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Original Article

Lactic Acid Bacteria Antagonism of Acid-tolerant and Antibiotic-resistant Non-staphylococcal Pathogenic Species Isolated from a Fermented Cereal Beverage using Baird-Parker Agar

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ABSTRACT

Background and Objectives: Fermented foods may contain acid-tolerant and antibiotic-resistant pathogenic microorganisms. Pathogens can be antagonized by lactic acid bacteria, resulting in improved microbiological safety. This study assessed the antagonistic effects of selected lactic acid bacteria against pathogenic bacteria isolated from a cereal fermented food.

Materials and Methods: Eight presumptive staphylococci isolated from a commercially produced cereal fermented beverage on Baird-Parker agar supplemented with tellurite were identified using 16S rRNA sequencing. Acid resistance of the isolates was assessed using acidified media (brain heart infusion broth of pH 3.6 and 1.5% acidity) for 48 h followed by taking plate counts. Antibiotic resistance (19 antibiotics) was assessed using the disk diffusion assay. A spot-on-the-lawn method was used to assess antagonistic effects of *Lactobacillus plantarum* MNC 21, *Lactococcus lactis* MNC 24, *Weissella confusa* MNC 20 and *Lactobacillus rhamnosus* yoba 2012 against *Lysinibacillus macroides*, *Bacillus subtilis*, *Enterococcus faecalis* and *Escherichia coli*.

Results: Presumptive staphylococci were identified as *Lysinibacillus macroides* (n = 1), *Bacillus subtilis* (n = 2), *Enterococcus faecalis* (n = 4) and *Escherichia coli* (n = 1). These isolates were acid-tolerant (from 6.3 ±0.9 log cfu/mL at 0 h to 3.6 ±0.9 log cfu/mL at 48 h), antibiotic-resistant (multiple antibiotic resistance index of 0.1–0.5) and their growth was inhibited by the lactic acid bacteria (inhibition zone diameters of 14–24 mm).

Conclusions: The lactic acid bacteria cultures of MNC 20, MNC 21, MNC 24 and *Lactobacillus rhamnosus* yoba 2012 can be used in various food fermentations to inhibit growth of bacterial pathogens; thus, improving product safety.

Keywords: Antagonism, acid-tolerant, antibiotic-resistant, lactic acid bacteria, Baird-Parker Agar

Introduction

Lactic acid fermented foods are popular for their unique sensory attributes, nutritional values, shelf stability and safety (1, 2). Shelf stability and product safety are attributed to the antimicrobial compounds produced by the lactic acid bacteria (LAB) (3). A number of cereal-based fermentations are generally dominated by LAB. In these types of fermentations, processors rely on wild microorganisms in the environment and raw materials as well as utensils to begin the process (4). This may create chances for undesirable spoilage and/or pathogenic microorganisms to multiply in the product, especially if production hygiene is poor. To practice more control of the fermentation, starter cultures have been identified for various fermented foods (4, 5).

Obushera is a fermented sorghum-millet beverage, traditionally from south-western Uganda. It is increasingly becoming common in various urban centers because of its cultural, social and perceived health benefits. The unregulated nature in which the beverage is commercially produced presents serious food safety concerns. A recent study reported the presence of acid-tolerant and antibiotic-resistant virulent *E. coli* in commercially produced *Obushera* (6). This was attributed to the fact that processors solely rely on spontaneous fermentation and do not carry out post-processing pasteurization. Moreover, they usually

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work in poor hygiene conditions (7). Previous studies have reported presence of various pathogenic bacterial species in fermented cereal beverages. In general, *E. coli*, *Staphylococcus aureus*, *Klebsiella aerogenes*, *Pseudomonas aeruginosa*, *Salmonella* spp., *Shigella* spp., *Citrobacter* spp. and *Proteus* spp. have been detected in *Ogi*, a fermented cereal beverage from Nigeria (8, 9). Another study reported presence of *E. coli* and *Staphylococcus* spp. in more than 50% of the *Obushera* samples (10). These observations reveal the need for improving process hygiene and adopting starter cultures with antimicrobial properties.

