Antimicrobial activities of bacterial probiotic cultures against liver abscess causing pathogens in beef cattle

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Introduction

Liver abscesses:

- Liver abscesses occur in beef cattle fed high-grain and low-roughage diets.
- The prevalence of liver abscesses is highly variable, but the average ranges from 10 to 20%, and cattle with liver abscesses show no clinical signs and are detected only at slaughter.
- Liver abscesses are of significant economic concern to the beef industry because of liver condemnation and reduced body weight gain and efficiency of beef production.
- *Fusobacterium necrophorum* subsp. *necrophorum*, which originates from the rumen, is the primary etiologic agent, and *Trueperella pyogenes* is the secondary pathogen.
- Recently, occurrence of *Salmonella enterica*, particularly serotype Lubbock, in liver abscesses has been reported.

Prevention:

- Tylosin, a macrolide, is the most widely used in-feed antibiotic to reduce the incidence of liver abscesses.
- Because macrolides are medically important antibiotics, there is considerable effort to seek a replacement for tylosin.
- Among antibiotic alternatives, probiotics have gained wide acceptance in the cattle industry because they are considered natural and generally recognized as safe (GRAS) products.
- Bacterial probiotics include species of: *Lactobacillus*, *Enterococcus*, *Bifidobacterium*, *Pediococcus*, *Bacillus*, *Leuconostoc*, and *Propionibacterium*.
- These bacteria secrete certain products, such as lactic acid, ethanol, short-chain fatty acids, hydrogen peroxide, bacteriocins, etc. to exert the antimicrobial activity.

Objectives

- To determine the antimicrobial activities of culture supernatants of bacterial probiotic cultures on pathogens that cause liver abscesses.
  - Macro-broth dilution
- To evaluate antimicrobial activities of probiotic bacterial cultures on liver abscess causing bacterial pathogens in a ‘rumen-simulating environment’.
  - *In-vitro* rumen fermentation
  - Most Probable Number method
  - Quantitative PCR

Materials and Methods

Preparation of probiotic cultures:

- Probiotic cultures tested included *Bacillus pumilus, Bacillus subtilis, Lactobacillus acidophilus, Lactobacillus buchneri, Lactobacillus helveticus, Lactobacillus rhamnosus, Pediococcus acidilactici,* and *Pediococcus pentosaceus.*
- Probiotic bacterial species were cultured in broth (MRS or Mueller-Hinton) to obtain turbidities matching 0.5 McFarland Standards.
- Bacterial supernatant was prepared by centrifugation of culture suspension for 15 min at 4°C and 7,500 rpm.
- Resultant supernatant was filter sterilized and stored at -20°C in aliquots before and after pH adjustment (~7.0).

Macro-broth dilution method:

- Both *Fusobacterium* subspecies were cultured in PRAS-BHI broth and *Trueperella pyogenes* and *Salmonella* Lubbock were cultured in Mueller-Hinton broth with and without probiotic culture supernatants.
- Probiotic culture supernatants were tested both with and without adjusting the pH to 7.0.
- Bacterial concentration is determined by two methods:
  - Obtaining the optical density at 0, 6, 12, 24, and 48 hours of incubation.
  - Serial dilution and spread plating at 24 and 48 hours after incubation to determine the viable bacterial count.
- Experiment was repeated with different bacterial inoculum preparations.

In-vitro rumen fermentation assay:

- The experiment was done by using 100 ml serum bottles to evaluate the antimicrobial activity of *Lactobacillus helveticus* on ruminal *Fusobacterium* (subsp. *necrophorum* and *funduliforme*) concentration using glucose, sodium lactate and cattle feed (no tylosin) as substrates.
- Ruminal fluid was collected 4 -5 h post-feeding from a ruminally-cannulated animal, and strained through 4 layers of cheese cloth.
- *Fusobacterium* culture was added at a concentration of 1×10^8 CFU/ml to the strained rumen fluid followed by probiotic bacterial culture. The control serum bottles received no probiotic strain.
- Each serum bottle flushed with CO_2 and sealed with rubber stoppers and incubated at 39°C.
- At 0 and 24 h, the pH of the strained rumen fluid was recorded and concentration of *F. necrophorum* was determined by the most probable number and qPCR methods.
- The assay procedure for each substrates was carried out in replicates on different days using new ruminal samples collected from the same animal.

Statistical analysis:

- Data were analyzed by using SAS (v. 9.4; Cary, NC).
- The PROCGLM procedure was used to fit the least squares in a linear model.
- Analysis of variance was performed on the log transformed MIC values.
- The model included the fixed effect of tested probiotic bacterial cultures and replication as a random effect.
- MPN and qPCR data were log transformed prior to analysis and PROC MIXED procedure was used to analyze the data.

Results

Table 1: PH of the bacterial probiotic culture supernatants used to test their antimicrobial activity against the liver abscess causing bacterial pathogens

Probiotic bacterial strains	pH - Unadjusted	pH- Adjusted
Bacillus pumilus	5.56	7.07
Bacillus subtilis	6.15	7.04
Lactobacillus acidophilus	3.97	7.02
Lactobacillus buchneri	4.68	7.01
Lactobacillus helveticus	3.77	7.01
Lactobacillus rhamnosus	3.85	7.01
Pediococcus acidilactici	3.95	7.03
Pediococcus pentosaceus	3.98	7.05

Macro-broth dilution method:

Lactobacillus helveticus pH-Unadjusted

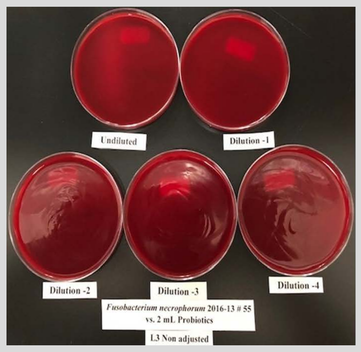

Figure 1A: Inhibition effect of *Lactobacillus helveticus* culture unadjusted supernatant (2 ml) on the growth liver abscess bacterial pathogens

