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ANTIMICROBIAL SUSCEPTIBILITY PATTERNS OF LAB ISOLATED FROM WAKALIM, A TRADITIONAL ETHIOPIAN FERMENTED SAUSAGE

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ABSTRACT

Wakalim *is a traditional Ethiopian spiced fermented beef sausage. In this study, 168 lactic acid bacteria strains isolated during the fermentation of* wakalim *were evaluated for their antimicrobial resistance patterns. All of the isolates tested were sensitive to Amp, Pen, Ery, Cep, Aml, and Tet. Among the* Lactobacillus *isolates, the most frequent resistance was noted for methicillin (96%), followed by resistance to streptomycin (85%), vancomycin (72%), kanamycin (47%), and gentamycin (38%). The most frequent resistance among* Pediococcus *isolates was observed for vancomycin and streptomycin (96% each), followed by resistance to kanamycin (84%), gentamicin (55%), and methicillin (30%). A total of 13 multiple drug resistance (MDR) patterns were detected. About 42% of the isolates showed MDR to four drugs, 29% to five drugs, and 2% to six drugs. The most frequent MDR pattern was Van/Str/Kan/Gen/Met and was seen in 42% of the isolates. This pattern was seen in* Lactobacillus, Pediococcus, *and* Weissella *isolates, but was the most frequent pattern in* Pediococcus *isolates (41%). Our* Lactobacillus *isolates showed 10 different MDR patterns, with Van/Str/Gen/Met (21%) and Van/Str/Met (19%) being the most frequent patterns. Our isolates were not reservoirs of transferable resistance genes for tetracycline, erythromycin, ampicillin, penicillin G, cephalothin, and amoxicillin.*

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PRACTICAL APPLICATION

Intrinsic resistance of lactic acid bacteria (LAB) to many antibiotics may be considered as advantageous for those with probiotic potential. This would help to sustainably utilize the strains in the intestine to maintain the natural balance of intestinal microflora during antibiotic treatments. However, there is the danger of transferring multiple drug resistance to pathogens in the intestinal environment. The susceptibility of our LAB isolates, on the other hand, to the most frequently used clinically important antimicrobials is beneficial as it minimizes the chances of disseminating resistance genes to pathogens both in the food matrix and/or in the gastrointestinal tract. It could, thus, be concluded that our isolates are not reservoirs of transferable resistance genes for tetracycline, erythromycin, ampicillin, penicillin G, cephalothin and amoxicillin. This is particularly important because our traditional fermented sausage product, wakalim, is also consumed without any further heat treatment, and some of the LAB isolates exhibited *in vitro* probiotic potential.

INTRODUCTION

A large variety of antibiotics are currently being used in human and veterinary medicine, but their efficacy has been threatened by microbial resistance. Currently, there is concern over the possible spread of resistance determinants to antimicrobials (Florez et al. 2005).

Lactic acid bacteria (LAB) from fermented products may act as reservoirs of antimicrobial-resistance genes that could be transferred to pathogens, either in the food matrix or in the gastrointestinal tract (Florez et al. 2005). Genes conferring resistance to several antimicrobials (i.e., chloramphenicol, erythromycin, streptomycin, tetracycline and vancomycin) located on transferable genetic elements (plasmids and transposons) have already been characterized in lactococci (Perreten et al. 1997) and lactobacilli (Gfeller et al. 2003) from foods. As a result, some species of LAB commonly used in the food industry or naturally occurring in raw food materials are commonly resistant to glycopeptide antibiotics including vancomycin (Tynkkynen et al. 1998).

Resistance to common antibiotics by LAB could be intrinsic or acquired (Mathur and Singh 2005). Lactobacilli, pediococci and Leuconostoc spp. have been reported to have a high natural resistance to vancomycin (Hamilton-Miller and Shah 1998). The resistance of LAB to vancomycin may be due to the presence of D-Ala-D-Lac as the normal dipeptide in their peptidoglycan (Florez et al. 2005). For a number of lactobacilli, a very high frequency of spontaneous mutation to kanamycin and streptomycin was found (Curragh and Collins 1992).

Currently, there are lots of data on the prevalence of antibiotic resistance and the mechanisms implicated in clinical bacteria, but information about the antibiotic susceptibility/or resistance in LAB isolated from food is limited (Cataloluk and Gogebakan 2004; Florez et al. 2005). Particularly, there is no report on the antibiotic resistance patterns of LAB isolated from traditional Ethiopian fermented foods. Those strains isolated from traditional fermented foods could have applications as starter cultures for large scale production or as probiotics to enhance the health of consumers. However, strains intended for use in the food systems as starters or probiotics should be carefully examined for antimicrobial susceptibility (Teuber et al. 1999). The aim of this study was, therefore, to evaluate the current resistance or susceptibility of some LAB isolated from wakalim, a traditional fermented sausage.