Studies have shown potential contribution of selected LAB such as *Lactobacillus* (*Lb.*) *plantarum* MNC 21, *Lactococcus* (*L.*) *lactis* MNC 24 and *Weissella* (*W.*) *confusa* MNC 20 in production of *Obushera* that is consistent in quality and safety. The three LAB were isolated from *Obushera* and had desirable starter culture characteristics (5). They were not only high and fast lactic acid producers but also produced *Obushera* with a sensory profile similar to the traditionally fermented one. A recent study reported antimicrobial characteristics of *Lb. plantarum* MNC 21, *L. lactis* MNC 24 and *W. confusa* MNC 20 against *S. aureus*, *Escherichia* (*E.*) *coli* and *Salmonella* (*S.*) *enterica* (6, 10). In another study, LAB (MNC 20, 21 and 24) were reported to bind aflatoxin B1; thus, lowering its bioavailability (11). Therefore, these cultures are currently promoted for use in the commercial production of *Obushera*.

Despite evidence showing antagonistic effects of the *Obushera* starter cultures against bacterial pathogens, the scope was limited to *S. aureus*, *E. coli* and *S. enterica* (6, 10). In a recent unpublished study, the authors recovered eight presumptive staphylococci on Baird-Parker agar supplemented with tellurite. These isolates had peculiar biochemical and morphological characteristics that were not characteristics of staphylococci. Therefore, the aim of this study was to establish the identity of the presumptive staphylococci and assess their acid stability and antibiotic susceptibility. The study investigated antimicrobial effects of *Lb. plantarum* MNC 21, *L. lactis* MNC 24 and *W. confusa* MNC 20 against the eight presumptive staphylococci from *Obushera*. Therefore, this study has provided further evidence on the antimicrobial activity of LAB starters from *Obushera.*

Materials and Methods

Lactic acid bacteria

Lb. plantarum MNC 21 (GenBank accession no. JF512470), *L. lactis* MNC 24 (GenBank accession no. JF512471) and *W. confusa* MNC 20 (GenBank accession no. JQ754455) were isolated from *Obushera* (12). Furthermore, *Lb. rhamnosus* yoba 2012 (originally, *Lb. rhamnosus* GG) (Yoba for Life Foundation, Amsterdam, the Netherlands) was provided by the Uganda Industrial Research Institute, Kampala, Uganda. The LAB were separately propagated as described previously (13). Briefly, from the stock (sterile Ringer's solution with 15% glycerol stored at -20 °C), 0.1 mL of each culture was incubated in 100 mL of sterile MRS broth (Laboratorios CONDA, Madrid, Spain) at 30 °C for 24 h. Cells were washed, recovered by centrifugation at 7,500× g for 10 min and suspended in 100 mL of sterile Ringer's solution (Oxoid, Hampshire, England).

Presumptive staphylococci

In this study, eight presumptive staphylococci isolated from commercially produced *Obushera* in a previous study were used. Cultures were isolated on Baird-Parker agar (Laboratorios CONDA, Madrid, Spain) supplemented with tellurite egg yolk emulsion (Oxoid, Hampshire, England), making them presumptive staphylococci. However, despite forming black colonies on the agar, their morphological and biochemical characteristics (Table 1) were not typical of staphylococci. The presumptive staphylococci were initially stored in sterile Ringer's solution with 15% glycerol stored at -20 °C. For further analyses, isolates were propagated by independently inoculating 0.1 mL of the stock in 100 mL of sterile brain heart infusion (BHI) broth (Laboratorios CONDA, Madrid, Spain) and incubating at 30 °C for 48 h. Cultures were washed and recovered by centrifuging at 7,500× g for 10 min and then suspended in 10 mL of sterile Ringer's solution (Oxoid, Hampshire, England).

Table 1. Biochemical reactions and cell morphologies of the presumptive staphylococci isolated from *Obushera*

Isolate	Gram stain	Oxidase test	Catalase test	Indole test	Cell morphology
BMC 4	+	-	+	-	Rods
BMC 5	+	-	+	-	Rods
BMC 22	+	-	-	-	Cocci
BMC 32	-	-	+	+	Rods
BMC 35	+	-	-	-	Cocci
BMC 39	+	-	-	-	Cocci
BMC 41	+	-	+	-	Rods
BMC 45	+	-	-	-	Cocci

Note: Staphylococci are Gram positive, oxidase negative, catalase positive, indole negative cocci

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DNA extraction, polymerase chain reaction and sequencing