Lactobacillus helveticus pH-Adjusted

Figure 1B: Non inhibition effect of *Lactobacillus helveticus* culture adjusted supernatant (2 ml) on the growth liver abscess bacterial pathogens

In-vitro rumen fermentation assay:

Table 2: Effect of *Lactobacillus helveticus* and its supernatant on ruminal *F. necrophorum* counts *in-vitro* at 0 and 24 h when using sodium lactate as a substrate

Components	0 h				24 h			
	pH	qPCR		MPN/ml	pH	qPCR		MPN/ml
		Fn	*Ff*			*Fn*	*Ff*	
RF+ Buff + Na-L	7.08	0	7.70E+03	2.19E+04	7.12	0	5.25E+03	1.24E+02
RF + Buff + Na-L + *Fn*	7.18	3.99E+07	0	2.95E+04	7.17	2.30E+07	0	2.08E+03
RF + Buff + Na-L + *Fn* + *Lh*	7.03	3.81E+07	5.65E+03	2.94E+04	7.01	3.48E+07	5.20E+03	1.83E+05
RF + Buff + Na-L + *Fn* + *Lh*-S	6.25	4.29E+07	4.53E+03	4.38E+03	6.31	2.73E+07	0	0

RF: Ruminal fluid **Buff:** McDougal’s Buffer **Na-L:** Sodium Lactate
***F n*:** *F. necrophorum* ***L h*:** *Lactobacillus helveticus* ***L h*-S:** *Lactobacillus helveticus*- Supernatant

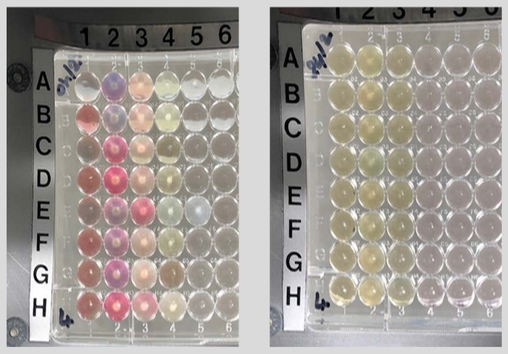

Figure 2: Effect of *L. helveticus*-supernatant on the concentration of *F. necrophorum* in ruminal fluid at 0 and 24 hours by the Most Probable Number (MPN) method

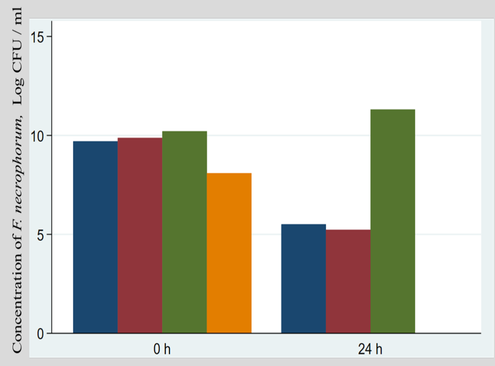

Figure 3: Effect of *L. helveticus*-supernatant on the concentration of *F. necrophorum* in ruminal fluid at 0 and 24 hours by the Most Probable Number (MPN) method

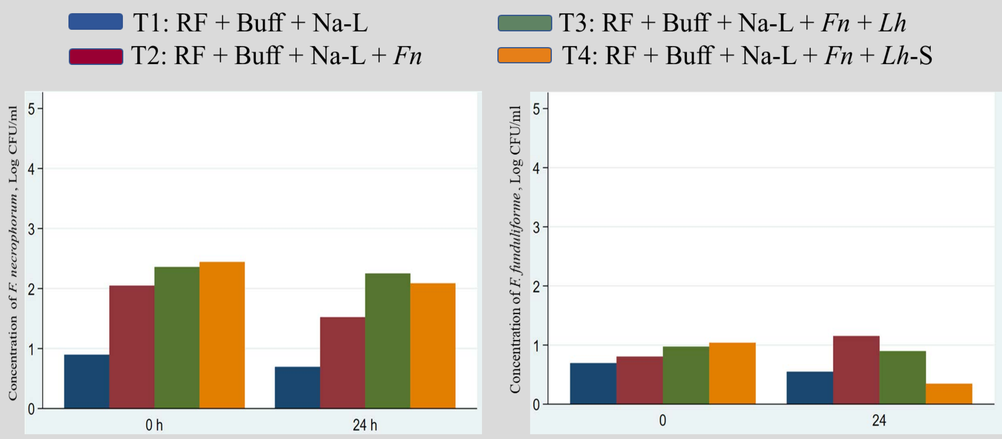

Figure 4: Effect of *L. helveticus* bacterial culture on the concentration of *F. necrophorum* in ruminal fluid at 0 and 24 hours by qPCR

Figure 5: Effect of *L. helveticus* bacterial culture on the concentration of *F. funduliforme* in ruminal fluid at 0 and 24 hours by qPCR

Summary

- *Lactobacillus acidophilus, L. helveticus, L. rhamnosus,* and *Pediococcus pentosaceus* culture supernatants inhibited the growth of *F. necrophorum* in a dose-dependent manner.
- *Lactobacillus helveticus* culture supernatant inhibited the growth of *F. necrophorum* in an *in-vitro* rumen fermentation system.
- Probiotic culture supernatants that inhibit the pathogens may have the potential to control liver abscesses.
- Further studies are ongoing to investigate different concentrations and combination of probiotic culture supernatants against the liver abscess causing bacterial pathogens.

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