Effect of Dietry Tannic Acid on the Facultative Gut Microflora of the Red Squirrel

KARA EBERLY and ERIC MOULD
Department of Biology, Saint Mary's College, Notre Dame, Indiana 46556
and
DOUGLAS DUFF
Department of Biology
Indiana University, South Bend, Indiana 46334

Introduction

The polyphenolic compounds (tannins) have been the subject of considerable study because of their action in inhibiting ruminant digestion when they are present in feeds(1,11). Polyphenolics have also been reported to inhibit digestion in monogastric animals. However, the native diets of some animals, including the red squirrel, are high in polyphenolic compounds. In digestion studies with red squirrels fed laboratory diet supplemented with tannic acid, one of the less toxic polyphenolics, dry matter and lipid digestion were found to be unaffected by up to 5 percent dietary tannic acid after a two week period of adaptation to the diet(12). This ability to adapt to a high tannic acid diet could be the result of changes in enzymatic levels in the squirrels, changes in the gut microflora, or both.

The well-documented sensitivity of many bacteria to tannic acid, as well as several reports of bacteria and fungi which can tolerate tannic acid or even use it as a carbon source(3-11,13-16,18,19), led to the examination of the effects of dietary tannic acid on gut microflora. If the gut microflora were essentially unchanged in quantity and composition, then a bacterial role in adaptation to dietary tannic acid would be highly unlikely. Changes in the microflora might reflect sensitivity to tannic acid without any effect on digestion. In this case, however, microflora composition could prove to be indicative of the animal's natural diet at the time of evaluation. On the other hand, floral changes could be highly significant to the squirrel's ability to utilize tannic acid containing diets.

Fecal samples were chosen because bacteria from the whole gastrointestinal tract were of interest; fecal samples should contain bacteria from the stomach and small intestine as well as colon organisms; and a series of diets could be tried on the same animals. Total bacterial counts were chosen to evaluate the overall effects on gut bacteria since they should reflect primarily populations of anaerobes. Finally, a series of selective and differential media were chosen to quantitate the populations of the predominant facultative organisms.

Materials and Methods

Red squirrels (*Tamiasciurus hudsonicus*) were live trapped and housed in 45 cm by 45 cm by 25 cm metal cages on the same cage rack. Thus there was no direct contact between animals, and opportunities to coprophagy were reduced. However, there was no attempt to strict isolation, nor was the diet presterilized. Squirrels were fed *ad libitum* on a balanced rodent ration from Bio-Serv Inc. which contained 18.5% protein, 5.05% fat, 2.99% ash. The diet was supplemented with 0, 0.5, 0.75, 1.0, 2.0, or 5.0 percent tannic acid which replaced undigestible fiber. Tap water was provided *ad libitum*.

Fecal samples were collected within 24 hours after capture and after 7 days on the given diet. Other collection times are indicated in the results. Samples

were aseptically weighed and uniformly suspended in sterile saline at a 1/100 dilution. Further ten-fold dilutions were made in trypticase soy broth and plated on trypticase soy agar (TSA), KF streptococcal agar(KF), Mitis-salivarius agar(MI), Levine's eosin methylene blue agar(EMB), Salmonella-shigella agar (SS), Mannitol-salt agar (MS), and Rogosa SL agar(SL). After plates were incubated 48 hours at 37C in candle jars, characteristic colonies were selected, gram-stained, restreaked, and identified as to group by conventional bacteriological methods. A sample of bacteria diluted 1/100 in saline was preserved in ten percent formalin and counted on a Petroff-Hauser counter for total counts. A total of 9 squirrels were evaluated in three different experiments. In two experiments increasing amounts of tannic acid were fed sequentially. In the third experiment the control diet was followed immediately by five percent tannic acid. Data were analyzed using a one-way analysis of variance.

Results

Direct bacterial counts on squirrels which had received the control diet for 7 and 13 days averaged 3.5×10^{11} bacteria per gram of feces. Total counts averaged 4.2×10^{11} per gram feces on days 1 and 7 after a change to a 5% tannic acid diet and 3.5×10^{11} per gram feces on days 2 and 7 after a return to the control diet. There were no statistically significant changes in direct counts. Total facultative counts for the control diet at 7 and 13 days averaged 8.5×10^{8} or .04 % of the total count. Therefore, the direct counts represent chiefly anaerobes, and the total anaerobe population was not significantly altered by the tannic acid in the diets.

Quantitative changes were observed in the facultative gut microflora. There was approximately a 1000 fold decrease ($p < .05$) in the counts of the genus *Lactobacillus* when tannic acid was included in the diet (Table 1 and Figure 1).