Genomic DNA of the eight bacterial isolates was extracted from pure colonies using GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich, Missouri, USA) based on the manufacturer's instructions. Amplification of 16S rRNA genes was carried out as previously described by Mukisa et al. (5) using 1F (5′-GAGTTTGATCCTGGCTCAG=3′) and 5R/1492R (5′-GGTTACCTTGTTACGACTT-3′) universal primers (Macrogen, Seoul, South Korea). The PCR was set up in a total volume of 50 μL, including 0.2 μM of each primer, 25 μL of 1× master mix with standard buffer (New England Biolabs, MA, USA), 4 μL of DNA and sufficient amounts of sterile nuclease-free water. Initial denaturation was carried out at 94 °C for 3 min, followed by 30 cycles of denaturation (94 °C, 30 s), annealing (55°C, 30 s), extension (72 °C, 3 min) and final extension (72 °C, 10 min). The DNA was purified using QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) based on the manufacturer's instructions. This was followed by sequencing with similar primers, using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) and ABI 3730xl DNA Analyzer (Applied Biosystems, USA). Sequencing was carried out by Macrogen Europe, Amsterdam, the Netherlands. Identification was carried out by performing a nucleotide sequence database search at National Centre for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST).

Acid tolerance

Acid tolerance was assessed by adding each isolate to 10 mL of lactate acidified BHI broth (pH 3.6, titratable acidity of 1.5%) to give a final cell concentration of 5–7 log cfu/mL. Broth was incubated at 25 °C and cells enumerated at 0 and 48 h. Cultures were enumerated by surface plating on Baird-Parker agar (Laboratorios CONDA, Madrid, Spain) supplemented with tellurite egg yolk emulsion (Oxoid, Hampshire, England). Plates were incubated at 30 °C for 48 h.

Antibiotic resistance

Susceptibility of the isolates to nineteen antibiotics was assessed as described in the Kirby-Bauer disk diffusion method (14). Briefly, fresh culture suspensions were standardized to 0.5 McFarland (equivalent to 8 log cfu/mL). Using sterile cotton swabs, pre-poured plate count agar (Laboratorios CONDA, Madrid, Spain) plates were swabbed with the culture suspensions and incubated at 37 °C for 1 h. Antibiotic discs were set on the agar surface and incubated at 37 °C for 24 h. Diameters of the inhibition zone were measured in mm. Isolates were categorized as resistant, intermediate or susceptible based on the guidelines of the Clinical and Laboratory Standard Institute (2013). The multiple antibiotic resistance (MAR) index was computed as a/b, where a was the number of antibiotics; to which, the isolate was resistant and b was the total number of antibiotics; to which, the isolate was exposed (15). The antibiotic discs were purchased from Bioanalyse, Ankara, Turkey. These included ampicillin (10 µg), amoxicillin (25 µg), amoxicillin-clavulanic acid (30 µg), colistin (10 µg), gentamicin (10 µg), cephalexin (30 µg), ceftriaxone (30 µg), ciprofloxacin (5 µg), chloramphenicol (30 µg), erythromycin (15 µg), nitrofurantoin (300 µg), kanamycin (30 µg), levofloxacin (15 µg), metronidazole (10 µg), penicillin G (10 µg), rifampicin (5 µg), trimethoprim-sulphamethoxazole (25 µg), tetracycline (30 µg) and vancomycin (30 µg).

Antimicrobial activity

Antimicrobial activities of *Lb. plantarum* MNC 21, *L. lactis* MNC 24, *W. confusa* MNC 20 and *Lb. rhamnosus* yoba 2012 against the presumptive staphylococci isolates were assessed using spot-on-the lawn method (16) with some modifications. Briefly, sterile pre-poured plate count agar plates were spotted with 10 μL of 6 log cfu/mL of each LAB and incubated at 30 °C for 24 h. Plates were overlaid with 10 mL of molten plate count agar at 45 °C seeded with 4 log cfu/mL of each presumptive staphylococcus isolate and incubated at 30 °C for 24 h. The antimicrobial activity was assessed by measuring diameter (mm) of the zone of inhibition around the colonies.

Statistical analyses

All experiments were carried out in triplicates. Data were analyzed using XLSTAT Software (17). One-way analysis of variance was used to test for significant differences ($p < 0.05$) between the means of acid tolerance (microbial counts) and antimicrobial activity (inhibition zone diameters). Post-hoc analysis was carried out using the least significant difference (LSD).

Results

Molecular identification

Identities of the eight presumptive staphylococci based on 16S rRNA sequencing are shown in Table 2. They were all non-staphylococcal species, identified as *Lysinibacillus* (*Ls.*) *macroides* (n = 1), *Bacillus* (*B.*) *subtilis* (n = 2), *Enterococcus* (*En.*) *faecalis* (n = 4) and *E. coli* (n = 1).

