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Treatment of Diarrhoea in Humans Using *Lactobacillus salivarius* PROBIOTIC Isolated from Human Saliva

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Abstract

In this study, *Lactobacillus salivarius* was isolated from human saliva by culture technique using MRS medium, identified through relevant biochemical protocols, and compared to a known control of *Lactobacillus acidophilus* sourced from Puritan Pride Inc., Qadelade, New York, USA. Primary isolation of microorganisms of interest was carried out at Cheznik Diagnostic and Research laboratories while animal preparation and treatment were carried out at Animal Farm, Mgbakwu Awka. Twenty wistar rats were used as the test animals and another twenty for positive control. The animals in the test group were induced to diarrhea using 1 ml of castor oil following 12-hour fasting while the control group was fed with daily feed and distilled water. The test animals upon production of diarrhoeic stool were fed with 1×10^9 cfu/ml (410 mg/dl w/v) of the *Lactobacillus sp* isolate in distilled water. The diarrhoea was resolved within 24 hours of treatment. The average weights of the animals taken just after preparation and early treatment (T_x) showed appreciable loss in weight among the test animals possibly due to stress compared to initial weight at T_0. However, upon continued treatment with the isolate, obvious weight gain (Tf) compared to T_x was observed. The results of the haematological data at the final analysis showed proximal values and consistency to that of the control group following administration of the *Lactobacillus sp* isolate. The routine general health data of the test animals showed marked improvement upon treatment with the isolate. A test of significance at 0.05 showed a positive agreement ($p < 0.05$). The isolated *Lactobacillus sp* showed marked sensitivity to erythromycin, ceftazidine, and chlorompheni-

col antimicrobial drugs just like other known probiotic strains. No known health hazard or death was recorded. From the study, it was observed that saliva can also proffer a good site in addition to the gut and ileum for the isolation of probiotic microorganisms. It has also shown that the presumptive *Lactobacillus salivarius*, though strain-specific, possesses probiotic properties effective in resolving most gut issues but especially in diarrhoeic cases.

Keywords

Probiotics, *Lactobacillus salivarius*, MRS Medium, Wistar Rats, Diarrhoea

1. Introduction

In recent years, the incorporation of probiotic bacteria into foods has received increased scientific interest for health promotion and disease prevention. This has attracted much interest amongst customers and the Food Industry as more probiotic bacteria are now incorporated into foods. The concept of probiotics is very ancient. The first recorded probiotic was fermented milk for human consumption. After that, probiotics became popular in animal nutrition [1].

According to FAO/WHO, probiotics are live microorganisms which when administered in adequate amounts confer a health benefit on the host. There is a task however underlining this process and this is that generally as safe (GRAS) status of new organisms with no previous history has to be confirmed by safe studies using target animals prior to being incorporated into feed products [2]. There is no general guideline for the safety assessment of a novel probiotic strain at this stage and the type of tests that should be included has warranted much controversy More recent studies have promoted probiotic specific safety evaluation criteria, especially the infectivity, metabolic activity and immune functions of a probiotic strain [3]. Probiotics are commonly consumed as part of fermented foods. Examples are yoghurt, pickles, sauerkraut, kenkey, tempeh, cheese, palm wine, etc [4].

Probiotics can also be administered as dietary supplements. They are thought to be beneficial to the host by maintaining the intestinal microbial balance, inhibiting pathogens and toxins producing tissues and cells as well as improving blood circulation [5] as is the case with spirulina. Recent evidence suggests that probiotic effects are strain-specific which means a beneficial effect produced by one strain cannot be assumed to be provided by another strain, even when they belong to the same species [6]. Other aspects of health in which probiotics are useful include; treatment of Crohn's disease, prevention and treatment of diarrhea, prevention and treatment of lactose intolerance, lowering of serum cholesterol, improvement of immune function, prevention of colon cancer, reducing incidences of yeast infections and vaginitis.

It must be able to colonize the mucosal cells of the intestine or gut. Must tolerate the low pH of the gastric and pancreatic fluids. Furthermore, it must dem-

onstrate good inhibitory properties against some selected potential harmful microorganisms. Finally, it must have good sensory properties and be isolated from the same species [6] [7].

