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Antagonistic impact of Lactobacilli on *Escherichia coli* in the gut of *Labeo rohita* fingerlings fed with bakery wastes

Abdul Rehman[1], Fayyaz Rasool[1*], Shakeela Parveen[2], Aqsa Tahir[3], Mati Ullah[1], Syed Shafat Hussain[1], Nimra Khalid[1], Harun Arslan[4]

[1]Department of Fisheries & Aquaculture, University of Veterinary and Animal Sciences, Lahore, Pakistan
[2]Department of Zoology, Wildlife and Fisheries, University of Agriculture Faisalabad, Pakistan
[3]Faculty of Veterinary & Animal Sciences, Pir Mehr Ali Shah, Arid Agriculture University, Rawalpindi, Pakistan
[4]Department of Aquaculture, Faculty of Fisheries, Ataturk University, Erzurum, Turkiye

Abstract

The present study was designed to check the feed conversion ratio (FCR) and, changes in gut microbiota of *Labeo rohita* fingerlings fed with different bakery product diets. The beneficial growth promoting lactobacilli and pathogenic *Escherichia coli* bacteria were isolated from the gut of *Labeo rohita* fingerlings and evaluated the antagonistic impact of lactobacilli on *E. coli*. The study was conducted for three months using five circular tanks of 5 feet diameter in two replicates. The experimental tanks were named as D1, D2, D3, D4 and D5 based on different types of diets. Fingerlings of the D1 tank were fed with control feed of Oryza Organics containing 20% crude protein. The D2 tank was fed with an experimental diet containing 25% cream cake as a basic ingredient. The D3 tank was fed with an experimental diet with 35% cream cake as a basic ingredient, while the D4 tank fingerlings were fed with a diet with 25% biscuits as a basic ingredient, and D5 tank fingerlings with a diet with 35% biscuits as a basic ingredient. The feed was provided according to the body weight of the fish twice a day. By using the staining method and biochemical test, lactobacilli and *E. coli* were identified from the gut of the fingerlings. To confirm the diagnosis of identified bacteria, 16S rDNA gene sequencing was used. Highest growth was observed (FCR, 1.200 ± 0.028) in D4 followed by that in D1 (1.360 ± 0.014), D2 (1.450 ± 0.014), D5 (1.512 ± 0.002), and D3 (1.595 ± 0.007), respectively. The antagonistic activity of lactobacilli against *E. coli* was also recorded. In diet D4, the lactobacilli concentration was highly significant, i.e., $9.7 \times 10^7 \pm 2.4 \times 10^7$ cfu/g and that of *E. coli* was found as $1.1 \times 10^7 \pm 1.4 \times 10^6$ cfu/g followed by D5 having $6.6 \times 10^7 \pm 1.6 \times 10^7$ cfu/g lactobacilli and $1.9 \times 10^7 \pm 2.5 \times 10^6$ cfu/g *E. coli*, D2 with $2.4 \times 10^7 \pm 1.9 \times 10^6$ cfu/g lactobacilli and *E. coli* $1.6 \times 10^7 \pm 2.1 \times 10^6$ cfu/g, D1 with $2.4 \times 10^7 \pm 1.9 \times 10^6$ cfu/g lactobacilli and *E. coli* $1.8 \times 10^7 \pm 1.8 \times 10^6$ cfu/g, and D3 with $9.1 \times 10^6 \pm 1.8 \times 10^6$ cfu/g lactobacilli and *E. coli* $1.4 \times 10^7 \pm 1.4 \times 10^6$ cfu/g.

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Introduction

Aquaculture is an important industry and it primarily relies on feed, and only feed accounts for more than 60% of total cost incurred on this field. The provision and consumption of plentiful nutritionally

***CONTACT** Fayyaz Rasool, fayyaz.rasool@ue.edu.pk; drfayyaz1980@gmail.com, Department of Zoology, University of Education, Faisalabad Campus, Faisalabad, Pakistan

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balanced palatable feed by fish is the major module of success of fish culture. Like other terrestrial animals, balanced nutrients are required by fish to grow, reproduce, and maintain itself active. These nutrient requirements can be accomplished through a choice of food items, based on feeding behavior of an animal, and price and availability of food (Das et al., 1991; Alfred et al., 2020). Due to presence of higher protein contents, amino acid profile, vitamins, and mineral and fatty acid contents, fishmeal has been considered the best diet. While on the other hand, as substitutes of fish meal, plant protein sources such as soybean, cereals, oil-seed cakes and maize by-products have been practiced. Alternatively, 2.25 times higher energy contents exist in fat and oil with respect to that in cereals being a regular component of the fish feed (Markovic et al., 2016).

