

Effect of Varied Culture Conditions on Crude Supernatant (Bacteriocin) Production from Four *Lactobacillus* Species Isolated from Locally Fermented Maize (Ogi)

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Abstract This Lactic acid bacteria (LAB) predominates the micro flora of fermented products. They produce metabolites that inhibit the growth of food borne pathogens and spoilage microorganisms. The isolation and identification of LAB from fermented maize (Ogi) and the effect of varied culture conditions on crude supernatant production and activity was evaluated. Four (4) isolates of bacteriocin producing *Lactobacillus* species (*L. lactis*, *L. fermentum*, *L. casei* and *L. plantarum*) with antibacterial activity against *Salmonella typhimurium* (ATCC 14028) and *Shigella dysenteriae* (ATCC 23351) were subjected to varying growth medium conditions. The crude supernatant production was tested at different physical and cultural conditions such as temperature (25, 30, 35 and 40°C), pH (5, 6, 7 and 8), sodium chloride (NaCl) concentration (2, 4, 6 and 8%) and incubation duration (12, 24, 48 and 72 hours). The optimum bacteriocin production judged by their different zones of inhibition of crude supernatant was recorded at temperature, 30°C and then 35°C. There were significant differences between all the incubation temperatures at $P < 0.05$. Duration of incubation showed highest crude supernatant activity after 72 hours. Furthermore, optimal conditions for crude supernatant production were observed to be highest at pH 6.0 followed by 5.0 and then 2% NaCl concentration. There were significant differences between the zones of inhibition of crude supernatants produced against the indicator organisms at various media pH and salt concentrations at $P < 0.05$. These crude supernatants may have a potential use in reducing contaminations during industrial processes, as food preservatives and may help in improving the gastro-intestinal tract by fighting off pathogenic bacteria.

Keywords: varied, culture, crude supernatant, bacteriocin, production, lactobacillus, fermented, maize, Ogi

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1. Introduction

Food is any substance or mixture of substances both solid and liquid, which are intended for human consumption or ingestion for their nutritional support for the body or pleasurable benefits. It usually consists of plant or animal origin, which contains essential nutrients such as carbohydrates, fats, proteins, vitamins or minerals and is ingested and assimilated by an organism to produce energy, stimulate growth and maintain life [1,2].

The lactic acid bacteria (LAB) are a group of Gram positive bacteria, non-respiring, non-spore forming, cocci or rods, which produce lactic acid as the major end product of the fermentation of carbohydrates. They are the most important bacteria in desirable food fermentations, being responsible for the fermentation of sour dough bread,

sorghum beer, all fermented milk, cassava (to produce garri and fufu) and most "pickled" (fermented) vegetables [3,4]. Lactic acid bacteria occur naturally in several raw materials like milk, meat and flour used to produce foods. LAB is used as natural or selected starter cultures in food fermentations in which they perform acidification due to production of lactic acids. Protection of food from spoilage and pathogenic microorganisms by LAB is through producing organic acids [5]. The LAB produces an array of antimicrobial substances (such as organic acids, diacetyl, acetone, hydrogen peroxide, reuterin, anti-fungal peptides and bacteriocins [6]. Bacteriocins are ribosomally synthesized extracellularly released low molecular-mass peptides or proteins (usually 30–60 amino acids) which have a bactericidal or bacteriostatic effect on other bacteria [7,8]. The bacteriocins produced by LAB offer several desirable properties that make them suitable for

food preservation: (i) are generally recognized as safe substances [7], (ii) are not active and nontoxic on eukaryotic cells, (iii) become inactivated by digestive proteases, having little influence on the gut microbiota, (iv) are usually pH and heat-tolerant, (v) they have a relatively broad antimicrobial spectrum, against many food-borne pathogenic and spoilage bacteria, (vi) they show a bactericidal mode of action, usually acting on the bacterial cytoplasmic membrane: no cross resistance with antibiotics, and (vii) their genetic determinants are usually plasmid-encoded, facilitating genetic manipulation [9].

