IVESTIGATING THE RUMEN MICROFLORA OF MONOTERPENE TOLERANT GOATS

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

Investigating the Rumen Microflora of Monoterpene Tolerant Goats

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The Juniper plant is an invasive species in western areas of the United States. Camphor, a monoterpene that is abundant in Juniper is found to be 5 times more abundant in intolerant monoterpene goats when consumed than tolerant monoterpene goats. Media with the addition of camphor is investigated during this study to identify suitable growing conditions to compare tolerant and intolerant monoterpene rumen microflora. An ethanol and camphor solution is swabbed and injected into different media plates. The solution is also added to TSA broth before solidification. Intolerant rumen microflora bacteria is grown on each and compared. Results showed significant growth when camphor is added to the TSA broth before solidification into a plate.

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NOMENCLATURE

TSI	Triple Sugar Iron Agar
TSA	Tryptic Soy Agar

CHAPTER I

INTRODUCTION

Juniper plant species (*Juniperus spp.*) have become an invasive species in the western United States, such as in West Texas where the raising of meat goats is a major agricultural industry. Juniper foliage contains monoterpenes. The consumption of juniper foliage is toxic in goats, which show the clinical signs of decrease feed consumption, anorexia, weight loss and decrease in production (Riddle et al., 1996). Toxicity to the ingested monoterpenes is variable within the goat production; research has shown that there is a subpopulation of goats in West Texas that is able to tolerate ingestion of Juniper foliage (Campbell et al., 2010). Camphor ($C_{10}H_{16}O$) is an important monoterpene found in Juniper species and is not water-soluble. In some studies, camphor is shown to be the primary carbon source for bacteria (Bhuvaneswari, 2013).

In a study by Campbell et al., (2010) the pharmacokinetics of camphor, a major monoterpene in Juniper, was investigated between goats that were tolerant and goats that were intolerant to Juniper consumption. Bioavailability of camphor, after oral administration, was 5 times greater in the intolerant Juniper goats than in the goats that are tolerant. Maximum concentration was 6 times greater in the intolerant goats. The researchers concluded that physiological mechanisms were responsible for reducing the phytoyoicosis and internal mechanisms are a large part in distinguishing between the tolerant and intolerant goats (Campbell et al., 2014). An interesting result was that the elimination kinetics of camphor between the two goat groups were similar, which indicated that both groups of goats eliminated

the camphor similarly. This suggests that the cause of the decreased camphor is more likely pre-hepatic and could due to degradation and elimination by gastrointestinal microflora.

Another study looked at different concentrations of Juniper fed with urea and studied the ewes' rumen fluid after ingestion to analyze the effect on rumen bacteria. This experiment showed results of modest changes in the rumen bacteria diversity and the lamb performances (Ishaq et al., 2017). Recent research has shown that the rumen microbiome differs between normal goats and goats that can tolerate a greater consumption of Juniper foliage (Brady et al., 2018).

Our group believes that the rumen microbial population within monoterpene tolerant goats is responsible for the degration and decreased bioavailability of the monoterpenes. Our goal is to culture and identify the bacteria within the rumen microflora that will degrade camphor. The objective of the experiment is to develop a media that incorporates camphor so that it is selective for these bacteria.

CHAPTER II

METHODS

Media Preparation and Bacterial Cultures

Initially, Triple Sugar Iron (TSI) broth and agar was augmented with differing amounts of camphor dissolved in different vehicles. The augmented media descriptions are in Table 1.

Table 1. Media within each plate is 5 mL of Triple Sugar Iron Agar (TSI).

Plate	Protocol
A	1 mL walnut oil and no camphor
B	TSI broth with 1.5 mg camphor in 1 mL walnut oil
C	TSI broth with 15.2 mg camphor in 1 mL walnut oil
D	TSI broth with 152 mg camphor in 1 mL walnut oil
E	TSI broth with 1.5 mg camphor in 1 mL ethanol
F	TSI broth with 15.2 mg camphor in 1 mL ethanol
G	TSI broth with 152 mg camphor in 1 mL ethanol

Bacterial Cultures

Rumen fluid collected from a necropsied goat was filtered through tripled layered cheesecloth. An aliquot of rumen fluid was pipetted into the different media preparations. Bacterial growth in the broth was sub-cultured on to Blood agar, MacConkey agar, and Trypticase soy agar that had the surface swabbed with 94.2 mg camphor in 1 mL of ethanol.

Other Augmented Media

To allow diffusion of camphor concentrations within the media, 92.4 mg camphor in 1 mL ethanol was injected into a wet Tryptic Soy Agar (TSA) plate. The plate was not autoclaved because the camphor would evaporate in the heat. The plate sat at room temperature for four hours to give time to solidify.