Due to escalating demand of fish meal and its limited supply, soybean meal, and oil-cakes are used extensively in aqua-feed. High market prices of these key ingredients have shifted pressure on cereal grains. Moreover, plant-based feed ingredients in pelleted and dough form are also acquired by fish. Therefore, it is the need of the hour to look at alternatives available in the country. There have been good efforts in research on finding sources of protein in aquaculture diets other than traditional ones (Kalita et al., 2008; Olsen and Hasan, 2012; Imran et al., 2018).

Nutritional requirements of fish are not only accomplished by utilizing waste materials as a fish feed, but it also lowers down the intensity of organic environmental pollutants. Presence of digestible carbohydrate contents (cookies, bread, cakes, rusks, etc.) and their mixture in bakery waste enables them to be used as energy sources. White meal is an essential component of bakery waste; it can be replaced with cereals in a limited amount (Hardy, 2010). In general, 2,981 kcal/kg of net energy is present in the bakery meal, which is almost close to that of maize, i.e., 2,672 kcal/kg (National Research Council, 2012).

Some large-scale studies have been conducted on microbiota extracted from the gut of marine and freshwater fish (Cahill, 1990; Ringoe and Birkbeck, 1999; Hansen and Olafsen, 1999; Ghosh et al., 2002; Yukgehnaish et al., 2020). The microbial gut contents can be defined as an ability of indigenous autochthonous or transient allochthonous to stand and colonize the mucus layer of digestive tract (Ringoe and Birkbeck, 1999), and it examines that fish gastrointestinal (GI) tract containing bacterial population at dense level (Austin, 2006). In general, it is observed that diverse enzyme producing bacteria present in the gastrointestinal tract of host animals have association with metabolism (Rowland et al., 2018). So, it has been recommended by the scientists that gut microbiota might be favorable to fish in nutritional point of view (Ghosh et al., 2002; Kar and Ghosh, 2008).

Therefore, to develop fish quality and acceptability thereby improving aquaculture, it is essential to be aware of pathogenic gut microbiota which would allow determining their role in fish health. In some previous reports, pathogenic gut microbiota like fecal coliforms, *Streptococci*, *Vibrio*, *Pseudomonas*, *Aeromonas* and *Enterobacter* have been observed from the gastrointestinal tract of Rohu fish (Ghosh et al., 2010; Rahman et al., 2010).

All types of pathogenic bacteria can badly affect the economy of a country and can hinder the development of aquaculture by triggering health concerns to humans and fish. Many fishes are found to be consolidated with infectious pathogens, which can act as vectors of fish borne diseases with outbreak of human beings (Novoslavskij et al., 2016).

The present study was conducted with a primary objective to compare feed conversion ratio (FCR), and isolate the beneficial growth promoting lactobacilli and pathogenic *E. coli* bacteria from the gut of *Labeo rohita* fingerlings. The antagonistic impact of lactobacilli was analyzed on *E. coli* in the gut of *Labeo rohita* fingerlings fed with bakery wastes, and control feed of Oryza Organics (20% CP). Moreover, for molecular confirmation of the isolates was done by 16S rDNA sequencing.

Material and Methods

Study area and experimental plan

The present study was conducted at the Department of Fisheries & Aquaculture UVAS, C-Block, Ravi Campus Pattoki. The experiment was conducted in 5-circular tanks each of 152 cm diameter with two replicates. The tanks were designated based on diets. Five types of feeds were used as experimental diets; fish in D1 was fed with the control diet of Oryza Organics with 20% CP; D2 and D3 were fed with 25% and 35 %, respectively, having cream cake as basic ingredient. While fish in the experimental groups D4 and D5 were fed with 25% and 35%, respectively, having biscuits as a key ingredient (**Table 1**). The tanks were disinfected with $KMnO_4$ before stocking. After acclimatization, the fish was stocked at biomass ratio of 1.0 g/gallon of water in each tank and optimum growth conditions were maintained up to 90 days.

Table 1. Feed formulation of experimental diets

	D1 20% CP (Control diet)	D2 25% (Cream cake)	D3 35% (Cream cake)	D3 25% (Biscuit)	D4 35% (Biscuit)
Oryza Organics	100	0	0	0	0
Cream cake	0	25%	35%	0	0
Rica polish	0	50%	40%	50%	40%
Fish meal	0	24%	24%	24%	24%
Vitamins mixture	0	1%	1%	1%	1%
Biscuit	0	0	0	25%	35%
Total	100%	100%	100%	100%	100%

Physicochemical parameters

In order to achieve best results, the physicochemical parameters such as dissolved oxygen (DO), pH, total dissolved solids (TDS), salinity, and electrical conductivity (EC) were monitored and maintained at an optimum range by applying appropriate measures.

