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Title: Wound-healing Potentials of Dietary *Lactobacillus plantarum* and *Psidium guajava* leaf meal in *Clarias gariepinus* Juveniles

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Abstract

This experiment was aimed at assessing healing processes in juvenile Clarias gariepinus (Burchell 1822) fed varying levels of Lactobacillus plantarum (Strain 762) and Psidium guajava (Linneaus) meal. Eighty-four C. gariepinus with mean body weight of 125g were used. Fish were randomly selected in completely randomised design. They were distributed into 21 aquaria of 40L capacity of water. A wound model adopted was a standard 10 mm2 incision made on lateral and caudal regions. Fish were fed using 42% crude protein diet at 0, 40, 60, and 80 of L. plantarum (cells-1000mL) and guava leaf meals (g-1000g) respectively. Wound closures were observed daily for quantitative measurements. Healing rates were measured on the regions at 0, 3, 6, 9 and 12 days. Data were analysed using descriptive statistics and ANOVA at P<0.05. The results showed a better healing rate at lateral parts across treatments with complete healing recorded on the 12th day. The fastest healing rates and percentage healing were in fish fed LPA-Basal diet+40 cells-1000mLL. plantarum per kg of feed and GMC- Basal diet+ 80 g-1000g guava meal per kg of feed. These results reveal L. plantarum at lower and P. guajava at higher concentrations had faster healing performance than the control.

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Keywords: Clarias gariepinus, Lactobacillus plantarum, Psidium guajava, Wound-healing.

Introduction

Several commercial fish species have been intensively cultured in enclosed or narrow spaces such as tanks, cages or ponds under high density (Kaileh et al., 2007). This practice has led to negative effects of cultured fish species including African catfish. However, defensive mechanism of African catfish which gives it its venomous characteristics is attributed to the presence of a single, sharp and stout sting in front of the soft-rayed portion of the pectoral fins (Weston et al., 2005). Consequently, African catfish uses the mechanism of their pectoral and dorsal well developed articulated stings to inflict severe wounds on their victims (Junqueira, 2006). Often their cannibalistic characteristic is displayed in circumstances of hunger-induced stress and overcrowding. Moreover, when skin is impaired, opportunistic micro-organisms infect leading to delayed wound- healing (Weber et al., 2012).

With the intensification in aquaculture to meet global food consumption, high stocking density is necessitated in ponds for optimum catfish production. Consequently, fish incur wounds/injuries which pre-dispose them to infections and mortality (Weber et al., 2012). Emphases are now on feeds that have both growths promoting and wound healing potentials (Castillo et al., 2014). However, in most nations, the role of medicinal plants is highly important in primary health care. Anti-inflammatory and wound healing properties have been revealed in several plants including, *Tamarindus indica* Pulp (Adeniyi et al., 2018), *Mallotus oppositifolius, Momordica charantia* (Agyare et al., 2014) and *Phyllanthus muellerianus* (Boakye et al., 2018).

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The guava tree, *Psidium guajava* Linnaeus in the family Myrtaceae, is a tropical hardwood plant that can reach a height of 10 m (Martins et al., 2009). It is a native of Mexico and extends throughout South America, Europe, Asia and Africa and it is used in the treatment of fever, pains, dysentery, respiratory disorders, gastroenteritis, diarrhoea, diabetes and hypertension (Martins et al., 2009). Guava leaves are also used as medication in the treatment of wounds, ulcers, toothache and for the relief of rheumatic pain (Gutiérrez, 2008). However, the significant role of *P. guajava* in aquaculture in relation to wound- healing has not been explored.

Similarly, *Lactobacilli* are normal intestinal microflora of animals and humans which are classified as probiotics. They are Gram-positive lactic acid bacteria and non-pathogenic (Martin et al., 2007). Their culture supernatants have been shown to possess wound-healing properties and antimicrobial properties (Bleau et al., 2005). They usually enhance energy and are known to remedy common cold, prevent cancers, and lower cholesterol level (Reid, 2006). They likewise enhance inflammatory response during tissue repair in animals and stimulate proliferation of embryonic cells (Brackett and Halper, 2005). However, fermented dairy products containing *Lactobacillus* have been used in traditional medicine in the modulation of microbial ecology in prevention of infections caused by pathogenic bacteria, stimulation of the immune system and in gastrointestinal disorders normalization (Brooks, 2007). *Lactobacilli* are also known to be therapeutic, help in alleviating urinary tract infections in the treatment of diarrhoea, irritable bowel syndrome and atopic eczema (SenthiKumar et al., 2013). Howbeit,

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the significant role of *L. plantarum* as probiotics in relation to wound healing in aquaculture is yet to be explored.

