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African Journal of Microbiology Research

Full Length Research Paper

Characterization of tannase-producing bacteria in cowpea *(Vigna unguiculata L.Walp)*

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Bacteria populations isolated from Cowpea were screened on tannic acid agar medium and determined by zones of hydrolysis after 48 h of incubation at 35°C with three bacteria isolates with isolate codes; DVN 5, DVN 8 and DVN 17a, identified as *Enterococcus faecalis, Enterococcus sp.* and *Staphylococcus sp.* respectively observed as having the highest zones of hydrolysis and subjected to further screening. Tannase production was carried out via submerged fermentation at 37°C and 120 rpm for 120 h. Tannase activity was measured using the Dinitrosalicyclic method and DVN 5 showed the greatest activity; its enzyme was labeled A1 and was subjected to further enzyme characterization. The optimum condition for tannase activity was determined using a number of parameters such as temperature, pH, effect of substrate concentration, effect of various cations and effect of cation concentration. Optimum conditions were therefore ascertained at a temperature of 60°C, a pH of 9.0, substrate concentration of 0.8 g and a Mg^{2+} concentration of 1 mM. The Michealis-Menten constant (Km) value for the hydrolysis of tannic acid was approximately 0.0037 mg/ml while the maximum velocity (Vmax) was approximately 9.1408. This study established that tanninolytic bacteria species can be isolated from cowpea and used to produce tannase in appreciable quantities.

Key words: Cowpea, bacteria, tannase, enzymatic activity, optimum conditions.

INTRODUCTION

Tannase (EC 3.1.1.20) or tannin-acly hydrolase is an inducible enzyme that catalyzes the hydrolysis of tannins to release gallic acid and glucose (Belur et al., 2011; Banerjee et al., 2012; Jana et al., 2013; Lekshimi et al., 2021). It is produced by plants, animals and microbes (mostly bacteria and fungi). Tannins are a group of naturally occurring astringent plant phenolic compound distributed in different parts of vascular plants (Belmares et al., 2004, Kwan et al., 2022). Tannins are the fourth most abundant plant constituents (Mansor et al., 2019) and provide antimicrobial protection to plants (Mohapatra et al., 2006; Raitanen et al., 2020). Cowpea (*Vigna unguiculata*) is a member of the *Leguminosae* family. It has relatively low-fat content and a total protein content that is two to four times greater than cereal and tuber crops. The protein in cowpea grain is rich in amino acids,

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lysine and tryptophan, compared to cereal grains.

Cowpea can be used at all stages of growth as vegetable crop and the leaves contain significant nutritional value (Nielson et al., 1993; Ahenkora et al., 1998; Kouam et al., 2018). The aim of this study was to diversify the potential use of cowpea in Nigeria for enzyme production rather than simply for consumption.

MATERIALS AND METHODS

Chemicals

The chemicals used throughout the study were of analytical reagent grade manufactured by Oxoid.

Sample collection

A total of fifty cowpea seeds were purchased from the Poka market in Epe, Lagos State. They were transported in nylon bag to the Department of Microbiology Laboratory of Augustine University, Ilara-Epe, Lagos.

Sample preparation

The Cowpea seeds were put in a plastic container and dampened by placing it under a tap in the sink of the laboratory with the tap being turned on just enough for drops of water to flow out. They were then kept in a drawer in the laboratory to allow for microbial growth and dampened again every 24 h to promote the growth of microorganisms. This was done for a week at room temperature of 27^0C.

Isolation of bacteria

The Cowpea seeds with the most observable microbial growth and spoilage were pounded using a mortar and pestle. The grounded seeds (3.68g) were dissolved in 10mls of deionized water to prepare the stock sample solution. The stock was serially diluted up to 10^{-2} and 10^{-4} and plated using both Plate Count Agar and Tryptone Soy Agar and appropriately labeled and incubated at 35^0C for 48 h. After incubation, samples were further sub-cultured into nutrient agar to produce pure bacteria isolates.

Cultural, morphological and biochemical characteristics of isolates

The isolate was sub-cultured on nutrient agar medium. Colony characteristics such as size, shape, texture, consistency and transparency were noted down. Gram staining was carried out for the isolate. Biochemical tests such as; Indole test, Methyl red /Voges-Proskauer test, Motility tests were carried out on the isolates

Sugar fermentation tests

Selected bacteria isolates were analyzed for the ability to break down certain sugars, that is; glucose, sucrose, galactose, lactose, etc.

Isolation and screening of tannase producing bacteria

Screening of tannic acid bacteria was carried out according to the method of Nadaf and Ghosh (2011), whereby pure isolates were inoculated into a defined medium containing 0.1% tannic acid as the main carbon source. Isolates 5, 8 and 17 were chosen as the best and subjected to secondary tannase production.

Secondary screening

Secondary screening was done to estimate the enzyme activity. Submerged fermentation was performed on the 3 isolates in a screening sterilized media having following composition (g/L): Tannic acid 5.0; Peptic digests of animal tissue 5.0; Sodium chloride 5.0; Beef extract 1.50 and Yeast extract 1.50. The pH was adjusted to 7.5 (+/-0.2). The medium contained 0.5% (w/v) tannic acid as sole source of carbon and acted as substrate for tannase producing bacteria (Brahmbhatt and Modi, 2015). 48-h old cultures grown in nutrient broth were inoculated into the screening media at (2% (v/v)). Erlenmeyer flasks were used to incubate the isolates at 37^0C for 5 days and were kept in a rotatory shaker (SEARCHTECH INSTRUMENTS, ZHP-100) at 120 rpm.

Tannase production

The crude enzyme was collected after intervals of every 24 h, bringing a total of 15 crude enzyme solutions, 5 solutions for each of the 3 bacteria isolate chosen; isolate AUI 5 was labeled **A1-A5** corresponding to the number of days each enzyme solution had fermented before recovery, isolate AUI 17a was labeled **B1-B5** and AUI 8 was labeled **C1-C5**. The crude enzyme broth was aseptically removed from the flask and filtered through Whatman filter paper no.4. 1.5 ml of culture filtrate was collected and subjected to centrifugation using a refrigeration centrifuge (SEARCHTECH INSTRUMENTS, TGL-16R) at 12,000 rpm for 10 min at 4^0C. The supernatant was collected as crude enzyme and used for enzyme assays.

Tannase assay

Tannase activity was determined by estimating the reduced glucose liberated using 3, 5-dinitrosalicylic acid reagent (Miller, 1959; Dhiman et al., 2022). Standard curve was studied by preparing dilutions up to 1200 g/ml of glucose solution. The activity was determined by using the supernatant obtained after centrifugation and pre-incubating it with 0.1M acetate buffer (pH 5.0) containing 0.5% tannic acid as substrate. 1 ml of crude enzyme was reacted with the equal amount of substrate which was dissolved in the buffer and incubated for 30 min at 37°C followed by incubation in boiling water bath for another min to deactivate the enzyme-substrate activity. From this 2 ml system, 1 ml was withdrawn and reacted with 3,5-dinitrosalicylic acid reagent (DNSA) and finally the system was diluted by adding 10 ml distilled water and absorbance was measured at 540 nm on spectrophotometer (Model VIS-723N). The formula to calculate activity was: Enzyme activity (U/ml) = Microgram of glucose produced / V x T, where microgram of glucose can be obtained from the standard graph, V is the aliquot of enzyme sample and T is the time of hydrolysis (Brahmbhatt and Modi, 2015). Static as well as shaking conditions were studied by Miller method. One unit of tannase activity was defined as the amount of enzyme releasing 1µmol of glucose per minute under assay conditions.

