Elimination of Antibiotic Resistant *Enterobacteriaceae via* Combined Application of Direct Electric Current, Alternating Electric Current and 2-Thiocyanomethylthio benzothiazole

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Abstract

Multidrug-resistant *Enterobacteriaceae* originating from animals' intestinal tracts may be found on salted and soaked hides/skins. The presence of proteolytic, lipolytic, multidrug-resistant *Enterobacteriaceae* on hides/skins and the resulting destructive processes results in economic losses to the leather industry and creates health hazards for workers. To minimise this destructive bacterial presence, the bactericidal effect of combined application of direct or alternating electric current and an antibacterial agent against multidrug-resistant *Enterobacteriaceae* were examined in this study.

Multidrug-resistant *Citrobacter freundii, Citrobacter koseri, Enterobacter cloacae, Serratia rubidaea, Serratia marcescens, Serratia plymuthica, Morganella morganii, Proteus mirabilis, Providencia rettgeri,* isolated from soaked hides/skins, were used in the present study. Among the test isolates, *Proteus mirabilis, Serratia marcescens, Serratia plymuthica, Serratia rubidaea*, were protease and lipase producer strains. All test isolates were resistant to critically important antimicrobials which are used in both human and veterinary medicine. Bacterial effect of combined application of 508mA/cm^2 direct electric current, 454mA/cm^2 alternating electric current and an antibacterial agent (2-(thiocyanomethylthio) benzothiazole) against the mixed culture of these micro-organisms were investigated in nutrient broth containing 3% NaCl. After application of six cycles of the combined electric current treatment against the mixed culture of multidrug-resistant *Enterobacteriaceae* treated with (2-(thiocyanomethylthio) benzothiazole) was completely killed within five hours. The Log_{10} reduction of the mixed culture at the end of the experiment was 7.55.

In conclusion, the combined application of direct electric current, alternating electric current and antibacterial agent may be used in soak liquors to eliminate proteolytic, lipolytic, multidrug-resistant *Enterobacteriaceae* in the leather industry.

1 INTRODUCTION

Sheepskins and cattle hides harbour different species of the family *Enterobacteriaceae* which are found in human and animal intestines, soil, water and decaying vegetation.[1,2] *Enterobacteriaceae* are commonly transferred from faeces onto hides/skins during the skinning operation on the slaughterline via direct hide-to-faeces contact and hand/equipment contaminated by faeces.[3,4] The presence of these bacteria on hides results in significant damage to hides resulting in economic loss and poses a health hazard to industry workers. When animals are alive, the skin of the animals controls the growth of these micro-organisms due to its low moisture content and acidic pH. After slaughter, these enteric species find a suitable environment to grow and increase in numbers on raw hides and skins. To prevent the bacterial growth on hides and skins, these organic materials are temporarily preserved with salt. In spite of the salting process used to preserve hides and skins, different species of the family *Enterobacteriaceae* were isolated from salted hides and skins.[5,6] In the previous study carried out with 10 salted hides cured in England, Australia and Turkey, 106 isolates of the family *Enterobacteriaceae* such as *Citrobacter amalonaticus, Citrobacter freundii, Edwardsiella tarda, Enterobacter aerogenes, Enterobacter amnigenus, Enterobacter agglomerans, Enterobacter cloacae, Enterobacter gergoviae, Enterobacter liquefaciens, Enterobacter intermedius, Enterobacter sakazakii, Escherichia coli, Hafnia alvei, Klebsiella pneumoniae ssp. ozaenae, Klebsiella oxytoca, Proteus mirabilis, Salmonella paratyphi A, Salmonella choleraesuis ssp. arizonae, Salmonella typhimurium, Serratia marcescens, Yersinia pseudotuberculosis* and *Yersinia ruckeri* were recovered from these samples.[5] In another experimental study, 27 hide isolates of the family *Enterobacteriaceae* (*Cedecea lapagei, Enterobacter sakazakii, Enterobacter cloacae, Escherichia vulneris, Escherichia coli, Ewingella americana, Klebsiella pneumoniaea ssp. ozaenae, Klebsiella oxytoca,*

