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Comparison of nitroethane, 2-nitro-1-propanol, lauric acid, Lauricidin® and the Hawaiian marine algae, Chaetoceros, for potential broad-spectrum control of anaerobically grown lactic acid bacteria

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The gastrointestinal tract of bovines often contains bacteria that contribute to disorders of the rumen, and may also contain foodborne or opportunistic human pathogens as well as bacteria capable of causing mastitis in cows. Thus there is a need to develop broad-spectrum therapies that are effective while not leading to unacceptably long antibiotic withdrawal times. The effects of the CH4-inhibitors nitroethane (2 mg/mL), 2-nitro-1-propanol (2 mg/mL), lauric acid (5 mg/mL), the commercial product Lauricidin® (5 mg/mL), and a finely ground product of the Hawaiian marine algae, Chaetoceros (10 mg/mL), were compared in pure cultures of Streptococcus agalactia, Enterococcus faecium, Streptococcus bovis, and in a mixed lactic acid rumen bacterial culture. Lauricidin® and lauric acid exhibited the most bactericidal acidity against all bacteria. These results suggest potential animal health benefits from supplementing cattle diets with lauric acid or Lauricidin® to improve the health of the rumen and help prevent shedding of human pathogens.

Keywords: Lauric acid, Lauricidin®, nitroethane, Chaetoceros, rumen bacteria.

Introduction

The bovine gastrointestinal tract can be a reservoir for a variety of unwanted bacteria, which if allowed to proliferate, can be detrimental to the health of the ruminant. Among these bacteria, Streptococcus bovis and acid-tolerant lactobacilli contribute to a multitude of ruminal metabolic disorders and other disease maladies. Streptococcus bovis is a well-known ruminal organism that under optimal conditions can outgrow other rumen flora and produce large amounts of lactate and capsular polysaccharides which can lead to rumen acidosis and bloat.[1–3] Streptococcus and Lactobacillus increases have been observed in cattle with bloat. They are believed to contribute to the increase in capsular polysaccharides leading to bloat of the rumen because the polysaccharide can create a stable foam from the gases produced during fermentation; the foam defeats the natural anti-foaming agents in the rumen, causing a build-up of gas that cannot be belched normally.[2,4,5]

Mastitis, an infection of the udder, is the most common illness in dairy cattle and also the most economically devastating.[6] Economic losses associated with mastitis have been estimated at $1.2 to 1.7 billion per year in the United States alone.[7] Streptococcus agalactiae (group B Streptococcus, GBS), a Gram-positive obligate pathogen is the most common cause of bovine mastitis.[8] Although GBS is susceptible to a variety of antimicrobial agents, the increased number of multiple drug-resistant pathogens and the production of milk contaminated with antibiotics make treatment with traditional antibiotics a poor choice.[9] Enterococcus spp are important causative agents of enteric disease in food-producing animals.[10,11] Additionally, enterococci are also a known cause of mastitis,[12] and the most commonly isolated enterococcus is Enterococcus faecium.[13] Hershberger et al.[14] surveyed the antibiotic resistance of enterococci from animals. Although they found no vancomycin-resistant strains they did find antibiotic resistant rates in E. faecium that were 2%, 0% and 55% resistant to Quinupristin/dalfopristin, gentamicin and ciprofloxacin.
Materials and methods

**Bacterial strains and chemicals**

*Streptococcus agalactiae* was provided without strain identification by Dr. Max Paape (USDA, Beltsville, MD); *Streptococcus bovis* strain JB1, originally provided by the late Dr. James Russell (USDA, Ithaca, NY) and *Enterococcus faecium* 1.3[27] were strains from our culture collection (Southern Plains Agricultural Research Center, ARS-USDA, College Station, Texas 77845). Sodium laurate (sodium dodecanoate), nitroethane and 2-nitro-1-propanol were purchased from Sigma-Aldrich (St. Louis, MO). The marine algae *Chaetoceros* was provided by Dr. Jon Kabara (Bradenton, FL, USA). The marine algae *Chaetoceros* was produced and harvested from an open continuous microalga culture system at the Anuenue Fisheries Center, Sand Island, Oahu, Hawaii by Dr. Jaw Kai Wang[28] and contained (sodium dodecanoate), nitroethane and 2-nitro-1-propanol, lauric acid, Lauricidin R, and the marine algae *Chaetoceros* against *S. bovis, E. faecium, S. agalactiae* and a mixed lactic acid bacterial culture from the rumen.

