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Biochemical and Microbiological Evidence for Fermentative Digestion in Free-Living Land Iguanas (*Conolophus pallidus*) and Marine Iguanas (*Amblyrhynchus cristatus*) on the Galápagos Archipelago

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ABSTRACT

Herbivorous lizards are potentially capable of high digestive efficiency, but the presence of an indigenous microbial population has been implied from measurements of activity rather than directly studied. This study is the first to provide direct biochemical and microbiological evidence for fermentative digestion in free-living land iguanas (*Conolophus pallidus*) and marine iguanas (*Amblyrhynchus cristatus*) from the Galápagos archipelago. In marine iguanas, the stomach and large capacious colon contained ca. 32% and 60%, respectively, of the weight of total gut content. Total volatile fatty acid concentration was ca. 150 and 180 mM, respectively, for marine and land iguanas. Molar proportions of acetate, propionate, and butyrate (80.3%, 9.5%, and 3.5%) in land iguana fecal samples were similar to those for marine iguanas. Examination of fecal samples using confocal and transmission electron microscopy, as well as cultivable counts, revealed a dense and diverse population of bacteria, with spores prominent. Total culturable counts of anaerobes (2.22×10^8 g⁻¹ wet weight of fecal material) outnumbered aerobes on average by a factor of ca. 700. Combined, these results strongly support the contention that these unique herbivorous lizards are largely dependent on the presence and metabolic activities of a resident bacterial pop-

ulation in order to hydrolyze and ferment plant polymers that are indigestible to the host.

Introduction

Herbivory is relatively rare in reptiles being restricted to turtles and tortoises (order Chelonia) and the lizards (Troyer 1983; King 1996). Most herbivorous lizards belong to the order Iguania in the families Iguanidae (especially the subfamily Iguaninae) and Agamidae (King 1996). In the past, extremely successful reptilian herbivores such as dinosaurs and other ancient reptiles existed, and this raises the question as to why a successful and widespread group of animals such as lizards has not radiated more prominently into herbivorous niches. Is it because of thermoregulatory constraints as a consequence of being ectotherms or because they lack adaptive specializations required for herbivory and effective utilization of plant material? Evidence is accumulating that repeatedly shows that reptiles can be efficient and sophisticated herbivores. Adaptations for herbivory that have been suggested include large size and slow metabolic rate (Szarski 1962; Sokol 1967; Nagy 1982; Zimmerman and Tracy 1989; King 1996), scissor-like teeth for cropping vegetation (Throckmorton 1973; Mattison 1989), the presence of gastroliths to assist in mechanical disruption of feed (King 1996), as well as a large colon with anatomical adaptations to restrict outflow and long transit times through the intestinal tract (Iverson 1980, 1982).

Herbivorous lizards are potentially capable of high digestive efficiency, but this varies depending on diet, transit time, and other factors (Zimmerman and Tracy 1989; Van Marken Lichtenbelt 1991; reviewed by King 1996). The presence of an indigenous microbial population has been implied from measurements of activity (Foley et al. 1992) rather than directly studied. On the basis of studies that document the importance of anaerobic bacteria, fungi, and ciliate protozoa to digestion in other herbivores, principally mammalian herbivores and insects, their significance in herbivorous reptile digestion should be intuitive. However, to date, only one study measured hindgut bacterial populations in the green iguana (*Iguana iguana*). Colony counts ranged from three to 24×10^9 g⁻¹ hindgut content, and the dominant bacterial species were of the genera *Clostridium* and *Leuconostoc* (McBee and McBee 1982). It was es-

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timated that green iguanas obtain 30%–40% of their energy requirements from hindgut fermentation (McBee and McBee 1982). Microbial fermentation was estimated to contribute 47% of the digestible energy intake in the herbivorous agamid lizard *Uromastix aegypticus* (Foley et al. 1992).

Thus, these herbivorous lizards appear largely dependent on the presence and metabolic activities of a resident microbial population in order to hydrolyze and ferment plant polymers that are indigestible to the host (Prins and Kreulen 1991; Mackie et al. 1997, 2000a, 2000b). This study is the first to provide direct biochemical and microbiological evidence for fermentative digestion in free-living land iguanas (*Conolophus pallidus*) and marine iguanas (*Amblyrhynchus cristatus*) from the Galápagos archipelago.

Material and Methods

Animals and Sampling

A field trip to the Galápagos Islands of Santa Fe (Meido; 90°02'W, 0°50'S) and Caamaño (a small islet in Academy Bay, Santa Cruz; 90°17'W, 0°46'S) during January 2001 facilitated the collection of fresh samples from land and marine iguanas. Caamaño is easily accessible by boat from Charles Darwin Research Station (CDRS), Puerto Ayora, Santa Cruz. At the time, typical dry La Niña climatic conditions prevailed on the Galápagos. The sampling trip terminated a few days before the oil spill at Puerto Bacuerizo on the island of San Cristobal on January 16, 2001 (Wikelski et al. 2001).

Marine Iguanas (*Amblyrhynchus cristatus*). Fecal collection was carried out on the islands of Santa Fe ($n = 12$) and Caamaño ($n = 7$). Fresh fecal material was collected from individual marine iguanas at the time of defecation by scooping the combined feces and white uric acid deposit into a sterile 15-mL screw-cap tube. These collections were possible because of the tame nature of the lizards, enabling the sampler to stand within an aggregation of animals and observe as defecation occurred. These lizards were not noosed, and hence no animal measurements were made. In addition, the gut anatomy was examined and digesta from each major gut compartment obtained by retrieving two lizards after being preyed on by the Galápagos hawk (*Buteo galapagoensis*) on Santa Fe Island. Measurements of pH, gut length, and weight of the total and separate gut compartments were recorded after excising the gastrointestinal tract from each of the hawk kills.

