Comparative Characterization Of Endemic Lactic Acid Bacteria Of Enterococcus Genus

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Abstract: The diversity of multidrug-resistance (MDR) of pathogenic strains to antibiotics, most widely used for treatment of human diseases in the Republics of Armenia and Nagorno Karabakh were examined. It was shown, that difference of resistance of pathogens to antibiotics depends on their isolation sources. It was shown, that bacteriocin containing partially purified preparations, obtained from different strains of Enterococcus faecium and durans species, isolated from various samples of matsun, salted cheese and other acid milk products from milk of different domestic animals from rural households, inhibited the growth of multidrug-resistant bacteria belonging to different taxonomic groups with different efficiency.

Keywords: Antimicrobial biopreparation, multidrug resistance of pathogens, antimicrobial inhibition, intra-species diversity

Introduction

Antibiotic are normally used to treat microbial diseases since 50 years ago. However, use of antimicrobials in both human and animal populations over the past several decades has led to the emergence of multidrug-resistant (MDR) bacterial populations that are resistant to many commercially available drugs. Some of the more problematic drug-resistant pathogens encountered today include methicillin-resistant Staphylococcus aureus, multidrug-resistant Streptococcus pneumoniae, and vancomycin-resistant Enterococcus spp. among the gram-positive bacteria and multidrug-resistant Acinetobacter baumannii, Klebsiella pneumoniae, Escherichia coli and Pseudomonas aeruginosa among the gram-negative bacteria [21]. Methicillin-resistant Staphylococcus aureus (MRSA) is a significant public health problem worldwide [14]. Proteus mirabilis causes 90% of Proteus infections and can be considered a community-acquired infection. Proteus vulgaris and Proteus penneri are easily isolated from individuals in long-term care facilities and hospitals and from patients with underlying diseases or compromised immune systems [6]. P.aeruginosa exhibits the highest rates of resistance for the fluoroquinolones, with resistance to ciprofloxacin and levofloxacin ranging from 20 to 35% [11]. Acquired resistance to first-line antimicrobial agents increasingly complicates the management of extra-intestinal infections due to Escherichia coli, which are a major source of illness, death. The food supply, including poultry products, may transmit antimicrobial drug–resistant Escherichia coli to humans [17]. So, the continued development of new classes of natural antimicrobial agents, possessing antibacterial activity, has become of increasing importance for medicine and veterinary. The obtaining and investigation of biological properties of proteinoceous antibiotics, on the basis of probiotic lactic acid bacteria (LAB), shown, that bacteriocins, metabiotics and peptides of LAB represent bactericides have a broad range of activity and are excellent candidates for development of new prophylactic and therapeutic substances to complement or replace conventional antibiotics. The increasing interest in these compounds has stimulated the isolation of new strains of probiotic LAB –bacteriocin producers and the characterization of novel peptides [5], [10]. It is known, that most of human probiotics consist of Lactobacillus spp. and Bifidobacterium spp., whereas less information exists about the effectiveness of enterococcal strains as probiotics. In animals, enterococcal probiotics strains have been used for treatment of diseases such as diarrhea or antibiotic associated diarrhea, inflammatory pathologies that affects colon such as irritable bowel syndrome (IBS), or immune regulation. Susceptibility to clinically relevant antibiotics of Enterococcus strains isolated from food stuffs is very important for consumer health. E. faecium is one common species used as probiotic in animal feed. For example, E. faecium reduced the portion of piglets suffering diarrhea and improved their performances [24], or reduced the intestinal colonization by enteropathogenic bacteria [19]. E. faecium Paraghurt® has demonstrate its efficacy in lowering the symptoms associated with IBS in a clinical study as well as E. faecium PR88 and the multi-strains probiotic ProSymbioflor® (E. faecalis and E. coli) [18]. The use of E.durans TN-3 alleviates DSS colitis through the induction of T-reg cells and the restoration of the diversity of the gut microbiota [9]. The aim of this study was comparative investigation of antimicrobial activity of partially purified preparations from Enterococcus genus strains against multidrug resistant pathogens isolated from different sources of patients.

Materials and methods

Microbial strains and growth media. Enterococcus durans P13, Ent. durans M44, Ent. durans M42, Ent. durans AG 76, Ent. faecium M22, Ent. faecium AE 225-9, Ent. faecium KA 3, Ent. faecium KAP-1, Ent. faecium KV15-1 and Ent. faecalis AV 222, were isolated from different dairy products from Republics of Armenia (RA) and Nagorno Karabakh (NKR). LAB strains deposited with the Microbial Depository Center (MDC) at the SPC “Armbiotechnology” NAS RA. Pure cultures of LAB were maintained as frozen stocks at -20^{o}C in the MRS broth containing 40% Glycerol. Lactic acid bacteria strains were grown in MRS agar and broth (Merck (Germany), HiMedia (India)).

