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In vitro assay for production of organic acids and diacetyl by Lactococcus spp. isolated from food source

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Abstract The present study was aimed at evaluating production of organic acids and diacetyl by five isolates of *Lactococcus* using high performance liquid chromatography and head space gas chromatography mass spectrometry. The *Lactococcus* isolates were *L. garviae* K2, *L. piscium* SU4, *L. lactis* subsp. *cremoris* E22, *L. plantarum* L7 and *L. lactis* subsp. *hordniae* E91. Production of organic acids and diacetyl were monitored in growth medium for 48 h. The pH of growth medium was below 4 from 24 h to 48 h and the minimum value of 3.67 was recorded for *L. piscium* SU4 at 48 h. No detection of lactic acid was recorded by the isolates until 12 h after which concentration increased with progression of incubation time; overall, the highest concentration of 21.94 g/10^7 CFU was recorded for *L. lactis* subsp. *cremoris* E22 at 48 h. A similar observation was recorded for acetic acid production by the isolates; however concentrations of acetic acid were lower than lactic acid. *L. lactis* subsp. *cremoris* E22 also recorded the highest concentration of acetic acid (7.69 g/10^7 CFU) at 42 h. Production of diacetyl by the *Lactococcus* isolates was detected at 6 h and increased progressively during incubation with the highest concentration of 71.99 µg/10^7 CFU being recorded at 24 h for *L. lactis* subsp. *hordniae* E91. In conclusion, production of higher concentrations of organic acids and diacetyl by *L. lactis* subsp. *cremoris* E22 and *L. lactis* subsp. *hordniae* E91 respectively suggest they could be better biopreservative agents in food preservation.

Keywords lactic acid; acetic acid; diacetyl; Lactococcus; biopreservative agents

Introduction

Organic acids are significant as natural preservatives and for sensory characteristics of many food products. Some organic acids have been implicated as possible factors in the cure and prevention of certain diseases; lactic acid has been related to the inhibition of certain pathogenic bacteria in yogurt. Orotic acid is a growth factor for lactobacilli and has been identified as a possible factor in milk that could help reduce the incidence of cholesterolemia in humans [1]. Lactic acid is an organic acid with a wide range of industrial applications. Lactic acid is not naturally present in foods, but is produced during fermentation of foods by lactic acid bacteria. These foods include sauerkraut, pickles, olives, and some meats and cheeses [2]. Lactic acid is used in foodstuffs for acidification, to increase flavor and aroma, and is a potent microbial inhibitor. Lactic acid is applied to a diverse range of foodstuffs, including meats, fish, vegetables, cereals, and cake products. In fermented drinks it is used to contribute specifically to aroma and preservation [3]. Acetic acid is one of the oldest chemicals known to humanity and is produced naturally during spoilage of fruit and certain other foods by the presence and activity of microorganisms. In culture media, acetic acid is more inhibitory against *Listeria* than lactic acid and addition of acetic acid usually results in greater cell destruction.

The use of lactic acid bacteria (LAB) in the manufacture of fermented food products has received considerable interest and is very common in the meat industry [4]. Mostly used strains are homofermentative LAB such as *Lactococcus*, *Pediococcus* and some species of *Lactobacillus* which produce lactic acid as the major carbohydrate metabolite giving the characteristic taste and flavour development as well as preservation of the fermented meat products [5-6]. The heterofermentative types of LAB such as *Leuconostoc* would produce other metabolites in addition to lactic acid and can lead to defect in the fermented meat sausage [7].