Growth studies

To check the effect of feeds on growth performance of fingerlings of *L. rohita*, growth parameters including wet body weight, total length, gain body weight, feed conversion ratio (FCR), and feed conversion efficiency (FCE) were recorded weekly.

Sample collection for microbial analysis

From each treatment, 25 *Labeo rohita* fingerlings were dissected and gut content of each fish was homogenized in a saline solution prepared by dissolving 1.0 g of NaCl in 100 mL of distilled water and was serially diluted up to 6-fold. One µl of each dilution was poured on the Macconkey and MRS agar and incubated for 24 h at 37 °C. Colonies of *E. coli* formed on the Macconkey agar and lactobacilli on the MRS agar were enumerated. The colonies developed on cultured plates were identified morphologically using the gram staining technique (Bergey and Holt, 1994). Biochemical tests such as Gram staining, endospore staining, motility test, capsule staining, glucose fermentation test, oxidase test, catalase test, indole test, urease test and Voges-Proskauer test were performed for confirmation of bacteria at species level following the protocol described elsewhere (Sivasubramanian et al*.,* 2012).

Antagonistic activity of gut isolates

The antimicrobial properties of the isolated bacterial strains of *Lactobacillus* and *E. coli* from *Labeo rohita* fingerlings fed with bakery wastes of different percentage were assessed using the well diffusion techniques previously described by Sivasubramanian et al. (2012).

DNA extraction and molecular study

DNA extraction of the isolates was carried out using kits (ZymoBIOMICS DNA KITS, USA) following the protocol of the manufacturer. Molecular identification of bacterial isolates was done using PCR primers targeting the *eae* gene specific for pathogenic *E. coli,* F (5'-TCAATGCAGTTCCGTTATCAGTT-3') and R (5'-GTAAAGTCCGTTACCCCAACCTG-3') (Miri et al., 2017). For PCR amplification of *Lactobacillus* group specific gene, F (5'-AGCAGTAGGGAATCTTCCA-3') R (5'-ATTYCACCGCTACACATG-3') primers were used (Rauta et al., 2013; Aslam et al., 2016). Agarose gel electrophoresis was used to separate mixtures of DNA according to molecular size by applying electric field and a gel documentation system (Michl et al., 2017).

Statistical analysis

The SAS statistical computer software version 9.1 was used for working out Pearson's Correlation matrix between the physicochemical parameters and fish morphometrics. Analysis of variance (ANOVA) for comparing the growth among the control and treated tanks was worked out, and the t-test was performed for comparing bacterial species (Eaves et al., 2004).

Results

Physico-chemical and growth properties of experimental groups

Physico-chemical properties

The parameters of all experimental groups were recorded twice a day throughout the trial. There was a significant ($P < 0.05$) difference in the values of physico-chemical parameters as shown in **Figure 1**.

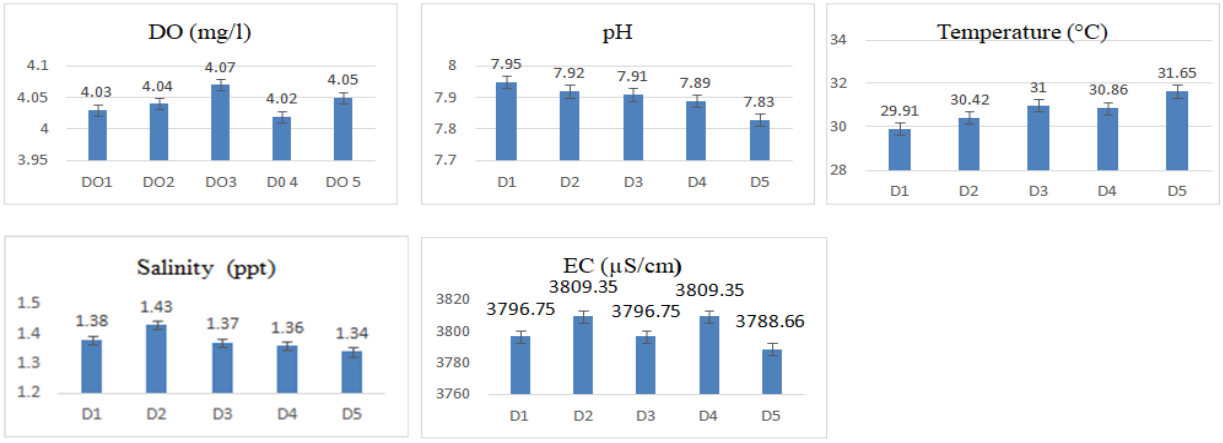

Figure 1. Physico-chemical properties of all experimental groups

Growth parameters

The results obtained from the growth parameters after analysis are given in **Table 2**. The (FCR) feed conversion ratio of the D4 (25% Biscuit) group was found the best, while other experimental groups were rated as sufficient. Comparison of all growth parameters is shown in **Figure 2**.