The inhibitory spectrum of bacteriocins can be narrow and confined to closely related species, or it can be relatively broad, inhibiting a range of target organisms, including food-spoilage and pathogenic bacteria, such as *Listeria monocytogenes*, *Bacillus cereus*, *Clostridium tyrobutyricum*, methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant enterococci. In general, bacteriocins act mainly by pore formation in target cell membranes, or by inhibiting cell wall synthesis or enzyme activities in the cytosol (RNAse or DNAse) [9].

Ogi (Akamu) is a product of fermented maize (*Zea mays*) widely eaten in Africa [10,11]. Similar maize preparations in Ghana are referred to as “Akana” or “Kenkey”. Ogi is often marketed as a wet cake formerly wrapped in leaves but presently in transparent polythene bags. Gelatinized Ogi (a porridge) called “pap” is mainly used as a breakfast meal for adults and weaning food by low income earners who cannot afford the more expensive imported weaning foods [12]. In most parts of Africa especially in Nigeria, children are fed with mashed adult foods. These foods are bulky and this therefore reduces food intake by a child, often resulting in malnutrition. The development of nutritionally balanced calorie less dense, low bulk and easily digestible weaning food becomes necessary. This involves the use of simple but time consuming traditional technology called fermentation [13]. The traditional fermentation method employed in Ogi production is a wild process and microorganisms are not controlled [14]. Microbiological analyses have shown the presence of several genera of bacteria, moulds and yeasts in the fermented maize product-Ogi [15,16].

In the present study, different culture conditions were adjusted for crude bacteriocin (crude supernatant) production using *Lactobacillus* isolates from locally fermented maize (Ogi) to determine optimal fermentation conditions for crude supernatant production.

2. Materials and Methods

2.1. Fermented Products

Ten (10) fermented maize (Ogi) samples bought from Oshodi and Odo markets, Lagos State, Nigeria were analyzed.

2.2. Test Organisms

Pure strains of pathogenic gram negative bacteria responsible for food infections; *Salmonella typhimurium* (ATCC 14028) and *Shigella dysenteriae* (ATCC 23351) were obtained from the Nigerian Institute of Medical Research laboratory (NIMR) Yaba, Lagos, Nigeria and maintained on agar slants at 4°C in the refrigerator.

2.3. Isolation of Lactic Acid Bacteria from Ogi

One (1) gram of a 72hrs fermented Ogi was transferred into 5ml peptone water (Merck, Germany) and serially diluted (10 fold dilutions). Then 1ml of each of the dilution was aseptically transferred into sterile Petri dishes (Pyrex and Anumbra) and 15ml of de Man Rogosa Sharpe (MRS) medium (Merck, Germany) was added using pour plate technique then incubated at 37°C for 48hrs in an anaerobic flask (Oxoid). After incubation, colonies with different morphologies were randomly selected using a flamed platinum wire loop, streak plated and sub – cultured on MRS agar plates to obtain pure colonies. All isolates were examined for Gram reaction, production of catalase and oxidase activity.

2.4. Identification of LAB Isolates

Isolates were identified using the following tests: ammonia production from arginine, CO₂ production from glucose and growth at different pH values, growth at different NaCl concentrations and carbohydrate fermentation. LAB isolates were tested for characteristics of Gram staining, cell morphology, colony morphology, motility, carbon dioxide production from glucose, growth at 10°C and 45°C, growth at pH of 4.4 and 9.6, growth in 6.5% and 18% NaCl, catalase reaction by 3% hydrogen peroxide and carbohydrate fermentation [17].

2.5. Characterization of Isolates

LAB strains were characterized according to methods recommended by several authors [18,19]. All strains were initially subjected to Gram staining, catalase test, growth at 10 and 45°C in MRS broth, gas production from glucose, arginine hydrolysis [20] and growth in the presence of 2%, 3%, 4%, 6.5% and 10% of NaCl. The fermentation of carbohydrates (starch, amygdalin, arabinose, cellobiose, fructose, galactose, glucose, lactose, maltose, mannitol, mannose, melizitose, melibiose, raffinose, rhamnose, ribose, sucrose, salicin, sorbitol, trehalose and xylose) was performed in MRS broth (prepared without sugars) containing 1% solution of carbohydrate and added to 0.025% bromocresol purple as pH indicator. Results were recorded after 48 h of incubation at 30°C.