Species identification was confirmed by 16S rDNA gene sequencing method. To amplify the DNA from isolated LAB, the method of 16S PCR was employed using universal primers for Enterobacteriaceae, and marker Genladder (100 bp, plus 1.5 kb, GENAXXON, Bioscience) [22]. Nucleotide sequence of the obtained amplified 16S rDNA was determined by ”MACROGEN” (Korea). Strain identification was performed using the online BLAST software (www.ncbi.nlm.nih.gov/BLAST).

Test culture. Conditionally pathogenic bacteria from the culture collections of Department of Microbial Depository Center (MDC) and Laboratory of Biosafety of food products at SPC "Armbiotechnology" were used. Antibiotic resistant pathogenic bacteria strains were isolated from infected patients (feces, urine, wounds, blood, throat, etc) in the Infection Hospitals of Stepanakert and were investigated in the Stepanakert Center for Hygiene and Epidemiology. Bacteria were grown in Nutrient agar (Himedia, India) at pH 7.2 for 16 hours and at 37°C, then harvested and suspended in the Nutrient broth at $2.2x10^6$ CFU/ml.

Determination of antimicrobial activity For determination of antimicrobial activity the spot-on-lawn method was applied. Antimicrobial activity was assessed by measuring the size of the inhibition zone (diameter) of test culture growth (Ø, mm) after 24 h incubation in thermostat at 30° C. Antimicrobial activity calculated according to Parente [15] and expressed in arbitrary units (AU/ml).

Purification of antibacterial compounds by ion-exchange chromatography and obtaining of antimicrobial preparation (AMP). For separation of biomass, cultural liquids (CL), obtained after overnight growth of investigated LAB were centrifuged at 6,000 g during 20 min, at + 4°C. Obtained cell free culture liquids were purified by ion-exchange chromatography method [1].

Determination of resistance to antibiotics. To determine resistance of LAB strains to antibiotics, the method with antibiotic disks was applied [2]. The standard antibiotic disks of Amikacin 30 µg, Ofloxacin 5 µg, Ceftazidime 30 µg, Doxycyclin 10 µg, Ciprofloxacin 5 µg, Gentamicin 120 µg, Cefalotin 30 µg, Cefazolin 30 µg, Azithromicin 15 µg, Cefuroxime 30 µg, Augmentin 30 EG 10, Tetracyclin,30 µg, Nalidixic acid,30 µg (Liofilchem s.r.l. Roseto, Italy) were used.

Results

More than 400 different strains of lactic acid bacteria (LAB) isolated from various samples of matsun, salted cheese made of milk of cow and other domestic animals from rural households of several regions of Armenia and Nagorno Karabakh Republics. LAB strains were characterized by cell morphology, carbohydrate fermentation. Following the results of microscopy, LAB strains, isolated from Armenian matsun samples were mainly presented by rod-shaped bacteria (60 %) and coccoid forms were about 40 %. While LAB strains, isolated from samples from NKR were mainly presented by coccoid cells (about 86 %). For preliminary selection, time of milk fermentation and presence of antimicrobial activity against conditionally pathogenic Gram-negative Salmonella typhimurium G-38 and Gram-positive Bacillus subtilis G17-89 were tested. Strains of LAB, possessing high antimicrobial activity (at pH=6,0) were chosen for future investigations. It was shown, that strains of lactic acid bacteria, isolated from matsun from cow milk from different regions, differ by their physiological, biochemical properties, have high probiotic activities, growth rate, viable at pH =3,0-8,0 and inhibit the growth of microorganisms, belonging to different taxonomic groups [8]. The influences of partially purified AMP, obtained from different strains of Enterococcus genus on the growth of MDR pathogenic bacteria were examined. The investigation results are presented in the Table 1. As it seen from the given results, AMP of LAB, isolated from different sources, inhibited the growth of pathogenic bacteria with different efficiency. Highest activity against MDR pathogens shown bacteria, isolated from goat milk products. This can be resulting from differences in nature and amount of antibacterial compounds, produced by LAB. The diverse efficacy of growth inhibition may probably also relate to the different mechanisms of action of the substances towards bacteria cell membrane. Above all, they explain it with the presence of specific receptor proteins required for binding to bacteriocins and their transport into the bacteria [13]. Thus, statistical results proved that strains of strains of Enterococcus genus, isolated from cow milk dairy products display less efficiency against antibiotic resistant bacteria in comparison with LAB, isolated from other domestic animals milk dairy products. AMP from domestic animal LAB inhibited the growth of pathogenic strains of Klebsiella sp. with high efficiency. In recent years, the frequency of food poisoning in Armenia, caused by dairy and meat products, has increased dramatically. According to the National Bureau of Expertise (Yerevan, RA) data, the main pathogens are Escherichia coli Staphylococcus aureus species [12]. More than 70 strains of human pathogens were isolated from infected patients (feces, urine, wounds, blood, throat, etc). The investigation of differences in antibiotic resistance of human pathogens, isolated from different sources was of interest. The multidrug resistance of human pathogenic strains of Salmonella spp, Staphylococcus spp, Pseudomonas aeruginosastrains, Proteus spp., Klebsiella sp. and E.coli strains to antibiotics (N=20), widely used in medical practice in Armenia and NKR was determined. Average results of sensitivity of MDR pathogens to antibiotics, listed above are summarized in Figure 1. Obtained results showed, that antibiotic resistance of bacteria belonging to different species depends on their isolation sources. Highest number of MDR bacteria was isolated from blood, urine and feces. The Ps. aeruginosa and Klebsiella sp. were more resistant to the action of antibiotics (about 50%), then strains of E.coli and Proteus sp. species. As it seen strains of Staph. aureus sp., isolated from feces, were more sensitive to the action of antibiotics, than Staph. aureus sp., isolated from other sources. It was shown that the probiotic culture E. faecium L3 possess diverse ability to inhibit the growth of Streptococcus sp. belong to different genus [23]. This may explain the occurrence of MDR found in the strains isolated from hospital environment [18]. Thus, the efficiency antibiotics on the pathogens of same genus depend on their isolation sources. We have previously shown that the AMP, obtained from CL of Lactobacillus and Enterococcus genus, isolated from dairy products from cow milk, showed different efficiency against several species of Salmonella and E.coli genus, isolated from different food products from RA [12], [7]. The comparative investigation of influence of antibiotics and AMP obtained from CL of LAB of Enterococcus genus, isolated from dairy products from different domestic animals milk, on the MDR pathogens, isolated from infected patients were of interest. Results are shown in the Figure 2 on the example of two different strains of St, aureus (a, b) and E. coli (c, d). The obtained data show high stability of the investigated different strains of the genus Enerococcus. The high genetic diversity in Enterococcus group suggests adaptations for specific mutations in different environments. It should be noted that the

