Bacteria and digestive enzymes in the alimentary tract of the giant African land snails, *Archarchatina marginata* and *Achatina achatina*

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Abstract

A study was carried out to investigate the bacteria flora in the gut of the Giant African Land Snails (GALS), Archarchatina marginata and Achatina achatina. Microflora cultures from snail gut contents were prepared to isolate and identify microorganisms within the snail digestive tract. Enzyme assays were carried out on a few of the microorganisms to determine the presence and level of enzymatic activities. Results showed that a wide range of bacteria inhabit the alimentary tract of GALS. Bacteria isolates from the stomach (Bacillus cereus and Pseudomonas syringae) and mouth regions (Aeroccus viridians and Azobacter chroococcum) in the two species of GALS effectively digested starch, cellulose and casein. However, Bacillus species from the stomach region in A. achatina exhibited the highest enzymatic activities for cellulase (10.00 µmol/min/ml at 18 h), protease (23.97 µmol/min/ml at 18 h) and amylase (9.56 µmol/min/ml at 24 h) during incubation. Consequently, results from the present study confirmed that amylolytic, cellulolytic and proteolytic bacteria within the digestive tract of the two species of GALS do aid in the snail's digestive processes.

Keywords: Microorganisms, snail gut, enzyme activity and Giant African Land Snails

Introduction

An understanding of the physiology of digestion in the snail would encompass detailed study of the gut anatomy, biochemistry and activities of resident microorganisms within the snail alimentary tract. Snails are well equipped with a wide array of digestive enzymes, particularly carbohydrases (Flari and Lazaridou-Dimitriadou, 1996). Reports showed that terrestrial gastropods feed upon fresh plants with high protein and calcium contents (Chevalier *et al.*, 2003) and may also participate, with other soil invertebrates, in the decomposition of leaf litter (Hatziioannou *et al.*, 1994). Consequently, they need a large set of polysaccharide depolymerases and glycoside hydrolases for the digestion of plant materials. The gastrointestinal tract of the land snail *Achatina fulica* is known to harbor metabolically active bacterial communities (Pawar *et al.*, 2012). Therefore the choice of cereals and plants by snail farmers should take into account the fermentative abilities of the intestinal microbiota (Charier *et al.*, 2006). The dependence of pulmonates on microbial activity within their gut would explain their extraordinary efficiency in plant fibre digestion (Charrier and Daguzan, 1980). Adedire *et al.* (1999) revealed the presence of microbial species

such as *Rothia, Corynebacterium, Pseudomonas, Proteus, Enterobacter, Bacillus, Streptococcus, Flavobacterium and Klebsiella* species in the intestinal tract of *Archachatina (Calachatina) marginata* Swainson. The objective of this study therefore, was to determine and compare the type of microflora and enzymatic activities of a few microorganisms in different regions of the alimentary tract of *A. marginata* and *A. achatina*.

Materials and methods

Experimental animals and sample collection

The identification of the gut anatomy of Giant African land Snails was carried out in the Snail Research Unit of Animal Physiology Laboratory, College of Animal Science and Livestock Production (COLANIM), Federal University of Agriculture, Abeokuta, Nigeria. Enzyme assays and microbial analysis were carried out at the Enzyme Biochemistry and Microbiology Laboratories respectively of the School of Science, Federal University of Technology, Akure, Nigeria. Twelve adult snails of 150 – 250 g liveweight (*6 each of A. marginata* and *A. achatina species*), bought from Oje market Ibadan, were sacrificed for this experiment. Snails were dissected according to the methods of Segun (1975). After dissection, the different organs/regions of the digestive tract were exposed in order to obtain scrapings of the epithelia of different portion of the gut. Each portion of the snail gut was wiped with sterile moist cotton swabs separately. The different sterile moist swabs were soaked in a preparation of 13 g of nutrient broth powder in 1litre of deionized water,

Preparations of micro-flora cultures from snail gut content

Media used for isolation of bacteria was Nutrient agar. The media was prepared according to manufacturer's instruction and sterilized in autoclave at 121□ for 15 min. A portion of each region of the gut was streaked onto nutrient agar plates, using inoculating loop and incubated for 18-24 h at 37□ for bacterial growth. Individual colonies observed were sub-cultured on nutrient agar plates for another 18-24 h and finally grown on agar slants to preserve the pure cultures. The bacterial isolates were characterized based on colonial morphology, cultural characteristics and biochemical tests as described by Oyeleke and Manga (2008). The isolates were identified by comparing their characteristics with those of known taxa using the Bergey's manual of determinative bacteriology (Holt et al., 1994).

