

## Mutualistic Fermentative Digestion in the Gastrointestinal Tract: Diversity and Evolution<sup>1</sup>

RODERICK I. MACKIE<sup>2</sup>

*Department of Animal Sciences and Division of Nutritional Sciences, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801*

**SYNOPSIS.** All animals, including humans, are adapted to life in a microbial world. Anaerobic habitats have existed continuously throughout the history of the earth, the gastrointestinal tract being a contemporary microniche. Since microorganisms colonize and grow rapidly under the favorable conditions in the gut they could compete for nutrients with the host. This microbial challenge has modified the course of evolution in animals, resulting in selection of complex animal-microbe relationships that vary tremendously, ranging from competition to cooperation. The ecological and evolutionary interactions between herbivorous dinosaurs and the first mammalian herbivores and their food plants are reconstructed using knowledge gained during the study of modern living vertebrates, especially foregut and hindgut fermenting mammals. The ruminant is well adapted to achieve maximal digestion of roughage using the physiological mechanism at the reticulo-omasal orifice which selectively retains large particles in the reticulo-rumen. However, the most obvious feature of all ruminants is the regurgitation, rechewing and reswallowing of foregut digesta termed rumination. Foregut fermenting mammals also share interesting and unique features in two enzymes, stomach lysozyme and pancreatic ribonuclease which accompany and are adaptations to this mode of digestion. The microbial community inhabiting the gastrointestinal tract is represented by all major groups of microbes (bacteria, archaea, ciliate protozoa, anaerobic fungi and bacteriophage) and characterized by its high population density, wide diversity and complexity of interactions. The development and application of molecular ecology techniques promises to link distribution and identity of gastrointestinal microbes in their natural environment with their genetic potential and *in situ* activities.

### INTRODUCTION

The gastrointestinal tract is a specialized tube divided into various well-defined anatomical regions extending from the lips to the anus. However, for the purposes of this contribution concerning mutualistic fermentative digestion, discussion is restricted to the stomach (rumen-reticulum, crop, gizzard), small intestine and large intestine (cecum and colon). By definition, foregut fermentors comprise animals with a pre-gastric fermentation chambers such as the rumen, reticulum, and omasum of ruminants and diverticula or fermentative sacs of other ruminant-like mammals. Hindgut fermentors are defined as those animals with large fermentation compartments in the cecum, colon and rectum. Large populations of microorganisms inhabit the gastrointestinal tract of all animals and form a closely integrated ecological unit with the host. This complex mixed, microbial culture comprising bacteria, ciliate and flagellate protozoa, anaerobic phycomycete fungi as well as bacteriophage can be considered as the most metabolically adaptable and rapidly renewable organ of the body which plays a vital role in the normal nutritional, physiological, immunological and protective functions of the host animal. Development of microbial populations in the alimentary tract of higher animals commences soon after birth. The processes involved in the establishment of microbial populations are complex, involving succession of micro-

organisms and many microbial and host interactions eventually resulting in dense, stable populations inhabiting characteristic regions of the gut.

Physical and chemical conditions within the gut of different animals may differ considerably but are usually relatively constant in a single species on a given diet. This is the case for homeothermic animals, in which, allowing for irregularities in the intake of food, factors such as temperature, oxygen, acidity and moisture vary little with time. In poikilothermic animals temperature can be a major variable.

The detailed composition of the gut contents of most animals is extremely complex. To date, the microbial environment in the rumino-reticulum has been closely defined and allowing for variation in the nature and amount of food ingested serves as a good model for other gut ecosystems, both herbivores and non-herbivores. The hindgut environment is more constant in terms of physical and chemical composition with nutrients for ceco-colonic bacteria being provided by undigested dietary polysaccharides and endogenous secretions and tissues such as mucopolysaccharides, mucins, epithelial cells and enzymes.