Consumer concern over the possible negative health effects of some food preservatives associated to the increasing demand for foods having long shelf-life has resulted in increasing pressure on food industry to remove chemically synthesized additives and to provide more natural alternatives for the assurance of food safety and extension of shelf-life. These consumer-led trends have fuelled a renewed search for preservatives derived from plant, animal and microbial sources. In fact, in the last two decades, numerous scientific papers have been published about the antimicrobial properties of compounds derived from herbs and spices, fruits and vegetables, animal tissues and micro-organisms [8]. Among the substances of microbial origin, diacetyl seems to be a good candidate due to its selective antimicrobial activity and absence of toxicity against humans and animals. In fact, it is produced mainly by yeasts and LAB, microorganisms used as starter cultures in food industries for the products of several fermented foods. Moreover, it is widely known as a generally recognized as safe (GRAS) flavoring compound [9]. Diacetyl contributes to the flavor of many fermented foods, especially in fermented dairy and bakery products [10]. Diacetyl is volatile and has antimicrobial properties; widely used as an antimicrobial dip for utensils and surfaces in the food and pharmaceutical industries [11]. The physiological reason for the production of diacetyl is not clearly understood. It is hypothesized in the literature that diacetyl is synthesized to reduce the toxicity of pyruvate. The metabolic pathway leading to the formation of diacetyl and acetoin has been well characterized. The cells form active acetaldehyde from pyruvate and thiamine pyrophosphate by pyruvate oxidase. The active acetaldehyde condenses with another molecule of pyruvate and forms a-acetolactate, catalysed by a-acetolactate synthase and then the a-acetolactate is converted to diacetyl by a-acetolactate oxidase [12].

Lactococcus is one of the genera of LAB, and the genus comprises five species including the well-known *Lactococcus lactis* that contains three subspecies (*lactis*, *cremoris* and *hordniae*) mainly found in milk products. Isolations of species of the genus have been widely reported, especially from dairy [13-14] and meat [15-17. In this study, production of organic acids and diacetyl by five isolates of *Lactococcus*, isolated from beef in a previous study [18], were evaluated in growth medium using HPLC and HS-GCMS.

Materials and methods

Source of Lactococcus spp. and culture conditions

The *Lactococcus* spp. used in this study consisted of five isolates that have been isolated and identified from Nigerian beef in a previous study [18]. Their growth media and conditions are shown in Table 1.

Table 1: *Lactococcus* and indicator strains used, culture conditions and sources

Name	Media	Temp (^oC)	Source
Lactococcus garviae K2	M17	30	Olaoye, 2014b
L. piscium SU4	M17	30	Olaoye, 2014b
L. lactis subsp. *cremoris* E22	M17	30	Olaoye, 2014b
L. lactis subsp. *hordniae* E91	M17	30	Olaoye, 2014b
L. plantarum L7	M17	30	Olaoye, 2014b

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

Quantitative estimation of organic acids using high performance liquid chromatography (HPLC)

A method based on HPLC described by Olaoye *et al*. [19] was used to assay for the organic acids produced by the *Lactococcus* isolates *in vitro* with slight modification. The full detail of the modified method is described as follows.

An inoculum of a *Pediococcus* isolate (100 µl each, representing ~ 10^6cfu/ml) of 24 h M17 broth cultures (grown at 30^{o}C), adjusted to same optical density, was transferred into a 30 ml sterile M17 broth in universal bottle, and incubated at 30^{o}C in a shaking incubator (200 rpm) for 48 h. Starting from 0 h, 15 ml of sample was removed and centrifuged at 3,500 x g for 15 min, at 6 h intervals of incubation. The cell free supernatant (CFS) was obtained and filter sterilized by passing it through a 0.2 µm syringe filter (Sartorius AG 37070 Goettingen, Germany).

The organic acids were extracted from the CFS using the following procedure. The Cartridge (Strata X 33u Polymeric Reversed Phase, 30mg/ml, Phenomenex, UK) was conditioned by slowly passing 1 ml of absolute methanol (HPLC grade) through, followed by 1 ml of 10% (v/v) methanol in NaH_2PO_4:Methanol, 98:2 (Fernandez-Garcia and McGregor, 1994). The mixture of the HPLC mobile phase (10% Methanol in NaH_2PO_4:Methanol, 98:2) and sample supernatant (in ratio 3:1, i.e 900µl mobile phase and 300µl supernatant) was then slowly passed through the pre-conditioned cartridge. Few drops (200 - 250µl) were allowed to run off while the remaining (950 - 1000 µl) was collected in an Eppendorf tube for injection into the HPLC system. Samples for analyses were prepared in three replicates.

Uninoculated M17 broth, prepared as the samples, was used to set a baseline for measurement of the organic acids. Standard concentrations (g/l) of 0.5, 1.0, 2.5, 5.0, 7.5, 10, 12.5, 15, 17.5 and 20 of the lactic and acetic acids were prepared and analyzed by HPLC. Results were used to plot standard curves from which the concentrations of the acids in the samples were measured (Figures 1 and 2). All data for the concentrations of lactic and acetic acids were normalized as g/10^7 CFU.

