

*Original Contribution***BIOMASS YIELD AND ANTIMICROBIAL SUBSTANCES PRODUCTION IN LIMITING – NUTRIENT CULTURES OF LACTOBACILLUS SPECIES****I. F. Fadahunsi\***

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**ABSTRACT**

Lactic acid bacteria had been reported to produce antimicrobial compounds such as lactic acid, hydrogen peroxide and diacetyl which are inhibitory to pathogenic bacteria. Attempt was therefore made in this study to screen for the antimicrobial property of these metabolites and to optimize their production based on growth nutrient formulation or by varying some components of the growth medium (De Mann Rogosa Sharpe i.e MRS) Four species of lactobacillus were isolated from fermented beverages such as ogi, kunnuzarki and pito and were identified as *L. plantarum*, *L. delbrueckii*, *L. reuteri* and *L. acidophilus*. The result obtained from the screening of the antimicrobial compounds showed that some indicator organisms were inhibited while some were resistance to the metabolites of the LAB species. The nutrient limiting study revealed that varying glucose concentration significantly affected the biomass yield but did not show significant effect on the production of antimicrobial compounds. ( $p > 0.05$ ) However, the effect of varying peptone concentration revealed that there was significant effect on the antimicrobial compounds secreted while the biomass yield were insignificantly affected. At 1.0% concentration of peptone (control) a biomass of  $20.00 \pm 0.14$  mg/ml was recorded and the corresponding antimicrobial compounds produced were  $19.00 \pm 0.16$  g/l of lactic acid,  $0.360 \pm 0.14$  g/l of hydrogen peroxide and  $1.90 \pm 0.26$  g/l of diacetyl, were recorded in *L. Plantarum* while at 0.5% concentration of peptone, a biomass of  $19.68 \pm 0.43$  mg/l was produced while the corresponding antimicrobial compounds were  $25.00 \pm 0.48$  g/l of lactic acid,  $0.54 \pm 0.17$  g/l of hydrogen peroxide and  $2.80 \pm 0.71$  g/l of diacetyl. However, at 0% concentration of peptone a biomass of  $19.59 \pm 0.39$  mg/l was noted while the corresponding antimicrobial compounds were  $17.00 \pm 0.74$  g/l of lactic acid,  $0.33 \pm 0.12$  g/l of hydrogen peroxide and  $1.70 \pm 0.22$  g/l of diacetyl. This trend was equally observed in all the species used. The limiting effect of tween-80 revealed that there was significant difference on both the biomass and antimicrobial substances produced. It could be concluded from this study that metabolites produced by LAB possess inhibitory properties and the production of these substances can be optimized by varying the concentration of some of the components constituting the growth medium.

**Key words:** Lactobacillus species, antimicrobial substances, biomass yield, nutrient-limiting.

**INTRODUCTION**

The antimicrobial properties of Lactic acid bacteria are of special interest in developing strong competitive starter cultures and utilization as biocontrol or biopreservative agent.

A subgroup of naturally occurring antimicrobial compounds are produced by lactic acid bacteria, (e.g *Lactobacillus*, *Lactococcus*, *Pediococcus* species) (1). They are used to inhibit spoilage microorganisms and, thus, prolong shelf life and preserve food quality. The antimicrobial

effect of lactic acid bacteria has been appreciated by man for more than 100 years (2). These compounds produced by LAB exhibit broad spectrum antagonistic property to microorganisms (3). The characteristics aroma, flavor and sensory qualities of fermented food emanated from the action of the metabolites of lactic acid bacteria (4) used to ferment or produced them.

The higher the concentrations of the antimicrobial agent, the more rapid microorganisms are destroyed, though in some situations a lower concentration is sufficient to use.

