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Gut pH, Histology and Microbial Enzymes Production of the Mouth and Stomach in *Achatina achatina*.

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Target Audience: Snail farmers, commercial snail producers

Abstract

*The African land snail, Achatina achatina digestive activities were studied by determining pH, gut morphology, and preparing microflora cultures from the gut content. The microorganisms within the digestive tract were isolated, identified and enzyme assays were carried out on two of the isolates to determine the presence and levels of enzyme activity. Results revealed that Bacillus aureus (from the stomach region), and Aeroccus viridians (from the mouth region) effectively digested starch, cellulose and casein. However, B. aureus from the stomach region exhibited the highest enzymatic activities for amylase (9.39µM/min/ml), cellulase (10.13µM/min/ml), and protease (**24.23µM/min/ml**) all at 18h of incubation. The study provided insight into gut micro-environment, morphology and bacteria activities aiding digestive processes within the gut of Achatina achatina. The enzymes production capabilities of the bacterial organisms isolated from the gut of A. achatina could find use in industrial production of items such as laundry detergents, paper, wine, biofuel and degradation of biomass.*

Keywords: *Bacterial organisms; gut pH; enzymes; land snails; histology.*

1. Description of problem

The biochemical and microbial activities within the alimentary tract of *Achatina achatina* are physiologically adapted to its diet. Snail farmers need to understand well the physiology of digestion in the giant African land snails in order to provide adequate nutrition even at commercial levels that will be well suited to snails' digestive processes. In addition, enzyme producing bacteria from the snail gut could find use in the commercial biochemical industries. The anatomical and histological features of the mouth and stomach were reported in other species of mollusks (1, 2, 3, 4) however, only limited studies described the histology and microbial enzymes aiding digestion in the mouth and stomach in A. achatina. Giant land snails are well equipped with a wide range of digestive enzymes (5). Reports showed that land snails often eat plants high in protein and calcium nutrients (6), and they also partake in

leaf litter decomposition just like other invertebrates living on the soil (7). The snail's ability to decompose plant materials requires the use of a number of glycoside and polysaccharide hydrolyzing enzymes during digestion. The African land snails exhibit great efficiency in the hydrolysis of herbaceous feed materials because they depend on the activities of microorganisms within their gastrointestinal tract (8). Studies of the bacteria species found within the digestive tract of *Achatina fulica* showed mainly Firmicutes and Proteobacteria (9, 10). Some authors suggested that the microorganisms and snail host share a symbiotic relationship that unfolds with evolution. (8). Intestinal bacteria organisms facilitate the process of fermentation within the hosts gut (**11**), while soluble cellulose and chitin are particularly broken down by these species. Other reports revealed that microbial organisms are transient populations which interact with snail host when soil is ingested with food materials (8) they reported that the microbial species found within the host intestinal tract was similar to those present in the soil environment. (12) showed that *C. aspersus* do not have an endogenous microbial flora and that the microbes were taken in with plant materials and more especially with faeces. Studies have implicated diet, physiological state and season as factors that can influence the type and population of the bacterial floral in the gut of snails and other animals, (9, 10). Report for animals like bovine and fish, showed that growth and developmental stage influences microbiomes within the host (9, 13). The microflora may also be important to the snail's immune system (14). Different groups of bacteria isolated from snails could be exploited for industrial use (8). The focus of this study therefore, was to ascertain the gut pH and gut histology, presence and enzymatic activities of a few microorganisms in different parts of the alimentary tract of *Achatina achatina* which may find use in industries and guide in snail diet formulation.

