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SELECTION OF *Lactobacillus fermentum* OVL AS POTENTIAL PROBIOTIC AGENT

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ABSTRACT

Lactobacillus fermentum strain OVL isolated from Kunnu, a locally fermented beverage produced from sorghum, was assessed as a probiotic organism. *In vitro* antagonistic property of the organism shows that it can effectively inhibit the growth of some indicator bacteria. The strain was examined for resistance to antimicrobial agents and low pH. The probiotic strain, OVL was administered by orogastric dosing to albino rats (*Rattus norvegicus*). Faecal samples of rats were collected before, during, and after consumption. Lactobacilli and *E. coli* counts were determined in the faeces. This study reveals that *L. fermentum* OVL increased the faecal lactobacilli counts and reduced the *E. coli* counts in the faeces of rats in groups MDL and PDL that were dosed with the strain. *Lactobacillus fermentum* strain OVL may be considered to be a potential probiotic for animals and eventually for man.

INTRODUCTION

The composition of the gut microflora of healthy animals remains steady, but if the stability is broken, the pathogenic microorganisms are able to colonise the gut, leading serious infections. The stability of the gut can be broken by the following factors: sudden change in the animal[1], deprivation of water[2], trips[3], and antibiotic administration and radiation[1]. To prevent infection, a steady gut microflora composition must be maintained, especially for the dominant flora of which lactic acid bacteria are important representative.

Ehrmann *et al.*[4] suggested that the possible mechanisms for the beneficial effects of lactic acid bacteria on the gastrointestinal disturbances are by two modes: production of antimicrobial substances such as lactic acid, bacteriocins, and hydrogen peroxides; and adherence to the mucosa and co-aggregation to form a barrier which prevents colonisation of pathogens. For an organism to exhibit these mechanisms, it must be able to adhere to the intestinal epithelium to overcome potential huddles, such as the low pH of the stomach, the presence of bile acids in the intestines and the competition against other microorganisms in the gastrointestinal tract [5,6].

Lactobacilli represent a very important group of lactic acid bacteria. The justification for the use of lactobacilli stems from studies that show that when the gut flora develops after birth, as lactobacilli increases other components of the flora decreases[7] Walker and Duffy suggested that the current perspective on biotechnical application of probiotic products require further *in vitro* and *in vivo* investigation to evaluate the potential of using wild type organism or those obtained by genetic engineering. Probiotic organisms are found to be abundant in fermented foods and members of the genus *Lactobacillus species* are the most common.

The objective of this study was therefore to characterise *Lactobacillus fermentum* OVL, isolated from kunnu, a cereal based fermented beverage, for potential application as probiotic agent using *in vitro* and *in vivo* data.

MATERIAL AND METHODS

Source of *Lactobacillus species*

Lactobacillus species were isolated from Kunnu purchased from Akure Central Market. The Kunnu sample was diluted in normal saline (0.85g Nacl/100ml of water) and 0.1ml of 10^{-5} dilution were plated on de Mann Rogosa and Sharpe (MRS) agar, (Lab M, Lancashire, UK). The plates were incubated anaerobically at 37^0 C for 2 days. Discreet colonies were subcultured to obtain pure culture.

Identification of Isolates

The isolates were characterised using colonial, morphological, and biochemical properties. Standard methods described by Cowan and Steel[9] and Parker and Collier[10] were used for identification.

Detection of Inhibitory Activity

The agar well diffusion method described by Schillinger and Lucke[11] was used to screen the inhibitory activity of the isolates against the indicator bacteria. This involves seeding sterile tryptone soya agar (Oxoid) with indicator bacteria in triplicates. Fifty microlitre (50ìl) of the cell free supernatant of the isolates were introduced into the wells. The indicator bacteria used are *Escherichia coli* NCIB 86, *Bacillus cereus* NCIB 6349, *Pseudomonas aeruginosa* NCIB14070, and *Klebsiella pneumoniae* NCIB18950.

Sensitivity of the Isolates to Antibiotics

Susceptibility of isolates to antibiotics was tested using the disk diffusion method described by Chang et al[12]. Sterile antibiotic disk (Sens Test 8, G.T. Manchester, England) was aseptically placed on solidified MRS agar plates that had been seeded with the isolates. The plates were incubated anaerobically at 37^0 C for 24 hours. The plates were examined for zones of inhibition around each disk to determine the sensitivity of the isolate to different antibiotics in the disk.