Table 1. Morphological and biochemical characteristics of bacteria strain AUI 5 (*Enterococcus faecalis).*

Isolate	Colony morphology	Gram'S stainZ	MSA	MOT	IND	S.H	URE	TDA	CAT	VP	MR	CIT	SM
AUI 5	Cocci, single orientation, round colony shape, cream pigmentation, transluscent, smooth surface	Positive	+	-	-	-	-	-	+	+	-	+	-

+ = Positive result; **-** = negative result; MSA= Mannitol salt agar; G/+ve= Growth with mannitol production; CIT = Simmon citrate test; TDA= Tryptophan deaminase test; MOT= Motility test; M.R = Methyl Red; S.H = Starch hydrolysis; V.P = Vogues Proskauer; URE = Urease test; CAT = Catalase test; IND = Indole production; SM = Skimmed milk test.
Source: Authors

Protein estimation and specific enzyme activity

Protein estimation of the enzyme samples was carried out according to Lowry et al. (1951). Absorbance was taken at 750nm; 1ml of the isolate was used. Specific enzyme activity was calculated by dividing the total enzyme activity by the protein content. It denoted the purity of the enzyme solutions. Enzyme A1 from bacteria sample AUI 5 showed the greatest activity and was subjected to further characterization tests.

Tannase characterization

The effect of various physio-chemical factors like pH, temperature, substrate concentration, cations and cation concentration on tannase activity were assayed. For evaluating the effect of the above factors on tannase activity, the bacteria culture was grown on nutrient medium (Muslim et al., 2015; Dayana et al., 2021).

Effect of temperature, pH and Substrate (Tannic Acid) Concentration on tannase activity

The effect of temperature and pH on tannase activity was measured by standard assay as described by Muslim et al. (2015) with incubation temperatures ranging from 30^0C to 75^0C while pH ranged from 4.0 to 9.0 and substrate concentrations ranged from 0.2 to 2.0%

Effect of cations and cation concentration

The effect of various cations at 1mM concentrations were studied using their water soluble salts. The cations were Cu^{2+}, K^+, Mg^{2+} and Ca^{2+}. The soluble salts of each cation were Copper (II) Sulphate, Potassium Nitrate, Magnesium Sulphate heptahydrate and Calcium Chloride respectively. Tannase activity was measured by standard assay (Muslim et al., 2015). The cation with the highest positive impact on the tannase enzyme from the cation assay was used in the cation concentration test. Concentrations were varied from 2mM to 10mM, with 1 mM used as a relative value. Tannase activity was measured by standard assay (Muslim et al., 2015).

Kinetic determination

Initial reaction rates of tannase were determined at different substrate concentrations ranging from 0.2 to 2% tannic acid. The kinetic constant (Km) was estimated following the method of Lineweaver and Burk (1934) for tannase from isolate code AUI 5 and enzyme designation A1 (Ranaldi et al., 1999).

RESULTS AND DISCUSSION

Cultural, morphological and biochemical characteristics of isolate

The Cultural, morphological and biochemical characteristics of Bacteria strain AUI 5, identified as *Enterococcus faecalis* were ascertained and the results are represented in Tables 1 and 2.

Isolation and screening of tannase producing bacteria

16 morphologically different bacteria colonies were obtained from the degraded Cowpea sample used in this investigation. Out of the 16, isolates AUI 5, 8 and 17a exhibited the highest zones of tannic acid hydrolysis, with AUI 5 and 8 having the highest at 21 mm and 20 mm respectively (Figure 1), while 17a had 13 mm. They were thus selected for secondary tannase screening to determine their enzyme activity. The isolate AUI 5 its tannase enzyme, A1, showed a specific activity of 0.0043 U/mg.

Tannase assay

The result of this investigation revealed that bacteria isolates from Cowpea produced tannase. The investigation revealed tannase production after 120 h incubation from all three chosen isolates for secondary screening (Figure 2).

This is supported by Schons et al. (2012) who also reported tannase production after 120 h. Enzyme isolates

Table 2. Sugar fermentation test results of bacteria strain AUI 5.

Isolate	GLU	FRU	GAL	MAL	SUC	LAC	MANT	D-XYL	GLY	TRE	RAF	MAN	SAL	ARA
AUI 5	A	A	A	-	+	-	A+	A+	A+	A+	-	A+	A+	A+

GLU = Glucose - = neither acid nor gas produced; GAL = Galactose; A = Acid produced only; FRU = Fructose; A+ = Acid produced (durham tubes absent); MAL = Maltose; + = Acid and Gas Production; SUC = Sucrose; TRE = Trehalose; LAC = Lactose; RAF = D-Raffinose; MANT = Mannitol; M AN = D-Mannose; D-XYL = D(+) Xylose; SAL = Salicin; GLY = Glycerol; ARA = L-arabinose.
Source: Authors.

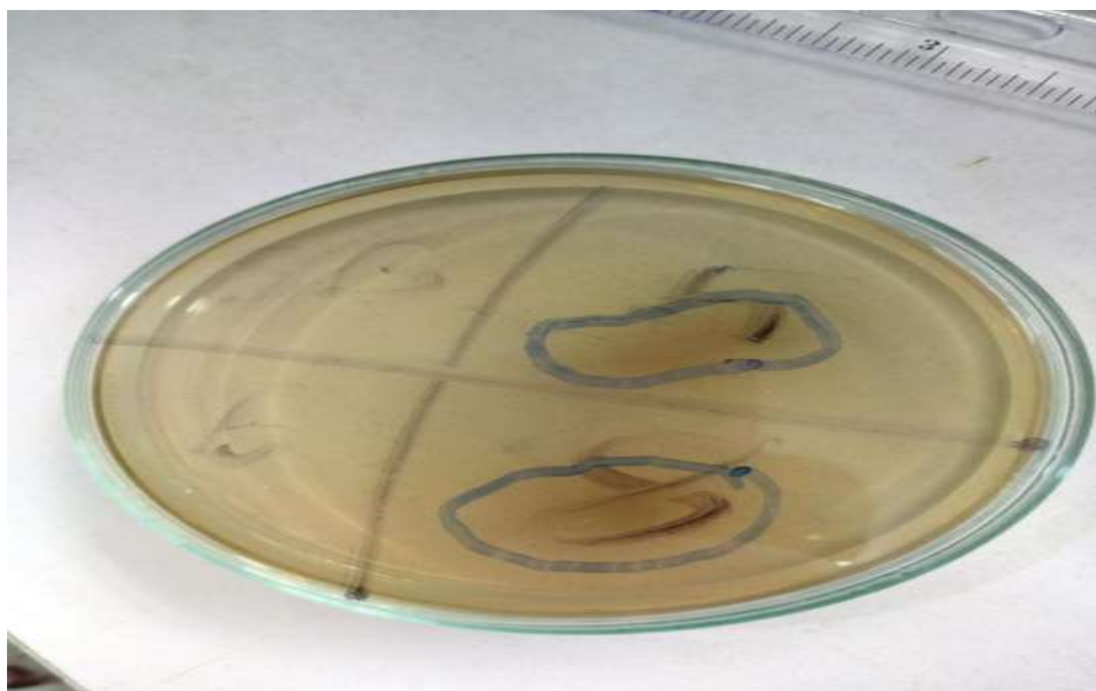

Figure 1. Marked zones of tannic acid hydrolysis for AUI 5 (above) and AUI 8.
Source: Authors.

