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A study on the effect of bacteriocin production from lactic acid bacteria in poultry nutrition

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ABSTRACT
Objective: This research was carried out to isolate lactic acid bacteria from dairy products, to examine the isolated lactic acid bacteria for the production of bacteriocin, its purification as well as its use as supplement in poultry nutrition to improve the growth performance of the birds.
*Methodology and results***:** Five (5) samples of cow milk and five (5) samples of fresh yoghurt were procured from the market; each sample was serially diluted to the third fold and plated on De Man Rogosa Sharpe agar (MRS) agar. Pure discrete colonies of *Lactobacillus* spp were isolated and sub-cultured on MRS broth. *Lactobacillus* spp isolated from Cow Milk and Fresh Yoghurt was screened using agar well diffusion. *Lactobacillus* isolates which showed high zone of inhibition against the test organisms (*Staphylococcus aureus* and *Escherichia coli*) were designated positive. These isolates were centrifuged at 20,000 rpm ($\leq 4^0$C) for 30 min and their supernatant was adjusted to pH of 7.1 with Na_2CO_3 to remove lactic acid effect, before filter sterilization using membrane filter (0.2µm). Five (5) mg/ml catalase was added to eliminate peroxides effect. The solution was tagged as bacteriocin crude extract (BCE) which was infused into the poultry feed. Ten (10) three weeks old chicks were separated into two groups (5 each). "Group A" as the control and "Group B" as test. The test chicks had 5ml of bacteriocin infused into their daily feed while the control chicks were fed without any infusion of bacteriocin in their feed. The chicks were weighed every seven (7) days to compare the weight gain between the control and test chicks. At the end of the study, it was found that the test chicks weighed 900g when compared to the control chicks, which weighed 830g.
*Conclusion and application of results***:** This therefore means that bacteriocin produced by lactic acid bacteria aids in the weight gain of poultry. This suggests that bacteriocin could be considered a major growth booster in poultry feed and should be applied in its feed formulation.
Keywords: Bacteriocin, Cow milk, Fresh yoghurt, Lactic Acid Bacteria, *Lactobacillus*

INTRODUCTION

Bacteriocins are peptides or proteins ribosomally synthesized by bacteria that inhibit or kill other related or unrelated microorganisms (Leroy and De Vuyst, 2004; Cotter *et al*., 2005). From the first days of life, microorganisms successively colonize the digestive tract of the chicken. Naturally, occurring succession of intestinal bacteria leads to establishing the climax community, and by means of competitive exclusion inhibits the pathogens from entering the intestines. However, this process may be disturbed in intensive production conditions as birds have little chance to acquire properly balanced intestinal microflora (Józefiak *et al.,* 2004; Rehman *et al.,* 2007). Many of the bacterial species classified by now in broiler digestive tract produce bacteriocins, though the majority has not been investigated yet. Their activity is considered an important tool of native bacteria GIT colonization. Additionally, many strains used as dietary probiotics are also capable of bacteriocin production (Stern *et al.,* 2006). Thus, the use of pure bacteriocins as feed additive could be a useful boost of intestinal bacteriocin concentrations and may improve the efficacy of bacteriocin producing bacteria present in the GIT (Bordignon *et al.,* 2011; Musikasang *et al.,* 2012; Robyn *et al.,* 2012; Shin *et al.,* 2008). In 2005, Svetoch *et al*. described class IIA bacteriocins produced by *Bacillus circulans* and *Paenibacillus polymyxa* as being toxic to *Campylobacter jejuni in vitro*. These results were then confirmed in several *in vivo* experiments, which demonstrated that infection of 1-day-old chicks with *C. jejuni* could be suppressed by feed supplemented with bacteriocins (Stern *et al.,* 2005). Portrait *et al*. (2000) isolated *Fusobacterium mortiferum*, (FM1025) from poultry caeca, showing "*in vitro*" activity against *Salmonella enteritidis*, *Salmonella wien*, *Shigella exneri*, *Pseudomonas aeruginosa* and *Pseudomonas stutzeri*. Audisio *et al*. (1999) characterized a bacteriocin producing strain of *Enterococcus faecium* J96 isolated from the GIT of free-range chickens, and showing activity against *Salmonella pullorum*. A more recent study of Grilli *et al*. (2009) focused on the application of bacteriocin produced by *Pediococcus pentosaceus* FBB61 as a poultry feed supplement and the performance showed positive effects on the birds, in that the bacteriocin improved the growth and feed conversion ratio of broiler chickens. Thus, this research sort to study the effect of bacteriocin on poultry nutrition and their weight gain by Isolating Lactic Acid Bacteria (LABs) from dairy product, screening the LABs for bacteriocin production as well as its purification.