Table 2. Identities of the presumptive staphylococcal isolates from *Obushera* based on 16S rRNA sequencing

Isolate code	Closest relatives	Identity score (% sequence similarity[a])	*E* value
BMC*			
4	*Lysinibacillus macroides*	97	0.0
5, 41	*Bacillus subtilis*	98	0.0
22, 35, 39, 45	*Enterococcus faecalis*	97-98	0.0
32	*Escherichia coli*	97	0.0

[a]Percent similarity with related sequences from the NCBI database. *Numerical code for a specific isolate

Acid tolerance

Figure 1 shows tolerance of the isolates to the acidified broth (pH 3.6, titratable acidity of 1.5%). The average counts of the isolates significantly decreased ($p < 0.05$) from 6.3 ±0.9 log cfu/mL to 3.6 ±0.9 log cfu/mL after incubating in acidified broth for 48 h. Acid tolerance of the isolates was in the order *B. subtilis* BMC 41 > *En. faecalis* BMC 45 > *E. coli* BMC 32 > *En. faecalis* BMC 22 > *B. subtilis* BMC 5 > *Ls. macroides* BMC 4 > *En. faecalis* BMC35 > *En. faecalis* BMC 39. Decreases in microbial cells ranged 1.4–4.4 log cfu/mL for the most acid-tolerant to the most acid-labile isolates in lactate acidified BHI broth (pH 3.6, titratable acidity of 1.5%).

Antibiotic susceptibility

Table 3 shows the number of antibiotic susceptible, intermediately susceptible and resistant bacteria while Table 4 shows the bacterial MAR indices. All the bacteria were resistant to colistin and metronidazole but susceptible to amoxicillin-clavulanic acid, ciprofloxacin, chloramphenicol, nitrofurantoin and levofloxacin. *Ls. macroides* BMC 4 and *B. subtilis* BMC 5 had the highest MAR index (0.50). In contrast, *E. coli* BMC 32 had the lowest MAR index (0.10). Five of the eight isolates had MAR indices ≥ 0.2.

