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Efficacy of Probiotic Lactobacillus Casei in Bio-control of Escherichia Coli O157:H7 in Nono

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Abstract:
The use of chemical preservatives and non-therapeutic antibiotics for milk preservation has been an aged long practice. Although, there are specified dosages for their uses, overall fact that they are synthetic raises a need for the adoption of bio-control measures which are deemed to be natural. Lactobacillus casei is a probiotic organism indigenous to Nono. Nono is a fermented milk beverage consumed in Nigeria. This research aimed at the use of L. casei as a bio-preservative against E. coli O157:H7 in Nono. L. casei and E. coli O157:H7 isolates were obtained from Nono and identified with 16S rDNA sequencing. The Lactobacillus isolate was assessed for probiotic abilities. It was used in ratios of 1:1, 1:2, 1:3, 1:4, and 1:5 against E. coli in Nano preservation. The time course and pH changes were monitored over a period of 24 h. There was notable decrease in the E. coli counts within 24 h with 1:5 ratio of E. coli to L. casei giving the highest contaminant reduction. Likewise same 1:5 ratio gave the highest L. casei count over the 24 h monitoring. pH monitoring showed that acidity increased with increase in ratio of L. casei, and 1:5 ratio had the highest acidity of 4.0±0.1. This research thus has shown that L. casei can act as a bio-control agent for the preservation of Nono from E. coli contamination. Its activity has been shown to be modulated by competitive inhibition and decrease in pH.

Keywords: *Bio-preservative, Nono, L. casei, E. coli O157:H7*

1. Introduction

Lactobacillus casei is a Gram positive anaerobic rod which belongs to the lactic acid bacteria (LAB) group. It is a facultative homo-fermenter found indigenous in dairy products such as *Nono* and cheese. *Nono* is a locally fermented milk beverage consumed in Nigeria. It is a functional food which contains high numbers of Lactic acid bacteria (LAB) which includes *L. casei*. It is usually enjoyed with a sorghum cake known as *Fura* and thus it is usually called *Fura D'Nono*. Despite its role as a functional food, unhygienic and unaseptic milking, processing, packaging and vending procedures, could render the food product susceptible to contamination with *Escherichia coli* and other enteric pathogens. *Escherichia coli* is one of the top seven pathogens of public health concern (CDC, 2014). *E. coli* O157:H7 is considered an emerging disease pathogen which causes infections such as hemorrhagic gastrointestinal disease and haemolytic uremic syndrome in humans (Isibor, *et al*., 2013).

L. casei is well-known for its wide probiotic values such as bacteriocin production, bile tolerance, acid tolerance, ability to have a good attachment to the intestinal walls, immune modulation, cholesterol regulation, non-toxicity, antibacterial activity *inter alia*; and has been given Generally Recognized as Safe (GRAS) status, as there has not been any established risk to humans (Gaynor, 2012). Hence adopting it in food preservation comes with an added advantage when ingested along with the food it is meant to preserve, thus boosting the health status of the end food consumers. Bio-preservation or bio-control is a measure employed as an alternative to the use of chemical preservatives and antibiotics for food preservation. Thus, this research aimed at the use of *L. casei* as a bio-control agent against *E. coli* proliferation in *Nono*.

2. Methods

2.1. Bacterial Strains, Culture Conditions and Characterization

Lactic acid bacteria were isolated from different samples of *Nono* by cultivating the strains on De Mann Rogosa and Sharpe Agar (Merck) at 30^0C, pH 6.5 for 24 h in an anaerobe jar. The strains were screened for the ability to produce antibacterial substances against *E. coli*, tolerate acidic pH, bile and were checked for xylene adherence as described by Pringsulaka *et al*., (2012).

E. coli strains were isolated from same milk product by cultivation on Eosin methylene blue agar at 37^0C for 24 h. Isolates were screened for the ability to ferment sorbitol, and the non-sorbitol fermenting isolates were subcultured on Sorbitol McConkey agar with BCIG (Oxoid) and incubated for 24 h at 37^0C as described by Isibor *et al*., (2013).

2.2. Selection and confirmation of Choice Isolates

Lactic acid bacteria with the most zone of inhibition to *E. coli* isolates was selected for the preservation study while the *E. coli* isolate was randomly selected from the Sorbitol McConkey agar plate. The isolates were confirmed using 16s rDNA sequencing at Macrogen Laboratories South Korea.

2.3. Standardization of Pure Cultures of Isolates

A 0.25 ml aliquot of pure cultures of *L. casei and E. coli* O157:H7 were grown in 25 ml MRS broth and Nutrient broth respectively and incubated in an anaerobe jar (for *Lacotbacillus*) and aerobically (for *E. coli*) without agitation at 30^0C for 24 h. The cultures were serially diluted using 1 in 10-fold dilution to achieve concentrations of 10^8 cfu/ml for *L. casei* and10^6 cfu/ml for *E. coli* according to the method described by Hartmann *et al*., (2011).