2.6. Detection of Inhibitory Activity of Crude Bacteriocin from Selected Isolates

Selected LAB isolates were grown in MRS broth at 37°C for 24 hrs. Cell free crude supernatant of each isolate was obtained by centrifugation at 3,000xg for 20 min. The supernatant was adjusted to pH 6.5 with 1M NaOH and subsequently filter sterilized through a 0.2µm membrane filter (Whatman, Germany). Inhibitory activity was determined using agar well diffusion assay [21]. Inhibitory effect of the hydrogen peroxide in the crude supernatant was eliminated by reacting with 5mg/ml catalase added. Suitable agar medium containing 1% agar (45°C) was inoculated with each of the two indicator strains. Agar wells of 5 mm diameter were cut and the filter-sterilized crude supernatant (20µl) was added into each well. The plates were incubated at 37°C for 24hrs. The inhibition zones around the wells were measured.

2.7. Effect of Varying Culture Conditions on Crude Supernatant Activity

The selected lactic acid strains were subjected to different culture conditions to derive optimum conditions for crude supernatant production. To study the effect of varying culture conditions, growth and crude supernatant production and thus bacteriocin activity was estimated at varied temperatures (25, 30, 35, and 40°C) pH (5.0, 6.0, 7.0 and 8.0), sodium chloride (NaCl) concentrations (2.0, 4.0, 6.0 and 8.0% w/v) and duration of incubation (12, 24, 48, and 72hrs) in MRS broth. All samples were collected after 48hrs, except for those measuring incubation time effects before inhibitory activity was determined by agar well diffusion assay as described above.

2.8. Data presentation and statistical analysis

Data were represented as means ± standard error of mean as well as bar charts. Two-way analysis of variance and Bonferroni's multiple comparison tests using GraphPad Prism (Version 6.0) software were used to analyze data. Values were considered significant when P<0.05.

3. Results

3.1. Isolation of Lactic Acid Bacteria

In this study, *Lactobacillus* strains producing antimicrobial compounds were isolated from fermented maize (Ogi). Four (4) *Lactobacillus* species met basic characteristics of Lactobacilli, and showed antibacterial activity against both indicator organisms. They include *L. lactis*, *L. fermentum*, *L. casei* and *L. plantarum*.

3.2. Identification of Isolates

The general properties of 7 LAB strains isolated from Ogi were determined by phenotypic characterization. There production of gas from glucose, hydrolysis of aesculin, arginine and citrate hydrolysis, growth at different temperatures and at different levels of salt were also put into consideration, species were arranged in groups by the carbohydrates fermentation profile (Table 1 and Table 2).

3.3. Optimization of Culture Conditions

The broth medium containing each isolate was incubated at various temperatures; 25.0, 30.0, 35.0 and 40.0°C and the bacteriocin harvested were tested against *Salmonella typhimurium* and *Shigella dysenteriae* (Figure 1 and Figure 2 respectively).

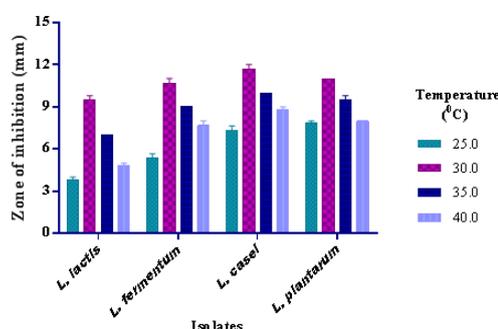


Figure 1. Zone of inhibition (mm) of crude supernatant from different *Lactobacillus* isolates cultured at varied temperatures (°C) against *Salmonella typhimurium*

Table 1. Physiological characteristics of lactic acid bacteria strains isolated from Ogi

Species number	Gas from Glucose	Motility	Hydrolysis of			Growth at different temperatures (°C)					Production of		Growth at different pH			Growth in the presence of NaCl (%)				
			ADH	Citrate	Aesculin	10	15	40	45	50	Acetoin	Dextran	6.5	9.2	9.6	2	3	4	6.5	10
1	-	-	+	-	+	-	-	+	-	-	v	-	+	+	-	+	+	+	+	-
2	+	-	+	-	v	-	+	+	-	-	-	-	+	v	v	+	+	+	+	-
3	+	-	-	-	+	-	+	+	v	-	-	-	+	-	-	+	+	+	+	-
4	+	-	-	-	+	-	+	+	-	-	-	-	+	-	-	+	+	+	+	-