Gram negative enteric bacilli, staphylococci, yeasts, and enterococci were also isolated. However, there was no statistically significant relationship of their population sizes to tannic acid in the diet (Table 2). The mean log enteric count on the control diet was 6.9 with a standard deviation of 1.24. However, patterns with individual animals were quite variable; 4 squirrels showed moderate to extensive decreases with increased tannic acid levels; 1 showed a progressive increase; and 3 showed no pattern at all with wide fluctuations in enteric levels. Four squirrels failed to have detectable levels (greater than 1000/g feces) of enterococci; 2 only developed them at the 5% tannic acid level; 1 had an erratic pattern; and 2 showed moderate increases in enterococcus levels. Variations among animals were also seen with the staphylococci and yeasts. Although there was

TABLE 1. *Fecal Lactobacillus counts of red squirrels fed artificial diets with variable tannic accid content for a period of 7 days.*

DIET % Tannic Acid	LOG VIABLE COUNT[a] Per Gram Feces (Mean ± standard dev.)	NUMBER
0	7.90 ± 0.54	6
0.5	5.51 ± 1.01	3
0.75	6.10 ± 0.84	6
1.00	5.44 ± 1.71	6
2.00	5.23 ± 0.47	5
5.00	4.70 ± 1.42	8

[a] *Lactobacillus* counts were made from MI, KF, and TSA plates, which gave consistently higher counts than Rogosa SL medium.

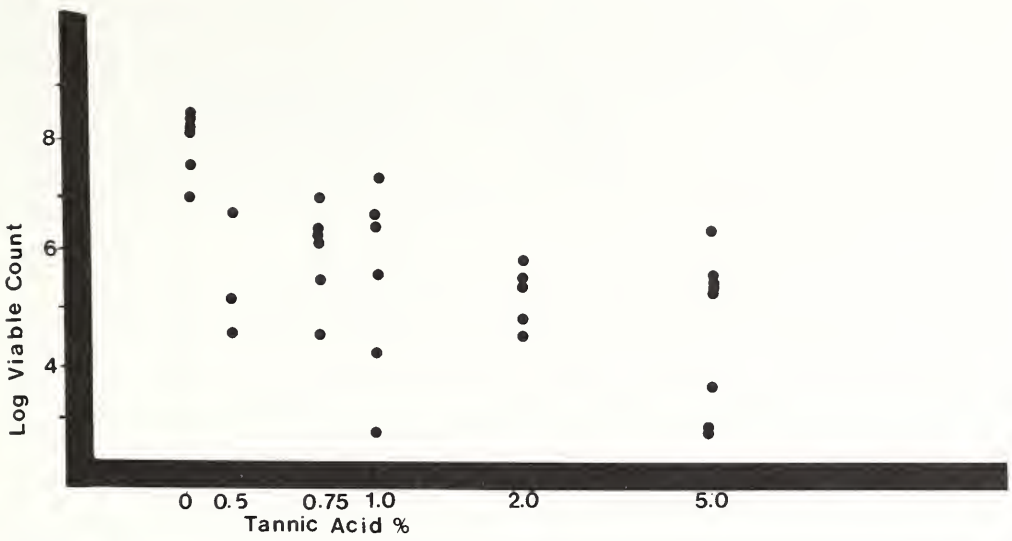

FIGURE 1. *Seven-day fecal Lactobacillus counts of red squirrels fed artificial diets with variable tannic acid content.*

no decrease in population levels of staphylococci or yeasts in squirrels with detectable populations (1000 or more), there appeared to be a tendency towards reduced numbers of animals with detectable levels in the presence of higher dietary tannic acid levels (Table 2). However, these reductions were not statistically significant at the 5% level (Chi square test).

One important question concerned whether the results obtained at 7 days on a diet represented a period when lactobacillus counts were still falling or a time at which they had reached their lowest levels. Secondly, we needed to know how rapidly populations returned to control levels. Lactobacillus counts were made at 7 and 13 days on the control diet; then animals were switched to a 5% tannic acid diet, and samples were assayed on days 1 and 7 on the 5% diet. Finally, animals were returned to the control diet and samples were assayed on day 2 (Table 3). It appears that the 7 day sample underestimates slightly the initial effect of tannic acid on levels of fecal lactobacilli but does provide a reasonable time point for comparing the effects of various dietary tannic acid levels. Recovery of the population levels appeared to be essentially complete in 2 days.

Finally, comparisons between population levels at capture and after 7 days on the control diet showed only 2 instances where an organism which had not been present in detectable levels at capture became detectable on the new diet. A staphylococcus reached population levels of 1.8×10^5 in a squirrel which had had less than 10^3 /g feces at capture, and a squirrel with undetectable lactobacillus levels at capture reached populations of 1.5×10^8 / g. Populations of enterics and lactobacilli each increased in 5 of 6 squirrels when they were put on the control diet.