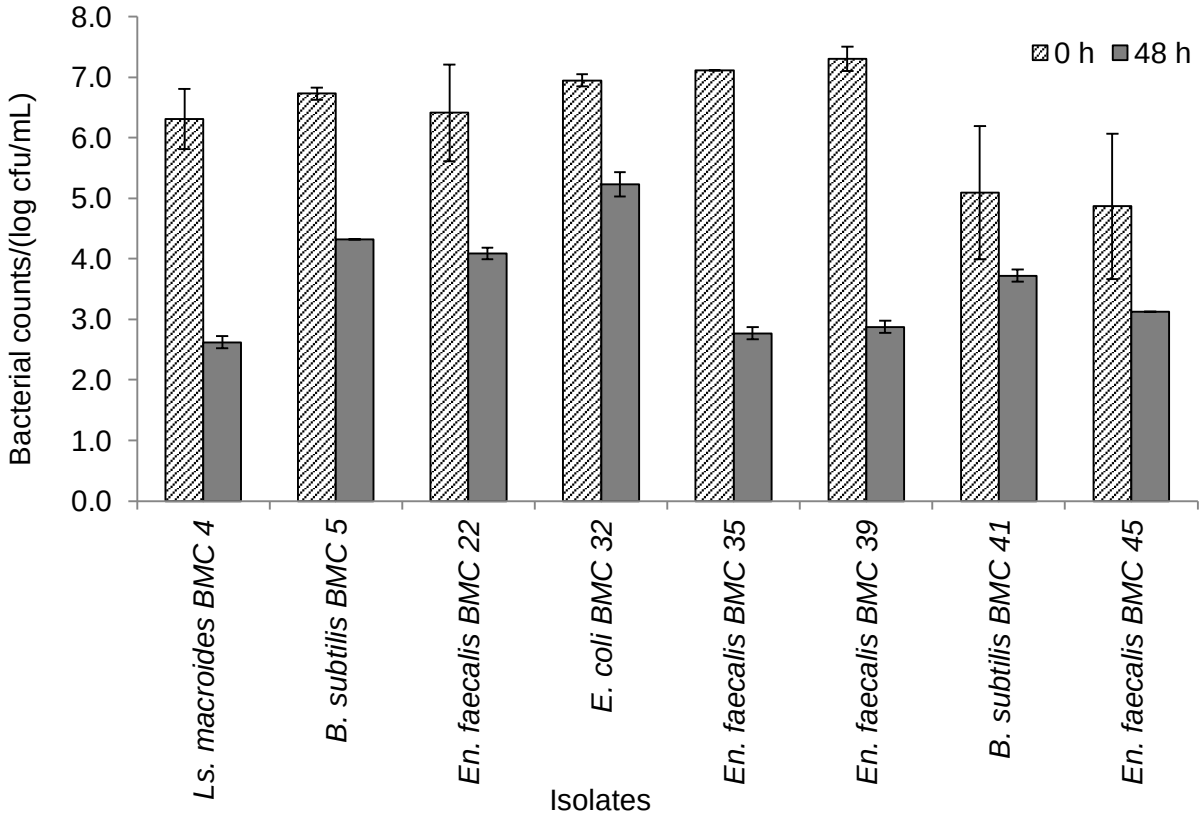

Figure 1. Changes in the bacterial counts between 0 and 48 h of incubation in lactate acidified brain heart infusion broth (pH 3.6, titratable acidity of 1.5%). Values are means of three independent assessments. Error bars represent standard deviations

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Table 3. Prevalence of antibiotic susceptibility in presumptive staphylococcal isolates (n = 8) from *Obushera*

Antibiotic	Susceptible	Intermediate	Resistant
Ampicillin, 10 µg	6		2
Amoxicillin, 25 µg	6		2
Amoxicillin-Clavulanic acid, 30 µg	8		
Colistin, 10 µg			8
Gentamicin, 10 µg	5	3	
Cephalexin, 30 µg	2		6
Ceftriaxone, 30 µg	7		1
Ciprofloxacin, 5 µg	8		
Chloramphenicol, 30 µg	8		
Erythromycin, 15 µg	5	1	2
Nitrofurantoin, 300 µg	8		
Kanamycin, 30 µg	4	3	1
Levofloxacin, 15 µg	8		
Metronidazole, 10 µg			8
Penicillin G, 10 µg	6		2
Rifampicin, 5 µg	2	1	5
Trimethoprim-sulphamethoxazole, 25 µg	6		2
Tetracycline, 30 µg	7	1	
Vancomycin, 30 µg	6		2

Table 4. Antibiotic resistant profiles and multiple antibiotic resistance (MAR) indices of the presumptive staphylococcal isolates from *Obushera*

Bacterial isolate	Antibiotic resistance profile	Number of antibiotics bacterium is resistant to	MAR index
Lysinibacillus macroides BMC 4	AxAmCtClEMetPRaStxVa	10	0.50
Bacillus subtilis BMC 5	AxAmCtClEMetPRaStxVa	10	0.50
Bacillus subtilis BMC 41	CtMetRa	3	0.15
Enterococcus faecalis BMC 22	CtClCroMet	4	0.20
Enterococcus faecalis BMC 35	CtClMet	3	0.15
Enterococcus faecalis BMC 39	CtClKMet	4	0.20
Enterococcus faecalis BMC 45	CtClMetRa	4	0.20
Escherichia coli BMC 32	CtMet	2	0.10

Ampicillin (Am) 10 µg, Amoxicillin (Ax) 25 µg, Ceftriaxone (Cro) 30 µg, Trimethoprim-sulphamethoxazole (Stx) 25 µg, Colistin (Ct) 10 µg, Cephalexin (Cl) 30 µg, Metronidazole (Met) 10 µg, Rifampicin (Ra) 5 µg, Erythromycin (E) 15 µg, Penicillin G (P) 10 µg, Vancomycin (Va) 30 µg, Kanamycin (K) 30 µg. A total of 19 antibiotics were used.

Antimicrobial activity

Antimicrobial activity of the *Obushera* LAB against the eight presumptive staphylococcal isolates is summarized in Figure 2. The LAB inhibited all the presumptive staphylococci isolates (inhibition zone diameter >11 mm) (18). Extent of inhibition varied significantly ($p < 0.05$) for each LAB. *Lb. rhamnosus* yoba 2012 and *L. lactis* MNC 24 had the highest inhibition (24 mm) against *E. coli* BMC 32. The lowest inhibition (14 mm) was seen for *Lb. rhamnosus* yoba 2012 and *W. confusa* MNC 20 (against *En. faecalis* BMC 45) and *Lb. plantarum* MNC 21 (against *B. subtilis* BMC 5 and *En. faecalis* BMC 45).

Discussion

Results from 16S rRNA sequencing of the presumptive staphylococci (Table 2) were similar to results of the biochemical and morphological characteristics (Table 1), which indicated that the eight bacterial isolates were actually non-staphylococcal species. This was not surprising because although BPA supplemented with tellurite egg yolk emulsion was designed to selectively enumerate *S. aureus* in foods, *S. epidermidis* and *S. saprophyticus* as well as other bacterial species could grow on the media and decrease tellurite to form characteristic black/gray colonies (19). The other bacterial species that could grow on this media included *Bacillus* spp., *E. coli*, *E. faecalis*, *Micrococcus* spp. and *Proteus* spp. (19, 20). Similar to staphylococci, the other species formed dark colored colonies on the media (19, 20). Although the appearance of *Lysinibacillus* spp. on BPA is not documented, *Ls. macroides* forms black colonies on the media based on the results.

