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DOMINANT CULTURABLE BACTERIAL MICROBIOTA IN THE DIGESTIVE TRACT OF THE AMERICAN BLACK VULTURE (*CORAGYPS ATRATUS* BECHSTEIN 1793) AND SEARCH FOR ANTAGONISTIC SUBSTANCES

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ABSTRACT

Strict and facultative culturable anaerobic bacteria from the digestive tract of six American black vultures (*Coragyps atratus* Bechstein 1793) were isolated and identified. After capture, the birds received a non-contaminated diet for one week to eliminate possible allochthonous microorganisms. Then, specimens collected from tongue, stomach and intestines were weighed, submitted to decimal dilution in an anaerobic chamber, inoculated into culture media and incubated aerobically and anaerobically at 37ºC for enumeration, isolation and identification. Isolated bacteria were submitted to tests to detect possible antagonisms between them. The total bacterial population along the digestive tract ranged from 3.46 ± 0.39 log CFU/g in the stomach to 10.75 ± 0.37 log CFU/g in the distal intestine. Some bacteria were isolated for the first time from the digestive tract of *C. atratus*: *Actinomyces bovis*, *Lactobacillus cellobiosus*, *Micrococcus luteus*, *Neisseria sicca*, *Clostridium bifermentans*, *Enterobacter agglomerans*, *Peptostreptococcus* sp., *Sarcina* sp., *Serratia odorifera*, and *Shigella flexneri*. Associations between microorganisms were observed during isolation on two occasions, one involving *A. bovis* and *N. sicca*, and the other involving *A. bovis* and a Gram-negative rod. Hetero-, iso- and autoantagonisms were observed, suggesting the ecological role of these indigenous microorganisms in terms of population auto-control and environmental barrier in the digestive tract of carrion-feeding birds.

Key words: Culture, microbiota, digestive tract, antagonistic substance.

INTRODUCTION

The American black vulture (*Coragyps atratus* BECHSTEIN 1793) is a bird highly associated with human activity, having an ample geographic distribution throughout the American Continent. Its scavenging habits make the bird an interesting subject of study, especially for its bacteriologic characteristics. Since scavenging birds feed regularly on decaying carcasses of animals that have succumbed to infectious diseases, the thought arose that these birds probably were highly resistant to pathogenic microorganisms and their toxins. As examples, in scavenging birds high resistance against *Bacillus anthracis* (12) and oral doses of *Clostridium botulinum* toxin, normally lethal for other animals, have been demonstrated (7).

Several mechanisms may explain this resistance: a) the specific absorption mechanism in the digestive tract of the bird; b) the physicochemical and physiological conditions in the digestive system (pH, oxygen concentration, intestinal transit), c) the constitution of the digestive epithelium, and/or d) the

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presence of a complex microbiota in the digestive ecosystem (6,7,12,19,20). Anti-microbial agents may be secreted by the liver or gastric epithelium, or produced by microorganisms of the normal microbiota. Another hypothesis may be the modulation of the production and/or action of toxins by the indigenous microbiota and/or by the host. As an example, mortality and intestinal cytotoxin production during an experimental infection with *Clostridium difficile* were highly reduced in gnotobiotic mice previously inoculated with bacteria from the normal digestive ecosystem (3). Similarly, in the absence of indigenous microbiota, germfree animals are more susceptible to the botulinal neurotoxin (10).

The importance of the intestinal microbiota to the welfare of the host was recognized early in the history of microbiology. As a result, the nature of the gastrointestinal microbiota of many animal species is well documented in the literature. However, very few data exist on the digestive tract microbiota of carrion-feeding birds in general and *C. atratus* in particular. In the few studies found in the literature on the fecal microbiota of the Whiteback griffon vulture (*Gyps africanus*) and *C. atratus* (6,19), the focus was generally more epidemiological than bacteriologic, except for a report on *Cathartes aura* (20). Moreover, none of these studies involved qualitative or quantitative determinations of the bacteria present in the various portions of the digestive tract or distinguished the autochthonous from the allochthonous microbiota.

The present study concerns the enumeration, isolation and identification of the culturable indigenous microbiota in the digestive tract of the American black vulture (*C. atratus*), as well as antagonistic substances produced by the isolated bacteria.