Land Iguanas (*Conolophus pallidus*). Fresh fecal material was obtained from land iguanas ($n = 7$) on the island of Santa Fe after noosing the lizards. Individual lizards were weighed, and snout vent length (SVL) and temperature were recorded. Gentle tactile stimulation around the anal vent resulted in release of flatus followed by defecation of large, leafy, cigar-shaped pellets that were collected directly into sterile 50-mL screw-cap tubes.

Sample Treatment. Each fecal and ingesta sample was divided into one untreated portion for microbiological analysis, an acid-preserved sample (5.0 g material + 5.0 mL 1.0 M HCl) for analysis of ammonia, and an alkali-preserved sample (5.0 g material + 5.0 mL 1.0 M NaOH) for individual and total volatile fatty acid (VFA) analysis (Mackie et al. 1978). For some samples, material was preserved with formol-saline or added to Karnovsky's fixative for confocal and electron microscopy. All samples were stored at 4°C in a portable solar-powered refrigerator on the island of Santa Fe and during storage in the laboratory at CDRS. The samples were then transported by hand back to the laboratories at the University of Illinois on ice in a cooler box.

Microbiological Analysis

Methanogens were detected qualitatively by examination of wet mounts on glass slides using fluorescence microscopy for specific F_{420} fluorescence (Doddema and Vogels 1978). Direct microscopic counts were carried out on air-dried, crystal violet-stained Reichl slides spotted with 20- μ L aliquots of the decimal dilutions (10^{-2} and 10^{-3}) prepared for cultivable enumeration as described by Mackie and Wilkins (1988). Electron microscopy was carried out on material preserved in Karnovsky's fixative. On return to the laboratory, the fixed material was treated with 1% (w/v) OsO_4 and then dehydrated and embedded in Epon 812 epoxy resin. Thin sections were viewed and photographed under a JEOL 100C electron microscope at 80 kV. Confocal scanning laser microscopy was performed using an Olympus BX50 Confocal Microscope and $\times 60$ objective lens. Confocal illumination was provided by a Kr/Ar laser fitted with a long-pass 560–600-nm filter (red fluorescence signal). The fluorescent image was overlaid on the diffraction image using Version 3.0.30 Fluoview software.

Fecal counts of marine iguanas for total culturable anaerobes, total aerobes, and selected polysaccharide degraders were performed using an anaerobic chamber (Coy Laboratory Products, Ann Arbor, Mich.) and routine medium preparation protocols in our laboratory for analysis of gut microbiota as described previously (Mackie et al. 1978; Meyer and Mackie 1986; Mackie and Wilkins 1988). Briefly, subsamples (1.00 g wet weight) of iguana feces or macrophytic algae were diluted 1 : 10 with cold anaerobic diluent and shaken with sterile glass beads. Each sample was serially diluted, and the highest dilutions (10^{-5} through 10^{-7}) were used to inoculate nutrient agar plates designed for enumeration of total aerobes, total anaerobes, and a variety of selective media for polysaccharide-degrading bacteria. Plates were incubated for 48 h (aerobes) or 5–7 d (anaerobes) at 37°C (iguana body temperatures range from 20° to 40°C, depending on thermal environment, but are generally 35°–38°C when basking in the sun; Christian and Tracy 1983; Wikelski et al. 1993, 1997). After enumeration of colonies, isolates from the highest dilutions showing growth were picked

onto maintenance slants and examined microscopically for purity and later characterization.

Biochemical Analysis

Fecal and compartmental pH on individual animals was measured on fresh material or in situ, respectively, with a portable meter. Fecal and ingesta samples were preserved for analysis of VFA and NH_3 with 1 M NaOH and 1 M HCl, respectively, and transported back to the laboratory at the University of Illinois. Total and individual VFAs were determined using gas chromatography (Erwin et al. 1961) and ammonia by the phenol-hypochlorite method (Chaney and Marbach 1962).

Results

Digestive Tract Morphology

The two marine iguanas recovered from hawk kills on Santa Fe provided the only opportunity to examine and describe gut anatomy and morphology (Fig. 1). The large, tubular stomach was thin walled and distended with macrophytic algae (W1, 100% red [*Gelidium* and *Centroseras* sp.] algae; W2, 95% red and 5% green [*Ulva* sp.] algae). The stomach was muscular in

its posterior (pyloric) region and contained a collection of gastroliths (17 in W1 and five in W2; Table 1). The small intestine was relatively short. The hindgut comprised a small dilatation or "cecal bulb" by the ileocolonic junction followed by a large, capacious colon (Fig. 1, right). The colon was partitioned into as many as 10 or more segments by transverse mucosal folds (Fig. 1, right). The extreme posterior of the colon was constricted into a bulblike cloaca. A few nematodes were found in the colon of lizard W1. Both lizards were in good body condition (body condition index = 44.5 and 48.1; Table 1) with deposits of abdominal fat.

Morphometric Analysis of Gut Length, Weight, and Content

When total length of intestine was expressed as a percentage of SVL, the values were 327% for lizard W1 and 233% for lizard W2. Average values for the stomach, small intestine, and colon were 46%, 113%, and 124% of SVL for the two lizards (Table 1). The total weight of gut content was 37.5% and 23.8% of empty body weight (total body weight minus gut plus its digesta content). The length of the stomach, small intestine, and colon as a percentage of total gut length was 13% and 21.4%, 42.6% and 37.1%, and 45.4% and 42.9% for lizards W1

