AGRICULTURAL RESEARCH FOUNDATION
FINAL REPORT
FUNDING CYCLE 2014 – 2016

TITLE: Acquisition of immune-resistance to urushiol through daily consumption of milk from goats fed poison oak

RESEARCH LEADER: Massimo Bionaz

COOPERATORS: ***Dr. Claudia Ingham*** (Department of Animal and Rangeland Sciences) and ***Prof. Lisbeth Goddik*** (Food Sciences department). Additional collaborators were Prof. ***David Hendrix*** from the Biochemistry and Biophysics department and Prof. ***Duo Jiang***, Statistics department, both at Oregon State University. The trial was led by a STEM leader student (***Emily Sahagun***). There were additional undergraduate students helping Emily (***Sarah Akers***, ***Randi Wilson***, and ***Chelsey Naito*** from the department of Animal and Rangeland Sciences and ***Jennifer Belveal*** from the Department of Biology). Claudia Ingham, Jennifer Belveal and ***Kristine Gomez*** (department of Animal and Rangeland Sciences) helped carry out the pilot study. Dr. ***Rolando Solensky*** (Corvallis Clinic), was an original co-PI in the grant, but he was not involved in the pilot study and the main experiment presented in this report because it did not involve a clinical trial.

IMPORTANT NOTE:
The original objective of this proposal was changed because of the inability to proceed with the request from FDA to perform the proposed study. Therefore, in order to use the grant for a similar purpose we re-designed, in agreement with the ARF, the whole experiment and proposed new objectives. During the process of FDA approval we performed a pilot study where we demonstrated that milk does not contain urushiol, the toxicant present in poison oak and responsible for the contact dermatitis. Data from the pilot study are available in Table 1 and discussed in details in the previously submitted interim report.

Table 1. Urushiol content in various samples. Except for the poison oak which is the mean of 4 samples representing 4 different collections, the samples from the animals (feces, blood, plasma, and milk) are from 2 Saanen lactating goats. Data are in mg or urushiol/g or mg of urushiol/mL of fresh material.

Sample		HDTC	HDDC	HDCC	HDC	Total
Poison oak	mg/g	4.65±1.04	0.39±0.24	0.50±0.34	0.56±0.51	***6.10±1.00***
	%	*73.6±20.0*	*7.7±5.0*	*7.0±5.2*	*11.7±9.8*	
Feces	mg/g	0.32±0.07	0.03±0.02	0.01±0.00	0.05±0.02	***0.42±0.11***
	%	*77.8±3.4*	*7.1±2.4*	*2.9±0.5*	*12.2±1.9*	
Blood	mg/mL	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	***0.00±0.00***
	%	*n/a*	*n/a*	*n/a*	*n/a*	
Plasma	mg/mL	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	***0.00±0.00***
	%	*n/a*	*n/a*	*n/a*	*n/a*	
Milk	mg/mL	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	***0.00±0.00***
	%	*n/a*	*n/a*	*n/a*	*n/a*	

HDTC = Heptadecatrienylcatechol; HDDC = Heptadecadienylcatechol; HDCC = Heptadecenylcatechol; HDC = Heptadecylcatechol

SUMMARY:
One of the recent discoveries is the presence of small exosomes containing RNA in milk. One of the most important RNA components in these exosomes are micro RNAs (miRNA). The miRNA play an important regulatory role in cells by decreasing the expression of genes (especially impeding the translation of mRNA into protein) and are highly conserved between species. Researchers have found that exosomes of milk are highly enriched in miRNA that can affect the immune system (Pauley and Chan, 2008). If it is true that milk from goats fed poison oak affects the immune response to urushiol, then the miRNA in milk can be good candidates to evaluate. In addition, prior studies have shown that miRNA in milk exosomes are resistant to many treatments but the amount of miRNA is decreased by pasteurization (Chen et al., 2010, Pieters et al., 2015); therefore, there is the possibility that any beneficial effect of feeding poison oak is reduced by pasteurization by decreasing the amount of miRNA. Considering the items above, the new hypothesis of the proposal is ***that feeding poison oak affects miRNA in milk exosomes involved in regulating immune response-related genes and miRNA abundance is negatively affected by pasteurization***.