MATERIALS AND METHODS

Isolation of Lactic Acid Bacteria

Wakalim is a spiced traditional Ethiopian fermented beef sausage commonly prepared from lean meat, fat, salt, garlic, onion and other spices. For isolation and characterization of LAB, 25 g of samples were aseptically removed from fermenting wakalim and homogenized in 225 mL of 0.1 mL sterile peptone-water using a Stomacher lab blender (Stomacher 400, Seward, London, UK). Appropriate dilution (0.1 mL) of homogenized samples were surface plated in duplicate on pre-dried surfaces of MRS agar and incubated under anaerobic condition, using anaerobic jar (BBL, GasPak Anaerobic Systems) for 2 to 3 days at 30–32C. About 10 to 15 colonies were randomly picked from countable plates of MRS agar, purified and further identified using API 50CH (Biomeriuex, Marcy I'Etoile, France). All LAB strains used in this study were isolated from the natural fermentation of wakalim.

A total of 168 isolates of LAB were used in this study. The isolates belonged to the dominant LAB populations and consisted of Lb. plantarum1 (11), Lb. pentosaceus (3), Lb. paracasei subsp. paracasei (4), Lb. pentosus (4), Lb. brevis1 (5), Lb. fermentum (23), Lact. lactis subsp. lactis (10), Ped. pentosaceus1 (60), Ped. pentosaceus2 (15), Leuconostoc lactis (3), Weissella viridescens (4), and some other members of the genus Lactobacillus (18) and Pediococcus (8).

Antimicrobial Susceptibility Testing

The isolates were tested for their susceptibility to different antibiotics on MRS agar (pH 7.4 ± 0.2) (Rojo-Bezares et al. 2006) following the standardized disc diffusion technique with 12 Oxoid drug discs: amoxacillin (Aml), (25 μg); ampicillin (Amp), (10 μg); cephalothin (Cep), (30 μg); clindamycin (Cli), (2 μg); erythromycin (Ery), (15 μg); gentamycin (Gen), (10 μg); kanamycin (Kan), (30 μg); methicillin (Met), (5 μg); penicillinG (Pen), (10 μg); streptomycin (Str), (10 μg); tetracycline (Tet), (30 μg); and vancomycin (Van), (30 μg).

Standardization of Inocula

Pure culture was inoculated into a tube containing 4 to 5 mL of MRS broth. The broth culture was incubated at 32C for 18 to 24 h under anaerobic condition (BBL, GasPack System). After incubation, the turbidity of the actively growing broth culture was adjusted with sterile physiological saline solution to obtain turbidity approximately comparable with cell density of about 1×10^8 cfu/mL using McFarland's turbidity standards (Andrew 2001).

Inoculation of Test Plates

MRS agar plates (Oxoid) were swabbed with the standardized culture suspensions using a sterile cotton swab. The predetermined antimicrobial discs were dispensed onto the surface of the inoculated agar plate using a disk dispenser (Oxoid, Antimicrobial Susceptibility Test System, UK). After 36 h of incubation at 30–32C under anaerobic condition in an anaerobic jar (BBL, GasPak System), the diameters of the inhibition zones were measured. The strains were classified as sensitive or resistant following the cut-off points given by the manufacturer. For the purpose of interpretation, those intermediate cases were considered sensitive (National Committee for Clinical Laboratory Standards 2000; Rojo-Bezares et al. 2006).

Data Analysis

Data were analyzed using Stastical Package for Social Sciences (SPSS for Windows; version 10.0, SPSS Inc, Chicago, IL, USA).

RESULT

Almost all isolates belonging to the various genera were sensitive to Amp, Pen, Ery, Cli, Cep, Aml and Tet. Among the Lactobacillus isolates, the most frequent resistance was noted for met (96%), followed by resistance to str (85%), van (72%), kan (47%) and gen (38%). The most frequent resistance among Pediococcus isolates was observed for van and str (96%, each) followed by resistance to kan (84%), gen (55%) and met (30%). The few isolates of Lactococcus, Leuconostoc and Weissella showed varying resistance against few drugs (Table 1).