### TYPES OF ANIMAL-MICROBE RELATIONSHIP

Anaerobic habitats have existed continuously throughout the history of the earth, the gastrointestinal tract being a contemporary microniche (Fenchel and Finlay, 1995). In fact, all animals, including humans, are adapted to life in a microbial world. Microbial populations have been described in herbivores, omnivores and carnivores and in all zoological classes including insects, fish, reptiles, birds, rodents, lagomorphs, pigs, horses, elephants, marsupials, sheep, goats, cattle,

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<sup>2</sup> E-mail: r-mackie@uiuc.edu

camels, antelopes, monkeys and humans, and even dinosaurs. The complexity of animal-microbe relationships varies tremendously, ranging from competition to cooperation. The animal alimentary tract has evolved as an adaptation enabling the animal to secure food and limit consumption by other animals. This allows the retention and digestion of ingested food, followed by absorption and metabolism of digestion products, whilst feeding and other activities continue. Since microorganisms grow rapidly under favorable conditions in the gut they could become serious competitors for the animal's food. This microbial challenge has modified the course of evolution in animals, resulting in selection for varied animal-microbe relationships (Hungate, 1976, 1984). The evolutionary strategy in the first case has been to compete with the resident microbes and in the second to cooperate with them.

#### *Competition model*

In the competition model, exemplified by carnivorous animals, host and microbe are competitors for the same food. Immunological and other adaptations delay consumption of the host by the resident microbes and prevent invasion of animal tissues by microorganisms in the gastrointestinal tract. Microbicidal concentrations of acid are secreted preventing attack of ingested feed, allowing the host's digestive enzymes to act followed by absorption of enzymic digestion products. However, a slower rate of passage, together with rapid growth, results in a large microbial population in the hindgut.

#### *Cooperation model*

The abundance of carbohydrate in plant cell walls (cellulose and hemicellulose) is the basis for the evolution of the cooperation model. The carbohydrate polymers of plant cell walls are indigestible by most animals (including all mammals), but can be hydrolysed and fermented by the microbial partner, with the resultant end-products of fermentation plus microbial cells being utilized by the host animal. The most widely known and economically important example of a cooperative animal-microbe relationship is found in ruminants in which the capacious, continuously fermenting rumen delays passage of digesta, allowing time for solubilization of fiber components by microorganisms. One cost of this relationship is the breakdown or sacrifice of dietary protein by rumen microorganisms before digestion by the animal's own enzymes. However, poor quality protein is upgraded through conversion to microbial protein (Tamminga, 1979).

#### *Combination model*

The combined competition-cooperation model of animal-microbe interactions avoids this difficulty since the host enzymatic breakdown products are absorbed before the microbial fermentation takes place. Thus, the host obtains not only the nutrients digested by its

own enzymes but also fermentation products from materials its enzymes cannot digest. This type of interaction occurs in horses, elephants, hyraxes, rodents, and lagomorphs (hares and rabbits) but is probably best exemplified in the termites.

The difference between this and the competition model lies in the extent to which anatomical modifications of the host allow longer retention of the digesta in the hindgut with consequent increased solubilization and fermentation. In both models the microbes act after the host has absorbed the nutrients made available through its own enzymes and in both models there is marked microbial activity in the hindgut. A disadvantage of the combination model is that, although the host absorbs the fermentation end-products, the microbial cells themselves cannot be used as a nutrient source. Some animals have overcome this deficiency by consuming the faeces or cecal content containing the microbes using a strategy termed coprophagy or cecotrophy respectively. These models of existing animal-microbe relationships are useful when trying to determine the evolution of mutualistic fermentative digestion in the gastrointestinal tract.

#### EVOLUTION OF MUTUALISTIC FERMENTATIVE DIGESTION

Recently much interest has been generated in reconstructing the diets of herbivorous dinosaurs and assessing ecological and evolutionary interactions between these reptiles and their food plants. Of considerable importance to this discussion are the physiological correlates of herbivory absent from the fossil record but possibly provided by consideration of herbivory in living vertebrates (Farlow, 1987).