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Proteus vulgaris, Proteus penneri, Raoultella ornithinolytica, Raoultella planticola, Serratia liquefaciens, Serratia odorifera, Serratia plymuthica, Serratia ficaria, Serratia marcescens, Serratia rubidaea, Yersinia enterocolitica) were recovered from five salted cattle hides (Dubai, Turkey, Israel) and 28 skin isolates of the family *Enterobacteriaceae* (*Enterobacter cloacae, Enterobacter sakazakii, Escherichia vulneris, Escherichia coli, Citrobacter koseri, Klebsiella oxytoca, Klebsiella pneumoniaea ssp. ozaenae, Proteus vulgaris, Proteus penneri, Raoultella ornithinolytica, Raoultella planticola, Serratia liquefaciens, Serratia odorifera, Serratia plymuthica, Serratia ficaria, Serratia marcescens, Serratia rubidaea, Yersinia enterocolitica*) were recovered from five salted sheepskins imported from Australia, Lebanon, U.S.A. and South Africa.[6] When the antibiotic resistance profiles of these isolates were investigated, seventy percent of the salted hide isolates and sixty-eight percent of the salted sheepskin isolates exhibited resistance to three or more antimicrobial agents (chloramphenicol, amikacin, streptomycin, tobramycin, kanamycin, gentamicin, amoxycillin-clavulanate, ampicillin, ampicillin-sulbactam, piperacillin-tazobactam, cefoxitin, ceftriaxone, ceftazidime, cephalothin, cefuroxime sodium, ciprofloxacin, ofloxacin, trimethoprim-sulfamethoxazole, aztreonam, nalidixic acid, tetracycline).[7] While resistance of the isolates to ampicillin, ceftriaxone, cefuroxime sodium was detected as 45%, resistances to nalidixic acid, piperacillin-tazobactam, chloramphenicol, ceftazidime were found respectively to be 42%, 38%, 35%, 33%. Moreover, resistance of some isolates against ampicillin-sulbactam (29%), trimethoprim-sulfamethoxazole (25%), amoxycillin-clavulanate (25%), cefoxitin (20%), cephalothin (16%), tetracycline (16%), and tobramycin (13%) was observed. Although resistance to kanamycin, streptomycin, gentamicin, ciprofloxacin, amikacin, imipenem, meropenem, and ofloxacin of the isolates was uncommon, resistance to aztreonam was fairly common.[7] In addition to salted cattle hides and sheepskins, 26 strains of the family *Enterobacteriaceae* were isolated from soaked sheepskin and cattle hide samples treated with sodium dimethyl dithiocarbamate.[8] When resistance profiles of soaked sheepskin isolates (*Citrobacter freundii, Citrobacter koseri, Cronobacter sakazakii, Enterobacter amnigenus, Enterobacter cloacae, Morganella morganii, Proteus mirabilis, Providencia rettgeri*) against 24 antibiotics were examined, some of the sheepskin isolates were found to be resistant to tobramicin (8%), meropenem (8%), imipenem (8%), trimethoprim-sulfamethoxazole (15%), cefoxitin (15%), ampicillin/sulbactam (15%), kanamycin (15%), tetracycline (31%), chloramphenicol (46%), piperacillin/tazobactam (46%), cefuroxime sodium (46%), nalidixic acid (54%), ciprofloxacin (54%), ceftazidime (61%), streptomycin (62%), cephalothin (62%), amoxycillin/clavulanate (62%), ampicillin (69%), ceftriaxone (69%), and aztreonam (92%).[9] When resistance profiles of soaked cattle hide isolates (*Citrobacter koseri, Cronobacter sakazakii, Morganella morganii, Providencia rettgeri, Serratia marcescens, Serratia plymuthica, Serratia rubidaea*) against 24 antibiotics were investigated, some of the cattle hide isolates showed resistance to kanamycin (10%), piperacillin/tazobactam (20%), ciprofloxacin (20%), ceftazidime (20%), cephalothin (20%), cefoxitin (20%), ampicillin/sulbactam (20%), nalidixic acid (30%), chloramphenicol (30%), ceftriaxone (40%), aztreonam (50%), streptomycin (60%), tetracycline (70%), cefuroxime sodium (70%), amoxycillin/clavulanate (80%), ampicillin (80%).[9] It was observed that eighty-three percent of the salted hide isolates and eighty-six percent of the salted sheepskin isolates showed resistance to five or more antimicrobial agents tested.[9]

