carro et al., 2014). To our knowledge, few if any of these currently available commercial biological products report the inclusion of rumen anaerobes in their probiotic formulations. The use of novel probiotics based on rumen microbes would broad the spectrum of effective alternatives to prevent the colonization and growth of enteric pathogens.

Rumen has rich microbiota that plays a crucial role in digestion and fermentation (Yufeng et al., 2023). The rumen microbiome preserves the health of its host by destroying harmful by products of fermentation. If the structure of the rumen microbiota is disordered, the health of ruminants is threatened (Yufeng et al., 2023). A study about the gastrointestinal microbiome of ruminants (Xie et al., 2021) showed that anaerobic bacteria as *Prevotella* and *Fibrobacter* were predominant in the stomach, while *Bacteroides*, *Clostridium, Alistipes* and *Ruminococcus* were prevalent in the large intestine.

The main factor hindering the development of probiotic formulations containing rumen anaerobes could be related to the significant challenge of protecting and preserving anaerobic bacteria from exposure to toxic atmospheric oxygen. A suitable carrier that preserves the viability and biological activity of probiotics throughout the product's storage time needs to be found. For this reason, the aim of this study was to develop a stable emulsion that maintain microbial viability of anaerobic ruminal probiotics for newborn calves as a health- and growth-promoting anti-diarrheic. In this emulsion, the internal phase was the bacteria fermentation broth, and the external phase was oil with surfactants, acting as a protective coat for the probiotic microbes.

MATERIALS AND METHODS

Microorganisms

Three of the four anaerobic probiotic bacteria strains, *Ruminococcus flavefaciens* Rf, *Butyrivibrio fibrisolvens* B9, and *Streptococcus bovis* C2, were isolated from Colombian criollo cattle rumen. The fourth anaerobic probiotic bacterial strain, *Fibrobacter succinogenes* Fs, was isolated from the cecum of a native Colombian capybara (*Hydrochoerus hydrochaeris*). The bacteria were stored in a 15% glycerol solution at -80°C at the AGROSAVIA Colombian Bank of Microorganisms in the Collection of Microorganisms of Interest in Animal Nutrition (Mosquera, Colombia). Revived strains were sub-cultured twice in M10 anaerobic bacteria culture broth (Eller et al., 1971) and incubated anaerobically (100% CO_2) in an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI, USA) for 24 h at 39 ± 2°C.

Biomass Production

Three batches of each individual strain were incubated anaerobically in 600 mL of M10 at 39 ± 2°C until the respective optimal growth times were reached. Previously, the microbial growth kinetics were determined for each of the probiotic strains to establish optimal fermentation times, following the methodology of Junco and Rodríguez (2001). (Data not showed). Isolates *F. succinogenes* Fs, *B. fibrisolvens* B9, and *S. bovis* C2 reached maximum biomass concentrations after 8 h of culturing, respectively. In contrast, *R. flavefaciens* Rf reached a maximum concentration after 24 h of culturing.

Culture pH was measured with an electrochemical analyzer, Consort™ C931 (Consort bvba, Turnhout, Belgium). Bacteria colony forming units (CFU) were measured using the Hungate roll-tube technique (Hungate, 1969) and M10 medium. A CO_2 gas phase was maintained during a 48h incubation at 39 ± 2°C.

Development of the Emulsion

An emulsion is a system formed for two phases: A oil phase and an aqueous phase. In our case, the aqueous phase was probiotic bacteria and oil phase was compound for oil, and surfactants.

The probiotic carrier emulsion was developed in our laboratory, based on the hydrophilic-lipophilic balance (HLB) method (Griffin, 1949; Jin et al., 2008; Pimentel-Moral et al., 2018). The oil phase in the carrier emulsion consisted of sunflower oil (PremierTM, Harinera del Valle, Valle de Cauca, Colombia) at 47.25 mL/100mL of total emulsion volume; with HLB = 7, surfactants Tween 20 (Bell Chem International, Longwood FL, USA) at 2.83 mL/100 mL, and HLB = 16.7 and soy lecithin (Danisco, Du Pont, México) at 9.17 mL/100 mL; with HLB = 4.0. Sodium bicarbonate (0.75% w/v) (Merck KGAa, Darmstadt, Germany) was added to maintain stable pH.

