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## LACTOBACILLUS CASEI ISOLATES FROM KUNUN ZAKI: THE PROBIOTIC POTENTIAL AGAINST INDUCED SHIGELLOSIS IN EXPERIMENTAL RATS

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### ABSTRACT

Two different volumes of *Lactobacillus casei* isolates (0.4ml of  $2.0 \times 10^{10}$  CFU/ml and 0.4ml of  $4.0 \times 10^{10}$  CFU/ml) from a local beverage (*kunun zaki*) cultured on MRS agar were orally administered to different groups of shigella-free experimental rats. Both groups were subsequently dosed with 0.4ml  $1.0 \times 10^5$  CFU/ml of *Shigella dysenteriae* and studied along with set control groups. The results from the rectal temperature, feed consumed, weight gained and biochemical tests revealed that *L. casei* isolates from *kunun zaki* exhibited impressive potential against bacillary dysentery. The probiotic efficacy of the *L. casei* isolates tends to be dose dependent.

**Keywords:** *Lactobacillus casei*, *Shigella dysenteriae*, *kunun zaki*, probiotics, shigellosis

### INTRODUCTION

Gastrointestinal diseases, characterized by stomach and intestinal inflammation (gastroenteritis), are often triggered up when there is a disruption of the complex ecosystem of the gastrointestinal tract. These infections are common, especially in the developing countries where the associated mortality rate is particularly high in infants and young children [1]. The loss of water and electrolytes from the body can lead to severe dehydration, which is the principal risk factor causing fatality in young children, especially the malnourished, hypoglycemic and those in poor health condition [2]. Dysentery, one of these gastrointestinal diseases, is associated with species of bacteria from the enterobacteriaceae family and this term is usually restricted to shigella infections. According to Levine *et al* [3], shigellosis or bacillary dysentery results from acute inflammatory reaction of the intestinal tract caused by the shigella organism which constitutes of four species as follows: *S. sonnei*, *S. flexneri*, *S. dysenteriae* and *S. boydii*. Shigellosis continues to be a major health problem worldwide, causing an estimated 1 million death and 165 million cases annually [4]. Beside these, it is reported that shigella causes approximately 580,000 cases of bacillary dysentery annually among travelers and military personnel from industrialized countries [5]. Individuals with HIV/AIDS are reported to be more frequently infected with *Shigella spp* [6]. To further compound the situation, the causative agents are reported to be resistant to commonly available antimicrobial drugs which poses an increasing problem [2]. Sequel to these problems, this work was designed to investigate the probiotic effect of *Lactobacillus casei* isolated from a local beverage, *kunun zaki*, in the treatment of dysentery induced in rats by *Shigella dysenteriae*. We had reported the probiotic activity of *L. casei* from human breast milk [7]. Probiotics which are live microorganisms that when administered in adequate amounts confer health benefits on the host [8], are available in fermented diary products,

other probiotic fortified foods, tablets, capsules, powders and sachets. Some common examples of Lactic acid bacteria (LAB) being used as probiotics are *Bifidobacterium infantis*, *Lactococcus lactis*, *Bacillus coagulans*, *Lactobacillus reuteri*, *Lactobacillus acidophilus* and *Lactobacillus casei*. In this work, the probiotic potentials of *Lactobacillus casei* was demonstrated against *Shigella dysenteriae* *in vivo*.

## **MATERIALS AND METHODS**

### **General Asepsis**

All methods including media preparation and sample collection were carried out under aseptic conditions. Workbench was made aseptic by cleaning with sterilizing reagents, flaming the environment via a lit gas burner.

### **Sample Collection**

*Kunun zaki* samples used for this research were collected in refreshment spots on the University of Jos campus. Clinical strain of *Shigella dysenteriae* was obtained from Mr. Felix of the Department of Microbiology, University of Jos. Four weeks old albino rats (*Rattus norvegicus*) were obtained at the Department of Physiology, University of Jos, Nigeria. The rats were placed on commercial feed (growers mash) for a week to acclimatize. At the end of this period, the rats weighed between 87.5g and 97.5g.