MATERIALS AND METHODS

Birds

Six adult *C. atratus* vultures were utilized in this study. The birds were captured using a rope-trap in Contagem, Belo Horizonte and Ibiá areas, Minas Gerais state, Brazil, after special scientific collecting permits were obtained from the Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA license 021/96, protocol P-15640/94). In order to eliminate possible allochthonous microorganisms, the birds were fed a non-contaminated controlled diet for six days before sacrifice by ether inhalation. This diet consisted of tap water and bovine meat (lung) inspected by the Sanitary Surveillance for the absence of pathogenic microorganisms according to current legislation. The meat was kept under refrigeration in the laboratory and, before being offered to the birds, was submitted to superficial decontamination under UV for 20 min. The meat was manipulated with the use of gloves and sterilized material for cutting the pieces to be offered (10 cm long and 3 to 4 cm wide).

Necropsy

Before sacrifice, the birds were examined for general health aspects (general appearance, mucosal appearance and physical integrity) by one of the author (L. A. Lima, VMD), looking for any sign of disease or pathology. The necropsy was performed under a laminar flow hood where the entire digestive tract of the birds was removed with the extremities closed. Sets of sterilized dissection instruments were utilized for each portion of the digestive tract to avoid cross-contamination and fragments of the tongue, stomach and intestines were removed. Due to the absence of histologic information about the digestive tract of *C. atratus*, an arbitrary subdivision of the intestines was adapted from the study of Salanitro *et al.* (18). The intestines, which had an average length of 120 cm, were divided into proximal intestine (right below the stomach), medial intestine (approximately 40 cm after the stomach) and distal intestine (right above the cloaca). Each digestive tract fragment was 0.5 cm^2 and was taken from equivalent portions of each bird.

Enumeration

The fragments were weighed, macerated in 2 mL of prereduced anaerobically sterilized fluid (Ringer-PRAS) and introduced into an anaerobic chamber containing 10% H_2, 5% CO_2 and 85% N_2 (Forma Scientific Company, Marietta, OH, USA) where decimal dilutions were performed in regenerated sterile buffered saline. Amounts of 0.1 mL from each dilution were spread onto the following culture media: Blood Agar supplemented with hemine (5 mg/mL), menadione (1 mg/mL) and yeast extract (0.5%), and Bacteroide Bile Esculine Agar (11). Portions of relevant dilutions were transferred from the chamber for plating onto Blood Agar and MacConkey Agar and subsequent aerobic incubation. The Petri dishes were incubated at 37ºC for one week inside the anaerobic chamber, after which colonies were counted. Under aerobic conditions, the dishes were incubated at the same temperature for 24 h. Total anaerobic counts and counts for each morphologically different colony on all media and atmospheric conditions were performed in duplicate.

Isolation and identification of microorganisms

After enumeration, the dishes containing 30 to 300 colonies were used to isolate three to four specimens of each colony presenting different macroscopic appearance. These different morphotypes were isolated on appropriate medium and submitted to microscopic examination, respiratory test and initial biochemical and physiological tests (catalase, oxidase and motility). Using the previous results as a guide, some microorganisms were inoculated into Adolfo Lutz Institute Medium (IAL) to obtain a presumptive identification for Gram-negative rods. Using a single tube of this medium, it is possible to determine indole production, L-tryptophan deamination, presence or absence of sucrose and glucose fermentation, gas

production, H_2S production, urea hydrolysis, L-lysine decarboxylation and motility (15). Biochemical tests were also carried out using the API 20 A, API 20 STREP and API 50 CH identification system kits (bioMérieux, Marcy-l'Etoile, France) for anaerobes, streptococci and carbohydrate metabolism of microorganisms, respectively. The BBL Crystal E/NF identification system kit (Becton Dickinson Microbiology Systems, Cockeysville, MD, USA) was used for enterobacteria and non-fermentative microorganisms. Additional biochemical tests were done when necessary.