Probiotics can be in powdered form, liquid form, gel, paste, granules or available in the form of capsules, sachets, etc. Probiotics can be bacteria, moulds, yeast. But most probiotics are bacteria mainly lactic acid bacteria. Few are from the yeast and fungi family especially *Saccharomyces sp* and *Aspergillus sp* respectively. A probiotic may be made out of a single bacterial strain or it may be a consortium as well. e.g. LB17 "live" probiotic contains 17 strains of lactic bacteria (10 lactobacillus + 2 bifidobacterium), digestive enzymes, amino acids, vitamins and minerals.

Many of the above mentioned non-pathogenic microorganisms have been studied extensively in the recent past for their respective probiotic effects on human population. Some are isolated from the gut e.g. *Lactobacillus acidophilus*, *L. casei*, *L. plantarum* (first isolated from saliva), have been shown to have a potential probiotic effect. As part of the on-going studies, this study undertakes to evaluate the treatment of antibiotic-induced diarrhea in Wistar rats (*Rattus rattus*) using probiotic *Lactobacillus salivarius* isolated from human saliva.

1.1. Statement of the Problem

Chemotherapies and synthetic antimicrobic drugs until now are used as prophylaxis and therapeutics to combat certain health disorders and infectious ailments. Most of these are not without some adverse affects especially among the chemotherapies. The mortality rate is on the increase despite the prevalence of various kinds of these drugs with the average life span of an African drastically reduced to 45.9 years [8]. Again, multiple resistances to these drugs by microorganisms are on the increase. Some antimicrobial drugs induce diarrhoea in some patients being treated with them. It becomes necessary therefore, to explore bio-natural alternatives with good therapeutic and prophylactic values which are not subject to microbial resistance and with highly minimal or no adverse effect. And which specifically can be used to prevent diarrhoea. To this effect, a lot of non-pathogenic microorganisms (especially LAB) have indeed proven to be useful and capable of occupying this niche.

1.2. Objectives

This study intends to diversify the population of existing probiotic agents as well as explore other sites in the body system other than the gut and ileum for possible isolation of probiotic bacteria. Specifically, to evaluate the probiotic effect of the *Lactobacillus salivarius* cultivated from human saliva with respect to the treatment of diarrhoea in a rat model regarding:

- Measurement of general health;
- Heamatological analysis;
- Bacterial translocation.

2. Materials and Methods

2.1. Materials

2.1.1. Microorganisms

Lactobacillus species from the oral cavity (mouth lining, tongue and teeth) with potential probiotic effect include *Lactobacillus plantarum* and *Lactobacillus salivarius*. Positive Control Microorganisms (*Lactobacillus acidophilus*) were sourced from Puritan Pride Inc., Qadelade, New York, USA.

2.1.2. Media

Media used include De Mann Rogosa and Sharpe (MRS) broth and agar, peptone broth, and tomatoes juice agar (**Table A2**).

2.1.3. Animals

In this study, 40 (8-wk old) Wister rats (*Ratus ratus*) bred at the Animal farm Mgbakwu Awka were chosen. Animals were divided at random into two groups (n: 20), one for each treatment. Animals were housed at a controlled temperature range of about 24˚C ± 2˚C, Relative humidity range of about 55% ± 10%, Air changes per hour 10 - 15 ACH, Light: dark cycle 12:12 hours. The rats were sensitive to noise so the noise level was maintained at less than 85 db. They were offered a commercial diet (Vital feed) containing adequate nutrition. Feed was provided *ad libitum* in stainless steel wire- bar lid feeders. The chlorinated Water was provided as drinking water (chlorine concentration 10 - 12 ppm) which was free from *Pseudomonas aeruginosa*. Beddings were made of hard wood covered with saw dust which was evacuated and replaced at interval. The rats were housed in a stainless steel cage with stainless wire. The size of the cage was 12 × 11 × 7.5 as described by the National Institute of Health Guide for care and use of laboratory animals, USA [9].

2.2. Experimental Design

Double blinded experimental design; an experimental procedure in which neither the subjects of the experiment nor the persons administering the experiment know the critical aspects of the experiment used to guide against experimenter bias and placebo, was employed including two treatments [10].

2.3. Method

2.3.1. Sample Collection

Samples of saliva were collected aseptically from 10 different individuals and collected into a pool using the standard universal container.