### **In vivo Feeding**

The rats were divided into 4 groups labeled thus; the negative control (NC), positive control (PC) group, treatment groups TG<sub>1</sub> and TG<sub>2</sub>. Groups TG<sub>1</sub>, and TG<sub>2</sub> were orogastrically dosed with 0.4ml 2.0 x 10<sup>10</sup> CFU/ml and 0.4ml 4.0 x 10<sup>10</sup> CFU/ml of *L. casei* respectively followed by infection with 0.4ml 1.0x10<sup>5</sup>CFU/ml of *Shigella dysenteriae* while the PC group received only 0.4ml 1x10<sup>5</sup> CFU/ml *Shigella dysenteriae*. The treatment was done once per day for 2 days. The rats were further fed with their normal feed for 7 days post treatment. The initial and final weights of the rats were recorded and the data gathered were used to calculate final weight gained.

### **Colony Morphology and Microscopy**

The method adopted was as described [2]. Briefly, growths on culture plates were purified (to obtain single distinct colonies per plate) via sub culturing a distinct colony on MRS Agar. Pure colonies were given isolate Code numbers and observed macroscopically noting the colony shape, color elevation, margin and size. The cultures were smeared and gram stained. Under the microscope the gram reaction and morphology of each isolate was noted.

### **Motility Test**

5ml of peptone water was inoculated with the test organism and incubated at 37° C for 24 hours to obtain bacterial isolate. A drop of peptone water containing the organism was placed on sterile slide, covered with cover slips and observed microscopically using the low magnification objective. This was carried out to determine the presence of motile and non-motile isolates.

**Determination of Cell Concentration (CFU/ml) of Administered isolates.**

The bacterial cell count was done as described [9]. Briefly, one milliliter of each bacterial sample were sub cultured in peptone water and made in single fold dilutions using sterile distilled water in sterile test up to the 7<sup>th</sup> (for *L.casei*) and 4<sup>th</sup> (for *S. dysenteriae*) dilution. 1ml of the diluted sample was pipetted into sterile petri dishes before the addition of 20 – 25ml of molten agar. Each was allowed to solidify after gentle swirling to enhance proper mixing of the inoculum in the agar and finally incubated under suitable conditions. The number of viable cells in colony forming unit per milliliter was determined using the equation:

$$\text{CFU/ml} = \text{dilution factor} \times \text{volume of dilution factor used} \times \text{total number of colonies.}$$

**Biochemical Tests**

The isolates obtained were then subjected to biochemical tests which comprises of the various tests needed to identify each of the bacterial isolate up to their suspected levels. These tests exploit the ways different bacteria species react to the presence of certain chemicals. Such tests include catalase, oxidase and sugar fermentation.

**Catalase Test**

The catalase enzyme acts as a catalyst in the breakdown of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) to oxygen and water. The test was carried out based on the method described by Cheesbrough [2]. Hydrogen peroxide was placed on a slide and with the aid of a sterile wire loop a colony was emulsified and placed on the slide containing the Hydrogen peroxide. Effervescence is indicative of a positive reaction.

**Oxidase Test**

This test depends on the presence of oxidase in bacterial system which catalyzes the transport of electrons between an electron donor in the bacteria and a redox dye. This was tested using the method of Collee *et al.*, [10]. Colonies of the organism were picked with a wooden stick and dabbed on a wet strip of filter paper which had been soaked with oxidase reagent. The appearance of a purple color after about 10 seconds indicates a positive oxidase reaction.

**Sugar Fermentation Tests**

The sugar fermentation test was done as described by Seeley and Vandemark [11]. Eight different broths were made of 8 different sugars (Manitol, glucose, fructose, xykiose, galactose, sucrose, and lactose), in peptone water and inoculated with colonies of the isolates. Durham tubes then were inverted into the reaction tubes for gas production. These tubes were racked and incubated at 37°C for 48 hours. A positive result was observed for turbidity and air trapped in the Durham tubes.

**Determination of Anti-shigellosis Effects of *Lactobacillus casei***

After the foregoing treatment, the experimental rats were under constant observation for any of the signs and symptoms associated with bacillary dysentery based on the method described [2] as follows:

### **Fecal Characteristics**

Fecal characteristic such as color and texture were observed in the feces of rats on the different treatments. The fecal samples were collected prior to ingestion (day 0) and up to 5 days after ingestion.