Table 2. Growth parameters of different experimental groups

Parameters	Treatments				
	D1	D2	D3	D4	D5
Initial weight (g)	6.78±0.007^{a}	6.77±0.007^{a}	6.755±0.007^{b}	6.73±0.007^{c}	6.70±0.007^{d}
Final weight (g)	29.20±0.141^{b}	27.80±0.141^{c}	25.80±0.141^{e}	38.65±0.070^{a}	26.67±0.035^{d}
Net weight gain (g)	22.41±0.148^{b}	21.025±0.148^{c}	19.04±0.134^{e}	25.23±0.516^{a}	19.97±0.042^{d}
Net weight gain (%)	330.25±2.61^{b}	310.35±2.48^{c}	283.85±4.454^{e}	374.65±7.28^{a}	297.85±0.919^{d}
Specific growth rate (%)	1.613±0.002^{b}	1.589±0.002^{c}	1.552±0.002^{e}	1.75±0.001^{a}	1.57±0.000^{d}
Feed conversion ratio	1.360±0.014^{d}	1.450±0.014^{c}	1.595±0.007^{a}	1.20±0.028^{e}	1.51±0.002^{b}

D1 (Control diet); D2 (Cream cake 25%); D3 (Cream cake 35%); D4 (Biscuit 25%); D5 (Biscuit 35%). Mean values in each column or row sharing different letters differed significantly at $P < 0.05$.

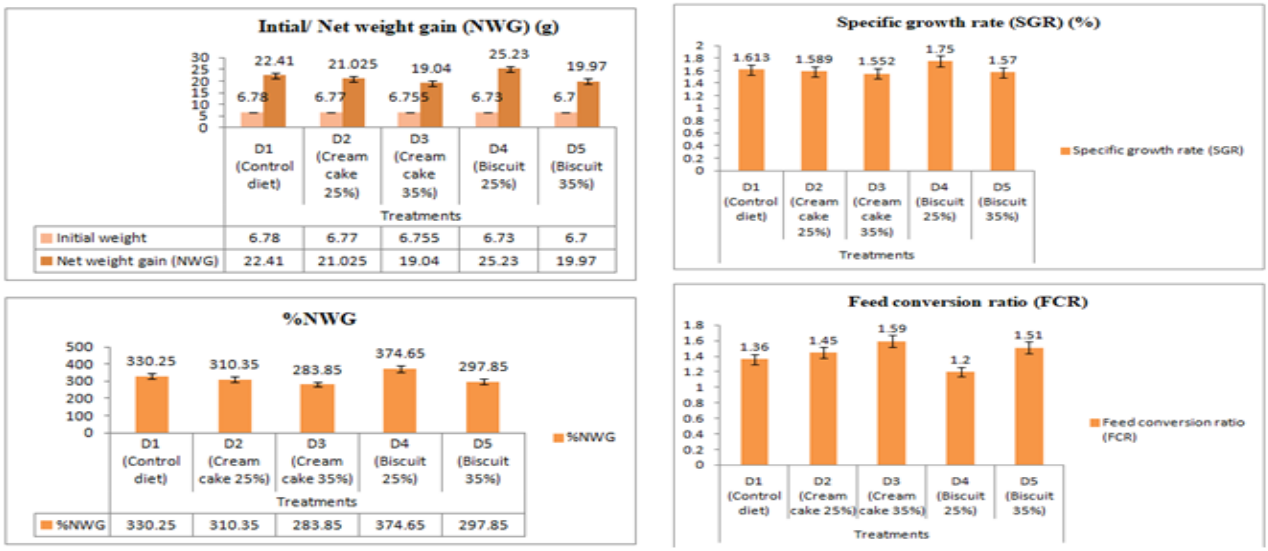

Figure 2. Growth parameters of all experimental groups

Proximate records

A significant difference ($P < 0.05$) was found between the treatments regarding growth parameters. The protein was recorded as 62% in D3, which was highly significant compared with those of D2 and D1 (**Table 3**).