Bacterial antagonism

The bioassay for antagonistic diffusible substances was carried out by the double-layer method originally described by Kelner (8). The inoculum was prepared in Tryptic Soy Broth (TSB). Eighteen-hour cultures were spotted simultaneously with a micropipette (2 mL at equidistant points) onto the surface of basal Tryptic Soy Agar (TSA) layered on Petri dishes and allowed to dry. After incubation at 37ºC for 18 h, inside or outside the anaerobic chamber depending on the bacteria, the cells were killed by exposure to chloroform for 30 min. Residual chloroform was allowed to evaporate and the Petri dishes were overlaid with 4 mL of TSA soft agar (0.75%) which had been inoculated with 10 mL of a 24 h culture of the indicator strain. After 18 h of incubation at 37ºC under aerobiosis or anaerobiosis, the plates were evaluated for the presence of zones of growth inhibition and the diameter of the halos were measured with the help of a caliper. To exclude the possibility of residual chloroform interference in the growth of indicator strains, the evaluation of antagonistic activity was performed without its use. The assays were performed by testing bacteria from the same digestive portion as producer and indicator strains. Hetero-, iso- and autoantagonisms were defined, respectively, as the growth inhibition of a producer strain against taxonomically unrelated strains, against isolates of the same species and against itself.

Three other indicator strains not belonging to the *C. atratus* digestive ecosystem were also utilized: *Escherichia coli* K_{12} Sm, *Pseudomonas aeruginosa* and *Staphylococcus aureus* ATCC 25923.

RESULTS

The total culturable indigenous microbiota varied throughout the digestive system of *C. atratus*. The mean population levels were of 6.52 ± 0.14, 3.46 ± 0.39, 7.98 ± 0.49, 10.16 ± 0.40 and 10.75 ± 0.37 log CFU/g, respectively for the tongue, stomach, proximal intestine, medial intestine and distal intestine.

From the different portions of the digestive tract of the six birds, a total of 45 different bacteria of the dominant indigenous microbiota were isolated. These colonies were identified as belonging to thirteen different bacterial genera, corresponding to seventeen species. Table 1 shows the bacterial species isolated from each portion of the digestive tract of *C. atratus* and their respective population level and frequency. The following bacteria were found: *Actinomyces bovis, Lactobacillus cellobiosus, Micrococcus luteus, Neisseria sicca, Staphylococcus epidermidis, Staphylococcus saprophyticus, Streptococcus pyogenes, Clostridium bifermentans, Clostridium* sp., *Enterobacter agglomerans, Escherichia coli,* metabolically inactive *Escherichia coli, Escherichia fergusonii, Peptostreptococcus* sp., *Sarcina* sp., *Serratia odorifera* and *Shigella flexneri*. Metabolically inactive *E. coli* is defined (5) as an immobile Gram-negative rod glucose positive and negative for H_2S production, urease, lysine decarboxylase, gas production in carbohydrate fermentation and sucrose fermentation

During the bacterial isolation procedures, two strains recovered from two birds and apparently presenting a single macroscopic appearance proved, after Gram staining, to consist in fact of two different bacteria. In the first case, the association found in the tongue of one bird was between *A. bovis* and *N. sicca*. The second case of association consisted of *A. bovis* and a Gram-negative unidentified rod found in the proximal intestine of a second vulture. These associations were considerably stable, resisting to various attempts of separation. In the second association, the microorganisms were so intimately dependent that dissociation led to the death of one of them.

Table 2 shows only positive results obtained from the antagonistic assays using the 45 isolates from the digestive tract of *C. atratus*. In these experiments, all bacterial isolates from the same portion of the digestive tract were used as producer and indicator strains, being tested with themselves and to three bacterial reference strains. A total of 25 antagonistic activities (subdivided into 20 heteroantagonisms, two isoantagonisms and three autoantagonisms) were observed in the assays. For the most part, the antagonistic activity was present in enterobacteria (48%), followed by staphylococci (32%), *A. bovis* (16%) and *M. luteus* (4%). The antagonistic activities of enterobacteria were principally concentrated in the stomach and in the proximal and medial intestines, while antagonistic activities of staphylococci were only found on the tongue and in the distal intestine. No bacterial isolates of the same species (*S. epidermidis, A. bovis, E. agglomerans, E. fergusonii, E. coli*) obtained from different portions of the digestive tract showed the same antagonistic spectrum.

DISCUSSION

The absence of a specific methodology for the study of the digestive microbiota of wild birds, especially the scavenger ones, led us to adopt a classic procedure used for the investigation of the microbiota from domestic birds and mammals (4,18). The controlled diet prescribed for the birds for

Table 1. Bacteria isolated from different portion of the digestive tract of *Coragyps atratus*.