+: Positive reaction; -: Negative reaction; v: variable. 1. *Lactobacillus lactis*; 2. *Lactobacillus fermentum*; 3. *Lactobacillus casei*; 4. *Lactobacillus plantarum*

Table 2. Carbohydrate fermentation of lactic acid bacteria strains isolated from

Specie number	Starch	Amygladin	Melizitose	Sorbitol	Trealose	Arabinose	Raffinose	Xylose	Ribose	Sucrose	Rhamnose	Cellubiose	Melibiose	Salicin	Mannitol	Mannose	Fructose	Galactose	Glucose	Lactose	Maltose
1	+	-	-	-	V	-	-	V	+	-	-	+	-	+	-	-	+	+	+	+	+
2	-	-	V	-	-	+	V	-	+	V	-	-	-	-	+	-	+	V	+	V	+
3	-	+	V	-	+	-	-	-	-	-	-	+	+	+	+	+	+	+	+	-	V
4	-	+	+	+	+	-	-	-	+	+	-	+	-	+	+	+	+	+	+	+	+

+: Positive reaction; -: Negative reaction; v: variable. 1. *Lactobacillus lactis*; 2. *Lactobacillus fermentum*; 3. *Lactobacillus casei*; 4. *Lactobacillus plantarum*

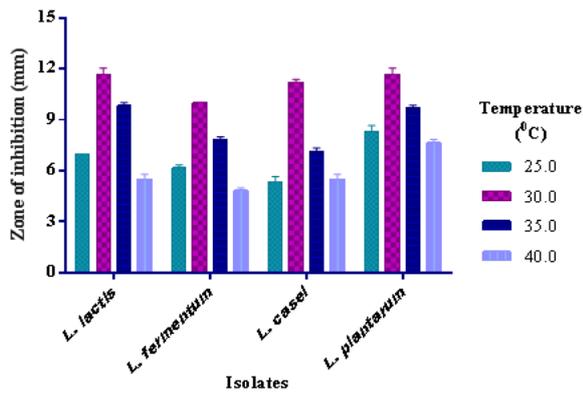


Figure 2. Zone of inhibition (mm) of crude supernatant from different *Lactobacillus* isolates cultured at varied temperatures (°C) against *Shigella dysenteriae*

The effect of varied incubating duration (Hrs) on bacteriocin production was noted as different zones of inhibition were observed among the various isolates against the indicator organisms (Figure 3 and Figure 4).

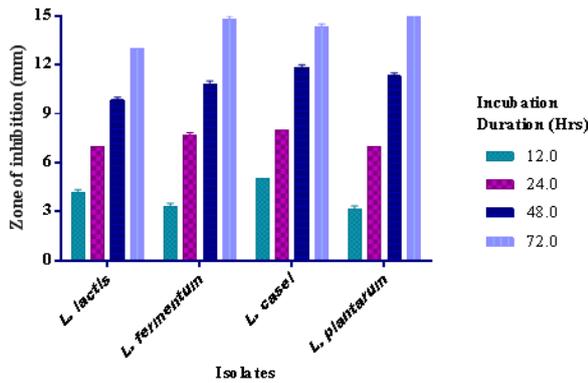


Figure 3. Zone of inhibition (mm) of crude supernatant from different *Lactobacillus* isolates cultured at varied incubation durations (Hrs) against *Salmonella typhimurium*

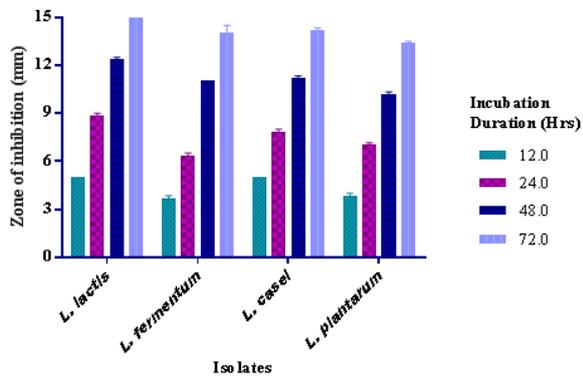


Figure 4. Zone of inhibition (mm) of crude supernatant from different *Lactobacillus* isolates cultured at varied incubation durations (Hrs) against *Shigella dysenteriae*