OBJECTIVES:
By using lactating Saanen goats with kids, the objectives of the proposal were to assess the effect of feeding poison oak on 1) milk yield and composition, and 2) feed intake and body weight of goats and kids, and 3) abundance of various miRNA in milk exosomes. Furthermore, an additional objective 4) was to assess the effect of pasteurization on abundance of various miRNA in milk exosomes.

PROCEDURES:

Figure 1.Goats were maintained in single pens at the OSU Sheep Center

Animals. Use of animals was approved by the OSU Institutional Animal Care and Use Committee with protocol #4695. We used 6 lactating Saanen goats with the following features: 78.3±6.1 Kg of body weight; 56±9 day in milk; 3.6±1.1 kg of milk/day. The goats were kept at the OSU Sheep Center in single pen (Figure 1). Goats were still nursed by 2 kids/goat (except two goats, one had 3 kids and one only 1 kid). Goats were randomly assigned to treatment and control in each time period based on body weight, milk yield, number of kids, sex of the kids, and milk composition. The trial started 15 June 2016 after 1 week of adaptation to the pen. The pens were set so to have the possibility to separate the does from the kids by using fences. The part of the pen only allowed for the does presented ~~a~~ portable feed bunk that was used for feeding poison oak or the hay control and a container with free availability of a commercial goat mineral mix. The does were kept in this

part of the pen for 3h in the morning and 3h in the afternoon to allow for consumption of the poison oak or the hay control. For the rest of the day, the does were allowed to access the other part of the pen where the kids were, so to allow the kids to nurse the does. That section of the pen had a fixed feed bunk where ad libitum alfalfa was provided for the does and the kids. Both parts of the pen had continuous water availability.

Experimental design. Goats were used in a cross-over design with two 3 weeks feeding periods. The cross-over designed allowed to use all the goats for each treatment so to increase statistical power. With this design we had a total of 6 goats per group (i.e., 3 goats were used as treatment and 3 as control for 3 weeks and then the treatments were switched for 3 weeks) (Figure 2). A known amount of poison oak or hay control was provided fresh twice a day at approx. 8AM and 5PM and residuals removed and amount recorded prior each feeding.

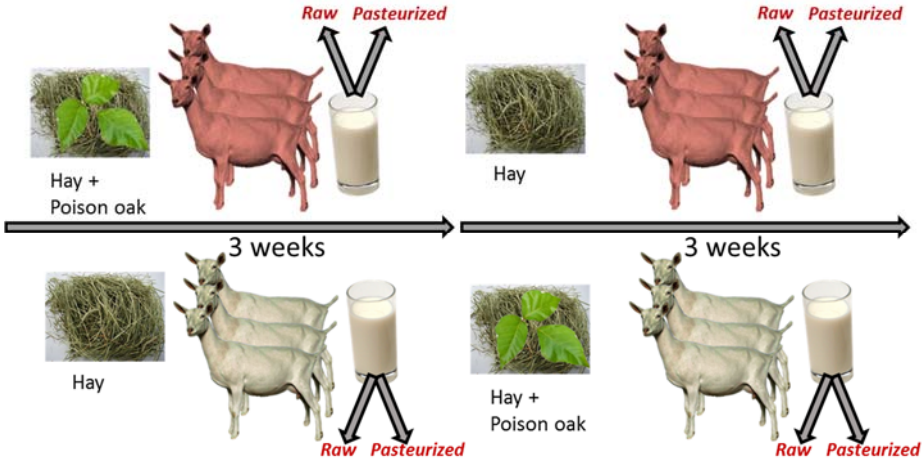

Figure 2. Experimental design

Measurements. During the 3 weeks of the treatment, body weight was recorded weekly for both does and kids. The dry matter intake was measured daily accounting for the treatments (poison oak or hay control) and ~~a~~lfalfa. A sample of alfalfa and orchard hay used as hay control was sent to a commercial laboratory for analysis (Dairy One, Ithaca, NY). Milk yield was measured in the morning just before starting the trial (time 0), and then at 7, 14, and 21 day of treatment. To provide accurate measurement of the milk yield, kids were removed at 8PM of the day before and does completely milked. Does were kept separated from the kids until milked again in the morning at 8AM.