The wound healing in humans and animals involves the process of inflammation, cell proliferation and contraction of the collagen lattice. This process may however be disrupted by the presence of oxygen free radicals or microbial infection (Gonçalves et al., 2008). In view of these, the study was aimed at evaluating the wound healing potentials of *L. plantarum* and guava leaf meal (*P. guajava*) at their different inclusions levels in the diets of juvenile African catfish.

Materials and methods

Ethical statement

The experimental procedure followed the University of Ethical Committee for Animal Use in Research (UI/ACUREC/App/2017/0028). This was in conformity with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-83) and the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Probiotic and plant additive diets preparations

Fresh guava leaves (*P. guajava*) were collected and identified in Forestry Research Institute of Nigeria (FRIN) with voucher number 110937. After harvest of the leaves, they were washed with clean water, air dried for twenty-one days and processed into meal. Samples of Lactic acid bacteria (LAB) were collected from fermented cow milk (wara). Identification of LAB was assumptously carried out by physiological and biochemical tests. These were further confirmed through molecular characterisation as *L. plantarum* Strain 762 (Agaliya and Jeevaratnam, 2013).

Fish feed ingredients were purchased from a reputable fish feed store and basal diet of 42% crude protein was prepared using Pearson Square Method. The single cell proteins of *L. plantarum* Strain 762 and *P. guajava* leaf meal were added to the experimental feed relative to each treatment. The dry feed ingredients were well grinded, mixed and pelletised with water and a binding agent (starch). The extruded pellets of 2 mm in size were sun-dried and stored in neoplastic bags made in Nigeria until experimental use.

The Ingredients used included Fishmeal, Groundnut cake, Soybean meal, Wheat middlings, Yellow maize, Guinea corn, common salt, Palm oil, vitamin and mineral premix. Eight experimental diets were prepared including two control diets by incorporating *L. plantarum* and *P. guajava* leaf meal at different concentrations into the diets as follows:T_m1 and T_m2- were control experiments of basal diet of 42% crude protein level. T_m3, T_m4, T_m5- consisted of basal diet with additional 40cells$^{-1000\ mL}$, 60$^{-1000\ mL}$, 80$^{-1000\ mL}$of *L. plantarum* per kg of feed.T_m6, T_m7, T_m8- consisted of basal diet with additional 40g$^{-1000\ g}$, 60$^{-1000\ g}$, 80$^{-1000\ g}$ of guava meal per kg of feed.

Experimental set-up

The weight of fish was taken for each treatment at day 0 and day 12 of the experiment. The experiment was conducted at the Department of Aquaculture and Fisheries Management Wet Laboratory of the University. Ninety-six *C. gariepinus* juveniles (12 for each treatment) with average body weight of 125±0.2 g were selected at random from each replicate treatment for the experiment. These were selected from previously fed fish with experimental diets for 84 days. Fish were randomly distributed into seven treatments groups. Four fish per replicate were distributed into rectangular plastic aquaria produced by neoplastics Nigeria of dimensions 50cmx34cmx27 cm of 40 L capacity of water. Twenty-fourplastic aquaria were used for the experimental set-up. Fish were acclimatized for 2 weeks after feeding experiment and confirmed to be of good general health based on complete physical examination before the commencement of the experiment.

Wound Creation: Wound Creation: The caudal and lateral areas of fish were aseptically prepared. These processes were carried out by swabbing the two areas with absorbent cotton wool (Vandana Surgi Pharma, India) immersed in absolute ethanol (Fred Holmberg & Co AB 99%, Sweden) and placed on sterilised slabs. These two areas were selected to assess the differences in the healing rates on the fish bodies. The epidermis and sub-epidermis tissues of *C. gariepinus* at various body parts are known to have different thickness, hence different healing rates on fish bodies (Kurokawo et al. 1993). Sterilised surgical blades (Kiato, India) were used for incision. A standard template (a measured thick stick, drawing the site on a well uniform 10 mm square quadrant) was placed on the fish and surgical cuts of 10 mm^2 were incised on the fish towards the dermis to the lateral part, above the pelvic fin and around the caudal region (Bello et al., 2013). Fish were returned to the aquaria and fed to satiation for 12 days (Bell, 2002; Abo, 2004).