2.4. Bio-preservation Tests and their Time-course Studies

The methods of Hartmann *et al*., (2011) and Pringsulaka *et al*., (2012) were adopted to assess the efficacy of different volumes of *L. casei* in reducing viable counts of *E. coli* O157:H7 in Nono. 10 ml of fresh Nono sample was dispensed into five groups of three sterile test tubes per group and pasteurized in a water bath at 60^oC for 20 minutes. 1 ml of 10^6 cfu/ml of *E. coli* was introduced into the test tubes with varied volumes of 1 ml, 2 ml, 3 ml, 4 ml and 5 ml of 10^8 cfu/ml of *L. casei*. The set up was allowed to stand for 24 hours at room temperature and microbial counts in cfu/ml were calculated in time intervals of 4 h, 8 h, 12 h, 16 h and 24 h. The pH of the mixtures was also noted for same time intervals.

2.5. Statistical Analyses

One sample T-test was used to analyze means using SPSS version 21.

3. Results

3.1. Probiotic Screening of Lactobacillus Isolate

The examined *Lactobacillus* isolate had good probiotic attributes which included cell free supernatant and crude bacteriocin inhibition against *E. coli* as shown in Table 1.

3.2. Molecular Typing of Isolates

The *Lactobacillus* and *E. coli* isolates were identified as *Lactobacillus casei* and *E. coli* O157:H7 respectively with 16s rDNA sequencing.

3.3. Bio-preservation Tests and their Time-course Studies

There was a marked decrease in the *E. coli* count over the 24 h monitoring period with group E (1:5 ratio) having the least *E. coli* count as shown in figure 1. Group E also had the highest lactic acid bacteria count as shown in figure 2. Table 2 shows the pH variations in the set-ups and it was observed that acidity increased with increase in time and also with increase in the volume of microorganisms.

Screening Parameters	Results
Antibacterial activity against *E. coli*	+ (11.5 mm diameter zone of inhibition).
Tolerance to Acidity	+
Tolerance to Bile	+
Cell Hydrophobicity Test	+
Crude Bacteriocin activity against *E. coli*	+ (12 mm diameter zone of inhibition).
Microaerophilic Growth	+
Tolerance to 10% NaCl	+

Table 1: Probiotic Attributes of Lactobacillus Isolate

+ = Positive

- = Negative

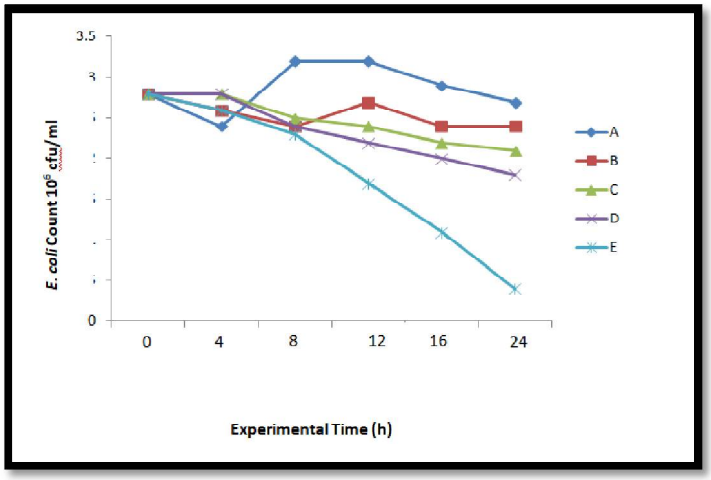

Figure 1: E. coli O157:H7 Count in the Bio-Preservation Of Nono
A = 1:1 Ratio, B=1:2 Ratio, C=1:3 Ratio, D=1:4 Ratio and E= 1:5 Ratio of E. Coli O157:H7 To L. Casei.

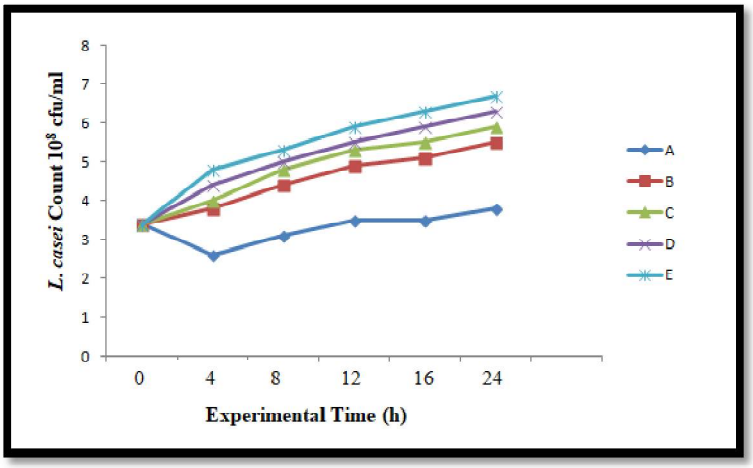

Figure 2: L. Casei Count in the Bio-Preservation of Nono
A = 1:1 ratio, B=1:2 ratio, C=1:3 ratio, D=1:4 ratio and E= 1:5 ratio of E. coli O157:H7 to L. casei