Twenty-five milliliters of each bacterial culture broth were combined in equal proportion (C2, B9, Rf and Fs), in a total volume of 100 mL. A volume of 150 mL oil phase was added to this and mixed at 9,000 rpm with an Ultraturrax IKA T18® homogenizer (IKA-Werke), with a CO_2 stream

at 800 psi and a flow rate of 10 CFH. A portion (10 mL) was drawn into in a 10-mL HDPE syringe, then stored for six months at 4 ± 2°C. At 0, 2, 3, and 6 months, bacterial CFU/mL were determined by the Hungate roll-tube technique. Emulsion pH was measured with a Consort™ C931 (Consort bvba) and contamination was evaluated (filamentous molds and aerobic bacteria) at each time.

Animal management and treatments

An animal efficacy assay of the emulsion was carried out on a dairy farm located in Sopo, Cundinamarca, Colombia, after verification of compliance with the animal welfare standards explicit in the "Five Freedoms", established as a guide to ensure appropriate animal care in livestock production systems (Webster, 2001). The selection criteria for calves were based on results of the evaluation of their vitality following the APGAR scale (Lange et al., 2018). Once selected, the calves were managed under the corresponding farm management protocols.

In Vivo Efficacy

Selected female Holstein calves were included in the study at the time of birth, all newborn calves were treated according with the particular managment in the farm, and randomly placed in three experimental groups: group 1 was given freshly prepared probiotic emulsion; group 2 was given the probiotic emulsion stored for 6 months at 4 ± 2°C; and group 3, the control, no treated with probiotic emulsion. The start of the probiotic dosing was initiated after the calf received colostrum from the mother (at 24 to 72 h after birth), called day 1 (D1). Each calf received a total of 12 10-mL oral doses with the first doses given over ten consecutive days and subsequent doses given on day 15 (D15) and day 30 (D30). In total, 78 calves were evaluated with 39 animals in group 1, 20 animals in group 2 and 19 animals in group 3. Calf weights were determined monthly, using an electronic scale. Response variables were analyzed as body weight gain over monthly for 3 months, considering the farm and treatment effects, and were expressed as daily weight gain. Also, the time of weaning and the number of diarrhea episodes were determined by direct observation.

Statistical analysis

For the stability study of probiotic emulsion, we follow a completely randomized experimental design and all measurements were performed in triplicate from three syringes. The viability expressed as colony forming unity per mL (CFU/mL) of emulsion were Log10 transformed. Statistical significance of the results was determined using one-way analysis of variance (ANOVA), and mean comparison test (Tukey, confidence level of 95%).

For the *in vivo* efficacy test, a completely randomized design with a 3 × 2 factorial was followed for the trial. Daily weight gain was subject to an ANOVA and comparison of means LSD with a 95% confidence level. The time of weaning and the number of diarrhea episodes were subject to a Kruskal-Wallis one-way analysis of variance analysis with a 95% confidence level.

RESULTS AND DISCUSSION

Six months of storage at 4 ± 2°C of the prepared probiotic emulsion resulted in a significant reduction ($P < 0.05$) of viable cell counts, from 1.95×10^9 CFU/mL at time zero to 3.02×10^8 CFU/mL at six months. However, viable cell counts were never less than 1×10^8 CFU/mL, that is higher than the recommended dose for the majority of probiotic strains (1x10^6 CFU/g of feed) (Simon, 2005; Vinderola et al., 2000). Additionally, this minimum value was established in previous studies (data not shown), which revealed the minimum viable cell count needed for a formulation to be effective. Also, following storage for six months at 4 ± 2°C, the emulsion showed no signs of instability, such as clotting or phase separation.

The emulsion pH was between 7.32 and 7.57 after six months of storage. These results suggest that emulsion pH was stable for six months, although strains B9 and C2 are butyric and lactic acid producers, respectively. With respect to contaminants, at time zero, the levels of aerobic bacteria and filamentous fungi were less than 1×10^3 CFU/mL. After six months of storage, the contaminating levels for aerobic bacteria and fungi were less than 10^4 CFU/mL, with neither *Salmonella* sp. nor *Escherichia coli* detected in the preparations.

Table 1 shows the effects of probiotic emulsion in Holstein calves. No differences were detected between calf weight gain for the control treatment (no probiotic emulsion given) and the freshly made or stored probiotic emulsions during the first month of evaluation ($P = 0.4192$). However, for the second and third months, weight gain was greater ($P < 0.05$) in calves treated with both the fresh probiotic and stored, compared to untreated control calves. Additionally, weaning time and frequency of diarrhea for animals treated with fresh and stored emulsions showed lower ($P < 0.05$) values compared to the control treatment.