Wound - healing assessment

The wounds inflicted were photographed using phone camera (Nokia Asha 210, USA). Wound closure was observed on daily basis for visual assessment and quantitative measurements. Measurement was carried out using measuring Ruler (beginner dual-scale ruler, China) at 0, 3,

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6, 9 and 12 days in wound area in two-dimensional healing. Percentage healing, daily healing rates and change in wound area were calculated as follows:

$$\%\ \text{healing} = \frac{\text{change in wound area (area of regenerated tissue)}}{\text{inital wound area}}\ \text{x100}$$

(Ammar et al., 2015)

$$\text{Daily healing Rates} = \frac{\text{Total percentage of area healed}}{\text{Number of days measurement was taken}}$$

(Bells, 2002)

$$\text{Change in wound area (mm)} = \text{Initial wound area on 0th day} - \text{Wound area left on nth day } (n = 3,6,9,12)$$

Skin biopsy was taken from the treatment groups (caudal and lateral) at day 3, 6 and 12 for routine histological processing for microscopic examination.

Statistical analysis

This experiment was subjected to one-way analysis of variance (ANOVA) using the SPSS (Statistical Package for Social Sciences, 2006 version 20.0). The individual means were compared using Duncan multiple range test (DMRT)

Results

Daily healing rates of juvenile *C. gariepinus* fed *L. plantarum* and guava (*P. guajava*) leaf meal after 12 days of wound-incision.

Figures 1 to 4 revealed the results of the daily healing rates of juvenile *C. gariepinus* after 12 days of wound-incision. From figures 1-3, the daily healing rates of treatments LPA, LPB and GMC were significantly higher (P<0.05) than those of control, LPC,GMA and GMC at the caudal regions on the third, sixth and ninth days. On the lateral region, there was no significant difference (P>0.05) across all the treatment groups on the third, sixth and ninth days. Figure 4 revealed complete healing on the twelfth day with no significant difference (P>0.05) on the caudal region across all treatments. There was complete healing at the lateral region across all treatments groups on the twelfth day with no significant difference (P>0.05).

Percentage (%) healing of juvenile African catfish (*C. gariepinus*) fed *L. plantarum* and guava (*P. guajava*) leaf meal at various inclusion levels.

Tables 1-4 revealed the percentage (%) healing and change in wound area at caudal and lateral regions of juvenile African catfish (*C. gariepinus*) fed *L. plantarum* and guava (*P. guajava*) leaf at various inclusion levels. Table 1 revealed a significant difference (P<0.05) at the caudal region having Basal diet +80cells$^{-1000mL}$*L. plantarum* per kg of fish (80LPc) inclusion level with (25.2±19.8) % healing rate as compared with the lateral region (14.3±3.6) % and the control (10.0±2.5) %.

The percentage wound-healing were higher (P<0.05) at the lateral and caudal regions in fish fed inclusion Basal diet+ 80 g^{-1000g} guava meal per kg of feed (80PGLM) (84.8±1.4 and 78.6±2.3) % compared to the control at the lateral and caudal regions (53.8±9.3 and 42.2±2.4) on the sixth day (Table 2). There was no significant difference (P>0.05) at the caudal and lateral parts between all the inclusions and control on the ninth day (Table 3). Complete healing across all treatment groups at the caudal and lateral regions was revealed on the twelfth day (Table 4).

Histological Examination of juvenile African catfish (*C. gariepinus*) fed *L. plantarum* and guava (*P. guajava*) leaf meal at various inclusion levels.

Plate 1 revealed hyperaemia and scab tissue formation at caudal part on the third day, these were characterized by granulation tissue and inflammatory cells on the epidermis while Plate 2 revealed wound-healing at the lateral part after 3 days with intense hyperaemia and inflammatory response on the epidermis. Plate 3 revealed a reduced surface keratinocytes on the epidermis of the caudal part on the sixth day, while Plate 4 revealed wound-healing with grossly reduced surface area at lateral part and the appearance of normal skin morphology on the sixth day. Plate 5 revealed completely healed wound at caudal and lateral parts on the twelfth day.