The effect of varied medium pH and Sodium chloride concentration on bacteriocin production was affected as different zones of inhibition were observed among the various isolates against the indicator organisms (Figure 5 and Figure 6; Figure 7 and Figure 8) respectively

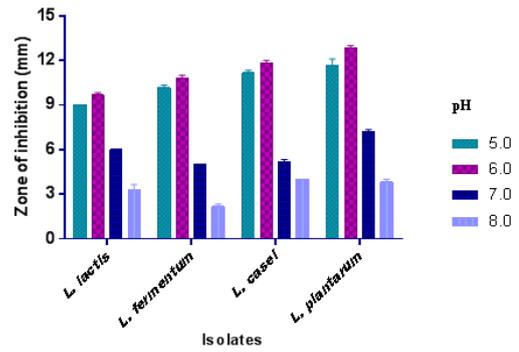


Figure 5. Zone of inhibition (mm) of crude supernatant from different *Lactobacillus* isolates cultured at varied media pH against *Salmonella typhimurium*

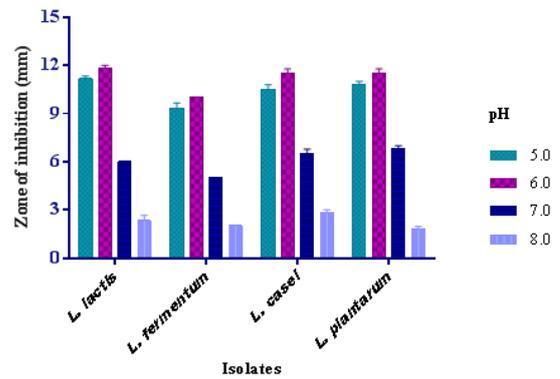


Figure 6. Zone of inhibition (mm) of crude supernatant from different *Lactobacillus* isolates cultured at varied media pH against *Shigella dysenteriae*

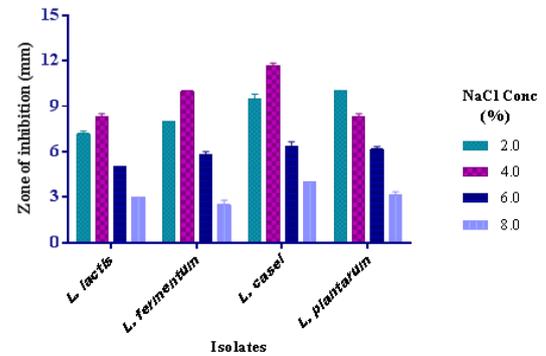


Figure 7. Zone of inhibition (mm) of crude supernatant from different *Lactobacillus* isolates cultured at varied media sodium chloride concentration against *Salmonella typhimurium*.

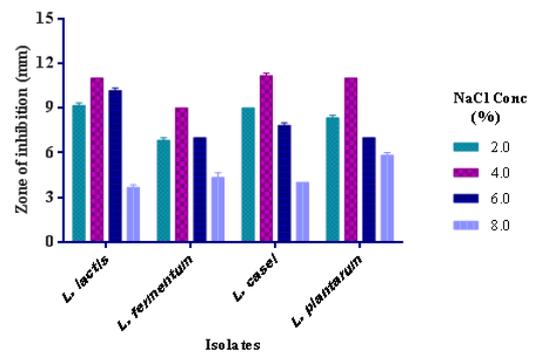


Figure 8. Zone of inhibition (mm) of crude supernatant from different *Lactobacillus* isolates cultured at varied media sodium chloride concentration against *Shigella dysenteriae*

4. Discussion

These identified *Lactobacillus* species were in agreement with those earlier identified from similar fermented food products by [22]. The isolates were then tested for antibacterial activity against the indicator organisms (*Salmonella typhimurium* and *Shigella dysenteriae*). The crude supernatant activity from the isolates that showed antibacterial activities were further tested after the culture conditions were varied to determine the effect of different cultural conditions on the antibacterial action of the crude extract produced. Parameters such as incubation duration and temperature, media pH and salt concentrations were varied.