Table 3. Proximate analysis (%) of fishes

TrT	Ash	Fat	Moisture	Protein	Carbohydrates	Dry matter	Fiber
D1	16.2 ± 1.02^{d}	7.13 ± 0.06^{a}	0.13 ± 0.01^{d}	52.9 ± 0.36^{b}	4.15 ± 0.09^{d}	99.9 ± 2.65^{c}	0.22 ± 0.07^{c}
D2	12.1 ± 1.03^{b}	7.62 ± 0.03^{d}	0.31 ± 0.02^{b}	51.9 ± 0.36^{a}	4.17 ± 0.23^{c}	99.7 ± 2.97^{d}	0.21 ± 0.04^{d}
D3	15.2 ± 0.13^{e}	7.83 ± 0.15^{a}	0.29 ± 0.01^{c}	51.9 ± 0.36^{e}	4.01 ± 0.17^{e}	99.7 ± 2.45^{a}	0.21 ± 0.02^{e}
D4	17.2 ± 0.13^{d}	8.83 ± 0.15^{c}	0.49 ± 0.01^{a}	53.8 ± 0.12^{c}	6.23 ± 0.12^{a}	99.9 ± 2.23^{a}	0.24 ± 0.05^{a}
D5	16.1 ± 0.40^{c}	7.81 ± 0.31^{b}	0.37 ± 0.03^{e}	52.5 ± 0.12^{d}	5.98 ± 0.16^{b}	99.8 ± 2.75^{b}	0.23 ± 0.03^{b}

TrT (Treatments); D1 (Control diet); D2 (Cream cake 25%); D3 (Cream cake 35%); D4 (Biscuit 25%); D5 (Biscuit 35%). Mean values in each column or row sharing different letters differed significantly at $P < 0.05$.

Bacterial enumeration and antagonistic activity of *E. coli* and lactobacilli

Bacterial enumeration

The colonies of *E. coli* and lactobacilli were enumerated as cfu/g of the sample. The data in cfu/g of all samples are given below in **Table 4**.

Table 4. Colonies (cfu/g) of *E. coli* and *lactobacilli* of different experimental groups

Feed	*Lactobacillus*	*E. coli*
D1	$2.4 \times 10^{7} \pm 1.9 \times 10^{6}$	$1.8 \times 10^{7} \pm 1.8 \times 10^{6}$
D2	$2.4 \times 10^{7} \pm 1.9 \times 10^{6}$	$1.6 \times 10^{7} \pm 2.1 \times 10^{6}$
D3	$9.1 \times 10^{6} \pm 1.8 \times 10^{6}$	$1.4 \times 10^{7} \pm 1.4 \times 10^{6}$
D4	$9.7 \times 10^{7} \pm 2.4 \times 10^{7}$	$1.1 \times 10^{7} \pm 1.4 \times 10^{6}$
D5	$6.6 \times 10^{7} \pm 1.6 \times 10^{7}$	$1.9 \times 10^{7} \pm 2.5 \times 10^{6}$

D1 (Control diet); D2 (Cream cake 25%); D3 (Cream cake 35%); D4 (Biscuit 25%); D5 (Biscuit 35%).

Antagonistic activity

The antagonistic activity of lactobacilli against *E. coli* is shown in **Table 5**. The antagonistic effect was observed to be highest in D4 followed by that of D5. While D1, D2 and D3 showed a decreasing trend.

Table 5. Antagonistic activity (cfu/g) of lactobacilli against *E. coli* of different experimental groups

Diet	Bacteria	Mean activity ± SEM	*P* at 0.01
D1	*E. coli*	$1.8 \times 10^{7} \pm 1.8 \times 10^{6}$	0.024162^{*d}
	Lactobacilli	$2.4 \times 10^{7} \pm 1.9 \times 10^{6}$	
D2	*E. coli*	$1.6 \times 10^{7} \pm 2.1 \times 10^{6}$	0.010248^{*c}
	Lactobacilli	$2.4 \times 10^{7} \pm 1.9 \times 10^{6}$	
D3	*E. coli*	$1.4 \times 10^{7} \pm 1.4 \times 10^{6}$	0.093606^{e}
	Lactobacilli	$9.1 \times 10^{6} \pm 1.8 \times 10^{6}$	
D4	*E. coli*	$1.1 \times 10^{7} \pm 1.4 \times 10^{6}$	0.001165^{*a}
	Lactobacilli	$9.7 \times 10^{7} \pm 2.4 \times 10^{7}$	
D5	*E. coli*	$1.9 \times 10^{7} \pm 2.5 \times 10^{6}$	0.008261^{*b}
	Lactobacilli	$6.6 \times 10^{7} \pm 1.6 \times 10^{7}$	

D1 (Control diet); D2 (Cream cake 25%); D3 (Cream cake 35%); D4 (Biscuit 25%); D5 (Biscuit 35%).

Bacterial isolation and identification

Isolation of *E. coli* and lactobacilli

From serially diluted plates used for enumeration, the putative colonies of *E. coli* and lactobacilli were isolated and pure cultured on TSA, EMB and Casein agar, incubated for 24 h at 37 °C, and then colonies of *E. coli* and lactobacilli observed. The bacteria produced colonies on a specific agar which are shown in **Figure 3**.