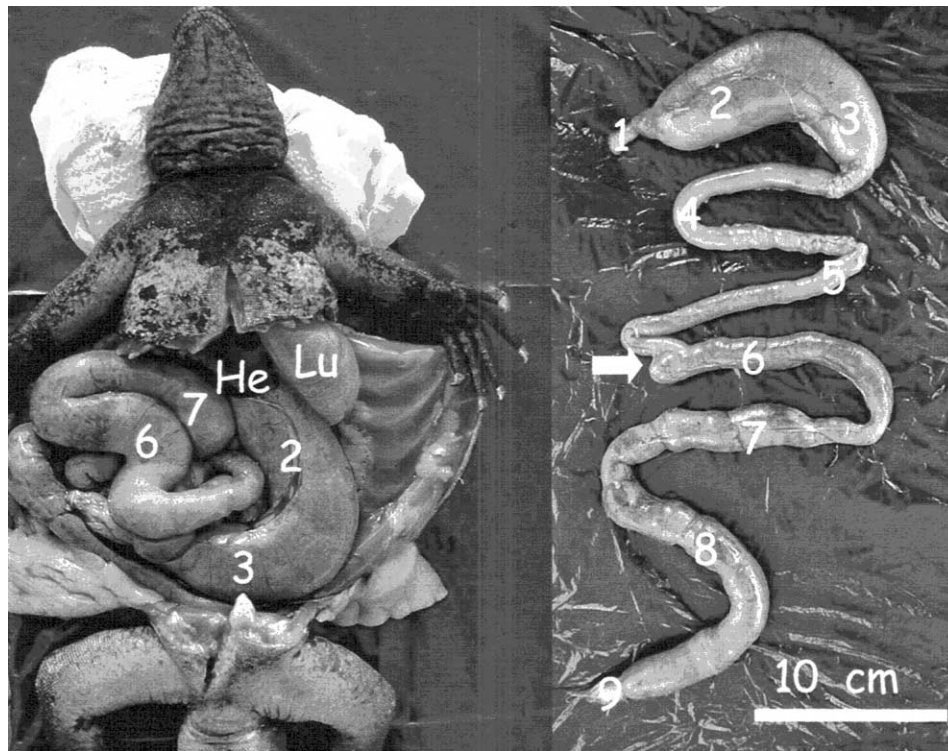


Figure 1. Gastrointestinal topography (left) and gut anatomy (right) of marine iguana (*Amblyrhynchus cristatus*). The specimen was obtained by retrieving a hawk kill. Organs and gut compartments are labeled as follows: He = liver; Lu = lung; 1 = esophagus; 2 = stomach (pyloric region); 3 = stomach (pyloric region containing gastroliths); 4 = small intestine (proximal); 5 = small intestine (distal); 6 = colon (proximal); 7 = colon (middle); 8 = colon (distal); 9 = cloaca; arrow = cecal bulb.

Table 1: Morphometric analysis of gut length, mass, and content of two marine iguanas obtained by retrieving hawk kills

Measurement	W1	W2
Total body mass (kg)	1.60	1.30
Snout vent length (cm)	33	30
Body condition index	44.5	48.1
Total gut length (cm)	108	70
Total gut weight (g)	420	250
Stomach:		
Length (cm)	14	15
Weight (g)	126	83
Gastroliths (<i>n</i>)	17	5
Mean L × W (mm)	12 × 8	13 × 8
Weight (g)	15.9	3.6
Small intestine:		
Length (cm)	46	26
Weight (g)	21	23
Colon:		
Length (cm)	49	30
Weight (g)	273	130

Note. Body condition index was calculated as (body mass/snout vent length³) × 10⁶.

and W2, respectively. In contrast, the percentage of the weight of gut content in each of these gut compartments was 30.0% and 33.2%, 5.0% and 9.2%, and 65.0% and 52.0% for lizards W1 and W2, respectively.

Biochemical Evidence for Digestion in Land and Marine Iguanas

Fecal concentrations of metabolites for free-living marine and land iguanas on the island of Santa Fe and for free-living marine iguanas on the island of Caamaño are reported in Table 2. For marine iguanas, the total concentration of VFA obtained as the sum of individual VFA was 134.6 mM for Santa Fe and 167.5 mM for Caamaño. Values for pH, which were 6.2 and 5.8 for the fecal collections from the two islands, reflected these acid concentrations. Analysis of individual VFA confirms the presence of all the end products normally found in analysis of mammalian gut fermentations. Acetate concentrations were highest (94.3 and 143.6 mM for the two sites), followed by propionate (11.1 and 16.9 mM) and butyrate (5.7 and 3.0 mM). Interestingly, concentrations of C₄-C₅ branched- and straight-chain VFA (isobutyrate, isovalerate + 2-methyl butyrate, valerate) were all relatively high when compared with mammalian gut fermentations, ranging from 3.0 to 5.5 mM. The ratio of C₂/C₃ was 8.5 for the marine iguana sites as well as the land iguana sample set. For land iguanas sampled on the island of Santa Fe, total VFA concentration was higher (178.8 mM) than that of marine iguanas from the same island. Molar proportions

of acetate, propionate, and butyrate (80.3%, 9.5%, and 3.5%) in land iguana fecal samples were similar to those for marine iguana. Also, concentrations of branched-chain C₄-C₅ VFA were high (4.3 and 5.2 mM).

Analysis of pH, VFA, and ammonia concentrations in gut compartments of the two marine iguanas provide further biochemical evidence for fermentative microbial digestion (Fig. 2). Values for pH were lowest in the pyloric region of the stomach and increased to ca. 6.0 in the small intestine. The highest pH value (6.23) was recorded in the distal colon (CO 3) of lizard W1 (Fig. 2A). Total and individual concentrations of VFA were lowest in the stomach and increased dramatically in the colon, with concentrations of total VFA in the range of 200–350 mM in segments CO 1 and CO 2 (Fig. 2B). A similar pattern was found for NH₃-N concentrations except that concentrations increased to 10–12 mM in the last two colonic segments as VFA concentrations decreased (Fig. 2C). Several large peaks on these gas chromatograms remain to be identified, and thus total concentrations and molar proportions of VFA are preliminary at this stage. Insufficient sample obtained from the two small intestinal segments precluded metabolite analysis from these sites.

Microbiological Evidence for Digestion in Marine Iguanas

Examination of fixed samples using confocal microscopy revealed a dense and diverse population of bacteria, with spores prominent (Fig. 3C, 3D). A diversity of marine diatoms, many still intact, was also evident in the stomach and fecal material from marine iguanas (Fig. 3A, 3D). No visual evidence for the presence of characteristic ciliate protozoa was seen, but morphological forms similar to chytridiomycete fungi were observed in both land and marine iguana fecal material (Fig. 3B). Methanogenic bacteria were present in wet mounts of fresh fecal material from both land and marine iguanas on the basis of characteristic greenish yellow F₄₂₀ fluorescence.