Sensitivity of the Isolates to Different pH

The resistance of the isolates to different pH of 2, 3, 4, and 5 were investigated using the method of Garriga et al[13]. Overnight broth cultures of the isolates were centrifuged at 5000**g** for 10 minutes. The pellets were resuspended in different saline solution and were diluted (1/10) in sterile distilled water at pH2, 3, 4, and 5. After 3 hour of incubation in the different pH regimes, the cultures were plated on MRS agar and incubated at 37^0 C for 48 hours to determine counts of surviving cells.

Cultivation of *Lactobacillus species*

Four *Lactobacillus species* were isolated from Kunnu, *Lactobacillus fermentum* strain OVL was selected as a potential probiotic strain because of its high degree of resistance to pH, antibiotics, and antimicrobial activity against the indicator bacteria. The isolate was grown in MRS broth and incubated at 37^0 C for 2 days to obtain large cell concentration. The cells were washed and resuspended into rehydrated skim milk (10% w/v), lyophilised, and stored at -2^0 C until use.

***In vivo* Feeding Trials**

Sixteen (16) albino rats (*Rattus norvegicus*) aged 4 – 6 weeks obtained from a research farm in Ile Ife, Nigeria, were used in the feeding trial. The rats were divided into 4 groups of 4 rats each. Group PD were fed on diet composed of (%/diet) casein 10; fats 5, vitamin mineral premix 5, and corn starch 80, while group MD were fed on diet on the diet above but the casein was substituted with 10% mushroom (*Plerotus sajor caju*) protein. Groups PDL and MDL were dosed with 0.3ml of $5.9x\ 10^{13}$ cfu/g of *Lactobacillus fermentum* strain OVL in addition to being fed on diet PD and MD respectively.

Faecal Bacterial Count

Faecal samples were collected and investigated at days 0, 7, 14, and 28. Serial dilutions of faeces were plated on MRS agar to select lactobacilli and on Eosin methylene blue (EMB) agar (Lab M, Lancashire, UK) to select *E. coli*. The colony forming units on the plates were recorded after incubation at 37^0 C for 24 hours.

Data Analysis

Data gathered from faecal analysis were processed using one-way analysis of variance and means were compared using Duncan Multiple Range test.

RESULTS AND DISCUSSION

The current perspective in probiotic study is the selection of wild type organisms and those obtained by genetic engineering. The selection process involve *in vitro* and *in vivo* investigation of new isolates to verify if they have probiotic potential[8]. Some of the criteria used in the selection of new isolates are: the ability to persist, adherence to cells, and antagonistic activities against pathogens[4]. Fermented products had been found to be good sources of probiotic organisms, and *Lactobacillus fermenum* OVL isolated from Kunnu, a fermented beverage was used for this study.

The isolate inhibited the indicator bacteria with zones of inhibition varying from 1mm to 4mm (Table 1). The inhibitory effect of the isolate was more pronounced against *Klebsiella pneumoniae*. The ability of this isolate to inhibit the growth of these bacteria especially *E. coli* and *Shigella dysenteriae,* two enteric pathogens, is a very good criterion. Salminen *et al*[14] reasoned that if antimicrobial activities could be transferable to *in vivo* conditions, it seems beneficial for the maintenance of the intestinal microflora. The ability of lactobacilli to produce toxic metabolites such as lactic acid, hydrogen peroxide, and bacteriocin had been suggested as being responsible for their ability to inhibit other bacteria[15].

Lactobacillus fermentum OVL displayed a good resistance to antibiotics (Table 2). Ability of

Table-1: Antagonistic activities of *Lactobacillus fermentum* OVL towards indicator bacteria

Indicator bacteria	Activity*
Bacillus cereus NCIB 6349	+
Escherichia coli Type 1 NCIB 86	+
Pseudomonas aeruginosa NCIB 14070	+
Klebsiella pneumoniae NCIB 18950	++
Shigella dysenteriae (Clinical isolate)	+

*Clear zones; +clear zones of 1 mm or more; ++ clear zone of 3 mm or more

Table - 2 : Sensitivity of *Lactobacillus fermentum* OVL to antibiotics

Antibiotics	Resistances	MIC (µg/ml)
Amoxycillin	+	10
Ampicillin	+	10
Chlortetracycline	+	30
Erythromycin	-	5
Penicillin	+	1
Streptomycin	+	30
Tetracycline	+	30
Gentamycin	+	10