Milk samples were collected in 50 mL tubes containing a preservative and shipped to Willamette DHIA, Salem, for component analysis. Additional milk samples were collected for analysis of urushiol content using HPLC. At the end of the 3 weeks treatment for each period of the cross-over, milk from each goat was collected in two 50 mL tubes. One tube was preserved at 4C and the other tube was pasteurized at 72°C for 30 min. From both tubes miRNA from milk exosomes was isolated by subsequent centrifugation as follow: twice at 3,000xg for 10 min, once at 12,000xg for 60 mins, once at 35,000xg for 60 mins, and once at 70,000xg for 60 mins with transfer of the supernatant in new tubes without disturbing the pellet between

centrifugations. The supernatant was then filtered using a 0.8µm syringe filter prior purification using the QIAGEN ExoEasy kit for isolation of miRNA. The final miRNA was quantified using a spectrophotometer. The purified miRNA was sent to the Center for Genome Research and Biocomputing at Oregon State University for RNA sequencing using Illumina HiSeq 3000. The miRNA has been sequenced and we are performing bioinformatic analysis; however, final results are not yet available for the present report and only preliminary data are presented.

Statistical analysis. Data were arithmetically corrected based on the control group at baseline prior statistical analysis so to have the same average between treatment and control at time 0. Data were checked for outliers and normal distribution. For the final analysis, Proc GLIMMIX of SAS was used with time, treatment, and time x treatment interaction as main effect and goats as random variables. Statistical significance was determined with $p \leq 0.05$ and tendency with $0.05 \geq p \leq 0.10$.

Poison oak collection and feeding. Poison oak was collected from the Inavale Farm (Philomath, OR) twice a week. For each collection, personnel used coveralls, gloves, masks, glasses, and hats to avoid contact of the skin with the plant (Figure 3). Each collection took in average 3h and a mean±SD of 3.8±1.6 kg of poison oak leaves/person were collected (1.3 kg/h per capita). The poison oak was preserved in a plastic tub at the OSU Sheep Center. Dry matter intake of the poison oak was assessed each day while dry matter of alfalfa and orchard grass hay was assessed weekly by high energy microwave for 10 min.

Figure 3. Collection of poison oak at Inavale farm (Philomath, OR). From left to right Jennifer Belveal, Emily Sahagun, and Chelsey Naito. The collection was performed using a coverall, gloves, a hat, and boots. Only leaves with stems were collected.

SIGNIFICANT ACCOMPLISHMENTS:

The variation of the dry matter of poison oak was relatively large due to the preservation in open air in the summer [ranged from 13 to 67%]. From Figure 4, it is possible to see a consistent increase in dry matter as time passed after collection of fresh poison oak. Often the fresh collected poison oak was provided the day after the collection and/or the fresh poison oak was mixed with the old poison oak. These data highlight the importance of measuring dry matter of the poison oak daily.

