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Effects of *Tamarindus indica* (Linnaeus 1753) pulp-fortified diets on the gut microflora and morphometry in African catfish *Clarias gariepinus* (Burchell 1822)

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ABSTRACT

The global criticism on the adverse effects of synthetic antibiotics including destabilization of gut microflora necessitated research into natural alternative like phytobiotics. Tamarind pulp is a phytobiotic known for its antimicrobial, growth-promoting and antioxidants properties, with little information on the mechanism of its growth promotion in fish. Therefore, this study investigated the effects of tamarind pulp (TP) meal as feed additives on the growth, gut microflora, and morphometry in Clarias gariepinus. The fish were fed with diets fortified with graded levels (0.0%, 0.5%, 1.0%, 1.5% or 2.0%) of TP or 0.2% oxytetracycline (OTC). Fish weight gain, feed conversion ratio (FCR), gut microflora and morphometry were determined. The weight gain of fish rose ($P < 0.05$) with increasing levels of TP, while the FCR reduced significantly, compared to those fed the control diets. The total viable counts and enterobacteriaceae from the gut of fish fed OTC-fortified control diet was lower, compared the fish fed unfortified control (0.0% TP) diet and diets containing TP. Fish fed 1.0-2.0% TP-fortified diets had significantly higher yeast counts, compared to those fed other diets, while on the other hand higher ($P<0.05$) Streptococcus sp was obtained from fish fed the control diets. The thickness of muscular lining of the base of villi, villi height and absorption area were higher ($P < 0.05$) in fish fed OTC-fortified (C2) diet, compared to other treatments, while lower crypt depth were obtained in fish fed TP-fortified diets. The correlation analyses showed moderate negative ($R = -0.539$ at $P < 0.05$) relationship between FCR and population of enterobacteriaceae while a very strong positive relationship ($R = 0.848$ at $P < 0.01$) existed between FCR and population of haemolytic Streptococcus. The enhanced growth in fish fed TP-fortified diets could be accredited to the reduced crypt depth and colonization of yeast and reduced Streptococcus counts. Therefore the application of tamarind pulp meal at 2% is recommended for enhanced growth and healthy gut microflora composition.

Introduction

The composition and population of microflora in the gastrointestinal tract play an important role in the growth and health status of animals. High exposure of fishes to exchanges of microflora between the environment and the digestive system increases the risks for frequent destabilization of gut microflora, which can affect the optimal performance of the system. The presence of health-enhancing gut microflora prevents excessive production of potentially harmful microorganisms in the gut (Romero and Navarrete, 2006). Gut microflora play significant in digestion by stimulating the enterocytes to synthesize gut peptides which enhance secretion of digestive enzymes such as proteolytic, amylolitic, cellulolytic, and chitinolytic enzymes which are important for digestion of nutrients (Bairaji *et al*., 2002; Gutowska *et al*., 2004; Butt and Volkoff, 2019) and consequently improve nutrient utilization and growth performance. The intestinal tracts of healthy fish harbour microbiota which have been investigated by several authors (Navarrete *et al*., 2008; Adel *et al*., 2016; Boonanuntanasarn *et al*., 2017; Hostins *et al*., 2017; Huyben *et al*., 2017) due to their

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assumed importance in digestion, nutrition and disease control.

The global concern on the consequence of the increased use of synthetic antibiotics for food animals, including the risk of chemical residues in food fish, the development of resistant pathogen strains, and disruption of gut microflora (Sajid *et al.*, 2011; Gent *et al.*, 2012; Carison *et al.*, 2017; Zhou *et al.*, 2018) warrants suitable alternatives. The caution on utilization of synthetic antibiotics as feed additives has accelerated evaluation of natural alternative feed additives; one of which is phytobiotic additives. Phytobiotic additives may have beneficial effects on stimulation of appetite, enhancement feed conversion, stimulation of immune system, and regulation of gut microflora of farmed animals; although the mechanism of action may vary among the phyto-additives and fish species (Erener *et al.*, 2011). Utilization of some phytobiotics has been reported to increase growth of gut beneficial microbiota such as *Lactobacillus*, *Bacillus*, yeast, proteolytic bacteria and / or reduction in *E. coli*, *Aeromonas*, *Vibrios*, and other potential pathogenic organisms in fish (Honghai *et al.*, 2004; Giannenas *et al.*, 2012; Ramesh *et al.*, 2013). *Clarias gariepinus* is a commercial tropical farmed fish species and the most-cultured species in Nigeria, widely cultured intensively in other African countries, as well as in many parts of Europe and Asia due to its fast growth, high consumer acceptance and adaptability to a wide range of environmental conditions and high market value (Al-Dohail *et al.*, 2009; Strauch *et al.*, 2018; Adeniyi, 2020).