A total of 13 multiple drug resistance (MDR) patterns were detected among our LAB isolates. About 42% of the isolates showed MDR to four drugs, 29% to five drugs and 2% to six drugs. The most frequent MDR pattern was Van/Str/Kan/Gen/Met and was seen in 29% of the isolates. This pattern was seen in Lactobacillus, Pediococcus and Weissella isolates but was the most frequent in Pediococcus isolates (48%). The most frequent MDR pattern against four drugs was Van/Str/Kan/Met (21%) and against three drugs was Van/Str/Met (14%) as shown in Table 2.

Our Lactobacillus isolates showed 10 different MDR patterns, including resistance to five drugs. MDR to Van/Str/Gen/Met and Van/Str/Met were among the most frequent with values of 21% and 19%, respectively. Among the pediococci, the most frequent pattern was Van/Str/Kan/Gen/Met with a value of 48%. The few isolates of Lactococcus, Leuconostoc and Weissella had one MDR pattern each (Table 3).

DISCUSSION

Our LAB isolates showed marked resistance to five of the 12 antibiotics tested in this study and the highest was against streptomycin and vancomycin. Likewise, most LAB investigated by Aymerich et al. (2006) displayed resistance to vancomycin. The antibiotic resistance frequency of our isolates to Streptomycin was similar to that reported by Hummel et al. (2007), but the frequency of resistance to Gentamycin was much higher than that in our study. In addition, none of our isolates revealed resistance to Ampicillin, Penicillin, Erythromycin, Amoxacillinl and Tetracycline although low-level of resistance to these drugs was recorded by other investigators (Hummel et al. 2007). Contrary to our observation, Gevers et al. (2003) isolated tetracycline-resistant lactobacilli from several fermented sausages and found *tet*(M) to be the only resistance genotype.

Various strains of lactobacilli were reported to be resistant to high levels of vancomycin (Tynkkynen et al. 1998; Goldstein et al. 2000; Florez et al. 2005). Natural resistance of lactobacilli to kanamycin, gentamicin, streptomycin and vancomycin was also documented by Danielsen and Wind (2003). The resistance of LAB to vancomycin may be due to the presence of D-Ala-D-Lac as the normal dipeptide in their peptidoglycan (Florez et al. 2005). Lactobacilli, pediococci and leuconostocs which were intrinsically resistant to high

TABLE 1.
RESISTANCE PATTERNS OF LACTIC ACID BACTERIA ISOLATED FROM WAKALIM

Isolates	Number of isolates	Number of resistant isolates											
		Amp	Pen	Van	Ery	Str	Cli	Cep	Kan	Aml	Gen	Tet	Met
Lactobacilli	68	0	0	49	0	58	0	0	32	0	26	0	65
Lactococci	10	0	0	10	0	10	0	0	0	0	10	0	10
Pediococci	83	0	0	80	0	80	3	0	70	0	46	0	25
Leuconostoc sp.	3	0	0	3	0	3	0	0	3	0	0	0	3
Weissella sp.	4	0	0	4	0	4	0	0	4	0	4	0	4
All isolates	168	0	0	146 (87%)	0	155 (92%)	3 (2%)	0	109 (65%)	0	86 (51%)	0	109 (65%)

Aml, amoxacillin; Amp, ampicillin; Cep, cephalothin; Cli, clindamycin; Ery, erythromycin; Gen, gentamycin; Kan, kanamycin; Met, methicillin; Pen, penicillin; Str, streptomycin; Tet, tetracycline; Van, vancomycin.

TABLE 2.
PATTERN OF MULTIPLE DRUG RESISTANCE OBSERVED IN LACTIC ACID BACTERIA ISOLATES

Number. of drugs in pattern	Multiple drug resistance pattern	Number. of resistant isolates
6	Van/Str/Cli/Kan/Gen/Met	3
5	Van/Str/Kan/Gen/Met	49
4	Van/Str/Kan/Met	35
	Van/Str/Gen/Met	24
	Str/Kan/Gen/Met	7
	Van/Str/Kan/Gen	4
3	Van/Str/Met	23
	Van/Str/Kan	7
	Kan/Gen/Met	3
	Str/Gen/Met	2
2	Kan/Met	5
	Str/Met	5
	Van/Met	2

Aml, amoxacillin; Amp, ampicillin; Ceph, cephalothin; Cli, clindamycin; Ery, erythromycin; Gen, gentamycin; Kan, kanamycin; Met, methicillin; Pen, penicillin; Str, streptomycin; Tet, tetracycline; Van, vancomycin.

levels of vancomycin had peptidoglycan precursors terminating with D-lactate instead of D-alanine (Billot-Klein et al. 1994; Handwerger et al. 1994).