Electron microscopic examination of fecal material preserved in Karnovsky's fixative was carried out, and representative examples of these observations are presented in the micrographs of thin sections (Fig. 4). In general, electron microscopy revealed bacteria with differing morphology with many spores in the sections (Fig. 4). In addition, zones of hydrolysis surrounding algal leaf blades were clearly visible (Fig. 4B). Cross sections of marine diatoms can also be seen in the electron micrographs (Fig. 4D).

The colony counts of different functional groups of culturable bacteria in marine iguana feces obtained on the island of Santa Fe are presented in Table 3. The total culturable counts of anaerobic bacteria averaged 2.22×10^8 g⁻¹ wet weight of fecal material (Table 3). The direct microscopic counts averaged 5.85×10^9 g⁻¹ wet weight, indicating that as little as 3%–7% of the total bacteria has been cultured using media developed for enumeration of indigenous gut bacteria in the rumen eco-

Table 2: Fecal metabolite concentrations (mM; and volatile fatty acid molar proportions) of free-living land (*Conolophus pallidus*) and marine (*Amblyrhynchus subcristatus*) iguanas on the islands of Santa Fe and Caamaño, Galápagos archipelago

Metabolite	Marine	Molar (%)	Land	Molar (%)
Santa Fe:				
Acetate	74.3 ± 38.3	70.1	143.6 ± 27.2	80.3
Propionate	11.1 ± 3.5	8.2	16.9 ± 5.8	9.5
Isobutyrate	3.6 ± 2.2	2.7	4.3 ± 1.2	2.4
Butyrate	5.7 ± 2.9	4.2	6.3 ± 4.3	3.5
Isovalerate	3.0 ± 1.5	2.2	5.2 ± 4.2	2.9
Valerate	4.5 ± 2.7	3.3	2.3 ± 2.1	1.3
Total VFA	134.6 ± 17.9	...	178.8 ± 35.0	...
NH ₃ -N	4.3 ± 2.1		1.8 ± 1.3	
pH	6.2 ± .3		7.4 ± .4	
Caamaño:				
Acetate	129.0 ± 20.4	77.0		
Propionate	12.9 ± 2.1	7.7		
Isobutyrate	5.0 ± 2.5	3.0		
Butyrate	10.6 ± 2.6	6.3		
Isovalerate	5.5 ± 1.1	3.3		
Valerate	4.4 ± 2.6	2.6		
Total VFA	167.5 ± 18.9	...		
NH ₃ -N	1.0 ± .6			
pH	5.8 ± .3			

Note. Values are reported as mean ± SD g⁻¹ wet weight fecal material. For Santa Fe marine population, *n* = 12, and for land population, *n* = 7. For Caamaño marine population, *n* = 7.

system. Total culturable counts of anaerobes outnumbered aerobes on average by a factor of 687 (range 334–1,048). Total viable anaerobic and aerobic counts on algal leaf material collected have not been determined accurately but were less than the lowest dilution plated (10⁻⁵) in the experiments.

Numbers of anaerobic polysaccharide-degrading bacteria ranged from 1.5 to 10 × 10⁷ and 6.7 to 50 × 10⁶ g⁻¹ wet weight fecal material for guar gum and agar-degrading bacteria (Table 3). On average, these numbers constitute 25.8% and 6.8% of the total anaerobic count for guar gum and agar degraders. Examination of the zones of hydrolysis on the agar plates showed two distinct forms, and it was decided to enumerate agarose degraders in feces of two iguanas. These were approximately threefold lower than agar degraders on average. Also, because marine macrophytic algae are highly sulfated, counts of sulfate-reducing bacteria were determined on the same two fecal samples with lactate as the added carbon and energy source to the medium. Counts were ca. 3 × 10⁷ g⁻¹ fecal material on this medium. However, colonies (or their centers) did not turn black, indicating sulfate reduction by the isolated colonies, although cream- to white-colored colonies were easily visible and could be enumerated. Thus, these counts are reported as lactate-utilizing bacteria until this issue is resolved. Numerous

isolates were made from the various media but have not been systematically purified and characterized to date. However, microscopic examination of a number of the isolates indicates a high proportion of *Clostridium* spp. among the predominant isolates. McBee and McBee (1982) reported that the dominant fecal bacterial species in three green iguanas were of the genus *Clostridium*, whereas in the other eight iguanas examined, the dominant bacteria were of the genus *Leuconostoc*.

Discussion

Most herbivorous mammals that subsist on plant fiber depend on a symbiotic association with a complex microbial population resident in a specialized portion of the digestive tract (McBee 1977; Mackie et al. 1997). This complex microbial population breaks down plant cell wall material and nonstarch polysaccharides that are indigestible to the host herbivore. During this anaerobic fermentation process, volatile fatty acids are produced, microbial protein and vitamins are synthesized, and other nutrients are supplied, thus providing energy and nutrients for the host that it could not obtain directly from its food. In return, the host provides the microbial population with substrate supply and end product removal, as well as conditions

