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Antibiotic susceptibility patterns of biofilm producing gram negative bacilli isolated from Kilis local cheese (*Food–related antibiotic resistance*)

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Abstract. In present study, it was investigated to determine the presence of Gram–negative enteric bacilli from dairy products including raw milk and cheese traditionally manufactured in Kilis. The antibiotic susceptibility profiles and ability of biofilm forming of strains isolated from Kilis local cheese were also researched. Totally, 30 Gram negative enteric bacilli that are members of the family *Enterobacteriaceae* was isolated from Kilis cheese sample. Out of 30 isolates, 11 strains (37 %) were revealed to be biofilm producer by Congo red agar analysis. 100 % of these isolates showed sensitivity to aztreonam, cefazolin, cefepime, ceftazidime, cefuroxime axetil, chloramphenicol, gentamicin, meropenem streptomycin and tetracycline. On the other side, 11 strains were resistance to clindamycin, erythromycin and penicillin. MAR indexs of these enteric isolates were found as 0.23. Our results clearly indicate that antibiotic resistance of biofilm–forming *Enterobacteriaceae* spp. in Kilis cheese may adversely affected the safety and quality of local foods and human health.
Keywords: Antibiotic resistance, Biofilm, Enteric bacteria, Food quality.

Introduction

Antibiotics, natural, synthetic, or semi–synthetic substances, are powerful medicines that fight some infections for protection global health. Antibiotics have been extensively used for enhancing the life expectancy of patients and the decline in bacterial infectious disease mortality.

However, the widespread use of antibiotics results in emergency of antimicrobial resistance among bacteria [STAVROPOULOU *et al.*, 2019]. The spread and increase of antibiotic resistance are recognised as a serious global problem worldwide for human, animal health and the environment by the World Health Organization [DIAS *et al.*, 2018].

The overuse of antibiotics for the rapid growth of food–producing animals causes the transfer of antibiotic resistance bacteria along the food chain [FOUNOU *et al.*, 2016]. Additionally, the resistance genes in bacteria move from food to human community by the various ways such as food processing (starter cultures, probiotics, bio conserving microorganisms and bacteriophages) and agriculture production. In brief, food is a vector that transfers antibiotic resistance bacteria and genes coded this resistance to human [VERRAES *et al.*, 2013].

One of the major factors promoting to antibiotic resistance is three–dimensional living structure formed by microorganisms embedded in a slimy extracellular matrix [DIAS *et al.*, 2018; GEBREYOHANNES *et al.*, 2019].

Biofilm–associated antimicrobial resistance is disseminated by genetic material exchange included conjugation, transduction, and transformation with varying from organism to organism [SALA *et al.*, 2012].

Especially, the mixed bacterial biofilm produced by several species of Gram–negative bacteria contaminated foods enhances the resistance to specific antibiotic substances compared to planktonic cells [GERBA, 2015].

And bacterial biofilms on food contact surfaces (stainless steel, glass,

rubber and polyurethane) can adversely influenced the safety and quality of food [GALIE *et al.*, 2018] in the dairy industry.

In addition, spoilage enzymes such as protease and lipases produced by biofilm release into the dairy products. This leads to the reduction in quality of raw milk and products [WEBER *et al.*, 2019].

The aim of the present work was to investigate the antibiotic susceptibility profiles of biofilm forming Gram negative enteric bacteria isolated by dairy products collected from Kilis local market.

Material and methods

Enteric bacilli isolation

Raw milk and dairy products were collected from 3 different local markets, Kilis. All samples were aseptically transported to our microbiology laboratory. 1 mL of raw milk sample was mixed with 9 mL sterile peptone water.

Each cheese product (10 g) was homogonized by gentle mixing with 90 mL sterile peptone water. Homogenized dairy samples were incubated at 37 °C for 24h. Then, the resulting bacterial suspensions were serially diluted in sterile physiological saline up to 10^8.

Two–fold serial dilutions of the samples were inoculated in MacConkey agar for isolation of Gram–negative enteric bacilli. Enteric bacteria having the ability to ferment lactose were screened according to the appearance of pink colonies on agar plate. The selected isolates were subcultured in nutrient agar for further analysis and stored at 4°C.

Biofilm formation and bacteria identification

For investigating biofilm formation of Gram–negative enteric bacilli, congo red analysis (brain heart infusion broth (BHI) supplemented with 5 % sucrose and 0.8 g L^{-1} congo red stain) was performed [MATHUR *et al.*, 2006]. The enteric bacteria were inoculated on congo red agar plate and incubated at 37 °C for 24h. Subsequently, colony colors of bacilli were observed. The dark red or blackish colonies with dry or crystalline consistency were evaluated as biofilm producers [DA COSTA LIMA *et al.*, 2017].

The biofilm producers' strains were identified by applying morphological and biochemical methods and comparing with standard description reported in Bergey's Manual of Determinative Bacteriology.