The HPLC system and chromatographic conditions used were same as previously described [19-21].

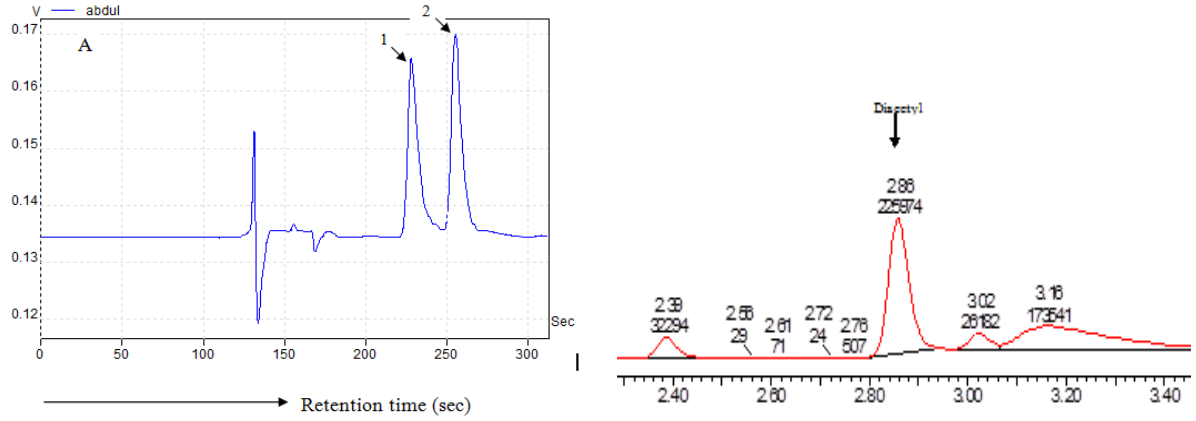

Figure 1: A) Chromatograms of standard concentrations of lactic acid (1) and acetic acid (2) generated by HPLC; B) Ion chromatograms and peak size of diacetyl standards as generated by GC

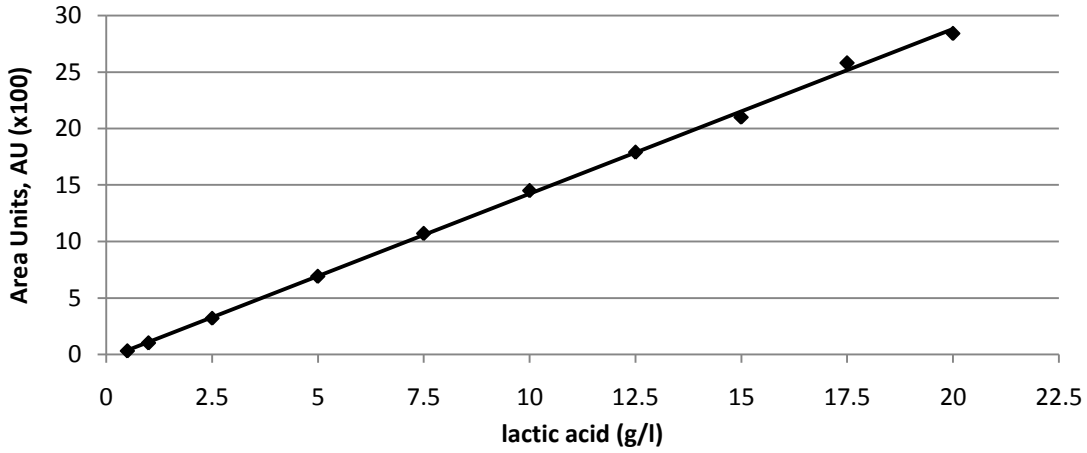

Figure 2: Curve of standard concentrations of lactic acid against area units (AU) measured in HPLC

Measurement of diacetyl using headspace gas chromatography mass spectrometry (HS-GC MS)

For the production of diacetyl (DA) by the *Lactococcus* isolates a method using head-space analysis and gas chromatography–mass spectrometry (GC-MS) was used [21]. Sample preparation was the same as that used for the organic acids to obtain CFS. Based on non-detection of DA in the CFS by the GC during preliminary trials (probably due to low concentration), the CFS was spiked with a known concentration of DA and recovery attempts were then made. A recovery concentration of DA above 100% was assumed to be due to that present in the CFS originally. Preparation of standard concentrations of DA was made in blank M17, analysed by GC and the results were used to plot a standard graph (Figure 3), which was used to measure the concentrations of DA in the broth supernatants of the *Lactococcus* isolates. Blank MRS broth was used to set a baseline for measuring DA.

