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Antimicrobial activity and antibiotic resistance of LAB isolated from Sudanese traditional fermented camel (*Camelus dromedarius*) milk gariss

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Abstract

Fourteen strains of *Enterococcus* species, 3 strains of *Lactobacillus* species and two strains of *Streptococcus* isolated from 6 different *gariss* samples collected from Kordofan and Khartoum production sites, Sudan were screened for antibacterial activity against *Staphylococcus aureus* ATCC 25923 and *E.coli* ATCC25922 and for their antibiotic resistance against 9 antibiotics. The results indicated that all the 19 strains of LAB isolated from *gariss* were found to show no antimicrobial effect on both *Staphylococcus aureus* ATCC 25923 and *E.coli* ATCC25922. As intrinsic resistance of Enterococci for antibiotics, there were 57.14% (8 isolates) showing resistance to at least one antibiotic and some even to almost all antibiotics studied, one strain (*E. durans R05*) resistant to penicillin. *S. acidominimus was* resistant to only Ciproflaxin while *S. thermophilus* was resistant to Chloramphenicol, Erythromycin and Ciproflaxin; *L. plantarum BJG32* was resistant Chloramphenicol, Erythromycin and Ciproflaxin; *L. pentosus S2LPO2* was resistant Erythromycin and Ciproflaxin, while *L. plantarum BJ6* was not resistant to any one of antibiotics investigated..

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Introduction

The primary antimicrobial effect exerted by Lactic Bacteria (LAB) is due to a combination of many factors e.g. production of lactic acid which reduce pH acid, production of various antimicrobial compounds, which can be classified as (i) low-molecular-mass (LMM) compounds such as hydrogen peroxide (H_2O_2), carbon dioxide (CO_2), diacetyl (2,3-butanedione), uncharacterized compounds, and (ii) high-molecular-mass (HMM) compounds (inhibitory substances such as bacteriocins which are responsible for the most antimicrobial activities). All of them can antagonize the growth of some spoilage and pathogenic bacteria in foods. The levels of production of organic acids by LAB depend on species or strain, culture composition and growth conditions (Jay, 1982; Klaenhammer, 1988; Piard and Desmazeaud, 1991, 1992; Lindgren and Dobrogosz, 1990; Ogunbanwo, 2005; Ammor *et al.,* 2006). Antimicrobial resistance is an increasing problem worldwide, with the effective treatment of bacterial infections being compromised. The extensive use of antibiotics in both human and animal treatment has created a selective pressure for acquisition of resistance phenotypes that can be transmitted via food (Zhou *et al.,* 2012). Mixed cultures of lactic acid bacteria are more effective in antimicrobial compound production compared to individual cultures, and the stimulation ability of some pathogenic bacteria was found to be higher than that of LAB (Rathnayaka, 2012).

In the last decade, increasing concern has arisen about the safe use of LAB cultures for food and feed applications, in the light of the latest knowledge about their possible role as an antibiotic resistance gene reservoir. Particular concern is due to the evidence of a widespread occurrence in this bacterial group of conjugative plasmids and transposons. On the basis of these premises, an increasing amount of research has been directed to the study of antibiotic resistance in LAB. Most studies carried out until now about antibiotic resistance in LAB regard members of the genus *Enterococcus*, which holds a peculiar position among food microorganisms. Enterococci are widespread and play a pivotal role in traditional fermented foods to the same extent, or even more, than other LAB (Ogier and Serror, 2008; Clementi and Aquilanti, 2011), while in some references Enterococci are common causes of nosocomial infections and are ranked second (after staphylococci) as aetiological agents of hospital-associated infections in US hospitals, with *Enterococcus faecalis* and *Enterococcus faecium* (Hidron, *et al.,* 2009).All the properties that define a strain as a good probiotic, the antibiotic resistance and the ability of such strain to act as a donor of antibiotic resistance genes must be carefully assayed as well, genes conferring resistance to tetracycline, erythromycin and vancomycin have been detected and characterized in *Lactococcus lactis*, Enterococci and *Lactobacillus* species isolated from fermented meat and milk products. A number of initiatives have been recently launched by various organizations across the globe to address the biosafety concerns of starter cultures and probiotic microorganisms. The studies can lead to better understanding of the role played by the dairy starter microorganisms in horizontal transfer of antibiotic resistance genes to intestinal microorganisms and food-associated pathogenic bacteria (Majhenič and Matijašic, 2001; (Mathur and Singh, 2005). Antimicrobial substances are produced by a wide range of bacteria, including dairy starter cultures. Lactic acid bacteria (LAB) can produce antimicrobial substances with the capacity to inhibit the growth of pathogenic and spoilage microorganisms; the main threat associated with these bacteria is that they may transfer resistance genes to pathogenic bacteria, which has been a major cause of concern to human and animal health (Akpinar, *et al.,* 2011; Zhou *et al.,* 2012). One of the methods of the selection for the potentiality of the starter culture strains was the investigation of the safety aspects and technological attempt for the evaluation of the LAB strains to be selected as starter culture for production of *gariss* under controlled conditions. As *gariss* (traditional fermented camel milk of Sudan) one of the important health care of dairy products of the traditional origin, the present