Antibiotic susceptibility test of biofilm producing strains

This test was carried out on Mueller Hinton Agar (MHA) by Kirby–Bauer disc diffusion assay according to the Clinical Laboratory Standard Institute (CLSI) guidelines [BAUER *et al.*, 1966].

The commercially antibiotics discs including Aztreonam (ATM; 30 mcg), Cefazolin (CZ; 30 mcg), Cefepime (FEB; 30 mcg), Ceftazidime (CAZ; 30 mcg), Cefuroxime axetil (CXA; 30 mcg), Chloramphenicol (C; 30 mcg), Clindamycin (CD; 2 mcg), Erythromycin (E; 15 mcg), Gentamicin (GEN; 10 mcg), Meropenem (MRP; 10 mcg), Penicillin (P; 10 mcg), Streptomycin (S; 10 mcg) and Tetracycline (TE; 30 mcg) were tested for all strains. The turbidity of the overnight bacterial culture was adjusted to 0.5 McFarland standard reference range.

Following the inoculation, the plates were incubated at 37 ºC for 12–24 h.

Then, the diameter of inhibition zone was measured and the results were evaluated as sensitive, intermediate resistant or resistant by comparing to CLSI standard results.

All antibiotic susceptibility analyses were carried out in triplicate.

Results and discussion

Totally, 30 Gram negative enteric bacilli, members of the family *Enterobacteriaceae*, were isolated from cheese sample collected from Kilis local markets. Isolates were identified by morphological (Gr staining) and biochemical test (indole, methyl red, voges proskauer, citrate, catalase and oxidase test systems).

Regarding the genus found, all bacteria isolated from dairy products were characterized as enteric bacilli. Similar findings related to *Enterobacteriaceae* isolated from traditional cheese samples have been reported. Guven and collab., declared isolation of Gram–negative enteric bacilli belong to *Klebsiella*,

Enterobacter and *Escherichia* genera from Urfa cheese, Turkey [GUVEN *et al.*, 2008].

In the other study, the growth of *Escherichia* and *Klebsiella* genera from production to storage of the cheese named "wara" in Nigeria was demonstrated [OGBOLU *et al.*, 2014]. Mladenović and collab., indicated that the presence of Gram–negative enteric bacilli (*Klebsiella*, *Enterobacter, Serratia* and *Escherichia* spp.) in traditional Serbian cheese [MLADENOVIĆ *et al.*, 2017].

Results expressed by Ardıc and collab., that enteric bacilli species were isolated from in Urfa cheese traditionally produced by using raw milk are similar to our findings [ARDIC *et al.*, 2007].

Among 30 isolates, 11 strains (37 %) with rough black colonies were classified as biofilm producer by CRA analysis (Figure 1). The residual strains (73 %) with smooth red colonies were described as non–biofilm producers.

In addition to the presence and identification of *Enterobacteriaceae* genera in milk products, some studies have focused on virulence factors such as antibiotic resistance [AMADOR *et al.*, 2009] and biofilm formation of theirs [MLADENOVIĆ *et al.*, 2018]. The results obtained in this research are similar to the other studies which determined biofilm forming ability of enteric bacteria, *E. coli*, isolated from artisanal cheese in Southern Brazil [PARUSSOLO *et al.*, 2019].