2.3.2. Cultural Technique

According to available research [11] [12], the samples collected were plated (spread plate method) on the De Mann Rogosa and Sharpe (MRS) agar as well as tomato juice agar [13]. Control *Lactobacillus salivarius* was also plated. The plates were incubated at 39˚C for 24 - 48 h at microaerophilic temperature and CO_2-enriched environment. Colonies showing characteristic morphology were

subcultured into sterile peptone broth. This was followed by identification protocols e.g. morphological appearance, gram staining, biochemical characteristics, etc against both the pure isolates and positive control strain of *Lactobacillus salivarius*.

2.3.3. Carbohydrate Fermentation

This was used to test the ability of the isolates to utilize simple sugar like maltose as a carbon source. Triple Sugar Iron agar (TSI) containing maltose was poured into a bijou bottle and allowed to be set in a slant. Suspected Lb specie isolated from the MRS agar and incubated in a sterile peptone broth was used to inoculate the slant and incubated for 24 - 48 hrs. The presence of yellow coloration confirmed positive test [14].

2.4. Molecular Identification Assay

Isolates were identified based on their 16S rRNA gene sequences. Amplification and Sequencing of 16S rRNA gene were performed at Macrogen Inc. U.S.A.

2.5. Animal Preparation

The test animals for probiotic treatment were subjected to at least 12 hours of fasting and later induced to diarrheic condition using 1 ml castor oil administered orally/animal.

2.6. Probiotic Treatment

Animals were daily fed the 10^9 cfu//ml or (410 mg/100ml) of *lactobacillus sp* suspension in sterile distilled water plus a commercial diet for 30 days (**Table A1**).

2.7. Non Probiotic Treatment

Animals were fed daily with sterile distilled water plus a commercial diet for 30 days. New feed stock of the *Lactobacillus sp* was generated each day in peptone broth.

At the expiration of the 30 days of treatment, animals were anaesthetized humanely by an overdose of chloroform for blood collection, to determine bacterial translocation examination. Hematological analyses were performed before (zero time) and after 30 days of feeding treatment while the vital signs of the animals were routinely monitored.

2.8. Total Leukocyte (WBC) Count

Using a clean Pasteur pipette, 0.38 ml of Turk's fluid (WBC dilution fluid) was transferred into a clean test tube. 0.02 ml of the blood aliquot was added to the test tube and mixed properly to make 1:20 dilution. Neubaeur counting chamber was filled with the sample mixture and counted under the microscope using a low power objective (×10 objective) [15].

2.9. Differential Leukocyte Count

A small amount of the blood aliquot was dropped 1 cm from the end of a clean slide.

Using a spreader (slide with a smooth edge) the drop was spread up to two third of the length to make a thin film. The blood film was allowed to dry and stained with diluted Geimsa (1:40) stain. After drying, the film was covered with immersion oil and viewed using oil immersion objective (×100 objective). The different leukocytes were counted using a differential counting machine or tally system [13] [15].

2.10. Packed Cell Volume (Haematocrit) Test

The haematocrit test is a procedure that determines the percentage of red blood cells (RBCs) in whole blood. Usually employed to check anaemia. The blood sample aliquot was used to fill heparinised capillary tubes. One end of each capillary tube was sealed. The tubes were spurned in haematocrit centrifuge at 12,000 r.p.m for 5 minutes, after which the capillary tubes were read on a haemotocrit reader to measure the packed cells over the plasma column [12] [13] [14].

2.11. Measurement of General Health (Vital Signs)

The general health appearance of the animals was daily monitored using a scoring system of 1:5 feed intakes and body weights were recorded once a week. The occurrence of diarrhea and vomiting was monitored daily. Other vital signs monitored were bright eyed alert, smooth coat with sheen, hyperventilating, non reactive to stimulus, and cold paws [9].

2.12. Statistical Analysis

This was carried out by analysis of data using Pearson Product Moment and Spearman coefficient to determine the significant difference ($p < 0.05$) between the data sets of the experimental treatment; used to state the reliability indices employed, how the data were correlated to arrive at specific results, Test-retest reliability and consistent conclusion [16].

3. Results

3.1. Morphology/Biochemical Reactions of the Isolates

Three growths "X", "Y", "Z" were observed on MRS plate while two, "X" and "Z" were on tomato juice agar. "X" was observed to be whitish in color, smooth but biconcave edges, and less than 1 mm. "Y" was whitish in color, smooth with round edges, and less than 1 mm in size. "Z" was creamy, larger in size (up to 3 mm) and mucoidal (**Table 1**). The growth was observed to be more on MRS agar than on Tomato juice. While the control (X) was found to be catalase (−), "Y" and "Z" were found to be catalase (−) and (+) respectively. By this result, "Z" was presumptly excluded from likelihood of being *Lactobacillus sp*.

