Turkish Journal of Agriculture - Food Science and Technology, 4(10): 887-892, 2016

Turkish Journal of Agriculture - Food Science and Technology

www.agrifoodscience.com,
Turkish Science and Technology

Antimicrobial Activities of Five Strains Of *Lactococcus* Isolated from Beef Against Indicator Organisms of Public Health Significance

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A R T I C L E I N F O

Article history:
Received 27 June 2016
Accepted 28 September 2016
Available online, ISSN: 2148-127X

Keywords:

Lactococcus
Antimicrobial agents
Spoilage and pathogenic organisms
Antimicrobial activities
Public health significance

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A B S T R A C T

Five strains of *Lactococcus*, including *L. garviae* K2, *L. piscium* SU4, *L. lactis* subsp. *cremoris* E22, *L. plantarum* L7 and *L. lactis* subsp. *hordinae* E91 were screened for production of antimicrobial agents. The strains were also analysed for antimicrobial activities against spoilage and pathogenic organisms, including *Staphylococcus* aureus, *Salmonella* Typhimurium, *Escherichia coli*, *Yersinia enterocolitica*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Listeria monocytogenes*, *Bacillus cereus* and *Pseudomonas fluorescens*. Result revealed that *L. piscium* SU4, *L. plantarum* L7 and *L. lactis* subsp. *cremoris* E22 had lactic acid production (g/10^7 colony forming units, CFU) of 7.23, 7.20 and 7.19. The value of 6.83 recorded as lactic acid produced by *L. garviae* K2 was significantly different from those obtained for others. The highest acetic acid production (3.55 g/10^7 CFU) was recorded for *L. garviae* K2 while *L. piscium* SU4 had the lowest (2.99 g/10^7 CFU). *L. lactis* subsp. *hordinae* E91 had diacetyl production of 71.99 (µg/10^7 CFU), which was higher than those recorded for other *Lactococcus* strains. Test for antimicrobial activities showed that *Escherichia coli* NCTC 86, *Yersinia enterocolitica* NCTC 10460, *Pseudomonas aeruginosa* NCIMB 10848, *Bacillus cereus* NCTC 21113 and *Pseudomonas flourescens* recorded higher susceptibilities to the antimicrobial action of the *Lactococcus* strains than others; zones of inhibition of 5 mm and above were recorded for the indicator organisms compared to lower values (<5 mm) obtained for others. *Listeria monocytogenes* also showed medium susceptibility (zones of inhibition <5 mm) to the antimicrobial activities of the *Lactococcus* strains. It was concluded that the *Lactococcus* strains could be effective in the control of spoilage and pathogenic organisms; their antagonism recorded against *Listeria monocytogenes*, *E. coli*, *Salmonella* Typhimurium and *Staphylococcus aureus* may be of public health significance.

Introduction

The lactic acid bacteria (LAB) comprise a group of Gram positive, non-sporulating, cocci or rods, and are catalases lacking organisms. They produce lactic acid as the major end product during the fermentation of carbohydrates, and only grow in complex media where fermentable carbohydrates and higher alcohols are used as an energy source, mainly to form lactic acid (Olaoye and Ntuen, 2011). Homofermentative LAB degrade hexoses to lactic acid (lactate), whereas heterofermentative LAB degrade hexoses to lactate and additional products such as acetic acid (acetate), ethanol, CO2, formic acid (formate), or succinic acid (succinate). LAB is widespread in most ecosystems and is found in soil, water, plants, and animals. They are responsible for many food fermentation processes, but they are also commonly found on non-fermented foods such as dairy products, meat products, seafood, fruits, vegetables, cereals, sewage, and in the genital, intestinal, and respiratory tracts of humans and animals. LAB are widely used as protective cultures in the food industry for the production of fermented foods, including dairy (yogurt, cheese), meat (sausages), fish, cereals (bread and beverages such as beer), fruit (malolactic fermentation processes in wine production) and vegetables such as sauerkraut, kimchi and silage (Olaoye and Ntuen, 2011).

