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Bio-preservative activities of *Lactobacillus acidophilus* U1during fermentation of fresh minced goat meat

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ABSTRACT
Objective: To assess the effect of bacteriocinogenic *Lactobacillus acidophilus* U1 (LacU1) isolated from pygmy goat meat on food borne pathogens and spoilage microorganisms during the fermentation of minced goat meat.
Methodology and results: Bacteriocin obtained from LacU1 was purified and characterized. Survival of food spoilage and pathogenic bacteria artificially inoculated aseptically in fermenting minced goat meat was investigated. The bacteriocin showed inhibitory activity against both spoilage and pathogenic microorganisms with activity of 12800AU/ml against *Enterococcus faecalis*. Development of an off-odour was prevented in minced goat meat inoculated with isolate *Lac*U1. In the fermenting goat meat, there was a sharp decrease in the population of inoculated pathogens within the first 48 h and a decrease to complete extinction at 72 h for *Staphylococcus aureus* and *Escherichia coli*, and 96 h for *Listeria denitrificans* and *Enterococcus faecalis*. The bacteriocin was heat stable at 60°C, with optimum activity of 12800(AU/ml) at pH 2 to 6. The activity was unstable or lost after treatment with proteolytic enzymes
Conclusions and application of findings: The use of bacteriocinogenic *L. acidophilus* U1 in meat as a starter culture can prevent proliferation of pathogenic and spoilage microorganisms which may cause deterioration when meat on sale is exposed to high temperature. This effect would extend the shelf life of meat products.

Key words: *Lactobacillus acidophilus* U1, bacteriocin, bio-preservation, meat

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INTRODUCTION

Meat is highly regarded in many parts of the world as a nutritious and highly desirable source of protein, fat, essential minerals, B vitamins and iron (Price & Schweigert, 1971). However meat is highly susceptible to spoilage and has been frequently implicated in the spread of food borne diseases (Ingram & Simensen, 1980). Microbial contamination can lower the quality of fresh meat, shorten its shelf life and result in economic loss and probably health hazards (Ichraq *et al*., 2004). In many developing countries inappropriate slaughtering facilities and techniques causes unnecessary loss of meat as well as valuable by-products thus reducing availability of animal protein.

Meat composition depends upon the animal species, the degree of fattening, the processing, packaging and storage method.

Pygmy goat meats are popular in West-Africa where there is a strong tradition of consuming lean meat (Devendra & Owen, 1983). In these areas, a number of methods are used to preserve meat including salting, cooking, drying and smoking. Fermentation is an inexpensive method that could also be used for preservation of meat and meat products. However, there is a dearth of information on the preservation of minced goat meat by fermentation. This study was undertaken to bridge the gap in knowledge using bacteriocinogenic *Lactobacillus acidophilus* as starter culture.

MATERIALS AND METHODS

Bacterial strains: Lactic acid bacteria were isolated from pygmy goat meat randomly collected from four abattoirs in south-western part of Nigeria. Ten grams of goat meat were added to 90 ml of sterile diluent containing 0.1% peptone water and homogenized for 30 s. From appropriate ten – fold dilutions, isolation of Lactic acid bacteria was carried out on MRS agar and incubated anaerobically at 30^0C for 48 h. Cultures were purified by repeated streaking on fresh media. Strains were characterized using AP1 50CH strips and AP1 50CHL medium (AP1 systems, Biomerienx sa, France). The pathogenic microorganisms used as indicator organisms were obtained from the culture collection of the Medical Microbiology Laboratory, University College Hospital, Ibadan, Nigeria.

Screening for bacteriocin production: *Lactobacillus* strains selected as test organisms were cultured in MRS broth (pH 5.5) for 48 h at 30°C in an anaerobic jar. Extraction of bacteriocin was carried out using the method of Schillinger & Lacke (1989). Inhibitory activity due to hydrogen peroxide was eliminated by the addition of 5 mg catalase/ml (C 100 bovine liver, Sigma) (Daba *et al*., 1991). The culture supernatant was purified according to the method of Ogunbanwo *et al*. (2003). Antagonistic activity against gram positive and gram negative bacteria and *Candida albicans* were determined using a well diffusion assay. The plates were examined for clear zone of inhibition around the well and the zone of inhibition was measured and recorded (Schillinger & Lacke, 1989). The antimicrobial activity of the bacteriocin was defined as the reciprocal of the highest dilution showing inhibition of the indicator organism and was expressed in activity units per ml (AU/ml). The most efficient strain was selected for use in subsequent meat preservation tests. The purified bacteriocin sample from the selected strain was characterized with respect to thermal and pH stability, susceptibility to denaturation by enzymes and treatment with dissociating agents (Ogunbanwo *et al*., 2003).