Considering that the four rumen bacteria are anaerobes, the main factor that can affect cell viability is oxygen. The presence of oxygen is harmful to most anaerobic probiotics, mainly due to the potential reaction of oxygen with water, forming reactive oxygen molecules that would attack proteins, lipids, and nucleic acids, and cause death (Ahn et al., 2001). This strongly affects the storage stability of probiotics in dairy-based products (Yeo et al., 2011). It is likely that the oil (dispersant phase) in the emulsion used

Table 1: Biological responses in neonate calves given preparations of the probiotic emulsion

Treatment	Number of calves	Birth weight (kg)*	Biological responses				
			Weight gain month 1 (kg)	Weight gain month 2 (kg)	Weight gain month 3 (kg)	Weaning (Days)	Diarrhea episodes (diarrhea/ calf/month)
No emulsion Control	39	36.92 ± 5.94	46.0 ± 7.02	60.28 ± 9.63^{b}	97.44 ± 7.50^{b}	113.92 ± 22.90^{a}	5.92 ± 3.48^{a}
Fresh emulsion	20	35.70 ± 5.27	48.32 ± 4.75	74.5 ± 10.43^{a}	105.02± 6.64^{a}	98.85 ± 2.70^{b}	1.90 ± 1.59^{b}
Emulsion stored for 6 months at 4 ± 2°C	19	35.47 ± 5.14	48.76 ± 5.54	75.44 ± 8.63^{a}	105.41 ± 11.29^{a}	96.68 ± 4.33^{b}	2.26 ± 1.37^{a}

*Means ± SD in columns and with different superscripts are significantly different ($P < 0.05$

in this study covered the water droplets (dispersed phase) from the fermentation broth of each bacterium. The oil decreased or prevented oxygen in the environment from contacting the bacteria.

Aerotolerant species adhering to these tissues are not restricted to the lumen surface of epithelial cells, but are found in the intercellular spaces, on superficial cells and in the underlying tissues (Dinsdale et al., 1980). Recently, Li et al. (2020) showed that members of Cristensenellaceae, Muribaculaceae, *Prevotella*, *Butyrivibrio,* and *Treponema* are predominant on the rumen wall epithelium. These microbial communities would physiologically act as biological filters of the toxic oxygen that may impair cellulolytic activity by strict anaerobes such as *Ruminococcus* and *Fibrobacter*. As indicated above, the fatty acid (FA) composition of the bacterial cell membrane interacts differently when exposed to surfactants, and the same would happen in the presence of different concentrations of molecular oxygen. Oxygen transport across the bilayer structure depends on both diffusion and solubility in the bilayer, which is closely related to the content of cholesterol and unsaturated fatty acids on the cell membrane (Möller et al., 2016; Li and Buckin, 2019).

The sunflower oil component of the carrier emulsion is a mix of saturated and unsaturated fatty acids (FA): stearic, oleic, linoleic, and linolenic acids. The three-dimensional structures of these molecules may exhibit different properties that influence molecular interactions affecting the capacity to trap free oxygen present in the formulations. The storage stability of the mixture suggests that the emulsion could protect the viability and biological activities of ruminal bacteria over time. Even though cell viability was reduced significantly after 6 months at 4 ± 2°C, viable cells were retained at a therapeutic concentration (1x10^{8} CFU/mL). Subsequent to dosage, there were observed positive effects on neonate calves for the variables evaluated, weight gain and diarrhea episodes.

Presented here is a stable emulsion formulation that is easily prepared with a low number of operational steps and is low-cost in comparison to other technologies such as lyophilization, spray drying, and encapsulation. These methods can be used to separate the cells from adverse environmental conditions and reduce injury by providing a protective coating (Yeo et al., 2011). These findings are very important, as working with probiotics pose unique challenges; bacteria often die during processing and shelf life is unpredictable. Also, probiotics are extremely susceptible to environmental conditions, such as oxygen, processing and preservation treatments, acidity, and salt concentration, which collectively affect the overall viability of probiotic microbes (Mandal and Hati, 2017). This means of preparation also addresses the industrial concerns of complexity and cost effectiveness of mass production (Mitropolou et al., 2013). Hence, the proposed emulsion can be prepared in an efficient manner on an industrial scale, while addressing the important variables of efficient and protective inclusion of the probiotic bacteria (Huckle and Zhang, 2011).