LAB is expected to produce of lactic acid from carbohydrate sources rapidly in adequate concentrations. The primary antimicrobial effect exerted by LAB is the production of lactic acid resulting in reduction of pH; the levels of production of organic acids by LAB depend on the species or strain which are normally affected by growth medium composition and conditions (Lindgren and Dobrogosz, 1990). In addition, they produce various low-molecular-mass compounds such as hydrogen peroxide (H_2O_2), carbon dioxide (CO_2), diacetyl (2,3-butanedione), uncharacterized compounds and high-molecular-mass compounds like bacteriocins. All of these can antagonize the growth of some spoilage and pathogenic bacteria in foods and have been explored in the control of most unwanted organisms, but their production is variable among strains of LAB (Olaoye and Onilude, 2011). Many LAB produce a range of

antimicrobial substances that are generally active towards other LAB (especially closely related strains) and food borne pathogens such as *Listeria monocytogenes*, *Staphylococcus aureus*, *Clostridium perfringens* and *Bacillus cereus* (Noonpakdee *et al.*, 2003). LAB comprise of many genera including *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus* and *Streptococcus* (Olaoye, 2014). The genus *Lactococcus* comprises many species, and they have been isolated from several food sources, especially dairy and meat.

The present study reports the production of some antimicrobial agents by some strains of *Lactococcus* which have been isolated from beef in a previous study (Olaoye, 2014). Their antimicrobial activities against known spoilage organisms of food and pathogenic organisms are also reported, with the objective of proposing suitable strains for use in the biopreservation of food as a results of the generally regarded as safe (GRAS) status of LAB.

Materials and Methods

Source of Lactococcus Strains, Spoilage/Pathogenic Organisms and Culture Conditions

The *Lactococcus* strains used in the present study consisted of five microbial isolates that have been isolated and identified from Nigerian beef in a previous study (Olaoye, 2014). The indicator spoilage and pathogenic organisms used in challenging the antimicrobial activities of the *Lactococcus* strains were obtained from the Food Microbiology laboratory of the Department of Microbiology, University of Ibadan (Table 1). They include *Staphylococcus aureus*, *Salmonella* Typhimurium, *Escherichia coli* NCTC 86, *Yersinia enterocolitica* NCTC 10460, *Pseudomonas aeruginosa* (NCIMB 10848), *Klebsiella pneumoniae* U11468, *Listeria monocytogenes* NCTC 11994, *Bacillus cereus* NCTC 21113 and *Pseudomonas fluorescens*.

The optimal growth temperatures and culture medium of the different organisms are shown in Table 1.

Measurement of Technological Properties Contributing to Antimicrobial Activities of the Lactococcus Strains

Organic acids (lactic and acetic acids): The slightly modified HPLC method described by Olaoye *et al*. (2008) was used to measure the organic acids production by the *Lactococcus* strains *in vitro* assay. This involved the use of an inoculum (100 µl) each of the *Lactococcus* strains, representing approximately 10^6CFU/ml in 30 ml sterile M17 broth in universal bottle (Inoculum was taken from cultured strains previously grown in M17 broth at 30°C for 24 h, with optical density adjusted to same value each); the universal bottles were incubated at 30°C in a shaking incubator (200 rpm) for 24 h. Starting from 0 h, samples were taken every 6 h during incubation measurement of organic acids. Samples (15 ml) were centrifuged at 3,500 x g for 15 min, and the cell free supernatants (CFS) were obtained and filter sterilized, using 0.2 µm syringe filter (Sartorius AG 37070 Goettingen, Germany). Uninoculated M17 broth, similarly treated as other samples, was used to set baseline for measurement of the organic acids. Standard concentrations (g/l) lactic and acetic acids were prepared and analysed by HPLC, results of which were used to plot standard curves from which the concentrations of the acids in the samples were measured. Concentrations of lactic and acetic acids were normalized as g per 10^7 CFU.

The HPLC system and chromatographic conditions used were same as previously described (Olaoye et al., 2008). The HPLC system used consisted of LC-10ADVP pump (Shimadzu, UK), equipped with injection valve of 20µl capacity; UV detector (SpectroMonitor 3000, LDC/Milton Roy, Florida, USA); Data recorder (Picolog for windows, Release 5.12.1, St Neots, UK); C18 analytical column (a reverse phase Techsphere ODS-2 5U, 250mm length, 4.6mm internal diameter).