Discussion

This study assessed the wound- healing performance of *L. plantarum and P. guajava* diets at varying levels on juvenile *C. gariepinus*. Procedurally, there are four steps involved in wound-healing. These include: Haemostasis, inflammation, proliferation, and tissue remodeling or resolution (Gosain and DiPietro, 2004). It was observed in this experiment that the control, *L. plantarum* diet and *Psidim guajava* leaf meal showed fast healing rates at all levels after twelve days of wound creation. Nonetheless, the highest change in wound area (mm^2) and percentage healing (%) were in fish fed dietary *L. plantarum* (40LPc) and dietary *P. guajava* (80PGLM) compared to the control and other treatment groups. In similar

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studies, (Weston et al., 2005; Peral et al., 2009) doses of *Lactobacillus fermentum* LP3 and *L. plantarum* AT4 administered to animals (such as mice and rabbits) and man helped in accelerating healing of burns and leg ulcers. In another study, it was reported that *Lactobacillus* rhamnosus Gorbach-Goldin strain (G-G strain) and *Lactobacillus gasseri* strain ATCC 33323 hastened the healing of gastric ulcers in rats. The mechanism of wound-healing in animals may be due to the production of immunoglobulin (IgG, IgM, IgA) (Lahteinen et al., 2014). In addition to this, they help in the absorption of antigens released from dead microbes, they likewise stimulate cells mediated immunity (Dongarra et al., 2013). According to Rask et al., (2013), it was observed that during wound-healing, probiotics induce macrophages, lymphocytes and natural killer cells. During this process, degranulation of heterophils and oxidative burst regulation occurs to hasten wound- healing (Rask et al., 2013). This may have probably enhanced the wound-healing process of fish fed dietary probiotics in this present study (Rask et al., 2013).

Howbeit, the healing mechanism of probiotics such as *L. plantarum* may be influenced by the presence of plantaricin and bacteriocin. This was substantiated with the research carried out by Umeh et al., (2005). These workers observed an inhibitory activity of *L. plantarum* on the bacteria that often complicate wounds and burns in animals. The above stated report agrees with other reports that there was a faster tissue repair with the treatments which had probiotics inclusions at various levels as compared with the control (Nowroozi et al., 2004). The faster wound-healing observed in fish fed dietary *L. Plantarum* at all levels compared with control might have been enhanced by the influence non-specific immune activity in *L. plantarum* (Salvador et al., 2012).

Likewise, the healing observed in fish fed dietary *P. guajava* at various inclusion levels in this study, may be due to the presence of phytochemical constituents (Setufe et al., 2018). These include saponins, tannins, terpenoids, alkaloids, cardiac glycosides and phenols in *P. guajava* which may have influenced the fast healing rate of the experimental fish. The anti-inflammatory potential of *P. guajava* leaf meal might have further enhanced the fast wound-healing activity in experimental fish compared with the control fed the basal diet (Szynanski et al., 2006). Mbuh et al. (2008) and Ogbonnia et al. (2008) also reiterate the fact that tannins react with proteins to induce tanning effect in animals, as this proline – rich- protein complexes help in the synthesis of cell protein essential for the treatment of inflammations and tissue wounds. Wound-healing process was faster at the lateral region of juvenile *C. gariepinus* than at the caudal part might be due to the movement of migratory fronts (epidermis from wound edges) on the sixth and ninth days. The concentration of epidermis at the lateral region might have heightened the faster wound closure of fish compared with the caudal region. Thus, the visible fast wound-healing at the lateral region (Guerra et al., 2008).

Optimal intensification of wound healing can only occur when bio-physiological processes occur sequentially at a particular time and duration. Otherwise, healing may be impaired or delayed (Mathieu et al., 2006). According to a study carried out by Akanmu et al., (2016), it was observed that *Lactobacillus fermemtum* enhanced wound- healing in *Heterobranchus* *bidorsalis* juveniles . On this premise, the faster healing rate at the lateral part might have also been due to cell differentiation and concentration of tissue-specific stem cells within the region compared with the wound at caudal part of fish (Venkaiah and Lakshmipathi, 2000).