The effect of varied incubating temperatures on crude supernatant production by the isolates in de Man Rogosa Sharpe broth was determined by its antibacterial activity against the indicator strains. The result showed that antibacterial activity was highest at 30.0°C followed by 35.0°C as seen in Figure 1 and Figure 2. There were significant differences between the different zones of inhibition produced for all the incubating temperatures (at $P < 0.05$). This was in agreement with the work of [23] and [24], which showed that bacteriocin production was affected by different incubating temperatures. The maximum crude supernatant activity recorded at 30°C suggests that ambient growth temperature is most ideal for their production by *Lactobacillus* species.

The effect of varied incubating duration (Hrs) on crude supernatant production was affected as different zones of inhibition were observed among the various isolates against the indicator organisms (Table 3 and Table 4). Optimum crude supernatant production was observed after 72 hours judged by the zones of inhibition against the indicators. There were observable reduction crude supernatant activities as incubation time dropped. There were significant differences between the various incubation times (at $P < 0.05$). This result was in complete agreement with [25], which showed that incubation time affects bacteriocin production.

The results obtained in this study regarding crude supernatant activity from media incubated at varied pH values (Figure 5 and Figure 6) showed optimum activity at pH 6 followed closely by pH 5 for both indicator organisms. There were clear significant differences between pH 7 and 8 but not in pH 5 and 6 as both showed similar zones of inhibition for all the isolates against indicator organisms. This result was consistent with the reports of [26], which showed influence of pH on growth of vaginal *Lactobacilli*. This pH tolerance is an extremely important feature since the isolates have the ability to survive, grow and produce bacteriocins under acidic and alkaline conditions.

Every microorganism has a minimal, a maximal and an optimal pH for growth and metabolism. Microbial cells are significantly affected by the pH of their immediate environment because they apparently have no mechanism for adjusting their internal pH.

Furthermore, the effect of varied medium percentage sodium chloride (NaCl) concentration on crude supernatant production and activity was also evaluated. Highest zones of inhibition and consequent optimum crude supernatant production was observed at NaCl concentration of 4%, but started reducing as salt concentration increased further

(Figure 7 and Figure 8). There was significant difference between zones of inhibition obtained at all the salt concentrations. This was in agreement with [25] who studied cultural parameter for bacteriocin production.

Besides the strong acid medium in the stomach, the probiotic microorganisms taken orally have to defend against the bile salt in the gastrointestinal tract [27]. Hence, bile tolerance is considered to be one of the important properties required for high survival and as a consequence of probiotic activity. The decrease in crude supernatant production as salt concentration increased could be attributed to stress on the isolates.

Microbial food safety is an increasing public health concern worldwide [28,29] and many gram negative bacteria like *Escherichia coli*, *Salmonella* serovars, *Campylobacter* species, *Shigella* species etc, have been implicated in food borne diseases [25]. Alternate methods for controlling pathogenic bacteria by the production of antimicrobial peptides called bacteriocins are now highly considered. Bacteriocins from lactic acid bacteria have attracted much attention and have been the subject of intensive investigation due to their ability to act as a bio-preservative agent, which led to their incorporation into foods, particularly in the dairy foods and also in human therapeutics [30,31,32,33].

5. Conclusion

This study thus suggests the use of *Lactobacillus* species isolated from Ogi in bacteriocin production. Crude supernatants from *Lactobacillus* species harnessed under different culture conditions have shown different antimicrobial potencies. These findings could be applied in the food and pharmaceutical industries to further enhance maximum bacteriocin production at optimal levels to replace conventional antibiotics in combating pathogens that are vastly acquiring antimicrobial resistance. Further work can be done to ascertain the antibacterial effect of the purified forms of the crude supernatants from *Lactobacillus* species from Ogi to obtain pre bacteriocins and ways in which they could be used in industrial processes to arrest contamination during industrial processes and in local fermented food preservation.

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