Table 1 *Lactococcus* strains and other microorganism used, sources and growth conditions

Name	Media	Temp (^{o}C)	Source
Lactococcus garviae K2	M17	30	Olaoye, 2014
L. piscium SU4	M17	30	Olaoye, 2014
L. lactis subsp. *cremoris* E22	M17	30	Olaoye, 2014
L. lactis subsp. *hordinae* E91	M17	30	Olaoye, 2014
L. plantarum L7	M17	30	Olaoye, 2014
Staphylococcus aureus	BHI	37	Food Microbiology laboratory, Dept of Microbiology, University of Ibadan, Nigeria
Salmonella Typhimurium	BHI	37	"
Escherichia coli NCTC 86	BHI	37	"
Yersinia enterocolitica NCTC 10460	BHI	37	"
Pseudomonas aeruginosa (NCIMB 10848)	NB	30	"
Klebsiella pneumoniae U11468	BHI	37	"
Listeria monocytogenes NCTC 11994	BHI	30	"
Bacillus cereus NCTC 21113	NB	37	"
Pseudomonas fluorescens	NB	30	"

M17, growth medium for cultivation of *Lactococcus*; BHI, brain heart infusion; NB, nutrient broth

Analysis was performed at ambient temperature with a mobile phase consisting of 0.02M NaH_2PO_4 (adjusted to pH 2.55 ± 0.02 with H_3PO_4) and methanol (98% v/v) and delivered at a flow rate of 1ml/min. The samples were detected by their UV absorbance monitoring at 220 nm with sensitivity setting of 0.002 absorbance units full scale (AUFS). The pressure of pump was 13.5±0.5 Mpa. Sample injections into HPLC were made using a 50 µl stainless steel syringe holder, with a maximum of 20 µl being injected into the injection valve, while the remaining was collected as waste through the waste outlet.

Diacetyl: Measurement of diacetyl (DA) production by the *Lactococcus* strains was accomplished by the use of headspace analysis and gas chromatography–mass spectrometry (GC-MS) as described by Olaoye et al. (2008). Sample preparation was done as described for the organic acids. A known concentration of pure DA was added into the CFS and recovery attempts were then made, a recovery concentration of DA above 100% was assumed to be due to the quantity of DA in the original CFS and added concentration of the pure compound. Preparation of standard concentrations of DA was made in blank M17, analysed by GC and the results were used to plot a standard graph, from which the concentrations of DA in the samples were measured. Blank M17 broth was used to set a baseline for measuring DA.

Concentrations of DA were expressed as µg/10^7 CFU.

Antimicrobial Activities of the Lactococcus Strains Against Indicator Organisms

Detection of antagonism of the *Lactococcus* strains against indicator organism was done by placing a drop (20 µl) of BHI broth (in which each of the strains has been grown for 18 h) on petri dish plates already inoculated with indicator organisms. Plates were inoculated at 30°C for 24 h after which they were examined for occurrence of antagonism (Figure 1).

The paper disc assay method (Gurira and Buys, 2006) was used for measuring zones of inhibition (Figure 2). A sterile filter paper disc (Whatman AA, 6 mm, Fisher Scientific, UK) was soaked in CFS for 30 min, and then applied on plates previously seeded with BHI or Nutrient broth (with 0.7% agar) containing 50 µl of indicator organisms. The plates were incubated overnight at 30°C for 24 h and zones of inhibition were measured. Clear zones extending for 1 mm or more were considered as positive for inhibition (Noordiana et al., 2013).

Antagonism by the *Lactococcus* strains against the indicator organisms was also detected by adding 500 µl of CFS of the strains to BHI broth (already inoculated with indicator organism) at 5 h during incubation. The optical density (OD) of the indicator organism was monitored during incubation, before and after addition of CFS, at 580 nm to detect likely presence of antagonism (Noordiana et al., 2013; Olaoye, 2015).

Statistical Analysis

The data obtained were analysed using the means of three replicates of each sample. Means were separated and analysed using the *t*-test in data analysis functionality of Microsoft Excel 2010 to determine differences. Significant differences between samples were determined at $P<0.05$.