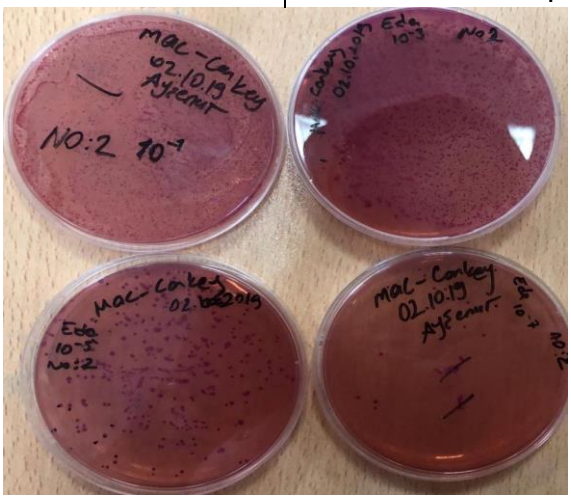

Figure 1. Colony observation of Enterobacteriaceae strains on MacConkey agar

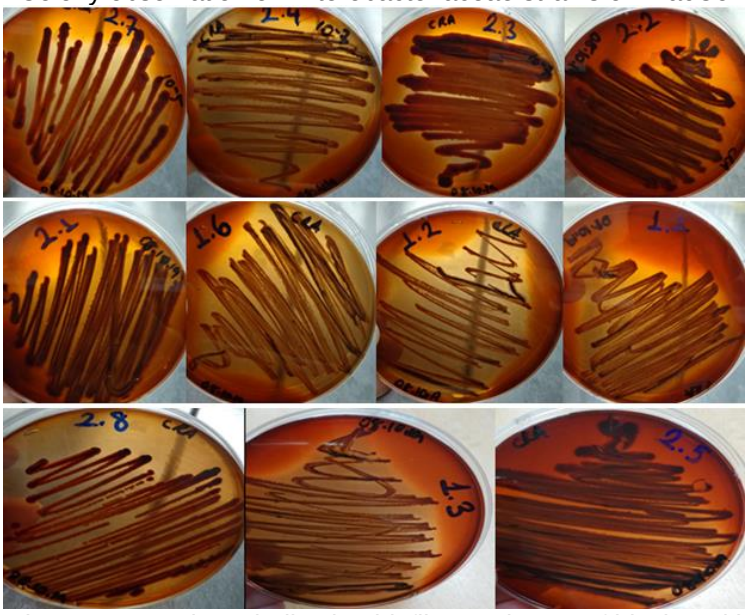

Figure 2. Colony colors on CRA plates indicating biofilm producers of black and brown colonies

Table 1 shows the percentage of susceptibility to different antibiotic of foodborne isolates. Among screening 13 standard antibiotic, 100 % of isolates

were determined the resistance to clindamycin, erythromycin and penicillin. In addition, all tested isolates (100 %) were sensitive to aztreonam, cefazolin, cefepime, ceftazidime, cefuroxime axetil, chloramphenicol, gentamicin, meropenem streptomycin and tetracycline.

The sensitive to two or more antibiotics and the resistance to at least one antibiotic were observed for all enteric strains. The formula used for the multiple antibiotic resistant (MAR) indexs calculations were as follows: Number of antibiotics that isolate was resistance/total tested number of antibiotics for each isolate. According to this formula, MAR indexs of all enteric isolates were recorded as 0.23.

Table 1.

Antibiotic susceptibility pattern of Gram–negative enteric isolates from Kilis cheese

Antibiotics	% Resistance	% Intermediate	%Sensitive
Aztreonam (ATM)	0	0	100
Cefazolin (CZ)	0	0	100
Cefepime (FEB)	0	0	100
Ceftazidime (CAZ)	0	0	100
Cefuroxime axetil (CXA)	0	0	100
Chloramphenicol (C)	0	0	100
Clindamycin (CD)	100	0	0
Erythromycin (E)	100	0	0
Gentamicin (GEN)	0	0	100
Meropenem (MRP)	0	0	100
Penicillin (P)	100	0	0
Streptomycin (S)	0	0	100
Tetracycline (TE)	0	0	100

All investigated enteric strains were susceptible to aztreonam and meropenem inhibited cell wall of bacteria by binding covalently to PBPs compared with the other tested β–Lactam antibiotic, penicillin. Besides, resistance to antibiotics belongs to extended–spectrum cephalosporins in different generation were not observed.

Highly sensitive rates (100 %) to chloramphenicol, gentamicin, streptomycin and tetracycline disrupting the steps in protein synthesis with the different action mechanisms were detected. On the other hand, strains were developed resistance to clindamycin and erythromycin blocked bacterial protein synthesis.

The isolation of *Enterobacteriaceae* members showing multiple antibiotic resistance from different dairy products sold local market in German, Egypt and Turkey was emphasized by many researchers [HAMMAD *et al.*, 2009; ODENTHAL *et al.*, 2016; TEKINER and OZPINAR, 2016].

Similarity, Hleba and collab., noted that *Enterobacteriaceae* genera isolates from cheese in Slovika were susceptibility to streptomycin, chloramphenicol and gentamycin [HLEBA *et al.*, 2011].

Contrary to our findings, 24 % resistance to tetracycline was observed.

In the other study, Gaffer and collab., recorded that all *Enterobacteriaceae* species obtained from Damietta cheese were resistance to penicillin [GAFFER *et al.*, 2019].

On the other hand, 100% susceptibility to meropenem was observed. These findings was supported to our study. The low (40 %) aztreonam resistance despite of high β lactamic group resistances was found as contrast to our results.

The frequency of antibiotic resistance among *Enterobacteriaceae* strains isolated from Kilis cheese demonstrates the contamination based on unhygienic applications in production processing and sale of local foods.

The resistance observed against clindamycin, erythromycin and penicillin may be explained that the overuse of these antibiotics in livestock induces the

development of resistant bacteria in dairy product of Kilis animal origin.

Also, the poor personal hygiene during production and selling process contributes contamination with organisms showing antibiotic resistance of raw foods. However, the capability of biofilm fomation of enteric strains leads to reduce antibiotic susceptibility and spread resistance genes from organism to organism.

The virulence factors such as antibiotic resistance and biofilm forming enteric isolates may adversely affect the safety and quality of Kilis local cheese.

Conclusions

Our study indicated that Kilis cheese traditionally produced from raw milk was contaminated with biofilm forming bacteria belong to family *Enterobacteriaceae*, probably originated from production and selling process. Further research should investigate the control of potential pathogenic bacterial strains threatening human health in Kilis cheese.

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