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ORIGINAL ARTICLE

Quantification of enterobacteriaceae in faeces of captive black rhinoceros (*Diceros bicornis*) in relation to dietary tannin supplementation

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Summary

Free-ranging browsing herbivores ingest a range of secondary plant compounds, such as tannins, with their natural diet. As many of these substances have been shown to have antibacterial properties, it could be speculated that a lack of such compounds in captive zoo diets could favour the growth of potentially pathogenic intestinal bacteria. The effect of a supplementation of a conventional diet (N, consisting mainly of grass hay and/or lucerne hay and pelleted compound feeds) fed to eight captive black rhinoceroses (*Diceros bicornis*) from three zoological institutions with either tannic acid (T), a source of hydrolysable tannins, or quebracho (Q), a source of condensed tannins, was investigated. The number of faecal colony forming units (CFU) of *Enterobactericeae* was determined by colony count of dilution series from fresh faeces applied to MacConkey agar plates. Tannins were added to the diets at approximately 5–15 g/kg dry matter, depending on the varying intake of roughage and compound feeds by the animals. There was no difference in the number of CFU between diets N ($95.0 \times 10^5 \pm 225.3 \times 10^5$/g fresh faeces) and T ($164.3 \times 10^5 \pm 225.1 \times 10^5$/g fresh faeces); in contrast, diet Q led to a significant reduction in CFU ($4.3 \times 10^5 \pm 6.5 \times 10^5$/g fresh faeces) compared with the other diets. These findings suggest that condensed tannins could have the potential to reduce the number of potentially pathogenic intestinal bacteria, and that the deliberate inclusion of tannin sources in the diets of captive wild animals should be further investigated. The fact that tannic acid, shown to have antibacterial effects in various *in vitro* studies, did not have an effect in this study, emphasizes that the relevance of tannin supplementation for intestinal health must be verified *in vivo*.

Introduction

In the wild, many free-ranging animals consume diets that include secondary plant compounds, such as tannins. Whereas these substances have been understood primarily as repellents or defense against herbivory, due to toxicity or inhibition of digestion, potentially positive effects have attracted attention more recently (Clauss, 2003). Tannins could enhance antioxidant status (Clauss et al., 2003, 2006b), and there is evidence that they reduce pathogenic organisms. On the one hand, tannins have been shown to be effective against helminth parasites *in vitro* (Athanasiadou et al., 2001) and *in vivo* in sheep (Niezen et al., 1995), goats (Kabasa et al., 2000) and red deer (Hoskin et al., 2000). On the other hand, tannins have been found to be potent bacteriostatic and bacteriocidal substances in *in vitro* assays for a large range of pathogenic bacteria (Scalbert, 1991; Chung et al., 1998b), including *Enterobacteriaceae* such as *Escherichia coli*, *Salmonella*, or *Proteus* spp., or obligate anaerobes such as *Clostridium* spp. (Hara and Watanabe, 1989; Schrägle and Müller, 1990; Baumann and Müller, 1993; Sotohy et al., 1995). However, there have been only few successful attempts to demonstrate a bacteria-reducing effect of tannins *in vivo*. Whereas Baumann et al. (1997) and Sotohy et al. (1997) found an inhibiting effect of some, but not all tannin-containing browse species investigated on the number of colony-forming units of *Clostridium perfringens* in sheep, Aschfalk et al. (2000) could not repeat these findings. In pigs, green tea polyphenols reduced the total bacterial number while increasing that of *Lactobacillus* sp. (Hara et al., 1995), and in chickens, green tea polyphenols reduced the number of Enterobacteriaceae while again increasing that of *Lactobacillus* spp. (Hara, 1997). In humans, green tea polyphenols reduced the number of *Clostridium* spp. (Okubo et al., 1992).

Given the effect of secondary plant compounds of the intestinal microflora, and the difference in the dietary consumption of such compounds between free-ranging and captive individuals of a species, it could be expected that captive animals differ in the composition of their faecal microflora from free-ranging conspecifics (Berry, 1998). To our knowledge, few targeted investigations in wildlife species have been performed. The only systematic evaluation known to us is the one by Schales et al. (1993b) who demonstrated a much higher number of faecal bacteria in captive capercaillies (*Tetrao urogallus*) than in free-ranging individuals. Additionally, they identified only a small number of *E. coli* and no *Clostridium spp*. in free-ranging birds, whereas both groups were frequent in captive animals. Capercaillies consume mainly conifer needles in the wild, and in a parallel study, Schales et al. (1993a) demonstrated an antibacterial effect of secondary plant compounds isolated from conifer needles.

As *Salmonella* spp. and *E. coli* have been reported to be important pathogens in captive rhinoceroses (Windsor and Ashford, 1972; Clausen and Ashford, 1980; Ramsay and Zainuddin, 1993; Kenny, 1999), the group of the Enterobacteriaceae is of clinical relevance. in this species. In a study on the influence of dietary tannins on the digestive physiology of captive black rhinoceroses (Clauss et al., 2006a, 2007), we decided to perform a bacteriological screening of the faeces of the animals on the different feeding regimes. Because the investigations had to be carried out at different facilities, a simple method was chosen investigating only this one bacteria group as a whole, thus avoiding more complicated isolation or even particular anaerobic procedures.