The control Lactobacillus "X" was found to be gram (+) short rods, while "Z" was found to be gram (+) large oval shapes with buddings typical of yeast (possibly not lactobacilli). "Y" on the other hand was found to be gram (+) or (−) rods (**Table 1**).

Table 1. Result of morphology/biochemical reactions of the Isolates.

Isolate	Morphology	Catalase test	Gram reaction	Carbohydrate utilization
X	Bi-concave edge, yellow colonies	(−)	G (+) short rod/coccobacillus	(+)
Y	Smooth-edge white colonies	(−)	G (+) rod with variable staining	(+)
Z	Muffy-edge white colonies	(+)	G (+) oval shape with buddings	(+)

While control Lactobacillus "X" and "Y" were able to utilize maltose sugar incorporated in a peptone agar slant contained in a bijou bottle by giving off yellow slant, 'Z" was also observed to have exhibited a (+) utilization test to maltose sugar test (Table 1).

3.2. Antimicrobial Sensitivity Test

The control Lactobacillus "X" and presumptive Lactobacillus isolate "Y" showed marked sensitivity to chloramphenicol, gentamycin, ceftazidine, and erythromycin. A summary of the result is contained in Table 2 below.

3.3. Result of Average Weight of Animals

Weight of the individual experimental animal at zero time, during diarrhea stooling, and after treatment with presumptive *Lactobacillus sp* were summarized in Table 3 and graphically represented in Figure 1. While the control animals maintained steady weight, the test animals expectedly showed loss of weight before and beginning of treatment owing to the induced stress. However, the weights were regained with continued administration of the *Lactobacillus* isolate.

3.4. Result of General Health Routine Monitoring

The average weight and other vital signs monitored weekly and daily from the animal groups are presented in Table 4 using the 1:5 score system. (cf Figure A1). The statistical result using the Pearson package shows a significant difference as in Table A3.

3.5. Haematological Analysis Result

The results of average total white blood cell, differential, and haematocrit analysis on the test animal blood samples during the diarrhoeic and probiotic treatment stages were presented in Table 5. The packed cell volume (PCV) values show a marked increase during diarrheic period due to loss of fluid, As well white blood cell (WBC) and Differential counts both showed marked increase in leucocytes especially neutrophil (Table 5). Test of significance using Spearman statistical package at the degree of freedom (D/F) = 5 confirms the significance (Table A4).

Table 2. Result of antibiotic sensitivity test.

Isolates	Chloramphenicol	Gentamycin	Erythromycin	Ampicillin	Ceftazidine
X	S	S	S	V	S
Y	S	V	S	R	S
Z	R	R	R	R	R

S = Sensitive, R = Resistance, V = Variable reactions.

Table 3. Average weight of animals at various stages of the experiment.

SN	Description	Average weight (g) W_0	W_x	W_f	SN	Description	Average weight (g) W_0	W_x	W_f
	Blue head	114	**106**	**116**		Black fore leg (left)	**115**	**110**	**118**
	Blue tail	148	**141**	**151**		Black hind red (right)*	**117**	**118**	**120**
	Blue ear	130	**121**	**132**		**Black jaw ***	**125**	**126**	**128**
	Blue fore leg (left)	125	**118**	**122**		**Green head**	141	**136**	**140**
	Blue fore leg (right	120	**111**	**119**		**Green tail***	118	**119**	**123**
	Blue hind leg (right)*	129	**130**	**134**		**Green ear**	123	**118**	**125**
	Blue hind leg (left)	**109**	**111**	**115**		**Green fore leg (left)**	**108**	**109**	**113**
	Yellow head*	**124**	**126**	**131**		**Green fore leg (right)***	128	**129**	**133**
	Red tail*	**134**	**135**	**137**		**Green hind (left)**	135	**127**	**133**
	Red ear	119	**109**	**117**		**Green jaw***	120		
	Red fore leg*	121	**123**	**126**		**Red head***	123	**124**	**130**
	Red fore leg (left)	140	**132**	**141**		**Yellow tail***	106	**108**	**111**
	Red hind leg (right)*	112l	**113**	**117**		**Yellow ear**	144	**138**	**147**
	Red hind leg (left)	108	**102**	**112**		**Yellow for hand**	122	**117**	**126**
	Black head*	115	**117**	**121**		**Yellow fore hand (left)**	128	**130**	**133**
	Black tail	119	**110**	**121**		**Yellow hind leg left**	113	**107**	**123**
	Black ear*	131	**133**	**137**		**Yellow hind leg (right)**	118	**119**	**125**
	Black fore leg (right)	125	**116**	**128**		**Yellow jaw***	127	**122**	**134**
	Brown head*	107	**111**	**118**		**Brown jaw***	128	**119**	**130**
	Brown tail	119	**113**	**131**		**Purple head***	112	**114**	**120**