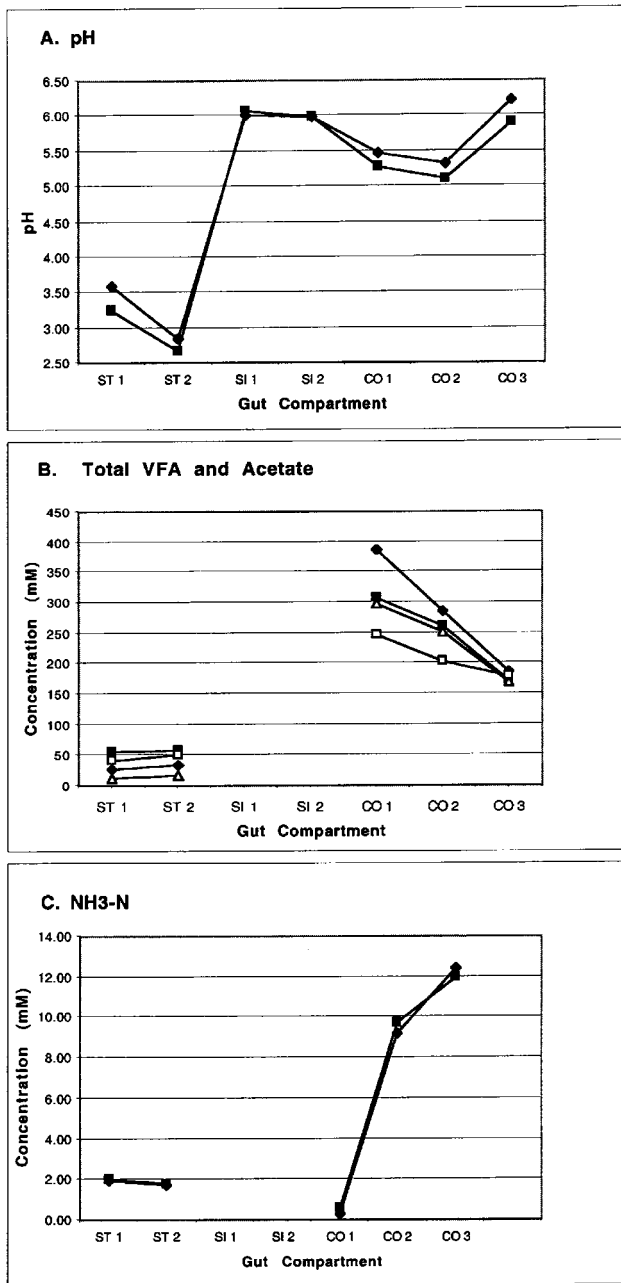


Figure 2. Measurements of pH (A), total volatile fatty acid (VFA) and acetate concentration (B), and NH₃-N concentration (C) in gut compartments of two marine iguanas (diamonds, W1; filled squares, W2). Total VFA (diamonds, W1; filled squares, W2) and acetate (triangles, W1; open squares, W2) and NH₃-N were not measured in the small intestine because of insufficient sample. Gut compartments are as follows: ST 1 = stomach (pyloric region); ST 2 = stomach (pyloric region containing gastroliths); SI 1 = small intestine (proximal); SI 2 = small intestine (distal); CO 1 = colon (proximal); CO 2 = colon (middle); CO 3 = colon (distal).

of moisture, temperature, and pH that are close to optimal for growth. Anaerobic conditions are created by the metabolic activity of the microbes themselves (Hungate 1985).

Herbivory in lizards is rare, and only approximately 2% of all lizard species are truly herbivorous (Pough 1973; Zimmerman and Tracy 1989). The majority of herbivorous lizards occur in two families, the Agamidae and Iguanidae. The most well studied of the iguane lizards is the green iguana (*Iguana iguana*), which lives solely on a herbivorous diet throughout its life (Rand 1978; Iverson 1982; Troyer 1984a). Indeed, some of the correlates required for fermentative digestion have been described for green iguanas, namely, a large, partitioned colon (Iverson 1982) populated by anaerobic bacteria (McBee and McBee 1982; Troyer 1982); studies demonstrate that green iguanas can digest 54% of cell wall constituents (Troyer 1984b) and supply 30%–40% of their energy budget from hindgut fermentation (McBee and McBee 1982). Microbial fermentation was estimated to contribute 47% of the digestible energy intake in the large, desert-dwelling, herbivorous agamid lizard *Uromastyx aegypticus* (Foley et al. 1992). In addition, ¹⁴C was detected in respired air from *U. aegypticus* following oral doses of ¹⁴C cellulose, confirming not only that cellulose was digested but also that the lizards gained oxidative energy from cellulose degradation. However, this research area of herbivory in lizards remains largely unexplored. An additional limitation to the study of herbivorous reptiles under natural or free-living conditions is that many species are endangered in their native habitats, so collection of adequate samples for examination of the digestive tract, its contents, and function is difficult. A field trip to the Galápagos Islands of Santa Fe and Caamaño during January 2001 facilitated the collection of fresh fecal samples from free-living land and marine iguanas and provided a rare opportunity to study biochemical and microbiological evidence for fermentative digestion in these unique herbivorous lizards.

The proportional capacities of the total digestive tract, the stomach, and small intestine and the colon or fermentative compartment for *A. cristatus* are compared with values for *I. iguana* and mammalian herbivores in Table 4. Proportional capacity was expressed as the ratio of organ content to empty body mass. The total gut capacity of *A. cristatus* represents 30.7% of its body mass and 17.0% for the colon for the two lizards available for study. These values are a little higher than those reported for *I. iguana* (18.5% and 11.8%; Troyer 1984b). These values are similar to those reported for ruminants and large nonruminants (elephants, horses, warthogs, and capybara) and larger than the relative gut capacities of small nonruminants (rabbits, guinea pigs, and voles; Parra 1978). Thus, marine iguanas are equipped with a capacious colon or a fermentative compartment that contains a large proportion of the total content within the intestinal tract. In addition, a large proportion of the total gut content is contained in the stomach, which serves as a storage compartment for the macrophytic

