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Effect of anaerobic bovine colostrum fermentation on bacteria growth inhibition

Efeito da fermentação anaeróbica do colostro bovino na inibição do crescimento de bactérias

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ABSTRACT

*Efficient handling programs that provide high quality colostrum in adequate amounts to dairy farm calves are needed to assure their health and survival. Replacers (or milk substitutes) often become necessary when colostrum presents inadequate quality, or in order to break the cycle of infectious disease transmission. In this study we aimed to assess the effect of anaerobic fermentation processing (colostrum silage) on bacterial that represent interest to animal health. Colostrum samples were inoculated with cultures of **Brucella abortus**, **Escherichia coli**, **Leptospira interrogans serovar Copenhageni**, **Mycobacterium bovis**, **Salmonella Enteritidis**, **Salmonella Typhimurium**, **Staphylococcus aureus**, and **Bacillus cereus** and then subjected to anaerobic fermentation. On the first day, and every seven days until 30th days after fermentation, the samples were cultured and colony forming units counted. At seven days of fermentation, **B. abortus**, **L. interrogans**, and **M. bovis** were not detected. At 14th days of fermentation, **E. coli**, **S. aureus**, **S. Enteritidis** and **S. Typhimurium** were no longer detected. However, we were able to detect both lactic acid bacteria and **B. cereus** until 30th days of fermentation. From this study we suggested that anaerobic fermentation processing can inhibit important bacteria that cause economical losses for the cattle industry. The observations suggested that colostrum silage is a promising form to conserve bovine colostrum.*

Key words: *colostrum silage, lactic acid bacteria, calves, milk substitutes*.

RESUMO

*Eficientes programas de manejo que permitem o fornecimento de colostro de alta qualidade e em quantidades adequadas para bezerros são necessários para garantir a sua saúde e sobrevivência. Substitutos do leite tornam-se frequentemente necessários quando o colostro apresenta qualidade inadequada ou a fim de quebrar o ciclo de transmissão de doenças infecciosas. Este estudo teve como objetivo avaliar o efeito da fermentação anaeróbica do colostro bovino (silagem de colostro) sobre bactérias de interesse para a saúde animal. Alíquotas de colostro foram inoculadas com culturas de **Brucella abortus**, **Escherichia coli**, **Leptospira interrogans serovar Copenhageni**, **Mycobacterium bovis**, **Salmonella Enteritidis**, **Salmonella Typhimurium**, **Staphylococcus aureus** e **Bacillus cereus** e submetidas ao processo de fermentação anaeróbia. No primeiro dia e a cada sete dias até o 30º dia de fermentação, as alíquotas foram cultivadas e analisadas pela contagem de unidades formadoras de colônias. Aos sete dias de fermentação, unidades formadoras de colônias de **B. abortus**, **L. interrogans** e **M. bovis** não foram detectadas e unidades formadoras de colônia de **E. coli**, **S. aureus**, **S. Enteritidis** e **S. Typhimurium** não foram observadas no 14º dia de fermentação. No entanto, **B. cereus** e bactérias ácido lácticas foram detectadas até o 30º dia de fermentação. Este estudo sugere que o processo de fermentação anaeróbia do colostro bovino proporciona efeitos inibitórios sobre as bactérias avaliadas, constituindo-se num promissor método de conservação do colostro bovino.*

Palavras-chave: *silagem de colostro, bactérias lácticas, bezerros, substitutos do leite.*

INTRODUCTION

Colostrum, the first milk produced by the cow after birth, is rich in nutrients, immunoglobulin, minerals, vitamins and bioactive substances (GODDEN, 2009; KURALKAR & KURALKAR,

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2010). Lack of hygiene in preparing both the cow and milking equipment, excessive time between milking and suckling, mastitis, all can predispose colostrum contamination by microorganisms (STEWART et al., 2005). Bacteria such as ***Mycoplasma*** spp., ***Mycobacterium*** spp., ***Campylobacter*** spp., ***Salmonella*** spp., ***Listeria monocytogenes***, and ***Escherichia coli***, may be present in colostrum due to contamination of the mammary gland, post-milking contamination, or bacterial proliferation through improperly stored colostrum (STABEL et al., 2004). ***Staphylococcus aureus***, viral bovine diarrhea, and viral bovine leukemia have been added to this list (MCGUIRK & COLLINS, 2004). However, it is important to note that cows with infectious diseases and/or zoonosis must not be placed on the milking line in order to prevent the transmission of pathogens to humans and animals (BRASIL, 2011).