Tamarindus indica L (tamarind) is a tree widely available in the tropical and subtropical regions of the world (Bhadoriya *et al.*, 2011). Tamarind contains tartaric acid, tamarindienal, epicatechin, and polyphenolic compounds amongst others. Previous studies (Fabiyi *et al.*, 1993; Tabuti 2008; Kuru 2014; Arshad *et al.*, 2019) focused on the chemical composition and antimicrobial properties of tamarind pulp and its relevance in human nutrition and medicine with little information on its utilization in aquaculture. However, our previous studies have demonstrated growth-promotiong (Adeniyi *et al.*, 2018a; Adeniyi *et al.*, 2020) in catfish and tilapia as well as wound-healiing (Adeniyi *et al.*, 2018b) properties of tamarind pulp in catfish. Information on the effects of *T. indica* on gut microflora*,* gut morphometry and their correlation with feed utilization is scarce. Hence, the present study aimed to investigate the effect of dietary *T. indica* pulp (TP) meal on the population of some microflora and gut morphometry and their relationship with feed efficiency in *C. gariepinus*.

Materials and Methods

Experimental fish and diets

The African catfish, *C. gariepinus*, were obtained from a hatchery in Ilorin, Kwara State, Nigeria, acclimated for two weeks, and fed with commercial feed (45% crude protein). The fish (n=540, average weight 5.75±0.1g) were distributed into 6 treatments in triplicates at the stocking rate of 30 / replicate in 100-L tank. The experimental diets consisted of two controls: the unfortified control, ($C_{1;}$ 0.0%) and fortified (C_2) containing 0.2% oxytetracycline (0.2% OTC) and four TP meal-fortified diets (≈40% crude protein; ≈390kcal/100g) at 0.5%, 1.0%, 1.5% or 2.0% inclusion levels (Adeniyi et al., 2018a);.The fish were fed at the rate of 3% of their body weight daily for 12 weeks; the daily ration were hand-fed to the fish twice daily in the morning (8:30-9:30 h)and evening (4:30-5:30 h).

Growth performance and feed utilization parameters

The following parameters were estimated after the feeding trial:

Weight gain (WG, %) = (Weight gain (g) / Initial weight (g)) x 100

Feed conversion ratio (FCR) = Feed intake (g) / Weight gain (g)

Condition factor = Fish weight (g) / (Standard length, cm)^3

Fish productivity index = Weight gain (g) x Fish survival (%) / FCR x 10

Determination of gut microflora in the experimental fish

The effect of dietary treatment with OTC and tamarind on the population of some gut microflora in the experimental fish was determined. At the end of the feeding trial, three fish were randomly selected from each treatment. The skin of each fish was cleaned with 70% ethanol to reduce incidental organisms. The fish were then dissected with sterile scissors and the gut aseptically removed, weighed and homogenised with mortar and pestle. Each of the homogenate was made up to 10mL with sterile peptone water (Oxoid, Hampshire, England) and mixed thoroughly. The mixture was serially diluted to 10^{-10}, out of which 0.1mL of each dilution was spread-plated in sterile Petri dish in triplicate using selective agar (Adel *et al.*, 2016; Boonanuntanasarn *et al.*, 2017). Sterilised plate count agar (Oxoid, Hampshire, England), MacConkey agar (Oxoid, Hampshire, England), yeast extract agar (Oxoid, Hampshire, England), de Man, Rogosa and Sharpe agar (MRS; Oxoid, Hampshire, England), and blood agar were spread on different plates in triplicates to enumerate the population of the gut total viable count (TVC), enterobactriaceae, yeast, lactic acid bacteria and haemolytic *Streptococcus* species, respectively. The plates were then incubated at 37°C for 24 hours, after which the total colony forming units (CFU) was counted; CFU/g was calculated and expressed as Log_{10}CFU/g (Ramiah *et al.*, 2014).