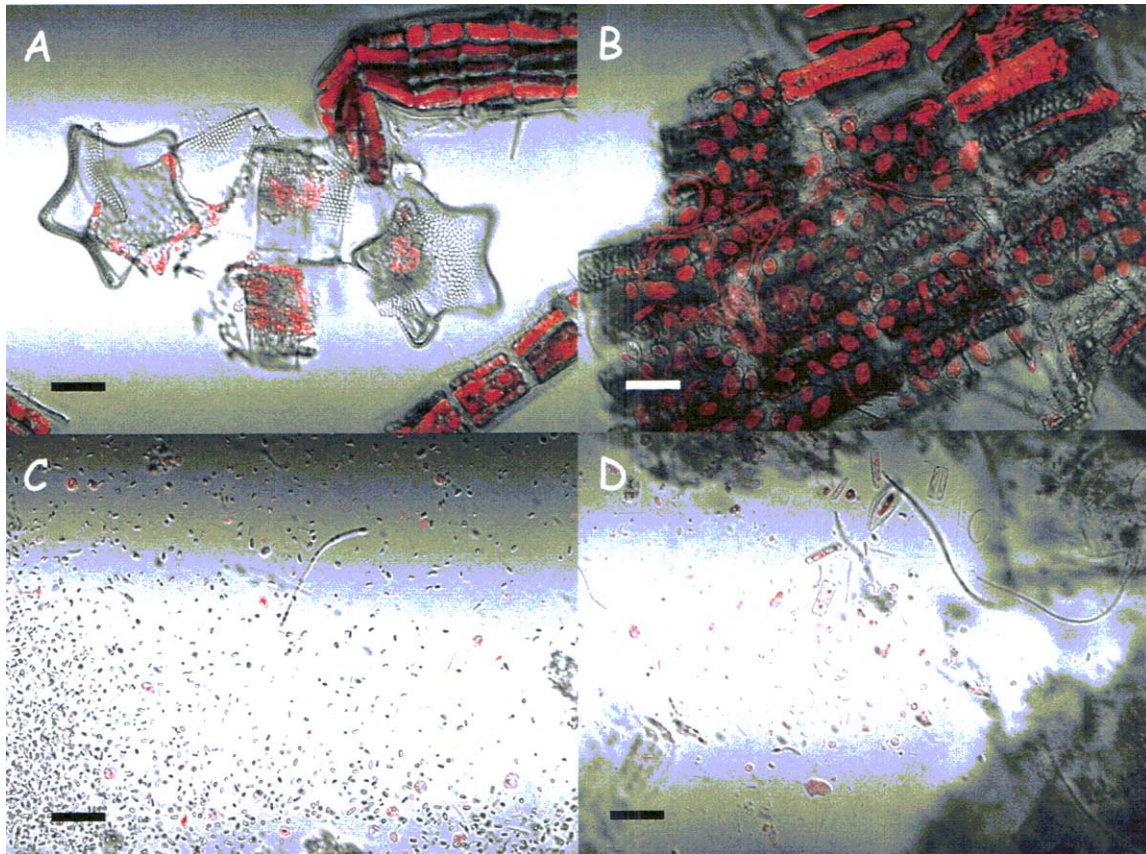


Figure 3. Confocal scanning laser microscopic images of stomach (*A, B*) and colonic (*C, D*) samples obtained from marine iguana W1 preserved in Karnovsky's fixative. Stomach samples demonstrated a high proportion of intact macrophytic algal material (*B*) and marine diatoms (*A*). In contrast, the colonic samples showed a dense and diverse population of bacteria with spore formers evident (*C*) as well as the presence of marine diatoms and degrading amorphous plant material (*D*). Red fluorescent material in macrophytic algae and diatoms represents chlorophyll, which is prominent in the stomach (*A, B*) but is largely degraded in the colon and feces (*C, D*). Magnification was $\times 600$ for all four fields, and scale bars on the figures represent $10 \mu\text{m}$.

algae collected during short but intense feeding periods at low tide (Wikelski and Trillmich 1994).

Gastroliths were present in the pyloric region of the stomach in both the marine iguanas sampled. These gastroliths numbered 17 and five and weighed 15.9 and 3.6 g in lizards W1 and W2. Gastroliths are thought to participate in further trituration of plant material, and our discovery confirms previous anecdotal records (Eibl-Eibesfeld 1956) of their presence in marine iguanas. Their presence has not been reported in other contemporary iguanine lizards. However, deliberate consumption of stones and sediment has been reported for crocodiles, lizards, and turtles (Sokol 1971). Geophagy has been observed in several iguanines, notably *Iguana iguana* and *Ctenosaura pectinata*, as well as in *Gopherus agassizii*, *Testudo hermannii*, and *Testudo elephantopus* (Rick and Bowman 1961; Sokol 1971). It has been suggested that herbivorous dinosaurs swallowed large stones that collected in a gizzard-like compartment for grinding of masticated plant material (Bakker 1980). Such stomach

stones or gastroliths have been reported from the gut (or nearby) of prosauropod, sauropod, and ornithomimid dinosaur fossils (referenced in Farlow 1987). Although there has been controversy over how widespread the use of gastroliths was in dinosaurs, Farlow (1987) suggests that this was a common practice.

Metabolite levels (both VFA and NH_3) represent the net result of production and utilization. When the rate of production exceeds utilization, concentrations increase and vice versa. VFAs are produced as end products of the anaerobic fermentation of carbohydrates by the resident microbiota in the gastrointestinal tract. The highest levels were recorded in the proximal colon (Fig. 2*B*, CO 1) and declined in the mid-colon and rectum (CO 2, CO 3), indicating that VFAs are produced and absorbed in the hindgut of *A. cristatus*. Levels of VFA in the stomach and presumably the small intestine, for which insufficient sample was available, were low, indicating that little digestion of structural carbohydrates occurs in these