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They are non-pathogenic bacteria that play a vital role in our everyday life from production of wholesome foods, vitamins and prevention of certain diseases due to their antimicrobial property(5). LAB grows under anaerobic conditions, (6) mesophylic in nature and can grow at temperature ranging from 5<sup>0</sup>C – 45<sup>0</sup>C (7) These organisms are weakly proteolytic and lypolitic which are of interest to the food industry. The conventionally used phenotypic method in the identification of LAB (8) is gradually giving way to genetic techniques such as 16s rDNA sequencing which allows for a more consistent and accurate method of identification of individual strains (9) In addition to the organic acids (lactic acid, acetic acid), they also produced antimicrobial substances such as hydrogen peroxide, diacetyl and bacteriocins (10). Innovative approaches had been attempted as an alternative to antibiotic in the treatment of gastro-intestinal diseases and these include using livebio-therapeutic agents such bacterial isolates of LAB (11).

LAB competes with other microbes by modifying the microenvironment with their metabolic end products (12, 13). Consumption of LAB has some beneficial effects which include enhancing the immune system (14) improving health of the intestinal tract, synthesizing and enhancing bioavailability of nutrients, reducing symptoms of lactose intolerance (15) reducing risk of certain cancers and decreasing the prevalence of allergy in susceptible individuals

(16) This study was therefore carried out to screen for antimicrobial activities of lactic acid bacteria and investigate the effects of varying glucose, peptone and tween-80 concentrations on the production of biomass and antimicrobial compounds in some selected species of *Lactobacillus* isolated from traditionally fermented African foods.

## MATERIALS AND METHODS

### Isolation procedure

Isolation of *lactobacillus* spp. from fermented Kunni-zarki, pito and ogi was out carried by employing the serial dilution method of Meynell and Meynell (17) and using the pour plate method of Harrigan and McCane (18). The medium employed for isolation was de Mann Rogosa and Sharpe agar. The pH of the medium was adjusted to 5.5 and incubation was carried at 37<sup>0</sup>C anaerobically for 48h.

### Method of identification

Identification of pure cultures of the isolates was carried out using the API 50CH strips and API50CH medium (API system MontalieuVercieu France).

### Screening for Antimicrobial activity

The agar well diffusion method of Schillinger and Lucke (19) was employed. The plates were observed for zone of inhibitions which were indicated (+) where observed and negative (-) where not seen.

**Table 1.** Nutrient limiting formulation

Number/ code	Glucose (g/l)	Peptone (g/l)	Tween-80 (ml/l)
*1	20	10	1
2	10	10	1
3	0	10	1
4	20	5	1
5	20	0	1
6	20	10	05
7	20	10	05

\* Control

The nutrient limiting formulation is shown in **Table 1**. MRS broth (De mann Rogosa and sharpe medium) was prepared by varying the

concentrations of glucose, peptone and tween-80 components of the medium (20). Each medium preparation was dissolved per liter of distilled

water and 50ml aliquots was dispensed into 150ml Erlenmeyer flasks in four replicates and sterilized at 121°C for 15mins. The flasks were separately inoculated with 0.1ml (5.0x10<sup>4</sup>cfu/ml) 24h old culture of *L.plantarum*, *L.reutei* and *L.delbrueckii* and *L. acidophilus* and incubated anaerobically at 37°C for 48h.

### Biomass measurement

Broth in each Erlenmeyer flask was centrifuged (Bechman J-MI model) and the supernatant was decanted into sterile McCartney bottles while the pellets were dried in an oven at 80°C for 24h. This was weighed to obtain the dry biomass, Un-inoculated flask served as control.

### Quantitative measurement of Antimicrobial compounds

**Lactic acid:** Three drops of phenolphthalein indicator were added to 25ml of each supernatant, in 150ml Erlenmeyer flask and titrated with 0.1M sodium hydroxide until a pink colour appeared. One ml of 0.1M sodium hydroxide solution is equivalent to 90.80g of lactic acid (21).

**Diacetyl:** This was determined by transferring 25ml of supernatant into 150ml Erlenmeyer flask and 7.5ml of hydroxylamine solution was used for residual titration with 0.1M hydrochloric acid (HCl) to a greenish yellow end point using bromophenol blue as an indicator. The equivalent

factor of hydrochloric acid to diacetyl is 21.5mg (21).