All of the studies mentioned above showed that *Enterobacteriaceae* on both salted and soaked hides/skins were not killed by conventional applications in the leather industry. In the previous studies, antibacterial activity of direct electric current and alternating electric current against *Enterobacteriaceae* colonising intravascular catheters,[10] goat meat surface,[11] beef surface,[12] in water samples taken from river and sea[13] and hide brine curing liquors[14,15] has been demonstrated by researchers. The viable cell counts of *Proteus mirabilis, Klebsiella pneumoniae, Escherichia coli* which belong to the family *Enterobacteriaceae, Staphylococcus aureus, Staphylococcus epidermidis* colonising intravascular catheters were reduced *via* 10mA direct electric current.[10] Saif *et a*l. (2006) reported that *E. coli* O157:H7 (10^8CFU/mL) found on meat surfaces was killed by a pulsed direct current square wave electric signal with 10, 20, 30mA/cm^2 current levels, at 30, 50, 80% duty cycles and 1kHz frequency within 32 minutes, respectively. In addition, the antibacterial activity of direct electric currents of 15mA/cm^2, 30mA/cm^2, and 45mA/cm^2 at 1, 10 and 100kHz; 30%, 50% and 70% duty cycles, 2, 8, 16 minutes treatment durations against *E. coli* O157:H7 (ATCC 700728) found on beef surfaces (105 CFU/mL) was examined by Mahapatra *et al.* (2011). Researchers stated that 98.9% reduction of *E. coli* O157:H7 found on beef surfaces was obtained with 45mA/cm^2 at 1kHz frequency, 70% duty cycle and 16 minute treatment.[12]

In our previous study, the antibacterial activity of 0.5A, 1A, 1.5A, 2A alternating electric currents to multidrug-resistant *E. coli* ATCC 25922 and multidrug-resistant fecal *E. coli* MAAG 1405 which was isolated from a stream, were separately investigated in the water samples collected from the Sarısu River, Ayamama River, Black Sea, and the Marmara Sea in Turkey. These strains were inactivated in 5 minutes in Sarısu River water (0.5 A), 1-3 minutes in marine waters (1 A), 5-10 minutes in Ayamama River water (1A) *via* alternating electric current treatment.[13] Furthermore, 1.5A alternating electric current treatment for 15 minutes was found to be sufficient to kill proteolytic and lipolytic *Enterobacter cloacae* which belongs to the family *Enterobacteriaceae* in brine medium containing 25% NaCl.[14] In addition to these

studies, antibacterial activity of the combined treatment using 1.5A direct electric current and 2A alternating electric current to proteolytic hide isolate *Enterobacter cloacae* in Nutrient Broth containing 25% NaCl was examined. After the application of the electric current for 34 minutes, viable cell counts of *Enterobacter cloacae* were reduced from 1.88 x 10^7CFU/mL to 1.18 x 10^3CFU/mL and the Log_{10} reduction factor of *Enterobacter cloacae* was measured as 4.2.[15]

The presence of proteolytic and lipolytic multidrug-resistant *Enterobacteriaceae* on the soaked hides and skins causes economic loss and human health hazards for the leather industry. Hence, the objective of this research was to determine bactericidal effect of combined application of 508mA/cm^2 DC, 454mA/cm^2 AC, and antibacterial agent (2-(thiocyanomethylthio) benzothiazole) against *Serratia rubidaea, Serratia marcescens, Serratia plymuthica, Proteus mirabilis, Morganella morganii, Citrobacter koseri, Providencia rettgeri, Enterobacter cloacae* and *Citrobacter freundii* which were resistant to antibiotics used in human and veterinary medicine.