Screening for microbial cellulase and amylase enzyme activities

A loopful of grown culture of isolated colonies was inoculated on nutrient broth containing 1% carboxymethyl cellulose (CMC), for cellulase, and 1% starch, for amylase, in conical flasks. They were incubated at 37□ for 24 h allowing bacteria isolates to grow. After incubation, using dinitrosalicylic acid (DNSA) method (Bertrand et al., 2004) the cellulolitic and amylolytic activities were measured in the spectrophotometer. This was done by centrifuging the culture broth at 6, 000 rpm for 5 min. It was then filtered using Whatman's filter paper No. 1 and then 1 ml of the culture filtrate, 1 ml 1% CMC (for cellulase activity) and 1 ml 1% starch (for amylase activity) in 0.05 M citrate buffer, pH 4.8 incubated for 30 min. at 37□ then reacting with DNS reagent (to stop the growth reaction) and boiled for 5 min. It was then read with the spectrophotometer at 540 nm.

Microbial cellulase and amylase enzyme production and assay

The bacteria species was grown for production of cellulase and amylase in a minimum salt medium (500 ml) containing 0.05 g MgSO4, 0.5 g (NH4)2SO4, 1 g KH_2PO_4, 3.5 g K_2HPO_4, 0.25 g sodium citrate, supplemented with CMC as carbon source (for cellulase) and supplemented with starch (for amylase), in cooled distilled water (sterilized at 121□ for 15 min). The cultures were grown at 37□ for 30 h. Culture broth were sampled at 6 h intervals during growth to determine enzyme activity in relation to biomass yield by measuring at an absorbance of 540 nm with a spectrophotometer. Culture filtrate, obtained by filtration through Whatman No. 1 filter paper, served as the enzyme solution (Singh, 2003; El-Naghy et al., 1991). For cellulase, 0.5 ml of culture supernatant fluid was incubated with 0.5 ml 1% CMC in 0.05 M citrate buffer, pH 4.8 at 40□ for 30 min, while for amylase, 0.5 ml of culture supernatant fluid was incubated with 0.5 ml of 1% starch in 0.05 M acetate buffer, pH 5.0 at 40□ for 30 min. The reducing sugar products were assayed by the dinitrosalicylic acid (DNSA) method (Bertrand et al., 2004), using glucose as the sugar standard. Cellulase and amylase activities were assayed by the determination of reducing sugar released from CMC and starch respectively.

Microbial protease enzyme production and assay

Bacteria isolates were grown in culture medium containing 2.5 g glucose, 3.75 g peptone, 2.5 g MgSO4, 2.5 g KH2PO4, 0.05 g FeSO4, 3 g casein in 500 ml cooled distilled water in a conical flask (sterilized at 121□ for 15 min,) and incubated for 24 h. The culture broth was sampled every 6 h, during growth. The protease activity was assayed by the method of Lovrien *et al.*, (1985). 3 ml of reaction mixture containing 0.5% casein in 2.95 ml of 0.1 M Tris-HCl buffer, pH 8.0 and 0.1 ml of each enzyme was incubated at 40□. After 30 min, the reaction was stopped by adding 3 ml of cold 10% trichloroacetic acid. After 1 h, each of the culture filtrate was centrifuged at 6,000 rpm for 5 min to remove the precipitate and absorbance of the supernatants was read with spectrophotometer at 540 nm. The amount of amino acids released was calculated from a standard curve plotted against a range of known concentrations of tyrosine. One unit of enzyme (u/ml/min) was defined as the amount of enzyme that liberated 1.5 g tyrosine per ml per minute during assay (Bertrand *et al.*, 2004).

Data analysis

Data were subjected to descriptive analytical methods using line graphs.

Results and discussion

The list of microorganisms isolated from the different regions of the alimentary tract of GALS is shown in Table 1. The characteristics of the wide range of bacteria organisms found in the different regions of the snail gut are also presented in Table 2. There was a very wide range of bacteria microorganisms isolated from the alimentary tract of GALS. However, there were some bacteria species that were found to be common to both species of snail, namely: *Bacillus spp.*, *Micrococcus spp.*, *Staphylococcus spp.*, *Aerococus spp.*, *Erwinia spp.* and *Klebsiella spp.* Bacteria isolates, one each from the mouth and stomach regions of *A. marginata* and *A. achatina* species, were separated for further incubation and screening for their enzymatic activities. The organisms were: *Aeroccus viridians* isolated from the mouth of *A. achatina*, *Azobacter chroococcum*

Table 1: List of bacteria isolated from the different regions of the alimentary canal of GALS