Gut morphometry

The entire intestines of sampled fish from each treatment were removed and immediately placed in 10% formaldehyde. Samples of each intestine were routinely sectioned to 5um thickness and stained with haematoxylin and eosin on slides and dehydrated. Five intact well oriented villi were selected for each intestinal cross section per slide based on the presence of intact lamina propria (Awad *et al.*, 2009). Villi height, villi width, propria width, crypt depth, and thickness of the underlying muscle were measured using a binocular microscope (Olympus, USA) with a micrometer rule and digital camera. The morphometrics were converted to centimetre (cm) and the absorption area was calculated as the product of villi height and width while the well pronounced goblet cells were also counted.

Statistical analysis

The data were analyzed using one-way analysis of variance (ANOVA) while Duncan multiple range test was used to compare differences among means at 5% probability level. The correlation between weight gain and feed conversion ratio against population of some gut flora and morphometry in the fish was done at 5% or 1% level. The statistical analysis was done using the SAS (Statistical Analysis System, 2010) software.

Results

Effects tamarind pulp on fish performance

Inclusion of tamarind pulp in the diets of *C. gariepinus* enhanced ($P<0.05$) weight gain (Figure 1) and reduced feed conversion ratio (Figure 2), compared to the fish fed with the two control diets, while the highest weight gain ($P<0.05$) among the tamarind-fortified group was at 2.0% inclusion level. The overall performance of the fish, as reflected by the productivity index (Figure 3) and condition factor (Figure 4), significantly increased with dietary tamarind pulp, compared to the fish fed the control diets.

Effects of dietary tamarind pulp on the gut microflora

The total viable counts (TVC) and enterobacteriaceae (Figure 5) from the gut of fish fed C_2 (OTC-fortified) diet was lower, compared the fish fed C_1 diet and diets fortified with TP. The population of Yeast and *Streptococcus* sp. is presented on Figure 6. Fish fed 1.0-2.0% TP-fortified diets had significantly higher yeast counts, compared to those fed other diets, while on the other hand higher ($P<0.05$) *Streptococcus* sp. was obtained from fish fed the control diets. The number of *Streptococcus* species seemed to reduce ($P>0.05$) in the gut of the fish as TP inclusion level increased. Fish fed C_2 (diet containing OTC) had the lowest microbial load except for *Streptococcus*. Lactic acid bacteria were not found in all the fish examined.

The correlation analysis between feed conversion ratio and some gut flora (Table 1) shows that there was moderate negative relationship ($P<0.05$) between FCR and population of enterobacteriaceae: the higher the FCR, the lower the enterobacteriaceae. Also, a positive weak ($R=0.392$, below 50%) relationship was established between the population of yeast and enterobacteriaceae in the gut of the *C. gariepinus* fed with tamarind pulp additive, indicating that although population of yeast rose as that of the Enterobacteriaceae increased, their dependence on each other is weak. On the other hand, a very strong ($P<0.01$) positive relationship ($R=0.848$) existed between FCR and *Streptococcus*, indicating that higher population of *Streptococcus* correlated with higher FCR and vice versa. There was moderate negative correlation between Enterobacteriaceae and *Streptococcus* as well negative weak correlation between yeast and *Streptococcus*, showing that increase in *Streptococcus* resulted to reduction in Enterobacteriaceae and yeast.