work was design to study the antimicrobial and antibiotics susceptible of LAB strains isolated from *gariss* samples collected from Kordofan and Khartoum production sites using well and disc diffusion methods.

Material and methods

Lab strains

Strains were isolated from *gariss* samples and identified phenotypically and genotypically (using genus specific primer multiplex PCR and GDDE-PCR fingerprinting method data not shown) (*Enterococcuc faecium 75, E.hirae B3, E.faecium G130, E.hirae MP1, E. faecium NWL, E. durans R05, E. dispar DLS3002, E. faecium CK1013, L. pentosus S2LPO2, L. plantarum BJG32, Streptococcus thermophilus, S. acidominimus, E. faecium CB6, E. durans R03, E.sanguinicola UPAA71, E. faecalis 45689, E. mundtii MDEYAN, L. plantarum BJ6 and E. hirae CECT4081*).

Antibacterial screening

The strains of LAB were cultured on MRS and M17 broth for three days at 30°C. The antibacterial activity was made by using well diffusion method (Kelly *et al.,* 1996). In agar TSA (Tryptic Soy Agar Difco, Detroit, USA) flooded by the pathogenic strain *Staphylococcus aureus* ATCC 25923 and *E.coli* ATCC25922 (incubated aerobically at 37°C for 24h in nutrient broth) three wells of 5 mm in diameter were performed. The wells were filled with 60-80 µl of filtered supernatant. The supernatant was firstly centrifugated at 10,000 rpm for 20 minutes and secondly filtered on a 0.2mm pore size sterile disposable syringe membrane filter, according to Kalalou *et al.,* (2004). The filtered supernatant was neutralized by sterilized 0.1 N NaOH (121°C for 15minutes) to obtain a pH of 6.5. Few drops of catalase solution (Biomerieux, Marcy Etoile, France) were added to eliminate the hydrogen peroxide. After 24 hours incubation at 37°C, the diameters of inhibition zones appearing around the wells were measured (average of two diameters perpendicular), the experiment was repeated twice.

Antibiotic resistance test

Resistance to antibiotics was tested by disc diffusion methods. The assay was carried out according to Khali (2009), multiple discs on the same plate was used to eliminate differential effects from growth time and temperature. Pure isolates of lactic acid bacteria were cultivated in liquid medium MRS (MRS broth biokar France). A Petri dish of 25 ml of Muller Hinton medium (Difco, USA) was prepared. The liquid cultures of the strains were incubated one day at 37°C, and then they were diluted by sterile NaCl (9% w/w) to reached a count of 1×10^8 UFC/ml. One milliliter by Petri dish was spread on the medium and the surplus was drained. The Petri dish was left for 15 minutes to have dry surface of medium. After that the antibiotic discs (6 discs per plate) the Petri dish were incubated (24h) at 37°C. After incubation, the diameter of the zones of inhibition was measured. The disc diffusion zone diameter was interpreted by using a table of values of each antibiotic based on the methods studied by Standardisation de l'Antibiogramme en Médecine Vétérinaire (2008) with modifications (table.1). The investigation concerned on all the nineteen different strains of LAB which identified earlier phenotypically and genotypically (the data not shown).