**Hydrogen peroxide:** Twenty ml of 0.1M dilute sulphuric acid was added to 25ml of the supernatant and was titrated against 0.1M potassium permanganate solution. A decolourization of the sample was regarded as the point. One ml of 0.1M potassium permanganate is equivalent to 1.7mg of hydrogen peroxide (21).

### RESULTS

Four species of lactobacillus were isolated from ogi, pito and kunni-zarki. Their identities were confirmed as *L. plantarum*, *L. delbrueckii*, *L. acidophilus* and *L. reuteri*.

**Table 2** shows Susceptibility of indicator organisms to antimicrobial substance produced by LAB Spp. the antagonistic pattern of the metabolites produced by the LAB species against selected bacterial pathogens while **Table 3** shows the effect of varying glucose concentrations on biomass yield and antimicrobial compounds production by Lactic acid bacteria.

**Table 4** shows the effect of limiting peptone concentration on biomass yield and antimicrobial production by Lactic acid bacteria.

**Table 5** shows the effect of varying tween-80 concentration on biomass yield and antimicrobial compounds production by Lactic acid bacteria.

**Table 2.** Susceptibility of indicator organisms to antimicrobial compounds produced by LAB Spp

	<i>S. aureus</i>	<i>E. coli</i>	<i>S. sonnei</i>	<i>S. flexneri</i>	<i>S. dysenteriae</i>	<i>S. enteritidis</i>	<i>S. typhi</i>	<i>S. typhimurium</i>	<i>V. cholerae</i>
<i>L. plantarum</i>	-	+	+	++	++	-	-	++	+
<i>L. delbrueckii</i>	-	++	+	+	++	-	-	+	+
<i>L. acidophilus</i>	+	+	+	-	-	+	-	-	++
<i>L. reuteri</i>	+	++	+	-	+	+	-	+	-

The antimicrobial screening procedures showed that they produced antimicrobial compounds such lactic acid hydrogen peroxide and diacetyl which inhibited some of the indicator microorganisms tested but did not inhibit some. Metabolites obtained from *L. plantarum* and *L. delbrueckii* inhibited *E. coli*, *S. sonnei*, *S. flexneri*, *S. dysenteriae*, *S. typhimurium*, and *V. cholera* but did not inhibit *S. aureus*, and *S. typhi*,

while *L. acidophilus* inhibited, *S. aureus*, *E. coli*, *S. sonnei*, *S. enteritidis* and *V. cholerae* but did not show inhibition to *S. flexneri*, *S. dysenteriae*, *S. typhi*, *S. typhimurium*. Inhibition zones were seen around *S. aureus*, *E. coli*, *S. sonnei*, *S. dysenteriae*, *S. enteritidis* and *S. typhimurium* while there was no inhibition zone around *S. flexneri*, *S. typhi*, and *V. cholerae* when the metabolites from *L. reuteri* was tested (**Table 2**).

**Table 3.** Effect of limiting glucose concentration on biomass yield and antimicrobial compounds production by Lactic acid bacteria

Antimicrobial/Biomass		<i>L. plantarum</i>	<i>L. reuteri</i>	<i>L. acidophilus</i>	<i>L. delbrueckii</i>
Lactic Acid (g/l)	G1	19.00±0.16a	14.00±0.30a	19.00±0.44a	15.00±0.36a
	G2	18.80±0.16a	13.70±0.14a	18.70±0.36a	14.60±0.39a
	G3	18.50±0.56a	13.60±0.06a	18.50±0.30a	14.50±0.08a
Hydrogenperoxide (g/l)	G1	0.360±0.14a	0.280±0.10a	0.310±0.14a	0.262±0.02a
	G2	0.358±0.07a	0.278±0.13a	0.307±0.29a	0.261±0.06a
	G3	0.365±0.11a	0.275±0.04a	0.306±0.07a	0.260±0.11a
Diacetyl(g/l)	G1	1.90±0.26a	1.40±0.39a	1.60±0.04a	1.30±0.08a
	G2	1.86±0.11a	1.36±0.0.8a	1.59±0.20a	1.30±0.02a
	G3	1.85±0.22a	1.35±0.08a	1.55±0.10a	1.25±0.00a
Biomass (mg/l)	G1	20.00±14a	12.00±13a	19.00±16a	13.00±0.07a
	G2	17.00±0.11b	9.00±0.28b	17.00±0.71b	10.00±0.11b
	G3	7.05±0.47c	5.00±0.07c	8.00±0.36c	4.00±0.01c

Means followed by the same letter(s) are not significant different ( $p > 0.05$ ) Duncan's multiple

range tests. Each value represent mean of three replicates.

