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Antibacterial Action of Essential Oils of *Artemisia* as an Ecological Factor

II. Antibacterial Action of the Volatile Oils of *Artemisia tridentata* (Big Sagebrush) on Bacteria from the Rumen of Mule Deer

JULIUS G. NAGY AND ROBERT P. TENGERDY

Department of Fishery and Wildlife Biology and Department of Microbiology, Colorado State University, Fort Collins, Colorado 80521

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Rumen microorganisms of wild and captive deer were subjected to increasing amounts of volatile oils. The oils had a marked antibacterial effect on the rumen bacteria when the concentration reached approximately 16 μliters of oil per 10 ml of rumen fluid nutrient broth. The gross reactions of rumen bacteria obtained from wild, as well as captive, deer to the volatile oils seemed to be of the same magnitude; thus no adaptation by the bacteria to the oils was apparent.

An area of approximately 422,275 square miles is covered with sagebrush in the western part of the United States (1). These semievergreen plant species are nutritionally important, especially during late winter, when other plant species either are covered with snow or are of little nutritive value. Range livestock, such as sheep and cattle, and wild animals, such as deer and antelope, consume varying amounts of sagebrush. Excessive amounts of sagebrush in the diet, however, can cause digestive disturbances in some ruminant species (3). It is possible that the volatile oils present in sagebrush species exert antibacterial action against the rumen bacteria and consequently interfere with proper digestion (6).

In a previous paper (7), the antibacterial action of the volatile or essential oils of certain sagebrush species (*Artemesia tridentata* and *A. nova*) was investigated. We found in our experiments that these oils possess a wide spectrum of antibacterial properties. In this article, we discuss the antibacterial action of the volatile oils of *A. tridentata* (big sagebrush) against cultured deer rumen bacteria.

MATERIALS AND METHODS

Volatile oils of *A. tridentata* were obtained according to the procedure of Nagy et al. (6). Usually, we used the techniques of Hungate (4) for anaerobic culturing of rumen bacteria. The compositions of the various media employed for culturing deer rumen microorganisms were those described by Bryant and Burkey (2), with some modifications (presented in Materials and Methods).

We obtained rumen contents of mule deer in three different series of experiments. In the first experiment, three wild deer from the Cache la Poudre River drainage (northern Colorado) were shot during late March and early April and were then taken to the laboratory where the rumen contents were processed 4 to 6 hr after death. All of the rumen contents contained sagebrush (*A. tridentata*) leaves and twigs. For the second trial, deer rumen contents were obtained from the Walden, Colorado, area deer herd. This herd is known to winter almost exclusively on sagebrush. However, only one animal was used in this trial.

In the third trial, the rumen contents of a yearling male and female deer were tested. These animals were raised from a few days of age in captivity, and they received a diet of concentrate and alfalfa hay ad libitum. During the trials, the animals were fed twice daily while confined in a 4 × 8 ft cage; they consumed 700 to 800 g of concentrate per day. Rumen samples were extracted by a stomach tube (inside diameter, 1 cm) with the aid of a vacuum pump. To facilitate the removal of the rumen contents, the concentrate was run through a Wiley mill before feeding. During the trials, each animal was used twice as rumen fluid donor.

Throughout the experiments, two types of media were used—rumen fluid nutrient broth (RFNB) and rumen fluid agar medium (RFAM). The composition of the anaerobic diluting solution that we used to dilute rumen contents prior to inoculation, as well as the composition of RFNB and RFAM, were similar to those outlined by Bryant and Burkey (2). However, there were two main differences: (i) a higher percentage of rumen fluid (60%) and consequently a lower percentage of water were used in the media, and (ii) bacteria were grown in media made within the same rumen fluid from which the inoculum was obtained. The latter procedure was necessary because a preliminary finding showed that bacteria of wild

deer grew very erratically in a medium containing rumen fluid obtained from a different wild deer. Hungate (5) discussed similar observations, but in more detail. We added Tween 80 (0.5%) to the RFNB to obtain better dispersion of the volatile oils.

Rumen fluid for the two media was prepared in the following manner: rumen contents were pressed through four layers of cheesecloth and were then centrifuged for 5 min at 12,100 × *g* with a Servall SS-3 automatic superspeed centrifuge. The supernatant fluid was poured off and this fluid was centrifuged again for 1 hr at 37,000 × *g*. We collected the supernatant liquid, and placed appropriate amounts into round-bottom flasks (500 ml) containing the other ingredients for the particular medium. The media were sterilized, and cysteine-HCl and sodium carbonate solution were added; then 9-ml portions of the media were transferred to tubes. We used 12.5 × 1.2 cm test tubes for RFNB and 14.8 × 1.5 cm test tubes for RFAM, with rubber stoppers. The final *p*H of the media was 6.5.