#### *Dentitions and gastric mills*

Features one would expect to see in herbivorous dinosaurs that provide some evidence of diet include dentition. Herbivorous mammals masticate their fodder in order to comminute feed into small particles exposing a large surface area for microbial enzymatic attack. Although some dinosaurs (*e.g.*, ceratopsids and hadrosaurids and to a lesser extent hypsilophodontids and iguanodontids) had rather specialized masticatory dentition, other herbivorous dinosaurs (*e.g.*, prosauropods, sauropods, pachycephalosaurs, stegosaurs and ankylosaurs) had simple bladelike or peglike teeth adequate for cropping vegetation but not mastication (Farlow, 1987).

Herbivorous, granivorous and insectivorous birds have a highly specialized, muscular gizzard with an inner lining of hard cuticle. In many species the cuticle thickens into hard plates that grind together. In herbivorous birds this action is enhanced by the presence of sand and stones. In ostriches and emus these consist of pebbles 2–3 cm in diameter. Some 200 pebbles, collectively weighing 2.5 kg were recovered from the gut region of a moa skeleton (extinct large flightless bird) in New Zealand. Although grit is not essential for digestion of plant material it increases dry matter digestibility and efficiency of digestion. Deliberate

stone and soil swallowing has been reported for modern snakes, lizards, turtles and crocodylians serving to macerate digesta. In crocodylians stomach stones most likely serve as ballast (Taylor, 1993), in lizards and turtles pebbles may well aid in comminuting plant tissues and chitinous skeleton of insects. It has been suggested that herbivorous dinosaurs swallowed large stones that collected in a birdlike gizzard grinding the poorly masticated herbage. Such stomach stones or gastroliths have been reported from the gut regions or found nearby of prosauropods, sauropods and ornithomorphs (Taylor, 1993). Just how widespread this practice was amongst dinosaurs is unknown but could have been fairly common. If this was the case it would have enhanced their ability to digest coarse herbage. It is worth noting that herbivorous reptiles can attain digestive efficiencies roughly comparable to those found in herbivorous mammals by subjecting feed to digestive processes (including microbial fermentation in some species) for longer periods of time. However this strategy is not feasible for endothermic herbivores with a rapid metabolic rate and having limited time available to extract energy and nutrients. Thus the main advantage of mastication or gastric grinding is to decrease the length of time needed to attain a digestibility that could be approximated simply by a longer residence time in the animals' digestive tract (Farlow, 1987).

#### *Digestive physiology of herbivorous dinosaurs*

On the basis of the adaptations commonly found in living vertebrate herbivores, it is likely that herbivorous dinosaurs had relatively long capacious guts. Furthermore, it is likely that most herbivorous dinosaurs employed a mutualistic gut microbiota. The previously discussed benefits of large size to a mammalian herbivore probably also accrued to large herbivorous dinosaurs. If dinosaurs had lower metabolic rates than birds and mammals, then big dinosaurs probably had low mass specific metabolic rates and consequently slow turnover rates of gut contents than smaller dinosaurs. Large dinosaurs could have obtained their daily energy requirements from a low quality fiber diet presumably due to a slow rate of passage and long exposure of digesta to fermentative digestion than smaller dinosaurs. As with mammals, herbivorous dinosaurs of different body size probably selected different diets. Thus, smaller herbivorous dinosaurs likely consumed fodder of lower fiber/higher cell soluble content than their larger relatives in order to sustain their relatively higher metabolic rates resulting from a smaller body size. However, ectothermy may have reduced the dietary differences between large and small herbivorous dinosaurs without eliminating the differences altogether (Farlow, 1987).

Discussion of herbivorous dinosaur diets thus far has been based on provision of energy and protein and (most likely) fodder quality in terms of these nutrients. Of equal importance was the allelochemical defenses of plants they ate (refer to Rosenthal and Janzen

(1979) for modern herbivores). Such toxins can markedly reduce the digestibility of plant matter or directly affect the herbivores metabolism. It has been suggested (Guthrie, 1984) that ruminants are adapted to dealing with type 1 defenses (qualitative toxins or allelochemicals such as alkaloids) which occur in unpredictable, ephemeral plant species or tissues while quantitative defenses (type 2 such as tannins) are typical of more predictable, available and accessible plant species which are handled better by monogastric hindgut fermenters. Farlow (1987) argues that given their size it is likely that most dinosaurs fed mainly on plants that were reasonably predictable, accessible and available and thus protected by quantitative defenses. Smaller herbivorous dinosaurs, however, may have fed to a greater extent than their larger kin on plants defended by qualitative toxins. Also, if herbivorous dinosaurs had lower metabolic rates than birds and mammals of comparable size this may have reduced dependence on elaborate detoxification mechanisms.