MATERIALS AND METHOD

The sample size was determined by using Cochran's sample size formula**, $n_o = Z^2pq/e^2$**

Where: e is the desired level of precision (i.e. the margin of error), p is the (estimated) proportion of the population which has the attribute in question, q is 1 – p. The z-value is found in a Z table.

Two hypotheses were considered, a null hypothesis: H0: $\mu = 0$ and an alternative hypothesis: H1: $\mu = \mu^*$

Microbial isolation: Standard methods of culture media preparation was carried out according to methods of Cowan and steel (1974). Tenfold serial dilutions were performed up to 10^3 and aliquots (0.1ml) of each dilution were poured-plated on De Man Rogosa Sharpe (MRS) agar and nutrient agar and then incubated at 30^0C for 24 hours. The same procedure was also carried out for the fresh yoghurt samples.

Screening of Isolates for Bacteriocin Production: *Lactobacillus* isolates from CM and FY were screened initially using agar well diffusion methods. Aliquots (50uL) of 48hr old

pure cultures of *Lactobacillus* isolates were inoculated into wells (7-mm diameter) of fresh nutrient agar culture plates seeded with either *Staphylococcus aureus* or *E. coli* as indicator organisms. Isolates with zone of inhibitions were designated as positive or otherwise negative. Only positive designated isolates were cultivated in 250ml of MRS broth for 48 hours at 30^{o}C with pH adjusted to (7.1) and stored at 4^{O}C prior crude extract purification and quantification assays.

Purification of bacteriocin: Protocols for crude extract of bacteriocin was sequential by centrifuging primed culture (48 hr old) at 20,000 rpm ($\leq 4^0$C) for 30 mins. Supernatant was adjusted to pH of 7.1 with Na_2Co_3 to remove lactic acid effect, before filter sterilization using membrane nylon filter (0.2µm) and 5mg/ml catalase was added to eliminate peroxides effect (Kacem *et al*., 2005). The solution was tagged as bacteriocin crude extract (BCE) which was used for the antibacterial assay.

Antibacterial Assay: The antibacterial activity of the BCE (Bacteriocin Crude Extract) was determined using agar well diffusion method described by Mohammed *et al*., (2016) with slight modifications. From each BCE, four dilutions were made and labelled as 1.0 Au/ml, 0.75 Au/ml, 0.5 Au/ml, and 0.25 Au/ml. From each concentration, 5µl of the sample was drawn and discharged into 7-mm diameter wells in petri dishes (with 4 wells per plate: 3 sample and 1control) the agar was seeded with 1ml of test organisms. Sterile distilled water was used as negative control on each plate while ciprofloxacin was used as positive control. All plates were incubated at 37°C for 24 h and inhibition zone diameters were measured using a meter rule.

Animal Studies

Chicks: Three weeks old chicks (Broilers) were purchased from Poultry Company, at Thinkers Corner Enugu. They were distributed into two pens with each pen containing five (5) birds for a period of nine (9) weeks. Each of the bird groups was housed in a seperate pen situated at 80cm from the floor to limit the consumption of faeces. The cycle of light was natural environment (12 hours of daylight) and the temperature during the treatment, was maintained with a propane lamp heating at 32^{0}C in the first week, 30^{0}C in the second week and 26^{0}C in the third week. From day 21, the temperature was maintained between 20 and 24^{0}C.

Experimental Design: The chicks were divided in to two groups. The first group (test) comprised of five chicks, which were fed with the test bacteriocin infused in their feed, while the other group (control) also consist of five chicks but were only fed with the broiler feed with no infusion of bacteriocin. The bacteriocin was allowed to defrost (it was frozen for preservation) and then 5ml was measured using a pipette and mixed with the feed. Using an end-over-end mixer in a ratio of 5ml bacteriocin/25g feed. The bacteriocin was added weekly to the poultry feed as recommended by Guerra *et al*., (2007).

Preparation of Bacteriocins for Application to Poultry feed: The bacteriocin crude extract (BCE) was used as bacteriocin treatment. The feed used in this research was a commercial formulated feed pellet obtained from the poultry industry. Bacteriocin Crude Extract (5ml) was mixed with the Feed. The BCE was added as feed supplement for the test chicks, while the feed pellet without BCE was used as the control.

Growth Evaluation: The chicks were weighed every 7th day of feeding continuously. Growth Performance (GP), Feed Consumption Ratio (FCR), was determined using the following equation:

$$GP = 100 \times \frac{Wt - W_0}{W_0}$$

The feed consumption ratio was calculated using the equation below:

Feed Consumption Ratio = Feed consumption
(FCR) Weight gain

Where W_t is the final body weight, W_0 is initial bodyweight.

