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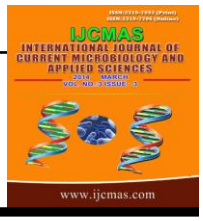

Original Research Article

Isolation and identification of lactic acid bacteria from rhizosphere soils of three fruit trees, fish and ogi

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Keywords

Rhizosphere soils; yellow maize gruel; *Leuconostoc*.

A B S T R A C T

A total number of twenty one (21) bacterial isolates was obtained from rhizosphere soils of mango, banana, and guava trees, gastrointestinal tracts and gills of fish, fish pond sediment and water as well as yellow maize gruel (ogi). Out of these isolates, seventeen (17) were found out to be LAB. Eleven of the LAB isolates were identified to be *Lactobacillus* species, three were *Streptococcus* species, two were *Lactococcus* species and the remaining one was *Leuconostoc* species. . All the LAB isolates possessed the ability to grow at a low pH of 3.0; eight of the isolates grew at 10°C, ten of the isolates grew at 15 °C while five of the isolates grew at 45 °C. Eight of the isolates had the ability to grow at 4% NaCl while only *Lb. casei* isolated from different sources grew at 6.5% NaCl.

Introduction

Lactic acid bacteria (LAB) consist of a number of bacterial genera within the phylum Firmicutes. The genera *Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Lactosphaera, Leuconostoc, Melissococcus, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus* and *Weissella* are recognized as LAB (Jay, 2000; Ercolini *et al*., 2001; Holzapfel *et al*., 2001). Lactic acid bacteria (LAB) have played a long and important role in food technology. The LAB include a wide variety of cell types and physiological and biochemical characteristics. They are often associated with animal oral cavities and intestines e.g. *Enterococcus faecalis* and plant leaves *Lactobacillus, Leuconostoc* (Savadogo *et al*., 2006). They occur naturally in fermented food (Caplice and Fitzgerald, 1999) and have been detected in soil, water, manure and sewage (Holzapfel *et al*., 2001). Lactic acid bacteria are regarded as a major group of probiotic bacteria (Collins *et al*., 1998; Tannock, 1998; Schrezenmeir and de Vrese, 2001). Isolations of LAB, from the products of milk, fermented foods and plants have been frequently reported but studies on the isolation from soil remain scarce (Chen *et al*., 2005), although it is well known that spore-forming LAB exist

in soil (Suzuki and Yamasato 1994; Yanagida *et al.* 1997). Therefore, the present investigation was carried out to compare the LAB isolates from rhizosphere soil with those of fish and its environ together with that of one of the Nigerian fermented maize known as "ogi'.

Materials and Methods

Moist garden soils were collected from fruit trees (mango tree, banana tree and guava tree) at 15cm depth from agricultural farm of Federal University of Technology, Akure (FUTA), Nigeria. Yellow maize gruel "Ogi" (yellow) was purchased from Oba market in Akure while Tilapia fish (*Oreochromis mossambicus*) as well as fish pond sediment, and its water were collected from FUTA fish farm.

Isolation procedure

One gram of soil samples and placed into 9ml of sterile distilled. Nine- fold serial dilutions were then made from the mixed solution and 1ml from the last 3 dilutions (10^{-7}, 10^{-8}, 10^{-9}) were pipetted and plated on de Man Rogosa and Sharpe (MRS) agar plates by pour plate method (Awan and Rahman, 2005). The plates were then incubated at 37^{0}C for 48 hours anaerobically. Morphologically distinct and well isolated colonies were picked and transferred to new MRS agar plates by streaking to obtain pure colonies.

Also, tilapia (*Oreochromis mossambicus)* fish collected from FUTA fish pond was washed with sterile distilled water to remove the unwanted particles. It was then dissected to remove the gastro intestinal tracts; gills and intestine and was homogenized in the same sterile distilled water for centrifugation. After centrifugation, the supernatant was taken and serially diluted until nine-fold. An aliquot (1ml) from last three dilutions (10^{-7}, 10^{-8}, 10^{-9}) were pipetted and plated on de Man Rogosa and Sharpe (MRS) agar plates by pour plate method (Awan and Rahman, 2005).