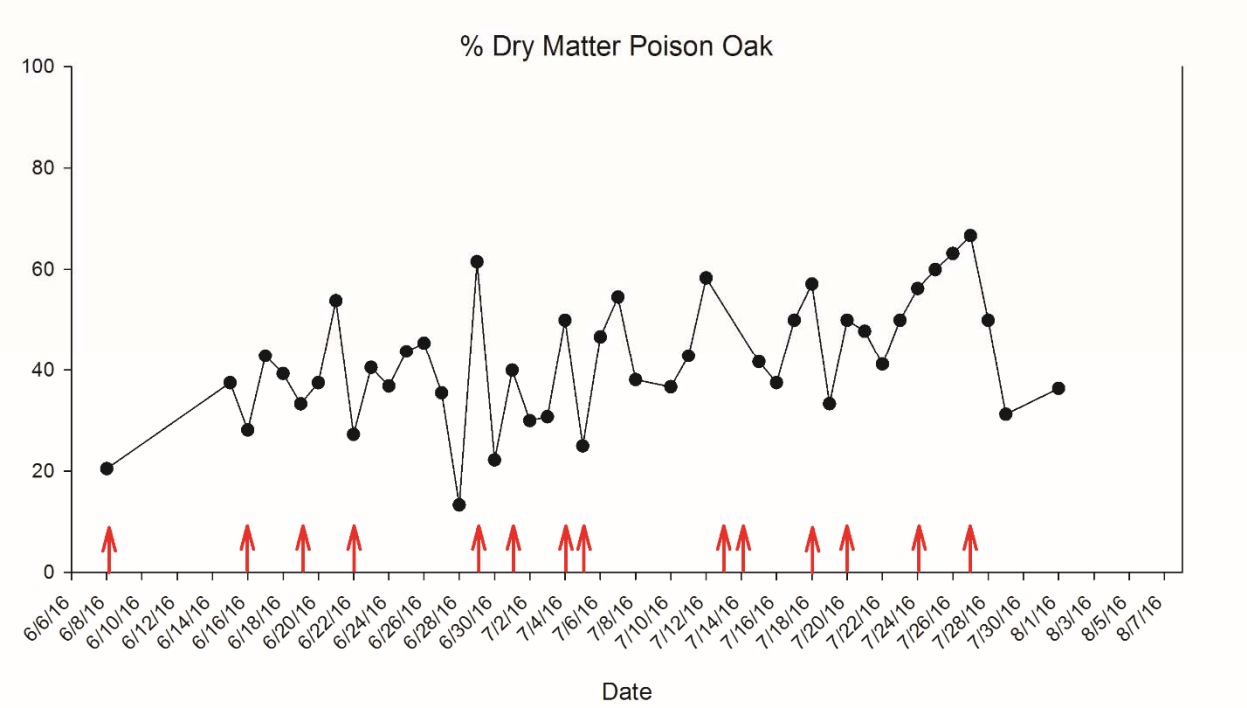

Figure 4. % dry matter of poison oak during preservation. In red arrow are indicated the date of collection of poison oak

Most of the goats ate poison oak well with one goat eating up to 0.8 kg of dry matter (1.7 kg/d as is). We had to add some molasses to aid in eating poison oak by some goats (#2 and #25, see Figure 5). Even with the molasses, the goats did not eat the poison oak well, despite~~s~~ leaving the animals with only poison oak for most of the day. The variation in amount of poison oak eaten by each goat, and the proportion of it in the whole diet, is shown in Figure 5. We observed an overall significant increase in amount of poison oak eaten by the goats from the beginning to the end of the trial with an average±SD of 0.32±0.18 kg of poison oak (DM basis) [range 0.04 to 0.78 kg] eaten daily, corresponding to 12.1±6.4% of the daily feed intake [range 1.5 to 36.3%]. Our data indicate that there is large variation in the amount of poison oak consumed by the goats and only a proportion of the goats (in our case 4 out of 6) consumed the poison oak without problems. It is unclear the reason for the variation observed.