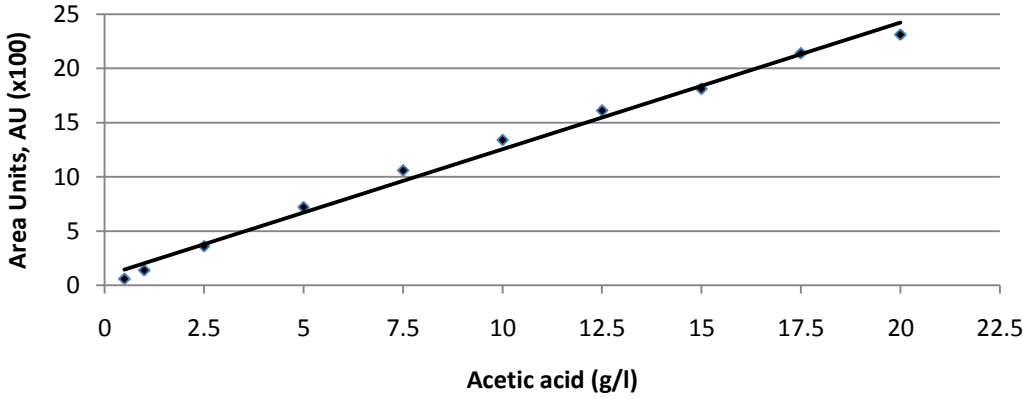

Figure 3: Curve of standard concentrations of acetic acid against area units (AU) measured in HPLC

Measurement of colony forming units of Lactococcus during growth

The colony forming units (CFU) of the *Lactococcus* isolates were measured by taking 10ml of medium (M17 broth) in which the isolates were being cultivated at six hourly intervals and diluted into 90 ml of maximum recovery diluent. It was shaken using vortex machine to obtain 10^{-1} dilution, from which further dilutions were made. One milliliter (1 ml) of appropriate dilutions were then plated into petri dishes containing solidified M17 agar and then incubated at 30^{o}C for 24 h. Colonies of *Lactococcus* that emerged on the agar media were counted and results expressed in logarithm of CFU per milliliter (i.e log CFU/ml).

Measurement of pH in growth medium of Lactococcus

pH of growth medium of *Lactococcus* spp. was monitored during growth at 6 h intervals. The pH was then measured by a pH meter (pH 212 Microprocessor, Hanna Instruments, USA) using the method of Marugg *et al*. [22].

Statistical analysis

Results which depend on growth time were analyzed according to a completely randomized design with three replicates. Data were subjected to variance analyses and differences between means were evaluated by Duncan's multiple range test using SPSS statistic programme, version 10.01. Significant differences were expressed at $p < 0.05$.

Results and Discussion

The five *Lactococcus* isolates being evaluated for production of organic acids and diacetyl in the present finding were isolated and identified from beef in a previous study [18]. They included *Lactococcus garviae* K2, *L. piscium* SU4, *L. plantarum* L7, *L. lactis* subsp. *hordniae* E91 and *L. lactis* subsp. *cremoris* E22 (Table 1). They were cultivated on M17 medium (liquid broth and solid agar), known to be suitable for cultivation *Lactococcus* [18,23].

The HPLC assay for lactic acid and acetic acid in the broth supernatants of the *Lactococcus* isolates gave good separation of the acids (Figure 1A). The assay indicated that retention times (RTs) of approximately 230 and 255 sec were obtained for the respective acids (Figure 1A). The RTs of the organic acids were lower than those reported in previous studies. Zotou *et al*. [20] reported RTs of 360 sec and 390 sec for the respective acids in wine, while Fernandez-Garcia and McGregor [1] obtained 720 and 840 sec when measuring the lactic and acetic acids respectively in yoghurt. The differences in the RTs may be attributed to possible variation in the HPLC methods and/or conditions used in the assay procedures by the various researchers. In the present finding, the lower RTs recorded could reduce the time required for analysis of the organic acids, thereby allowing an increased throughput of samples.