-: Susceptible; +: Not susceptible

Table - 3 : Tolerance of *Lactobacillus fermentum* OVL to acidic pH

pH	Survivor (cfu/ml)
Initial microbial load	2.04 ± 0.00^{b}
2	1.88 ± 0.06^{a}
3	2.00 ± 0.02^{b}
4	2.00 ± 0.01^{b}
5	2.03 ± 0.02^{b}

Values are means and std error of 3 replicates.
[a]Value lower and significantly different (P<0.05) from other treatments.

probiotic lactobacilli to resist antibiotic had been reported[16]. The observation shows that the isolate may be able to colonise the gastrointestinal tract in spite of treatment with common antimicrobial agents.

Strain OVL showed a high tolerance to pH 3, 4, and 5 (Table3). At pH 2, the strain OVL was able to grow which shows that it can adapt to acidic conditions in the stomach and intestine. Chang et al[12] had earlier reported that *Lactobacillus reuteri* BSA 131 was able to survive at pH 2.5 to 6.5 but none seemed to survive at pH1.0 and 1,5. The extremely low pH may be too lethal to their survival.

Strain OVL as a probiotic agent ought to bring about the maintenance of the gastrointestinal tract of the host. Mitsuoka[17] had earlier stated that the ability of probiotic strain to balance intestinal microflora of piglets could be assayed by two parameters. This composed of the detection of size of harmful bacteria population e.g. *E. coli* and of beneficial bacterial especially lactobacilli in the faeces. In Table 4, lactobacilli counts were higher and significantly different (P<0.05) in groups MDL and PDL dosed with strain OVL when compared with the groups that were not orally dosed with the isolate (PD and MD).

At the 21st and 28th day of feeding, faecal lactobacilli count increased by 1 log unit in groups PDL and MDL. The *E. coli* counts of faeces of rats in groups PDL and MDL reduced while there was a substantial increase in faecal *E. coli* counts of group PD (Table 5). However, the lactobacilli counts of rats' faeces fed diet MD, made up of mushroom, was found to be higher and significantly different (P<0.05) when compared to rats fed diet PD. This is an indication that the mushroom, *Plerotus sajor caju,* may promote the growth of beneficial lactobacilli in the gut. Mushrooms contain dietary fibres belonging to â-glucan, chitin, and heteropolysaccharides, which cannot be digested by animals. This dietary fibre had been found to promote the growth of fermentative species such as *Lactobacillus species*[8].

Table - 4 : Total counts of faecal lactobacilli during feeding trial

Groups	Day 0	Day 7	Day 14	Day 28
PD	6.94 ± 0.01^{b}	6.63 ± 0.04^{a}	6.67 ± 0.02^{a}	6.66 ± 0.07^{a}
MD	6.87 ± 0.01^{a}	6.80 ± 0.01^{b}	6.80 ± 0.08^{b}	6.81 ± 0.02^{b}
PDL	6.85 ± 0.01^{a}	7.08 ± 0.06^{b}	7.27 ± 0.01^{c}	7.29 ± 0.00^{c}
MDL	6.90 ± 0.01^{b}	6.72 ± 0.32^{a}	7.33 ± 0.04^{c}	7.32 ± 0.03^{c}

Values are means and std error of 4 replicates. Values with the same superscript along column are not significantly different (P>0.05).

Table - 5 : Total counts of faecal *E. coli* during feeding trial

Groups	Day 0	Day 7	Day 14	Day 28
PD	6.98 ± 0.00^{b}	6.98 ± 0.02^{c}	6.97 ± 0.02^{c}	7.05 ± 0.05^{c}
MD	7.01 ± 0.01^{c}	6.99 ± 0.03^{d}	6.79 ± 0.04^{b}	6.78 ± 0.02^{b}
PDL	7.06 ± 0.01^{d}	6.68 ± 0.04^{a}	6.81 ± 0.03^{b}	6.37 ± 0.04^{a}
MDL	6.93 ± 0.01^{a}	6.81 ± 0.02^{b}	6.45 ± 0.03^{a}	6.44 ± 0.06^{a}

Values are means and std error of 4 replicates. Values with the same superscript along column are not significantly different (P>0.05).

Lactobacillus fermentum strain OVL showed a good promise as a probiotic agent based on the *in vitro* and *in vivo* data gathered from this study. This result shows that strain OVL may serve as a good probiotic agent in livestock and eventually in human.

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