Growth and bacteriocin production by *L. acidophilus* U1 over time: Growth, pH change and bacteriocin production by the test strain were observed over a period of time. Active culture of producer organism (1% v/v) was inoculated into 100ml aliquots of sterile MRS broth in Erlenmeyer conical flask. Inoculated flasks were incubated at 30°C for 24 h. Bacteriocin activity (AU/ml), pH and absorbance values at 580 nm were determined.

Meat fermentation: One kilogram of fresh pygmy goat meat was deboned, sliced and minced with a sterile meat mincer in a sterile flask and the pH was checked before inoculation (Ichraq *et al*., 2004). The selected strain (identified as *Lactobacillus acidophilus* U1) was used to inoculate the minced pygmy goat meat mixed with 50g of glucose in sterile plastic bags and fermentation was allowed to continue for a period of 7days at ambient temperature (26±1.0^0C). Minced goat meat without glucose and *Lactobacillus acidophilus* U1 served as control.

Determination of pH change and lactic acid production during fermentation: The pH changes during meat fermentation was measured after every 24 hour intervals for 7 days using a pH meter (Model 291MKZ) lactic acid production was determined using juice extract of the minced meat to which 3 drops of phenolphthalein were added as indicator and the quantity of lactic acid was determined by titration with 0.1 M NaOH until a pink color appeared. Each milliliter of 0.1 M NaOH is equivalent to 90.08 mg of lactic acid (A.O.A.C., 1990).

Food spoilage and pathogenic bacteria in fermenting meat: Pure cultures of food spoilage and the pathogenic bacteria *Staphylococcus aureus* NCTC 6571, *Listeria denitrificans* ATCC 14870, *Escherichia coli* NCTC 104/8 and *Enterococcus faecalis* EF1 with an initial microbial load of about $6.0x10^5$ c.f.u/ml were singly used to inoculate the minced goat meat in the presence and absence of bacteriocinogenic *Lactobacillus acidophilus* U1 in five (5) replicates. Serial dilutions of the fermenting samples were made in peptone water at various time intervals for the detection of the inoculated spoilage and pathogenic bacteria by

the pour plate method. The plates were incubated at 37^{0}C for 24 h and colonies typical of each organism were counted and biochemical tests carried out to confirm identity.

Organoleptic evaluation: Minced goat meat fermented with bacteriocinogenic *Lactobacillus acidophilus* U1 and unfermented minced goat meat (control) stored at 23±3°C for 14 days were evaluated for sensory parameters based on appearance, colour, odour, texture and overall acceptability. The ratings were presented on 9-point Hedonic scale ranging from 9 = high acceptability to 1 = low acceptability. Low acceptability indicates spoilage of the meat.

Data analysis: Data were subjected to analysis of variance (ANOVA) means separated by Duncan's Multiple Range Test.

RESULTS

The selected bacterial isolate was identified as *Lactobacillus acidophilus* U1. It produced bacteriocin which showed inhibitory activity against its competitors as well as spoilage and pathogenic microorganisms, with the largest spectrum of inhibition (14 mm) against *Enterococcus faecalis* EF1. However, *Candida albicans* was not inhibited by the bacteriocin produced by *L. acidophilus* U1 (Table 1).

Growth, pH and activity of bacteriocin produced by *L. acidophilus* U1 are shown in table 2.

As the incubation period increased, the growth of *L. acidophilus* U1 and production of bacteriocin by *L. acidophilus* U1 increased while the pH of the culture medium decreased. At the end of 24 h incubation period, bacteriocin activity was 12800 AU/ml and pH was 3.7±0.1 (Table 2).

The effect of pH, temperature, enzymes and surfactants on the inhibitory activity of bacteriocin produced by *L. acidophilus* U1 is shown in table 3. Bacteriocin activity was stable at acidic pH and decreased as the pH increased. The bacteriocin was heat sensitive with activity stable at 40 to 60°C, and reducing as the temperature increased from 80 to 121° C. The bacteriocin activity was unstable or lost after treatment with the proteolytic enzymes α-chymo-trypsin, Pronase E, pepsin, trypsin and proteanase K, whereas treatment with lipase, catalase, phospholipase C, lysozyme, α-amylase and dextranase had no effect on the activity of the bacteriocin. Exposure to surfactants resulted in an increase in the bacteriocin titre by at least one fold dilution with the exception of nonidet P-40, which led to total loss of bacteriocin activity.