Characterization and tentative identification of the isolates

Isolates were picked randomly at varying times from the plates and subcultured before being subjected to physiological and biochemical tests (Harrigan and McCance, 1976; Barnett *et al*., 1983; Sneath, 1986). The identification of lactic acid bacteria was carried out on overnight cultures of each isolate in MRS broth (Oxoid). All isolates were initially tested for Gram reaction, catalase enzyme and production of acid from glucose in Hugh and Lelfsons medium by oxidation or fermentation reaction (Harrigan and MacCance, 1976). Only Gram positive bacteria with catalase negative reactions were observed (Garvie, 1986; Kandler and Weiss, 1986; Schillinger and Lücke, 1987) and the representative isolates were purified by successive streaking onto the same agar. The isolates were then identified with reference to Bergey's manual of determinative bacteriology. Identification was based mainly on; gram and spore staining, absence of catalase, cultural and morphological characteristics (such as its elevation, shape, colour and texture of the colonies). Also, growth in 4% and 6% NaCl as well at 10°C, 15°C and 45°C and fermentation of different carbon sources was observed. *Lactobacilli* species were taxonomically classified following the discriminatory schemes of Kandler and Weiss (1986) and (Hammes *et al*. (1992). Isolates which were gram positive and catalase negative were

preserved in MRS broth medium as frozen stocks. This was prepared by mixing 0.5ml active cultures and 0.5ml MRS medium. Cultures were stored in triplicates and labelled according to their various sources of isolation.

Resistance to Low pH

Tolerance of isolated LAB to acidic pH was determined by growing all the isolates in acidic MRS broth. Active cultures were incubated for 24 hours in MRS broth. The cells were harvested by centrifugation, washed and resuspended in MRS broth and was poured in test tubes the pH was adjusted to 3.0 with 1ml HCl and 0.5ml NaOH. A 0.1ml of the broth was serially diluted and 1ml from the last 3 dilutions (10^{-7}, 10^{-8}, 10^{-9}) were pour plated on MRS agar plates. The plates were then incubated at 37^0C for 120 minutes anaerobically. The growth was evaluated by plate count method (Awan and Rahman, 2005). The growth was also monitored at OD620 using a T70 UV: VIS spectrometer.

Results and Discussion

Isolation and identification of lactic acid bacteria from collected samples

A total number of 21 bacterial isolates was isolated from soil samples, gill, intestine, fish pond water, fish pond soil as well as from ogi. Seventeen of the isolates were found out to be LAB while the 4 others isolates were yeasts. Morphological and biochemical details of these LAB isolates are shown in table 1. The LAB isolates were gram positive, catalase negative and non spore forming rods and cocci. Eleven of the LAB isolates were identified to be *Lactobacillus* specie, three were *Streptococcus* species, two were *Lactococcus* species and the remaining one was *Leuconostoc* species.

Resistance of LAB to NaCl and growth at different temperature range

Also, eight of the isolates had the ability to grow at 4% NaCl concentration while only *Lactobacillus casei* isolated from different sources grew at 6.5% NaCl concentration while other isolates could not grow, (Table 2). It was observed that eight of the isolates were able to grow at 10 °C, ten grew at 15 °C while five of the isolates were also able to grow at 45 °C (Table 2).

Screening of LAB isolates for resistance to low pH

Resistance to pH 3 is often used *in vitro* assays to determine the resistance to stomach pH. It was observed from this study that the total number of viable microorganisms enumerated at 3 hours by pour plate techniques and also growth monitored at OD620 that all the isolates possessed the ability to grow at a low pH. However, *Lactobacillus plantarum* isolated from moist guava soil showed the highest number of viable organism on the plate count followed by *Lactobacillus casei* isolated from moist banana soil while the least number of viable organisms on the plate count was observed on *Lactobacillus acidophilus* isolated from fish intestine Results, both cfu (colony forming units) values and OD620 are shown in table 3.

Lactic acid bacteria (LAB) which have played a long and important role in food technology including a wide variety of cell types and physiological and biochemical characteristics and isolation of this organism is required to determine the benefit it possess since it is commonly found in our environment. They are present in the fermented food, not only as visible cells and non-colony forming units,