Materials and methods

The feeding trials with captive black rhinoceroses have been described in Clauss et al. (2006a, 2007). Faecal samples were taken from eight animals from three facilities (Table 1). Three different zoo rations, consisting of varying proportions of roughage and concentrates, were fed (total n of trials = 24). Details of the diets are given in Clauss et al. (2006a, 2007). Adaptation periods for new diets lasted more than two months. Each animal received a basically identical diet three times; the diet normally fed at the respective institution (N, consisting mainly of grass hay and/or lucerne hay and pelleted compound

Table 1 Black rhinoceroses (*Diceros bicornis*) used in the feeding trials

Animal	Studbook no.	Sex	Age (years)	Facility
Emma	451	f	10	A
Quinto	430	m	11	A
Tisa	532	f	7	B
Taco	533	m	6	B
Mtoto*	150	f	31	C
Sabi	217	f	approximately 30†	C
Parky	318	m	19	C
Wanda	662	f	5	C

*Animal had an oral abscess and received a particular diet (Hatt et al., 2004).

†Exact age unknown as animal was caught from the wild.

feeds), a similar diet with an inclusion of 5 % tannic acid (a source of hydrolysable tannins; tannic acid powder pure DAB 7 FU Ph Helv USP, Merck, Darmstadt, Germany) in the pelleted diet compound (T), and again a similar diet with an inclusion of 5% quebracho (a source of condensed tannins; order no. 031040; Tannin Corporation, Peabody, MA, USA; non-purified, estimated condensed tannin content 75%; Robbins et al., 1991) in the pelleted diet compound (Q). This resulted in an additional tannin source intake of 5–15 g/kg DM, depending on the varying intake of roughage and compound feeds by the animals. Each animal served as its own control.

A fresh faecal sample was collected from each animal and diet period on three consecutive days (i.e. three samples per animal and diet period were analysed) immediately after defaecation, stored in uncontaminated plastic bags (without any additional agents) which were placed into styrofoam boxes for transportation, and brought to the respective microbiology laboratories within 2–4 h (differences between facilities, not diets, were due to different distances between the zoos and the laboratories); a sub-sample of approximately 10 g was homogenized by stirring in 100 ml of sterilized peptone-NaCl (6.0 peptone from casein, order no. 0006335894; Merck Eurolab, Darmstadt, Germany; 51.0 g NaCl, Merck; de-mineralized water up to a total volume of 6000 ml). For each homogenized sample, two dilution series up to a dilution of 10^6 were prepared. Of each dilution step, 0.1 ml was transferred onto a MacConkey agar plate (Difco 1818-17; Zurich: Oxoid, Basel, Switzerland; Cologne: Oxoid, Wesel, Germany; London: QCM Laboratories, London, UK). Plates were incubated at 37 °C for 24 h. After this time, the number of colony forming units (CFU) was counted on each plate, and the average number of CFU per faecal sample calculated.

There were eight animals, each fed three diets, with three faecal samples assessed per diet. Mean values per animal and diet period were calculated as averages of the values of individual faecal samples. The diet periods were compared using the exact Friedman test, followed by pair-wise, exact Wilcoxon tests. The Dunn/Sidak method served to adjust for multiple testing. All statistical calculations were performed using StatXact 6.2 (Cytel, Cambridge, MA, USA). The significance level was set to 0.05.

Results

As described before (Clauss et al., 2007), animals accepted the tannin-containing diets readily and did not show any signs of digestive upset. The average number (±SD) of *Enterobacteriaceae* CFUs per diet period were $95.0 \times 10^5 \pm 225.3 \times 10^5$/g fresh faeces for diet N, $164.3 \times 10^5 \pm 225.1 \times 10^5$/g fresh faeces for diet T, and $4.3 \times 10^5 \pm 6.5 \times 10^5$/g fresh faeces for diet Q. The Friedman test indicated significant differences between the diet periods ($p = 0.008$). In the pair-wise comparisons, there was no difference between period N and T ($p = 0.742$), but significant differences between N and Q ($p = 0.008$) and between T and Q ($p = 0.016$). In all individuals, the Q value was lower than the N value, and the Q value was similarly lower than the T value in all but one individual. In contrast, there was no common directional change between the N and the T value.

Discussion

Several limitations are inherent in this study. The large standard deviations indicate that in future studies, potential factors of variance, such as transport to a bacteriology lab, should be avoided, for example by using mobile incubation units. Any investigation should ideally be restricted to one single facility with a sufficient number of animals, and individual animals should be assessed in a cross-over design; the aim of a cross-over design is extremely challenging in a study like this one, where animals at different facilities are used, and individuals at each facility would have to receive different diets reliably over a long-term period. Given these limitations, the results of this study should only be regarded as an incentive for further studies on the effects of dietary tannins on microbiological characteristics of herbivore faeces and gut contents.