Digestive tract portion	Bacteria	Log CFU/g (±SD)	Occurrence /6 birds	Original site
Tongue	*Actinomyces bovis*	6.56 ± 0.02	5	a, b, c, d, f
	Lactobacillus cellobiosus	6.78	1	b
	Micrococcus luteus	6.30	1	a
	Neisseria sicca	6.57	1	b
	Staphylococcus epidermidis	6.46 ± 0.04	5	a, b, c, e, f
	Staphylococcus saprophyticus	6.43 ± 0.04	5	a, b, c, e, f
	Streptococcus pyogenes	6.53 ± 0.01	2	c, f
Stomach	*Actinomyces bovis*	3.34 ± 0.34	2	c, e
	Clostridium bifermentans	3.78	1	b
	Clostridium sp	3.27 ± 0.06	2	b, d
	Enterobacter agglomerans	4.35 ± 0.13	3	c, e, f
	Escherichia coli inactive	3.11 ± 0.07	3	c, e, f
	Escherichia fergusonii	3.40 ± 0.03	2	c, d
	Peptostreptococcus sp	3.26	1	b
	Staphylococcus epidermidis	3.41 ± 0.02	3	b, c, d
	Streptococcus pyogenes	3.0	1	c
Proximal Intestine	*Clostridium bifermentans*	8.08	1	c
	Enterobacter agglomerans	8.6 ± 0.01	2	a, b
	Escherichia coli	7.89 ± 0.01	2	b, f
	Escherichia coli inactive	7.9 ± 0.03	2	b, d
	Escherichia fergusonii	8.39 ± 0.07	4	a, c, e, f
	Lactobacillus cellobiosus	7.18 ± 0.03	3	c, e, f
	Peptostreptococcus sp	8.60	1	d
	Sarcina sp	7.28	1	c
	Serratia odorifera	7.51	1	b
	Staphylococcus epidermidis	8.36 ± 0.04	3	c, e, f
Medial Intestine	*Actinomyces bovis*	10.08	1	c
	Clostridium bifermentans	9.95	1	b
	Enterobacter agglomerans	10.52 ± 0.09	4	b, c, d, f
	Escherichia coli	9.20 ± 0.09	4	b, d, e, f
	Escherichia fergusonii	10.53 ± 0.06	2	b, e
	Micrococcus luteus	10.08	1	c
	Peptostreptococcus sp	10.55 ± 0.04	2	c, d
	Shigella flexneri	10.08	1	c
	Staphylococcus epidermidis	10.19 ± 0.12	2	c, d
	Staphylococcus saprophyticus	10.38	1	d
Distal Intestine	*Actinomyces bovis*	11.36	1	d
	Clostridium sp	10.95	1	e
	Enterobacter agglomerans	11.21 ± 0.08	2	d, f
	Escherichia coli	10.27 ± 0.19	3	a, b, f
	Escherichia coli inactive	10.90 ± 0.01	2	b, e
	Escherichia fergusonii	10.54 ± 0.04	2	d, e
	Peptostreptococcus sp	10.52 ± 0.03	3	c, d, e
	Staphylococcus epidermidis	10.43	1	b
	Staphylococcus saprophyticus	10.53 ± 0.09	4	b, c, e, f

a = bird #1, captured in March 26th 1996 at BHZOO, Belo Horizonte / Minas Gerais;
b = bird #2, captured in July 18th 1996 at Fazenda da Lage, Ibiá / Minas Gerais;
c = bird #3, captured in August 30th 1996 at Fazenda da Lage, Ibiá / Minas Gerais;
d = bird #4, captured in January 31st 1997 at Garbage deposit in Nova Contagem, Contagem / Minas Gerais;
e = bird #5, captured in March 27th 1997 at Garbage deposit in Nova Contagem, Contagem / Minas Gerais;
f = bird #6, captured in May 06th 1997 at Garbage deposit in Nova Contagem, Contagem / Minas Gerais.

Table 2. Antagonism between bacteria isolated from different portions of the digestive tract of *Coragyps atratus* and against three reference strains.