'A' and 'C' showed maximum tannase activity within 24-72 h whereas 'B' showed maximum activity at 24 and 120 h. Brahmbhatt et al. (2015) reported maximum tannase production within 72-96 h. Of all 15 enzyme solutions from A1 to C5, A1 produced the highest total activity (32.194 U/ml) and highest protein estimate (7402 µg/ml) (Table 3), and thus, had the highest specific activity. It was thus chosen for further tannase characterization tests.

Effect of temperature, pH and substrate (tannic acid) concentration on tannase activity

The optimum temperature for tannase in this study was at 60°C (Figure 3). Previous reports on temperature optima for tannase were between 20 to 60°C depending on the microbial source (Sharma et al., 1999; Mondal et al., 2001; Kaziecka-Burnecka et al., 2007; de Lima et al., 2022). Brahmbhatt et al. (2015) and Selwal et al. (2010) both reported tannase optima at 37°C. Kumar et al. (1999) reported maximum tannase activity at 30°C. Mondal et al. (2001) reported an optimum tannase activity at 40°C. Sabu et al. (2006) reported maximum tannase activity at 30°C. Das Mohapatra et al. (2006) reported maximum tannase activity at 35°C. de Lima et al. (2022) reported optimum activity at 30°C and 80% activity at 40 to 60°C. Amitabh et al. (2018) reported an optimal temperature range of 30 to 70°C. Liu et al. (2018) reported an optimum temperature of 40°C.

The optimum pH for tannase in this study was at 9.0 (Figure 4) showing a preference for alkaline environment, Belur et al. (2010) reported optimum tannase activity at pH 8.9, while Iwamoto et al. (2008) reported optimum

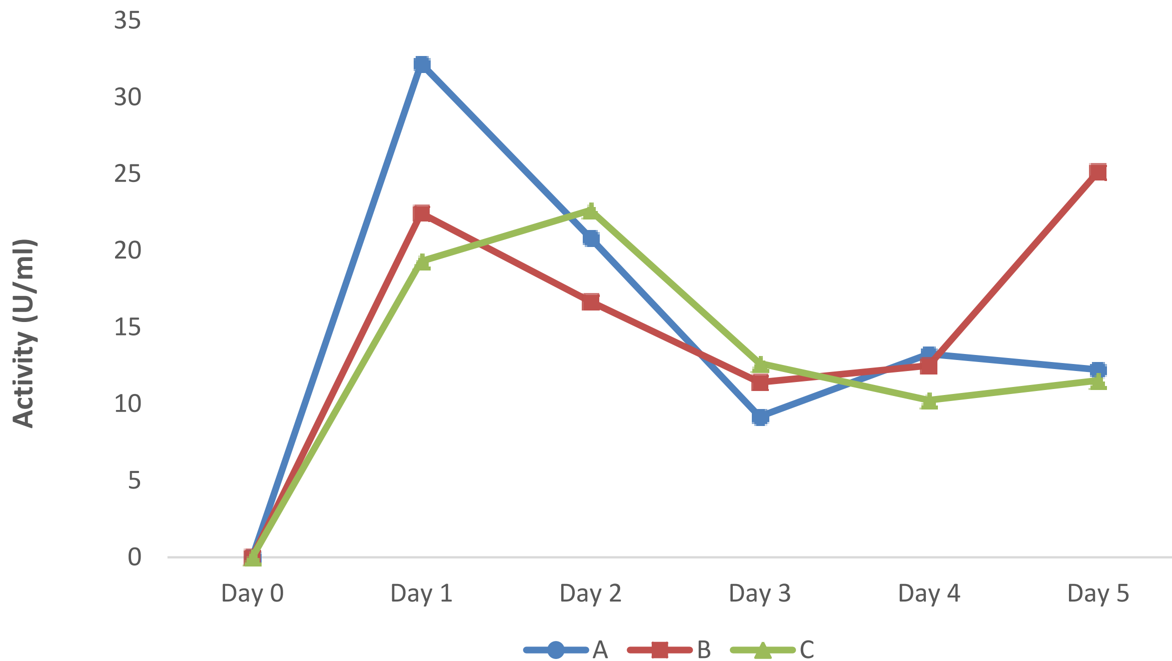

Figure 2. Total activity of tannase solutions in five days using submerged fermentation.
Source: Authors.

Table 3. Tannase assay results.

Enzyme code	Total activity (U/ml)	Protein (µg/ml)	Specific activity (U/mg protein)
A1	32.194	7402	0.0043

Source: Authors.

tannase activity at 8.0, however, generally, tannase exhibits optimum activity at acidic pH (Yao, et al., 2013), and several investigations have found tannase to be optimum at acidic pH range (Sharma et al., 1999; Mondal et al., 2000; Van de Lagemaat and Pyle, 2005; Sharma et al., 2011; Abdel-Nabey et al., 2011). Selwal et al. (2010) reported optimum pH of 5.5. Mondal et al. (2001) reported an optimum pH of 5.0. Ayed and Hamdi (2002) reported optimum pH at 6.0. Belur et al. (2010) reported optimum pH at 6.0. de Lima et al. (2022) reported optimum pH at 7.0. Amitabh et al. (2018) documented an optimal pH range of 3-10. Liu et al, (2018) reported an optimum pH at 7.0

The optimum tannic acid concentration for the enzyme in this study was 0.8% (w/v) (Figure 5). Abdel-Nabey et al. (2011) reported optimum tannase concentration to be at 2.0% (w/v). Seth and Chand (2000) reported maximum tannase activity at 3.5% (w/v). Ayed and Hamdi (2002) reported optimum tannase activity at 1.5% (w/v). Kanpiengjai et al. (2019) reported tannase activity at 1.0% (w/v).

Effect of cations and cation concentration

The effects of 4 cations ; Mg^{2+}, K^{+}, Cu^{2+} and Ca^{2+} using their soluble salts ($MgSO_4.7H_2O$, KNO_3, $CuSO_4$ and $CaCl_2$ respectively) (Figure 6) on the crude enzyme were estimated, Mg^{2+} was shown to promote the tannase activity the most, and thus was subjected to the cation concentration test.

K^{+} also promoted its activity whereas Ca^{2+}, Cu^{2+} inhibited it. Ca^{2+} has been reported as a tannase stimulator, which contradicts the investigation (Beniwal et al., 2010), Calcium Chloride (CaCl2) which was the soluble salt used in this study has been reported an a tannase inhibitor alongside Cu^{2+} which supports this study (Sabu et al., 2005; Wright 2005, Liu et al., 2018), which might explain why the enzyme was inhibited despite the presence of the calcium cation. K^{+} has been reported to stimulate tannase activity (Sabu et al., 2005; Wright, 2005). Magnesium was shown to have the maximum effect at 1 mM concentration in this investigation (Figure 7).