Snail gut region	Microorganism	
	AM	AA
Mouth	*Azotobacter chroococcum* *Kurthia zoopfii*	*Azomonas agilis,* *Aerococcus viridians*
Buccal mass	Shigella sp	*Micrococcus luteus*
Salivary gland	*Xanthomonas fragaride* *Micrococcus lutus*	*Erwinia herbicola* *Bacillus cereus*
Anterior oesophagus	*Aerococcus viridians* *Klebsiella rhinosderomalis*	*Gluconobacter oxydans* *Staphylococcus aureus*
Crop	*Streptococcus feacium* -	*Flavobacterium breve* *Bacillus megatarum* *Acinetobacter calcoaceticus*
Posterior oesophagus	*Proteus morganii* *Clostridium sporogenes*	-
Stomach	*Pseudomonas syringae* *Arthrobacter globifarmis*	*Bacillus aureus* *Citrophaga rubra*
Intestine	*Erwinia amylovora* *Corynebacterium zerosis*	*Bacillus coagulans* *Citrobacter freundii*
Rectum	*Aeromonas hydrophila* *Erwinia amylovora*	*Vibrio fisheai* *Micrococcus luteus*
Digestive gland	*Enterobacter cloacae*	*Klebsiella rhinosderomalis*
Crop juice	*Staphylococcus epidermidis* *Bacillus cereus*	*Staphylococcus aureus* *Bacillus cereus*

isolated from the mouth of *A. marginata*, *Bacillus cereus* isolated from the stomach of *A. achatina*, and *Pseudomonas syringae* isolated from the stomach of *A. marginata* species were screened, for their level of protein production, cellulase, amylase and protease activities. The production of protein by bacteria isolates from the gut of GALS is shown in Figure 1. Out of 4 bacteria spp. isolated and screened for their level of protein production. *Bacillus cereus* had the highest protein production (50.58 mg) at 24 h of incubation while *Pseudomonas syringae* had its peak protein production (33.10 mg) at 24 h of incubation. *Azotobacter chroococcum* had its peak protein production (32.18 mg) at 18 h of incubation while *Aerococcus viridians* had its maximum protein production (31.36 mg) at 24 h of incubation.

The cellulase activities of bacterial isolates from the gut of GALS are shown in Figure 2. *Bacillus cereus* had the highest activity of 10.00 μmol/min/ml at 18 h; which dropped to 8.08 μmol/min/ml at 24 h of incubation but rose to 8.46 μmol/min/ml at 30 h and thereafter declined. *Pseudomonas syringae* followed with a peak cellulase activity of 8.59 μmol/min/ml at 18 h of incubation period. *Aerococcus viridians* had a peak cellulase activity of 8.21 μmol/min/ml at 30 h of incubation, while *Azotobacter chroococcum* had the least cellulase activity peak of 3.46 μmol/min/ml at 18 h of incubation.

Figure 3 shows the amylase activities of bacterial isolates from the gut of GALS *Bacillus cereus* had the highest. The peak amylase activities were in the order, 9.56>8.96>7.93>2.44 μmol/min/ml for B*acillus cereus, Pseudomonas syringae, Azotobacter chroococcum*, Aerococcus *viridians* at 24, 24, 18 and 36 h of incubation respectively.

Table 2: Characteristics of bacteria organisms isolated from the alimentary tract of *A. marginata* and *A. achatina*.