Key: * = Control group, T_0 = Weight at zero time (before treatment), Tx = Av. Weight during Treatment, T_f = Av. Weight at the end of experiment.

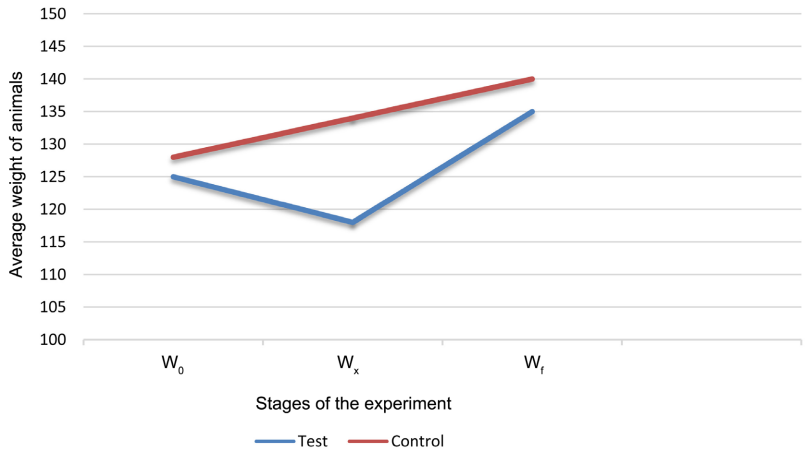

Figure 1. Graphical representation of the average weights of test animals in comparison with the control group.

Table 4. Average results of routine vital signs monitoring.

Week	Feed intake	Bright eye alert	Cold paw	Smooth coat	Hyper ventilating	Diarrhoea
Week 0						
Group 1	5	5	5	5	5	5
Griup2	5	5	5	5	5	5
1st - 2nd week						
Group1	5	5	5	5	5	5
Group2	3	2	2	3	2	2
3rd - 4th week						
Group1	5	5	5	5	5	5
Group2	5	5	4	4	5	4

Key: 1 = Very poor health, 2 = poor health 3 = Less healthy, 4 = healthy, 5 = Very healthy; Group 1 = Control; Group 2 = Test.

Table 5. Average result of haematological analysis on the test animals.

Time	Total white blood cell count ($\times 10^9$)	Differential white blood cell count ($\times 10^9$) Neut rophil	Lympho cyte	Mono cyte	eosino phyll	baso phyll	PCV (%)
Before antibiotic inducement	7.0	1.8	4.2	0.01	0.01	0.00	37
Diarrhoea stage	9.6	3.5	5.0	0.02	0.03	0.00	45
Post probiotic treatment	6.8	2.0	4.5	0.01	0.03	0.00	39

3.6. Molecular Analysis of Isolates

The analysis of 16S rRNA of the isolates shows a regular pattern as shown in Figure 2. Agarose electrophoretic separation of the nucleotides shows bands arrangement typical of *Lactobacillus sarivarius*.

4. Discussion

Probiotic microorganisms can be cultivated from other sites of the body other than the gut and ileum as shown by this study. They can be added to populate the colon and their amazing influence in improving the digestive health and overall wellness of the body by adhesion to human enterocyte-like Caco-2 cells is well noted [17] [18] [19].

Antibiotic Associated Diarrhoea (AAD) results from an imbalance in the colonic microbiota caused by antibiotic therapy. In this study, 0.41 g/ml (410 mg/ml (w/v)) of the *Lactobacillus salivarius* isolated from saliva, approximately 1×10^9 cfu/ml introduced into the drink of the animals prevented antibiotic-induced diarrhoea in Wistar rats (8 wks old).