In summary, it seems likely that most herbivorous dinosaurs, of whatever size, were hindgut fermenters. Finally, the probability that dinosaurs employed microbial fermentation may have implications for their thermal biology. If the relationship between body size and mass of fermentation contents described for mammals applies to herbivorous dinosaurs then the fermentation mass would have been considerable allowing the generation of thermoregulatory heat. Thus, they can be considered as having been, to some extent, fermentative endotherms.

#### *The first mammalian herbivores*

During the late Cretaceous and early Paleocene periods plant-eating mammals were frugivores, presumably because fruit can be more easily processed than foliage. Mammals did not invade the herbivorous niche until the Middle Paleocene (Collinson and Hooker, 1991). Evolution of large size was a prerequisite for the exploitation of leaves because of the need for a longer residence time in the gut for bacterial fermentation to obtain sufficient nutrients from foliage and herbage. In the late Cretaceous, dinosaurs occupied the herbivorous niche although grazers were still absent. The appearance of grazers in the Miocene is coincident with a similar radiation of grassland-forming grasses (Thomasson and Voorhies, 1990). Thus herbivore browsers first appear in the Middle Paleocene but they did not become significant until the late Eocene (Collinson and Hooker, 1991). Frugivory declined first with the appearance of herbivore browsing followed by an increase in grazers in post-Miocene at the expense of herbivore browsers. The earliest herbivores were ground dwelling (LGMs, large ground mammals) and achieved their dietary specializations largely through evolution from already large, ground-dwelling frugivores or, in the Paleocene, by a size increase from small insectivorous ancestors (Collinson and Hooker, 1991). Large size limited them to the ground. Most browsing herbivores in other locomotor

niches (SGMs, small ground mammals) changed their diet from frugivory without changing their locomotor adaptation. A period of nearly thirty million years existed in the vertebrate exploitation of leaves after dinosaur extinction and before the first few mammalian herbivores in the Middle Paleocene. This was followed by expansion of herbivores in the late Eocene when climates cooled and more open vegetation became established.

Hume and Warner (1980) published an excellent discussion on the evolution of microbial digestion in mammals. Since the fossil record provides no information on the morphology, physiology, biochemistry or microbiology of the gut much of the knowledge must be deduced from what is known about present-day animals coupled with the fossil record of animals and their probable feedstuffs and is therefore highly speculative. Microbial digestion surely arose long before mammals evolved. Microbial habitats exist in all regions of the gut from the mouth to the rectum and have been categorized as luminal, epithelial and cryptal (Savage, 1977). Large luminal populations of microbes develop in regions of the gut with relative stasis where retention time of digesta allows adequate microbial growth. In most deliberations only nutritional contributions to the host animal are considered. These are based on digestion of the plant cell wall by cellulases and hemicellulases provided by the microbial partner, the synthesis of microbial protein from poor quality dietary proteins and non-protein nitrogen mainly via ammonia as precursor, and the synthesis of B-vitamins and vitamin K. However, little consideration has been given to the protection provided by foregut fermentation resulting in transformation or modification of phytotoxins and mycotoxins in the diet (Mackie, 1987; McSweeney and Mackie, 1997 for review). Other contributions not normally considered in these deliberations are immunologic, physiologic and protective (see Tannock, 1997; Gaskins, 1997 for review). These arguments also support the theory that the development of foregut fermentation must have come after an initial development of the hindgut and that all foregut fermenters should have some fermentation in the hindgut (Hume and Warner, 1980).