**Culture conditions**

Pure cultures were grown in Brain Heart Infusion broth (BHI; Becton, Dickinson and Company, Sparks, MD, USA) prepared and distributed (10 mL per tube) anaerobically under 100 % N₂[29] to 18 × 150 mm crimp top tubes. The N₂ gas was deoxygenated via passage through a heated, reduced copper Hungate column designed to catalytically reduce any contaminating O₂ to water vapor.[29] Sodium laurate, Lauricidin® (0.05 g each) and 0.10 g of a ground product (approximately 1 mm particle size) of the marine algae were added as dry additions to tubes before addition of medium. These tubes and those containing no prior additive were closed with rubber stoppers, crimped and sterilized by autoclaving for 15 min at 15 psi pressure (121°C). Nitroethane and 2-nitro-1-propanol were added to tubes containing sterilized medium as a filter sterilized (0.22 μm) concentrated stock solutions. Nitroethane was prepared as a sodium salt as described by Majak et al.[30] to increase the solubility in aqueous solutions. Upon inoculation with 0.2 mL of overnight cultures grown in un-supplemented anaerobic BHI, treated and control cultures were incubated upright without agitation at 39°C for 24 h. Because cultures containing laurate and Lauricidin® were opaque and thus unsuitable for optical density determinations, viable cell counts were performed on all cultures to measure growth and survivability. Fluid samples collected at indicated intervals were serially diluted (10-fold) in anaerobic (N₂) phosphate-buffered saline (pH 6.8) and spread to BHI agar plates (Becton, Dickinson and Company). Inoculated plates were incubated at 39°C in a Bactron Anaerobic Chamber (Sheldon Labs Manufacturing Inc., Cornelius, OR, USA) under an N₂:CO₂:H₂ (90:5:5) atmosphere for 24 h.

**Mixed culture studies**

Tests for effects of supplements against lactic acid bacteria were performed via mixed culture of freshly collected ruminal fluid obtained from a cannulated Jersey cow maintained on a ryegrass pasture. Ruminal fluid was collected at approximately 10:00, strained through a nylon paint strainer[31] into a pre-warmed receptacle which was immediately closed and returned to the lab for distribution (10 mL per tube) to 18 × 150 mm crimp top tubes containing no additions (controls) or preloaded with dry additions of laurate or Lauricidin® or concentrated stock solutions nitroethane (sodium salt) or 2-nitro-1-propanol (Bozic et al.[32]) to achieve indicated concentrations. Tubes were incubated under 100 % N₂ gas at 39°C for 24 h and fluid samples collected at indicated intervals were serially diluted (10-fold) in anaerobic (N₂) phosphate buffered saline (pH 6.8) and spread to Rogosa agar (Becton, Dickinson and Company) plates. Plates were incubated for 24 h in the anaerobic chamber at 39°C and colonies enumerated.

**Statistical analysis**

Control and treated cultures were incubated in triplicate. Colony forming units were transformed to log₁₀ and means were calculated. Differences were determined by Students t test, with significance assigned at P < 0.05.[33]
Results

Effects of inhibitors on *S. agalactiae*

The effects of the chemicals tested on the growth of *S. agalactiae* are shown in Figure 1. Nitroethane had no effect on the growth of *S. agalactiae* during the 24-hour period studied. In contrast, the 2-nitro-1-propanol caused a steady decline in growth, with growth after 24 hours being almost 2 log CFU lower than the controls. Lauric acid and Lauricidin® both had a profound effect, with the levels of *S. agalactiae* being below detection limit at 6 hours. At 24 hours the lauric acid treated *S. agalactiae* remained less than 1 log CFU/mL and the Lauricidin® treatment had slightly more than 1 log CFU/mL. Chaetoceros produced an initial drop of approximately 1.5 log CFU/mL at 6 hours, but at 24 hours growth was back to the control level.

Effects of inhibitors on *E. faecium*

The effects of the tested chemicals against *Enterococcus faecium* are shown in Figure 2. As with *S. agalactiae*, the nitroethane had no effect on the growth of *E. faecium*. The 2-nitro-1-propanol and the Chaetoceros resulted in roughly 2 log CFU/mL reduction after 24 hours. Lauric acid and Lauricidin® both resulted in a nearly 5 log decrease recoverable colonies over the 24-hour test period.