Results and discussion

Antimicrobial effects of LAB isolated from gariss

All the 19 strains of LAB isolated from *gariss* samples were found to show no antimicrobial effect on both *Staphylococcus aureus* ATCC 25923 and *E. coli* ATCC25922.The same findings concerning LAB strains isolated from dairy products were observed by Herreros *et al.,* (2005) who screened thirty-one lactic acid bacteria isolated from Armada cheese for antimicrobial activity their results revealed that none of the strains showed antimicrobial activity against several pathogenic and spoilage reference strains.

Ebrahimi *et al.* (2011) evaluated the antagonistic activity exhibited by different Lactobacillus strains by the well-diffusion method. Most isolates did not inhibit indicator growth when the neutralized

supernatants were applied directly to the agar diffusion test. However, nonneutralized supernatants of those isolates inhibited growth of some indicator bacteria, i.e. *E coli and Staphylococcus aureus* and others.

Table 1. The Interpretation of Antibiotic resistance to LAB.

Antibiotic	Resistance	Intermediate susceptible	Susceptible
1- Ampicillin AM10µg	≤14	≥14	≥24
2- Penicillin P 6µg	≤14	≥14	≥24
3-ChloramphenicolC30µg	≤17	18-20	≥21
4-TetracyclineTE30µg	≤14	15-18	≥19
5-ErythromycinE15µg	≤15	16-20	≥21
6-Ciproflaxin CIP 5µg	≤ 16	17-22	≥23
7-Streptomycin STR 300µg	≤13	13-14	≥15
8-Vancomycin V30µg	≤14	15-16	≥17
9-FlumequineUB30µg	-*	-*	-*

*No interpretation data available for Flumequine.

Table 2. The Susceptibility of selected antibiotic on LAB isolated from *gariss*.

strain /antibiotic	P	AM	C	E	TE	STR	CIP	V	zone of ihibition of UB
E.faecium75	MS	SS	MS	SS	MS	MS	MS	SS	7
E.hirae B3	MS	SS	SS	MS	MS	MS	SS	SS	10
E.faecium G130	SS	SS	SS	SS	SS	SS	MS	SS	0
E.hirae MP1	MS	MS	SS	SS	MS	SS	R	SS	10
E.faecium NWL	SS	SS	SS	SS	SS	SS	SS	SS	14
E.dransR05	R	R	R	R	R	R	R	MS	9
E.dispar DLS3002	MS	MS	R	MS	MS	MS	R	SS	0
E.faecium CK1013	SS	SS	SS	R	SS	SS	MS	SS	9.5
L. pentosus S2LPO2	MS	MS	MS	R	SS	SS	R	SS	0
L. plantarum BJG32	MS	MS	R	R	SS	SS	R	SS	0
S. thermophilus	MS	MS	R	R	SS	SS	R	SS	7.5
S. acidominimus	MS	MS	SS	MS	SS	SS	R	SS	0
E.faecium CB6	MS	MS	MS	MS	R	SS	R	R	4.5
E.dransR03	SS	SS	MS	MS	SS	SS	MS	SS	10
E.sanguinicola UPAA71	MS	R	R	R	R	SS	R	SS	13
E. faecalis 45689	SS	SS	SS	SS	SS	SS	R	SS	4
E. mundtii MDEYAN	MS	SS	SS	R	SS	SS	MS	SS	0
L.plantarum BJ6	SS	SS	SS	MS	SS	SS	MS	SS	0
E.hirae ECT4081	MS	R	R	R	R	R	R	R	10

(No interpretation was found for UB). R: Resistant, MS: Intermediate Susceptible and SS: Susceptible.