**Table 4.** Effect of limiting peptone concentration on biomass yield and antimicrobial production by Lactic acid bacteria

Antimicrobial/Biomass		<i>L. plantarum</i>	<i>L. reuteri</i>	<i>L. acidophilus</i>	<i>L. delbrueckii</i>
Lactic Acid (g/l)	P1	19.00±0.44c	14.00±0.30c	19.00±0.44a	15.00±0.36c
	P2	25.00±0.48c	25.00±0.48c	18.70±0.36a	23.60±0.65a
	P3	22.00±0.74b	22.00±0.74b	18.50±0.30a	20.00±0.47b
Hydrogenperoxide (g/l)	P1	0.36±0.014c	0.36±0.014c	0.310±0.14a	0.31±0.56c
	P2	0.54±0.17a	0.54±0.17a	0.307±0.29a	0.55±0.24a
	P3	0.50±0.12b	0.50±0.12b	0.306±0.07a	0.40±0.05b
Diacetyl(g/l)	P1	1.90±0.26c	1.90±0.26c	1.60±0.04a	1.30±0.08c
	P2	2.80±0.71a	2.80±0.71a	1.59±0.20a	2.10±0.9a
	P3	2.60±0.22b	2.60±0.22b	1.55±0.10a	1.50±0.7b
Biomass (mg/l)	P1	20.0±0.11a	12.00±0.13a	19.00±16a	13.00±0.07a
	P2	19.68±0.43a	19.68±0.43a	17.00±0.71b	12.60±0.28a
	P3	19.59±0.39a	19.59±0.39a	8.00±0.36c	12.55±0.39a

Means followed by the same letter(s) are not significant different ( $p > 0.05$ ) Duncan's multiple

range tests. Each value represent mean of three replicates.

**Table 5.** The Effect of Limiting Tween-80 concentration on biomass yield and antimicrobial compounds production by Lactic acid bacteria

Antimicrobial/Biomass		<i>L. plantarum</i>	<i>L. reuteri</i>	<i>L. acidophilus</i>	<i>L. delbrueckii</i>
Lactic Acid (g/l)	T1	19.00±0.16a	14.00±0.30a	19.00±0.44a	15.00±0.36a
	T2	16.70±0.71a	11.00±0.51b	16.00±0.51a	12.00±0.30a
	T3	12.00±0.11c	7.00±0.38c	13.15±0.05c	11.00±0.72c
Hydrogenperoxide (g/l)	T1	0.310±0.14a	0.28±0.1a	0.310±0.14a	0.20±0.02a
	T2	0.39±0.04a	0.15±0.21b	0.24±0.17b	0.20±0.10b
	T3	0.23±0.11c	0.12±0.01c	0.15±0.05c	0.14±0.01c
Diacetyl(g/l)	T1	1.60±0.26a	1.40±0.31a	1.60±0.04a	1.3±0.80a
	T2	1.60±0.04a	1.30±0.25b	1.30±0.12b	1.2±0.80b
	T3	1.40±0.25c	1.00±0.63c	1.10±0.63c	1.00±0.6c
Biomass (mg/l)	T1	20.00±0.14a	12.00±17a	19.00±16a	13.00±0.07a
	T2	17.00±0.44b	9.00±0.61a	15.00±0.28b	11.00±0.14b
	T3	14.00±0.06c	5.00±0.60c	12.00±0.07c	9.00±0.27c

Means followed by the same letter(s) are not significant different ( $p > 0.05$ ) Duncan's multiple range tests. Each value represent mean of three replicates.