To ensure administration of contaminant free milking, researchers have recommended the use of commercial substitutes for colostrum (GODDEN et al., 2012; PRIESTLEY et al., 2013).

Healthy cows produce colostrum in amounts above the intake capacity of the calf (39 to 52kg) during the first four days post-partum (FOLEY & OTTERBY, 1978). Storing surplus colostrum by freezing (RAMÍREZ-SANTANA et al., 2012), aerobic acidification (FOLEY & OTTERBY, 1978), use of organic additives (GARCIA et al., 1981), or by anaerobic fermentation (SAALFELD et al., 2013) allows surplus colostrum to be employed in feeding more animals. Anaerobic fermentation (colostrum silage) preserves the nutritional constituents, and ensures the transfer of antibodies to calves (SAALFELD et al., 2013, 2014). Our group demonstrated that environmental microbial contamination of natural colostrum can be eliminated with 21 days of anaerobic fermentation, remaining only lactic acid bacteria (LAB) as viable (SAALFELD et al., 2013).

Considering that knowledge of microbiological parameters is a key to making use of anaerobically fermented colostrum as food to rear calves, the present study was elaborated to evaluate the inhibitory effects of anaerobic colostrum fermentation (colostrum silage) on bacteria of importance to animal health.

MATERIALS AND METHODS

Colostrum was obtained by mechanical milking of two Holstein cows from the Farmers' Training Center of EMATER - CETAC in Canguçu RS; complying with the hygiene rules laid down in Instruction 62 of the Ministry of Agriculture and Livestock (BRASIL, 2011). The colostrums milked on the second day postpartum, was transported (refrigerated, 4-6°C) to the microbiology laboratory at the Universidade Federal de Pelotas (UFPel) and divided into eight aliquots of 1,200mL. Each of these colostrum aliquots was inoculated with the following bacteria: ***Mycobacterium bovis*** BCG Pasteur ($5x10^6$CFU mL^{-1}), ***Brucella abortus*** vaccine strain B19 ($4x10^9$CFU mL^{-1}), ***Leptospira interrogans*** serovar Copenhageni ($1x10^7$ CFU/mL), ***Staphylococcus aureus*** ATCC 25923 ($5x10^9$CFU mL^{-1}) ***Escherichia coli*** (O141; K88ab; H4) ($6x10^7$CFU mL^{-1}), ***Salmonella Enteritidis*** ATCC 13076 ($5x10^{10}$CFU mL^{-1}), ***Salmonella Typhimurium*** ATCC 4028 ($6x10^{10}$CFU mL^{-1}) and ***Bacillus cereus*** ATCC 14579 ($6x10^7$CFU mL^{-1}). The inoculums criteria were based in the highest count found in contaminated milk samples by the diagnostic laboratory of School of Veterinary at the Universidade Federal de Pelotas. After bacterial inoculations, each of the eight aliquots was fractionated into five polyethylene terephthalate (PET) 226mL bottles, which were completely filled, closed and stored for anaerobic fermentation of the colostrum as previously described by SAALFELD et al. (2013). In addition, five aliquots were stored without bacterial inoculation, constituting the negative control of the experiment. All bacterial cultures were kindly provided by the Bacteriology Laboratory of the Technological Development Center (CDTec/UFPel). The counting of colony forming units (CFU mL^{-1}) for each bacterium was done by serial dilution (base 10 dilution to 10^{-10}), in duplicate, immediately after the initial inoculation, and upon each seventh day of fermentation, until 30 days of storage. A 1mL aliquot of each dilution was seeded by surface spreading with a Drigalski spatula. BHI agar (Brain Heart Infusion, Difco Il, USA) for ***B. cereus***; Chapmann agar (Difco Il, USA) for ***S. aureus***; MacConkey agar (Difco Il, USA) for ***E. coli***; BHI agar and MacConkey agar for ***S. Enteritidis*** and ***S. Typhimurium*** were used, with incubations at 37°C for 24 hours. For ***M. bovis*** (BCG) Middlebrook 7H10 culture medium (Difco, USA), was used with incubation at 35 to 37°C for 21 days. For ***L. interrogans*** the medium employed was Ellinghausen-McCullough-Johnson-Harris (EMJH) supplemented with 10% EMJH (Difco, USA), and incubation at 30°C for seven days. For ***B. abortus*** it was used Thayer-Martin agar, and sheep blood agar (Difco, USA), incubated at 37°C for 10 days. The control sample (without inoculation) was stroked on sheep blood agar (8%) (Difco, USA) and incubated at

37ºC for 24 hours, and on MRS agar (Man, Rogosa, and Sharpe; Biobras) incubated in microaerophilic environments at 37ºC for 48 hours to count the LAB. Colony forming unit counting was performed by a manual colony counter (Phoenix-Luferco) using plates having from 30 to 300 colonies. The LAB were submitted to Gram staining, catalase, maltose, VP (Voges-Proskauer), and nitrate tests for genera identification (KONEMAN et al., 2001; BARROS et al., 2009).