Strained rumen fluid (10 ml) was placed in a refrigerator until the media were ready for inoculation. At this time, dilutions were made up to 10^{-4} with the anaerobic diluting solution. By use of an autopipette, 1-ml amounts were transferred to tubes containing 9 ml of RFNB. Previously, essential oils of *A. tridentata* had been added to these tubes in amounts ranging from 0 to 20 μliters (in 2-μliter increments). The volatile oils contained no bacteria which would grow on any of the media used in these experiments. After the inoculation of these series, samples from the flask containing the 10^{-4} dilution were diluted to 10^{-7} and 10^{-8}. These dilutions were inoculated into melted and cooled tubes of RFAM from which the roll tubes were prepared. After incubating the roll tubes at 40 C for 3 days, we determined colony counts from average counts of two to four tubes. In experiments with RFNB, the medium was incubated for 24 hr. Dilutions were made from each tube, 1.0- and 0.1-ml amounts were transferred to roll tubes (prepared as previously described), and after 3 days of incubation colonies were counted.

RESULTS AND DISCUSSION

Colony counts on wild, as well as tame, deer rumen contents showed that 1 to 10 billion cultivable bacteria per ml of rumen contents were present. At the beginning of the incubation period, the inoculated RFNB contained 10,000 to 100,000 cultivable bacteria per ml of the broth.

Our results of colony counts of the bacterial survivors after 24 hr of fermentation with the oils of *A. tridentata* are presented in Table 1 and Fig. 1. In Table 1 the averages of these observations are given according to the locality of the animals. Statistical analysis of the data showed no significant differences between the colony counts from the rumen contents of wild deer obtained from the Cache la Poudre River drainage and the Walden area, or between captive male and female deer. However, we obtained statistically significant differences when the averages of all the wild rumen bacterial colony counts were compared with the averages of the captive deer. In Fig. 1 the average numbers of bacterial survivors from wild deer and from captive deer are plotted for comparison. Generally, the response of microorganisms from wild, as well as captive, deer to the action of the oils was similar. However, microorganisms from the captive deer seemed somewhat more sensitive. The number of surviving bacteria from the captive deer dropped considerably at 6 μliters of the oils per 10 ml of RFNB. At this level, there was very little decrease in numbers of bacteria obtained from wild deer. But above 16 μliters per 10 ml of RFNB, both populations decreased precipitously.

Roll tube colony counts determined from wild deer rumen contents illustrated the presence of 1 to 10 billion cultivable bacteria per ml of rumen contents. Gram stains and wet mounts indicated that the morphological types of bacteria present in the rumen of wild deer were similar to the microorganisms described by various investigators in domestic ruminants. However, the numerical representation of the organisms seemed different from that in cattle, sheep, and even tame deer. Some of the variations can be attributed to differences in the chemical composition of the ingested food; others are the consequence of the genetic makeup of deer, the manner of obtaining food, of supplying saliva, and of removing end products of fermentation. Identification of the microorganisms appearing in the rumen of deer was not the objective of this investigation. However, since almost no information is available on this subject, identification of the microorganisms is now underway. Various cocci, rods, and spirochetes were observed in the original rumen fluid, as well as in RFNB and RFAM. A gram-negative anaerobic organism with crescent-shaped cells was identified as a member of the genus *Selenomonas*. Flagellar stain demonstrated the presence of a tuft of flagella at the middle of the concave side of these organisms. We observed regularly at least one, but possibly two (a large and a small), members of the family *Spirochaetaceae* in the rumen contents of wild, as well as tame, deer. Apparently, these organisms grew better in a cellulose broth (Nagy, Ph.D. Thesis, Colorado State Univ., Fort Collins, 1966) than in the RFNB. Some microscopic counts of These organisms suggested that they were represented in smaller numbers per milliliter of rumen fluid in samples obtained from tame deer rather than from wild animals. The ciliate protozoan *Entodinium* sp. (8) also appeared in the rumen contents, but it was never alive after 24 hr of incubation in RFNB.

TABLE 1. *Colony count averages of rumen bacteria after 24 hr of incubation with increasing amounts of the essential oils of Artemisia tridentata*