Media including camphor was made to effectively disperse camphor throughout the entire plate. 100 mL of TSA media was created using 8 grams of TSA powder. The media was placed into a 250 mL Erlenmeyer flask and heated in a 1200W microwave for 3 minutes. It was then heated for another 2 minutes, stopping every 6 seconds to mix and to prevent it from boiling over. The media was then taken to a 50 deg C warm bath. While in the bath, 0.02 grams of camphor in 3 mL of ethanol was mixed into the TSA media. The mixture was poured into 4 plates creating a 20% camphor mix, 10% camphor mix, 5% camphor mix and 0% camphor mix, diluting each time with TSA media absent of camphor. The plates were then set at room temperature to cool before placing in 35 deg C conditions.

Bacterial Identification

Bacteria were cultured on Blood agar and sent to the Texas A&M University CVM Clinical Bacteriology Laboratory for identification. The method used was the matrix-assisted laser desorption ionization (MALDI) mass spectrometry.

CHAPTER III

RESULTS

The oil camphor solution added to the media broth was not suitable for our study shown in Table 2. The walnut oil did not dissolve or dissipate in the broth, but dispersed as oil droplets throughout the broth and over a 24-hours period produced an oil layer on top of the broth. The low and medium concentration of camphor in ethanol was suitable for further use.

Table 2. Media within each plate is 5 mL of Triple Sugar Iron Agar (TSI). Camphor is shown to dilute in ethanol. Plate E and F showed the best results.

Plate	Protocol	Results
A	1 mL walnut oil and no camphor	Oil does not dissolve in media
B	TSI broth with 1.5 mg camphor in 1 mL walnut oil	Oil does not dissolve in media
C	TSI broth with 15.2 mg camphor in 1 mL walnut oil	Oil does not dissolve in media
D	TSI broth with 152 mg camphor in 1 mL walnut oil	Oil does not dissolve in media
E	TSI broth with 1.5 mg camphor in 1 mL ethanol	Suitable
F	TSI broth with 15.2 mg camphor in 1 mL ethanol	Suitable
G	TSI broth with 152 mg camphor in 1 mL ethanol	Very milky, questionable if in solution

Media Results of Intolerant Goat Rumen Bacteria

After analyzing the dry media results, ethanol and camphor solution were used for the rest of the experiment. The maximum amount of camphor that was able to dissolve completely in 1 mL of ethanol was 92.4 mg. Using this information, 92.4 mg of camphor in 1 mL of ethanol was used with several different media/broths to observe the best growth of bacteria. Results in Table 3 show TSA plates demonstrated the most growth from the bacteria cultured off of plates E and F referred to in Table 1. Blood agar media also showed growth of bacteria from plates E and F and beta hemolyation. When observing growth on the TSA and Blood agar plates, bacteria cultured from plate F was rapid in growth, having an abundant amount of colonies within the

first 24 hours. However, bacteria cultured from plate E showed low growth, seeing first signs of bacteria colonies at 48 hours. This shows that the bacteria from plate E and F are different. Both bacteria were placed on a Blood agar plate for a Maldi test. The TSI plate E showed growth of Lactobacullus mucosae with a Maldi score of 1.94 The TSI plate F showed growth of Streptococcus galloyticus with a Maldi score of 2.08. If a Maldi score is ≥ 2.0, there is a strong possibility the identification given is correct.

Table 3. Bacteria cultured from TSI plates E and F. Used five different media/broths to compare growth.

Media/Broth	**Results**
TSA media with 92.4 mg of camphor in 1 mL of ethanol	Growth from recultured F plate within 24 hours Growth from recultured E plate within 48 hours
TSA broth with 92.4 mg of camphor in 1 mL of ethanol	Growth from recultured F plate
Blood Agar media with 92.4 mg of camphor in 1 mL of ethanol	Growth from recultured F and E plate
Trioglycollate broth with 92.4 mg of camphor in 1 mL of ethanol	No growth
MacConkey Agar media with 92.4 mg of camphor in 1 mL of ethanol	No growth

Other Augmented Media

When injecting 92.4 mg of camphor in 1 mL of ethanol into a wet TSA plate, the camphor solution diffused outward slightly before the TSA started to solidify at 45 deg C. The camphor solution did not solidify to the TSA plate after 4 hours in room temperature conditions and no bacteria could be cultured onto the unstable media.

Allowing complete mixture of camphor solution in wet TSA media ensures complete dispersal of the camphor. Results showed growth of Streptococcus galloyticus from 20% camphor, 10% camphor, 5% camphor and 0% camphor mixed into TSA plates.

CHAPTER IV

CONCLUSION

After conducting the different experiments, camphor is shown to be very difficult to place within media due to the lack of being soluble in water. When the 92.4 mg of camphor in 1 mL of ethanol was added to the broth at 50 deg C, the camphor was able to conform and disperse throughout the broth. This method was also able to show significant growth from intolerant goat rumen bacteria. Further research could investigate additional camphor being placed in the broth before solidifying and experimenting with different broths that can withstand the addition of camphor. Research should continue to investigate the microbiology component when comparing monoterpene tolerant and intolerant goats and compare rumen bacteria discovered within each goat when suitable media is found for the study.

In conclusion, camphor can successfully conform to TSA broth before solidifying, however research should continue to investigate the effectiveness of the media and what bacteria from monoterpene tolerant goats are able to grow in these conditions.

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