It was observed that at 2% glucose concentration (control G1) the biomass yield recorded was  $20.00 \pm 0.14$  mg/l which decreased significantly ( $P > 0.05$ ) to  $17.00 \pm 0.11$  mg/l at 1% glucose concentration (G2) and finally decreased significantly to  $7.05 \pm 0.74$  mg/l at 0% conc. (G3). The antimicrobial compounds production showed that at 2% glucose concentration (control G1) the lactic acid produced decreased insignificantly from  $19.00 \pm 0.16$  g/l to  $18.80 \pm 0.16$  g/l at 1% glucose conc. (G2) and decreased insignificantly to  $18.50 \pm 0.56$  g/l at 0% (G3). The hydrogen peroxide recorded at 2% glucose concentration (control G1) was  $0.36 \pm 0.14$  g/l which decreased insignificantly to  $0.358 \pm 0.07$  g/l at 1% glucose conc. (G2) and further decreased to  $0.3568 \pm 0.11$  g/l at 0% conc. (G3), while the diacetyl produced at 2% conc. glucose (control G1) decreased insignificantly from  $1.90 \pm 0.26$  g/l to  $0.186 \pm 0.11$  g/l at 1% conc. (G2) and further decreased to  $1.85 \pm 0.22$  g/l at 0% conc. (G3) The same trend was observed in all the tested Lactic acid bacteria (**Table 3**).

The result of varying peptone concentration on biomass yield and antimicrobial compounds production in *L. plantarum* is shown in **Table 4**. The biomass yield recorded at 1% peptone conc (P1 control) was  $20.00 \pm 0.11$  mg/l which decreased insignificantly to  $19.68 \pm 0.43$  mg/l at 0.5% peptone conc. (P2) and further decreased to  $19.59 \pm 0.39$  mg/ml at 0% peptone conc. (P3) The lactic acid produced at 1% peptone conc. (control P1) was  $19.00 \pm 0.44$  g/l which increased significantly to  $25.00 \pm 0.48$  g/l at 0.5% peptone conc. (P2) and decreased significantly to  $17.00 \pm 0.07$  g/l at 0% conc. (P3) while the hydrogen peroxide produced at 1% peptone conc. (P1 control) increased significantly from  $0.36 \pm 0.14$  g/l to  $0.54 \pm 0.17$  g/l at 0.5% peptone conc. (P2) which decreased significantly to  $0.33 \pm 0.12$  g/l at 0% peptone conc. (P3). However, the quantity of diacetyl produced at 1% peptone conc. (control P1) was  $1.90 \pm 0.26$  mg/l which increased significantly to  $2.80 \pm 0.71$  g/l at 0.5% peptone conc. (P2) and decreased significantly to  $1.70 \pm 0.22$  g/l at 0% peptone conc. (P3) The same trend was observed in all the tested Lactic acid bacteria.

**Table 5** shows the result of the effect of varying tween-80 concentration on biomass yield and

antimicrobial compounds production in *L. plantarum*. At 1ml conc. of tween-80 (control T1) the biomass recorded was  $20.00 \pm 0.11$  mg/l which decreased significantly to  $17.00 \pm 0.44$  mg/l at 0.5ml conc. (T2) which further decreased to  $14.00 \pm 0.06$  mg/l at 0% conc. of tween-80 (T3) The lactic acid produced at 1.0ml conc. of tween-80 (control T1) was  $19.00 \pm 0.16$  g/l which decreased to  $16.00 \pm 0.71$  g/l at 0.5ml tween-80 conc. (T2) and further decreased significantly to  $12.00 \pm 0.11$  g/l at 0% conc. of tween-80 (T3) while the hydrogen peroxide recorded at 1.0ml conc. of tween-80 conc. (control T1) was  $0.36 \pm 0.14$  mg/l, which decreased to  $0.29 \pm 0.04$  mg/l at 0.5ml conc. of tween-80 (T2), and further decreased significantly to  $0.23 \pm 0.11$  g/l at 0ml conc. of tween-80 (T3). The diacetyl monitoring showed that  $1.90 \pm 0.26$  g/l was produced at 1ml conc. of tween-80 (control T1) which decreased significantly to  $1.600.22$  g/l at 0.5ml conc. (T2) and further decreased to  $1.40 \pm 0.25$  g/l at 0ml conc. (T3) The same trend was observed in all the tested Lactic acid bacteria.