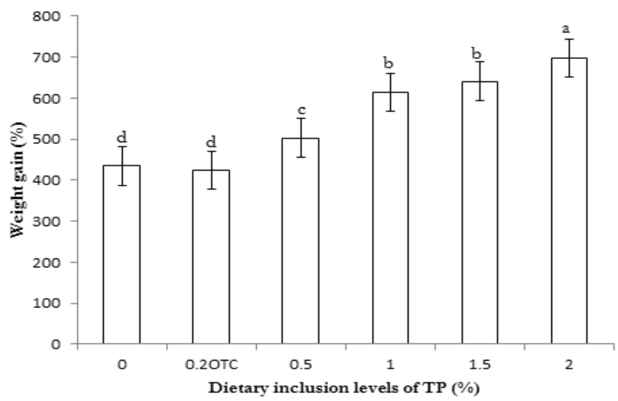

Figure 1. Weight gain of *Clarias gariepinus* fed diets fortified with tamarind pulp (TP) for 12 weeks.

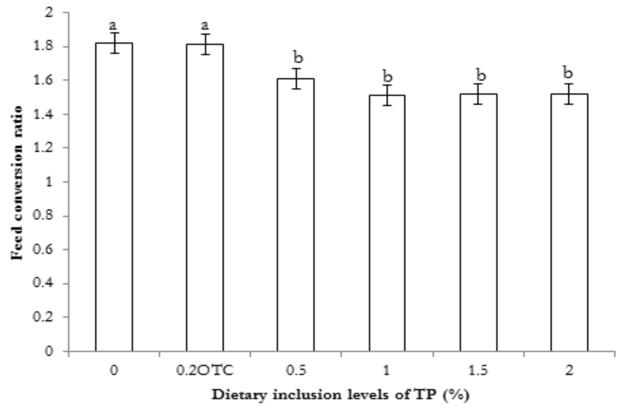

Figure 2. Feed conversion ratio of *Clarias gariepinus* fed diets fortified with tamarind pulp (TP) for 12 weeks.

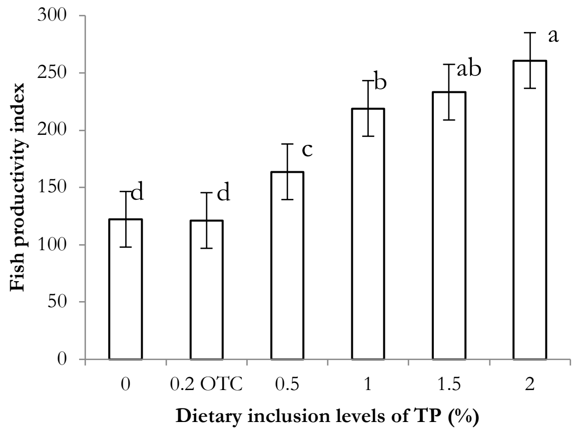

Figure 3. Fish productivity index of *Clarias gariepinus* fed diets fortified with tamarind pulp (TP) for 12 weeks.

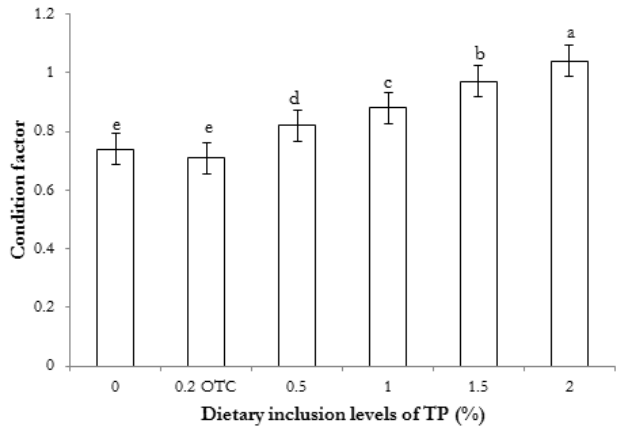

Figure 4. Condition factor of *Clarias gariepinus* fed diets fortified with tamarind pulp (TP) for 12 weeks.