#### DIGESTIVE PHYSIOLOGY OF VERTEBRATE HERBIVORES

Because of the nature of the plant cell wall and the difficulty in digesting it, herbivores have anatomical and/or physiological adaptations of the digestive tract to compensate for assimilation of this material. Herbivorous reptiles, birds and mammals usually have enlarged and/or elongated digestive tracts, often including fermentation chambers or sacs in the foregut or hindgut. Cecum-colon (hindgut) fermenters represent an older differentiation than foregut fermenters which in turn are older than ruminants (Langer, 1991). Bacteria, protozoa and anaerobic fungi inhabit these enlarged gut compartments as well as other sites in the gastrointestinal tract. The fermentative activity of these microbes results in the production of volatile fatty ac-

ids that are absorbed by the host animal and make a variable and in some cases considerable contribution to its nutritional economy.

There has been considerable debate on the relative merits of foregut and hindgut fermentation in mammals (Janis, 1976; Parra, 1978; Demment and Van Soest, 1985). The interrelationship between body size, diet and digestive strategy poses an interesting problem. The relationship between gut capacity and mass of fermentation contents is isometric with increasing body size regardless of whether they are foregut or hindgut fermenting mammals. Since an animal's mass-specific metabolic rate decreases with increasing body size while the ratio of gut capacity to body size remains almost constant, it follows that a large herbivore should have a slower turnover rate of its gut contents than a smaller herbivore. Hence, the mutualistic microbes of a larger herbivore will have longer residence time allowing greater fermentation or digestion of refractory plant material. Furthermore, a large animal's daily energy requirements can be supplied by fermenting and digesting a smaller fraction of its diet allowing it to survive on forage of lower quality than a small herbivore. In contrast, a very small herbivore's relatively higher metabolic rate with a rapid turnover makes it difficult for these animals to subsist on a high fiber diet and they consequently are typically hindgut fermenters.

Despite the sophisticated anatomical and physiological adaptations for herbivory these modifications are not essential. Some birds compensate for a lack of structural modification to the intestinal tract by consuming large quantities of grass *e.g.*, ducks, geese and the takahe (Morton, 1978). Even though emus lack well-developed digestive specializations for microbial fermentation and demonstrate high digesta passage rates, these birds digest plant cell wall constituents moderately well (35–45%) with significant levels of VFA production in the intestine with concomitant contribution to the energy economy of the bird. The lack of specialized fermentation chambers is offset by grinding coarse feed particles in the crop and exposure to acid conditions in the stomach prior to fermentation. However other ratites such as the ostrich and rhea, which routinely consume high-fiber, low-quality diets have more elaborate fermentation sites namely an elongated colon. Among mammals the giant panda has a slightly modified carnivore-like gut but compensates by consuming large quantities of fodder (Dierenfeld *et al.*, 1982).

#### *Special Features of the Ruminant and Molecular Evolution*

The ruminant is well adapted to achieve maximal digestion of roughage using the physiological mechanism at the reticulo-omasal orifice which selectively retains large food particles in the reticulo-rumen. Efficient separation of the fermentative from the acid-secreting region of the stomach may have allowed development of the most obvious special feature of the

ruminants, rumination, where foregut digesta is regurgitated, rechewed and reswallowed in a frequent regular pattern repeated 500 times per day, occupying a total time of more than 8 h, and involving more than 25,000 chews (Hume and Warner, 1980). Rumination occurs in all the Pecora and Tylopoda. In macropods regurgitation is more irregular and infrequent and involves much less chewing and has been termed mericism.

Nearly all foregut fermenters have a gastric or ventricular groove (*sulcus reticuli*) leading directly from the esophagus to the hind stomach (abomasum in ruminants). This ensures that during suckling milk is channeled directly to the abomasum bypassing wasteful ruminal fermentation. Foregut fermenting mammals also share interesting and unique features in two enzymes, stomach lysozyme and pancreatic ribonuclease, which accompany this mode of digestion.