From the result data (Table 3), Test animals lost appreciable weight within the first few days of treatment compared to weight at zero time, possibly due to induced stress (during fasting, and induced diarrhoea). However, with continuous feeding and treatment with the probiotic solution in distilled water, the Test animals began to regain weight gradually. This is possible as the probiotics are also regarded as Single cell protein; a good source of enriching diet that can enhance growth in addition to repopulating the gut's beneficiary bacteria [20].

The average data on routine health monitoring (Table 4) taken at zero time (T) showed that both groups were healthy and agile at the beginning of the experiment. As the treatment began; that is both negative and positive treatments on the Test group (1 - 2nd week), the Test group showed a marked decline in health including diarrhea as reflected by average values obtained (Figure A1), while the

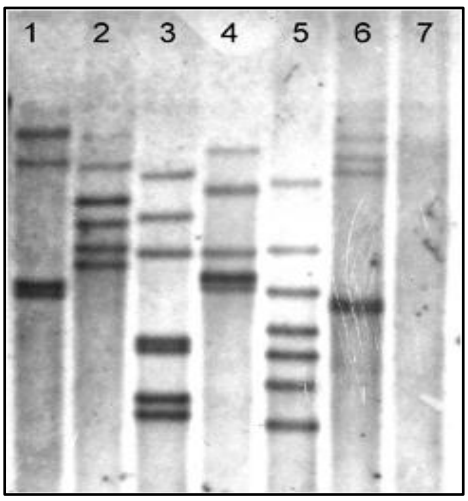

Figure 2. Probe hybridization of 16S rRNA gene copy number in probiotic *Lactobacillus salivarius* isolated from the human saliva.

control group remained stable. However, within the 3rd - 4th week, with adequate ingestion of the probiotic treatment by the Test group, the diarrhea was completely prevented and vital signs returned to normal health compared to the control group. A test of significance at 0.05 showed a positive agreement ($p > 0.05$).

As seen from the result data (**Table 5**), the average values of the full blood count – total white blood cell, differential count, and Packed Cell Volume obtained at zero time (T_0) were compared to the ones generated after treatment with probiotic lactobacillus (Y), although there were alterations in the values during the 1st two weeks with an increase in both WBC (leucocytosis) the significance test at 0.05 also show positive agreement ($p > 0.05$) (see **Table A4**), showing the important role probiotics play in maintaining the balanced immune system and normalizing altered immune function due to invasive bacteria or even toxins. Scientific studies [18] [21] confirm this finding.

No health implication or death of the animal was recorded among the test animals following administration of the probiotic *Lactobacillu salivariuss* rather they were observed to be active again and agile. This is supported by the bacterial translocation assay done as separate studies in Mongolia [21] which demonstrated the healthy benefit of *Lactobacillus casei Zhang.* Translocation is a pathogenicity factor of many pathogens in which they have the ability to adhere to the intestinal mucosal epithelium and transported (translocation) to other sites or organs like ileum, heart, liver, pancreas, etc. This was earlier confirmed by [22] in their study of probiotic as a useful agent against diarrhea.

The isolated and purified probiont can be used to enhance animal feed either by incorporation in feeds or administered through water. It also can be employed to improve human nutrition in yoghurt, milk and the likes. The dried concentrate of the probiont can be formulated into tablets (comprimes) or capsule or even in powdered form and administered therapeutically or synergistically with synthetic drugs to treat diarrhoea and other health problems. It is advisable to incorporate prebiotics in preparations of probiotics to help sustain the latter which must be consumed as live microorganisms. Prebiotics as fertilizers are used to enhance the growth of beneficiary microbionts. These are special plant fibres (containing oligofructose and inulin) that cannot be digested by the body.

Pharmaceutical companies and proprietories are invited to participate and invest into this bio therapy field.

The further study still recommended the strain specificity and safety assessment of the isolated *Lactobacillus salivarius* to formally establish the Generally Regarded As Safe (GRAS) status in line with WHO/FAO acceptable guidelines [23].

5. Conclusion

This study has indeed shown that the probiotic *Lactobacillus salivarius* strain

can actually be isolated from saliva and not just the gut alone. This shows the diversification of the sites of isolation of probiotics as well as the varieties and population of good microorganisms available for maintaining a healthy balance in gut flora as well as prophylactic and therapeutic use. Probiotic is indeed a child of necessity that could not have come at a better time in history than now when there is a global challenge of multiple microbial antibiotic resistances to most chemical and synthetic drugs that used to be therapeutically effective. Essentially, these probiotic strains could be developed as independent comprime (tablet) and capsule, or as active ingredients of food products that are ultimately intended for human consumption with prebiotics incorporated into them. Before this, it is advisable to undertake a detailed safety assessment of the probiotic strain both *in vivo* and *in vitro*.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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Appendix

Figure A1. Wistar rats producing diarrheic stool (arrow). (Rats look dull, weak and hyperventilating).