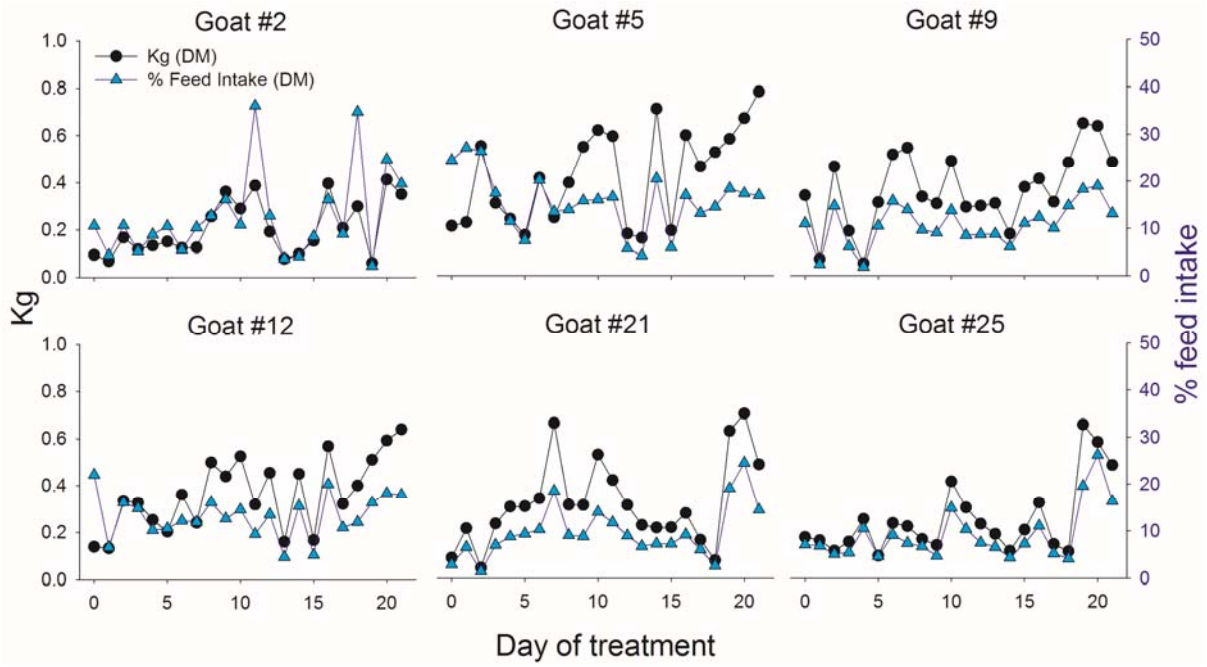

Figure 5. Kg of poison oak eaten/day and proportion of dry matter from poison oak on feed intake for all 6 goats in the trial

The poison oak did not affect milk yield, total solid in the milk, the energy content of the milk, overall feed intake of the goats or the kids, and the body weight of the kids; however, several parameters of the milk were affected by poison oak (Figure 6). Feeding poison oak also negatively affected body weight (76.1 vs. 74.1 in control vs. poison oak fed goats, $p<0.01$).

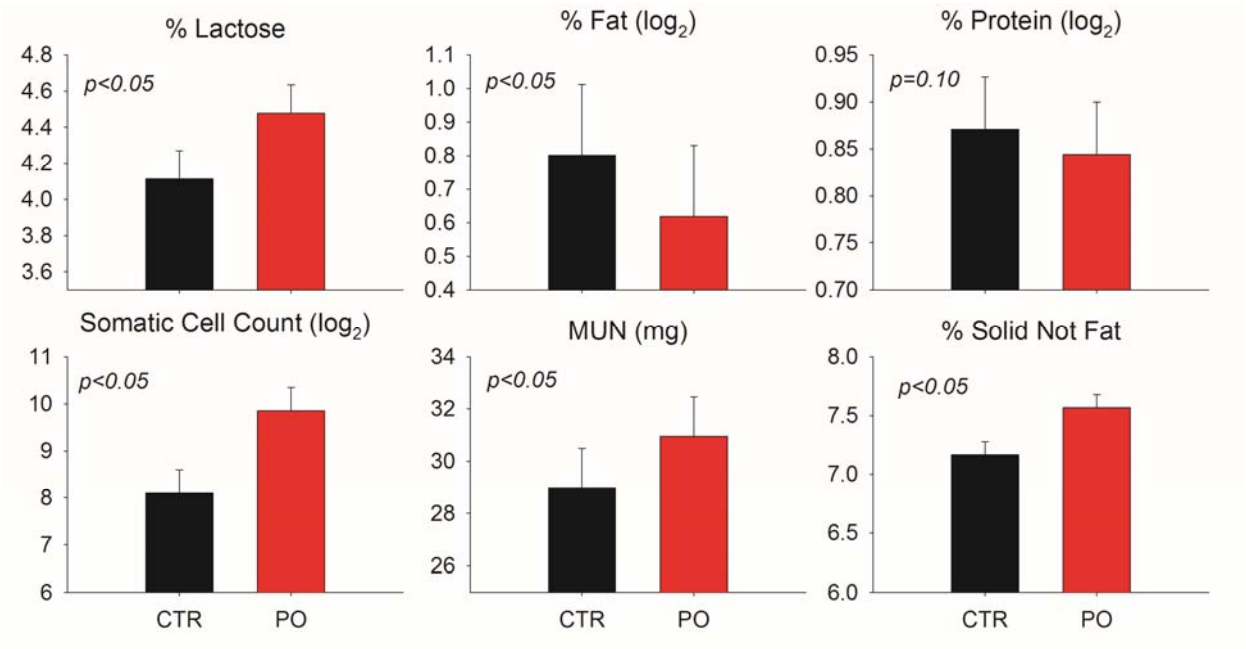

Figure 6. Milk components affected by feeding poison oak to lactating goats. MUN = milk urea nitrogen)