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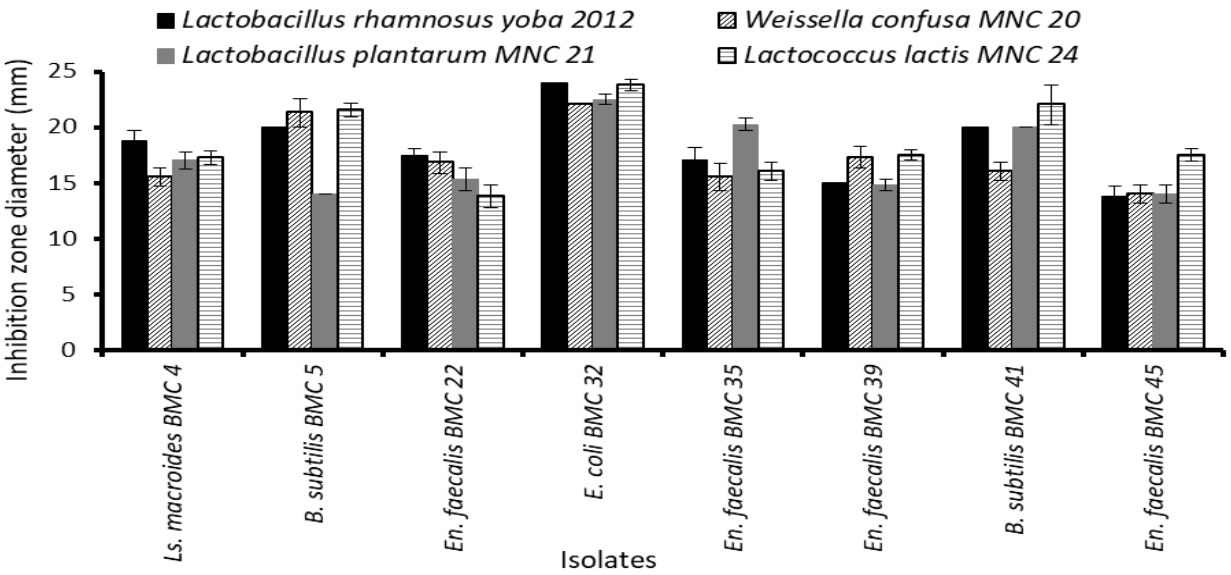

Figure 2. Inhibition of potentially pathogenic bacteria by the selected *Obushera* lactic acid bacteria. Error bars show standard deviations of four independent assessments

It is concerning that the presumptive staphylococci isolated from *Obushera* (Table 2) belonged to species of known pathogenicity (21–24). Bacilli are not only resistant endospore formers that are a nuisance to food processors, but also some species produce emetic toxins and enterotoxins responsible for diarrhea (24, 25). Although *B. subtilis* has not been known to cause illness, virulence genes in *B. subtilis* G7 isolated from a hydrothermal field have been reported (26). Thus, presence of *B. subtilis* in *Obushera* could indicate the presence of other pathogenic bacilli such as *B. cereus* and *B. licheniformis* (24). *En. faecalis* can invade the extraintestinal regions and cause bacteremia (21). Various *E. coli* strains can cause diarrhea, hemolytic uremic syndrome (HUS) and hemorrhagic colitis (HC) (27). Although *Lysinibacillus* spp. are rarely linked to human infections and are merely regarded as environmental contaminants, *Ls. sphaericus* and *Ls. fusiformis* have been involved in bacteremia in immunocompromised patients (22). Presence of such bacteria in *Obushera* deserves special attention and could also imply contamination of similar fermented foods. Indeed, pathogenic bacterial species in various acid fermented foods are documented (28–32).