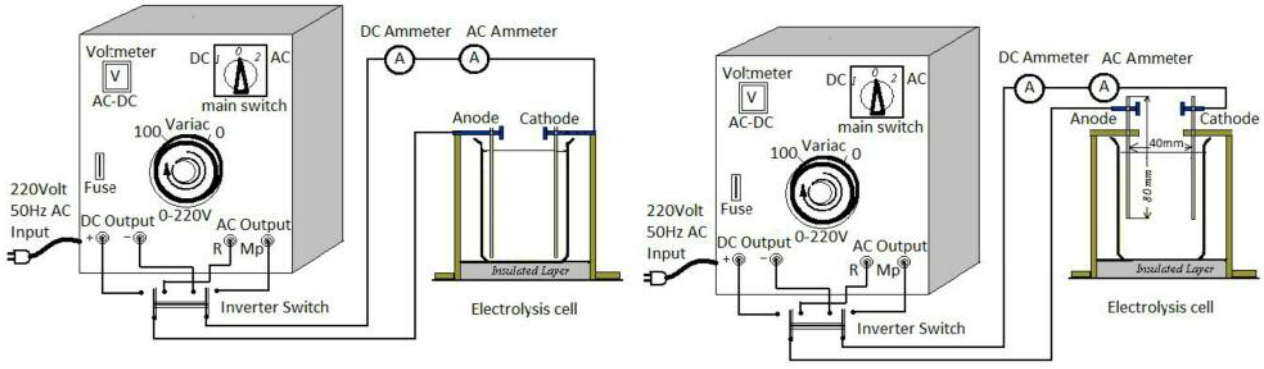
2 EXPERIMENTAL PROCEDURES

2.1 Test micro-organisms

In this study, multidrug-resistant *Serratia rubidaea, Serratia marcescens, Serratia plymuthica, Proteus mirabilis, Morganella morganii, Citrobacter koseri, Citrobacter freundii, Providencia rettgeri, Enterobacter cloacae*, originally recovered from soaked hides/skins treated with sodium dimethyldithiocarbamate in our earlier research,[8] were chosen as test micro-organisms. These isolates belonging to the family *Enterobacteriaceae* were identified using analytical profile index (API) 20E test kits which is specific test for identification of the family *Enterobacteriaceae* in our previous study.[8] While *Proteus mirabilis, Serratia marcescens, Serratia plymuthica, Serratia rubidaea* produced both protease and lipase enzymes, *Morganella morganii, Citrobacter koseri, Providencia rettgeri, Enterobacter cloacae* and *Citrobacter freundii* did not produced these enzymes.[8] *Serratia rubidaea, Serratia marcescens, Serratia plymuthica, Proteus mirabilis* were detected respectively to be resistant to 7 antibiotics (chloramphenicol, nalidixic acid, aztreonam, cefoxitin, piperacillin-tazobactam, ampicillin-sulbactam, ampicillin), 5 antibiotics (streptomycin, cefuroxime sodium, tetracycline, amoxycillin-clavulanate, ampicillin), 5 antibiotics (aztreonam, cefuroxime sodium, ceftriaxone, tetracycline, amoxycillin-clavulanate), 13 antibiotics (tobramycin, streptomycin, ciprofloxacin, imipenem, nalidixic acid, aztreonam, ceftazidime, ceftriaxone, cephalothin, tetracycline, amoxycillin-clavulanate, ampicillin-sulbactam, ampicillin). In addition, *Morganella morganii, Citrobacter koseri, Providencia rettgeri, Enterobacter cloacae* and *Citrobacter freundii* were respectively resistant to 9 antibiotics (streptomycin, chloramphenicol, cefoxitin, cefuroxime sodium, cephalothin, tetracycline, amoxycillin-clavulanate, ampicillin-sulbactam, ampicillin), 5 antibiotics (ciprofloxacin, nalidixic acid, aztreonam, ceftriaxone, ampicillin), 10 antibiotics (kanamycin, chloramphenicol, ciprofloxacin, nalidixic acid, aztreonam, ceftazidime, ceftriaxone, tetracycline, amoxycillin-clavulanate, ampicillin), 10 antibiotics (streptomycin, chloramphenicol, trimethoprim-sulfamethoxazole, nalidixic acid, aztreonam, cefuroxime sodium, ceftazidime, cephalothin, piperacillin-tazobactam, ampicillin), 11 antibiotics (streptomycin, chloramphenicol, ciprofloxacin, meropenem, aztreonam, cefoxitin, ceftriaxone, cephalothin, piperacillin-tazobactam, amoxycillin-clavulanate, ampicillin).[9,16,17]