Portion	Producer strain	Indicator strain (halo diameter in mm)	
		Isolated strain	Reference strain
Tongue	*S. epidermidis*	*A. bovis* (33)	*P.aeruginosa* (35)
		S. epidermidis (28)	*S. aureus* (41)
		S. saprophyticus (24)	*E. coli* K12 (29)
	A. bovis	*S. saprophyticus* (23)	*P. aeruginosa* (27)
	M. luteus	*S. saprophyticus* (18)	
Stomach	*E. agglomerans*	*E. agglomerans* (15)	
	E. fergusonii	*E. agglomerans* (34)	*S. aureus* (56)
		S. pyogenes (22)	*E. coli* K12 (51)
Proximal intestine	*E. agglomerans*		*P. aeruginosa* (29)
	E. coli inactive	*E. coli* (35)	
	E. fergusonii		*E. coli* K12 (26)
Median intestine	*E. coli*	*A. bovis* (26)	*E. coli* K12 (58
		M. luteus (61)	
Distal intestine	*A. bovis*		*E. coli* K12 (30)
	E. coli	*A. bovis* (57)	
	S. epidermidis	*S. saprophyticus* (29)	
	S. saprophyticus	*S. saprophyticus* (13)	

six days before sacrifice was important to eliminate the presence of allochthonous microorganisms normally associated with the ingestion of decaying animal carcasses, allowing the isolation of solely components of the autochthonous microbiota from the digestive tract.

The quantitative variation of the total population of microorganisms throughout the digestive tract was similar to that observed in the digestive tract of carnivores and other animals (14,16,17,18) with a higher concentration of microorganisms in the final portions of this tract. The lower level of the bacterial population in the stomach when compared to other birds and to non-human mammals (generally about 10^7-10^8 CFU/g of contents) was probably due to the highly acidic environment. The gastric contents have been shown to have a pH of the order of 1.0-2.0, which is a general feature of carnivorous birds. Only the pH of digesting stomach contents in the heron (*Ardea Cinerea*), kestrel *(Falco tinnunculus*) and barn owl (*Tyto alba*) ranges from 2.5 to 5.0. As observed in humans, the highly acidic conditions of the *C. atratus* stomach probably contribute to the breakdown of food and also constitute a potent barrier against microbial pathogens. The progressive increase of total microbial counts along of the small intestine may be explained by the simultaneous decrease of the intestinal transit flow from the duodenum to the ileum allowing the expansion of microbial populations.

Two aspects of the digestive microbiota of the American black vulture are unusual (Table 1). First, the high population levels of staphylococci which are apparent constituents of the dominant microbiota in all the portions of the digestive tract (until 10^{10} CFU/g in the distal intestine). These bacteria are sometimes present in the gastrointestinal microbiota, even in humans, but generally at lower population levels of about 10^3–10^4 CFU/g (4). Staphylococci are more frequently found in the cutaneous and upper respiratory tract ecosystems. The second unusual fact observed in this study was the presence of enterobacteria in the dominant populations from the stomach to the distal intestine. In other animals (all mammals and other birds), the enterobacteria generally belong to the sub-dominant populations (about 10 to 1000 fold lower than the dominant ones). However, in the few studies on the digestive microbiota (non-quantitative data obtained only for feces) of scavenger birds, enterobacteria were always isolated (6,19,20). Schlatter *et al.* (19), working with *C. atratus* in Chile, has also detected bacteria of the genera *Escherichia*, *Staphylococcus* and *Streptococcus*. Winsor *et al.* (20) also isolated and identified bacteria of the family *Enterobacteriaceae* from the intestinal microbiota of *C. aura*. The presence of enterobacteria in the digestive tract of domestic birds has been well described (1,2,9,14,18). The presence of a high population level of *S. flexneri* in the median intestine of one bird supports the importance of carrion-feeding birds in the spreading of bacterial pathogens as observed in other studies (6,19,20). Few anaerobic bacteria (*Peptostreptococcus sp.*, *Clostridium* spp., *Sarcina* sp.) were found in the digestive tract of *C. atratus* but when observed, these microorganisms reached high population levels. In spite of the use of a selective medium for *Bacteroides* (11), bacteria

from this genus were not isolated. The microbiota encountered in *C. atratus* demonstrated the similar presence of a large number of both Gram-negative rods and Gram-positive cocci. Among the bacteria isolated from the digestive tract of *C. atratus,* the following are reported for the first time: *A. bovis, L. cellobiosus, M. luteus, N. sicca, C. bifermentans, E. agglomerans, Peptostreptococcus* sp., *Sarcina* sp, *S. odorifera* and *S. flexneri*.