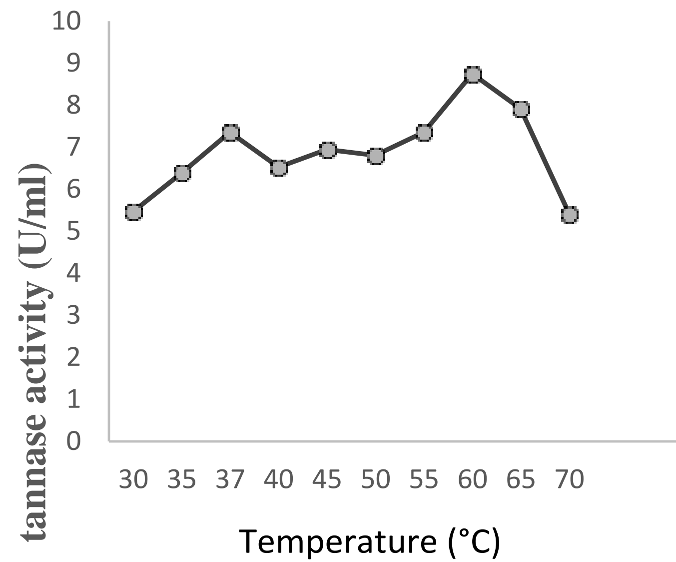

Figure 3. Effect of temperature on tannase activity.
Source: Authors.

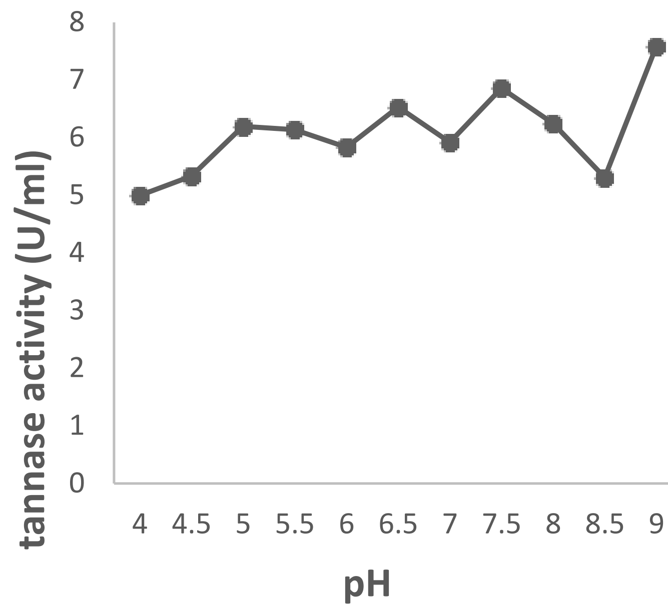

Figure 4. Effect of pH on tannase activity.
Source: Authors.

Kinetic determination

The apparent Km and Vmax value for the hydrolysis of tannic acid by tannase produced from enzyme A1 were 0.0037 and 9.1408 respectively. The values were calculated using the Lineweaver-Burk plot (Figure 8) based on the method of Lineweaver and Burk (1934).

Conclusion

Tannic acid media was used to isolate the bacteria having natural ability to degrade hydrolysable tannins by producing tannase. 16 bacteria strains were isolated from various tannin rich soil samples. Among them, three strains; AUI 5, AUI 8, and AUI 17a (*Enterococcus*

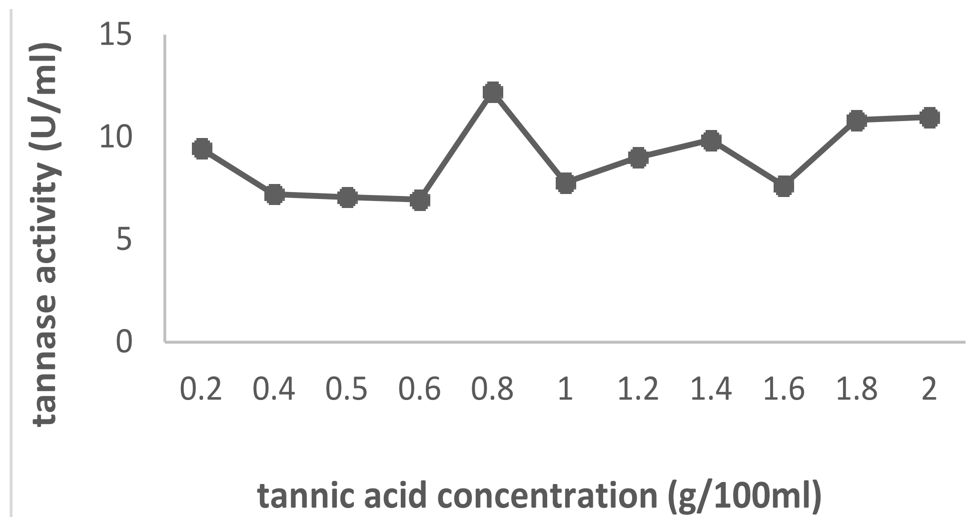

Figure 5. Effect of substrate concentration on tannase.
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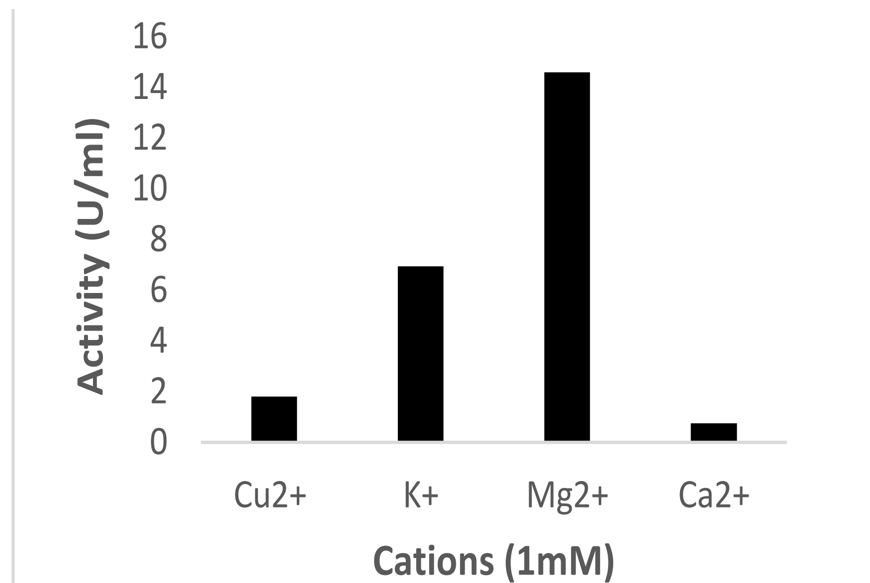

Figure 6. Effect of different cations on tannase activity (tannic acid).
Source: Authors.

faecalis, Enterococcus sp. and *Staphylococcus sp.* respectively) could hydrolyze tannin and were subjected to secondary tannase screening. The bacteria isolates were identified by morphological and biochemical characteristics. Strain AUI 5 with enzyme AI was selected as the most effective. In this investigation, it showed a tannase activity of 0.0043 U/mg protein after 24 h of incubation. The effect of various environmental factors on enzyme activity was carried out by growing the organism in varying conditions. This research work has established the fact that Cowpea (*Vigna unguiculata* L. Walp) is host to a number of tannase-producing bacteria from which the enzyme can be produced commercially. The optimum conditions from the tannase solution chosen has been determined as; 0.8%, 6°C, pH 9.0, 1mM Mg2+ for substrate concentration, temperature. It had a Km value of 0.0037 and a Vmax value of 9.1408. The low KM value indicates a high affinity by the enzyme for tannic acid.