Statistical Analysis: The data were statistically analysed using pre-packaged computer statistical software (SPSS 13.0) for windows. The one way and two way T-test were used in analysing the data.

RESULT AND DISCUSSION

A total of four lactic acid bacteria isolates were isolated with their Gram's reaction and colonial morphology as depicted in Table 1 below.

Table 1: GRAM'S Reaction and Colonial Morphology of Isolates

ISOLATES	COLONIAL MORPHOLOGY				CELL MORPHOLOGY		
	COLOUR	SHAPE	SIZE (MM)	MARGIN	GRAM'S REACTION	SHAPE AND ARRANGMENT	SIZE (MM)
LAB 1	Cream	Pin-point, circular, smooth and convex	0.8	Entire	Positive	Straight rod with rounded ends	0.9×3.0
LAB 2	White	Circular	1.2	Entire	Positive	Rods in chain	0.7×3.2
LAB 3	White	Circular, large, rough and irregular	2.1	Undulate	Positive	Rods with rounded ends	0.7×2.7
LAB 4	Creamish-white	circular	2.3	Entire	Positive	Rods	0.9×4.8

KEY: LAB 1= Lactic Acid Bacteria isolated from cow milk 4 (CM 4), LAB 2 = Lactic Acid Bacteria isolated from cow milk 5 (FCM 5), LAB 3 = Lactic Acid Bacteria isolated from fresh yoghurt 1, LAB 4 = Lactic Acid Bacteria isolated from fresh yoghurt 4

The result in Table 2 below showed the sugar fermentation pattern of lactobacillus isolates. This showed that all the lactobacillus isolates were galactose, lactose as well as sucrose positive but were indole negative.

Table 2: Sugar Fermentation Pattern of Isolates

Isolate	A	C	F	G	L	M	MN	MO	MI	RF	RH	S	SR	SU	T	X	CA	GG	AR	AE	NR	CU	MR	VP	IN	Organism
LAB 1	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	-	-	-	+	-	+	-	-	-	*L. plantarum*
LAB 2	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	-	-	-	+	-	-	+	-	-	-	*L. casei*
LAB 3	-	+	+	+	+	-	+	+	-	+	+	+	-	+	-	+	-	+	+	+	-	+	-	-	-	*L. acidophilus*
LAB 4	-	-	-	+	+	+	-	-	+	+	-	+	-	+	+	-	-	+	-	+	-	+	-	-	-	*L. lactis*

Key: LAB 1= Lactic Acid Bacteria isolated from fresh cow milk 4 (FCM 4), LAB 2 = Lactic Acid Bacteria isolated from fresh cow milk 5 (FCM 5),LAB 3 = Lactic Acid Bacteria isolated from Farm fresh yoghurt, LAB 4 = Lactic Acid Bacteria isolated from Habib yoghurt, + = positive reaction, _= negative, A= Arabinose, C=Cellobiose, F =Fructose, G = Galactose, L = Lactose, M= Maltose, Mn= Mannitol, Mo= Mannose, Mi = Melibiose, R=Raffinose, Rh=Rhamnose, S = Salicin, Sr = Sorbitol, Su = Sucrose, T = Trehalose, X=Xylose, CA = catalase test, GG = Gas from glucose, AR = Arginine hydroylsis, AE = Aesculin hydrolysis NR = Nitrate reduction, CU = Citrate utilization, MR = methyl red, VP = Vogues Proskaur, IN = Indole.

Lactobacillus isolate from cow milk samples (*L. plantarum)* had an antibacterial activity in agar-well diffusion against *E.coli* with inhibition zone diameter IZD as 10mm and *S.aureus* (12mm) while *L.acidophillus* isolates from fresh yoghurt sample recorded activity against *E.coli* (16mm) and *S. aureus* (14mm) as depicted in Table 3 below. The other *Lactobacillus* isolate did not show any activity against the test organisms, therefore, the *Lactobacillus* spp with the highest activity was used in the animal study.

Table 3: Antibacterial susceptibility of *Lactobacillus*

Organism/ concentration of bacteriocin	**Inhibition Zone Diameter (mm)**			
	1mg/ml	**0.75mg/ml**	**0.5mg/ml**	**0.25mg/ml**
L. plantarum				
E.coli	10.0	0.0	0.0	0.0
S.aureus	12.0	0.0	0.0	0.0
L.acidophillus				
E.coli	0	0.0	0.0	0.0
S.aureus	0	0.0	0.0	0.0
L. casei				
E.coli	16.0	0.0	0.0	0.0
S.aureus	14.0	0.0	0.0	0.0
L.lacti				
E.coli	0.0	0.0	0.0	0.0
S.aureus	0.0	0.0	0.0	0.0

KEY: *L. plantarum= Lactobacillus plantarum, L.casei = Lactobacillus casei, L.acidophillus = Lactobacillus acidophilus, L. lactis = Lactobacillus lacti.*

Table 4 showed the growth/weight performance of the chicks at different weeks. It was observed that the control chicks exposed to feed only weight less than the test chicks exposed to feed infused with bacteriocin.