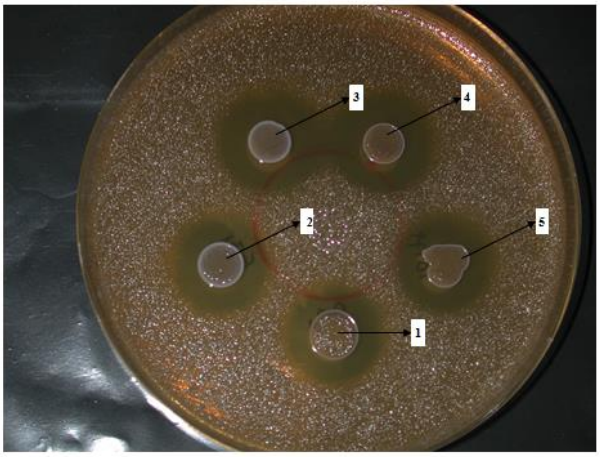

Figure 1 Antagonistic activities of the *Lactococcus* strains against *Yersinia enterocolitica* NCTC 10460
1, *Lactococcus garviae* K2; 2, *L. piscium* SU4; 3, *L. lactis* subsp. *cremoris* E22; 4, *L. lactis* subsp *hordinae* E91; 5, *L. plantarum* L7

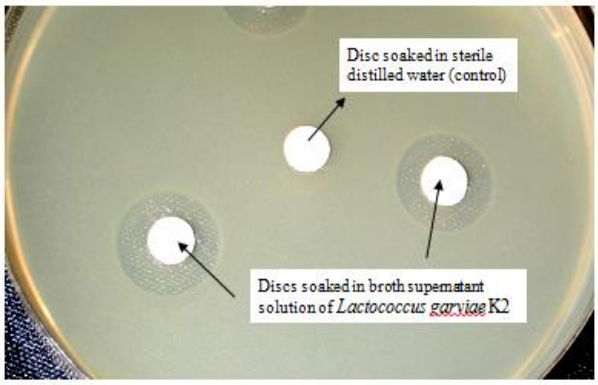

Figure 2 In vitro inhibition of Lactococcus garviae K2 against Klebsiella pneumoniae U11468, using the disc assay method.

Results and Discussion

The strains of *Lactococcus* used in this study were isolated and identified in a previous report (Olaoye, 2014). The indicator spoilage and pathogenic organisms used in challenging the antimicrobial activities of the *Lactococcus* strains were obtained from the Food Microbiology laboratory of the Department of Microbiology, University of Ibadan (Table 1). They include *Staphylococcus aureus*, *Salmonella* Typhimurium, *Escherichia coli* NCTC 86, *Yersinia enterocolitica* NCTC 10460, *Pseudomonas aeruginosa* (NCIMB 10848), *Klebsiella pneumoniae* U11468, *Listeria monocytogenes* NCTC 11994, *Bacillus cereus* NCTC 21113 and *Pseudomonas fluorescens*; only one strain each of the different organisms were used in this study.

Figure 1 shows the result of *in vitro* test for antimicrobial activities of the *Lactococcus* strains against *Yersinia enterocolitica* NCTC 10460, using the spot on agar method. All the strains displayed antagonism against the indicator organism *in vitro*, suggesting that they may be able to control its growth in food. In Figure 2, *Lactococcus garviae* K2 was shown to display antimicrobial activity against *Klebsiella pneumoniae* U11468 with the use of disc method. The disc method was used in measuring zones of inhibition of the *Lactococcus* strains against the different indicator organisms.

Presented in Table 2 are the concentrations of organic acids (lactic and acetic acids) and diacetyl produced by the *Lactococcus* strains in growth medium at 24 h of incubation. Results revealed that all the strains, with the exception of *L. garviae* K2, had lactic concentration higher than 7.00 g/10^7 CFU and no significant difference (P>0.05) was recorded in their values. However, concentration of 6.83 g/10^7 CFU was obtained for *L. garviae* K2, the value which was significantly lower (P<0.05) than those recorded for other strains. Production of acetic acid (3.55 g/10^7 CFU) was recorded for *L. garviae* K2, and this was significantly higher than those obtained for other *Lactococcus* strains (P<0.05). Production of organic acids has been noted as a key feature which contributes to antimicrobial activities of lactic acid bacteria against unwanted microorganism; and this has been reported in many research investigations (Sahnouni et al., 2014; Olaoye, 2014; Olaoye, 2015). Concentrations of lactic and acetic acids recorded for the *Lactococcus* strains were similar to those reported for other types of LAB in similar studies (Olaoye and Onilude, 2011; Olaoye, 2014). Olaoye and Onilude (2011) recorded lactic acid concentrations of between 5.55 to 7.99 g/10^7 CFU for *Pediococcus* strains of LAB during 24 h of growth, the values which were supportive of those recorded in the present study. The research workers also reported acetic acid concentrations of between 2.88 and 6.00 g/10^7 CFU for the LAB strains, which were also in support of those recorded in the present study. Furthermore, Olaoye (2014) reported acetic acid values of 2.31 to 4.22 g/10^7 CFU for some *Lactococcus* strains within 24 h of growth, and this was consistent with those recorded in the present report. The concentration of the organic acids was observed to correlate with antimicrobial activities of the *Lactococcus* strains against many of the spoilage and pathogenic organisms that were tested as shown in Table 3; this was discussed later in this section.