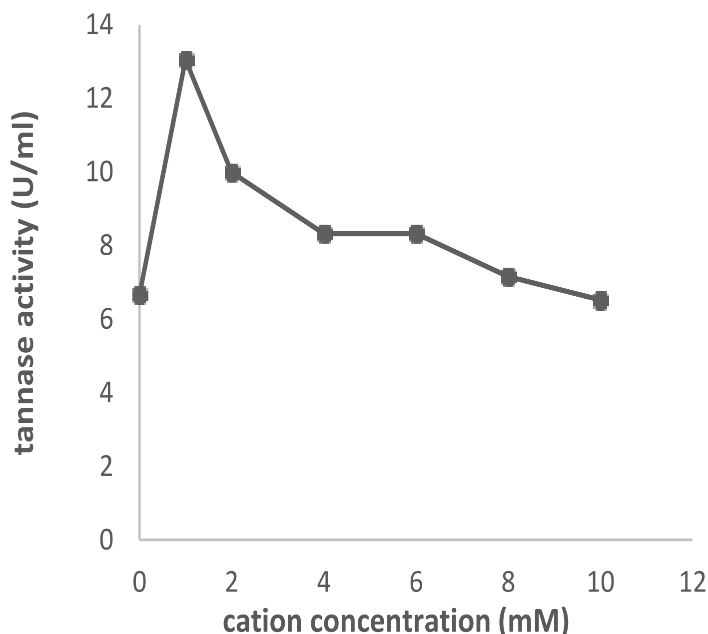

Figure 7. Effect on Mg^{2+} concentration on tannase.
Source: Authors.

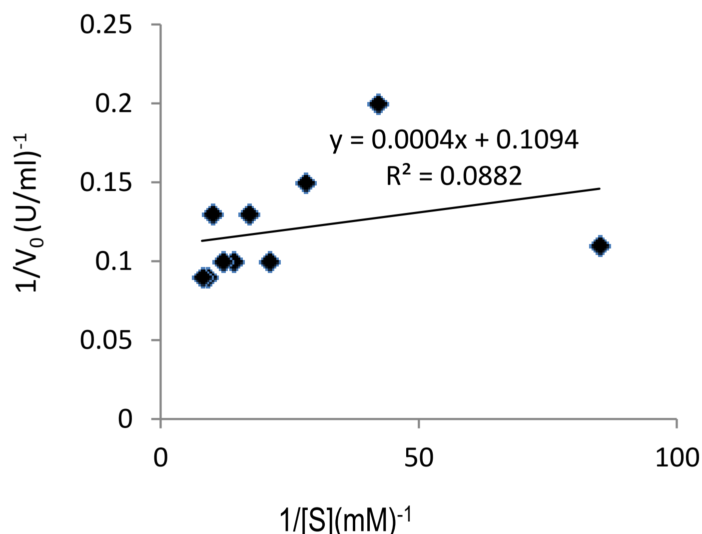

Figure 8. Lineweaver-burk plot for tannic acid hydrolysis activity.
Source: Authors.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Molecular analysis of zoonotic pathogens observed in *Thryonomys swinderianus* (Marsh cane rat) in the city of Daloa in Center Western Côte d'Ivoire

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Bush meat is a source of food and income for many people in Center Western Côte d'Ivoire. However, it can have adverse effects on the health of the population when food safety practices are not respected. The assessment of the health risk associated with the consumption of bush meat was carried out on 17 faeces samples of *Thryonomys swinderianus* (marsh cane rat) collected in the city of Daloa in Center Western Côte d'Ivoire. Coproscopy was performed on these faeces for the identification of intestinal parasites followed by DNA extraction from the isolated bacteria. This DNA was amplified by PCR using the 16S rDNA primer and sequenced. The resulting sequences were analysed using bio-informatics tools. This study revealed the presence of a diversity of parasites and bacteria pathogenic to humans in these animals. The *Trichuris trichiura* species is the most detected parasitic species in terms of intestinal parasites. The bacterial profile obtained is dominated by species belonging to the Enterobacteriaceae family, in particular *Klebsiella pneumoniae*, which is the most common species in the samples analysed. The presence of human pathogens in the faeces of wild animals demonstrates the zoonotic nature of parasitic and bacterial infections and the potential infectious risk of bush meat consumption.

Key words: *Thryonomys swinderianus*, Marsh cane rat, bush meat, zoonotic pathogens, health risk, molecular analysis, Center Western Côte d'Ivoire.

INTRODUCTION

Consumed wildlife or bush meat is a source of food and income for many populations in sub-Saharan Africa (Williamson and Backer, 2017; Chabi-Boni et al., 2019). Côte d'Ivoire, a country located in the West African tropical zone, is no exception. Indeed, the consumption of bush meat is becoming increasingly noticeable in all regions of the country in general and in the Center Western region in particular (Gonédélé et al., 2017; Yéboué et al., 2020).

In the Center Western region of Côte d'Ivoire, which is

the subject of our study, it appears that the wildlife commonly poached and sold in restaurants is largely made up of small mammal species, mainly rodents. The most poached species and the one most prized by the population in this part of the country is the marsh cane rat *Thryonomys swinderianus* (Yéboué et al., 2020). However, the consumption of this bush meat can have adverse effects on the health of the population when hunting, transport, handling and cooking do not follow food safety practices (Van Vliet et al., 2017).

According to the World Health Organization, more than 50% of new infectious diseases in humans are caused by pathogens from animals or animal products, 70% of which are from wildlife (OIE, 2012).

These diseases, known as zoonosis, can spread in a variety of ways between animal hosts and humans. These include shared vectors, indirect contact through exposure to rodent faeces in a peri-domestic environment, but especially through direct contact with an animal through handling, consumption, bites, scratches, body fluids, tissues and excreta (Wolfe et al., 2005; Johnson et al., 2020).

Côte d'Ivoire is not on the fringe of risk nations with regard to the spread of zoonotic diseases (Liégeois et al., 2009). However, few studies have focused on health risks since the Ebola fever episode in West Africa and the Covid pandemic.

The objective of this study is to highlight the health risks associated with the consumption of bush-meat through the parasitological and cyto-bacteriological analysis of faeces samples from specimens of the marsh cane rat *T. swinderianus* found in the city of Daloa located in the Center Western Côte d'Ivoire.

MATERIALS AND METHODS

Collection of samples

Samples of faeces from seventeen fresh specimens of *Thryonomys swinderianus* (marsh cane rat) were collected from restaurants in the city of Daloa in the Center Western Côte d'Ivoire during March and April 2020.

Isolation of intestinal parasites from bush meat

Faeces samples were analysed by coproscopy after enrichment with Willis' liquid (33% saturated aqueous NaCl solution of specific gravity 1.2), using the flotation technique as described by Degbe et al. (2018). The purpose of this technique is to concentrate the principle is based on the density of the NaCl solution used and that of the parasites. It relies on the use of solutions with a density higher than that of most parasite eggs (Degbe et al., 2018).

In a graduated beaker, about ten (10) g of faeces were carefully triturated with a small amount of 33% saturated salt water until the mixture was homogeneous.

The level of the homogenate was reduced to 60 mL by adding the NaCl solution used. The suspension column was then sieved to remove any coarse material. Approximately 50 mL of homogenate was collected and transferred to a tube.

Subsequently, a slide was placed on the surface of the liquid without trapping air bubbles. After about 30 min, the slide was removed from the surface of the solution and placed on an object slide for observation of the parasite elements. After observation under a light microscope (x10, x40) (WHO, 1994) was used for the identification of the parasites.

