Graduate student final project report submitted to The Ceres Trust.

Project Title:

Organic anthelmintics in sheep

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Summary:

Gastrointestinal nematode parasitism is one of the greatest threats to economic sheep production in the United States. With increased incidences of anthelmintic resistance and constraints of organic production, there is heightened interest in alternative natural dewormers, such as plants containing condensed tannins (CT). Therefore, the objective of this study was to evaluate the effect of organic fermented Pinot Noir (PN) grape extract on parasite level and performance in Katahdin lambs. Katahdin ewe and ram lambs (n = 45; 23.13 kg ± 0.60 BW) were stratified by fecal egg count, weight, and sex, and were allocated randomly to one of three treatments: 1) an oral dose (10 mL per 4.54 kg of BW) of fermented PN grape extract at 7 days (D7) intervals, 2) the same dose at 14 days (D14) intervals, or 3) control (C; oral dose of water at 14 day intervals). Condensed tannins were extracted, purified, and standardized from organic PN and were found to have a concentration of 0.20 mg/mL. Lambs were housed on pasture with no additional feed, for the duration of the 63-day study. End of study ($P = 0.05$) and change from beginning to end ($P = 0.04$) fecal egg counts were higher in C versus D7 and D14 lambs. Change in packed cell volume percentage from start of the study to end differed ($P = 0.05$) between D7 and D14 lambs. Average daily gain and total weight gain were greater ($P = 0.02$) for D7 and D14 lambs compared to C lambs. Start, end and change BCS and FAMACHA© scores did not differ ($P \geq 0.50$) across treatments. End monocytes and white blood cell counts were lower ($P = 0.05$ and $P = 0.03$, respectively) in D7 versus D14 lambs. Other blood parameter counts were similar between treatments. Therefore, fermented grape extract may be an effective organic and sustainable strategy for controlling nematodes and increasing performance in lambs.

Problem addressed:

Parasitic nematodes remain a major threat to the health and welfare of small ruminants worldwide. *Haemonchus contortus* parasitism is significant due to its ability to cause severe anemia and death in small ruminants (Hoste, 2006). The use of bioactive forages and phytotherapy to cure and control gastrointestinal nematodes (GIN) have been touted as a way to reduce the use of synthetic anthelmintics and decrease the incidence of parasite resistance in small ruminants. Many plant extracts are high in certain phenolics such as proanthocyanidins or condensed tannins (CT), and these compounds at certain levels have been shown to produce benefits in ruminants, such as better utilization of dietary protein, faster growth rates, higher milk yields, increased fertility, increased color and keeping quality of fresh meat, as well as, an improvement in animal health through prevention of bloat and a decrease in fecal egg counts (FEC) (Min and Hart, 2013). Many types of forage are high in CT such as chicory, birdsfoot

trefoil, sainfoin, and sericea lespedeza, but the amount consumed while grazing is difficult to measure. Nonetheless, the use of these plants has shown a reduction in FEC and a tendency for reduced worm burdens compared with those offered a diet containing low levels of CT (Heckendorn, 2007). The human food and drink industry contain examples of products, such as fermented grape extract, which have not been considered as a natural remedy for GIN. Research suggests that red grapes and red grape byproducts such as grape juice, fermented grape extract, and grape pomace have high levels of CT (Mattivi, 2009). Small ruminant producers seek a natural anthelmintic product that is easy to locate, cost effective, works well, and is easy to administer. The state of Missouri is known to have many vineyards, both organic and conventional. Vineyards produce several byproducts available for the producer to purchase such as juice or fermented product or dry byproducts such as grape pomace consisting of skin, seeds, stems, and pulp all which contain high levels of CT. The possibility of positive results could benefit vineyard proprietors with a sustainable use of byproducts that may not be fully utilized currently. The use of these CT rich products could make small ruminant production in the United States more sustainable by using fewer synthetic anthelmintics and by reducing the instances of parasite resistance.

Objective:

The objective of this study was to evaluate the effects of organic fermented Pinot Noir (PN) grape extract on parasite level and performance of Katahdin lambs.