Based on these data, it appears that poison oak changed the composition of the milk by decreasing % fat and increased % lactose with an overall larger solid not fat. The % protein

tended to decrease-but the SCC were increased by poison oak. In addition, the milk urea nitrogen was higher when poison oak was fed to goats. The change in % lactose and % fat in milk could be interpreted by a change in rumen fermentation products (i.e., propionic acid: acetic acid). Lactose production is driven by availability of glucose, which is derived by propionic acid, and milk fat from acetic acid; however, the larger lactose % and the lower % fat did not result in an overall larger synthesis of lactose or overall lower synthesis of milk fat (i.e., g of lactose/milking was only numerically higher [p=0.20] as well as g milk fat/milking was numerically lower [p=0.24] when goats received poison oak compared to hay control). Also, higher energy availability from the diet would have increased the synthesis of protein (Osorio et al., 2016), which did not happen with actually a numerical lower synthesis of milk protein. The higher MUN when poison oak was fed can indicate a higher availability of amino acids from the diet or a higher muscle breakdown. The latter is supported by a decrease in body weight observed. The reason for a large breakdown of the muscle protein is unclear; however, this would have resulted in a higher availability of amino acids for milk protein. The lack of increase in milk protein despite the possible larger availability of amino acids and no change (or higher) energy in the diet, is indicative of some more direct effect of the poison oak in the mammary. One possibility is an effect on transcription of genes. Therefore, we can infer that feeding poison oak may have induced the production of some components (or components derived from poison oak itself) that have affected the transcriptome of the mammary epithelia cells and, thus, the synthesis of lactose and milk fat (Osorio et al., 2016). Feeding poison oak also increased the somatic cells in milk. The somatic cells are mostly macrophages. Except one goat that had high SCC but not signed of clinical mastitis, none of the other goats had signed of clinical or subclinical mastitis. Thus, the increase in SCC can be interpreted as an increase in defense capacity of the mammary; however, further analysis need to be done to assess this. We were able to isolate high pure exosomes from goat milk (Figure 7).

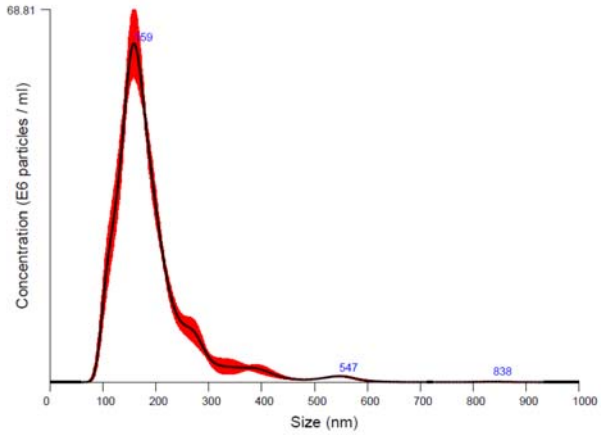

Figure 7. Nanosight analysis of exosomes isolated form goat milk. Exosomes are microscopic particle of around 150 nm in diameter. The analysis indicated we had a high purity of exosomes isolated form goat milk in our experiment.

The amount of total RNA isolated from milk exosomes was only numerically larger when poison oak was fed to the goats but the pasteurization significantly decreased the amount of RNA in milk exosomes in similar way in milk from goats fed poison oak or control (Figure 8).