At known times on two occasions during mammalian evolution, lysozyme appears to have largely abandoned its usual function which is to assist animals fight harmful bacteria and acquired a new function as a digestive enzyme in animals with foregut fermentation (Jollés *et al.*, 1989). Its new role is to lyse bacteria in the abomasum for nutritional purposes. On the most recent of these occasions, which began 15 million years ago, leaf eating monkeys (Colobinae) remolded lysozyme for functioning in stomach fluid (active at low pH and resistant to pepsin breakdown) and the rate of amino acid replacement increased (Dobson *et al.*, 1984; Stewart *et al.*, 1987). By contrast, on the other occasion, as cloven-hoofed animals (ruminants) recruited lysozyme for the same new digestive function, there was no apparent acceleration. Although because this lineage is at least 60 million years old there was an early period of fast evolution, allowing adaptation to functioning in the stomach environment, followed by a period when the rate of amino acid replacement became subnormal (Jollés *et al.*, 1989). Many mammals and birds have a single gene coding for lysozyme. In contrast ruminants have multiple genes for lysozyme. A traditional explanation for the origin of a gene family is that it provides a means of making more product. This explanation could apply to the ruminant lysozyme case, with the rise in gene number in advanced ruminants being viewed as the result of selection for the high levels of lysozyme in the stomach that may be necessary for efficient digestion of rumen bacteria. Lysozyme accounts for approximately 10% of stomach mucosal protein (Dobson *et al.*, 1984) and 10% of stomach mucosal mRNA (Irwin and Wilson, 1989), showing that the stomach genes are highly expressed in cows and other advanced ruminants, involving gene duplication as well as a change in gene expression. Work in progress on the genomic organization of ruminant lysozyme genes suggests that all of those genes reside on one chromosomal segment (Irwin *et al.*, 1992). The possibility that multigene families can accelerate adaptive evolution, by virtue of their capacity for bringing together functionally cou-

pled substitutions is emphasized in the review of Irwin *et al.* (1992).

The content of ribonuclease (RNase) in the pancreas varies greatly between species (Barnard, 1969). All ungulates, rodents and herbivorous marsupials had high amounts with low levels in all other mammals including hindgut fermentors such as equids, elephants and pigs. Barnard (1969) proposed that RNase developed in ruminants for degradation of bacterial RNA since a large fraction of ingested protein nitrogen is in the form of bacterial protein which must be digested in the small intestine to be of nutritional benefit. Interestingly, Barnard (1969) proposed a modified version of the nitrogen cycle of the ruminant, placing in context the role of pancreatic nuclease and included the cycling of phosphorus. Because of wide variations in activity and structure, RNase was suggested as a useful source of information on the evolution of a protein and on relationships between enzyme structure and activity. The abundance of RNase sequences from contemporary artiodactyls allows the reconstruction of the RNases that were the evolutionary intermediates in the most recent 40 million years of this evolution. Genes encoding the reconstructed proteins were obtained in the laboratory by site-directed mutagenesis from a synthetic gene for RNase, expressed in *E. coli* and the resulting proteins purified to homogeneity (Stackhouse *et al.*, 1990). The catalytic activities, substrate specificities and thermal stabilities of the reconstructed RNases were examined using parsimony analysis to assess the evolution of the reconstructed RNase family. These changes in molecular behavior of reconstructed RNases correspond to a point in the divergent evolution of mammals where digestive physiology of ungulates also underwent substantial changes, ultimately yielding artiodactyls with "true ruminant" foregut digestion (Jermann *et al.*, 1995). Foregut fermentation appears to have substantial adaptive value in many herbivorous environments and may have evolved convergently in marsupial kangaroos, the colobine monkey primates, and more than once within the artiodactyl lineage itself (Jollés *et al.*, 1989). The fact that a ribonuclease emerged with increased stability, decreased catalytic activity against duplex RNA (increased activity against small RNA substrates and ss RNA) and increased levels of expression at the same time as ruminant digestion emerged may be a coincidence but it also indicates that the ancestral molecules were not specialized digestive enzymes but played a non-digestive role such as RNase from the brain and seminal plasma (Jermann *et al.*, 1995). This research highlights the possibility that duplication of the RNase gene in the ruminants allowed tissue-specific expression and subsequent specialization of the enzymes, as is seen for ruminant lysozymes and should encourage more widespread use of evolutionary reconstruction as an experimental tool to direct site-directed mutagenesis.