A Mann-Whitney test was used to compare the means of the count results. The differences were considered significant at $P<0.05$.

RESULTS

Table 1 shows the CFU/mL results from the bacteria inoculated into the anaerobically fermented colostrum. After seven days of colostrum fermentation ***B. abortus***, ***L. interrogans***, and ***M. bovis*** were not detected. After 14 days of fermentation the same was evidenced for ***E. coli***, ***S. aureus***, ***S. Enteritidis*** and ***S. Typhimurium***. At the end of the evaluation period (30 days) only the fermented colostrum inoculated with ***B. cereus*** presented growth. In the control aliquots, after 14 days of fermentation, only LAB were present. An increase in LAB counts after seven days evaluation (10^9CFU mL^{-1}) was observed; which then declined at 14^{th} days (to 10^7CFU mL^{-1}) of fermentation. This same count remained stable until day 30 (Table 1). Morphological and biochemical analyses revealed the presence of gram-positive rods or coco bacilli, singly or in chains; the catalase and nitrate tests were negative; and the maltose and VP tests gave varied results. These features allowed us to classify the isolated LAB as probably of the ***Lactobacillus*** genus.

DISCUSSION

Colostrum is the main food source for newborn calves. However, microbial contamination can cause diseases and interfere with passive absorption of antibodies (GODDEN, 2009). A previous study by SAALFELD et al. (2013) demonstrated that anaerobic colostrum fermentation (silage colostrum) methods kept the nutritional properties necessary for calves after 21 days of fermentation, as well as inhibited the growth of ***Staphylococcus*** spp., ***Enterobacteriaceae***, yeast, and filamentous fungi which had been initially present in colostrum. The present study showed that after seven days of colostrum fermentation, growth of ***B. abortus***, ***L. interrogans***, and ***M. bovis*** were inhibited. Additionally, ***S. aureus***, ***E. coli***, ***S. Enteritidis***, and ***S. Typhimurium*** showed no growth after 14 days of fermentation. It is believed that microbial growth inhibition might be caused by the low pH of the fermented colostrum, which on average, has values of 4.0 to 4.3 (SAALFELD et al., 2013). Previous studies by PORTAELS & PALTYN (1982) showed that the optimum pH for ***M. bovis*** growth ranges from 5.8 to 6.5; for ***B. abortus*** 6.6 to 7.4 (PAULIN, 2003), and for ***Leptospira*** spp. 7.2 to 7.8 (AVILA et al., 1998). Additionally, it has been suggested that antimicrobial effects may be associated with antimicrobial substances, including bacteriocins, lactoferrin, transferrin, cytokines, and immunoglobulins (SUGIHARTO, 2015) present in the colostrum and which probably survive fermentation. Although, SAALFELD et al. (2014) demonstrated that antibodies remain preserved after the fermentation process, further studies are needed to evaluate the role of antimicrobial substances in fermented colostrum. In addition, in this study, was not our purpose to evaluate the mechanism(s) that may be involved in the growth inhibition observed.

In the present study, the growth of ***B. cereus*** displayed an initial decrease in bacterial counts, remaining constant at about $2.7x10^3$ CFU mL^{-1} until assessment day 30. It is believed that the behavior of this bacteria may be associated with its sporulation ability (KONEMAN et al., 2001), which makes it resistant to physical and chemical factors such as wide pH range (4.0 to 9.3) (STAACK, 2008). It is likely that the initial count decrease is due to the presence of vegetative cells at the start of the fermentation process. Nonetheless, the persistence of ***B. cereus*** after the fermentation period must be considered and assessed. Although most cultures of ***B. cereus*** are classified as saprobes (PERES-NETO & ZAPPA, 2011), and characterized as probiotics (TURNES, 1999), some strains may be associated with the occurrence of food-borne disease outbreaks in humans (DHAMA et al., 2013), while others may be associated with cases of mastitis (either subclinical or clinical of environmental origin) in cattle (PERES-NETO & ZAPPA, 2011).

