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INFLUENCE OF CELL-FREE SUPERNATANT OF LACTIC ACID BACTERIA AS ADDITIVE IN PRESERVATION OF PINEAPPLE JUICE

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Abstract: *This research explored the use of cell-free supernatant from Lactic Acid Bacteria (LAB) as a preservative in pineapple juice. LAB strains, Levilactobacillus brevis and Lactiplantibacillus spp., were sourced from healthy pineapple and soursop fruits, identified through standard biochemical analyses, and incorporated into the juice as freeze-dried supernatant. The sensory attributes of the juice (mouthfeel, taste, flavor, aroma, color, and overall acceptability) scored between 6.00 and 8.40. Interestingly, while juices with LAB extracts turned brown, the control retained its yellow hue, yet all samples were acceptable to assessors, with preferences ranked as follows: Control > Juice with Lactiplantibacillus spp. extract > Juice with Levilactobacillus brevis extract. The study also observed a slight pH increase in juices containing LAB extracts during the first three days, contrasting with the control's pH decrease within two days. Moreover, LAB-supplemented juice significantly inhibited pathogenic Escherichia coli survival at varying concentrations (2%–10%), comparable to sodium benzoate. This microbial reduction is attributed to compounds like lactic acid, hydrogen peroxide, diacetyl, and bacteriocin produced by LAB, with lactic acid playing a central role. Consequently, freeze-dried supernatant of these LAB strains shows potential as a natural preservative against Escherichia coli-induced diarrhea in pineapple juice.*

Keywords: *Biopreservative, Freeze-dried supernatant, Pineapple juice, Diarrhea*

1. Introduction

In order to ensure food quality and safety, there is need to control foodborne pathogens and spoilage microorganisms by natural means, without side effects on the consumers, therefore, natural preservation is expedient. Biopreservation is the natural process of prolonging and improving the safety and palatability of foods using living organisms such as plants, animals and microorganisms. This entails biological control as regards to microorganisms or their bioactive compounds to hinder the growth and multiplication of pathogens [1]. Microorganisms and their antimicrobial compounds such as the genus *Lactobacillus* are well established preservative that have been incorporated into foods for decades [2]. *Lactobacillus* utilization as biocontrol agents has gained a lot of attention resulting in a continual solution for food security [3-5].

Lactic acid bacteria (LAB) are diverse group of closely related bacteria having shared attributes, including the production of lactic acid, which is a byproduct of fermentation. Fermentation is an ancient food preservation technology. LAB have earned the 'generally regarded as safe' (GRAS) status and Qualified Presumption

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of Safety (QPS) by the American Food and Drug Agency (FDA) and the European Food Safety Authority (EFSA), respectively [6], because they are safe for consumption by humans likewise animals. During storage, they occur naturally and dominate the microflora of various foods [7]. They have become suitable for commercial development [8-9]. LAB species are used in many foods and feed industries, and these industries are consistently seeking potential strains to improve sensory and product quality [10].