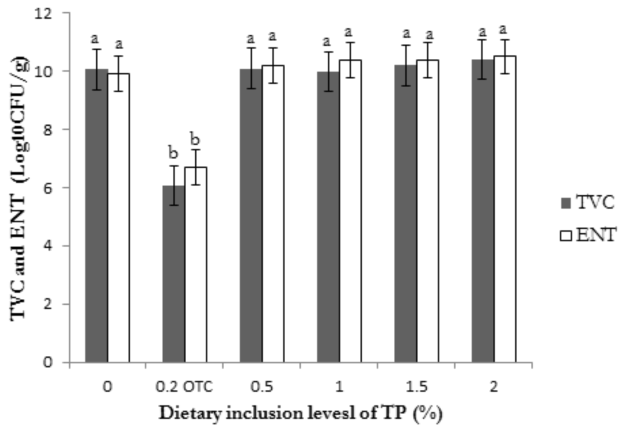

Figure 5. Total viable counts and Enterobacteriaceae in the gut of *Clarias gariepinus* fed diets fortified with tamarind pulp (TP) meal for 12 weeks.

Table 1. Simple correlation between feed conversion ratio and some gut microflora counts (CFU/g) in Clarias gariepinus fed diets fortified with tamarind pulp.

	FCR	TVC	Enterobacteriaceae	Yeast	*Streptococcus*
FCR	1.000		- 0.539*	- 0.262	0.848**
TVC		1.000	0.100	0.490	0.400
Enterobacteriaceae			1.000	0.392*	- 0.528*
Yeast				1.000	- 0.391*
Streptococcus					1.000

*Values significant at $P < 0.05$ **Values significant at $P < 0.01$; FCR = Feed Conversion Ratio TVC = Total viable counts

Effects of dietary tamarind pulp on the gut morphometry of *C. gariepinus*

The gut morphometry of *C. gariepinus* fed the experimental diets is presented on Table 2. The *C. gariepinus* fed C_2 (OTC-fortified) diet had higher ($P < 0.05$) villi height than in the fish fed with other diets. Dietary fortification with TP did not alter villi width ($P>0.05$), when compared with C_1 treatment. The villi width of *C. gariepinus* fed C_2 (OTC-fortified) diet was higher ($P < 0.05$) than the fish fed 0.5% TP. The propria width and crypt depth of fish fed with TP-fortified diets were lower ($P < 0.05$), when compared those fed with the control diets. Higher ($P < 0.05$) thickness of muscular lining of the base of villi (MT) and absorption area were obtained in fish fed OTC-fortified (C_2) diet, compared to other treatments. Also, the number of goblet cells from the epithelial layer of villi of the fish did not differ ($P < 0.05$) among the treatments.

Table 2. Gut morphometrics of *Clarias gariepinus* fed diets fortified with tamarind pulp meal for 12 weeks.

GM	Diets (% inclusion levels)					
	C_1 (0.0)	C_2 (0.2 OTC)	0.5	1.0	1.5	2.0
VH (cm)	1.04 ± 0.08^{bc}	1.38 ± 0.02^{a}	0.90 ± 0.03^{c}	0.95 ± 0.00^{bc}	1.10 ± 0.03^{b}	1.08 ± 0.09^{bc}
VW (cm)	0.12 ± 0.05^{ab}	0.19 ± 0.05^{a}	0.08 ± 0.01^{b}	0.09 ± 0.01^{ab}	0.10 ± 0.01^{ab}	0.09 ± 0.01^{ab}
PW (cm)	0.23 ± 0.01^{a}	0.24 ± 0.03^{a}	0.10 ± 0.01^{b}	0.12 ± 0.02^{b}	0.11 ± 0.01^{b}	0.15 ± 0.03^{b}
CD (cm)	0.22 ± 0.02^{a}	0.22 ± 0.03^{a}	0.14 ± 0.03^{bc}	0.11 ± 0.01^{c}	0.12 ± 0.02^{bc}	0.12 ± 0.08^{bc}
MT (cm)	0.16 ± 0.05^{b}	0.24 ± 0.03^{a}	0.12 ± 0.03^{bc}	0.10 ± 0.01^{c}	0.12 ± 0.00^{bc}	0.15 ± 0.02^{bc}
AA (cm^2)	0.13 ± 0.07^{b}	0.26 ± 0.07^{a}	0.07 ± 0.01^{b}	0.09 ± 0.01^{b}	0.11 ± 0.02^{b}	0.10 ± 0.02^{b}
GC	1.56 ± 0.09^{ab}	1.00 ± 0.50^{ab}	1.78 ± 0.05^{a}	0.78 ± 0.19^{ab}	0.56 ± 0.51^{ab}	1.11 ± 0.2^{ab}

Means with similar superscripts in a row are not significantly different at $P < 0.05$; GM = Gut morphometrics, OTC = Oxytetracycline, TP = Tamarind Pulp, VH = Villi height, VW = Villi width; PW = Propria width, CD = Cryptal depth, MT = Muscle thickness, AA = Absorption area, GC = Goblet cells numbers.