AM: Ampicillin10µg, P: Penicillin6µg, C: Chloramphenicol 30µg, TE: Tetracycline 30µg, E: Erythromycin15µg, CIP: Ciproflaxin 5µg, STR: Streptomycin 300µg, V: Vancomycin 30µg and UB: Flumequine 30µg.

Liu *et al.*, (2011) studied *Enterococcus faecium* isolated from *Byaslag* a traditional cheese of Inner Mongolia in China. The bacteriocin produced by *E. faecium* LM-2 showed a broad spectrum of activity against most tested strains in the genus *Listeria, Staphylococcus, Bacillus, Clostridium, Salmonella, Pseudomonas* and *Candida*, especially *L. monocytogenes, Staphylococcus aureus, E. coli, Bacillus cereus, Bacillus subtilis*, which were food spoilage or food borne pathogenic bacteria. Benkerroum *et al.*, (2004) studied the inhibitory activity of camel's milk and colostrums at 4°C and 20°C by the well diffusion assay against pathogen. The results showed that *Bacillus cereus* was resistant to the inhibitory activity present in camel's milk and to the colostrum, while *L. monocytogenes* LMG and

E. coli were the most sensitive as judged by the diameters of the inhibition zones.

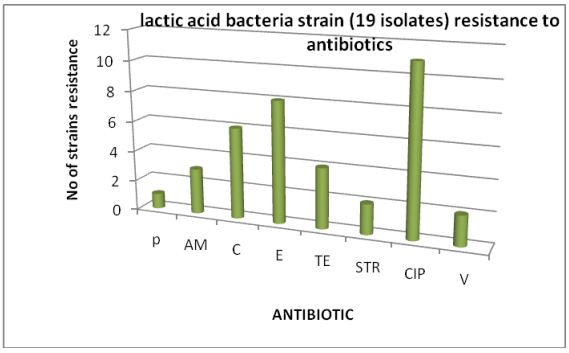

Fig. 1. LAB strains (19 isolates) resistance to antibiotics.

Antibiotic resistance of lab isolated from gariss samples

Enterococci group: Among Fourteen isolates enterococci, 57.14% (8 isolates) showed intrinsic resistance to at least one antibiotic and some even to almost all antibiotics studied; one strain (*E. durans R05*) was resistant to penicillin. This strain was resistant to all antibiotics investigated in present work except vancomycin; three strain (*E. durans R05, E.sanguinicola UPAA71* and *E. hirae CECT4081*) to Ampicillin, four strains (*E. durans R05, E. dispar DLS3002, E.sanguinicola UPAA71* and *E. hirae CECT4081)* to Chloramphenicol, five strains (*E. durans R05, E. faecium CK1013, E.sanguinicola UPAA71, E. mundtii MDEYAN* and *E. hirae CECT4081)* to Erythromycin, four strains (*E. durans R05, E. faecium CB6, E.sanguinicola UPAA71* and *E. hirae CECT4081)* to tetracycline, two strains (*E. durans R05* and *E. hirae CECT4081*) to streptomycin, seven strains (*E.hirae MP1, E. durans R05, E. dispar DLS3002, E. faecium CB6, E.sanguinicola UPAA71, E. faecalis 45689* and *E. hirae CECT4081*) to Ciproflaxin and two strains (*E. faecium CB6* and *E. hirae CECT4081)* resistant to vancomycin (Fig. 1).

Due to the ability of Enterococci to acquire new resistance determinants, multiply resistant strains have emerged in the last decade which exhibit resistant to tetracyclines, chloramphenicol, high levels of aminoglycosides, β-lactams, and vancomycin; the Enterococci isolated from *Hussuwa* (a sorghum- based fermented product that is consumed mainly in Northern Sudan) more than 50% of the strains showed resistance to at least one antibiotic and some even to three antibiotics (Yousif, 2003).