The transition in the cetaceans (whales, dolphins and porpoises) from terrestrial life to a fully aquatic

existence is one of the most enduring evolutionary mysteries. Previous paleontological and molecular evidence has indicated that cetaceans and artiodactyls constitute a natural clade within the subclass Eutheria (Novacek, 1992). Recent phylogenetic analyses of protein (11 nuclear-encoded protein sequences) and mitochondrial DNA (5) sequences indicate that cetaceans are not only intimately associated to the artiodactyls, they are in fact deeply nested within the artiodactyl phylogenetic tree. The results show that Cetacea are more closely related to the Ruminantia, than either ruminants or cetaceans are to members of the other two artiodactyl suborders, Suiformes (pigs, peccaries and hippopotamuses) and Tylopoda (camels and llamas). On the basis of the rate of evolution of mitochondrial DNA sequences and using paleontological reference dates for calibration, the whale lineage branched off a protoruminant lineage 50 million years ago (Graur and Higgins, 1994). By implication, the cetacean transition to aquatic life is inferred to be a relatively recent evolutionary event.

Graur (1993) reviewed the molecular phylogeny and higher classification of eutherian mammals based on DNA and protein sequences. Phylogenetic trees depicting relationships among 16 eutherian orders are presented (Graur, 1993). Evidence from sequence comparisons of mitochondrial DNA suggest that the artiodactyl family Bovidae is monophyletic and most tribes originated early in the Miocene with all extant lineages present by 16–17 million years ago providing an example of rapid cladogenesis, following the origin of families in the infraorder Pecora (Allard *et al.*, 1992). It has also been shown that Lagomorpha is significantly more closely related to Primates and Scandentia (tree shrews) than it is to Rodentia, invalidating the superordinal taxon Glires (Lagomorpha + Rodentia) (Li *et al.*, 1990; Graur *et al.*, 1996). The question arises, as in bacterial taxonomy and systematics, will molecular traits replace phenotypic grouping as the main tool of taxonomy and phylogeny in the future? Graur (1993) indicates such an event would have undesirable consequences and a more logical approach would be to utilize both data sets in a phylogenetically meaningful manner to provide insight into the process of evolution at both levels. He suggests using molecular data as the basis for characterization of the dynamics of morphological changes in evolution. Paradoxically molecular phylogeny may turn out to be the only means by which phenotypic traits retain their scientific value in taxonomy.

Most species of birds have obvious living relatives and are members of well-characterized groups. The hoatzin (*Opisthocomus hoazin*) is one of the few birds that differ in many ways so that its nearest surviving kin are uncertain. Of importance to this discussion is that the hoatzin is the only bird that used microbial foregut fermentation to convert cellulose into sugars for microbial fermentation. The hoatzin feeds primarily on tender young leaves, twigs and shoots of trees and marsh plants, which are ingested into a huge mus-

cular crop with a deeply ridged interior lining where active foregut fermentation occurs. The bony sternum and pectoral girdle are modified to accommodate the filled crop, and there is a callosity on the bone skin of the breast where the heavy crop is rested on a branch. The proventriculus and gizzard are small, and the lower esophagus is sacculated, which delays passage of particles into the lower gut where additional fermentation occurs in the paired ceca. The contents of the crop and esophagus can account for up to 10% of total body weight. The rate of food passage through the digestive system is rapid in many birds, but the hoatzin retains liquids for *ca.* 18 hr and solids for 24–48 hr, similar to retention times in sheep (Grajal *et al.*, 1989, 1991; Dominguez-Bello *et al.*, 1994). These birds also express high levels of a bacteriolytic lysozyme which is more similar in amino acid sequence to the rock pigeon than that of the domestic fowl (Kornegay *et al.*, 1994). Evolutionary comparison places them among the calcium-binding lysozymes rather than the conventional types. However, biochemical convergence and parallel amino acid replacements have been shown in hoatzin stomach lysozyme even though it has a different genetic origin from the mammalian examples and has undergone more than 300 million years of independent evolution (Kornegay *et al.*, 1994). DNA sequence evidence from the 12S and 16S rRNA mitochondrial genes and from the eye lens protein, aA-crystallin, indicates that the hoatzin is most closely related to the typical cuckoos and divergence occurred at or near the base of the cuculiform phylogenetic tree (Hedges *et al.*, 1995).