Materials and methods

The identification of the gut anatomy and histology of *A. achatina* was carried out in the Animal science laboratory while enzyme assays were carried out at the Enzyme Biochemistry laboratory; Twenty (20) adult snails of *A. achatina* species of 150 – 250g live weight range were bought from the snail markets, of which twelve were sacrificed for this experiment. The snails were dissected according to the methods of (15). The physiological measurements of the gut pH were carried out using pH meter. The excised gut organ was cut into regions (mouth, radula, buccal mass, Oesophagus, crop, salivary gland, stomach, intestine, digestive gland and rectum). Individual gut region was homogenized with the gastro-intestinal tract contents and an equal volume of phosphate buffer in a Potter homogenizer which was fitted with a Perspex pestle and surrounded by ice. The homogenate pH for each region of the snail gut was measured with the pH meter. For microbial studies, 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 13g of nutrient broth powder in 1litre of deionized water.

Preparation of micro-flora cultures from snail guts content was carried out as described by (17). Microbial protease

enzyme assay was done using the method of (18) and enzyme assay method by (19), and (20). Assay of products of reducing sugars was carried out by method of (21). The characteristics of these isolates were then compared with those of known taxa for identification (22). For light microscopy, the mouth and stomach of the dissected snails were removed and fixed in Bouin's fluid. Following fixation, the tissues were dehydrated, embedded in paraffin wax and stained with Haematoxylin/Eosin (23). Data were subjected to descriptive analytical methods using line graphs.

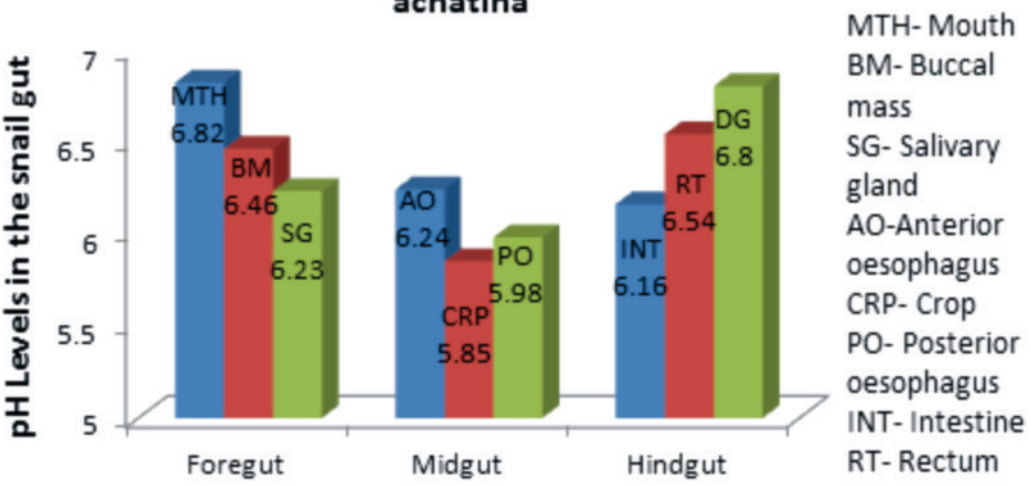
Results and discussion

The entire snail gut could be distinctly divided into three main parts; a foregut (mouth, buccal mass, salivary gland), midgut (anterior oesophagus, crop, posterior oesophagus, stomach) and hindgut (intesine, rectum, digestive gland). Similar apportioning was reported for snail gut observations carried out in earlier studies (1, 3, 24). The pH profile of the different gut regions in *Achatina achatina* is shown in Figure 1, results showed that the pH of the crop tissue was very acidic in *A. achatina.* This study agrees with earlier reports that the pH of the crop is more acidic than the pH of the other parts of the snail gut (24, 25, 26). The low crop pH may be attributed to the secretion of gut digestive enzymes and presence of food materials in the crop. The present study of *A. achatina* gut revealed an increase in pH up the gastrointestinal tract. Microorganisms have been generally implicated in the efficiency of food material digestion within the snail gut however their presence and efficiency may depend a lot on the physical and chemical characteristics such as the pH level encountered within the snail gut environment.