Conclusion

From this study, it has been revealed that the inclusions of dietary *Lactobacillus plantarum* and dietary *Psidium guajava* leaf meal in the diet of juvenile *Clarias gariepinus* enhanced fast wound-healing at dietary inclusion of LPA-Basal diet+40 cells$^{-1000mL}$*L. plantarum* per kg of feed and GMC- Basal diet+ 80 g^{-1000g} guava meal per kg of feed when compared to the control.

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Table 1: Changes in wound area of juvenile African catfish (*C. gariepinus*) fed with *L. plantarum* and guava (*P. guajava*) leaf meal at various levels on day 3

Means with same letter in column are not significantly different (P>0.05)

Key: C-Basal diet of 42% crude protein, 40LPc-Basal diet+40 cells$^{-1000mL}$*L. plantarum* per kg of feed, 60LPc-Basal diet +60 cells$^{-1000mL}$,80LPc- Basal diet +80cells$^{-1000mL}$*L. plantarum* per kg of fish, 40PGLM- Basal diet+ 40 g^{-1000g} guava meal per kg of feed, 60PGLM-Basal diet + 60 g^{-1000g} guava meal per kg of feed ,80PGLM- Basal diet+ 80 g^{-1000g} guava meal per kg of feed, CA-Caudal Area, LA- Lateral Area

Treatments	CA			LA		
	3days			3 days		
	Initial wound area (mm^2)	Change in wound area (mm^2)	%Healing	Initial wound area (mm^2)	Change in wound area (mm^2)	%Healing
Control	10.0	1.0±0.0	10.0±2.5[d]	10.0	2.0±0.0	16.6±3.2[b]
40LPc	10.0	2.0±0.1	18.3±5.8[b]	10.0	2.0±0.0	16.6±4.5[b]
60LPc	10.0	1.0±0.04	14.0±3.6[c]	10.0	2.0±0.2	14.8±8.4[c]
80LPc	10.0	2.0±0.2	25.2±19.8[a]	10.0	1.0±0.0	14.3±3.6[c]
40PGLM	10.0	1.0±0.1	8.4±8.0[e]	10.0	1.0±0.0	13.3±2.2[c]
6P0GLM	10.0	1.0±0.1	11.8±5.1[d]	10.0	2.0±0.0	20.0±0.0[a]
80PGLM	10.0	1.0±0.0	13.3±3.0[c]	10.0	1.0±0.0	13.4±4.1[c]

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Table 2: Changes in wound area of juvenile African catfish (*C. gariepinus*) fed with *L. plantarum* and guava (*P. guajava*) leaf meal at various levels on day 6

Treatments	CA			LA		
	6 days			6 days		
	Initial wound area (mm^2)	Change in wound area (mm^2)	%Healing	Initial wound area (mm^2)	Change in wound area (mm^2)	%Healing
Control	10.0	4.0±0.02^{b}	42.2±2.4^{c}	10.0	5.0±0.1^{b}	53.8±9.3^{c}
40LPc	10.0	8.0±0.01^{a}	79.3±1.4^{a}	10.0	8.0±0.1^{a}	80.0±5.6^{b}
60LPc	10.0	5.0±0.06^{b}	50.2±6.5^{b}	10.0	8.0±0.0^{a}	75.6±4.0^{b}
80LPc	10.0	8.0±0.04^{a}	79.5±4.2^{a}	10.0	9.0±0.0^{a}	84.3±0.8^{a}
40PGLM	10.0	7.0±0.04^{a}	74.0±4.3^{a}	10.0	8.0±0.0^{b}	82.0±2.8^{a}
60PGLM	10.0	8.0±0.02^{a}	78.6±2.3^{a}	10.0	8.0±0.0^{b}	84.1±4.2^{a}
80PGLM	10.0	7.0±0.03^{a}	74.1±2.9^{a}	10.0	9.0±0.0^{a}	84.8±1.4^{a}

Means with same letter in column are not significantly different (P>0.05)

Key: C-Basal diet of 42% crude protein, 40LPc-Basal diet+40 cells$^{-1000mL}$*L. plantarum* per kg of feed, 60LPc-Basal diet +60 cells$^{-1000mL}$,80LPc- Basal diet +80cells$^{-1000mL}$*L. plantarum* per kg of fish, 40PGLM- Basal diet+ 40 g^{-1000g} guava meal per kg of feed, 60PGLM-Basal diet + 60 g^{-1000g} guava meal per kg of feed,80PGLM- Basal diet+ 80 g^{-1000g} guava meal per kg of feed, CA-Caudal Area, LA- Lateral Area.