Table 4: Growth Performance of Chicks

DAYS	**WEIGHT OF CHICKS (g)**	
	CONTROL	**TEST**
0	82	82
7^{TH}	127	149
14^{TH}	201.6	239.9
21^{ST}	260	325
28^{TH}	327	403
35^{TH}	401	494
42^{ND}	478	600
49^{TH}	540	682
56^{TH}	720	800
63^{RD}	830	900

The feed consumption and feed consumption rate of the chicks (both the control and test) were also determined as shown in Tables 5 and 6 below. It was also observed that the test chicks showed higher rate of feed consumption than the control.

Table 5: Feed Consumption of Chicks

WEEKS	FEED CONSUMPTION OF CHICKS (g)	
	CONTROL	TEST
1	274	490.2
2	802.175	1078.336
3	1259.006	1748.406
4	1792.68	2544.49
5	2723.14	3617.568
6	3621.975	4990.509
7	4412.466	6000.022
8	9336.6	10945.125
9	11402.375	12968.28

Table 6: Feed Consumption Rate of Chicks (FCR)

WEEKS	FEED CONSUMPTION RATE OF CHICKS (g)	
	CONTROL	TEST
1ST	5.00	6.00
2ND	5.5	5.6
3RD	5.8	5.9
4TH	6	6.5
5TH	7	7.2
6TH	7.5	7.9
7TH	7.9	8.2
8TH	12.0	12.5
9TH	12.5	13.0

Lactobacillus isolates (*L. plantarum*, *L. fermentum* and *L. salivarius*) from fermented fruits and vegetables had broad antibacterial spectrum (IZD: 26–28 mm), against food-borne bacterial pathogens, as has been reported by Manzoor *et al*. (2016). Thus, the antagonism of *Lactobacillus* strain is pathogen specific. The antibacterial susceptibility test results (agar-well diffusion) employed in the current study, was in accordance with the results reported by Rahimifard and Naseri (2016) which demonstrated that agar well diffusion method was the best method to determine antagonism of LAB other than disk diffusion and spot on lawn method. The results of body weight gains support the finding of Kabir *et al., (*2002) who found that live weight gains were higher in bacteriocin fed group as compared to chicks having no bacteriocin. The feed consumption rate of the chicks showed that both the control and test chicks ate fairly same amount of food but those whose meal was infused with bacteriocin had better growth Performance than their control counterparts. The concept of antagonism of pathogenic bacteria using bacteriocin-producing *Lactobacillus* has been well documented, and the antibacterial property of such beneficial microorganisms has been considered as an important attribute in selecting bacteriocin for the maintenance of healthy microbial balance in the gut. Table 5 showed the quantity of feed consumed by test and control chicks, with the test chicks consuming a total of 12968.28g of feed and the control consuming a total of 1142.375g of feed. Table 6 showed that there was no significant difference between the feed consumption rate of the test and control chicks, as both the control and test birds ate same amount of food but those whose feed were infused with bacteriocin gained more weight than their control counterparts were. The use of bacteriocins from Lactic Acid Bacteria in poultry diet has become a phenomenon for maintenance of normal growth and health of birds. Feed-type bacteriocin products have been demonstrated to help and maintain a positive balance of intestinal micro flora

resulting in the improvements in health and weight of the chickens throughout their short life span (Ouwehand *et al.*, 2002). Thus, in agreement to the scientifically established facts, bacteriocin produced by lactic acid bacteria used in this study, aids in the weight gain of poultry.

CONCLUSION AND APPLICATION OF RESULTS

The use of bacteriocins in diet has become a phenomenon for maintenance of normal growth and quality health condition of birds. In addition, bacteriocins have proven to be effective as food preservatives and should be further explored as better natural alternatives to chemical additives. Bacteriocins have proven to be effective against *E. coli* and *S. aureus*; they have also currently proven helpful to livestock farmers in that they improve the quality of these particular livestock.

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AUTHORS CONTRIBUTION

FCO developed the manuscript with the statistical analysis involved. UMN conceived and designed the experiment. AOO performed the experiment.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests regarding the publication of this paper.

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