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Antibacterial activity of lactic acid bacteria isolated from fresh pepper and tomatoes against common food pathogens

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A B S T R A C T

This study aimed to investigate the antibacterial activity of lactic acid bacteria (LAB) isolated from fresh pepper and tomatoes against common food borne pathogens. Man Rogosa Sharpe (MRS) broth and agar were used for the isolation of LAB from the food products and whose antagonistic properties were tested against Bacillus cereus, Staphylococcus aureus, Streptococcus sp., E. coli, Klebsiella pneumoniae and Pseudomonas aeruginosa using well diffusion assay method. Four LAB species were isolated and these included Streptococcus pyogenes, Enterococcus faecalis, Lactococcus casei and Lactococcus fermentii. The percentage occurrence of LAB species ranged from 11.77% to 35.29%. E. faecalis exerted the strongest antibacterial activity against all selected pathogenic bacteria while L. casei showed the weakest activity. It was concluded that the isolated LAB showed remarkable inhibitory effect against tested pathogenic strains. It is therefore suggested that these potent isolates could be used as a natural bio-preservatives in different food products. However, further in vitro and in vivo studies are required, according to selection criteria, on their application in different food products.

Introduction

Lactic acid bacteria (LAB) are frequently isolated from fermented foods, diary/poultry products and gastrointestinal tracts of animals and humans. They may beneficially affect the host upon ingestion by a variety of proven mechanisms (Fayol-Messaoudi et al., 2005; Ljungh and Wadstrom, 2006). Some of the beneficial effects of lactic acid bacteria consumption include improving intestinal tract health (Parvez et al., 2006), enhancing the immune system (Agerholm-Larsen et al., 2000; Chang et al., 2000), synthesizing and enhancing the bioavailability of nutrients, reducing symptoms of lactose intolerance (Marteau et al., 2001), decreasing the prevalence of allergy in susceptible individuals and reducing risk of certain cancers (Saikali et al., 2004; Parvez et al., 2006). In addition, these organisms possess the potentials of acting as antibacterial agents especially in their ecological environment. This is, largely, due to the fact that they are capable to produce inhibitory substances such as bacteriocins, lactic acid, hydrogen peroxide, diacetyl, carbon dioxide and low molecular weight antibacterial substances (Piard and Desmazeaud, 1992; Khay et al., 2011). Adeniyi et al. (2006) reported varied inhibitory activities of lactic acid bacteria isolated from indigenous fermented diary foods against commonly encountered bacteria implicated in urinary tract infections. The killing activity of anti-Salmonella enterica serovar Typhimurium produced by Lactobacillus and Bifidobacterium strains in the presence of Luria broth (LB) has also been reported (Coconnier et al., 1993; Bernet- Camard et al., 1997; Lievin et al., 2000). Most recently, inhibition of Neisseria gonorrheae (NG) by the co- cultivation of LAB with NG was reported to have been due to the acidification of the medium (Graver and Wade, 2011) and low molecular weight antibacterial substances (Piard and Desmazeaud, 1992; Khay et al., 2011). Although, there is a lot of research on isolation and characterisation of LAB, only a few of them have focused on isolation from fruits and vegetables (Trias et al., 2008; Chen et al., 2010; Padmaja et al., 2011**;** Ravi et al., 2011) Lactic acid bacteria and their fermented food products are thought to confer a variety of important nutritional and therapeutic benefits on consumers, including antimutagenic and anticarcinogeinc activity (Friend and Shahani, 1984; Fernandes et al., 1987; Fernandes and Shahani, 1990;

Gilliland 1990; De Vuyst and Vandamme, 1994; Dodd and Gasson, 1994; Gibson, 1995; Hata et al., 1996; Danone, 2001 and Lee et al., 2004). Friend and Shahani (1984) reported that anti-cancer activity occurs when extracts of L. acidophilus, L. casei, and L. helveticus are used in treating sarcomas in mice. Shahani and Ayebo (1980) emphasized that L. acidophilus super strain DDS1 produced the strongest antitumour activity. Hosono (1986) reported that milk fermented with L. delbrueckii sp. Bulgaricus exhibited antimutagenic activities against 4NQO, a typical mutagen, and the water extract of dog faeces, a faecal mutagen, in vitro assay. Information on lactic acid bacteria from fresh pepper and tomatoes are limited. The isolation of lactic acid bacteria from fresh vegetables appears to be interesting since they can present affordable source of the organisms. These vegetables are readily available in Nigerian markets and are often served raw or as supplements to cooked foods especially rice. They form part of our major supplements in this part of the Country.