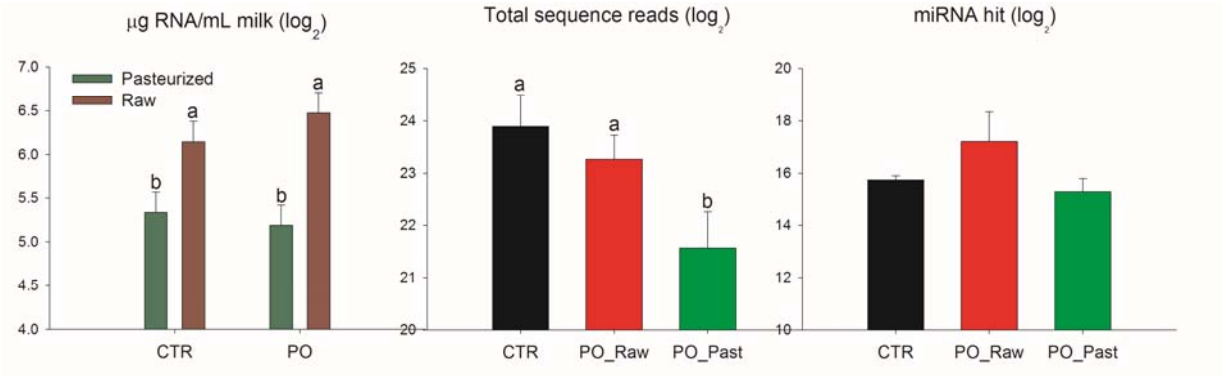

Figure 8. Amount of RNA isolated from milk exosomes, total number of sequence reads, and number of sequences that match annotated miRNA in goat. Different letter denote significant effect.

The reduction of RNA by pasteurization was 62.2±14%. These data are consistent with prior findings (Howard et al., 2015).

In exosomes isolated from the goat milk up to 50 million sequences were performed in each sample. The total number of sequences detected was significant lower in pasteurized compared to raw milk (Figure 7). Considering that the analysis was performed using the same amount of RNA, it is possible that the pasteurization had increased the degradation of RNA but with a less effect on micro RNAs (miRNA), as indicated by the lack of difference between the number of miRNA sequences (Figure 8). Thus, it appears that pasteurization augmented the degradation of all types of RNA but with a lower effect on miRNA. The reason for the lower degradation of miRNA compared to other RNAs present in milk exosomes is unclear. It is possible that the very low size of the miRNA (around 20 nucleotides vs. thousands in other types of RNA) helped to prevent degradation.

From the sequences we identified 158 previously known goat miRNA expressed in at the least half of the samples and 98 present in all the samples. The statistical analysis of the miRNA isolated from goats fed poison oak compared to control allowed to identify 18 miRNA significantly increased in milk exosomes by poison oak feeding (approximately 10% of identified miRNA). The bioinformatic analysis of these miRNA using http://www.mirdb.org/miRDB/index.html identified 883 genes in human that can be affected by these miRNA but only 89 with a high likelihood (i.e., target score ≥90). We have then performed the bioinformatic analysis using http://cpdb.molgen.mpg.de/CPDB/rlFrame and https://david.ncifcrf.gov/home.jsp to identify biological function that can be affected by the genes targeted by the 18 miRNA in human. We identified few biological functions related to RNA metabolism, protein synthesis, and, with lower likelihood, immune cell activation. We detected 23 miRNA with a significant proportional decrease due to pasteurization. The bioinformatic analysis of these miRNA revealed that these miRNA, if absorbed into the blood of people, can affect the expression of almost 400 genes, mostly involved in immune cells function, particularly the regulation of inflammation, transcription, and .

Overall, the data on the miRNA isolated from exosomes indicated that feeding poison oak can change the expression of around 10% of the miRNA in milk while pasteurization decrease around 60% of the RNA in milk exosomes plus decrease the expression of more than 10% of the miRNA which might be involved in controlling the immune system.

BENEFITS & IMPACT:

The findings from the present experiment are important considering the potential effect of miRNA in the immune system and, thus, in the response of subjects consuming milk. Important is the effect of feeding poison oak on milk composition indicating an effect that goes beyond the effect on energy content of the diet with a likely molecular effect that still need to be deciphered. The most important finding related to the main aim (i.e., evaluate the effect of milk from goat fed poison oak on the response to urushiol) is the effect of poison oak in milk miRNA. The implication of the preliminary findings is still unclear and further analyses are needed to identify if any of the miRNA observed can affect the immune cells, especially the one related to the contact dermatitis reaction. One of the major findings from the study was, besides the confirmation of a large decrease in RNA isolated from exosomes in pasteurized milk compared to raw milk, the negative effect of pasteurization of the milk on the abundance of miRNA that can have biological role in controlling the immune system. However, further studies are needed to investigate the real biological importance of such finding.

ADDITIONAL FUNDING RECEIVED DURING PROJECT TERM:

None

FUTURE FUNDING POSSIBILITIES:

Based on the results from the above data and data presented in prior reports, we plan to submit a proposal to run a clinical trial as originally proposed for the present grant. The final results from the RNA sequencing analysis will be determinant for the final decision of submitting such a proposal.

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