Among the diverse genera of LAB, *Lactobacillus* produces various organic acids like lactic acid, acetic acid and propionic acid that possess anti-microbial properties [11-12]. *Lactobacillus plantarum* obtained from soy milk also have strong antibacterial activity against *Escherichia coli (E. coli)* and other disease-causing bacteria [13]. Some LAB are found in the oral cavity, intestine and vagina of mammals, while some are present in dairy product, for example, milk [14]. LAB can be isolated from many raw fruits and vegetables [15-16]. LAB possess wide range of antimicrobial capacity, this is obviously due to the production of a variety of compounds including acids, hydrogen peroxide, diacetyl and bacteriocin [18]. These compounds could be added to various foods and fruit juice in order to prevent them from food-borne pathogens and spoilage microorganisms, thus increasing their shelf-life. The mechanism of action of these compounds includes their movement through the membrane of the target organisms in their hydrophobic non-ionized form and then reduction of the cytoplasmic pH, thereby causing metabolic activities to stop. Other factors that can increase the preservative action of the acids in LAB, includes the effect of pH, the level of the dissociation of the acid, and the particular effect of the acids itself on the microbes [19]. Freeze-drying time and cycle changes as a result of the strain involved and its formulation, but consistently takes a few days to be executed. The major merit of freeze-drying is that the process retains the probiotic cells at a reduced temperature to limit damage to the cells' integrity and bioactive compounds [20]. Fruit juices are healthy foods, containing essential nutrients which are necessary for body nourishment of all ages and have been reported to be a new and right medium for probiotic research [21]. Pineapple juice is produced by the extraction of pineapple pulp. It is a good source of sugars and vitamins especially vitamins C, B2 and B6 and has a pleasant flavor and refreshing sugar-acid balance [22]. Pineapple juice is sensitive to mould or yeast contamination, resulting in spoilage. There is increase in metabolic activity when cutting pineapple fruit, exposing them quickly to microbial contamination, thus reducing their shelf life [23]. Furthermore, LAB is reported to be natural preservatives for fresh produce and as food additives due to their fermentation compounds which are beneficial to the body especially when stored in cold temperatures [24-25]. The causative agents in microbiological spoilage of pineapple and papaya fruits are *Escherichia coli* and *Klebsiella* sp. [26]. Food pathogen such as *Escherichia coli* has the ability to cause serious gastroenteritis in consumers, and have a high level of drug resistance which is hazardous to human health. There is market preference for natural food additives such as Lactic acid bacteria because they are safe and have been found to be a suitable replacement of antibiotics for preventing different gastrointestinal disorders, without side effects compared to chemical additives, which can cause allergy, and other side effects such as disruption of the gut

microbial balance.
This research aimed to study the influence of freeze-dried cell-free supernatant of lactic acid bacteria as additive in preservation of pineapple juice.

2. Methodology

2.1 Sample collection

Fruit samples such as pineapple and soursop used in this study were obtained from the Bodija market and Oje market, Ibadan, Oyo State, Nigeria. The healthy fruits samples were collected into labeled sterile containers without washing and transported to the laboratory for microbial analysis. The lactic acid bacteria used in this study were obtained from the healthy fruits (pineapple and soursop). Only pineapple juice was used for the influence study because it is readily available and less expensive compared with soursop. The pathogenic strain, *Escherichia coli* was sourced from the diarrheic stool of a patient, at the University College Hospital, Ibadan, Oyo State.

2.1.1 Sample preparation

Fruit samples were prepared according to the method of Compaore [27]. Each 25 g sample was blended with 225 mL sterile distilled water. 1 mL of the homogenate was added into 9 mL of the distilled water in a test tube, labeled as 1:10 (10^{-1}) dilution. This process was serially continued in five additional test tubes, labeled 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}. The same procedure was applied to each sample, ensuring that the blender was thoroughly cleaned and disinfected with 70% ethanol in between uses to avoid cross contamination. Isolation and characterization of lactic acid bacteria ((*Lactiplantibacillus* spp. and *Levilactobacillus brevis)* were done using established microbiological and biochemical methods [28-31].

2.2. Influence of antimicrobial compounds from lactic acid bacteria (as additive in pineapple fruit juice) on the survival of food borne pathogen

2.2.1 Purification of antimicrobial compounds from lactic acid bacteria

Lactobacillus strains (*Lactiplantibacillus* spp. and *Levilactobacillus brevis)* having antimicrobial activity against pathogenic organisms were cultured in MRS broth (Hi-Media, India) at 37 ^{o}C for 48 hours**,** then subjected to centrifugation at 4000 rpm for 15 minutes at 4 °C, and the supernatant were filtered and sterilized using Millipore membranes filter (0.45 µm pore size, Merck, Sigma-Aldrich). The supernatants were freeze-dried by helding in a freezer at -30 ^{o}C and left overnight to be adequately frozen. The efficiently frozen samples were then loaded into the freeze-drying machine (Gallenkhamp UK) running under constant temperature of -65 ℃ and sample temperature of -45 ^{o}C.
Samples were left on the lyophilizer until sufficiently dried, and then lyophilized product was obtained [32].