Most probiotic products have a short life span of three months or less, even when stored at low temperature (Lee and Salminen, 1995; Astesana et al., 2018). No known literature presents findings on probiotic emulsion formulations based on anaerobic ruminal bacteria. However, with respect to research assessing storage stability for probiotic bacteria, the emulsion presented herein offers similar, or in some cases higher, performance.

Heidebach et al. (2010) encapsulated *Lactobacillus* F19 and *Bifidobacterium* Bb12 in casein-based microcapsules by enzymatic gelation followed by freeze-drying, and the encapsulation improved the survival rate of powdered cells during storage at 4 or 25°C and 11 or 33% relative humidity for up to 90 days. Soto et al. (2011) designed alginate macrocapsules coated with chitosan for *Lactobacillus casei,* where viability of cells was maintained at 1x10^{6} CFU/mL for 2 months refrigerating at 4 °C. Zhang et al. (2015)

showed that after storage for 63 days at 4°C, cell viability decreased almost 2.5 Log units. Similarly, Maldonado et al. (2016) demonstrated that *Lactobacillus johnsonii* CRL1693 remained viable after 120 days when mixed in lyoprotectors milk, milk þ-lactose, milk whey, and milk whey þ-lactose, wherein survival in milk whey and lactose was significantly affected by storage time. However, this strain did not survive when stored at 4°C for 180 days in any of the lyoprotectors assayed. To the best of our knowledge there are not probiotic formulations with anaerobic bacteria reported literature. Our emulsion is a novel carrier for this type of bacteria.

In our study, we found beneficial effects following the dosage of anaerobic ruminal bacterial strains, delivered in an oil emulsion, resulting in significant increases in body weight gain (BWG) and a reduction of diarrhea episodes. However, future studies, it would be important to design quantitative tests for each bacterium with the aim of evaluating individual behavior in the emulsion.

Ruminal bacteria and other microorganisms have been widely used as direct-fed microbial (DFM) to enhance cattle production (Krehbiel et al., 2003; McAllister et al., 2011; Khan et al., 2016; Stefanska et al., 2022). Young Bovine calves are promising targets for DFM since their stomach is physiologically and anatomically immature, limiting the types and abundance of the microbial species that will dominate their rumen compartment. With the proper substrates (fiber and feed concentrate), the rumen probiotic strains administered would proliferate and successfully establish in the developing rumen. In the current study young calves administered with formulated ruminal bacterial strains exhibited benefits in body weight gain and decreased diarrheal episodes.

Chiquette et al. (2007), using calves 21 to 35 d of age, showed that after repeated dosing with *Ruminococus flavefaciens* NJ, ruminal cellulolytic bacterial abundance was positively influenced, as well as improvements *in Sacco* digestibility of fibrous and feed concentrate. Another important finding was their observation that following dosage this cellulolytic bacterium persisted for nearly a week in the calves and at low levels. In a different study young lambs (4 weeks of age) were dosed with a mixture of *Ruminococcus* strains and species. Ruminal fibrolytic populations increased significantly and for extended periods post-dosing (Krause et al., 2000). The authors suggested, according to their findings, that the *Ruminococcus* administered would benefit the overall availability of fiber for microbial degradation (Krause et al., 2000). Krause et al. (2001) found that with increased ruminal dosing with the administered bacteria, there was no guarantee of probiotic strain persistence, however, administration of the probiotic bacteria did influence changes in other important fibrolytic bacteria such as *Fibrobacter succinogenes*.

CONCLUSION

The carrier emulsion protected cell viability and efficacy of anaerobic probiotic bacteria for 6 months of storage at 4 ± 2°C, thus this can be an alternative for carrying other anaerobe microorganisms. The bacterial mixture (B9, Rf, Fs and C2) improved weight gain, and prevented diarrhea episodes in weanling dairy female Holstein calves.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest. The agroproduct was designed and researched by Agrosavia scientists and licensed by a private commercial entity for distribution. The Agrosavia scientists who developed the agroproduct are not associated with the licensed private commercial entity.

NOVELTY STATEMENT

In this study, rumen anaerobic bacteria were formulated as an emulsion that was stable for six months. To our knowledge, few if any of these microorganisms are available in commercial biological products.

AUTHORS CONTRIBUTION

All authors contributed equally according to their experience and task and approved the final manuscript.

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