Table 1: Morphological and biochemical characteristics of LAB isolates

Isolate Code identity	Elevation	Colony texture	Edge	Colony colour	Colony shape	Carbon sources utilization											Probable
						GS	SS	Ct	G	L	F	M	S	A	GI	MI	
MBS	Raised	Shinny surface	Rough	Creamy	Short rods	+	-	-	-	+	+	+	+	-	+	+	*Lactobacillus casei*
MMS	Raised	Shinny mucoid surface	Smooth	Yellowish surround by white	Cocci in chains	+	-	-	+	+	+	+	+	-	+	+	*Streptococcus uberis*
MGS 1	Raised	Shinny mucoid surface	Smooth	Yellowish surround by white	Cocci in chains	+	-	-	+	+	+	+	+	-	+	+	*Streptococcus uberis*
MGS 2	Flat	Coarse surface	Smooth	Whitish	Rods	+	-	-	-	+	+	+	+	+	+	+	*Lactobacillus plantarum*
FPW	Raised	Shinny surface	Smooth	Creamy	Short rods	+	-	-	+	+	+	-	+	+	+	+	*Lactobacillus brevis*
FPS 1	Raised	Shinny surface	Smooth	Yellowish	Short rods	+	-	-	-	+	+	-	+	-	+	+	*Lactococcus lactis*
FPS 2	Flat	Coarse surface	Rough	Creamy	Cocci in chains	+	-	-	+	+	+	-	-	-	+	+	*Streptococcus lactis*
FPS 3	Flat	Coarse surface	Rough	Creamy	Rods	+	-	-	-	+	+	-	+	-	+	+	*Lactobacillus acidophilus*
FPS 4	Raised	Coarse shooting surface	Rough	Creamy	Cocci	+	-	-	+	+	+	-	+	-	+	-	*Leuconostoc citrovorum*
GII 1	Flat	Shinny surface	Rough	Yellowish	Rods	+	-	-	+	+	+	-	+	-	+	+	*Lactobacillus bulgaricus*
GII 2	Flat	Coarse surface	Rough	Creamy	Rods	+	-	-	-	+	+	-	+	-	+	+	*Lactobacillus acidophilus*
GIG 1	Raised	Shinny surface	Smooth	Yellowish	Short rods	+	-	-	-	+	+	-	+	-	+	+	*Lactococcus lactis*
GIG 2	Raised	Shinny surface	Smooth	Creamy	Rods	+	-	-	-	+	+	+	+	+	+	+	*Lactobacillus plantarum*
OGI 1	Flat	Coarse surface	Smooth	Whitish	Rods	+	-	-	+	+	+	-	+	-	+	+	*Lactobacillus fermentum*
OGI 2	Raised	Shinny surface	Smooth	Creamy	Short rods	+	-	-	+	+	+	-	+	+	+	+	*Lactobacillus brevis*
OGI 3	Raised	Shinny surface	Smooth	Creamy	Rods	+	-	-	-	+	+	+	+	+	+	+	*Lactobacillus plantarum*
OGI 4	Raised	Shinny surface	Rough	Creamy	Short rods	+	-	-	-	+	+	+	+	-	+	+	*Lactobacillus casei*

MBS – Moist banana soil; MMS – Moist mango soil; MGS – Moist Guava soil; FPW – Fish Pond Water; FPS – Fish Pond Soil; GII – Fish intestine; GIG – Fish Gills; OGI – Ogi; Gram staining; SS- Spore staining; Ct- Catalase; G- Glucose; L– Lactose; F- fructose; M- Mannitol; S- Sucrose; A- Arabinose; Gl- Galactose; Ml- Maltose; (+)- positive; (-)- negative

Table.2 Growth at different temperature and Resistance to NaCl

Isolate Code	Growth at different temperatures			Growth in NaCl		Probable identity
	10	15	45	4%	6.5%	
MBS	+	+	-	+	+	*Lactobacillus casei*
MMS	+	+	-	+	-	*Streptococcus uberis*
MGS 1	+	+	-	+	-	*Streptococcus uberis*
MGS 2	+	+	-	+	-	*Lactobacillus plantarum*
FPW	-	+	-	-	-	*Lactobacillus brevis*
FPS 1	-	-	+	-	-	*Lactococcus lactis*
FPS 2	+	-	-	+	-	*Streptococcus lactis*
FPS 3	-	-	+	-	-	*Lactobacillus acidophilus*
FPS 4	-	+	-	-	-	*Leuconostoc citrovorum*
GII 1	-	-	-	-	-	*Lactobacillus bulgaricus*
GII 2	-	-	+	-	-	*Lactobacillus acidophilus*
GIG 1	-	-	+	-	-	*Lactococcus lactis*
GIG 2	+	+	-	+	-	*Lactobacillus plantarum*
OGI 1	-	-	+	-	-	*Lactobacillus fermentum*
OGI 2	-	+	-	-	-	*Lactobacillus brevis*
OGI 3	+	+	-	+	-	*Lactobacillus plantarum*
OGI 4	+	+	-	+	+	*Lactobacillus casei*

but also with the primary and secondary metabolites they have produced during the fermentation process (Robinson, 1991). The MRS medium used was selective for the isolation of lactic acid bacteria since they are extremely fastidious. Lindquist (1998) reported that a medium that will support their growth must contain a fermentable carbohydrate and many growth factors. The results of the present investigation have shown that LAB were isolated from soil samples from the rhizosphere of three fruit trees from FUTA, intestine and gill of fish and its environ as well as from ogi. Isolation of different species of LAB obtained from different sources could be related to the different nutritional status of the isolation sites as observed by Chen *et al*. (2005).