2.3. Preparation of pineapple juice

Wholesome pineapples were peeled, blended and filtered with the muslin cloth to obtain the juice. The fruit juice samples (500 mL) were pasteurized at 80-82 ℃ for 10 minutes inside a water bath and subsequently cooled to 4 °C. The pasteurized juice sample inoculated overnight with a culture of the pathogenic strain *Escherichia coli* (1 x 10^8 colony forming unit/mL), and freeze-dried lactic acid bacteria metabolity were introduced into the juice as additive at different concentration, 2%, 4%, 6%, 8% and 10% of 20 mL of juice sample, juice without additive serve as negative control and positive control using sodium benzoate (0.2 g/mL) as chemical preservative.

2.4 Determination of survival potential of selected pathogen in pineapple juice

One millilitre of overnight culture containing 10^8 colony forming unit/mL of standard inoculums of pathogenic strain *Escherichia coli* was aseptically inoculated into 20 mL of prepared pineapple juice sample. Serial dilutions of the samples were made in sterile distilled water at various time intervals for the detection of the inoculated pathogen by the pour plate method.

The test pathogen survival and growth were monitored by periodically withdrawing 1 mL samples under aseptic conditions at 24-hour intervals (0-6 days) and incubating at 37 °C for 24 hours to enumerate microbial populations [33-34].

2.5 Sensory evaluation of the fruit juice sample treated with freeze-dried supernatant of lactic acid bacteria

An untrained panelist group comprising of fifteen members: 8 females and 7 males among students on Industrial attachment from various University and Polytechnic in Nigeria and staff of Nigerian Stored Products Research Institute, Dugbe, Ibadan, Oyo State, Nigeria, were involved in the sensory evaluation test. The ages of those that participated ranging from 20-53 years with over 80% of them were having post-secondary education. Also, the participants were informed that the fruit juice samples served was prepared from pineapple and additive from Lactic acid bacteria. The pineapple fruit juice treated with freeze-dried supernatant of lactic acid bacteria (*Lactiplantibacillus* spp. and *Levilactobacillus brevis)* were refrigerated, poured into transparent disposable cups and presented randomly to a panelist simultaneously. Panelists were equally presented with portable drinking water to rinse their mouth between sample tastings to ensure accurate flavor assessments. The panelists rated the pineapple fruit juice samples for mouthfeel, taste, flavor, aroma, color and overall acceptability (OVAC) based on a 9-point Hedonic scale where 1 and 9 represent "dislike extremely" and "like extremely" respectively [35].

3. Results and discussion

The sensory properties of pineapple juice with freeze-dried supernatant of LAB as additive was shown in Table 1. The sensory properties of pineapple fruit juice with freeze-dried supernatant of *Lactiplantibacillus* spp., *Levilactobacillus brevis* and the control ranged as follows: Mouthfeel (6.00-8.07), taste (6.00-7.93), flavor (6.07-7.20), aroma (6.53-7.80), color (6.33-8.40) and overall acceptability (6.53-8.13), respectively. Fruit juice samples supplemented with freeze-dried cell-free extract of *Lactiplantibacillus* spp. and *Levilactobacillus brevis* had brown coloration while the control was yellow. The sensory properties of pineapple fruit juice containing freeze-dried cell-free extract of *Lactiplantibacillus* spp., *Levilactobacillus brevis* likewise the control was all organoleptically accepted by the assessors. The order of preference for the fruit juice samples are: Control>555>666 i.e. control sample followed by fruit juice sample with cell free supernatant of *Lactiplantibacillus* spp. and then *Levilactobacillus brevis*.