Methods and Materials:

This project was conducted at Lincoln University Allen T. Busby Farm in Jefferson City, Missouri and was approved by the Animal Care and Use Committee (14-4). Organic fermented grape PN extract was provided by Badger Mountain Winery in Kennewick, Washington.

Organic Katahdin mixed-sex lambs (n = 45; 23.13 kg ± 0.60 BW) were weaned while grazing on fescue pastures and allowed to acquire a natural GIN infection. Prior to the 63-day trial, lambs were weighed, assigned a body condition score (BCS), and fecal egg count (FEC) were determined. Katahdin lambs were then stratified by FEC, weight, and sex, and were allocated randomly to one of three treatments: 1) drenched with organic fermented grape PN extract every 7 days (D7) at a rate of 10 mL per 4.54 kg of BW, 2) drenched with organic fermented grape PN extract every 14 days (D14) at a rate of 10 mL per 4.54 kg of BW, and 3) drenched with water every 14 days (C) at a rate of 10 mL per 4.54 kg of BW. The goal was to maintain animals above threshold for the duration of the 63-day study. Animals were removed from the study if they met 3 out of 4 criteria: 1) FEC of 4,000+, 2) FAMACHA© score of 4+, 3) PCV of 21% or less, or 4) a BCS below 2. For the duration of the 63-day trial lambs were grazed

on fescue pastures with ad libitum access to water and organic approved mineral supplements, with no additional feed added to the diet. Throughout the study lambs were maintained in a single group with ear tag numbers as the primary identification method.

Condensed tannins were extracted and purified from organic PN by the CT isolation method using Sephadex LH-20 gel filtration (GE Healthcare Bio-Sciences Corp, Piscataway, NJ; Strumeyer and Malin, 1975) then quantified by the Protein-Precipitable Phenolic method (Hagerman and Butler, 1978), which uses Iron Phenolate to detect tannins by UV Spectrophotometer (Beckman Coulter Inc., Model DU730, Fullerton, CA). Organic PN grape extract was found to have a concentration of 0.20 mg/mL of CT. Crude protein was measured at 1.6 mg/mL by sample. Protein Bound (PB) Quantification was obtained by the Naumann et al. (2014) method and found to bind 12.7 mg/mL of protein with a 32.8% binding capability. The polyphenol bioactivity of organic PN grape extract was measured by High Performance Liquid Chromatography (HPLC) testing, which found the level of combined Cyanidin and Dephinidin tannins to be 0.0007 mg/mL with 15.5% Galloylatted tannin bound.

Acid detergent fiber (ADF) and neutral detergent fiber (NDF) were determined using the Van Soest et al. (1991) method using an ANKOM^{200} Fiber Analyzer (ANKOM Technology, Macedon, NY) and crude protein (CP) was determined by Elementar Vario Macro Cube C/N Analyzer (Elementar Americas Inc., Donaustrabe, Germany) for fermented grape extract and all pastures.

During the 63-day trial, individual fecal samples were taken from the rectum of each animal, every 7 days. Fecal samples were processed within 24 hours for FEC by the modified McMaster procedure and quantified by using 2 gram sub-samples of fresh feces from each lamb (Whitlock, 1948). Individual blood samples were taken by jugular venipuncture into hematocrit tubes, with packed cell volume (PCV) determined using a HemataSTAT II Centrifuge (Separation Technology, Inc., Sanford, FL) within 6 hours of blood collection. Additionally, FAMACHA© scores (Hepworth et al., 2006) and BCS (Russell, 1991) were taken every 7 days.

Blood samples were taken by jugular venipuncture every 14 days into BD Vacutaine K3 EDTA 12 mg blood collection tubes. They were shipped to University of Arkansas in cold storage to maintain sample integrity and complete blood cell counts (CBC) were analyzed by an Abbott Cell-Dyn 3700SL Automate Hematology Analyzer (GMI Inc., Ramsey, MN) within 24 hours of collection.

Data was analyzed as a randomized design using repeated measure analysis of variance. Data was analyzed with PROC MIXED procedure of SAS (SAS Inst. Inc., Cary, NC). Animal was considered the experimental unit. Treatment means were reported as least square means with

the contrast statements of the mean of control versus D7 and D14, and the mean of D7 versus D14.