2.2 Electric current treatments and antibacterial application on the mixed culture of test micro-organisms

Each test isolate was grown on Nutrient Agar Medium (Merck, Darmstadt, Germany) containing 0.5% peptone, 0.3% meat extract, and 1.2% agar (pH7.0) for 24 hours at 37°C. Then, pure culture of each isolate was inoculated into the same nutrient broth medium and incubated for 24 hours at 37°C. The final density of each micro-organism was adjusted to 10^8CFU/mL by using sterile 0.85% saline solution. These bacterial suspensions were used to prepare the mixed culture of these isolates. Twenty mL of the mixed culture were added into the test medium containing 180mL sterile Nutrient Broth and 3% NaCl. A quantity of 100μL was taken from the test medium, serially diluted and spread evenly over Nutrient Agar surface to determine the viable cell counts of mixed culture. After incubation of the plates at 37°C for 24 hours, the colonies grown on Nutrient Agar Medium were counted. Before the experiment, temperature and pH of the test medium were respectively adjusted to 24°C and 7.0. The electrolysis cell consisted of a glass beaker having two internally attached platinum wire electrodes which were dipped into test medium. A variable alternating current source (VARIAC - VA: 2250, f: 50 Hz, Input: 220 V) was used to connect two platinum electrodes (80mm in length and 1mm in diameter, with a separation of 40mm). This source had a DC/AC main switch for DC and AC output power supply selection.[18,19] First, 508mA/cm^2 DC was applied for 2.5 minutes and this medium was left for 3 minutes without any treatment at room temperature. Then, 454 mA/cm^{2}AC was applied for 2.5 minutes and the medium was left for 5 minutes at room temperature. This treatment was conducted four times. Later, this medium was kept for 45 minutes at room temperature and the aforementioned electric current treatment was applied twice. After the treatment, 80mg of the antibacterial agent (2-(thiocyanomethylthio) benzothiazole) was added into 200mL test medium. Later, this medium was stored for seven hours at room temperature. Viable cell counts of the culture were determined after each cycle of electric current treatments, 45 minutes storage, and during the storage periods at room temperature (Fig 1).



a. DC treatment

b. AC treatment

Figure 1. Electrolysis cell used in this study.

3 RESULTS AND DISCUSSION

During the each cycle of electric current treatment, the test micro-organisms in the medium were killed gradually. After application of four cycles of electric current treatment combining both 508mA/cm^2 DC and 454mA/cm^2 AC, viable cell counts of the culture were reduced from 3.6 x 10^{7}CFU/mL to 1.0 x 10^{6}CFU/mL (1.55 Log_{10} reduction factor). Viable cell counts of the culture were detected as 5.0 x 10^5 CFU/mL (1.85 Log_{10} reduction factor) after 45 minutes storage. Significant reduction of viable cell counts of the mixed culture (4.6 x 10^{2}CFU/mL, 4.89 Log_{10} reduction factor) was detected after application of two cycles of DC treatments applied together with AC treatments. After addition of the antibacterial agent into the test medium treated with six cycles of electric current applications, the viable cells in the culture were killed gradually during storage. The viable cell counts of the culture were respectively detected as 3.0 x 10^2 CFU/mL (5.07 Log_{10} reduction factor), 1.7 x 10^2 CFU/mL (5.32 Log_{10} reduction factor), 1.0x10^{2}CFU/mL (5.55 Log_{10} reduction factor), and 8.0x10^{1}CFU/mL (5.65 Log_{10} reduction factor) within one hour storage, two hours storage, three hours storage, and four hours storage. Finally, all isolates of multidrug-resistant *Enterobacteriaceae* used in this study were completely killed within five hours storage at room temperature (Fig. 2).