The overall acceptability of the fruit juice by the assessors is not affected by any of the additives. Tropical fruit juices, mainly pineapple, might increase the aroma and flavor of the final product positively based on consumers' decision [21]. In similar research, probiotics have no negative effect on the overall acceptability of juice products e.g., pineapple juice enriched *Lactobacillus reuteri* [36], fermentation of fresh apple beverage by *Lactobacillus casei* [37], apple juice [38]. Interestingly, a noticeable color change (yellow to brown) was observed in fruit juice samples containing the freeze-dried supernatant of *Lactiplantibacillus* spp. and *Levilactobacillus brevis*. This color change could be due to the color of the freeze-dried supernatant, MRS broth in which the lactic acid bacteria were grown, while the aroma and characteristic flavor of *Lactiplantibacillus* spp. which was rated high by the assessors in comparison with *Levilactobacillus brevis* may be due to the quantity of diacetyl compound in *Lactiplantibacillus* spp. (0.77 g/L) which was higher than that of *Levilactobacillus brevis* (0.43 g/L) as studied and reported by Alimi [17].

Table 1.
Sensory properties of pineapple juice with freeze-dried, cell-free supernatant of LAB as additive

Sample	Mouthfeel	Taste	Flavor	Aroma	Color	Overall acceptability
555	$6.33^{a}\pm1.68$	$6.40^{a}\pm1.60$	$6.67^{ab}\pm1.18$	$6.87^{a}\pm1.36$	$6.33^{a}\pm1.99$	$6.87^{a}\pm0.83$
666	$6.00^{a}\pm0.93$	$6.00^{a}\pm1.00$	$6.07^{a}\pm1.22$	$6.53^{a}\pm0.74$	$6.40^{a}\pm1.24$	$6.53^{a}\pm1.09$
Control	$8.07^{b}\pm0.96$	$7.93^{b}\pm0.80$	$7.20^{b}\pm0.77$	$7.80^{b}\pm1.01$	$8.40^{b}\pm0.73$	$8.13^{b}\pm0.35$

mean values followed by different alphabet within a column are significantly different ($p\leq0.05$)
Ovac: Overall acceptability.
555: Fruit juice sample with *Lactiplantibacillus* spp.
666: Fruit juice sample with *Levilactobacillus brevis*.
Control: Pineapple fruit juice sample unto which no additive introduced

The survival of pathogenic bacteria in pineapple juice supplemented with freeze-dried *Lactiplantibacillus* spp. and *Levilactobacillus brevis* at varying concentrations (2%, 4%, 6%, 8%, and 10%) is presented in Figures I and II. The results indicate that the survival rate of *Escherichia coli* decreased in juice samples containing freeze-dried supernatant from *Lactiplantibacillus* spp. and *Levilactobacillus brevis* at all tested concentrations, similar to juice with sodium benzoate, when compared to the control (juice without any additive). Furthermore, the findings reveal that higher concentrations of *Lactiplantibacillus* spp. and *Levilactobacillus brevis* resulted in a greater reduction in the microbial load of pathogenic *Escherichia coli*.

Freeze-dried *Lactiplantibacillus* spp. and *Levilactobacillus brevis* isolated from healthy fruits demonstrated a strong inhibitory effect on pathogenic *Escherichia coli* across various concentrations. No growth of *Escherichia coli* was observed at 8% *Lactiplantibacillus* spp. on day 4, while counts for *Levilactobacillus brevis* ranged between 3.3–4.2 $\log_{10}$ cfu/ml. Additionally, no *Escherichia coli* growth was detected at 2%, 8%, and 10% concentrations of *Levilactobacillus brevis*. The freeze-dried cell-free supernatants of both strains exhibited significant inhibitory effects, with 8% and 10% *Levilactobacillus brevis* and 4% *Lactiplantibacillus* spp. completely eliminating *Escherichia coli.*

The freezed-dried cell-free supernatant of Lactic acid bacteria used in this study had significant impact on the pathogenic *Escherichia coli*, this could be traced to the synergistic action of the compounds from Lactic acid bacteria which include lactic acid, hydrogen peroxide, diacetyl and bacteriocin. The pH values of pineapple juice samples were monitored during storage, as shown in Table 2.