Results:

Natural GIN was apparent in all lambs with an average FEC of 43.8 ± 8.11 eggs per gram of feces. Two lambs were removed from the D14 treatment group by meeting three of four health threshold criteria. As displayed in Table 1, end of study ($P = 0.05$) and change from beginning to end ($P = 0.04$) FEC were higher in C versus D7 and D14 lambs. Change in PCV from start of the study to end differed ($P = 0.05$) between D7 and D14 lambs. Overall, FAMACHA© score was not different ($P \geq 0.50$) between treatment groups which suggests that using FAMACHA© scoring is subjective and may not be as precise.

As shown in Table 2, average daily gain and total weight gain were greater ($P = 0.02$) for D7 (5.4 kg) and D14 lambs (5.2 kg) compared to C lambs (4.2 kg). Start, end and change BCS averaged 2.5 and did not differ ($P \geq 0.50$) across treatments.

Monocytes (MONO) and white blood cell (WBC) counts were lower ($P = 0.05$ and $P = 0.03$, respectively) at the end of the study in D7 versus D14 lambs. A significant change ($P = 0.02$) was found in basophils (BASO) in D7 versus D14 lambs. A tendency ($P = 0.07$, $P = 0.09$, and $P = 0.09$, respectively) was found in change WBC, hemoglobin (HGB), and mean corpuscular hemoglobin concentration (MCHC%) in D7 versus D14 lambs. Other blood parameter counts were similar ($P \geq 0.10$) between treatments as displayed in Table 3.

Discussion:

Our results suggest that organic PN grape extract demonstrated a natural bioactive anthelmintic effect by producing a reduction in nematode FEC of pasture-grazed Katahdin lambs. Reduction in FEC may be due to temporary reduction of nematode numbers, reductions in female worm fecundity, reduced nematode excretion (measured by FEC), and/or egg output (Heckendorn et al., 2007; Hoste et al., 2006).

In this study, an increase in total weight gain and average daily gain in D7 and D14 lambs could suggest an added benefit of CTs ability to bind protein, causing a by-pass protein effect. The widely accepted explanation for positive effects of CT protein digestion and metabolism is that CT-protein complexes escape ruminal degradation and the protein is available in the abomasum (Reed, 1995). Moderate levels of 20 to 40 g CT/kg of dry matter bind to protein by hydrogen bonding at near neutral pH (pH 6.0 – 7.0) in the rumen to form CT-complexes, but dissociate and release bound protein at pH less than 3.5 in the abomasum (Min and Hart, 2013). Therefore, protecting dietary protein against degradation in the rumen subsequently

increases amino acid supply to the abomasum and small intestine, resulting in improved nutritional status of the animal and possible improved production (Min and Hart, 2013). Theoretically this could be because of an increase in bound protein due to greater bio-activity levels of Delphinidin and Galloylatted tannins, which have an increased ability to bind protein over other types of tannins. It could also be suggested that even though the amount of CT per milligram was less than many forages contain, it is more easily absorbed in the abomasum of the ruminant animal due to the solubility of the tannin in the organic PN.

It is possible that an enhanced immune response or resistance could be mediated by improved protein availability and could be partially responsible for any observed anthelmintic effects against *H. contortus* (Heckendorn et al., 2007). Some changes were found in CBC results, including a significant decrease in both MONO and WBC for D7 versus D14 at the end of the study. A significant change in BASO was found as well as tendencies for change in WBC, HGB, and MCHC% in D7 versus D14 lambs. Further exploration is needed to determine the anthelmintic properties and the biological processes by which CT changes the response of the host to the nematode; whether by direct or indirect means. It needs to be understood if the structural and chemical characteristics of the type of tannin are most important to the anti-parasitic activity or is it CT's ability to bind to protein and heighten animal nutritional plane, thereby increasing the animals' ability to fight GIN (Hoste et al., 2006). Research by Iqball et al. (2006) found when sheep were divided into three treatment groups of low CT, high CT, and a control, that high CT, facilitated protection of protein from degradation by rumen microbes, which minimized the effects of internal parasites. It was also found that it affected overall GIN numbers and increased animal performance by providing a direct effect on the parasite and also indirectly through improved protein supply.