Authors have reported acid-tolerant pathogenic bacterial species in acid fermented foods (6, 29, 30). Organic acids such as lactic acid are beneficial in food preservation because of their inhibitory effects against pathogenic bacteria (33). Organic acids penetrate the microbial cells in an undissociated form and then dissociate within the cytoplasm, lowering its pH. This causes structural damages to the cell membrane and macromolecules such as DNA. Moreover, it leads to loss of activity of acid-sensitive enzymes such as glycolytic enzymes; thus, severely decreasing ATP production (33). To counteract this, acid-tolerant microorganisms have developed a series of survival mechanisms. One of the mechanisms used is the proton pump such as the multiple-subunit F_1F_0-ATPase, which links production of ATP to the transmembrane proton motive force (34). The transmembrane proton motive force expels excess protons from the cells; hence, increasing the internal pH. The other mechanism includes changes in the cell envelop composition to decrease its permeability to organic acids (35). Some bacteria produce general shock proteins, chaperones and expression of transcriptional regulators, which are vital in the protection or repair of macromolecules such as DNA and proteins (36). Therefore, one or more of these adaptive mechanisms may be responsible for the acid tolerance (Figure 1). Moreover, the tolerance of bacteria to acid stress aggravates virulence and confers cross-protection against agents such as salt and heat in others (37).

Resistance of bacteria to antibiotics is either inherent or acquired (38). Overuse and misuse of antibiotics causes mutations of indigenous genes in bacteria, making them resistant to the drugs (28). Such bacteria use mobile genetic elements (MGE) such as transposons and plasmids to transfer antibiotic resistance genes to other microorganisms (38). Antibiotic resistance of pathogens increases patient hospital stay and thus medical expenses. Multidrug resistance is particularly worrisome because it can lead to eventual death of the patients. The enterococci were resistant to kanamycin, rifampicin, cephalexin, ceftriaxone, metronidazole and colistin (Table 4). Naturally, enterococci show moderate resistance to aminoglycosides (kanamycin, gentamicin and streptomycin). High resistances are linked to aminoglycoside-modifying enzymes which mutate the

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30S ribosomal subunit. Enterococci include chromosomally encoded enzymes capable of modifying kanamycin, resulting in resistance (39). This possibly explains the resistance of *En. faecalis* BMC 39 to kanamycin. While intrinsic enzymes-mediated high-level resistance is reported for kanamycin, there is still no evidence to suggest similar resistances for gentamicin and streptomycin. Therefore, these two antimicrobials are used to treat enterococci infections (40). Enterococcal resistance to rifampicin likely originates from exposure of commensal microbiota to the drug during treatment of other infections. This triggers chromosomal mutations, specifically in RNA polymerase B subunit *rpo*B gene, causing resistance (41). Enterococci are naturally resistant to cephalosporins such as cephalexins and cefriaxone (42). Although this is similar to the current results regarding cephalexins, the bacterial susceptibility to ceftriaxone cannot be explained. Enterococci seem naturally resistant to metronidazole because when it is used as part of an empiric regimen in critically ill patients, most bacteria are killed but enterococci are not affected (43). Colistin is effective against multidrug resistant Gram-negative bacteria (44); however, this antibiotic is not effective against enterococci (45).

The bacilli were resistant to colistin, metronidazole and rifampicin and *B. subtilis* BMC 5, specfically, was resistant to amoxicillin, ampicillin, cephalexin, erythromycin, penicillin G, trimethoprim-sulphamethoxazole and vancomycin (Table 4). This suggests strain differences or acquired resistance. Luo and Helmann (46) have stated that *B. subtilis* have high intrinsic resistance to various β-lactam antibiotics, but the mechanisms are not clear. This might explain *B. subtilis* BMC 5's resistance to amoxicillin, ampicillin, cephalexin and penicillin G. *Bacillus* resistance to erythromycin was due to *erm*J, *erm*D and *erm*K genes which causes methylation of the 23S rRNA macrolide binding sites (47–49). The nature of these genes is unclear. Adimpong et al. (50) have shown that the sites are located on plasmids. Based on Barbosa *et al.* (51) and Gryczan *et al*. (49), these sites are located on the chromosome. It is possible that the resistance is not inherent because Sun *et al*. (52) reported susceptibility of *B. subtilis* to erythromycin. Bacilli are naturally susceptible to rifampicin but can acquire resistance through *rpo*B gene mutation (53). Similarly, no naturally occurring vancomycin resistant *B. subtilis* is reported, suggesting that the resistance is acquired (54). Resistance of *B. subtilis* BMC 5 and BMC 41 to trimethoprim-sulphamethoxazole is supported by Fiedler *et al*. (55) in contrast to Sun *et al*. (52). Resistance might be due to mutational or recombinational changes in target enzymes and/or acquired resistance (56). It is not surprising that the bacilli were resistant to metronidazole and colistin. Metronidazole is effective against anaerobic and microaerophilic bacteria such as *Bacteroides* and clostridia whereas colistin is effective against Gram-negative bacteria (44, 57). Thus, *B. subtilis* may be naturally resistant to these two antibiotics. To the best of the authors' knowledge, susceptibility of *B. subtilis* to metronidazole and colistin has not been documented.