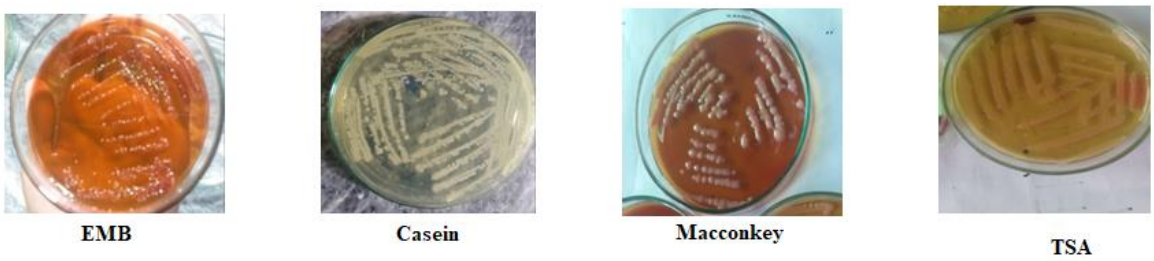

Figure 3: Bacterial colonies on different agars

Biochemical test identification of *E. coli* and lactobacilli

The pure culture of each bacterium was used for the biochemical test identification and following results were observed as shown in **Table 6** and **Figure 4**.

Table 6. Biochemical test identification of *E. coli* and lactobacilli

Biochemical test	*E. coli*	Lactobacilli
Gram staining	Negative (-ve)	Positive (+ve)
Endospore staining	non-spore forming	Negative (-ve)
Motility test	Motile	Negative (-ve)
Capsule staining	Variable	Negative (-ve)
Glucose fermentation test	Positive (+ve)	Positive (+ve)
Oxidase test	Negative (-ve)	Negative (-ve)
Catalase test	Positive (+ve)	Negative (-ve)
Indole test	Positive (+ve)	Negative (-ve)
Urease	Negative (-ve)	Negative (-ve)

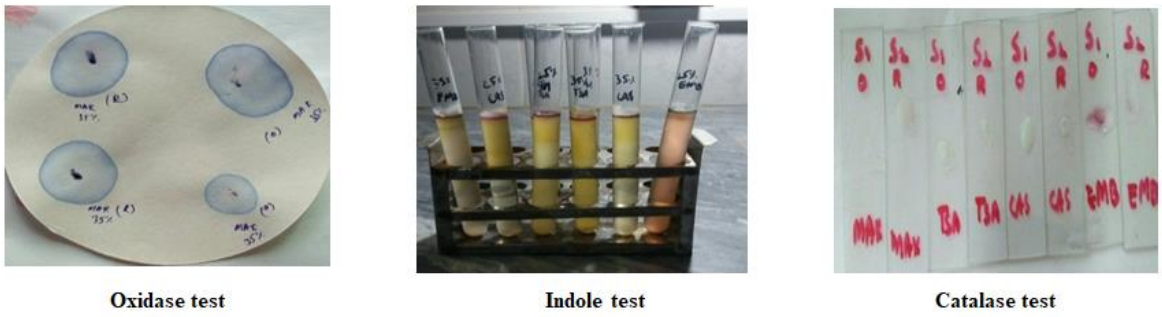

Figure 4: Biochemical tests for E. coli and lactobacilli

Molecular identification of *E. coli* and Lactobacilli isolates

DNA isolation

DNA was isolated by the organic method with a slight modification by adding NaCl. After DNA isolation, isolated DNA was run on 1% agarose gel for DNA confirmation as shown in **Figure 5**.

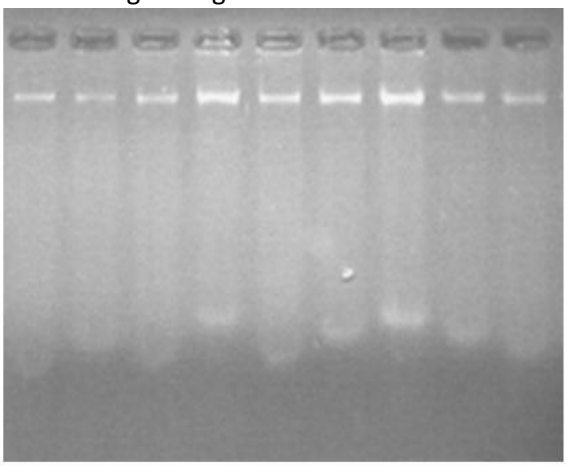

Figure 5: Wells 1, 2, 3,4, 5, 6, 7, 8, and 9 showed positive results for DNA Extraction

PCR amplification of *E. coli* specific gene and lactobacillus specific gene

The PCR amplification of *eae* gene and lactobacillus group specific gene gave products of 480 bp and 320 bp, respectively, as shown in **Figures 6** and **Figure 7**.