Table 1: Growth inhibition of various microorganisms by bacteriocin produced by *Lactobacillus acidophilus* U1.

Organism	Strain	Origin	*Inhibition zone*
Streptococcus thermophilus	IW4	Iru	-
L. brevis	OGW1	Ogi	-
L. plantarum	F1	Fufu	+ 4 (mm)
L. reuteri	PW1	Plam wine	+ 10 (mm)
L. acidophilus	U1	Meat	-
Micrococcus inteus	NCIBI96	Reference strain	+ 8 (mm)
Staphylococcus aureus	ATCC 14458	Reference strain	+ 6 (mm)
Staphylococcus aureus	NCTC6571	Reference strain	+ 8 (mm)
Staphylococcus epidermidis	NCTC5413	Reference strain	+ 8 (mm)
Listeria denitrificans	ATCC14870	Reference strain	+ 11(mm)
Listeria monocytogenes	587CHRL	Reference strain	+ 10 (mm)
Candida albicans	ATCC10231	Reference strain	-
Escherichia coli	NCTC10418	Reference strain	+ 12 (mm)
Escherichia coli	K12	Reference strain	+ 10 (mm)
Enterococcus faecalis	EF1	Reference strain	+ 14 (mm)
Salmonella typhimurium	ATCC13311	Reference strain	-

Key: - = resistant; + = sensitive; Zone of inhibition = mm

Table 2: Growth of *L. acidophilus* U1, pH of culture medium and bacteriocin production over time (mean±SD).

Time (h)	OD (580 nm)	pH	Bacteriocin Activity (AU/ml)
0	0.120±0.0	5.5±0.02	0.00±0.0
2	0.122±0.02	5.4±0.01	0.00±0.0
4	0.134±0.1	5.3±0.04	200±0.0
6	0.250±0.05	5.2±0.2	400±0.0
8	0.420±0.03	5.0±0.01	800±0.0
10	0.540±0.1	4.9±0.05	1600±0.0
12	0.630±0.01	4.7±0,1	3200±0.0
14	0.668±0.06	4.6±0.03	6400±0.0
16	0.721±0.1	4.4±0.01	12800±0.0
18	0.735±0.02	4.2±0.0	12800±0.0
20	0.765±0.07	4.0±0.1	12800±0.0
22	0.762±0.2	3.9±0.02	12800±0.0
24	0.760±0.05	3.7±0.1	12800±0.0

Table 3: Effect of pH, temperature, enzymes, and surfactants on activity of bacteriocin produced by *Lactobacillus acidophilus* U1.

Treatment	Bacteriocin Activity (AU/ml)	Temperature (^{0}C)	Bacteriocin Activity (AU/ml)
Untreated	12800	40	12800
pH		60	12800
2	12800	80	6400
4	12800	100	800
6	12800	121	400
8	6400		
10	1600		
12	400		
Enzymes		Surfactants	
Lipase	12800	Tween 20	25600
α - chymotrypsin	200	Tween 80	25600
Pronase E	400	Tritox X-100	25600
Pepsin	Not detected	Deoxycholic acid	25600
Catalase	12800	Sodium dodecyl sulphate	25600
Phospholipase C	12800	Nonidet P-40	Not detected
Trypsin	Not detected		
Lysozyme	12800		
α - Amylase	12800		
Dextranase	12800		
Proteinase K	Not detected		

As the fermentation progressed, the pH of minced goat meat inoculated with bacteriocinogenic *L. acidophilus* U1 decreased from pH 7.10±0.02 at 0 h to pH 4.12 ±0.01 after seven days. The pH of un-inoculated minced goat meat decreased from 7.16±0.03 to 6.95±0.01 during the same period. The quantity of lactic acid produced increased from 1.3±0.02 at day 0 to 6.0±0.1 g\l at day 7 in minced goat meat inoculated with bacteriocinogenic *L. acidophilus* U1 (Table 4).

Organoleptic evaluation of fermented and unfermented minced goat meat stored at 23^{0}C for 14 days revealed that as the storage time increased, the overall acceptability decreased. In minced goat meat inoculated with *L. acidophilus* U1, development of an off-odour was prevented while the non-inoculated meat had off-odour, which led to colour change during storage (Table 5).