A previous study reported that the decreasing pH of the colostrum at seven days of fermentation was accompanied by an increase in CFU mL^{-1} of LAB. These bacteria are able to split lactose into lactic acid, which renders the medium acidic (SAALFELD et al., 2013). In the present study, during the evaluation period, the LAB count stabilized at around 10^7CFU mL^{-1}, which was similar to that reported by SAALFELD et al. (2013), who evaluated samples of fermented

Table 1 - Bacterial count (mean CFU, ± SE) in anaerobic fermented bovine colostrum.

Microorganism	Day 0	Days of fermentation			
		Day 7	Day 14	Day 21	Day 30
Lactic acid bacteria (BAL)	$2,5 \times 10^{7a}$	$6,0 \times 10^{9} \pm 0.05^{b}$	$5,7 \times 10^{7} \pm 0.07^{a}$	$2,8 \times 10^{7} \pm 0.04^{a}$	$2,5 \times 10^{7} \pm 0.05^{a}$
Brucella abortus	40×10^{7}	ND	ND	ND	ND
Bacillus cereus	6×10^{7a}	$3,5 \times 10^{3} \pm 0.04^{b}$	$2,7 \times 10^{3} \pm 0.05^{b}$	$2,7 \times 10^{3} \pm 0.06^{b}$	$2,7 \times 10^{3} \pm 0.05^{b}$
Escherichia coli	6×10^{7a}	$3,0 \times 10^{3} \pm 0.05^{b}$	ND	ND	ND
Leptospira interrogans	1×10^{7}	ND	ND	ND	ND
Mycobacterium bovis	0.5×10^{7}	ND	ND	ND	ND
Salmonella Enteritidis	100×10^{7a}	$4,0 \times 10^{6} \pm 0.06^{b}$	ND	ND	ND
Salmonella Thyphimurium	100×10^{7a}	$5,0 \times 10^{5} \pm 0.03^{b}$	ND	ND	ND
Staphylococcus aureus	50×10^{7a}	$3,0 \times 10^{4} \pm 0.05^{b}$	ND	ND	ND

[a–b]Means lacking a common lowercase letter differ ($P < 0.05$), using a Mann-Whitney test; ND: not detected.

colostrum for up to 360 days of storage, showing that LAB remained viable throughout the studied period. Besides this pH action, the lactic acid bacteria have the ability to produce antimicrobial compounds called bacteriocins (BURITI & SAAD, 2007), inhibitory substances, such as hydrogen peroxide, diacetyl, oxygen metabolites, and others (PIARD & DESMAZEAUD, 1991). Many lactic acid bacteria play an important role in the production of fermented foods by inhibiting the growth of a wide variety of food spoiling organisms (JACK et al., 1995). Studies showed that bacterial genera considered as lactic acid, like ***Lactobacillus***, ***Bifidobacterium***, ***Enterococcus***, ***Pediococcus***, ***Lactococcus***, ***Leuconstoc*** and ***Streptococcus thermophilus*** have probiotic potential (HOLZAPFEL et al., 2001; GIRAFFA, 2004). However, microbial characteristics, and probiotic potential were not assessed in this study. Further studies are needed to identify whether fermented colostrum presents LAB with probiotic characteristics. Phenotypic classification of LAB colonies was made according to their physiological, morphological, and biochemical characteristics, assuming that, among other features, the lactic acid bacteria are gram-positive microorganisms, catalase negative, non-sporulating, and nitrate negative (BARROS et al., 2009).

Although colostrum fermentation process prevents growth of non-sporulating pathogenic bacteria, it still remains essential that producers pay attention to milking hygiene and herd sanitation to minimize bacterial contamination during harvesting and storage of colostrum.

CONCLUSION

The anaerobic colostrum fermentation process was able to inhibit the growth bacteria including ***B. abortus***, ***E. coli***, ***L. interrogans***, ***M. bovis***, ***S. Enteritidis***, ***S. Typhimurium***, and ***S. aureus***. These represent major bacteria of concern to the dairy industry, thus it is possible to suggest and recommend anaerobic colostrum fermentation as an alternative to provide colostrum to calves. The lactic acid bacteria which remained throughout the fermentation process likely kept retained their probiotic activity, adding the benefits of this technique.

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