The supplementation of quebracho, but not tannic acid, led to a significant decrease in the number of faecal *Enterobacteriaceae*. This finding was surprising, because tannic acid has been shown to have a high efficiency against a large variation of bacteria *in vitro* (Chung et al., 1998b). Nevertheless, in a feeding trial with chickens, tannic acid supplementation did not have any effect on the number of faecal bacteria (*Salmonella spp.*), either (Kubena et al., 2001). Similarly, although tannic acid has good antioxidant effects *in vitro*, it did not influence the faecal antioxidant status in the rhinoceroses of this study either, whereas quebracho did (Clauss et al., 2006b). As quebracho feeding also led to an increase in tannin-binding capacity in saliva for quebracho in the rhinoceroses (Clauss et al., 2005), salivary proteins did not prevent the effect on faecal *Enterobacteriaceae*. A possible reason for the absence of an effect of tannic

acid could be that, in contrast to the condensed tannins in quebracho, hydrolysable tannins – especially commercial tannic acid – are more easily degraded and/or absorbed in the gastrointestinal tract (Hagerman et al., 1992); tannic acid is hydrolysed into gallic acid, which is absorbed and excreted in the urine (Booth et al., 1959). Hagerman et al. (1992) stated that even although different tannin sources have similar effects *in vitro*, such as protein binding or iron chelation, the biological relevance of this finding must be tested in *in vivo* assays, as differences in resistance to neutralization or degradation by the animal organism will result in different effects *in vivo*. Thus, these authors demonstrated a significant difference between tannic acid and quebracho tannins on protein digestion in ruminants, with tannic acid showing little effect. The present study reaches a similar conclusion, as the *in vitro* effects of tannins on faecal *Enterobacteriaceae* are achieved *in vivo* by quebracho, but not by tannic acid.

Among the different mechanisms suggested for the antibacterial effects of tannin substances are the inhibition of bacterial enzymes and of oxidative phosphorylation, and the deprivation of substances required for microbial growth, including the chelation of dietary iron (Scalbert, 1991). Chung et al. (1998a) demonstrated that the antibacterial effect of tannic acid *in vitro* is due to iron chelation, that bacteria not dependent on iron for growth are not susceptible to the effect. The authors conclude that the antimicrobial effect of some other tannin substances are due to other causes presented by Scalbert (1991), such as the inhibition of extracellular bacterial enzymes, the deprivation of other required substrates other than iron, or the inhibition of oxidative phosphorylation. Given the fact that an increase in faecal antioxidant status, which was also effected by the condensed tannin source quebracho, can also be linked theoretically to iron chelation (Clauss et al., 2006b), these results are promising. Iron overload is an important health problem of captive black rhinoceroses (Smith et al., 1995; Paglia and Dennis, 1999; Dierenfeld et al., 2005) and the use of tannins for iron chelation has been discussed as a potential prophylactic measure. The results of this study suggest that condensed tannins might be more suitable for this cause, but also that any tannin source must be tested *in vivo* for its efficacy.

We can only speculate whether the observed decreased *Enterobacteriaceae* number could have a positive influence on the health of captive black rhinoceroses. Cases of both *Salmonella* and *E. coli* infection have been reported in this species in captivity (Ramsay and Zainuddin, 1993; Kenny, 1999). In a survey on the prevalence of salmonellosis in three captive rhinoceros species, Kenny (1999) found a prevalence of 10 % (of zoological institutions) if all species were combined. If the data was considered according to species, the browsing black rhinoceros had a distinctively higher incidence than the grazing white rhinoceros (*Ceratotherium simum*) or Asian rhinoceros (*Rhinoceros unicornis*). It is tempting to speculate that the browsing species, partially relying on the antibacterial effects of secondary plant compounds in the wild, is particularly susceptible to intestinal *Enterobacteriaceae*. However, in order to substantiate this assumption, not only data on the occurrence, but also on negative findings in faecal cultures of different rhinoceros species would be necessary.

The fact that faecal samples were investigated for bacteria in this study not immediately after the introduction of the tannin supplement, but 2–3 months later, suggests that the effect of the tannins on faecal microflora is not an acute but a lasting one. In correspondence to studies in other species, it can be expected that tannin supplementation will lead to an increase in tannin-resistant gut bacteria (Smith and Mackie, 2004), which have not been investigated in this study. In free-ranging browsing ruminants, such bacteria have repeatedly been isolated (Odenyo and Osuji, 1998; Odenyo et al., 2001). In order to further investigate the potential contribution of forage secondary compounds on the gut health of the black rhinoceros, a comparative study screening faeces of captive and free-ranging specimens qualitatively and quantitatively for a larger range of bacteria groups would be warranted.

In conclusion, the number of faecal *Enterobacteriaceae* was reduced by the addition of quebracho, but not tannic acid, to the pelleted diet compound in captive black rhinoceroses. The results indicate that *in vitro* results with tannins should be confirmed by *in vivo* assays. Tannins might contribute to overall gut health by controlling the number of potentially pathogenic bacteria. A potential, prophylactic effect of condensed tannin supplementation in captive browsing rhinoceroses should be further evaluated using tannin sources of more practical feasibility.

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