Oils (μliters/10 ml of medium)	Avg no. of bacterial survivors (per ml of medium) obtained from					
	Poudre deer	Walden deer	Captive deer		Wild (mean)	Captive (mean)
			Male	Female		
0	4.71×10^9	4.86×10^9	3.00×10^9	5.84×10^9	4.78×10^9	4.42×10^9
2	3.28×10^9	3.10×10^9	2.47×10^9	4.00×10^9	3.17×10^9	3.37×10^9
4	3.50×10^9	2.00×10^9	2.80×10^9	2.94×10^9	2.75×10^9	2.87×10^9
6	2.06×10^9	3.07×10^9	1.07×10^9	1.49×10^9	2.56×10^9	1.28×10^9
8	2.21×10^9	3.10×10^9	5.75×10^8	5.10×10^8	2.65×10^9	5.42×10^8
10	2.20×10^9	2.67×10^9	2.72×10^8	3.98×10^8	2.43×10^9	3.35×10^8
12	1.42×10^9	1.13×10^9	1.45×10^8	1.41×10^8	1.27×10^9	1.43×10^8
14	1.26×10^9	1.45×10^9	7.78×10^7	8.45×10^7	8.57×10^8	8.11×10^7
16	7.15×10^8	1.00×10^9	3.05×10^7	1.40×10^7	8.57×10^8	2.22×10^7
18	8.36×10^8	5.16×10^8	6.39×10^4	5.15×10^4	6.76×10^8	5.77×10^4
20	6.14×10^2	3.30×10^2	4.55×10^2	8.96×10^2	4.72×10^2	6.75×10^2

The addition of even small amounts of volatile oils restricted many organisms and encouraged the growth of a short-chain streptococcus. This organism apparently dominated the roll tubes with volatile oil concentrations of 6 to 16 μliters per 10 ml of RFNB. Red, lenticular (3 to 4 mm in diameter) colonies were formed, if the inoculum was extracted from wild deer. In the case of captive deer, the same organism was present without the red pigment. Because of its rapid growth and its ability to hydrolyze starch, we tentatively identified this gram-positive organism as *Streptococcus bovis*. Ample amounts of capsular material surrounded the RFAM colonies of this organism. Also, we observed within this material a variety of other bacteria. It is possible that the capsular material gave some protection to *S. bovis*, as well as to other bacteria, from the action of the volatile oils.

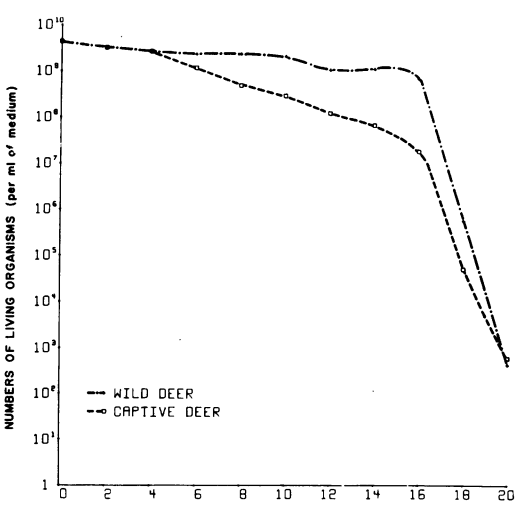

Fig. 1. *Antibacterial action of the essential oils of Artemisia tridentata against the rumen microorganisms of wild and captive deer.*

As previously mentioned, colonies of this streptococcus dominated the agar tubes containing up to 16 μliters of essential oils. However, at higher concentrations of oils these colonies disappeared, and the survivors consisted almost exclusively of small gram-negative micrococci.

The type of curve might explain the ability of ruminants to consume sagebrush species when the range contains a variety of other plants. Evidently, rumen microorganisms tolerated the essential oils up to a certain concentration. But when this concentration is exceeded, rumen microbial fermentation will slow down, and eventually digestive disturbances will occur. These results might also explain why the data of Dietz et al. (3) revealed that the digestibility of sagebrush increases if sagebrush is fed simultaneously with alfalfa hay.

In separate experiments we noted that current annual growth of *A. tridentata* leaves and twigs contained from 2 to 5% volatile oils, on dry-matter basis—depending on seasons, localities, and varieties of the species. In the experiments described in this paper, drastic reduction in the numbers of viable rumen bacteria occurred at about 16 to 20 μliters of the oils per 10 ml of medium. If one considers an average dry-matter content of 15% for deer rumen contents (D. E. Medin, Colorado Game, Fish and Parks Department, Fort Collins, *personal communication*), and if the diet consists of 100% sagebrush with an

oil content of 1 to 5%, then 10 ml of rumen contents would contain 15 to 75 μliters of essential oils. Depending on the volatile oil content of sage eaten, an amount of about 50% sagebrush in the diet is possibly a safe estimate of the amount which would be tolerable by deer. However, various interactions in the rumen and in the host animal might influence this estimate considerably in either direction.

Range nutrition studies have revealed that certain stands of sagebrush are preferred and utilized much more than others by range animals. *A. nova* (black sagebrush) with a volatile oil content of 1 to 2% is probably the most preferred. In view of the currently executed large-scale sagebrush eradication programs, the results presented suggest that a thorough examination of the possible relationship between the volatile oil content of sagebrush and utilization by range animals be carried out.

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