Materials And Methods

Source and Processing of Samples

Fresh pepper and tomatoes were purchased from different sellers in three local markets in Sagamu, Nigeria. These were packaged into sterile plastic containers, transported to the laboratory and processed immediately to prevent deterioration. Each sample was blended separately with a blender (Nakai Japan Magic Blender, Model 462). The blender compartment was flooded with boiled water after each blending and allowed to cool before loading the next vegetables. The blended samples were suspended in 20 ml of sterile Man Rogosa Sharpe (MRS) broth (Oxoid, UK), incubated in candle extinction jar at 37^0C for 24 hrs (De Man et al., 1960; Sarkono et al., 2010). The control consisted of un-inoculated, sterile MRS broth incubated under the same conditions as test cultures. The test cultures and the control were used to inoculate the MRS agar and incubated at the same conditions for another 48hrs. Isolated colonies with typical characteristics of LAB were picked from each plate and transferred to MRS broth. The cultures were identified according to their morphological, cultural, physiological and biochemical characteristics (Harrigan and Margaret McCance, 1970; Sneath et al., 1986; Fayol-Messaoudi et al., 2005). The biochemical tests used were Gram reaction; production of catalase and cytochrome oxidase; growth at 10°C, 45°C and 60°C for 1 week; growth at 10% NaCl, acid production from carbohydrates (1 % w/v) lactose, melebiose, raffinose; production of acid and gas from 1 % glucose; Hugh and Leifson (H&L) test in O/F medium; and production of ammonia from arginine (Holt et al. 1994).

Standard Strains

The standard strains used in this study were Bacillus cereus ATCC 27853, Staphylococcus aureus ATCC 29212, Streptococcus sp. ATCC 28910, E.coli ATCC 12900, Klebsiella pneumoniae ATCC 17800 and Pseudomonas aeruginosa ATCC 13315 all of which were procured from Federal Institute of Industrial Research, Oshodi (FIIRO), Lagos, Nigeria.

Production of bacteriocin-like inhibitory substance (BLIS)

Isolated cultures maintained were inoculated in 100 ml of MRS broth and incubated anaerobically at 37^oC for 24 h. The cells were killed by heating at 80^0C for 10 mins followed by centrifuging the broth at 10,000 rpm for 20 mins. Brand of centrifuge used was Alfa-Laval model 440 Hermetically sealed 316 SS solid disc bowl centrifuge SN 377615. The resulting cell debris that formed a pellet was discarded giving rise to a cell free supernatant. The pH of supernatant was adjusted to 5.0 with 1N NaOH, then concentrated to one tenth of the original volume by rotary flash evaporator and the solution thus obtained has been designated as BLIS. For synergistic activity, BLIS was mixed with 1ml of 1% of EDTA and filter- sterilized by 0.22 µm membrane filter paper (Millipore, India) to carry out the antimicrobial activity by well diffusion assay (Gomez et al., 2002; Vijai Pal et al., 2005).

Well diffusion assay method

0.1 ml of the 18 h old test cultures were inoculated onto nutrient agar plates by spread plate method. Four wells of diameter 8 mm were made in each of the plates. These wells were filled with 100 µl concentrated BLIS of Enterococccus faecalis, Streptococcus pyogenes, Lactobacillus fermenti and Lactobacillus casei with and without EDTA. The plates were incubated at 37° for 24hrs (Schillinger and Lucke, 1989). The inhibition zones were measured and recorded in millimeter.

Results And Discussion

Four (4) LAB species belonging to three genera were isolated from fresh pepper and tomatoes and these include Streptococcus pyogenes, Enterococcus faecalis, L. casei and L. fermentii (Table 1).

Table 1. Biochemical test for the identification of isolated Lactic acid bacteria

No.	GM/MOR	CAT	OXI	O/F	LAC	GLU	GROWTH AT 10^0C	GROWTH AT 45^0C	GROWTH AT 60^0C	10%NaCl	pH 9.6	NH_4 Arg	RAF	MEL	Most Probable Organism
1	+C	-	-	F	+	-	-	-	-	-	-	+	NA	NA	Streptococcus pyogenes
2	+C	-	-	F	+	-	+	+	+	+	+	+	NA	NA	Enterococcus faecalis
3	+C	-	-	F	+	-	+	+	+	+	+	+	NA	NA	Enterococcus faecalis
4	+C	-	-	F	+	-	+	+	+	+	+	+	NA	NA	Enterococcus faecalis
5	+C	-	-	F	+	-	+	+	+	+	+	+	NA	NA	Enterococcus faecalis
6	+C	-	-	F	+	-	-	+	-	-	-	-	NA	NA	Streptococcus viridans
7	+C	-	-	F	+	-	-	-	-	-	-	+	NA	NA	Streptococcus pyogenes
8	+C	-	-	F	+	-	+	+	+	+	+	+	NA	NA	Enterococcus faecalis
9	+C	-	-	F	+	-	+	+	+	+	+	+	NA	NA	Enterococcus faecalis
10	+C	-	-	F	+	-	-	-	-	-	-	+	NA	NA	Streptococcus pyogenes
11	+R	-	-	F	+	-	+	+	NA	NA	NA	-	-	-	Lactobacillus casei
12	+R	-	-	F	+	-	+	+	NA	NA	NA	-	-	-	L. casei
13	+R	-	-	F	+	-	-	+	NA	NA	NA	+	+	+	Lactobacillus fermenti