The typical chromatograms generated by the GC during assay for diacetyl (DA) in the broth supernatants of *Lactococcus* isolates are shown in Figure 1B. The RT obtained for the standards of DA by the GC was 286 sec. The use of GC has been applied to quantify DA from other media, such as cheese [24], wine [25] and beer [26].

Standard curve graphs were obtained from the area units of the different concentrations of lactic acid and acetic acid (Figures 2 and 3) as well as diacetyl (curve not shown); these were used in the measurement of the compounds in the respective broth supernatants of the *Lactococcus* isolates (by extrapolation of resulting area units in the standard curve).

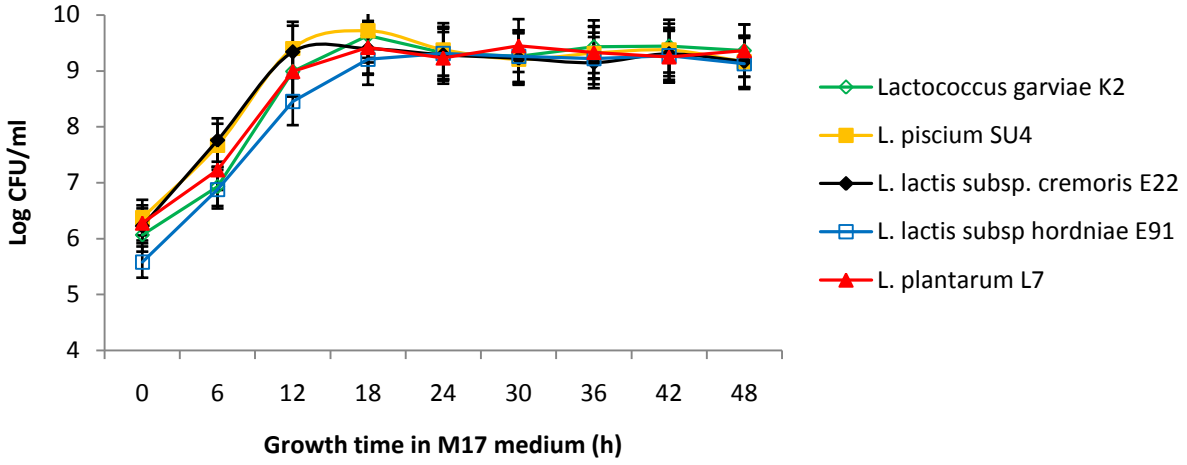

Figure 4: Number of cells (log CFU/ml) of Lactococcus isolates in M17 broth

Presented in Figure 4 is the graph showing the number of cells (log CFU/ml) of the *Lactococcus* isolates in M17 broth medium at different intervals of time during growth. The highest count of 9.72 was recorded for *L. piscium* SU4 at 18 h, followed by 9.63 for *L. garviae* K2 at same growth time. The number of cells of the *Pediococcus* isolates generally increased with increase in progression of growth time. Peak of most of the isolates was attained between 18 - 30 h of incubation. The result is consistent with the report of Olaoye *et al.* [19] on species of *Pediococcus* in MRS growth medium. The authors reported increase in the log CFU of the lactic acid bacteria as the growth period progressed,

Table shows the pH values of the *Lactococcus* isolates in growth medium. During growth period, pH of below 4 was recorded in the growth medium of all the *Lactococcus* spp; pH generally decreased with growth time and this could obviously be due to production of organic acids by the organisms causing lowering of pH in growth medium. Decrease in pH is normally associated with activities of lactic acid bacteria, and is an important factor by which they bring about antimicrobial action against other bacteria which may not be able to withstand low pH environment [27]; it is also an important factor in the control of undesirable microorganisms in food products [28-29]. The lowest pH value of 3.67 was obtained for *L. piscium* SU4 at 48 h, closely followed by 3.69 for *L. lactis* subsp. *cremoris* E22 at same growth time. Production of lactic acid was not detected in growth medium of the *Lactococcus* isolates until 12 h of incubation (Table 3). Lactic acid production by the isolates increased gradually with progression of

incubation time. Consideration of individual production of the acid indicates that highest values of 20.19, 21.28 and 21.94 (g/10^{7}CFU) were recorded for *L. garviae* K2, *L. plantarum* L7 and *L. lactic* subsp. *cremoris* E22 at 48 h, while 21.36 and 21.44 were obtained for *L. piscium* SU4 and *L. lactis* subsp. *hordniae* E91 at 42 h. The increase in the lactic acid production by the *Lactococcus* isolates with progression in incubation time may have contributed to the decrease in pH of the growth medium, as observed earlier (Table 2).