Streptococcus group: *S. acidominimus* was resistant to only one antibiotic (Ciproflaxin), while *S. thermophilus* was resistant to three antibiotics (Chloramphenicol, Erythromycin and Ciproflaxin). Dissemination of antibiotic resistance through the food chain is a serious public health issue, and resistance in fermented dairy products is a component of this paradigm; However, *Strep. thermophilus* isolates were much more frequently observed with combined resistance to chloramphenicol, lincomycin, kanamycin, neomycin, and gentamycin, without tetracycline and streptomycin (Zhou *et al.,* 2012).

Lactobacillus group: *L. plantarum BJG32* was resistant to three antibiotics (Chloramphenicol, Erythromycin and Ciproflaxin); *L. pentosus S2LPO2* was resistant to two antibiotics (Erythromycin and Ciproflaxin), while *L. plantarum BJ6* was not resistant to any one of antibiotics investigated. Many lactic acid bacteria (LAB) are resistant to antibiotics (Curragh and Collins, 1992; Adams and Marteau, 1995; Chateris *et al.,* 1998; Salminen *et al.,* 1998). However, some LAB may carry potentially transmissible plasmid-encoded antibiotic resistance genes, as shown for example in certain *L. fermentum*, *L. plantarum* and *L. reuteri* strains (Ishiwa and Iwata, 1980; Ahn *et al.,* 1992; Tannock *et al.,* 1994 and Fons *et al.,* 1997).

Majhenič and Matijašic (2001) stated that independently of the place of origin, LAB was, in general, sensitive to ampicillin, penicillin and erythromycin, and resistant to kanamycin and neomycin. Therefore, various species of LAB seem not to differ much in sensitivity/resistant pattern indicating that antibiotic susceptibility cannot serve as a criterion for LAB classification.

No data in previous work cited in literature indicated the effects of Flumequine UB30µg antibiotic on LAB. According to our work, there were seven LAB strains (*E.faecium G130*, *E.dispar DLS3002*, *L. pentosus S2LPO2*, *L. plantarum BJG32*, *S. acidominimus*, *E. mundtii MDEYAN and L.plantarum BJ6*) resistant to this antibiotic (i.e. the zone of inhibition was zero). *E. faecium NWL* (14mm) and *E. sanguinicola UPAA71* (13mm) have higher zone of inhibition among all strains investigated. *E.hirae B3, E.hirae MP1*, *E.durans R03* and *E.hirae CECT4081* have the same zone of inhibition (i.e.10mm). Zhou *et al.,* (2005) studied 18 different spectrum antibiotics on ten different strains of LAB, and they stated that all the 10 strains were sensitive to the gram positive spectrum antibiotics, erythromycin and novobiocin, the broad-spectrum antibiotics rifampicin, spectinomycin, tetracycline and chloramphenicol and the h-lactam antibiotics penicillin, ampicillin and cephalothin.

The resistance to the therapeutically used antibiotics ampicillin (three strains), penicillin (one strain) and streptomycin (two strains) was not high. Two strains were resistant to vancomycin and the presence of these vancomycin resistant enterococci in such Sudanese fermented dairy products is cause for concern; due to the problem of antibiotic resistance associated with some enterococcal strains, the use of enterococci as starter cultures was studied (Yousif, 2003).

Some resistant strains could inactivate the antibiotic by destroying or modifying the drug itself, so that it is no longer toxic. Some others may pump the drug out of the bacterial cell so that the concentration of the drug is too low to be effective. Second, the emergence of resistance in these organisms may have arrived through evolutionary events, such as mutations. Some resistant species may have an altered form of the target site of the drug (the place on the cell where the drug binds), so the antibiotic cannot find its target. Third, these resistance genes were located on the chromosome but were not incomplete to be detected. Other mechanisms of antibiotic resistance should also be considered; the resistance to some antibiotics may be mediated by transposons or plasmid-carried genes (Zhou *et al.,* 2012).

Conclusion

LAB isolated from *gariss* samples had no antimicrobial effect on both *Staphylococcus aureus* ATCC 25923 and *E. coli* ATCC25922, while the resistance to the therapeutically used antibiotics ampicillin (three strains), penicillin (one strain) and streptomycin (two strains) was not high. Two strains were resistant to vancomycin.

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