Table 3. Simple correlation between feed conversion ratio and some gut morphometrics in *Clarias gariepinus* fed diets fortified with tamarind pulp.

	FCR	Villi height (cm)	Villi width (cm)	Muscle thickness (cm)	Absorption area (cm^2)
Feed conversion ratio	1.00	0.39	0.501*	0.536*	0.466
Villi height		1.00	0.815**	0.789**	0.879**
Villi width			1.00	0.730**	0.990**
Muscle thickness				1.00	0.779**
Absorption area					1.00

*Values significant at P = 0.05; **Values significant at P = 0.01

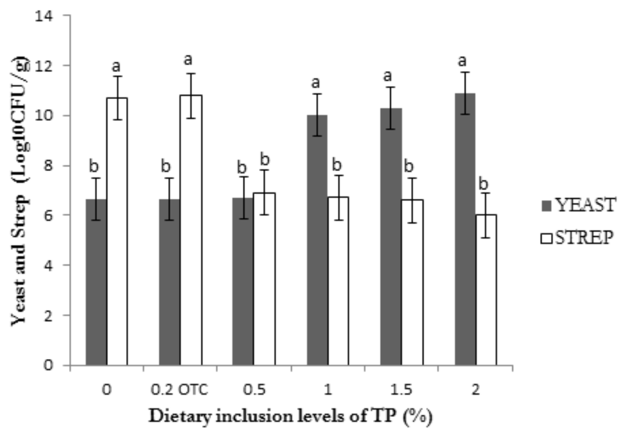

Figure 6. Yeast and *Strep (Streptococcus sp.)* counts in the gut of *Clarias gariepinus* fed diets fortified with tamarind pulp (TP) for 12 weeks.

Discussion

The higher number of enterobacteriaceae and reduction in the population of *Streptococcus* as shown in the correlation analysis might indicate the role of the enterobacteriaceae in digestion and maintenance of healthy gut ecology and consequently improvement of growth performance. The improved weight gain, lower feed conversion ratio, enhanced productivity and in fish fed TP-fortified diets in the present study might be attributed to the ability of TP to limit growth of *Streptococcus* sp., potential pathogenic species, but enhance growth of health-promoting gut microflora, components of entrobactetiaceae and yeast, which could have promoted digestion of feed and subsequently the growth performance. Intestinal microflora in the gut of healthy fish has been associated with digestive and immunomodulation activities (Bairaji *et al.*, 2002; Rauls *et al.*, 2004). Enterobacteriaceae family was equally reported as component of normal gut microflora of carps (Ye *et al.*, 2014) while several previous authors (Andlid *et al.*,1998; Gatesoupe 2007; Romero *et al.*, 2014; Huyben *et al.*, 2017; Boonanuntanasarn *et al.*, 2017) reported yeast as common intestinal microflora, playing significant role in immune stimulation, digestion of feeds, especially plant substrates, and growth promotion. Scalbert and Williamson (2000), Espin *et al.* (2017) and Karl *et al.* (2018) opined that gut microflora played significant role in biotransformation of polyphenolic compounds (e.g. flavonoids, a major components of tamarind pulp (Khairunnuur *et al.*, 2009; Adeniyi *et al.*, 2017; Adeniyi, 2018) known to have poor bioavailability in animals) to useful bioavailable compounds with antimicrobial, digestive and anti-oxidative properties.