Organism	Cell shape	Gram reaction	Spore	Nitrate reduction	Indole	Motility	Catalase	Glucose	Maltose	Sucrose	Lactose
Azotobacter chroococcum	ovoid	-ve	-ve	-ve	-ve		+ve	NC		YG	NC
Kurthia zoopfii	R	+ve	-ve	-ve	-ve	+ve	+ve		NC	Y	NC
Citrobacter freundii	R	-ve	-ve	+ve	-ve	+ve	+ve	YG	YG	Y	YG
Shigella sp	MLR	-ve		+ve	-ve	+ve	-ve	Y	Y	NC	Y
Xanthomonas fragaride	R	-ve	-ve		+ve	-ve	-ve	NC	NC	NC	NC
Micrococcus lutus	sphere	+ve		-ve	-ve	-ve	+ve	Y	Y	Y	NC
Aerococcus viridians	cocci	+ve	-ve	-ve	-ve	-ve	+ve	Y	Y	Y	YG
Klebsiella rhinosderomalis	R	-ve	-ve	-ve	-ve	+ve	-ve	NC	NC	NC	NC
Streptococcus feacium	cocci	+ve	-ve	-ve	-ve	+ve	-ve	NC	NC	NC	NC
Proteus morganii	LR	-ve	-ve	+ve	+ve	+ve	-ve	YG	Y	NC	NC
Clostridium sporogenes	LR	+ve	+ve	-ve	+ve	+ve		NC	NC	NC	NC
Pseudomonas syringae	cocci	+ve	-ve	-ve	-ve	-ve	+ve	NC	Y	Y	Y
Flavobacterium breve	R	-ve		-ve	+ve	-ve	+ve	Y		NC	NC
Erwinia amylovora	R	-ve			+ve		+ve	YG	NC	NC	NC
Corynebacterium zerosis	R	+ve	-ve	+ve	-ve	-ve	+ve	YG	Y	Y	NC
Aeromonas hydrophila	SR	-ve				-ve	-ve	Y	Y	NC	NC
Enterobacter cloacae	R	-ve	-ve		-ve	+ve	+ve				
Staphylococcus epidermidis	sphere	+ve			-ve	-ve		YG	YG	NC	NC
Bacillus subtilis	LR	+ve	+ve	+ve	+ve	+ve	+ve	Y	YG	NC	
Azomonas agilis,	ovoid	-ve	-ve	-ve	-ve	+ve	-ve	NC	Y	NC	Y
Citrophaga rubra	R	-ve	-ve	-ve	+ve	+ve	+ve	Y	NC	Y	NC
Acinetobacter calcoaceticus	R	-ve		-ve	-ve	+ve	+ve	YG	NC	NC	NC
Erwinia herbicola	R	-ve			+ve	+ve		Y	NC	NC	NC
Bacillus cereus	LR	+ve	+ve	+ve	-ve	+ve	+ve	Y			NC
Vibrio fischeri	R	-ve			+ve	+ve	+ve	Y	Y	Y	
Staphylococcus aureus	spheres	+ve		+ve	-ve	-ve		YG			
Bacillus coagularis	LR	+ve	+ve	+ve	-ve	+ve	+ve	YG			
Bacillus megaterium	R	+ve	+ve	+ve	-ve	+ve					

Legend: SR=Short rod, MLR=medium long rod, LR=long rod, YG=acid and gas production, NC=no change, Y=acid production, F=fermentation, OX=oxidation

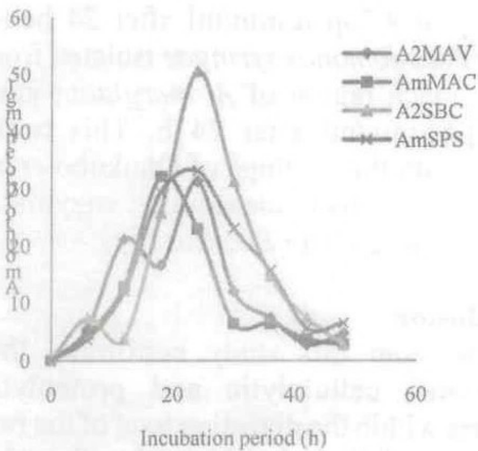

Figure 1: Production of protein by bacterial isolates from the gut of GALS

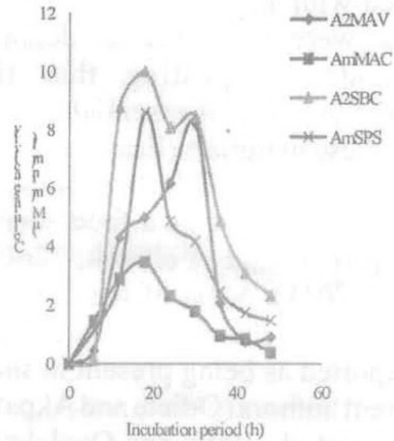

Figure 2: Cellulase activities of bacterial isolates from the gut of GALS.