Fig. 1. Effects of nitrocompounds, lauric acid, Lauricidin® or Chaetoceros on growth of *Streptococcus agalactiae in vitro*. a–d Different superscripts within 6-hour time indicate significant difference (*P* < 0.05). x–z Different superscripts within 24-hour time indicate significant difference (*P* < 0.05).

Fig. 2. Effects of nitrocompounds, lauric acid, Lauricidin® or Chaetoceros on growth of *Enterococcus faecium in vitro*. a–e Different superscripts within 6-hour time indicate significant difference (*P* < 0.05). x–z Different superscripts within 24-hour time indicate significant difference (*P* < 0.05).
Effects of inhibitors on S. bovis

*S. bovis* was relatively unaffected by most of the chemicals used in this test (Fig. 3). Nitroethane had no effect, while the 2-nitro-1-propanol and *Chaetoceros* produced modest effects of holding growth to about 1 or 1.5 log CFU/mL after 24 hours. In contrast, both lauric acid and Lauricidin® reduced growth to below the detection limit at both 6 and 24 hours.

Effects of inhibitors on mixed lactic acid bacteria from the rumen

The reaction of mixed lactic acid bacteria from the rumen after 24 hours incubation is shown in Figure 4. Only Lauricidin® exhibited a detectable effect, lowering growth at 24 hours by approximately 4 log CFU/mL. *Chaetoceros*, 2-nitro-1-propanol and lauric acid reduced growth only by 1 or 2 log CFU/mL at 24 hours.
Potential benefits of lauric acid in cattle diets

Discussion

Gram-positive bacteria of several genera are responsible for many disease processes in cattle. Many of these same bacteria are becoming resistant to currently used antibiotics, causing an emerging problem for the animal industry. Other antimicrobials with a broader spectrum of activity need to be developed.

The broad-spectrum antimicrobial activity of fatty acids, especially against Gram-positive bacteria, has been recognized for years. Medium- and long-chain fatty acids are thought to inhibit the growth of Gram-positive bacteria via absorption and disruption of cell membranes and Lauric acid is also known to inhibit the growth of Gram positive rumen microbes. Oils and pure fatty acids have been shown to be toxic to methanogens, and that toxicity has been noted to be proportional to degree of unsaturation.

Lauricidin is a glycerol monoester of lauric acid which exhibits bactericidal activity against Gram-positive bacteria similar to that of lauric acid. In the study reported here, lauric acid and Lauricidin were the most successful compounds tested in suppressing the growth of all organisms tested. Yabuuchi et al. found that in vitro growth of S. bovis was initially inhibited in a medium supplemented with lauric acid, but also found that over time S. bovis adapted to the lauric acid but continued to exhibit a time lag of 4 to 5 hours in growth. Anang et al. found that Lauricidin was more effective against the Gram positive pathogen L. monocytogenes than against Gram negatives such as Salmonella Enteritidis or E. coli O157:H7. Boddie and Nickerson tested post-milking teat germicides containing Lauricidin (1 %), lactic acid (6 %), and lauric acid (0.85 %) against Streptococcus agalactiae and reported numbers reduced by nearly 50 %.

The marine algae Chaetoceros is known to contain the highly unsaturated fatty acid hexadecatrienoic acid. Desbois et al. reported that hexadecatrienoic acid found in cell extracts of the diatom Phaeodactylum tricornutum exhibited antibacterial activity against Staphylococcus aureus.

One solution to the global crisis of antibiotic resistance is the discovery of novel antimicrobial compounds for clinical application. Additionally, there is a need for the development of new effective antibacterial agents which can be made inexpensively and which are safe for use in mammals. The compounds tested in this research have potential to fill this niche.

Conclusion

The primary objective of the present study was to compare the antibacterial effects of nitroethane, 2-nitro-1-propanol, lauric acid, Lauricidin, and the marine algae Chaetoceros against ruminal bacteria, including S. bovis, E. faecium, S. agalactiae and a mixed lactic acid culture. Lauric acid and Lauricidin were effective against all bacteria tested, although the mixed lactic acid bacteria had increased growth at 24 hours as compared to 6 hours.

References