Among the 45 bacteria isolated from the digestive tract of *C. atratus,* thirteen (29%) showed a certain level of antagonism. Heteroantagonism was more frequently observed (80%), followed by autoantagonism (12%) and isoantagonism (8%). However, it is well known that the production of an antagonistic substance depends upon a number of physicochemical and nutritional conditions such as optimal temperature and pH and composition of the culture medium. The conditions for *in vitro* assay are far different from those found in the digestive ecosystems. Most of these antagonisms were performed by enterobacteria (48%) and were principally concentrated in the stomach and in the proximal and median intestines. The antagonism of Gram negative microorganims was oriented against other Gram-negative bacteria (58% of the case) and this fact was also observed for Gram-positive bacteria (61.5%). The fact that the antagonistic spectrum of the same bacterial species (*S. epidermidis, A. bovis, E. agglomerans, E. fergusonii, E. coli*) varied depending on the digestive portion of the isolation suggests that these microorganisms are different strains of the same species.

The analysis of the results presented in Table 2 furnish material for speculation about the eventual occurrence of one of more antagonistic substances produced by the isolated microorganisms, which would act in an auto-control of the bacterial population of the digestive tract of *C. atratus*, and also in maintaining the balance of the digestive ecosystem of the bird, regulating the inter- and intraspecific relations of the various bacterial members that compose the digestive tract microbiota of *C. atratus*. However, the antagonistic interrelationships observed *in vitro* must be confirmed *in vivo*.

As a final consideration, it is also important to remember that the microbial interactions are essential to the establishment and maintenance of any ecosystem. The presence of microorganisms capable of inhibiting the growth of other microorganisms in the digestive tract of the *C. atratus* raises the possibility of a potent barrier effect produced by the microorganisms present in the digestive tract of scavenger birds, which inhibits the implantation of colonies of pathogenic microorganism in such birds. These microorganisms may act as a factor that contributes to the notable resistance to pathogenic microorganisms and their toxins presented by vultures.

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RESUMO

Microbiota bacteriana dominante cultivável no trato digestivo do urubu *(Coragyps atratus* Bechstein 1793)

As bactérias anaeróbias estritas e facultativas cultiváveis do trato digestivo de seis urubus (*Coragyps atratus* Bechstein 1793) foram isoladas e identificadas. Após a captura, as aves receberam uma alimentação de baixa contaminação durante uma semana para eliminar possíveis microorganismos alóctonos. A seguir, amostras colhidas na língua, estomago e intestinos foram pesadas, submetidas a diluições decimais numa câmara anaeróbia, inoculadas em meios de cultura e incubadas em aerobiose e anaerobiose a 37ºC para enumeração, isolamento e identificação. As bactérias isoladas foram usadas posteriormente como produtoras e reveladoras para detectar possíveis fenômenos de antagonismo. A população bacteriana total ao longo do trato digestivo variou de 3,46 ± 0,39 log UFC/g no estômago até 10,75 ± 0,37 log UFC/g no intestino distal. Algumas bactérias foram isoladas pela primeira vez do trato digestivo de *C. atratus*: *Actinomyces bovis, Lactobacillus cellobiosus, Micrococcus luteus, Neisseria sicca, Clostridium bifermentans, Enterobacter agglomerans, Peptostreptococcus* sp., *Sarcina* sp., *Serratia odorifera,* and *Shigella flexneri*. Associações entre microorganismos foram observadas durante o isolamento em dois casos, um envolvendo *A. bovis* e *N. sicca,* e o outro envolvendo *A. bovis* e um bastonete Gram-negativo. Hetero-, iso- e autoantagonismos foram observados, sugerindo um papel ecológico para esses microorganismos em termos de autocontrole populacional e de barreira ambiental no trato digestivo dessas aves.

Palavras-chave: urubu, microbiota, trato digestivo, substância antagonista.

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