Concentrations of diacetyl produced by the *Pediococcus* strains ranged between 31.25 and 71.99 µg/10^7 CFU, with *L. lactis* subsp. *hordinae* E91 having the highest value and the lowest was recorded for *L. lactis* subsp. *cremoris* E22. Diacetyl has been noted as a metabolite produced by LAB which contributes to their antimicrobial activities against spoilage and pathogenic organisms (Adejumo, 2014; Emerenini et al., 2014; Olaoye, 2015). Production of diacetyl by the *Lactococcus* strains in the present study may therefore be desirable towards control of unwanted organisms, especially spoilage and pathogenic types, in food products. As observed for the organic acids, concentration of diacetyl had direct correlation with the antimicrobial activities of the different strains against the indicator organisms and this was subsequently discussed in this section

Table 2 Measured technological properties contributing to antimicrobial activities of the *Lactococcus* strains

Property	*Lactococcus strains*									
	L. garviae K2		*L. piscium* SU4		*L. lactis* subsp *cremoris* E22		*L. lactis* subsp *hordinae* E91		*L. plantarum* L7	
	value	SD	value	SD	value	SD	value	SD	value	SD
Lactic acid (g/10^7 CFU)	6.83^b	2.37	7.23^a	2.21	7.19^a	1.04	7.11^a	2.36	7.20^a	1.04
Acetic acid (g/10^7 CFU)	3.55^a	1.03	2.99^b	0.36	3.15^b	0.75	3.24^b	0.79	3.20^b	1.20
Diacetyl (µg/10^7 CFU)	45.32^c	6.25	36.36^d	7.26	31.25^d	5.45	71.99^a	12.29	58.43^b	7.32

Values are means of three replicates; CFU, colony forming unit; SD, standard deviation; Values with different superscript letters across rows are significantly different (P<0.05).

Table 3 Zones of inhibition (mm) of the *Lactococcus* strains against spoilage and pathogenic organisms

Spoilage and Pathogenic Organisms	*Lactococcus strains*				
	K2	SU4	E22	E91	L7
Staphylococcus aureus	1.5^b	1.5^b	1.5^b	1.5^b	2.5^a
Samonella Typhimurium	2.0^c	3.5^b	5.0^a	5.0^a	3.5^b
Escherichia coli NCTC 86	6.0^b	5.0^b	10.0^a	12.0^a	12.0^a
Yersinia enterocolitica (NCTC 10460)	15.5^a	16.0^a	15.0^a	15.0^a	15.5^a
Pseudomonas aeruginosa (NCIMB 10848)	18.0^b	20.0^a	21.0^a	17.0^b	16.0^b
Klebsiella pneumoniae U11468	1.5^b	2.0^a	2.0^a	1.5^b	2.0^a
Listeria monocytogenes (NCTC 11994)	2.0^a	2.0^a	2.5^a	2.0^a	2.0^a
Bacillus cereus NCTC 21113	5.0^a	5.5^a	5.5^a	5.5^a	6.0^a
Pseudomonas flourescens	12.0^b	14.0^a	15.0^a	10.0^b	11.0^b

K2: *L. garviae* K2; SU4: L. *piscium* SU4; E22: *L. lactis* subsp *cremoris* E22; E91: *L. lactis* subsp *hordinae* E91; L7: *L. plantarum* L7; Values are means of three replicates; Values with different superscript letters across rows are significantly different (P<0.05).