### **Rectal Temperature**

Rectal temperatures of the rats were taken in the morning for sign of fever using a digital thermometer, which was inserted into the anus after it was sterilized. The thermometer was held in the anus for 1 – 2 minutes before readings were taken.

### **Feed Consumed**

The amount of feed consumed by the rats in each experimental case was obtained by weighing the amount of feed given in the morning and the amount of feed left the following morning.

### **Auxiliary Observatory Parameters**

These parameters include activity or agility of the rats and optical characteristics as these were pointers to ill health. A decrease in activity was reflected by lack of motion, sluggishness and the dullness of the eyes may result from abnormal conditions or pain.

### **Liver Function Tests**

Liver function test was carried out in order to check for any toxicological effects the administered test organism might have had on the experimental animals. The method involves measurement of enzymes (which include Aspartate amino transferase, Alanine amino transferase and alkaline phosphatase) that are indicators for damage to the liver of the experimental animals. The test parameters carried out were; albumen, bilirubin and total protein. Each parameter was tested for using the standard procedures of sample preparation and the absorbance read.

## **RESULTS**

At the end of the research carried out, lactic acid bacteria were confirmed to be associated with the local beverage, *kunun-zaki*. Table 1 shows the colony morphology and the microscopic feature of some of the isolates obtained from culture on Mann Rogosa and Sharp (MRS) Agar.

**Table 1: Colony Morphology and Microscopy of microbial Isolates from *kunun zaki* sample using MRS Agar.**

Isolate Code	Size	Color	Elevation	Margin	Gram Reaction	Shape	Arrangement	Sport	Motility	Spores	Inference
KP2c <sub>1</sub>	M	Round	White	Raised	Smooth	+	Ovoid	Single Rods	-	-	Lactobacillus
KP2c <sub>2</sub>	S	Round	White	Raised	Smooth	+	Thin Rods	Single	-	-	Lactobacillus Species
KP1c <sub>1</sub>	S	Round	Creamy White	Raised	Smooth	+	Large Rods	Single	-	-	Lactobacillus Species
KP4c <sub>3</sub>	S	Round	Creamy White	Raised	Crenate	+	Large Rods	Single	-	-	Lactobacillus Species
KP4c <sub>1</sub>	S	Flat	Creamy White	Slightly Raised	Smooth	+	Thin rods	Single	-	-	Lactobacillus Species
KP2c <sub>3</sub>	L	Round	White	Raised	Smooth	+	Thin Rods	Single	-	-	Lactobacillus Species

S- Small; M – medium; L – large

Table 2 shows the results of the biochemical analyses carried out to help further characterize the isolates. The biochemical features which became a basis for identifying *L. casei* was their inability to ferment xylose as well as none gas formation with glucose.

**Table 2: Biochemical Test on Some of the Isolates from *Kunun zaki***

Isolate	Catalase	Oxidase	Manitol	Manose	Fructose	Xylose	Galactose	Sucrose	Lactose	Glucose
KP2c <sub>1</sub>	-	-	+	+	+	-	+	+	+	+
KP2c <sub>2</sub>	-	-	+	+	+	-	+	+	+	-
KP4c <sub>1</sub>	-	-	+	+	+	-	+	+	+	+
KP2c <sub>3</sub>	-	-	+	+	+	-	+	+	+	+
KP1c <sub>1</sub>	-	-	+	+	+	-	+	+	+	+
KP4c <sub>3</sub>	-	-	+	+	+	-	+	+	+	+

The result of the clinical trial undertaken using experimental rats as subjects to assess the probiotic activity of *Lactobacillus casei* isolated from the local beverage was described on Table 3

**Table 3: Determination of Anti-Shigellosis Effect of *Lactobacillus casei***

Treatment Group	Rectal Temperature(°C)	Fecal Characteristics	Feed Consumed (g)	Inference
NC	36.2 ± 1.81	Brown soft pellets	14.7± 1.04	No sign of shigellosis
PC	41.8 ± 1.92	Light brown and loose	12.4 ± 2.20	Signs of shigellosis
TG <sub>1</sub>	36.2 ± 1.62	Brown soft pellets	14.2± 1.24	No signs of shigellosis
TG <sub>2</sub>	35.8 ± 2.80	Brown soft pellets	14.6±1.29	No signs of shigellosis

Table 4 reveals the levels of serum enzymes as well as levels of albumin, total protein and bilirubin in the experimental rats.