The list of microorganisms isolated from the different portion of the alimentary tract in *A. achatina* is shown in Table 1. The diversity of bacteria species found within the intestinal tract of *A. achatina* suggests that the snail is well adapted to different food materials. This observation has been reported in other snail species (9, 27). The gut microbiota in mammals are commonly adapted to diet of the host but in invertebrates more research is required to give better understanding of hosts and gut microbiota co- evolution (28). Snails are mainly herbivorous but some may eat dead insects and other snails. A, achatina may be utilizing its microbiota for survival, adaptation and dispersion in different ecosystems.

The characteristics of bacterial organisms found in the different portions of the snail intestinal tract are presented in Table 2. Although there were a number of bacterial microorganism isolated from the alimentary tract in *A. achatina*, only two bacterial isolates (one each) from the mouth and stomach regions of *A. achatina* species were separated for further incubation and screening for their enzymatic activities. The organisms namely: *Aerrococcus viridians* and *Bacillus aureus,* isolated from the mouth and stomach regions respectively, were then screened, for their levels of amylase, protease and cellulose activities. The amylase activities of the isolates from the mouth and stomach of *A. achatina* are shown in Figure 2. Bacterial amylase production was consistently higher in the stomach compared to the mouth region of the snail gut. *A. viridians* amylase activity was higher (1.03µM/min/ml) at 6h compared to *B. aureus* (0.77µM/min/ml) at the same incubation period. *B. cereus* had a higher

Figure 1: pH Levels in the diferent gut regions in A achatina



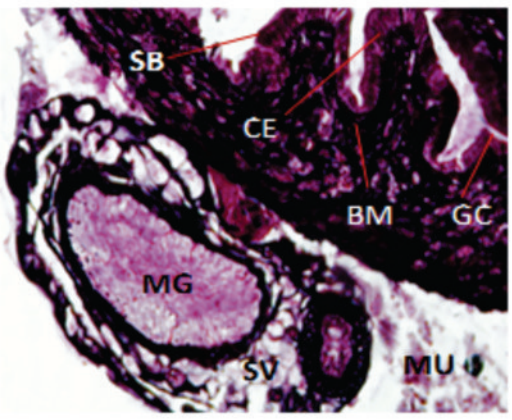


Plate1: Photomicrograph of the mouth in *A. achatina*: CE= columnar epithelium, MU=mucus, SV=secretory vesicle, MG=mouth gland, BM=basal membrane, GC=goblet cell, SB=striated border. Plate 1: H&E (X100)

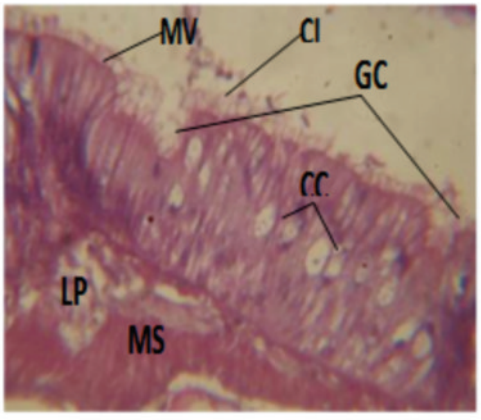


Plate 2: Photomicrograph of the stomach in *A. achatina*, GC= goblet cell, LP= lamina propia, CI=cilia, MS=muscle strands, MV= microvilli, CC=columnar cell. Plate 2: H&E (X100)

Table 1: List of bacteria isolated from the different regions of the gastro intestinal of A. achatina

Gut region	Bacterial Isolates
Mouth	*Aerococcus viridian*
Buccal mass	*Micrococcus luteus*
Salivary gland	*Erwinia herbicola*
Anterior oesophagus	*Staphylococcus aureus*
Crop	*Flavobacterium breve*
Posterior oesophagus	*Acinetobacter calcoaceticus*
Stomach	*Bacillus subtilis,*
Intestine	*Bacillus coagularis*
Rectum	*Vibrio fischeri*
Digestive gland	*Staphilococcus aureus*