Table 2: pH values in growth medium of *Lactococcus* strains

GT (h)	***Lactococcus garviae* K2**		***L. piscium* SU4**		***L. plantarum* L7**		***L. lactis hordniae* subsp. E91**		***L. lactis cremoris* subsp. E22**	
	value	**SD**	**value**	**SD**	**value**	**SD**	**value**	**SD**	**value**	**SD**
0	6.39	0.29	6.4	1.08	6.38	1.24	6.42	1.09	6.41	1.87
6	5.65	1.21	5.31	0.83	5.22	1.75	5.27	1.12	5.32	1.92
12	4.54	1.52	4.27	0.46	4.33	0.99	4.42	1.72	4.71	0.88
18	4.23	1.64	3.93	0.98	4	1.28	4.15	1.05	4.09	0.82
24	3.87	1.13	3.75	1.02	3.73	0.97	3.83	0.89	3.78	0.77
30	3.99	1.22	3.77	0.84	3.79	1.23	3.81	0.73	3.72	0.84
36	3.75	1.56	3.76	0.88	3.78	2.19	3.8	0.91	3.81	0.19
42	3.81	1.93	3.76	0.95	3.76	1.27	3.79	0.55	3.8	0.99
48	3.79	1.09	3.67	0.84	3.74	1.44	3.72	0.72	3.69	0.81

Values are means of three replicates; CFU, colony forming unit; GT, growth time; SD, standard deviation

Table 3: Concentrations of lactic acid (g/10^7 CFU) in growth medium of *Lactococcus* strains

GT (h)	***Lactococcus garviae* K2**		***L. piscium* SU4**		***L. plantarum* L7**		***L. lactis hordniae* subsp. E91**		***L. lactis cremoris* subsp. E22**	
	value	**SD**	**value**	**SD**	**value**	**SD**	**value**	**SD**	**Value**	**SD**
0	nd	-	nd	-	nd	-	nd	-	Nd	-
6	nd	-	nd	-	nd	-	nd	-	Nd	-
12	4.1	1.29	5.02	1.28	4.82	1.02	4.37	2.32	5.11	1.02
18	5.37	1.02	5.68	0.77	5.48	1.05	5.36	2.1	5.54	0.82
24	6.83	2.37	7.23	2.21	7.19	1.04	7.11	2.36	7.2	1.04
30	11.83	0.98	12.71	3.66	12.11	2.71	11.99	3.33	12.01	2.55
36	15.71	3.2	16.28	4.81	16.01	3.32	16.18	1.88	16.21	3.48
42	19.9	4.31	21.36	2.89	21.01	2.16	21.44	2.11	21.31	4.43
48	20.19	2.39	22.16	4.76	21.28	4.35	21.31	4.66	21.94	5.17

Values are means of three replicates; CFU, colony forming unit; GT, growth time; SD, standard deviation

Table 4: Concentrations of acetic acid (g/10^7 CFU) in growth medium of *Lactococcus* strains

GT (h)	***Lactococcus garviae* K2**		***L. piscium* SU4**		***L. plantarum* L7**		***L. lactis hordniae* subsp. E91**		***L. lactis cremoris* subsp. E22**	
	value	**SD**	**value**	**SD**	**value**	**SD**	**value**	**SD**	**Value**	**SD**
0	nd	-	nd	-	nd	-	nd	-	nd	-
6	nd	-	nd	-	nd	-	nd	-	nd	-
12	2.42	0.97	1.27	0.55	2.25	0.73	2.31	0.82	2.34	0.47
18	2.78	0.66	2.15	0.45	2.61	1.27	2.55	1.04	2.94	1.02
24	3.55	1.03	2.99	0.36	3.15	0.75	3.24	0.79	3.2	1.2
30	4.57	1.24	4.22	1.29	4.04	1.33	4.5	2.24	4.27	0.26
36	7.32	2.37	7.28	2.14	6.78	2.38	7.01	2.07	7.15	1.26
42	7.47	1.75	7.36	2.33	7.38	2.16	7.45	2.18	7.69	2.15
48	6.87	2.35	6.92	1.25	6.76	1.28	6.96	1.44	7.01	2.18