Time (h)			pH			
	A	**B**	**C**	**D**	**E**	
0	6.1±0.1	6.1±0.1	6.1±0.1	6.1±0.1	6.1±0.1	
4	6.1±0.1	6.1±0.1	5.8±0.1	5.6±0.02	5.6±0.02	
8	5.8±0.1	5.9±0.1	5.5±0.01	5.4±0.02	5.4±0.02	
12	5.5±0.25	5.7±0.25	5.4±0.01	5.1±0.01	4.9±0.01	
16	5.3±0.1	5.4±0.1	5.1±0.16	4.8±0.04	4.4±0.05	
24	5.3±0.1	5.1±0.1	4.8±0.03	4.4±0.02	4.0±0.1	

Table 2: Mean pH Changes in Nono during Efficacy testing of the Bio-preservative
A = 1:1 ratio, B=1:2 ratio, C=1:3 ratio, D=1:4 ratio and E= 1:5 ratio of E. coli O157:H7 to L. casei.

4. Discussion

Bio-control is a welcomed concept in food production and preservation and has been in practice for some period of time. With the world vehemently focusing on natural alternatives to the control of contaminations, infections and diseases, the use of bio-control measures is a focal method that cannot be overlooked or overemphasized. *Lactobacillus* species have been well researched and documented as probiotic microorganisms that also possess the ability to produce inhibitory substances commonly known as bacteriocin (Hyronimus *et al*., 2000). This corresponds with the probiotic attributes of *L. casei* as shown in table 1. *Lactobacillus* species are usually indigenous to dairy products especially the fermented ones. *Nono* is fresh milk which undergoes 24 h fermentation with starter culture locally called *Manshanu* (Makut *et al*., 2014). This starter culture is primarily the pre-ferment from the last fermentation batch that is stored and re-used to catalyze fermentation of new culture batches. A notable hazard threatening the safety of this product is the presence of enteric microorganisms (Abdulkadir and Mugadi, 2012). They contaminate the food, tamper with its wholesomeness and also constitute risks to the consumers. Different approaches boiling and pasteurization have been employed to tackle contamination issues associated with milk. However, heat treatment after fermentation of *Nono* will only result in the loss of beneficial microorganisms, especially the probiotics which would have been ingested. Therefore,

one may resort to the use of chemical preservatives or even antibiotics, but it is still noted that these preservatives are artificial and not so advised to be used irrespective of the fact that they are applied in controlled doses. Cases of antibiotic resistant microorganisms are on the increase and thus, antibiotic use is not a comfortable preservation method to be adopted. Bio-control using probiotic microorganisms indigenous to the product appears to be a potent control measure. This is because the technique behind its application is based on increasing the numbers of the probiotics against the contaminants in the product. This initiates quorum sensing amongst the probiotic organisms and aid in their competitive inhibition against the contaminants (Holzapfel *et al*., 2001).

E. coli O157:H7 is a contaminant isolated from *Nono* and it was also used in the establishment of bio-control efficacy of *Lactobacillus casei*. Both the bio-control agent and the contaminant co-habit in *Nono* and this basically is because they are both lactose fermenters. This observation raises a pertinent question as to why *L. casei* presence in *Nono* did not automatically inhibit the growth of *E.coli* in their natural habitat. A likely response to such situation is that in their natural habitat, they both make use of lactose and other nutrients in the milk to grow their cell numbers and competitive inhibition may not set in until there is nutrient depletion. Nutrient depletion could perhaps induce the *Lactobacillus* species to enter their stationary phase where bacteriocins and similar inhibitory substances are the produced (Joshi *et al*., 2006) and begin to inhibit the survival of *E. coli* in their natural habitat. This research however, provided a controlled environment for the growth of both organisms, while constantly increasing the ratio of the probiotic against the contaminant over a 24 h period at room temperature. Figure 1 showed that there was marked decrease in the *E. coli* count for all groups with group E showing the least count as time increased. Figure 2 also showed a marked increase in the *L. casei* count for all groups with group E showing the highest count as the hours of monitoring increased. Table 2 showed a decreasing pH value to acidic range in all the groups, reflecting the metabolism of the sugars in *Nono* (mainly lactose) and subsequent production of lactic acid from both microorganisms. However, Group E showed the highest acidity value of 4.0 at 24 h, which is not a very suitable pH of growth for the pathogen *E. coli* O157:H7. *E. coli* O157:H7 presence in *Nono* as reported in this research corresponds with the report of Abdulkadir and Mugadi (2012). It could be thus interpreted that the control of *E. coli* O157:H7 by *L. casei* was modulated by competitive inhibition based on increased cell numbers as well as by the influence of pH.

5. Conclusion

This study reveals that the incorporation of increased volumes of *L. casei* to *Nono* will aid in bio-preservation against *E. coli* and possibly, other enteric contaminants.

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