The value of FCR is very important in determining the biological value of any feed stuff in fish or animal nutrition: fish derive and utilize more nutrients from the diets at lower FCR value. The correlation analysis between FCR and *Streptococcus* indicated that increased in the population of this organism resulted to higher FCR, which might be associated with the reduced nutrient utilization and growth performance observed in fish fed with the control diets. The enterobacteriaceae and yeast might have contributed to the lower FCR and consequently the weight gain obtained in the fish fed TP-fortified diets. Previous studies (Zhang *et al*., 2018; Banu *et al*., 2020) showed that yeast-supplement diets enhanced growth of fish. Higher proteolytic bacteria were also found in the gut of *Channa striatus* fed with phytogenic-treated diets, than the control group (Ramesh *et al*., 2013). The enhancement of beneficial microflora (yeast) and inhibition of harmful one (*Streptococcus*) observed in the present study is in line with earlier studies on the effect of feed additives in which *Acinetobacter, Bifidobacterium, Lactobacillus*, *Bacillus* or yeast increased while *Aeromonas, Vibrios* or *E. coli* reduced in the gut of fish fed herbal-fortified diets (Luo *et al*., 2001; Honghai *et al*., 2004; Jang *et al*., 2007; Adeniyi *et al*., 2021) and shrimps fed probiotic-supplemented diets (Adel *et al*., 2016; Hostins *et al*., 2017).

The reduction in the crypt depth in *C. gariepinus* fed TP-fortified diets might have contributed to lower requirement for maintenance and higher utilization of nutrients for fish growth. Reduced cryptal depth in farm animal is associated with slower turnover of the intestinal surface and reduction in requirement for maintenance (Van-Nevel *et al*., 2005). The non-significant effects of tamarind pulp on the absorption area, villi height and width of the experimental fish, when compared to C_1 in the present study is similar to earlier observation on the effects of feed additives on some aquatic species (Daniels *et al*., 2010; Reyes-Becerril *et al*., 2014; Zahran *et al*., 2014). Also, the absorption area of villi did not increase significantly when European lobster larvae was fed with diets treated with *Bacillus* species, mannaoligosaccarides singly or in combination (Daniels *et al*., 2010). Dietary treatment of the diets of pacific red snapper microalgae or its combination with *Lactobacillus* did not affect microvilli significantly (Reyes-Becerril *et al*., 2014). Also, Zahran *et al*. (2014) did not observe significant effects on the intestinal histology of Nile tilapia fed diets fortified with *Astragalus* polysaccharides. On the other hand, Bello *et al*. (2012), Abdel-Tawwab *et al*. (2018) and Adeniyi *et al*. (2020) reported higher villi height and absorption area in fish fed diets containing phytoadditives, compared to the control treatment. Oxytetracycline-treated *C. gariepinus* had significantly higher villi height, villi width and subsequently higher absorption area than other experimental groups in the present study. Despite the significantly higher absorption area in fish fed OTC-fortified diet, the growth performance and nutrient utilization were lower than the TP-fortified treatments; this might be related to reduction in the gut yeast counts in OTC treatment. The results of the gut evaluation in this study showed the complementary roles of the gut microflora and morphometry in the digestion, absorption and utilization of nutrients.

The number of villi goblet cell in TP-fortified *C. gariepinus* was not affected by the experimental diets. Goblet cells produce mucins, which form a protective barrier layer on the intestinal epithelial surface. The barrier protects the epithelium from bacteria, toxins and threats from dietary constituents (Kim & Khan, 2013). The non-significant differences in the number of goblet cells in fish fed tamarind-fortified and control diets might indicate absence of harmful constituents in tamarind pulp. Contrarily, higher percentage of goblet cells was reported in rainbow trout fed diet containing 5g/kg diet phytogenic additive (Aquavac Ergosan) than fish in control treatment (Heidarieh *et al*., 2012) as well as in our previous study (Adeniyi et al., 2020) of tilapia fed TP meal.

Conclusions

The dietary fortification with tamarind pulp in enhanced African catfish productivity with highest at 2% inclusion level and that the mechanism of the performance enhancement could be accredited to the reduced crypt depth and enhanced yeast production and reduced *Streptococcus* counts. Therefore the application of tamarind pulp meal at 2% is recommended for enhanced growth and healthy gut microflora composition.

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