**Table 4: Liver function tests on experimental animals**

Treatment Group	Total protein (g/L)	Albumin (g/L)	Bilirubin ( $\mu\text{mol/L}$ )	Alkaline phosphatase ( $\mu\text{mol/L}$ )	Aspartate Aminotransferase ( $\mu\text{mol/L}$ )	Alanine amino transferase ( $\mu\text{mol/L}$ )
NC	36.40 $\pm$ 0.65	17.90 $\pm$ 0.14	2.72 $\pm$ 0.14	40.85 $\pm$ 0.21	48.00 $\pm$ 0.49	17.5 $\pm$ 0.19
PC	34.20 $\pm$ 0.66	15.30 $\pm$ 0.85	8.18 $\pm$ 0.70	80.00 $\pm$ 1.00	50.50 $\pm$ 1.3	9.0 $\pm$ 0.65
TG <sub>1</sub>	35.92 $\pm$ 0.09	17.30 $\pm$ 0.55	2.72 $\pm$ 0.16	39.95 $\pm$ 0.07	47.50 $\pm$ 0.48	16.0 $\pm$ 0.28
TG <sub>2</sub>	35.60 $\pm$ 0.32	18.65 $\pm$ 0.29	3.00 $\pm$ 0.04	40.40 $\pm$ 0.80	44.70 $\pm$ 1.18	17.0 $\pm$ 0.28

## DISCUSSION

The results from the macroscopic and microscopic analyses of colonies that grew in the culture plates (Table 1) and those obtained from Biochemical Tests (Table 2) were carried out in order to identify *Lactobacillus casei* among other bacterial species isolated from *kunun-zaki*. *L. casei* was characterized by its ability to ferment all sugars except xylose and also does not produce gas from glucose [12]. The result on Table 3 showed that the rectal temperature in the positive control group (infected with pathogenic *Shigella dysenteriae*) was higher (41.8°C), than those of the negative control group and tests groups, TG1 and TG2. Additionally, the fecal characteristics for the positive control was different (light brown and loose) from the rest experimental groups. These were signs of dysentery, unlike that in the positive control group. This was possibly so because of the protective mechanisms by *L. casei* such as; competitive inhibition, the generation of a non conducive acidic environment and the production of antibiotic-like substances (bacteriocins) as reported by Holzapfel and Wood [4]. The results of Table 4 showed that the serum ALP in the positive control group, that were experimentally infected with clinical strain of *Shigella dysenteriae* was higher (80.8iU/L) and significantly different ( $p < 0.05$ ) from the negative control group that were not given any treatment and also the test groups TG1 and TG2 (39.95 iU/L and 40.6iU/L that were orogastrically dosed with their respective concentrations of the test *Lactobacillus casei* in addition to the pathogenic *Shigella dysenteriae*. Serum ALP levels are known to be elevated in diseases of the bone, placenta and intestine [13, 14, 15]. Serum AST was also higher in the positive control (50.5 iU/L group but, it was not significantly different ( $p < 0.05$ ) from the rest treatment groups. AST is an enzyme that increases in activity in diseases such as severe bacterial infection, malaria, pneumonia, pulmonary infants and tumors to organs such as heart and muscle [16,17].

Serum ALT in the treatment groups was not significantly different ( $p < 0.05$ ) from the negative control which had the highest value of 17.5 iU/L. ALT level is predominantly a sign of liver damage [18,19]. Similarly the serum levels of Albumin, total protein and direct bilirubin in the treatment groups were not significantly different ( $p < 0.05$ ) from the negative control group. Although group TG2 had the highest value for albumin (18.68g/dl), it showed a lower value

for total protein (35.6g/dl) when compared with group TG1 (35.92g/dl). The positive control group had the highest value of 8.18mg/dl for direct bilirubin, which is indicative of hepatobiliary obstruction[20]. These results show that *Lactobacillus casei* had probiotic effect on the experimental rats at high concentration.

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