Discussion

Polyphenolic compounds appear to be important plant defenses against herbivores, and digestibility by herbivores is often reduced by high phenolic content (1,11). Native squirrel diets, however, contain large amounts of phenolic compounds. In controlled feeding studies using tannic acid supplemented diets, reduced dry matter and lipid digestibility were observed on the first diet fed (0.5% tannic acid), but then the animals appeared to adapt to tannic acid, and digestibility on the subsequent diets (0.75% to 5.0% tannic acid) returned to control levels (12).

TABLE 2. *Facultative fecal counts of red squirrels fed artificial diets with variable tannic acid content for a period of 7 days.*

DIET % Tannic Acid	PREVALENCE *Detectable* Total	LOG VIABLE COUNT Squirrels with counts only (/g feces)
GRAM NEGATIVE ENTERIC BACILLI[a]		
0	6/6	6.90 ± 1.24
0.5	3/3	3.95 ± 0.98
0.75	4/6	6.27 ± 0.72
1.0	5/6	6.25 ± 1.29
2.0	2/5	6.21 ± 1.77
5.0	6/8	6.81 ± 1.36
ENTEROCOCCI[b]		
0	3/6	5.98 ± 0.23
0.5	2/3	3.76 ± 1.07
0.75	2/6	6.04 ± 0.00
1.0	2/6	5.51 ± 0.99
2.0	1/5	3.30 ± 0.00
5.0	3/8	4.25 ± 1.23
STAPHYLOCOCCI[c]		
0	6/6	4.49 ± 0.54
0.5	2/3	5.52 ± 0.34
0.75	4/5	4.26 ± 1.07
1.0	3/6	3.85 ± 0.81
2.0	4/5	4.30 ± 0.86
5.0	3/8	5.14 ± 0.43
YEAST[d]		
0	3/6	5.86 ± 0.97
0.5	1/3	4.30 ± 0.00
0.75	3/5	5.66 ± 1.33
1.0	1/6	5.51 ± 0.00
2.0	0.5	— —
5.0	2/8	7.31 ± 0.30

a. Enteric counts were made from EMB and TSA plates.
b. Enterococcus counts were made from KF and MI plates.
c. Staphylococcus counts were made from MS plates.
d. Yeast counts were made from TSA, EMB, MI, and RSL plates.

One of the frequently reported actions of tannic acid is to bind to proteins thus reducing protein digestibility and inactivating enzymes (11). Therefore, the adaptation to dietary tannic acid could involve the increased secretion of digestive enzymes by squirrels. However, a number of aerobic and facultative bacteria and fungi have been reported which can degrade tannic acid, and some can use it

TABLE 3. *Fecal Lactobacillus counts of red squirrels fed artificial diets containing 0% and 5% tannic acid for varying time periods.*

DIET % Tannic Acid	DAY	LOG VIABLE COUNT[a] Per Gram Feces (Mean ± Standard div.)	NUMBER
0	7	8.24 ± 0.07	3
5	1	4.76 ± 1.87	3
5	7	5.78 ± 0.53	3
0	2	7.73 ± 0.22	3

[a] *Lactobacillus* counts were made from MI, KF, and TSA plates, which gave consistently higher counts than Rogosa SL medium.

as a sole carbon source (4-11,14,18,19). Therefore it is reasonable to examine the population levels of yeasts and bacteria in the gastrointestinal tract to determine their potential involvement. Bacteria have often been reported to vary in their sensitivity to tannic acid, so that even if they do not participate in tannic acid degredation, they may serve as indicators of dietary tannic acid (or polyphenolic levels). Fecal samples allow us to monitor bacteria from various parts of the gastrointestinal tract and of course permit repeated sampling from the same squirrel.

Total direct bacterial counts were made to determine if there were a quantitative effect on the overall anaerobe population, and none was found. The anaerobe counts, approximately 10^{11} /g of feces, are similar to those reported for rats, mice, and ground squirrels (2,17). This does not rule out changes in composition of the anaerobic flora; in fact such a change would be expected.

The facultative organisms isolated belonged to groups commonly found in the gastrointestinal tract and feces of mammals. Not all groups were represented in all squirrels at capture, which is not unexpected. The use of separate cages apparently prevented the free exchange of microflora. Therefore, the pattern of change in floral levels with increased dietary tannic acid often varied with the squirrel. Studies are presently underway in this laboratory to see if these differing patterns are due to differences in sensitivity of the isolates to tannic acid and/or their ability to utilize tannic acid as a carbon source.