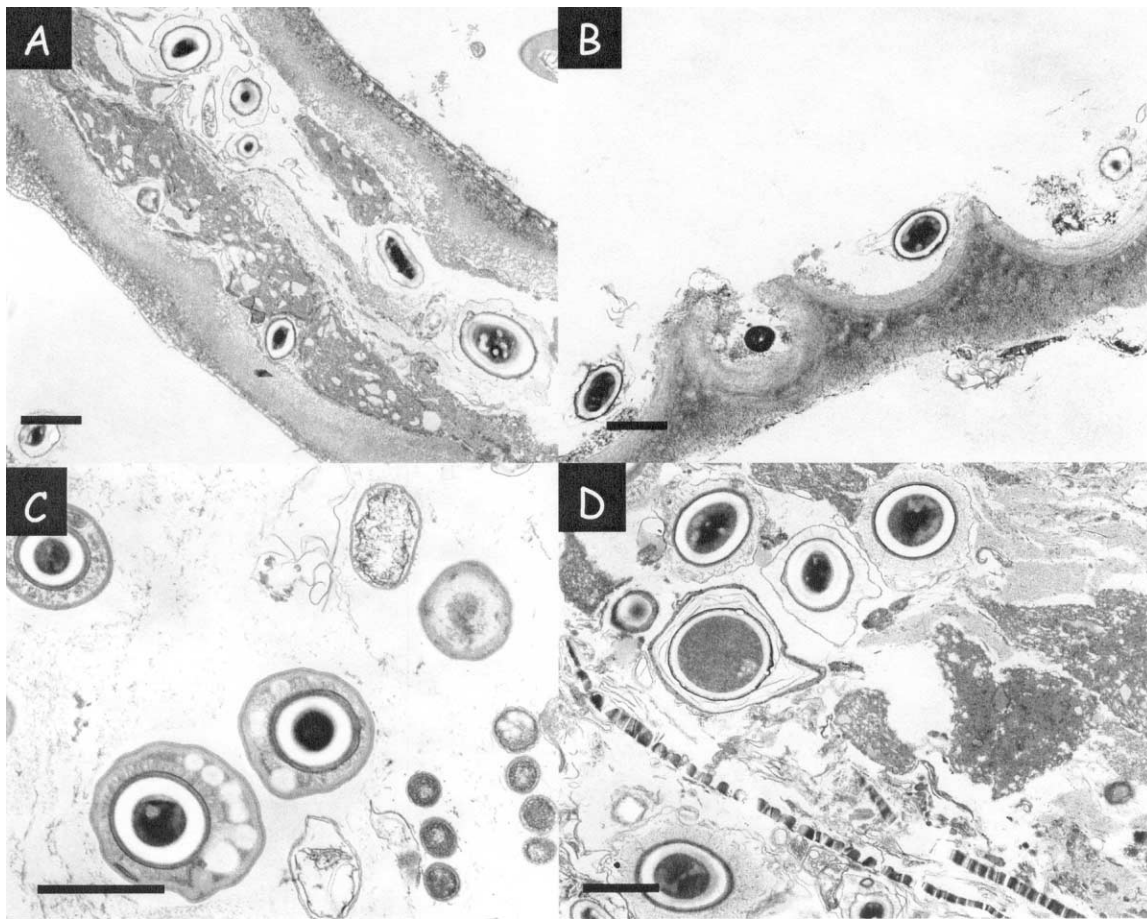


Figure 4. Transmission electron micrographs showing spore-forming bacteria in algal leaf material (A) with hydrolysis and digestion initiated from inside the plant material. Zones of hydrolysis (B) can clearly be seen surrounding spore-forming bacteria on the degrading plant material. C, D, Cross sections of plant material containing spore-forming bacteria. Magnification was $\times 8,000$ for A and B, $\times 10,000$ for D, and $\times 15,000$ for C. Scale bars represent $2 \mu\text{m}$.

segments (Fig. 2). Troyer (1984b) reported similar patterns of VFA levels in the gastrointestinal tract of *I. iguana*. The pattern for pH along the gastrointestinal tract increased from 2.5–3.0 in the stomach to 6.5–7.0 in the small intestine. The values in the colon decreased concomitantly with the production of VFA in this segment of the gut. Ammonia (expressed as $\text{NH}_3\text{-N}$) is produced by deamination of protein, peptides, and amino acids from dietary and endogenous sources as well as from urea and can be used as a biochemical index of fermentative digestion. The pattern of $\text{NH}_3\text{-N}$ concentrations in different gut compartments is consistent with this, that is, low in the stomach and increasing in the colonic segments, with the highest concentration in compartment CO 3.

Considerable evidence in support of microbiological indices of fermentative digestion has been obtained from this study. Observations under the light and confocal microscope demonstrated a dense and diverse population of bacteria in fecal samples from both marine and land iguanas. Morphological

forms similar to anaerobic chytridiomycete fungi commonly found in the intestinal tract of a range of herbivores were observed in low numbers. Ciliate protozoa typically found in fore-gut fermenters, especially ruminants, were not observed. Methanogenic bacteria can be identified by fluorescence microscopy of the F_{420} fluorescence, a unique coenzyme of methanogenesis. Interestingly, F_{420} fluorescing bacteria were observed in fecal material from both lizard species, with the numbers being higher in land than in marine iguanas. Hackstein and Van Alen (1996) included two zoo specimens (*I. iguana* and *Iguana delicatissima*) in their screen of 253 vertebrate species for methane emissions and detected high levels (ca. 300 nmol/g/h) of methane release from fecal material. These values were as high as or higher than those of most mammalian herbivores included in the survey.

Further examination of fecal samples from marine iguanas using electron microscopy confirmed the light and confocal microscopic observations concerning density and diversity of

Table 3: Direct microscopic and colony counts of different functional groups of culturable bacteria in fresh marine iguana feces obtained on the island of Santa Fe

Marine Iguana (no.)	Total		Polysaccharide Degraders			Lactate Utilizers	Direct Microscopic Count
	($\times 10^8$)	($\times 10^5$)	Guar Gum ($\times 10^7$)	Agar ($\times 10^6$)	Agarose ($\times 10^6$)	($\times 10^7$)	($\times 10^9$)
3	1.12	2.53	2.8	6.7	3.94
11	1.67	5.00	1.5	6.0	7.32
5	2.50	3.27	8.0	9.0	8.64
14	2.65	3.44	7.0	8.6	6.22
9	2.75	2.65	5.0	9.8	3.5	3.75	5.12
13	2.60	2.48	10.0	50.0	5.5	2.27	3.88
Mean \pm SD	2.22 \pm .66	3.23 \pm .96	5.72 \pm 3.23	15.0 \pm 17.2	4.5	3.0	5.85 \pm 1.91