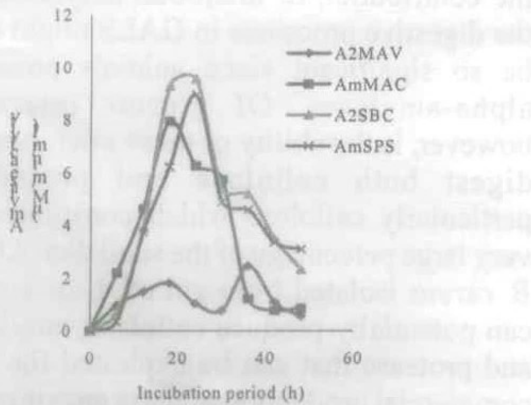

Figure 3: Amylase activities of bacterial isolates from the gut of GALS

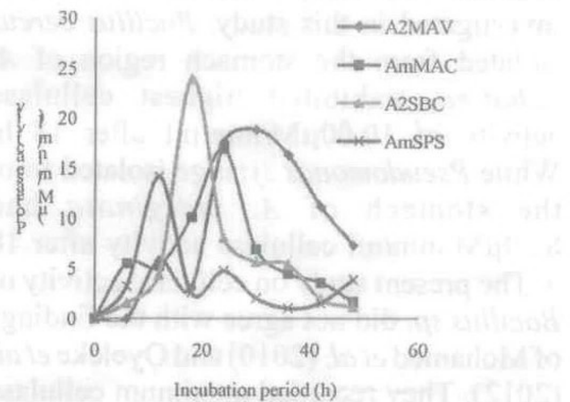

Figure 4: Protease activities of bacterial isolates from the gut of GALS

The activity of protease from bacterial isolates from the gut of GALS is shown in Figure 4. *Bacillus cereus* had the highest protease activity 23.97 µmol/min/ml at 18 h followed by *Aerococcus viridians* 18.84 µmol/min/ml at 30 h. *Azotobacter chroococcum* followed with a peak protease activity of 16.92 µmol/min/ml at 24 h of incubation period. *Pseudomonas syringae* had the least protease activity with a peak of 14.10 µmol/min/ml at 12 h of incubation. The production of protease by the bacterial isolates decreased with the passage of time.

The presence of a wide range of microorganisms in the gut of GALS is an indication of the snail's intestinal tract being a good source of nutrients for microbes. There exist a symbiotic relationship between the microbes and the snail. The importance of bacteria to the digestive process is not very clear. Several studies have identified bacteria from the snail gut which are able to degrade a variety of substrates including linamarin, carboxymethylcellulose, alginate, agarose and carrageenans (Erasmus *et al.*, 1997; Tanaka *et al.*, 2004; Kim *et al.*, 2007).

Snails treated with antibiotics to eliminate gut bacteria were still able to degrade polysaccharides suggesting that the bacteria assist but are not essential for the digestion of these materials (Erasmus *et al.*, 1997; Brendelberger, 1997). In some snail species bacteria are used as a food source (Garcia-Esquivel and Felbeck, 2006; Martin *et al.*, 2011). Most of the bacteria species reported in the present study had also been reported as being present in snail gut by different authors (Odiete and Akpata, 1983; Adedire *et al.*, 1999; and Oyeleke *et al.*, 2012).

The ability of gut bacteria isolates to produce digestive enzymes was also investigated in this study. *Bacillus cereus* isolated from the stomach region of *A. achatina*, exhibited highest cellulase activity of 10.00μM/min/ml after 18 h. While *Pseudomonas syringe* isolated from the stomach of *A. marginata* had 8.59μM/min/ml cellulase activity after 18 h. The present study on cellulase activity of *Bacillus sp.* did not agree with the findings of Mohamed *et al.* (2010) and Oyeleke *et al.* (2012). They recorded maximum cellulase productivity after 24 h for *Bacillus sp.* Disparity in findings on *Bacillus* spp may be due to the differences in the strains of bacteria species.

Protease activity, in *B. cereus* isolated from the stomach region of *A. achatina* was highest at 23.97μM/min/ml after 18 h, while *Aerococcus viridians* isolated from the mouth region of *A. achatina* had 18.85 μM/min/ml after 30 h. This report is in consonance with the findings of Oyeleke *et al.* (2012) but disagrees with findings by Wellingta *et al.* (2004). They reported highest protease activity by *Bacillus spp.* at 18 h and 9 h of incubation respectively, for *Bacillus spp.*

B. cereus isolated from the stomach region of *A. achatina* exhibited highest amylase activity at 9.56μM/min/ml after 24 hours while *Pseudomonas syringae* isolated from the stomach region of *A. marginata* gave 8.96 μM/min/ml after 24 h. This result agrees with the findings of Okukubo *et al.* (1964) that had maximum enzymatic activity after 24 h for *Bacillus* spp.

Conclusion

Results from this study confirmed that amylolitic, cellulolytic and proteolytic bacteria within the digestive tract of the two species of GALS do aid in the digestive processes. Obviously, most amylolytic bacteria possess extracellular amylases which they could use to digest starch, but the contribution of microbial amylases to the digestive processes in GALS might not be so significant since animals possess alpha-amylases. Of greater interest, however, is the ability of these microbes to digest both cellulose and proteins, particularly cellulose which constitutes a very large percentage of the snail diet. Also, *B. cereus* isolated from gut of *A. achatina* can potentially produce cellulase, amylase and protease that can be exploited for the commercial production of these enzymes in industries.

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