Conclusion:

Fermented grape extract may be an effective organic and sustainable strategy for controlling nematodes and increasing performance in lambs. The use of fermented grape extract is desirable because it is a sustainable resource, it is an easily assessable source of condensed tannins, and as a liquid it is straightforward to administer. The use of organic fermented grape extract could be applied by organic producers, helping to make organic small ruminant production more profitable. Additional research is needed to determine the extent of immunological response that may be seen by use of phenolics in the ruminant animal.

Outreach and Extension:

Dissemination of results occurred at the Midwest Organic & Sustainable Education Services (MOSES) Annual Conference where second place was won in the poster contest. This

research was published in the Organic Broadcaster newspaper. In addition, a publication is in progress for the Journal of Veterinary Parasitology and information about the project is anticipated being shared at the Missouri Organic Association annual meeting in 2016.

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Table 1. Effects of organic fermented grape extract on parasite level in Katahdin lambs.

	Treatment[1]				
Item	C	D7	D14	SEM[2]	Contrast[3]
Start FEC[4], eggs/g	43.0	39.6	48.7	8.11	ns
End FEC[4], eggs/g	50.6	28.1	24.7	9.57	W
FEC[4] change, eggs/g	10.5	-13.1	-18.5	10.82	W
Start FAMACHA©[5]	1.6	1.4	1.8	0.60	ns
End FAMACHA©[5]	1.5	1.5	1.5	0.12	ns
FAMACHA©[5] change	-0.2	-0.1	0.0	0.20	ns
Start PCV[6], %	34.2	31.4	33.4	1.31	ns
End PCV[6], %	36.3	37.0	36.8	1.05	ns
Change PCV[6], %	2.2	5.6	2.2	1.19	X

[1]C = Control, D7 = day 7 dose of organic PN, and D14 = day 14 dose of organic PN.

[2]SEM = Pooled standard error of means.

[3]Contrast statements: W = mean of Control lambs compared with the mean of D7 and D14 lambs ($P \leq 0.05$); X = mean of D7 compared with the mean of D14 lambs ($P \leq 0.05$); lowercase letters represent statistical tendencies ($P \leq 0.10$); ns = no significant difference ($P > 0.10$).

[4]FEC = Fecal egg count, eggs/gram.

[5]FAMACHA© = FAffa MAlan CHArt, used to determine anemic level of animal.

[6]PCV = Packed cell volume percent, estimation of animal hematocrit level.

Table 2. Effects of organic fermented grape extract on performance in Katahdin lambs.

	Treatment[1]				
Item	C	D7	D14	SEM[2]	Contrast[3]
Start BW, kg	23.8	22.7	23.4	1.06	ns
End BW, kg	28.0	28.2	28.9	1.05	ns
ADG, kg	0.07	0.09	0.08	0.006	W
Gain, kg	4.2	5.4	5.2	0.39	W
Start BCS[4]	2.9	2.9	2.7	0.14	ns
End BCS[4]	2.5	2.6	2.5	0.11	ns
BCS[4] change	-0.3	-0.3	-0.3	0.13	ns

[1]C = Control, D7 = day 7 dose of organic PN, and D14 = day 14 dose of organic PN.

[2]SEM = Pooled standard error of means.

[3]Contrast statements: W = mean of Control lambs compared with the mean of D7 and D14 lambs ($P \leq 0.05$); X = mean of D7 compared with the mean of D14 lambs ($P \leq 0.05$); lowercase letters represent statistical tendencies ($P \leq 0.10$); ns = no significant difference ($P > 0.10$).

[4]BCS = Body condition score.

Table 3. Effects of organic fermented grape extract on complete blood cell counts (CBC) in Katahdin lambs.