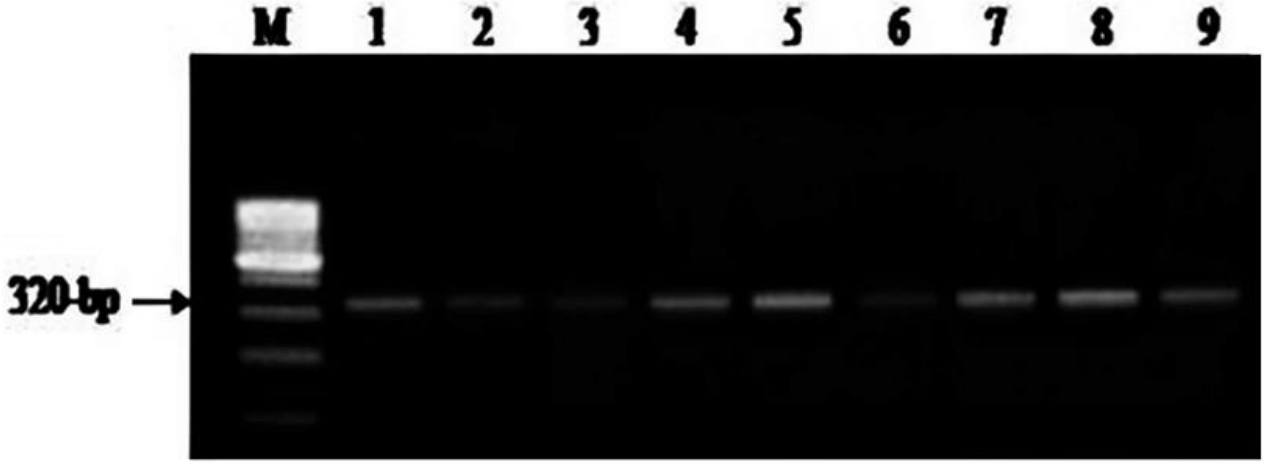

Figure 6: PCR results of *Lactobacillus* group specific

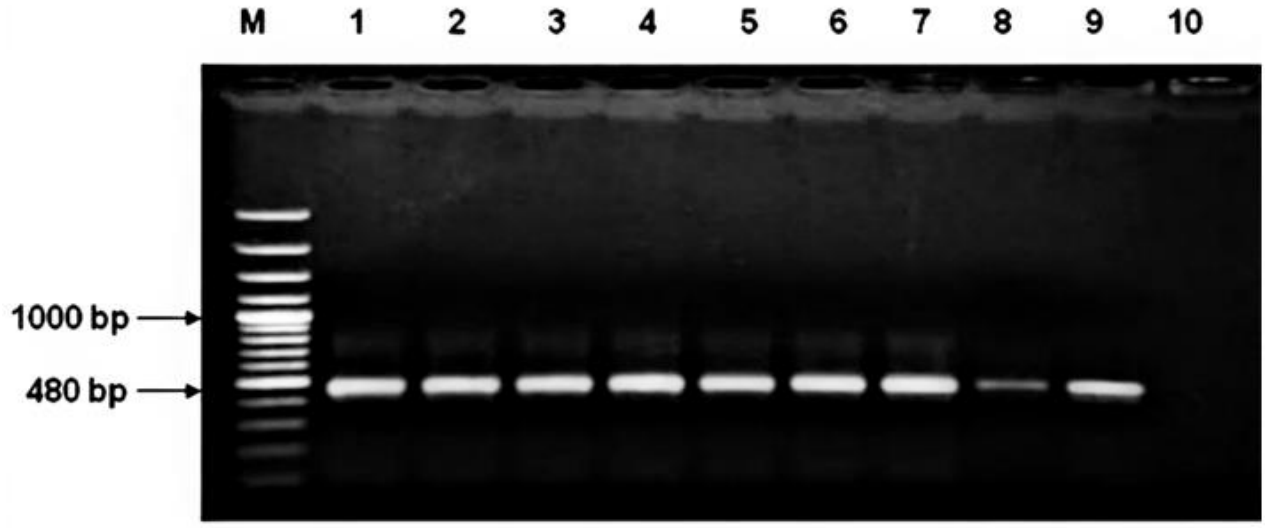

Figure 7: PCR results of EPEC specific genes

Discussion

In this study, we used bakery wastes, i.e., cream cake and biscuit each as 25% and 35% of feed ingredient and compared their growth effect with control (commercial) diet. The aim of the study was to utilize bakery wastes as value-added ingredients. After a successful feeding trial of 90 days, it was found that diet D4 (25% Biscuit) had the highest growth performance according to the growth data compared with the control and other treatment groups, followed by the control group which had the highest performance after D4. Whereas the remaining treatments had growth significantly lower than that of the control group, but still it was satisfactory. The final weight of D4 was highest among the treatment groups, i.e., 38.65 ± 0.070 (g). The other variables also showed a similar trend. However, FCR showed the results other way around (**Table 2**).