Only *Lactobacillus* populations showed a significant decrease in the presence of dietary tannic acid. This was anticipated since gram positive organisms have been reported to be more sensitive to tannic acid than gram negatives (18), and that has been confirmed in our laboratory. Also, the lactobacilli are found predominantly in the mammalian stomach(17), and that would be the area of highest exposure to tannic acid. Timed studies were done in an attempt to confirm this finding; and in fact lactobacillus populations were lower in all squirrels on day 1 after introduction of tannic acid into the diet than on day 7. This was not found to be the case with the other facultative microbes. Although we cannot say that day 7 represents a steady state condition, it does appear to represent a reasonable sampling time. Recovery of the *Lactobacillus* populations was essentially complete two days after return to the basal diet. Perhaps this is related to the reduced digestibility of the 0.5% tannic acid diet. If dietary tannins are responsible for a decrease in fecal lactobacilli, one could predict that freshly captured squirrels would have lower counts than after one week on the basal diet, and this was found to be the case.

In general, changes in dietary tannic acid levels did cause perturbations in the facultative gut microflora while overall anaerobe levels remained essentially constant. These changes may or may not be related to the ability of the squirrels to adapt to tannic acid in the diet. However, changes in lactobacillus counts do appear to be related to dietary tannic acid levels, and perhaps to the levels of other tannins.

Literature Cited

1. ARORA, S.P. 1978. Latest research findings on protein metabolism in ruminants. J. Nuclear Agric. Biol. 7:99-102.
2. BARNES, E. M. and G. C. BURTON. 1970. The effect of hibernation on the cecal flora of the thirteen-lined ground squirrel (*Citellus tridecemlineatus*). J. Appl. Bact. 33:505-514.

3. BASARABA, J. 1964. Influence of vegetable tannins on nitrification in soil. Plant and Soil 21:8-16.
4. BASARABA, J. 1966. Effects of vegetable tannins on glucose oxidation by various microorganisms. Can. J. of Microbiol. 12:787-794.
5. DESCHAMPS, A. M., G. MAHOUDEAU, M. CONTI, and J-M. LEBAULT. 1980. Bacteria degrading tannic acid and related compounds. J. Ferment. Technol 58:93-97
6. FRIEDRICH, H. 1956. Der Abbau von phenolischen Substanzen durch *Aspergillus niger*. Archiv fur Mikrobiologie 25:297-306.
7. HEDIN, P. A., O. H. LINDIG, P. P. SIKORWSKI, and M. WYATT. 1978. Suppressants of gut bacteria in the boll weevil from the cotton plant. J. of Econ. Entomology 71:394-396.
8. HENIS, Y. H. TAGARI, and R. VOLCANI. 1964. Effect of water extracts of carob pods, tannic acid, and their derivatives on the morphology and growth of microoorganisms. Appl. Microbiol. 12:204-209.
9. JACOB, F. H. and M-C PIGNAL. 1975. Interactions levures-tanins.II. Etude en milieu tannant de quelques levures hydrolysant l'acide tannique. Mycopathologia 57:139-148.
10. LYR, H. 1965. On the toxicity of oxidized polyphenols. Phytopathologische Zeitschrit 52:229-240.
11. MCLEOD, M.N. 1974. Plant tannins—their role in forage quality. Nutr. Abstr Rev. 44:803-815.
12. MOULD, E. D., D. DUFF, and K. EBERLY. 1982. Role of tannic acid on digestion by red squirrels. Proceedings of the Indiana Academy of Sciences, 92: in press.
13. RENNIE, R. J. 1977. Immunofluorescence detection of *Nitrobacter* in soil during NO2 oxidation in the presence of exotic chemicals. Microbios. Lett. 6:19-26.
14. RIPPLE, A. and J. KESELING. 1940. Uber tanninzersetzende Mikroorganismen. Arkiv fur Mikrobiologie 1:60-77.
15. SADANADAN, K. P. and S. P. ARORA. 1976. Influence of added tannic acid on growth and *in vitro* 32P—phosphate utilization by rumen microbes. Ind. J. Animal Sci. 46:593-597.
16. SADANADAN, K. P. and S. P. ARORA. 1979. Influence of tannins on microbial rumen fermentation pattern. J. Nuclear. Agric. Biol 8:1-3.
17. SAVAGE, D. C. 1977. Microbial ecology of the gastrointestinal tract. Ann. Rev. Microbiol. 31:107-33.
18. SMITH, D. G. Inhibition of swarming in *Proteus* spp by tannic acid. J. Appl. Bacteriol. 38:29-32.
19. WATSON, T. G. 1975. Inhibition of microbial fermentations by sorghum grain and malt. J. Appl. Bacteriol. 38:133-142.

Acknowledgments

We wish to acknowledge the support obtained from the Indiana Department of Natural Resources, the work of E. Renee Smith on characterization of squirrel intestinal microflora, and the assistance of Susan Seyfert on this project. We also wish to acknowledge the support of an Indiana University Grant in Aid for Research in purchasing artificial diets.