Results demonstrated that *E. coli* BMC 32 was resistant to colistin and metronidazole (Table 4). Although colistin is effective against Gram-negative bacteria, the resistance gene of *mcr*-1 has been reported in *E. coli* (58). These authors have reported that the colistin resistance gene is acquired by horizontal gene transfer. It is possible that *E. coli* BMC 32 had acquired colistin resistance genes. The *E. coli* was resistant to metronidazole because it could not uptake the antibiotic. Metronidazole uptake and killing of *E. coli* is mediated by anaerobic and DNA damage conditions (59, 60). *Ls. macroides* BMC 4 was resistant to ten antibiotics. Contrary to the current results, Sun *et al.* (52) reported susceptibility of *Ls. macroides* to ampicillin, vancomycin, trimethoprim sulphamethoxazole and rifampicin. Moreover, they reported intermediate susceptibility to erythromycin; in contrast to the present results. This verifies that *Ls. macroides* BMC 4 has acquired resistance to these five antibiotics. There is still limited data on the effects of various antibiotics on *Lysinibacillus* spp.

Resistance to (i) rifampicin by enterococci, (ii) erythromycin, rifampicin, vancomycin and trimethoprim-sulphamethoxazole by bacilli, (iii) colistin by *E. coli* and (iv) ampicillin, vancomycin, trimethoprim-sulphamethoxazole and rifampicin by *Lysinibacillus* (Table 4) was of concern because resistance to these agents was not inherently encoded. Moreover, the bacteria were potential pathogens, demonstrating that these antibiotics were ineffective in treatment of their infections. These microorganisms could transfer the acquired resistance to other pathogens. In contrast, their natural resistance to various antibiotics was not worrying since the resistance was not transferable. However, this nature of resistance should not be overlooked because it limits the treatment options. Five of the eight isolates had MAR indices ≥ 0.2 suggesting that these bacteria might have originated from sources that were highly associated with excessive use of the antibiotics tested (61). Resistance to antibiotics commonly used by humans shows that the isolates could have been of anthropogenic (human) origins and thus revealing involvement of processors in *Obushera* contamination. This link between human-based antibiotic resistance of foodborne pathogens and food processors has been supported by Ranjbar *et al.* (28).

LAB metabolites such as organic acids, bacteriocins, hydrogen peroxide, diacetyl and ethanol are critical in inhibition of bacterial pathogens; thus, contributing to food safety and preservation (3). The *Lb. plantarum* MNC 21, *L.*

lactis MNC 24 and *W. confusa* cultures are fast and high producers of lactic acid (14). This organic acid is likely the principle compound responsible for the inhibition of *Ls. macroides*, *B. subtilis*, *En. faecalis* and *E. coli* (Figure 2). These results are similar to those reported by other authors, who studied inhibition of pathogenic bacteria by LAB (6). Some pathogenic bacteria have the ability to survive low pH of acid fermentation by using acid tolerance response (ATR) (62). This is a phenomenon, where bacteria show increased resistance to acid stress due to prior exposure to mildly acidic environments (63). Therefore, LAB in the food should be present in high numbers of at least 6 log cfu/mL or g to avoid activating ATR in pathogenic bacteria (64). The bacteria are enough to rapidly produce sufficient quantities of acid to decrease pH below 4 for effective pathogen inhibition (31, 32). Therefore, presence of antagonistic LAB alone in foods is not adequate for ensuring product safety. Good hygiene practices during food processing are also critical to limiting pathogen contamination.

Conclusion

This study showed presence of acid-tolerant and antibiotic-resistant pathogenic bacteria in *Obushera*. It is of concern that some of the antibiotic resistances in this study were not naturally encoded. Bacteria with acquired antibiotic resistance are serious food safety concerns. This is aggravated by the possibility of the bacteria to transfer traits to other pathogens. *L. macroides*, *B. subtilis*, *E. faecalis* and *E. coli* were isolated on media designed for the isolation of staphylococci, suggesting possible overestimation of *S. aureus* counts or yield of false positives when enumerating or detecting staphylococci, respectively. Growth of these pathogenic species was inhibited by *Lb. plantarum* MNC 21, *L. lactis* MNC 24 and *W. confusa* MNC 20. Therefore, these LAB can be used in food fermentations to improve microbiological safety.

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