Table 3: Changes in wound area of juvenile African catfish (*C. gariepinus*) fed with *L. plantarum* and guava (*P. guajava*) leaf meal at various levels on day 9

Treatments	CA			LA		
	9days			9 days		
	Initial wound area (mm^2)	Change in wound area (mm^2)	%Healing	Initial wound area (mm^2)	Change in wound area (mm^2)	%Healing
Control	10.0	9.0 ± 0.0^{b}	94.2 ± 1.4^{ab}	10.0	10.0±0.0	100.0±0.0
40LPc	10.0	10.0 ± 0.0^{a}	99.2 ± 1.4^{a}	10.0	10.0±0.0	99.2±1.4
60LPc	10.0	10.0 ± 0.0^{a}	95.0 ± 2.5^{ab}	10.0	10.0±0.0	100.0±0.0
80LPc	10.0	9.0 ± 0.0^{b}	94.2 ± 1.4^{ab}	10.0	10.0±0.0	99.2±1.4
40PGLM	10.0	9.0 ± 0.2^{b}	91.7 ± 1.4^{b}	10.0	10.0±0.0	100.0±0.0
60PGLM	10.0	9.0 ± 0.2^{b}	90.8 ± 1.4^{b}	10.0	10.0±0.0	100.0±0.0
80PGLM	10.0	9.0 ± 0.1^{a}	95.8 ± 5.2^{ab}	10.0	10.0±0.0	100.0±0.0

Means with same letter in column are not significantly different (P>0.05)

Key: C-Basal diet of 42% crude protein, 40LPc-Basal diet+40 cells$^{-1000mL}$*L. plantarum* per kg of feed, 60LPc-Basal diet +60 cells$^{-1000mL}$,80LPc- Basal diet +80cells$^{-1000mL}$*L. plantarum* per kg of fish, 40PGLM- Basal diet+ 40 g^{-1000g} guava meal per kg of feed, 60PGLM-Basal diet + 60 g^{-1000g} guava meal per kg of feed ,80PGLM- Basal diet+ 80 g^{-1000g} guava meal per kg of feed, CA-Caudal Area, LA-Lateral Area.

Table 4: Changes in wound area of juvenile African catfish (*C. gariepinus*) fed with *L. plantarum* and guava (*P. guajava*) leaf meal at various levels on day 12

Treatments	CA			LA		
	12 days			12 days		
	Initial wound area (mm^2)	Change in wound area (mm^2)	%Healing	Initial wound area (mm^2)	Change in wound area (mm^2)	%Healing
Control	10.0	10.0±0.0	100.0±0.0	10.0	10.0±0.0	100.0±0.0
40LPc	10.0	10.0±0.0	100.0±0.0	10.0	10.0±0.0	100.0±0.0
60LPc	10.0	10.0±0.0	100.0±0.0	10.0	10.0±0.0	100.0±0.0
80LPc	10.0	10.0±0.0	100.0±0.0	10.0	10.0±0.0	100.0±0.0
40PGLM	10.0	10.0±0.0	100.0±0.0	10.0	10.0±0.0	100.0±0.0
60PGLM	10.0	10.0±0.0	100.0±0.0	10.0	10.0±0.0	100.0±0.0
80PGLM	10.0	10.0±0.0	100.0±0.0	10.0	10 .0±0.0	100.0±0.0

Means with same letter in column are not significantly different (P>0.05)

Key: C-Basal diet of 42% crude protein, 40LPc-Basal diet+40 cells$^{-1000mL}$*L. plantarum* per kg of feed, 60LPc-Basal diet +60 cells$^{-1000mL}$,80LPc- Basal diet +80cells$^{-1000mL}$*L. plantarum* per kg of fish, 40PGLM- Basal diet+ 40 g^{-1000g} guava meal per kg of feed, 60PGLM-Basal diet + 60 g^{-1000g} guava meal per kg of feed ,80PGLM- Basal diet+ 80 g^{-1000g} guava meal per kg of feed, CA-Caudal Area, LA-Lateral Area.