Isolation of bacteria on CHROMAgar™ orientation medium

The presence of pathogenic bacteria was also investigated in the faeces of the Marsh cane rat *T. swinderianus*. Ten (10) microlitres of faeces samples, previously grown in enrichment medium, were inoculated onto CHROMAgar™ Orientation agar by tight and loose streaks, using single-use loops. The inoculated media were then incubated at 37°C for 24 h in an oven. CHROMAgar is a chromogenic medium that allows colorimetric differentiation of bacterial agents present in faecal samples. Thus, it allows a directed identification of the bacterial species.

Identification of isolated bacteria

Morphological identification: Gram stain test

A differential identification of the isolated strains was performed. Gram staining is the basic differential identification test in bacteriology (Delarras, 2007). It makes it possible to differentiate between gram-negative bacteria (coloured purple) and gram-positive bacteria (coloured pink) and to study their morphological character, in particular the form (cocci or bacillus). To do this, a pure bacterial colony is spread on an object slide. After drying at room temperature, this preparation is subjected to the action of four reagents allowing contrast effects: A first dye (0.5% crystal purple), a mordant (1/3 Gram iodine obtained from Lugol's iodine) which complexes the dye, a differentiator (Gram differentiator) which is a decolourising solution and finally, a second dye (1% safranin solution).

The reading is made by microscopic observation with an X100 objective. The enterobacteria appear as pink coloured bacilli.

Biochemical identification

Identification based on the biochemical characteristics of the bacteria was carried out using the classical gallery system described by Le Minor and Richard (1993). This is a system consisting of several enzymatic and metabolic tests.

DNA extraction from the faeces of the marsh cane rat *T. swinderianus*, polymerase chain reaction (PCR) amplification and sequencing

Bacterial cultures were made in Luria Bertani (LB) broth.

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Table 1. 16S rDNA amplification programme.

Step	Temperature (°C)	Time	Cycle
Initial denaturation	94	2 min	1
Denaturation	94	30 s	
Annealing	46	40 s	35
Extension	72	1:30 s	
Final extension	72	10 min	1
Conservation	4	∞	

Source: Authors

Subsequently, 1 mL aliquots of LB were made in cryotubes. They were sowed with a well-isolated colony on CHROMAgar agar medium and incubated for 24 h at 37°C.

Extraction of genomic DNA from the bacteria in the faeces was performed according to the protocol using phenol-chloroform described by Chan and Goodwin (1995) and checked on a 1% agarose gel for quality control. The quantities of reagents were slightly modified and adapted to the conditions of this work.

The reaction mixture for each simplex PCR was prepared to a final volume of 50 µL containing: 1µl of each primer (F: 5'-GCAAGTCGAGCGGTAGCACAG-3' and R: 5'-CAGTGTGGCTGGTCATCCTCTC-3') (260 bp), concentrated to 10 pmol/µL (Eurogentec), 5 µL of Mg^{2+} PCR buffer (10X), 1.5 µL of $MgCl_2$ (25 mM), 2.5 µL of dNTPs (200 µM), 0.1 µL of Taq polymerase (5U/µL), and 35.9 µL of ultra-pure water. Three (3) µL of genomic DNA extract to be amplified was added to this mixture for transfer to the thermal cycler.

DNA was amplified according to the protocol shown in Table 1. PCR products representative of all bacterial species isolated were coded and sent to BGI TECH SOLUTIONS (HONG-KONG) for sequencing.

DNA sequences assignment

Chromas Lite® 2.01 software was used to make the DNA sequences received in the ABI format files analyzable. The forward and reverse sequences of the genes were assembled and then corrected using DNA Baser Assembler 5.15.0 software and Chromas Lite® 2.01 software. *In silico* analysis of the sequences and phylogenetic characterization of bacterial species are made. The sequences obtained were compared with existing reference sequences in the public genomic database of the National Center for Biotechnology Information (NCBI) using the BLAST (Basic Local Alignment Search Tool; http://www.ncbi.nih.gov) (Altschul et al., 1990).

Determination of phylogenetic relationships between bacterial species

The evolutionary history of the pathogens was deduced using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method on the 16S ribosomal DNA gene sequences. This method assumes an independent evolution of the different genetic profiles at a constant rate, with 1000 repetitions.

The optimal tree with the sum of the branch lengths = 2.77503562 is shown. The percentage of replicate trees in which the associated bacterial strains clustered in the bootstrap (1000 replicates) is indicated next to the branches.

The evolutionary distances were calculated using the Jukes-Cantor method and are in units of the number of base substitutions per site. This analysis was performed on 13 DNA sequences. Codon positions included were 1st, 2nd and 3rd. Positions observed in sequence pairs whose interpretation seemed uncertain were removed. There were a total of 269 positions in the final data set. These evolutionary analyses were performed in MEGA X software. Significance is observed for all statistical tests performed when the probability value (p) associated with the statistical tests is strictly less than 0.05.

Determination of the occurrence of microbial and parasitic agents

The occurrence of a micro-organism is the presence or absence in an analysed faeces sample. The percentage of occurrence was calculated in order to highlight the most represented microorganism in the intestinal microbiota of the animal under consideration.

Health risk analysis

The health risk associated with bush meat is highlighted by detecting and identifying the species of parasites and potentially pathogenic bacteria present in the faeces of *Thryonomis swinderianus* specimens and determining their occurrence.

RESULTS

Risk of parasitic infection

Analysis of the samples revealed the presence of four types of intestinal parasite eggs. These are: eggs of *Trichuris trichiura*, *Trichuris leporis, Trichostrongylus axei* and *Paraspidodera uncinata* (Figure 1).

Of the 17 samples of Marsh cane rat faeces analysed, eggs of *Trichuris trichiura* were detected in the majority (7; 41.2%), followed by eggs of *Trichuris leporis* (5; 29.4%), *Trichostrongylus axei* (3; 17.6%) and *Paraspidodera uncinata* (2; 11.8%).