Note. Counts are expressed per gram of wet weight fecal material.

the indigenous bacterial populations. Several other notable features were the presence of large numbers of spore-forming bacteria and visual evidence of the digestion of the macrophytic algal leaf blades, as evidenced by zones of hydrolysis surrounding spore-forming bacterial cells. The presence of high numbers of guar gum and agar-hydrolyzing bacteria was significant and demonstrates a direct selection for the indigenous bacterial population by diet containing a high proportion of dietary fiber (33%–75%, consisting of 20%–60% soluble polysaccharides; Fleury and Lahaye 1991; Lahaye 1991; Lahaye et al. 1993; Jimenez-Escrig and Cambrodon 1999).

Direct microscopic counts of bacterial cells in fecal samples from marine iguanas ranged from 3.9 to 8.6×10^9 g⁻¹ wet weight fecal material. These numbers are similar to the counts reported for *I. iguana* (3.3 to 23.5×10^9 g⁻¹ of content and 30×10^9 g⁻¹ for direct microscopic counts; McBee and McBee 1982). Total culturable anaerobic bacterial counts represented 2.3%–6.7% of the direct count. This is at the low end of the normal range for percentage of culturable bacteria. However,

this phenomenon, variously referred to as “cultivation bias” or “the great plate count anomaly,” could be due to a number of factors. The cultural conditions employed in these experiments were based on standard laboratory protocols adequate for growth of anaerobic bacteria from mammalian gut ecosystems where viable culturable counts range from 10% to 30% of the total direct microscopic count and may not be optimal for fecal anaerobes from marine iguanas. This could be improved by developing more specific habitat simulating media for this gut ecosystem on the basis of physicochemical measurements initiated in this article. However, this is only a partial solution because the two major problems faced by microbial ecologists studying the gastrointestinal community are the inevitable bias introduced by culture-based enumeration and characterization techniques and the lack of a phylogenetically based classification scheme (Raskin et al. 1997; Mackie et al. 2000a, 2000b). Indeed, most of our knowledge of gut bacterial communities has been derived using indirect microbiological techniques such as selective plate counts, selective enrichment, pure culture isolation,

 Table 4: Relative capacities of the digestive tract and fermentation compartments in *Amblyrhynchus cristatus*, *Iguana iguana*, and mammalian herbivores (after Parra 1978; Troyer 1984a)

Herbivore	Empty Body Mass (%)			
	Total Content	Stomach and Small Intestine	Cecum and Large Intestine	Rumen
Ruminants, all sizes	17.2	2.6	...	14.6
Nonruminants:				
Large	15.4	3.2	11.4	...
Small	7.6	2.9	4.7	...
Herbivorous lizards:				
<i>I. iguana</i>	18.5	8.7	11.8	...
<i>A. cristatus</i>	30.7	12.9	17.0	

and most probable number estimates. It is now recognized that a genotypically based classification scheme that also reflects natural evolutionary relationships is desirable when describing the bacterial community inhabiting the intestinal tract and feces. The application of nucleic acid (DNA and RNA)-based techniques can be used to detect, identify, and quantify bacterial populations in the gut and overcome detection and classification problems. Phylogenetic approaches enable identification and classification of bacterial, archaeal, and eukaryotic diversity as well as provide a link between phylogenetic and functional diversity. Thus, in the future, we propose to use a combination of classical culture-dependent and culture-independent molecular techniques to analyze microbial populations in fecal samples obtained from the endemic land iguanas (*Conolophus pallidus* and *Conolophus subcristatus*) and marine iguanas (*Amblyrhynchus cristatus*) of the Galápagos Islands and link microbiological results to the well-described ecology of these sister taxa (Rassmann 1997; Rassmann et al. 1997).

The research presented in this article clearly shows that marine iguanas are unique reptilian herbivores with a large colonic gut compartment specialized for fermentative digestion. The long retention time (7–10 d depending on body size and thermal environment; Wikelski et al. 1993, 1997; Wikelski and Wrege 2000) of nonstarch polysaccharides contained in the macrophytic algae that comprise the sole diet of these herbivorous lizards ensures that these substrates are exposed to bacterial digestion for extended times, allowing efficient degradation of alginates, laminarans, carrageenans, agar, sulfated fucans, and mannuronates indigestible to the host animal. The presence of a dense and diverse anaerobic bacterial microbiota in the hindgut results in the production of VFAs, mainly acetate, which contribute to the metabolizable energy requirements of *A. cristatus*. Fecal VFA and NH₃-N data for land iguanas strongly suggests that a similar strategy for fermentative digestion occurs in *C. pallidus*. However, in the case of the land iguana, the diet is more fibrous and would rely on extensive degradation of plant cell wall polymers by bacteria-hydrolyzing cellulose and hemicellulose (Christian et al. 1984). These results provide fascinating insight into the nutritional ecology of these herbivorous lizards found only on the Galápagos archipelago.

Our discovery of the prevalence and importance of microbial fermentation in Galápagos marine iguanas may also provide a mechanistic basis for the stress and mortality imposed on marine iguana cycles during natural and anthropogenic events such as El Niño–southern oscillation (Cooper and Laurie 1987; Laurie 1990; Laurie and Brown 1990a, 1990b; Romero and Wikelski 2001) and the recent oil spill (Wikelski et al. 2001, 2002). We hypothesize that after intestinal oiling, the reduction of gut bacterial populations and possible reduction in food intake, coupled with the associated decrease in digestive efficiency, are largely responsible for the >60% mortality in the Santa Fe marine iguana population (Wikelski et al. 2002). Although this hypothesis is currently untested, it highlights the

importance of studies directed at a mechanistic understanding of microbial digestion for survival and conservation in wild animal populations.

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