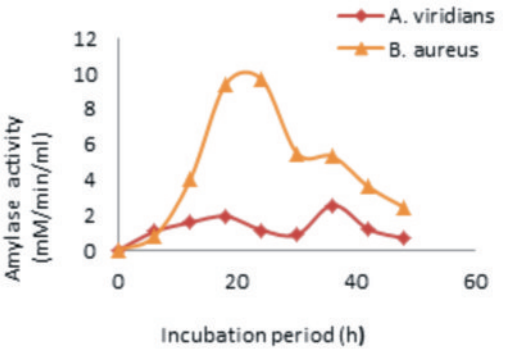


Figure 2: Amylase activities of bacterial isolates from the mouth and stomach in A. achatina

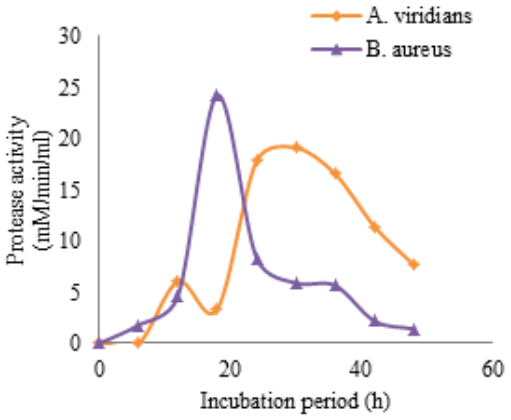


Figure 3: Protease activities of bacterial isolates from the mouth and stomach in A. achatina

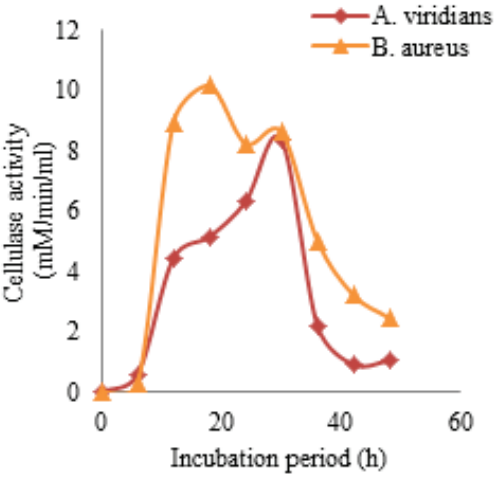


Figure 4: Cellulase activities of bacterial isolates from the mouth and stomach in A. achatina

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

Organism	CS	HLT	LAT	GLC	SCR	MN	ML	MTL	GR	SPR	GL	NR	OX	MR	VP	URS	CTL	IDL
Aerococcus viridians	CO		YG	Y	Y	YG	Y	-	+	-		-		-	-		+	-
Micrococcus lutus	SP	OX	NC	Y	Y	Y	Y	-	+		-	-	-				+	-
Erwinia herbicola	R	F	NC	YG	NC	NC	NC	+	-		-		-	+	-	+	+	+
Staphylococcus aureus	SP	F		Y	Y	Y	Y	-	+		+	+					+	-
Flavobacterium breve	R	OX	NC	Y	NC		NC	-	-		+	-	+			-	+	+
Acinetobacter calcoaceticus	R		NC	Y	Y	Y	NC	+	-		-	-	-	-	-		+	-
Bacillus subtilis	LR		NC	YG	NC	NC	YG	+	+	+	+	+		-	+			+
Bacillus coagularis	LR			YG		Y		+	+	+	+	+				-		-
Vibrio fischeri	R	F	NC	Y				+	-		+		+	-	+		+	+

amylase activity (9.65µM/min/ml) at 24h while *A. viridians* had a lower amylase activity with its initial peak (1.89µM/min/ml) at 18h and a latter peak (2.53µM/min/ml) at 36h of incubation. This result agrees with the findings of (29), who reported a maximum enzyme activity for *Bacillus* sp after 24h.