Although high proportions of our isolates were resistant to streptomycin, vancomycin, kanamycin methicillin and gentamicin, none of them displayed resistance toward ampicillin, penicillin, erythromycin, cephalothin, amoxicillin and tetracycline. Sensitivity to both ampicillin and cephalothin was also observed in some lactobacilli by Goldstein et al. (2000). On the contrary, the presence of potentially transferable genes conferring resistance to one or more of the antibiotics ampicillin, penicillin, erythromycin, cephalothin, amoxicillin and tetracycline were seen in several members of LAB (Perreten et al. 1997; Gfeller et al. 2003). Thus, the absence of any strain resistant to these antibiotics among our isolates was an indication that these populations neither possessed nor acquired the resistance gene so far.

Variation among our isolates in degree of resistance to different antibiotics could also be explained in terms of natural presence of resistance genes, mutation rate and reception of transferable resistance gene from other microbes. Moreover, the level of susceptibility of Lactobacillus species to various antimicrobial agents was shown to be species-dependent (Danielsen and Wind 2003). Accordingly, there could be several-fold variation between species with respect to susceptibility to vancomycin, tetracycline and clindamycin (Mathur and Singh 2005). Our lactobacilli were more variable than pediococci in their resistance to vancomycin. A report by Kastner et al. (2006)

TABLE 3.
DISTRIBUTION OF MDR AMONG THE VARIOUS GENERA OF LACTIC ACID BACTERIA

Genus	Number tested	MDR pattern	Number showing the pattern
Lactobacillus	68	Van/Str/Kan/Gen/Met	5
		Van/Str/Gen/Met	14
		Van/Str/Kan/Met	12
		Str/Kan/Gen/Met	7
		Van/Str/Met	13
		Van/Str/Kan	3
		Str/Gen/Met	2
		Kan/Met	5
		Str/Met	5
		Van/Met	2
Pediococcus	83	Van/Str/Cli/Kan/Gen/Met	3
		Van/Str/Kan/Gen/Met	40
		Van/Str/Kan/Met	19
		Van/Str/Kan/Gen	4
		Van/Str/Met	10
		Van/Str/Kan	4
		Kan/Gen/Met	3
Lactococcus	10	Van/Str/Gen/Met	10
Leuconostoc	3	Van/Str/Kan/Met	3
Weissella	4	Van/Str/Kan/Gen/Met	4

Van, vancomycin; Str, streptomycin; Kan, kanamycin; Gen, gentamycin; Met, methicillin; Cli, clindamycin; MDR, multiple drug resistance.

showed that Ped. pentosaceus, currently used as meat starter culture, was resistant to clindamycin, tetracycline, vancomycin and some six more antibiotics. In our case, resistance to clindamycin was observed only in Ped. pentosaceus 2 and at very low rate (2%).

A variety of multiple resistance patterns were observed, and the dominant pattern of resistance was Van/Str/Kan/Gen/Met. Multiple drug resistance is a possible phenomenon among LAB, and the resistance of strains of lactobacilli to 10 antibiotics and pediococcii to nine antibiotics has been reported by Kastner et al. (2006). Similar pattern of MDR within a population could indicate the flow of a gene, such as *tet*(K) genes, that confer resistance to multiple antibiotics.

A possible explanation for the susceptibility of all our isolates to those commonly used antibiotics in Ethiopia could be the lack of dissemination of transferable genes within the LAB population that encode resistance. Thus, the observed resistances to the other antibiotics could be the result of natural resistance of the isolates. Intrinsic resistance of LAB to many antibiotics was documented by Salminen et al. (1998). According to Kastner et al. (2006), many of the resistances in LAB are accounted to complex intrinsic features such as cell wall structure or metabolic properties.

Intrinsic resistance of lactic acid bacteria to many antibiotics may be considered as advantageous for those with probiotic potential. This would help to sustainably utilize the strains in the intestine to maintain the natural balance of intestinal microflora during antibiotic treatments. However, there is the danger of transferring multiple drug resistance to pathogens in the intestinal environment. The susceptibility of our LAB isolates, on the other hand, to the most frequently used clinically important antimicrobials is beneficial as it minimizes the chances of disseminating resistance genes to pathogens both in the food matrix and/or in the gastrointestinal tract. Thus, it could be concluded that our isolates are not reservoirs of transferable resistance genes for tetracycline, erythromycin, ampicillin, penicillin G, cephalothin and amoxicillin. This is particularly important because our traditional fermented sausage product, wakalim, is also consumed without any further heat treatment, and some of the LAB isolates exhibited *in vitro* probiotic potential (Bacha 2007).

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