Values are means of three replicates; CFU, colony forming unit; GT, growth time; SD, standard deviation

Production of acetic acid by the isolates assumed similar patters to that of the lactic acid (Table 4). Acetic acid production was however lower than lactic acid by the *Lactococcus* spp. the value of 7.69 (g/10^7 CFU) was recorded highest acetic acid concentration, and this was obtained for *L. lactic* subsp. *cremoris* E22 at 42 h. The lower production of acetic acid than lactic acid by the isolates may be advantageous in food biopreservation. This is because acetic acid has been implicated in imparting pungent off flavours to foods and is unsuitable for meat preservation [30]. The genus *Lactococcus* belong to the group of homofermentative LAB, and organisms in this group are known to produce lactic acid as the main metabolite of sugar metabolism, whereas organism belonging to heterofermentative group of LAB may produce considerably higher concentration of acetic acid than lactic acid.

Table 5 shows the concentrations (µg/10^7 CFU) of diacetyl in growth medium of *Lactococcus* isolates. There was early production of the compound by the organisms; concentrations of 5.51, 6.57, 5.58, 7.45 and 6.36 were recorded for *L. garviae* K2, *L. piscium* SU4, *L. plantarum* L7, *L. lactis* subsp. *hordniae* E91 and *L. lactis* subsp. *cremoris* E22 respectively at 6 h of incubation. The values increased with progression of time to yield highest production of 45.32, 36.36, 31.25, 71.99 and 58.43 for the respective *Lactococcus* isolates at 24 h, after which decline in concentrations was observed. Diacetyl has been reported to contribute significantly to exertion of antagonism by LAB against may spoilage and pathogenic organisms [12]. Hence, the *Lactococcus* isolates may be very useful as biopreservative agents during preservation of food products, especially in meat processing. Lanciotti *et al.* [11] reported antimicrobial activity of diacetyl against *Escherichia coli, Listeria monocytogenes* and *Staphylococcus aureus*. The production of diacetyl in the early stage of incubation by the *Lactococcus* isolates in this study is in support of Joyti *et al.* [12], who observed optimal production of the compound between 15-20 h of growth.

Table 5: Concentrations of diacetyl (g/10^7 CFU) in growth medium of *Lactococcus* strains

GT (h)	***Lactococcus garviae* K2**		***L. piscium* SU4**		***L. plantarum* L7**		***L. lactis hordniae* subsp. E91**		***L. lactis cremoris* subsp. E22**	
	value	**SD**	**value**	**SD**	**value**	**SD**	**value**	**SD**	**Value**	**SD**
0	nd	-	nd	-	nd	-	nd	-	nd	-
6	5.51	1.75	6.57	1.43	5.58	0.99	7.45	1.28	6.36	0.23
12	10.24	1.74	9.83	2.94	9.21	1.26	16.59	4.38	11.05	1.26
18	24.54	3.28	19.26	5.47	21.29	2.32	33.49	1.15	39.23	3.48
24	45.32	6.25	36.36	7.26	31.25	5.45	71.99	12.29	58.43	7.32
30	36.37	8.55	23.34	5.48	25.21	8.37	58.62	10.27	33.26	5.59
36	18.36	5.15	20.12	6.55	18.54	6.83	41.53	6.58	26.34	0.94
42	15.37	3.33	12.24	3.26	11.04	1.94	37.73	8.2	18.23	1.29
48	11.64	6.02	9.83	2.28	7.23	2.18	31.26	9.23	14.35	1.55

Values are means of three replicates; CFU, colony forming unit; GT, growth time; SD, standard deviation

This study concluded that the *Lactococcus* isolates produced considerable concentrations of lactic acid and acetic acid which may be useful in biopreservation of food products. The diacetyl produced by the isolates may also contribute to their antimicrobial activities towards inhibiting spoilage and pathogenic organisms in food products, thereby promoting safety and shelf life. However, analysis of acetic acid, lactic acid and diacetyl production by the *Lactococcus* spp. was carried out *in vitro* and it would be very useful to test the isolates on specific food products *in situ* in order to ascertain their effectiveness against specific spoilage and pathogenic organisms of food importance.

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