Antibiotic resistance profiles of our test isolates were ranged from 5 to 13 antibiotics which belong to eleven different antibacterial classes such as aminoglycosides (tobramycin, streptomycin, kanamycin); amphenicols (chloramphenicol); carbapenems (meropenem, imipenem); cephalosporins 1st generation (cephalothin); cephalosporins 2nd generation (cefuroxime sodium, cefoxitin); cephalosporins 3rd generation (ceftriaxone, ceftazidime); sulfonamides, dihydrofolate reductase inhibitors and combinations (trimethoprim-sulfamethoxazole); monobactams (aztreonam); penicillins-β-lactam/β-lactamase inhibitor combinations (ampicillin, amoxycillin-clavulanate, ampicillin-sulbactam, piperacillin-tazobactam);

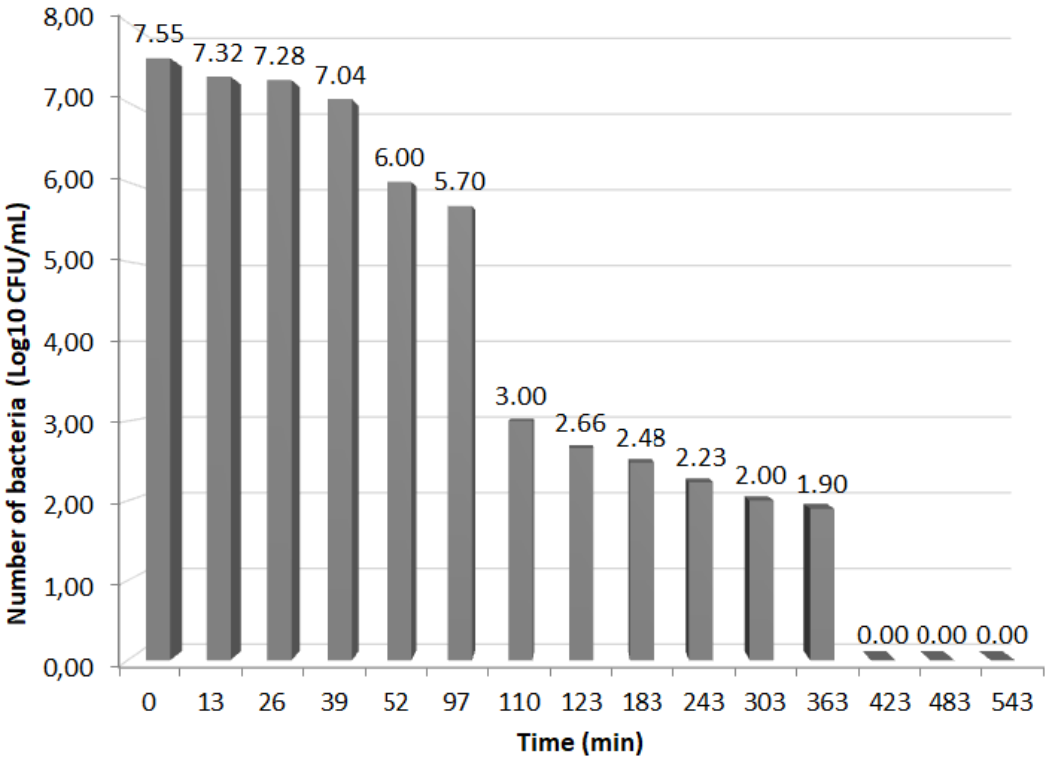


Figure 2. Log_{10} values of the mixed culture during the treatments and storage periods.

quinolones and fluoroquinolones (ciprofloxacin, nalidixic acid); tetracyclines (tetracycline).[9,16,17]

While ampicillin, amoxycillin-clavulanate, ampicillin-sulbactam, piperacillin-tazobactam, meropenem, imipenem, tobramycin, kanamycin, streptomycin, ceftazidime, ceftriaxone, aztreonam, ciprofloxacin and nalidixic acid were categorized as critically important antimicrobials according to the 5rd revision of the WHO list of critically important antimicrobials for human and veterinary medicine, cefalotin, cefoxitin, cefuroxime-sodium, trimethoprim-sulfamethoxazole, tetracycline, and chloramphenicol were reported as highly important antimicrobials for human and veterinary medicine.[20]