Risks to bacterial infections

In addition to intestinal parasites, analysis of faecal

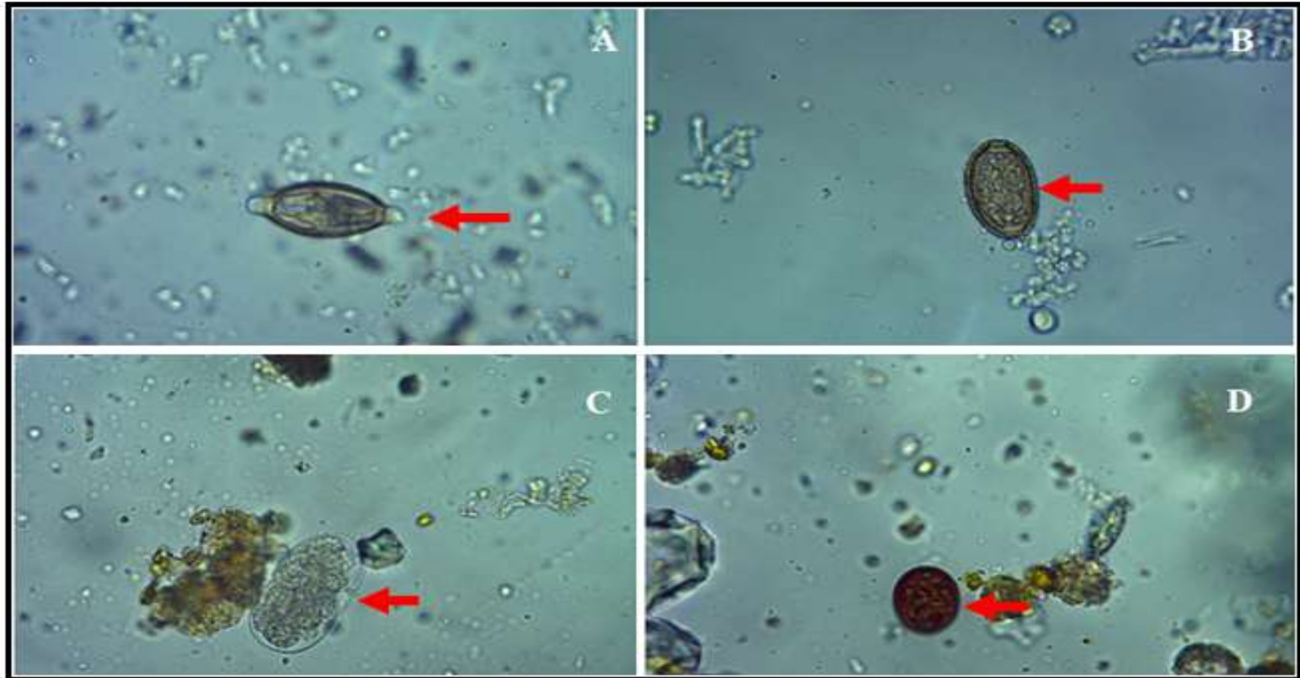

Figure 1. Parasite eggs isolated from the faeces of *Thryonomis swinderianus* (Marsh cane rat) collected in Center Western Côte d'Ivoire. A: Eggs of *Trichuris trichiura; B:* Eggs of *Trichuris leporis; C:* Eggs of *Trichostrongylus axei;* D: Eggs of *paraspidodera uncina.*
Source: Authors

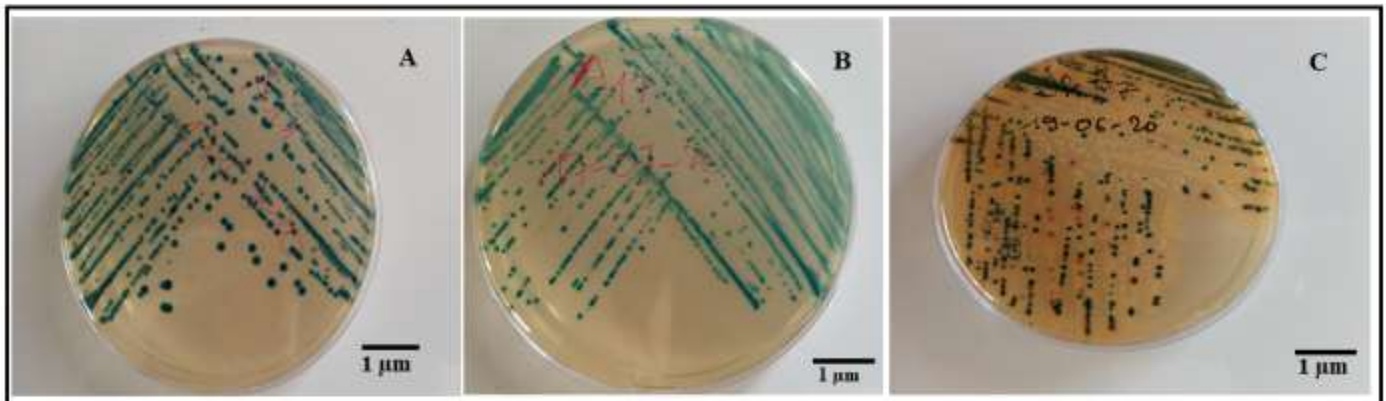

Figure 2. Gram-negative bacteria colony with metallic blue staining characterising the KESC group. KESC: *Klebsiella, Enterobacter, Serratia, Citrobacter; A and B: monobacterial infections; C: polybacterial infection.*
Source: Authors

samples revealed the presence of bacteria with infectious characteristics. On the basis of morphological and biochemical characteristics, the analyses described metallic blue staining Gram-negative bacteria characterising the KESC group (Figure 2). These bacteria belong to the Enterobacteriaceae family. Isolates of *Escherichia coli*, easily recognisable by their pink coloration, were also found in the intestinal bacterial flora of the Marsh cane rat. They are gram-negative. Also, polymicrobial infections are found (Figure 2).

In order to refine the characterisation of the gut microbiota of *Thryonomis swinderianus*, molecular typing of 16S rDNA was performed on 13 faeces samples. These Internal Transcribed Spacer (ITS) are characterised by DNA fragments of 260 base pairs.

Figure 3 shows the PCR amplified 16S rDNA

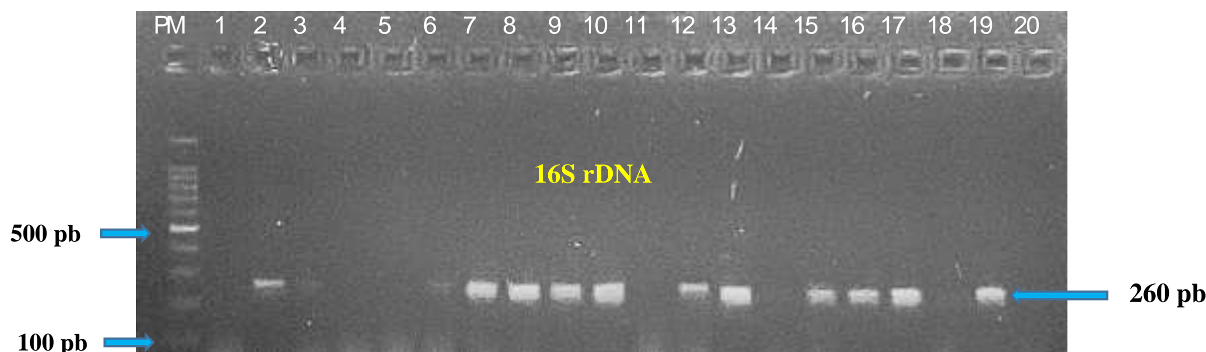

Figure 3. Electrophoresis gel showing PCR amplification products observed with the genetic markers 16S rDNA.
Source: Authors

fragments. The resulting PCR products were sequenced. Sequence analysis of the 16S rDNA gene, using the BLASTn programme, showed that the isolated bacterial strains are taxonically related to *Klebsiella* sp., *Enterobacter* sp., *Klebsiella pneumoniae, Burkholderia cepacia, Neisseria cineria, Erwinia endophytica, Serraria marcescens* and *Erwinia tasmaniensis* with percentages of identity ranging from 86.16 to 100%. These species are all enterobacteria.

The phylogenetic relationship of the bacterial strains reveals that the gut microbiota of *Thryonomis swinderianus* is composed of two groups (group A and group B) of bacteria (Figure 4). Group A comprises 61.54% (n= 8 strains) of the strains analysed. This group is predominantly composed of human pathogenic strains (87.5%; in red), whose close relatives are *Klebsiella pneumoniae*, *Enterobacter* sp. and *Burkholderia cepacia*. One isolate from the group was identified as an uncultured bacterium (black). In group B, there were 5 strains representing 38.46% of all strains analysed. The strains in this group are genetically related to species such as *Neisseria cineria*, *Erwinia endophytica*, *Klebsiella* sp., *Serratia marcescens* and *Erwinia tasmaniensis*. Species of potential interest in human pathology for this group are *Klebsiella* sp. and *Serratia marcescens* (Figure 4).