Table.3 Total LAB viable count on MRS agar (cfu/ml) at pH 3.0

Isolate code	Probable identity	10^{-7}	10^{-8}	10^{-9}	Growth in pH 3.0
MBS	*Lactobacillus casei*	112	133	154	++
MMS	*Streptococcus uberis*	54	99	129	+
MGS 1	*Streptococcus uberis*	92	148	133	++
MGS 2	*Lactobacillus plantarum*	121	155	172	++
FPW	*Lactobacillus brevis*	84	104	130	+
FPS 1	*Lactococcus lactis*	92	136	104	+
FPS 2	*Streptococcus lactis*	89	127	156	++
FPS 3	*Lactobacillus acidophilus*	141	124	111	++
FPS 4	*Leuconostoc citrovorum*	62	91	139	+
GII 1	*Lactobacillus bulgaricus*	72	111	153	++
GII 2	*Lactobacillus acidophilus*	72	94	122	+
GIG 1	*Lactococcus lactis*	94	120	142	+
GIG 2	*Lactobacillus plantarum*	56	94	121	+
OGI 1	*Lactobacillus fermentum*	83	109	138	++
OGI 2	*Lactobacillus brevis*	86	116	136	++
OGI 3	*Lactobacillus plantarum*	72	99	129	+
OGI 4	*Lactobacillus casei*	79	96	123	+

MBS – Moist banana soil ; MMS – Moist mango soil; MGS – Moist Guava soil ; FPW – Fish Pond Water; FPS – Fish Pond Soil; GII – Fish intestine; GIG – Fish Gills; OGI – Ogi

An explanation of the presence of lactic acid bacteria in the rearing water may be that water supplied in the inlet of the pond contains very little suspended organic matter and oxygen to saturation. It is feasible that they can thrive in the pond which increases their possibility to reach the gastrointestinal tract of the farmed fish because lactic acid bacteria require a very nutritious environment to grow (Kandler and Weiss, 1986), Low numbers of lactic acid bacteria (100 to 10^3 CFU/g) have been found in fish reared in ponds as observed by Ringø and Birkbeck (2000) and Spanggaard *et al*. (2000). The physicochemical properties of the fish pond water used in this study suggest that the water was very rich in some vital nutrients on which the growth of LAB is required. These required nutrients include magnesium and calcium (Data not shown). In this study, various species of lactic acid bacteria are present in relatively high number in the fish pond soil and water, but in low numbers in fish GIT.

Growth at different temperature is one of the criterions for the identification and screening of the isolates for probiotics properties. From the results it was observed that eight of the isolates were able to grow at 10 °C, ten of the isolates were able to grow at 15 °C while five of the isolates were also able to grow at 45 °C. Growth at different NaCl concentrations was also observed. Growth of isolates at different NaCl concentration was studied to know if it is also possible to administer probiotic strains through (drinking) water. Resistance to low pH is one of the major selection criteria for probiotics strains (Quwehand and Salminen, 1999; Çakır 2003). In this research, all the isolates were resistant to pH 3.0 during 3 hours. Although in the stomach, pH can be as low as 1.0, in most *in vitro* assays, pH 3.0 has been preferred because the time it takes during the digestion in the stomach is 3 hours (Çakır, 2003). *Lactobacillus plantarum* isolated from moist guava soil had the highest growth count during 3 hours incubation while *Lb. acidophilus* isolated from fish intestine had the lowest growth count. This may be due to the fact that a significant decrease in the viability of strains is often observed at pH 2.0 and below (Prasad *et al*., 1998).The results of the present investigation have shown that there is a diversity of lactic acid bacteria from soil samples from fruit trees of FUTA farm, intestine and gill of fish and its environs as well as ogi, a fermented Nigerian food. These lactic acid bacteria could be harnessed as probiotic substances since they are generally regarded as safe (GRAs).

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