Keys

GM/Mor: Gram stain and morphology; LAC: Lactose utilization; RAF: Raffinose utilization; CAT: Catalase; GLU – Glucose utilization; MEL: Melibiose utilization; OXI: Oxidase; NH4 – arg: Ammonia from arginine; +C: Gram positive cocci; O/F: Oxidative / fermentative; +R: Gram Positive rod; +: Positive reaction; -: Negative reaction; NA: Not applicable

The pH of control before and after incubation were 6.49 and 5.64 respectively while the pH range of all MRS broth cultures were within 3.30 and 3.92 (Table 2).

Table 2. pH Profile of Lactic Acid Bacterial Cultures from fresh pepper and tomatoes

Isolated LAB	pH of CFS
Streptococcus pyogenes	3.92
Enterococcus faecalis	3.33
Streptococcus viridians	3.3
L. casei	3.63
L. fermenti	3.42

Among the LAB isolated in this study, gram-positive cocci constituted 70.59% while gram-positive bacilli constituted the remaining 29.41%. The percentage occurrence of LAB species associated with fresh pepper and tomatoes ranged from 11.77% as obtained with L. fermentii to 35.29% with Enterococcus faecalis (Table 3).

Table 3. Number / percentage of lactic acid bacteria isolated from fresh pepper and tomatoes

Isolated/ Identified species	Number of LAB isolates	Percentage	Source
Total number of catalase negative bacteria	17		
Gram-positive cocci		70.59	
Streptococcus pyogenes	6	35.29	Fresh pepper
Enterococcus faecalis	6	35.29	Tomato
Gram-positive bacilli		29.41	
L. casei	3	17.64	Fresh pepper
L. fermentii	2	11.77	Tomato

BLIS of E. faecalis exerted the strongest antibacterial activity against all selected pathogenic bacteria while L. casei showed the weakest activity. The growth of E. coli was inhibited by all the LAB species isolated in this study. Growth inhibition was most pronounced in B. cereus except that resistance was shown to L. casei (Table 4).

Table 4. Antibiogram (zone of inhibition in mm) of BLIS against selected pathogens

Broth Culture	Gram negative bacteria			Gram positive bacteria		
	E. coli	K. pneumoniae	P. aeruginosa	B. cereus	S. aureus	Streptococcus sp
E. faecalis	14.0 $\pm$ 0.20	14.0 $\pm$ 0.20	13.0 $\pm$ 0.10	18.0 $\pm$ 0.65	17.0 $\pm$ 0.50	16.0 $\pm$ 0.20
S. pyogenes	10.0 $\pm$ 0.25	10.0 $\pm$ 0.03	11.0 $\pm$ 0.25	13.0 $\pm$ 0.20	NA	12.0 $\pm$ 0.30
L. fermenti	12.0 $\pm$ 0.30	10.0 $\pm$ 0.20	12.0 $\pm$ 0.25	14.0 $\pm$ 0.25	12.0 $\pm$ 0.35	13.0 $\pm$ 0.20
L. casei	7.0 $\pm$ 0.25	NA	NA	NA	NA	NA

Values are Mean ± SD of 3 determinations; NA – No activity

The growth of virtually all the test organisms were inhibited by the BLIS of the LAB species isolated in this study when 0.1% EDTA was introduced. BLIS of L. casei to which virtually all test organisms showed resistance was also found to exhibit some antibacterial activity with d introduction of 0.1% EDTA. BLIS of E. faecalis with 0.1% EDTA was found to exert the strongest antibacterial activity (Table 5).