DISCUSSION

The study of the health risk based on the analysis of marsh cane rat faeces samples revealed the presence of a diversity of microorganisms in these animals, notably parasites and bacteria. Four species of parasites were identified. These are *Trichuris trichiura*, *Trichuris leporis*, *Trichostrongylus axei* and *Paraspidodera uncinata*. Of these, *Trichuris trichiura* and *Trichostrongylus axei*, detected at rates of 41.2 and 17.6% respectively, are human pathogenic species.

Indeed, *T. trichiura* is a nematode responsible for trichocephalosis in humans. According to the WHO, this parasite infects more than one billion people, 220 million of whom are severely affected and one thousand of whom die each year (WHO, 2011). Trichocephalosis is a cosmopolitan parasite, most often without any symptoms. In tropical countries, poor rural areas with fecal peril, infestation can be massive and severe, especially in children (Sunkara et al., 2018). *Trichostrongylus axei* is an intestinal parasitic worm, generally found in ruminants, birds and primitive rodents, with a worldwide distribution (Anderson, 2000; Audebert and Durette-Desset, 2007).

In Africa (in the southern part of the continent), this species is one of the main parasites of its species found in ostriches (Smith, 2018). In these hosts, it causes decreased appetite and progressive weight loss. In addition, studies in Thailand have reported infection in humans, which appears to coincide with close contact with infected animals (Phosuk et al., 2013).

Infected humans present with stomach upset, abdominal bloating, diarrhoea and eosinophilia, which is a blood disorder due to increased eosinophils (Lattès et al., 2011; Wall et al., 2011). The presence and spread of *T. trichiura* and *T. axei* in the wild animal population in general and in marsh cane rat in particular, constitutes a health risk in case of contact with these animals. The zoonotic nature of these two pathogens therefore deserves special attention. Concerning the species *Trichuris leporis* and *Paraspidodera uncinata*, although human infection has never been demonstrated, they are responsible for serious infections in animals. Heavy infestation can cause anorexia, diarrhoea and weight loss associated with a rough coat, which could have serious consequences for the survival of the animals, and therefore for their health. Consequences for animal survival, and thus a threat to biodiversity (Schoeb et al., 2007; Bartholds et al., 2016).

Bacterial infections due to species such as *Klebsiella pneumoniae*, *Enterobacter* sp, *Serratia marcescens*, *Burkholderia cepacia*, *Neisseria cineria*, *Erwinia tasmaniensis* and *Erwinia endophytica* were detected.

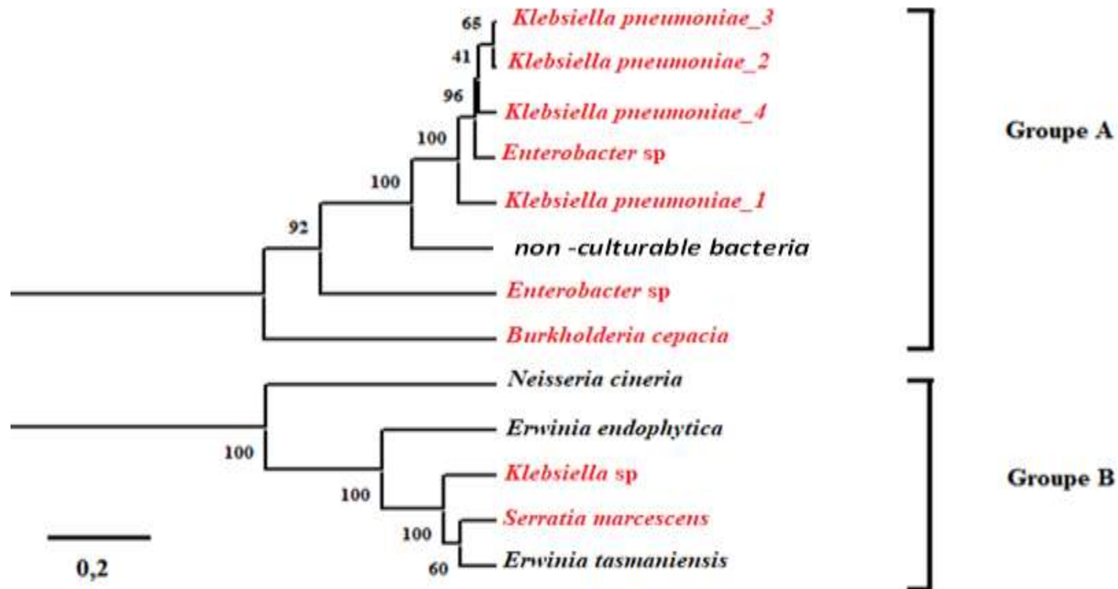

Figure 4. Dendrogram showing the phylogenetic relationship between the bacterial species identified from the 16S rDNA gene sequences. In red: Species accepted in human pathology; In black: Non-pathogenic species for humans or undefined.
Source: Authors

The bacterial profile obtained is dominated by species belonging to the Enterobacteriaceae family and known for their pathogenicity in humans. These are species of the genus *Klebsiella*, *Enterobacter* sp., *Burkholderia cepacia* and *Neisseria cineria*. *Klebsiella pneumoniae* is the most represented species of this family with an occurrence of 38.46% (in 5 samples out of 13 sequences analysed). This species has been medically recognised as one of the most important opportunistic pathogens, causing infections of the pulmonary system, urinary tract, circulatory system and soft tissues, acquired in the environment. Soft tissue infections acquired in hospitals or associated with healthcare worldwide (Hou et al., 2015). In addition, Vincent et al. (2010) and Jakobsen et al. (2012) have identified *Klebsiella* strains in pet feces. This study appears to be the first to demonstrate the presence of *K. pneumoniae* in wild animals in Côte d'Ivoire. The presence of human pathogenic bacteria in the faeces of wild animals shows a zoonotic character of bacterial infections.

The presence of "uncultured bacteria" in the faeces revealed by this study could be explained by the absence of genomic data, targeting the coding region of the ribosomal DNA gene sequence.

Conclusion

This study highlighted the health risks associated with the consumption of bush meat through the analysis of faecal samples of marsh cane rat *T. swinderianus* specimens. It revealed the presence of a variety of microorganisms in Marsh cane rat, including parasites and bacteria pathogenic to humans. The *Trichuris trichiura* species, responsible for trichocephalosis, is the most detected intestinal parasite. The bacterial profile obtained is dominated by species belonging to the Enterobacteriaceae family and known for their pathogenicity in humans. *K. pneumoniae* is the most represented species of this family. Although some authors have shown in their studies the presence of Klebsiella strains in the faeces of domestic animals. This study shows, for the first time, the presence of *K. pneumoniae* in wild animals in Côte d'Ivoire. The presence of human pathogens in the faeces of wild animals shows the zoonotic nature of these parasites and bacteria.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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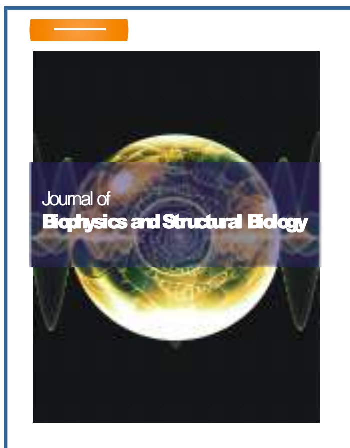

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