The survival patterns of food spoilage and food borne pathogenic bacteria inoculated into non-fermenting and fermenting minced goat meat are shown in figures 1a-1d. The growth of all the pathogens increased as the storage time increased in the non-fermenting minced goat meat. However, in the minced goat meat fermented with bacteriocinogenic *L. acidophilus* U1, there was a sharp decrease in the population of pathogens within the first 48 h and a decrease to complete extinction at 72 h for *Staphylococcus aureus* NCTC 6571, and *Escherichia coli* NCTC 10418, and 96 h for *Listeria denitrificans* ATCC 14870 and *Enterococcus faecalis* EF1.

Fig. 1a:

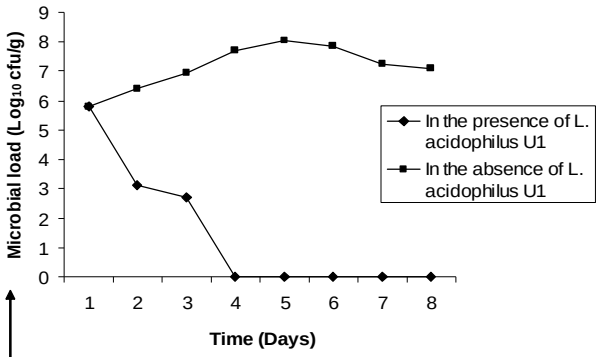

Fig. 1b:

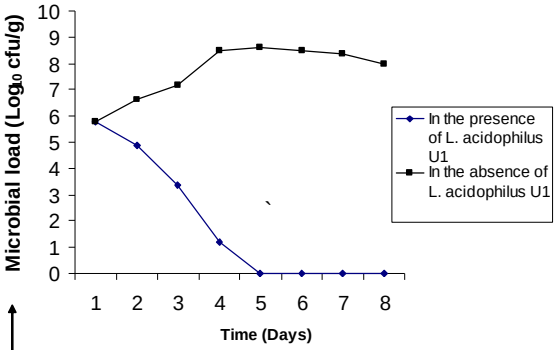

Fig. 1c:

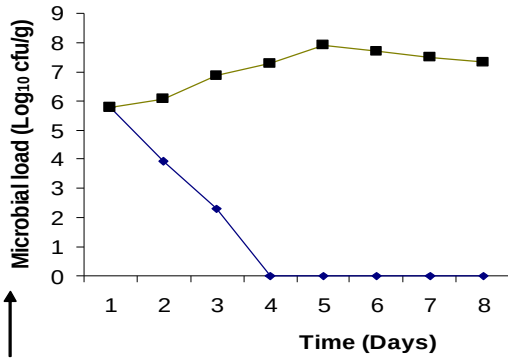

Fig. 1d:

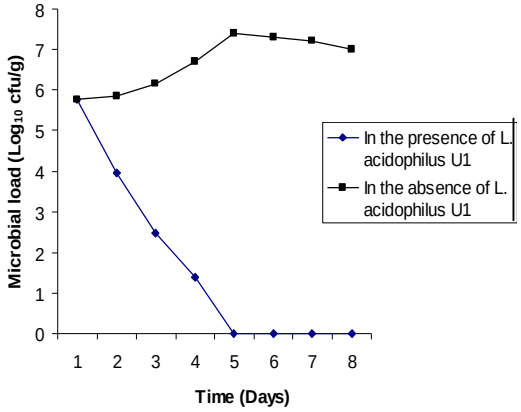

Figure 1: Survival of *S. aureus* NCTC6571 (a), *Listeria denitrificans* (b), *E. coli* NCTC10418 (c) and *E. faecalis* (d) in fermenting minced goat meat.

Table 4: pH changes and lactic acid production during fermentation of goat meat by *L. acidophilus* U1 (mean±SD).

Time (days)	Inoculated assay (Test)		Un-inoculated assay (Control)	
	pH	Lactic acid(g\l)	pH	Lactic acid (g\l)
0	7.10±0.02	1.3±0.02	7.10±0.05	1.3±0.01
1	6.20±0.05	2.5±0.01	6.97±0.02	2.2±0.1
2	5.65±0.01	3.8±0.04	7.00±0.01	1.9±0.02
3	4.92±0.01	4.6±0.2	7.09±0.01	1.5±0.05
4	4.20±0.02	4.9±0.05	7.20±0.02	1.1±0.02
5	4.52±0.01	5.4±0.01	7.16±0.03	1.2±0.01
6	4.37±0.04	5.7±0.02	7.00±0.01	1.9±0.01
7	4.12±0.01	6.0±0.1	6.95±0.01	1.1±0.02

Table 5: Organoleptic evaluation of fermented and unfermented minced goat meat stored at 4^{0}C for 14 days.