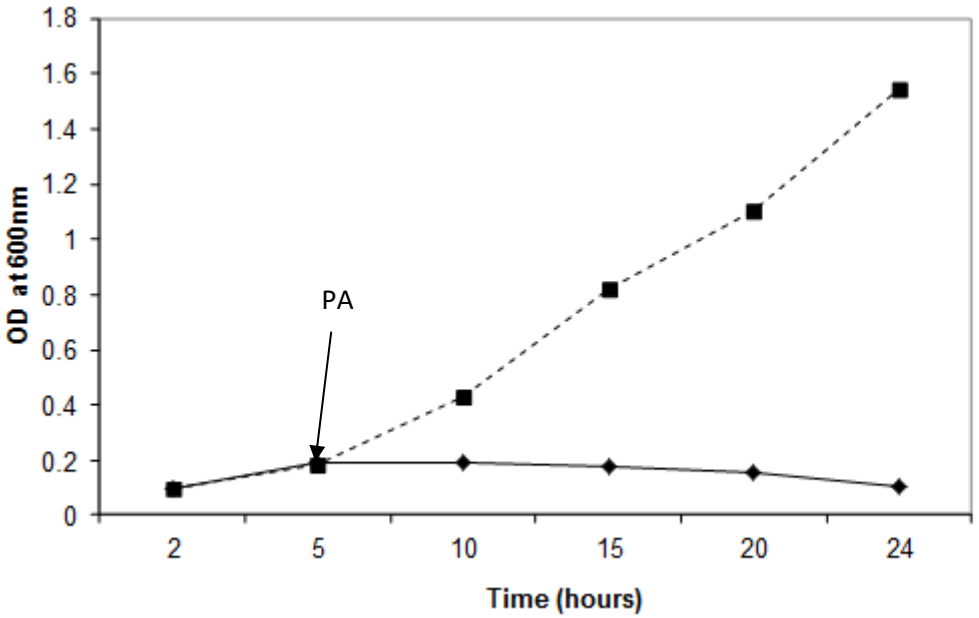

Figure 3 Effect of addition of CFS of *Lactococcus garviae* K2 on optical density (OD) of *Klebsiella pneumoniae* U11468 during growth; '--■-- ', OD of *K. pneumoniae* U11468 (no addition of *L. garviae* K2); '—◆— ', OD of *K. pneumoniae* U11468 after addition of *L. garviae* K2; PA, point of addition of broth supernatant solution of *Lactococcus garviae* K2