Birds have not made as much use of fermentative fiber digestion as have mammals with only about 3% of existing species regularly consuming herbage. Many avian herbivores consume large quantities of plant material but extract only the readily digestible components and the bulk of cell wall constituents are rapidly expelled without significant microbial fermentation. Even with the family Tetraonidae (grouse and ptarmigan) which utilize enlarged ceca as fermentation chambers, the contribution of fiber degradation to total energy expenditure is low with less than 20% of basal energy metabolism being derived from this source. It has been suggested that the weight reduction necessary for flight places a constraint on the size and weight of gut compartments. Thus, the birds which should be able to make the most use of fermentative digestion are the large flightless species such as the emu and ostriches not only because the constraints associated with flying are absent but also because in large animals it is possible to have large fermentation chambers relative to metabolic rate. Indeed, for the emu (*Dromaius novaehollandiae*) it was found that energy from digestion of neutral detergent fiber contributed up to 63% of standard metabolism and 50% of maintenance requirements (Herd and Dawson, 1984). This was achieved despite the fact that rate of passage of feed residues through the tract was rapid and the anatomy of the gastrointestinal tract was simple in structure

with small ceca and short colon. Recent research on the ostrich (*Struthio camelus*), the largest living flightless bird showed that the hindgut (long paired ceca and elongated colon) contained 58% of the total wet digesta in the entire intestinal tract with high concentrations of VFA (140–195 mM). Furthermore, the long retention times of fibrous feed in the intestinal tract (mean passage rate 40 hr) ensured exposure to microbial digestion for extended periods resulting in high digestibility of fiber and VFA production (Swart *et al.*, 1993a). Theoretical energy contribution of VFA was estimated to be as high as 76% of the metabolizable energy intake in growing chicks (Swart *et al.*, 1993b). These results confirm the importance of gut anatomy and physiology in providing a suitable environment for fermentative digestion and the possible role of evolution in the development of these structures.

#### MOLECULAR APPROACHES TO GUT MICROECOLOGY

The microbial community inhabiting the gastrointestinal tract is represented by all major domains of microbes, including the Bacteria, Archaea and Eucarya (Woese *et al.*, 1990) as well as viruses (bacteriophage), and characterized by its high population density, wide diversity and complexity of interactions. Despite this vast amount of knowledge, the basic prerequisites for ecological studies, namely enumeration and identification of community members have tremendous limitations.

These limitations can be overcome using modern molecular ecology techniques based on sequence comparisons of nucleic acids (DNA or RNA) can be used to provide molecular characterization while at the same time providing a classification scheme that predicts natural evolutionary relationships (Amann *et al.*, 1994, 1995). Rather than replacing the classical culture-based system, the new molecular techniques can be used in combination with the classical approach to improve cultivation, speciation and the evaluation of biodiversity. An example of the power of these modern molecular ecology techniques is provided by the analysis of 16S rRNA sequences (average length 1,500 nucleotides). The highly conserved regions of the rRNA molecule can serve as primer binding sites for *in vitro* amplification by PCR (Ludwig *et al.*, 1994). The more conserved regions are also useful, serving as targets for universal probes that react with all living organisms or for discriminating between broad phylogenetic groups such as the domains Archaea, Bacteria and Eucarya (Woese *et al.*, 1990). The more variable sequence regions are more appropriate for genus, species and even strain specific hybridization probes (Stahl and Amann, 1991; Raskin *et al.*, 1997). Thus the application of molecular ecology techniques based on nucleic acid probes for specific organisms (rRNA) as well as genes (DNA) and their expression (mRNA) will enable scientists to determine the exact role or function a specific organism plays in the gut ecosystem and its quantitative contribution to the whole. This is the ultimate goal of the microbial ecologist. The suc-

cessful development and application of these methods promises, for the first time, to link distribution and identity of gastrointestinal microbes in their natural environment with their genetic potential and *in situ* activities.

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