	Colour			Odour			Texture			Overall acceptability		
Samples	0d	7d	14d	0d	7d	14d	0d	7d	14d	0d	7d	14d
Fermented	8±0.2	6±0.4	5±0.2	8±0.1	7±0.2	6±0.5	7±0.2	6±0.3	5±0.1	8±0.2	7±0.4	5±0.2
Unfermented	9±0.1	4±0.2	2±0.1	8±0.3	5±0.4	1±0.2	8±0.1	5±0.2	3±0.2	9±0.5	5±0.1	2±0.1

Values are the means of fifteen replicates ± standard deviation

DISCUSSION

Lactobacillus acidophilus U1 isolated from pygmy goat meat was selected for this study based on its ability to produce bacteriocin with inhibitory activity against food spoilage and food borne pathogenic bacteria such as *Staphylococcus aureus*, *Listeria denitrificans*, *Listeria monocytogenes*, *Escherichia coli*, *Enterococcus faecalis* and *Micrococcus luteus*. Seauk – Hyun ko & Cheol Ahn (2000), isolated *Lactococcus lactis* KCA2386 from white Kimchi that produced bacteriocin which was active against not only other lactic acid bacteria but also a wide range of food pathogens.

The growth of *L. acidophilus* U1 and activity of its bacteriocin increased as the incubation period increased with bacteriocin activity of 12800 AU/ml at the end of 24 h incubation period. The bacteriocin activity detected in this study confirmed the report of Triona & Colin (1999) showing that most bacteriocins are produced during the active growth phase, and with a sharp decrease in activity at the end of the log phase due to protein degradation.

The treatment of active supernatant with catalase did not affect the initial bacteriocin titer, indicating that the inhibitory substance was not as a result of hydrogen peroxide, while the detection of inhibition when the active supernatant was adjusted to alkaline pH showed that inhibition was not due to low pH. However, the bacteriocin activity was unstable or lost after treatment with all the proteolytic enzymes used in this work, which suggests that the active substance could belong to bacteriocin class (Jimenz – Diaz *et al*., 1993). Exposure to surfactants resulted in an increase in the bacteriocin titre confirming that bacteriocin activity can be enhanced by the effect of chelators (Triona & Colin, 1999).

The decrease in pH of the fermenting minced goat meat inoculated with bacteriocinogenic *L. acidophilus* U1 would be due to the production of lactic acid by the organism. The increased production of lactic acid by *Lactobacillus species* has been attributed to lowered pH which permits the growth of lactic acid bacteria to the detriment of other competing organisms (Sanni *et al*., 1995).

The minced pygmy goat meat inoculated with bacteriocinogenic *L. acidophilus* U1 did not develop off-odour during storage. The improved stability of the meat could be due to production of bacteriocin coupled with the reduction of pH of the meat by the test organism during fermentation. According to Lindgren & Dobrogosz (1990), antimicrobial compounds such as lactic acid produced during fermentation cause a reduction of pH, but other substances such as hydrogen peroxide, formic acid, propionic acid, acetoin, diacetyl and bacteriocin are also produced. However, non-fermented minced goat meat showed severe deterioration characterized by off-odour and changes in colour, which could be due to high contamination, mincing effects and the high ambient temperature (Ichraq *et al.,* 2004).

The survival of spoilage and food borne pathogenic bacteria inoculated into non-fermented minced goat meat indicated that meat provides an excellent environment for growth of pathogens. Many pathogens can grow even in refrigerated storage, while others grow in ambient condition and when heat treatment is not sufficient i.e. when meat is not properly cooked. In the fermentation of minced goat meat, there was complete extinction of inoculated pathogens within 72 - 96 h of fermentation, which demonstrates the effectiveness of the bacteriocinogenic *L. acidophilus* U1.

Only few reports have been published about the bio-preservative activity of *L. acidophilus* strains, e.g. Kanatani *et al*. (1995) reported that *L. acidophilus* TK9201 produced bacteriocin that is active against different species of *Enterococcus*, *Lactobacillus*, *Pediococcus*, *streptococcus* and *L. monocytogenes*. The introduction of bacteriocinogenic *L. acidophilus* U1 in food as starter culture can be used to prevent proliferation of pathogenic and spoilage microorganism which may cause deterioration of meat on sale that is exposed to high temperature. In addition to direct application, genetic analysis of bacteriocin operons according (Triona & Colin, 1999) provides another opportunity for bacteriocin application, since the ability

to artificially over – produce the inhibitor would have cost benefits. We recommend that further toxicological research be carried out to strengthen the case for the safe use of bacteriocins in the food industry.

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