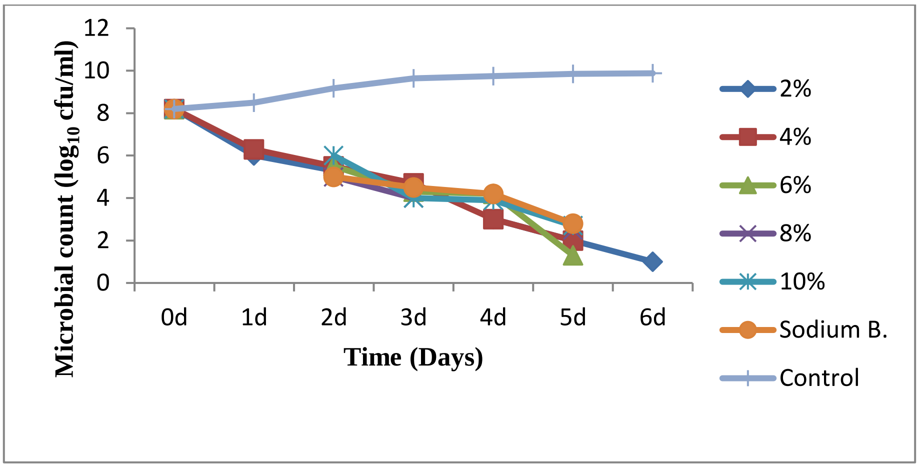

Fig. 1. Survival of *E. coli* in fruit juice mixed with freeze-dried supernatant of *Lactiplantibacillus* spp.

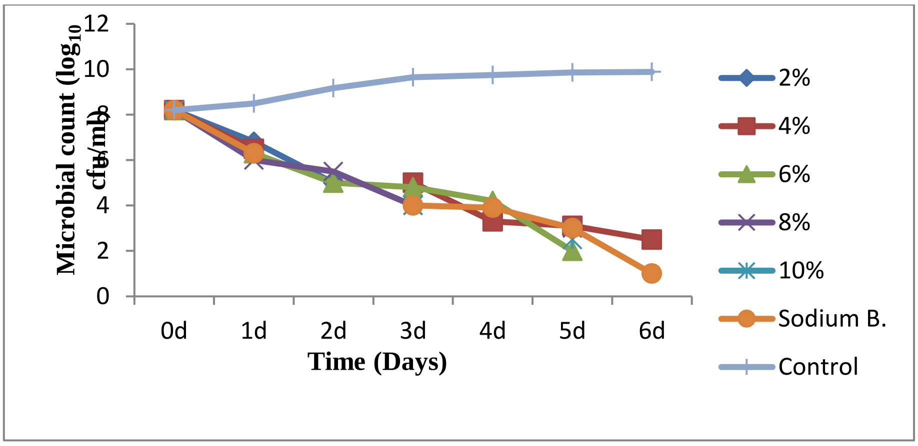

Fig. 2. Survival of *E. coli* in fruit juice mixed with freeze-dried supernatant of *Levilactobacillus brevis*

The study found that the pH of pineapple juice supplemented with freeze-dried *Lactiplantibacillus spp*. increased from 4.31 to 4.41, and that mixed with *Levilactobacillus brevis* rose from 4.32 to 4.40 during the first 72 hours (Day 1 to Day 3) of storage. In contrast, the pH of the control sample (juice without additives) decreased within two days. From Day 4 to Day 6, a gradual decline in pH was observed in the juice samples with freeze-dried *Lactiplantibacillus* spp. and *Levilactobacillus brevis.*

Table 2.

pH monitoring of pineapple juice samples during storage

Fruit Juice Sample	**Period of Storage (pH/Day)**					
	1	2	3	4	5	6
LP ((555)	4.31	4.38	4.41	4.28	4.00	3.88
LB (666)	4.32	4.37	4.40	4.26	4.10	3.94
C (777)	3.58	3.52	3.26	3.17	3.09	2.96

LP (555): Pineapple juice sample supplemented with *Lactiplantibacillus* spp.
LB (666): Pineapple juice sample supplemented with *Levilactobacillus. brevis*
C (777): Pineapple juice sample unto which no additive was added.