Table 5. Antibiogram (zone of inhibition in mm) of BLIS with 0.1% EDTA against selected pathogens

LAB/Test	Gram negative bacteria			Gram positive bacteria		
Organisms	E. coli	K. pneumoniae	P. aeruginosa	B. cereus	S. aureus	Streptococcus sp
EDTA alone	11.0 ± 0.10	10.0 ± 0.10	8.0 ± 0.10	12.0 ± 0.10	11.0 ± 0.20	9.0 ± 0.10
E. faecalis	23.0 ± 0.05	21.0 ± 0.02	20.0 ± 0.05	27.0 ± 0.03	24.0 ± 0.20	24.0 ± 0.20
S. pyogenes	14.0 ± 0.20	13.0 ± 0.30	13.0 ± 0.20	14.0 ± 0.25	10.0 ± 0.05	14.0 ± 0.70
L. fermenti	18.0 ± 0.30	16.0 ± 0.03	15.0 ± 0.20	18.0 ± 0.30	16.0 ± 0.25	16.0 ± 0.20
L. casei	12.0 ± 0.05	10.0 ± 0.02	9.0 ± 0.10	12.0 ± 0.02	12.0 ± 0.10	NA

Values are Mean ± SD of 3 determinations; NA- No activity

Thirty-six positive colonies were picked from MRS agar, only 17 were identified as LAB (Table 1). Six coccoid isolates were obtained from tomatoes and identified as E. faecalis corresponding to 35.29% of the LAB isolates; six coccoid isolates obtained from fresh pepper were identified as S. pyogenes (35.29%). The percentage occurrence of gram-positive bacilli was 29.41%, L. casei constituted 17.64%, while L. fermentii constituted 11.77%. El- Shafei et al. (2000) in their studies, isolated, screened and characterized 100 strains of bacteriocin-producing lactic acid bacteria from traditional fermented foods. Renata et al. (2004) also isolated Lactococcus lactis from meat and meat product. Kannappan et al. (2004) had observed that BLIS of LAB with EDTA combination inhibited Vibrio parahaemolyticus and E.coli. Kelly et al. (1991) reported that nisin inhibited Salmonella sp. in combination with EDTA. The BLIS produced by the isolates E. faecalis, S. pyogenes, L. fermenti in combination with EDTA were the effective inhibitor of all the pathogens tested as compared to L. casei. The BLIS of E. faecalis exerted the strongest antibacterial activity against the pathogenic organisms as shown by the various inhibition zones: B. cereus (18.0 ± 0.65 mm), S. aureus (17.0 ± 0.50 mm) and Streptococcus sp. (16.0 ± 0.20 mm). L. fermentii and S. pyogenes also showed good antibacterial activities except against S. aureus. L. casei exhibited the poorest antibacterial activity as no activity was detected against the entire test organisms except E. coli and with the smallest inhibition zone in this study (7.0 ± 0.25 mm). The gram-negative organisms namely, E.coli, K. pneumoniae and P. aeruginosa showed less inhibition compared to gram-positive organisms. This is in accordance with some of the earlier reports which showed that bacteriocins of LAB were more active against gram-positive organisms compared to gram-negative organisms (Jack et al., 1995; Patil et al., 2010; Savino et al., 2011). The reason for the observed activity may be due to the presence of an outer protective membrane in gram negative organisms, which covers the cytoplasmic membrane and peptidoglycan layer. The inhibitory action of LAB bacteria can be due to the accumulation of main primary metabolites such as lactic and acetic acids, ethanol and carbon dioxide. Additionally, LAB are also capable of producing antimicrobial compounds such as formic and benzoic acids, hydrogen peroxide, diacetyl, acetoin and bacteriocins such as nicin (Dracheva et al., 2007). The production levels and the proportions among those compounds depend on the strain, medium compounds and physical parameters. The inhibitory activities of LAB against Gram positive pathogens have been mostly shown to be due to the bactericidal effect of protease sensitive bacteriocins (Tannock, 2004). However, the antagonistic effects of LAB towards Gram negative pathogens could be related to the production of organic acids and hydrogen peroxide. EDTA, a metal chelating agent removes stabilizing cations from the outer membrane and these results in the partial loss of lipopolysaccharide layer and then membrane no longer functions as penetration barrier (Boziaris and Adams, 1999). A large number of lactic acid bacteria strains with different bioactive potentials especially in the form of antimicrobial properties have been identified from a variety of plant sources mostly in the form of fermented and pickled vegetables. These scientific evidences have been a motivating factor to choose a plant based fermented product prepared from different vegetables which could further confirm the results of this study.

Conclusion

It was concluded in this study that the isolated LAB showed remarkable inhibitory effect against both Gram positive and Gram negative pathogenic strains. However, the spectrum of inhibition was different from one organism to the other. These results suggest that this potent isolates could be used as a natural bio-preservatives in different food products. These positive outcomes would be a leading point towards application of simple worthy traditional methods in producing natural healthy food products while hoping that these friendly food groups would be added to daily diet of each individual to improve body immunity hence, checking indiscrimate intake of chemical antibiotics. However, further in vitro and in vivo studies are required, according to selection criteria, to investigate properties such as adhesion to mucosal cells of the gastrointestinal tract, bile salt and acid tolerance, bile salt hydrolase activity, viability, resistance to antibiotics, safety and organoleptic properties in order to empirically establish their application in different food products.

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