	Treatments[1]				
Item	C	D7	D14	SEM[2]	Contrast[3]
Start WBC[4], K/µL	10.20	9.95	9.56	0.73	ns
End WBC[4], K/µL	9.59	10.26	7.97	0.73	X
WBC[4] change, K/µL	0.61	-0.31	1.95	0.85	x
Start NEU[5], K/µL	3.67	3.12	3.22	0.38	ns
End NEU[5], K/µL	3.67	3.49	2.95	0.38	ns
NEU[5] change, K/µL	0.0	-0.37	0.50	0.45	ns
Start LYM[6], K/µL	3.16	3.56	3.26	0.34	ns
End LYM[6], K/µL	3.24	3.35	2.52	0.34	ns
LYM[6] change, K/µL	-0.08	0.21	0.76	-.43	ns
Start MONO[7], K/µL	2.64	2.57	2.34	0.28	ns
End MONO[7], K/µL	2.10	2.59	1.89	0.28	X
MONO[7] change, K/µL	0.54	-0.02	0.53	0.32	ns
Start EOS[8], K/µL	0.15	0.29	0.67	0.67	ns
End EOS[8], K/µL	0.26	0.42	0.27	0.67	ns
EOS[8] change, K/µL	-0.11	-0.13	-0.09	0.07	ns
Start BASO[9], K/µL	0.57	0.40	0.56	0.06	ns
End BASO[9], K/µL	0.32	0.41	0.33	0.06	ns
BASO[9] change, K/µL	0.25	-0.01	0.23	0.08	X
Start RBC[10], K/µL	10.35	9.53	10.16	0.60	ns
End RBC[10], K/µL	10.19	9.76	9.89	0.61	ns
RBC[10] change, K/µL	0.16	-0.23	0.66	0.78	ns
Start HGB[11], g/dL	10.19	9.42	9.97	0.53	ns
End HGB[11], g/dL	10.28	10.29	9.68	0.57	ns
HGB[11] change, g/dL	-0.09	-0.87	0.69	0.65	x
Start HCT[12], %	33.20	33.34	32.57	1.09	ns
End HCT[12], %	33.07	33.89	33.87	1.22	ns
HCT[12] change, %	0.13	-0.22	0.31	0.97	ns
Start MCV[13], fL	32.03	32.71	32.20	0.45	ns
End MCV[13], fL	32.45	32.66	31.83	0.47	ns
MCV[13] change, fL	-0.42	0.17	0.08	0.32	ns
Start MCH[14], pg	9.79	9.89	9.81	0.13	ns
End MCH[14], pg	10.08	10.11	9.77	0.14	ns
MCH[14] change, pg	-0.29	-0.19	0.02	0.09	ns
Start MCHC%[15], g/dL	30.59	30.24	30.53	0.24	ns
End MCHC%[15], g/dL	31.05	31.01	30.75	0.27	ns

MCHC%[15] change, g/dL	-0.46	-0.77	0.02	0.31	X
Start RDW[16], %	26.39	25.35	25.85	1.00	ns
End RDW[16], %	31.65	29.39	29.30	1.10	ns
RDW[16] change, %	-5.27	-4.16	-2.75	1.37	ns
Start PLT[17], K/µL	745.60	750.43	747.80	90.07	ns
End PLT[17], K/µL	582.60	514.47	541.34	92.30	ns
PLT[17] change, K/µL	163.00	235.96	191.97	108.57	ns

[1]C = Control, D7 = day 7 dose of organic PN, and D14 = day 14 dose of organic PN.

[2]SEM: Pooled standard error of means.

[3]Contrast statements: W = mean of Control lambs compared with the mean of D7 and D14 lambs ($P \leq 0.05$); X = mean of D7 compared with the mean of D14 lambs ($P \leq 0.05$); lowercase letters represent statistical tendencies ($P \leq 0.10$); ns = no significant difference ($P > 0.10$).

[4]WBC: White blood cells.

[5]NEU: Neutrophils.

[6]LYM: Lymphocytes.

[7]MONO: Monocytes.

[8]EOS: Eosinophils.

[9]BASO: Basophils.

[10]RBC: Red blood cells.

[11]HGB: Hemoglobin.

[12]HCT%: Hematocrit percentage.

[13]MCV: Mean corpuscular volume.

[14]MCH: Mean corpuscular hemoglobin.

[15]MCHC%: Mean corpuscular hemoglobin concentration percent.

[16]RDW: Red cell distribution width.

[17]PLT: Platelets.