The effect of bakery wastes was also evaluated on intestinal microbiota of the *Labeo rohita* fingerlings. The bakery wastes enhanced the intestinal *Lactobacillus* production as previously studied by Jini et al. (2011). *E. coli* occurs as antagonistic to lactobacilli. *Lactobacillus* is a nonpathogenic naturally occurring microbe, while *E. coli* is a pathogenic and a naturally occurring bacterium in the intestine. The maximum lactobacilli were recorded in the D4 diet (biscuit 25%) $9.7 \times 10^7 \pm 2.4 \times 10^7$ cfu/g, followed by those in D5 (35% Biscuit) 6.6×10^7 cfu/g, D1 (Control diet) and D2 (Cream cake 25%) 2.4×10^7 cfu/g, and minimum were observed in D3 (Cream cake 35%) 9.1×10^6 cfu/g. Whereas *E. coli* were present maximum in D5 (35% Biscuit) 1.9×10^7 cfu/g followed by those in D1 (Control Diet) 1.8×10^7 cfu/g, D2 (25% Cream cake) 1.6×10^7 cfu/g, D3 (35 % Cream cake) 1.4×10^7 cfu/g (**Table 4**). These results were like those of Punom et al. (2017) who isolated and cultured *E. coli* along with other pathogenic bacteria. The total

microbial count recorded by them was $5.24 \pm 2.02 \times 10^7$ cfu/g from the gastrointestinal tract.

The antagonistic activity of lactobacilli against *E. coli* was also calculated and it was observed that the lactobacilli against *E. coli* were present in maximum number in the samples of fishes fed with diet D4 with 25% biscuits as basic ingredients with *P* value of 0.001165, followed by 0.008261 in D5 (Biscuit 35%), 0.010248 in D2 (Cream cake 25%), 0.024162 in D1 (Control diet) and 0.093606 in D3 (Cream cake 35%) as shown in **Table 5**. In the same way, Buntin et al. (2008) isolated 160 lactic acid bacteria from fishes, shrimps and mollusks and observed inhibitory activity against *E. coli.* The antibacterial activity of culture supernatants was low while treating with catalase between pH 6.5-7.0. The 16S rDNA had homology of 98% (492/501 bp) in *Enterococcus faecium* SF, 98% (655/668 bp) in *Pediococcus pentosaceus* SL4 and 97% (691/712 bp) in *Pediococcus pentosaceus* LM2.

Furthermore, for molecular identification of bacterial isolates, DNA extraction was done using a kit (ZymoBIOMICS DNA KITS, USA), and gene specific PCR was carried out for identification of *E. coli* and lactobacilli. The identified *E. coli* was the same as isolated previously (Miri et al., 2017). However, the identified isolates of lactobacilli were similar to the isolates extracted earlier (Rauta et al., 2013).

Conclusion

The overall findings of the current study showed that the bakery wastes, i.e., biscuits and cream cake are valuable feed ingredients and can be utilized in fish feed, especially with 25% concentration. These ingredients promoted a significant increase in the population of beneficial bacterial colony ensuring better feed intake and better digestibility, which in return, gave better FCR and FCE.

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Declaration of Author(s), Editor(s) and Publishers

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Conflict of interest
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Contribution of authors
Study design: all authors. Conduction of experiments: AR. Data collection, analysis, and interpretation: AR, FY, SP, AT, MU, SSH, NK. Preparation of manuscript first draft: AR, FY, SP, AT, MU, SSH, NK, HA. Revision of manuscript: all authors.

Ethical approval
This work was approved by Institutional Ethical Review Board/Committee (IERB/C) of the University of Education, Faisalabad Campus, under approval number ZOOL/UEFC/303 dated 15-01-2021.

Handling of bio-hazardous materials
The authors certify that all experimental materials were handled with care during collection and experimental procedures. After completion of experiment, all materials were properly discarded to minimize any types of bio-contamination(s).

Availability of primary data and martials
As per editorial policy, experimental materials, primary data, or software codes are not submitted to the publisher. These are available with the corresponding author and/or with other author(s) as declared by the corresponding author of this manuscript.

Authors' consent
All authors contributed in designing and writing the entire review article. All contributors have critically read this manuscript and agreed for publishing in IJAaEB.

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