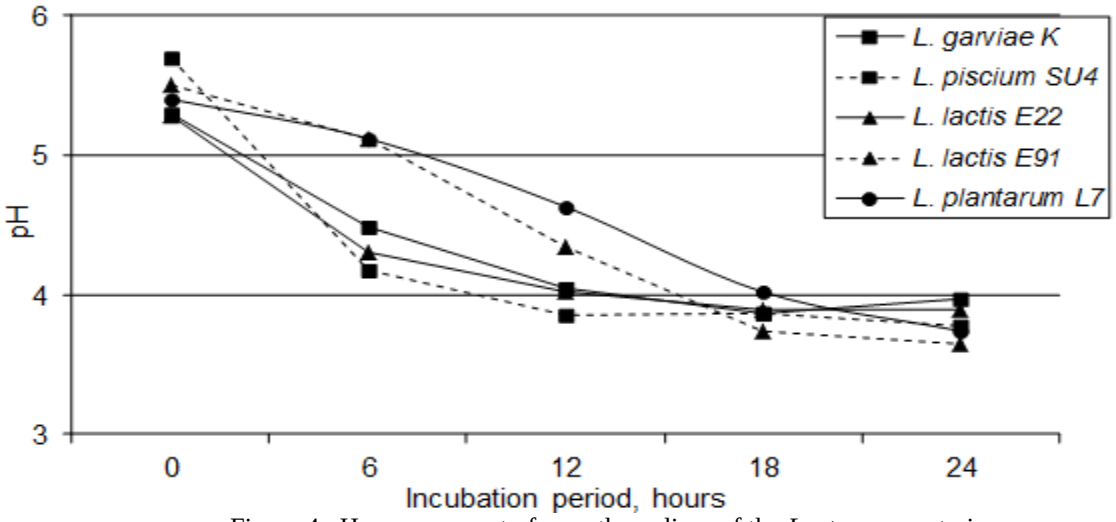

Figure 4 pH measurement of growth medium of the *Lactococcus* strain

The zones of inhibitions produced by the *Lactococcus* strains against the spoilage and pathogenic organisms during challenge tests *in vitro* are shown in Table 3. Among the indicator organisms, *Escherichia coli* NCTC 86, *Yersinia enterocolitica* NCTC 10460, *Pseudomonas aeruginosa* NCIMB 10848, *Bacillus cereus* NCTC 21113 and *Pseudomonas flourescens* showed higher susceptibilities to the antimicrobial activities of the *Lactococcus* strains; zones of inhibition of 5 mm and above were recorded against them while 5 mm and below were obtained for other indicator organisms. In support of the results of the present study, Adejumo (2014) and Khandare and Patil (2015) reported successful inhibition by some LAB strains against *Staphylococcus aureus*, *Samonella* Typhimurium and *Escherichia coli.* Sahnouni et al. (2014) and Khandare and Patil (2015) also noted inhibition of *Pseudomonas aeruginosa* by LAB in separate studies, thus further corroborating the result of the present study. In some research investigations, the abilities of LAB to control *Listeria monocytogenes* were reported by Olaoye and Onilude (2010), Olaoye et al. (2011) and Olaoye (2015); these reports justify the antimicrobial activities of the *Lactococcus* strains in the present study.

In this study, the antimicrobial activities of the *Lactococcus* strains recorded against *Staphylococcus aureus*, *Samonella* Typhimurium, *Escherichia coli*, *Yersinia enterocolitica* and *Listeria monocytogenes* may

be of public health significance because they have been reported to be pathogenic in nature (Ammor et al., 2006; Sahnouni et al., 2014; Khandare and Patil, 2015). Among other factors, production of organic acids and diacetyl by the *Lactococcus* strains may have contributed to their antimicrobial action against the indicator organisms, as earlier noted.

Confirmation that antimicrobial activities of the *Lactococcus* strains may be due to the extracellular metabolites produced by them in growth medium was carried out by the addition of the CFS of the strains into medium containing indicator organisms during growth. A typical graph of the measurement of their optical densities (OD) during growth, before and after addition of CFS of the *Lactococcus* strains is presented in Figure 3. From the graph, it was observed that OD of the indicator organisms decreased shortly after the addition of CFS compared with control without CFS; a difference of up to 1.0 unit in OD was recorded between them. This further justified the assertion that the antimicrobial activities of LAB were as a result of extracellular metabolites produced by them in growth medium, including organic acids and diacetyl (Ammor and Mayo, 2007; Olaoye and Onilude, 2011; Adejumo, 2014; Khandare and Patil, 2015).

pH determination in growth medium of the *Lactococcus* strains indicated that decrease in values was observed during growth (Figure 4); values generally decreased below 4.0 at 24 h of incubation in growth medium. The decrease in pH levels during growth may be attributed to production of organic acids by the *Lactococcus* strains. The pH values recorded for the different strains correlated directly with the concentration of organic acids produced by them; the organic acids produced by the *Lactococcus* strains were lower than 1 g/107 CFU at 6-9 h of incubation, during which period pH was higher than 4.0. Organic acid concentrations generally increased during incubation, and this prompted corresponding decrease in pH. Lowering of pH in food products due to growth of LAB is an important factor in the control of undesirable microorganisms (Kaban and Kaya, 2006). The reduction in pH recorded in the growth medium may also have contributed to the antimicrobial activities of the *Lactococcus* strains against the indicator organisms during the *in vitro* assay.

This study concluded that the antimicrobial action of the *Lactococcus* strains were effective in controlling some pathogenic and spoilage organisms *in vitro*. The antimicrobial actions recorded against *Listeria monocytogenes*, *Escherichia coli*, *Salmonella* Typhimurium and *Staphylococcus aureus* may be of public health significance as a result of their ability to cause diseases. It was also concluded that certain metabolites produced by the strains, especially organic acids and diacetyl, contributed to their antagonistic actions against the indicator organisms. Though the *Lactococcus* strains displayed antimicrobial activities against some spoilage and pathogenic organisms *in vitro*, it may be useful to evaluate their performance in food *in situ* in future studies.

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