The activity of protease from bacterial isolate from the gut of *A. achatina* is shown in Figure 3. In the stomach region there was a steep rise in protease production which reached a peak at 18h but dropped drastically immediately after the peak. *B. aureus* had the highest protease activity (24.23µM/min/ml) at 18h while *A. viridians* had a lower activity with a peak (19.10µM/min/ml) at 30h. This result is in consonance with the observation of (30), they reported that *bacillus sp* had the higher protease activity at 18 hours of incubation. (31) however reported a contrary observation that *Bacillus* sp had maximum enzyme activity after 9h. The production of protease by the bacterial isolates decreased with the passage of time.

The cellulase activity of bacterial isolates is shown in Figure 4. *B. aureus* had the highest cellulase activity (10.13µM/min/ml) at 18h; it dropped to 8.21µM/min/ml at 24h of incubation but rose to 8.59µM/min/ml at 30h and eventually starts declining while *Aerococcus viridians* have its peak cellulase activity 8.33µM/min/ml at 30h of incubation. The observation that *Bacillus aureus* had a higher enzymatic activity for cellulase is in agreement with the findings of (32) and (30), although the time for peak activity for cellulase in these reports differs from that obtained in this study. They reported that *Bacillus sp* reached maximum activity for cellulase after 24 hours. *The differences in observation of peak activities may be due to the variation in strains of Bacillus sp.*

The presence of a wide range of microorganisms in the gut of *A. achatina* is an indication of the snail's intestinal tract being a good source of nutrients for microbes and there exist a symbiotic relationship between the microbes and the snail. Earlier studies reported that microbes from the snail gut were able to degrade different substrates including carboxymethyl cellulose, agarose, alginate, laminarin, and carrageenans (10, 33, 34), even when antibiotics to remove intestinal bacteria was administered, snails were still able to hydrolze polysaccharides, thereby implying that bacteria organisms only assist in feed degradation and as such increasing the host efficiency. Most of the bacteria species reported in the present study had also been reported as being present in snail gut by different authors (30, 35).

The photomicrographs of the mouth and stomach regions in *A. achatina* (Plates 1and 2 respectively) with light microscopy of the mouth region showing the presence of a columnar epithelia, which provides a semi permeable barrier would only allow necessary ion transport, there are goblet cells which produces mucous secretions that coats and protects the surrounding surface from damage. The entire lamina propia is interlaced with muscle fibres. The presence of several glandular structures and mucous secreting cells were seen in the mouth epithelia in *A. achatina* indicating the digestive and absorptive functions of the mouth, apart from picking the food materials. The stomach wall exhibits the general pattern that is characteristic of the entire digestive tract: the mucosa, the submucosa, and the muscularis externa. Mucous cells were not seen in this region however, there were few intravillous spaces, microvilli and cilia on

long and narrow villi structures whose lengths depend on the height of the ridges in the sorting area. Also there were a large number of nucleated columnal cells, goblet cells and lipid droplets. This observation is in agreement with report that the lining epithelium of the stomach in molluscs is formed by thin ciliated columnar cells interspersed with secretory cells (1, 36). Stomach lipid droplets as observed in *A achatina* were very small and consequently not so important in fat storage, as was also reported for *A. depilans* (37). The histological observations of epithelial cells along entire digestive tract suggested that nutrient absorption, intracellular digestion and storage of reserves occur along the entire length of the snail gastrointestinal tract.

Conclusion

This study concluded that:

1. Amylolitic, cellulolytic and proteolytic bacteria within the digestive tract of *A. achatina* increased the efficiency in the digestive processes.
2. Since animals possess alpha-amylases, the contribution of microbial amylases to the digestive processes in *A. achatina* might not be so significant but of greater interest, is the ability of these microbes to digest both cellulose and proteins, particularly cellulose which constitutes a very large percentage of the snail diet.
3. *B. cereus* isolates from the intestinal tract of *A. achatina* can potentially produce amylase, protease, and cellulase, which may be utilized for the production of enzymes at a commercial rate in chemical industries.
4. Epithelial cells type along entire digestive tract suggested that nutrient absorption, intracellular digestion and storage of reserves occur along the entire length of the snail gastrointestinal tract.

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