In order to increase the preservation time (shelf stability) of pineapple juice, the influence of the antimicrobial compounds produced by *Lactiplantibacillus* spp. and *Levilactobacillus brevis* as additives to act as natural antimicrobial agents was studied. Over a six-day storage period, the survival rate of pathogenic *Escherichia coli* in pasteurized pineapple juice varied depending on the concentration (2%, 4%, 6%, 8%, and 10%) of the additives. A reduction in microbial load was observed across all concentrations when compared to the control. This inhibitory effect is likely attributed to the synergistic action of the bioactive compounds produced by lactic acid bacteria, including lactic acid, hydrogen peroxide, diacetyl, and bacteriocin. Among these, lactic acid is the primary organic acid generated during lactic acid fermentation [39]. Lactic acid demonstrates antimicrobial activity as a result of the diffusion of its non-ionized molecules into bacterial cells, where they target and disrupt intracellular enzymes [40]. Hydrogen peroxide has been identified as an antimicrobial agent in both water and food systems, with gram-negative organisms being particularly vulnerable [41]. Additionally, hydroxyl radicals generated by hydrogen peroxide can damage cell membranes, leading to increased lipid peroxidation and heightened membrane permeability [42]. Due to its strong oxidizing properties, hydrogen peroxide exhibits potent antimicrobial and antiviral effects, effectively targeting bacteria, fungi, molds, viruses, and bacteriophages [43]. Diacetyl effectively inhibits the growth of Gram-negative bacteria, such as *Escherichia coli*, by interfering with arginine utilization [44]. Compared to Gram-positive bacteria, Gram-negative bacteria exhibit greater susceptibility to the inhibitory effects of diacetyl [45]. Another study highlighted that a combination of peptide-based compounds (Bacteriocin) derived from *Lactiplantibacillus* spp. and *Lactobacillus lactis* subsp. lactis effectively reduced the microbial load of *Escherichia coli*, *Salmonella*, and *Shigella* on coated pineapple slices after a 5-day refrigeration period [46].

In a study, it was reported that *Lactiplantibacillus plantarum* identified by MALDITOF-MS, morphology, and biochemical characterization showed the best activity against *Escherichia coli* and *Staphylococcus aureus* [47].

The observed fluctuations in pH during storage were anticipated. As noted by Muhialdin et al. [48], the supernatant of certain Lactic Acid Bacteria (LAB) strains demonstrated antimicrobial activity across a wide pH range, from 3 to 9. The reduction in pH observed during the storage of fruit juice mixed with *Lactiplantibacillus* spp. and *Levilactobacillus brevis* is likely due to the activity of bioactive compounds produced by lactic acid bacteria, including lactic acid, hydrogen peroxide, diacetyl, and bacteriocins [8]. This allows fermentation to take place which inhibits the proliferation of some microorganisms thus, prolonging the shelf life and safety of the fruit juice.

4. Conclusion

Freeze-dried cell-free supernatants of *Lactiplantibacillus* spp. and *Levilactobacillus brevis*, derived from healthy pineapple and soursop demonstrated significant inhibitory effects on pathogenic *Escherichia coli* at various concentrations might be due to synergistic action of bioactive compounds in the Lactic acid bacteria strains. Notably, 8% and 10% freeze-dried cell-free supernatants of *Levilactobacillus brevis* and 4% freeze-dried cell-free supernatants of

Lactiplantibacillus spp. completely eradicated *Escherichia coli*. The inhibitory efficiency of the lactic acid bacteria strains in this study was influenced by factors such as species and strain type, concentration and nature of antimicrobial compounds, and environmental conditions. The freeze-dried supernatant of these LAB strains, *Lactiplantibacillus* spp. and *Levilactobacillus brevis* shows potential as a natural preservative against *Escherichia coli*-induced diarrhea in pineapple juice.

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