Table A1. Constituent of commercially produced pelletised Grower's feed per 25 kg.

General Ingredients	Specific nutrient requirement/25kg
Cereal/grains	Crude protein (15% min)
Premix (vitamins & minerals)	Fat (7% max)
Salt	Crude fibre (18% max)
Antitoxins	
Enzymes	Metabolisable energy (2550 Kcal/kg. min)
Vegetable proteins	Calcium (1.0% min)
Essential amino acids	Available phosphorus (0.35% min)
Antioxidants	
Prebiotics	

Feed composed by Grand cereals, subsidiary of UAC of Nig plc as Vital feed brand

Table A2. Media composition.

MRS media (g/l) at 25˚C	
Dextrose	20.00
Protease peptone	10.00
Beef extract	10.00
Yeast extract	5.00
Sodium acetate	5.00

Continued

Ammonium citrate	2.00
Dipotassium phosphate	2.00
Tween 80	1.00
HCl	1
Magnesium sulpfate	0.10
Manganese sulphate	0.05
pH	2.5 ± 0.2
Tomatoe Juice Agar (g/l)	
Tomato extract	10.00
Amino acid	1.00
Panthotenic acid	0.05
Tween 80	1.00
Thyamine	0.05
Magnesium sulpfate	0.10
Manganese sulphate	0.05
PH	4.5 ± 0.2
Peptone Broth (g/l)	
Tryptose	20.0
NaCl	1.0
Beef extract	10

Table A3. Statistical analysis of general routine health among test animals.

Sample No	Ave Results (Y)	$d\ \left(Y-\bar{X}\right)$	$d^2\ \left(Y-\bar{X}\right)^2$	SD ($\sqrt{d^2}/n-1$)
1	4	−0.45	0.2025	$\sqrt{d^2}/n-1=\sqrt{4.95}/19=2.22/19$ SD = 0.11 2SD = 2 × 0.11 = 0.22 - OCV = SD/X × 100 = 2.2% Correlation coefficient r = 1 − 0.22/4.95 = 0.955 Test of significance at 0.05: using Pearson Moment correlation $=\frac{\sum d}{\sum d^2}=\frac{0}{4.95}=0$ Calculated value = 0 Critical value = 0.3687 Since 0.3687 > 0, Accept the hypothesis
2	4	−0.45	0.2025	
3	4	−0.45	0.2025	
4	5	0.55	0.3025	
5	5	0.55	0.3025	
6	4	−0.45	0.2025	
7	5	0.55	0.3025	
8	4	−0.45	0.2025	
9	5	0.55	0.3025	
10	4	−0.45	0.2025	
11	5	0.55	0.3025	
12	5	0.55	0.3025	

Continued

13	4	−0.45	0.2025
14	5	0.55	0.3025
15	4	−0.45	0.2025
16	5	0.55	0.3025
17	4	−0.45	0.2025
18	4	−0.45	0.2025
19	4	−0.45	0.2025
20	5	0.55	0.3025
	$\Sigma 89 - X = 4.45$	$\Sigma 0$	$\Sigma 4.95$

Table A4. Statistical analysis of average haematological result among test animals.

X	Y	D $(X-Y)$	D^2 $(X-Y)^2$
7.0	6.8	0.2	0.04
1.8	1.9	−0.1	0.01
4.2	4.5	−0.3	0.09
0.01	0.01	0	0
0.01	0.03	0.02	0.0004
0	0	0	0
37	39	−2	4
$X = 7.15$			$\Sigma 4.14$

Using Spearman statistics

$$\text{Rho} = 1 - \frac{6\sqrt{\sum (x-y)^2}}{N(n-1)}$$

where N = frequency of data

$$= 1 - \frac{12.21}{42} = 1 - 0.290 = 0.71$$

Degree of freedom (DF) = 5

Level of significance = 0.05

Critical value (from table) = 1.000

Calculated value = 0.71

$P < 0.05$, and thus hypothesis accepted.