## DISCUSSION

Four species of *Lactobacillus* were isolated from ogi, kuunu-zarki and pito and they were identified as *L. plantarum*, *L. reuter*, *L. acidophilus* and *L. delbrucekii*. This finding corroborates the earlier submission of Okereke and Montiville (22) which indicated that *Lactobacillus* species are predominantly wide spread in nature including African fermented food. Species of *Lactobacillus* are capable of inhibiting other microorganisms by their abilities to produce antimicrobial compounds (23). Several workers have reported the antimicrobial activities of LAB filtrates towards gram positive indicators (24, 25, 26, 27). In addition lactic acid produced by LAB lowers the pH in their growth environment and this property converts the ability to antagonize other bacteria (28). It is reported by Dike and Sanni (29) that the undissociated form of lactic acid penetrates the cell membrane thereby protonating the cell cytoplasm with hydrogen ions and this mediates the antimicrobial effect of disintegrating gradient resulting in bacteriocidal activity of lactic acid on targeted bacteria. Diacetyl is a compound produced by LAB which is responsible for flavouring in fermented foods and is antimicrobial in nature (30). This compound acts in synergy with organic acids and hydrogen peroxide to enhance antimicrobial property (31). The of ability of LAB to produce antimicrobial compounds e.g hydrogen peroxide

is due to the absence of catalase in these microorganisms that causes the accumulation of hydrogen peroxide ( $H_2O_2$ ) which inhibits bacteria and fungi (32). The antimicrobial effect of hydrogen peroxide can be linked to its oxidizing effect on the cell membrane of the pathogens that come in contact with it and destruction of the cellular protein (28). During fermentation, the carbon dioxide produced creates an anaerobic micro environment which is toxic to aerobic bacteria, and lactic acid is also produced that contributes to a decrease in pH (33, 11, 34). The nutrient limiting study revealed that the variation of glucose concentration showed a significant increase on the production of biomass because as glucose concentration is increased, the biomass increased correspondingly. This observation might be due to the ability of LAB species to rapidly utilize glucose because they possess a relatively efficient phosphoenol pyruvate dependent phosphotransferase system for glucose transport and metabolism which resulted in the production of biomass (35). In addition glucose is considered the main carbon source by all microorganisms due to its size, fast uptake, metabolism and cellular energy conversion. Cang (36) also reported that low glucose concentration repressed formation of antibiotic like substances when included in their growth medium. The variation of peptone showed an insignificant effect on the production of biomass but a significant effect on the production of antimicrobial compounds. It was observed that peptone at 1% concentration stimulated the production of small quantities of antimicrobial substances, but at 0.5% peptone concentration, the highest concentration of antimicrobial substances was recorded. This occurrence might have emanated from the resultant stress the organisms were exposed to by limiting its peptone requirement. Tween-80 variation had effect on both biomass yield and antimicrobial substances produced. As tween -80 concentration was increased, the biomass yield and the antimicrobial substances produced increased correspondingly. This occurrence might be caused by the surfactant nature of Tween-80, which increases the permeability of the cell membrane and this could accelerate diffusion of nutrient into the cell leading (37) to an increased uptake of nutrient by the LAB species with concomitant increase in biomass yield and production of antimicrobial compounds.

## CONCLUSION

It could then be concluded that for the optimization of biomass production, and antimicrobial compounds, there is need to note that the composition of MRS (de Mann Rogosa and Sharpe) medium should consist of 20g of glucose, 5g of peptone and 1ml of tween-80 in 1000ml of distilled water in the presence of other components in the standard specified quantities. The need to optimize production of biomass and antimicrobial substances of *Lactobacillus* species arise from the fact that they are food grade microorganisms with antimicrobial activities which inhibits many Gram-positive bacteria associated with food spoilage and food borne illness (24, 38) and retain this property after heat treatment in foods (39, 40). They are nontoxic and nonpathogenic to animals and thus they are used to enhance safety and shelf life of many foods (41, 33).

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