In our previous study, the antibacterial effect of AC against both multidrug-resistant fecal *E. coli* MAAG 1405 and multidrug-resistant *E. coli* ATCC 25922 was investigated. While *E. coli* MAAG 1405 was found to be resistant to imipenem, amoxicillin-clavulanic acid, cefuroxime, cefotaxime, ampicillin, *E. coli* ATCC 25922 was resistant to amoxicillin-clavulanic acid, and ampicillin.[13] Both test strains (10^4CFU/mL) found in the water collected from Ayamama River were killed *via* 1A AC treatment 5-10 minutes, 1.5A AC treatment 3-5 minutes and 2A AC treatment for 1-3 minutes. When water samples collected from Black Sea, the Marmara Sea and Ayamama River were used as liquid media, these isolates were exterminated in 1 minute *via* 1A AC treatment. While the test strains in water of Sarısu River were annihilated *via* 0.5A AC in 5 minutes, 1A AC in 3 minutes, 1.5A and 2A treatments killed these strains in 1 minutes.[13]

Electric current treatments have also been used by the other scientists to kill Gram-negative micro-organisms such as *Vibrio parahaemolyticus* in seawater[18] and *Escherichia coli* O157:H7 on beef samples.[11,12] Park *et al.* (2003) applied 1.55mA, 10mA, 50mA, 100mA, 500mA, 1A and 2A DC to *Vibrio parahaemolyticus* ($1.0x10^4$CFU/mL) inoculated into seawater to examine bactericidal effect of DC. *Vibrio parahaemolyticus* in the seawater was killed *via* 500mA, 1A and 2A DC treatments within 100 minutes. In another study, researchers applied respectively 15mA/cm^2, 30mA/cm^2 and 45mA/cm^2 DC for 2, 8 and 16 minutes to beef samples inoculated with *Escherichia coli* O157:H7 in 0.15M NaCl solution. When the beef treated with 45mA/cm^2 DC for 16 minutes, reduction of 98.9% of *Escherichia coli* O157:H7 was observed.[12]

In another of our studies, the antibacterial activity of electric current application combining 1A DC and 1.5A AC with 1.5g/L of sodium dimethyldithiocarbamate against antibiotic-resistant microbial culture composed of Gram-positive and Gram-negative bacteria (*Pseudomonas putida, Enterobacter cloacae, Staphylococcus intermedius, Enterococcus avium, Bacillus lentus* and *Aerococcus viridans*) was tested in the test medium containing organic substances and 3% NaCl. Experimental results of the study showed that the antibiotic-resistant mixed culture was killed in 3 hours after antibacterial agent treatment.[21] Moreover, 0.5A DC, 1A DC, 0.5A AC, 1A AC were applied separately to the mixed culture of multidrug-resistant moderately halophilic bacteria (*Bacillus pumilus, Bacillus licheniformis, Gracilibacillus dipsosauri, Staphylococcus saprophyticus* and *Idiomarina loihiensis*) in brine solutions containing 25% NaCl.[22] These isolates were resistant to amikacin, streptomycin, spectinomycin, polymyxin B and oxolinic acid. While the mixed culture was exterminated within 1 minute *via* 0.5A DC, the mixed culture was annihilated within 15 minutes and 10 minutes *via* 0.5A AC and 1A AC treatments, respectively.[22]

4 CONCLUSIONS

This study investigates the bactericidal effect of a new treatment system composed of direct electric current, alternating electric current and 2-(thiocyanomethylthio) benzothiazole against the mixed culture of multidrug-resistant *Enterobacteriaceae* in NB containing 3% NaCl. This treatment system was found to be very effective in killing multidrug-resistant test strains such as *Proteus mirabilis* (13R), *Citrobacter freundii* (11R), *Providencia rettgeri* (10R), *Enterobacter cloacae* (10R), *Morganella morganii* (9 R), *Serratia rubidaea* (7R), *Citrobacter koseri* (5R), *Serratia marcescens* (5R) and *Serratia plymuthica* (5R), isolated from soaked hides/skins. Results of this study proved that proteolytic, lipolytic and multidrug-resistant *Enterobacteriaceae* damaged *via* six cycles of 508mA/cm^2 DC treatment applied together with 454mA/cm^2 AC can be eradicated completely by 2-(thiocyanomethylthio) benzothiazole.

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