differences in the sensitivity of Listeria, Clostridium, Propionibacterium, enterococcus sp., oral Streptococcus sp. to the action of lactic acid bacteria bacteriocins, described by other authors [20].

Discussion

So, isolation and study of new LAB is usually performed from a various dairy products. From traditional Greek cheese "Graviera" were isolated strains Enterococcus faecium KE 82, Lactococcus lactis subsp. Lactis M 104, which possess antimicrobial activity against Listeria monocytogenes [17]. The LAB isolates presumed to be present in Ethiopian donkey milk samples [4]. Goats' milk is also typically dominated by LAB, including species of Lactococcus, Lactobacillus, Leuconostoc and Enterococcus, as well as Enterobacteriaceae, Micrococcaceae, moulds (filamentous fungi) and yeasts. The microbial content of raw buffalo milk has been assessed, through culturing, and found to contain a large population of LAB, including lactococci and lactobacilli, as well as coliforms, E. coli, S. aureus and bacterial endospores [16]. Previously it was shown, that biodiversity of most of bacteria, isolated from dairy product matsun from Armenia, Georgia differ from NKR dairy product matsun microbial composition Matsun (syn. Matsoni, mazun, matsoon) is a traditional Caucasian fermented milk product largely used from ancient times. It is considered an analog of yogurt and prepared from milk of cow, sheep, goat, buffalo, or a mixture thereof. Essentially important is that the microbial composition of matsoni apparently differs from various Caucasian areas but is very stable and characteristic for the region of origin. Both production region and milk type influence matsoni microbiota, suggesting that the traditional production methods preserve the transfer of unique regional microbiota from batch to batch. Bacterial profiles were dominated by Lactobacillus and Streptococcus species [3]. Thus the prospect of the use of certain AMP, obtained from LAB, isolated from dairy products of domestic animals, against pathogenic strains is obvious. These AMP can be applied for long term use against different etiology antibiotic resistant pathogens for prevention or treatment of infectional diseases as an alternative to antibiotics.

Ethical Approval

This article does not contain any studies with human participants or animals performed by any of the authors."

Conflict of Interest

The authors declare that they have no conflict of interest.

References

[1] Aghajanyan A.E.,Tkhruni F.N., Hovhannisyan G.S., Eghyan K.A., Vardanyan A.A., Saghyan A.S. ″A method of producing and purification of bioinhibitors from culture liquids″. Patent RA № 2925 A, 27.04. 2015.

[2] Bauer A.W., Kirby M.M. , Sherris J.CTurk M. ″Antibiotic susceptibility testing by a standardized single disk method″, Am. J. Clin. Pathol vol. 45, no4, pp. 493-496, 1966.

[3] N.A. Bokulich, L. Amiranashvili , K. Chitchyan, N. Ghazanchyan , K. Darbinyan , N. Gagelidze, T. Sadunishvili, V. Goginyan, G. Kvesitadze, T. Torok , D.A. Mills, ″Microbial biogeography of the transnational fermented milk matsoni″ Food Microbiol , vol.5, pp.12-19, 2015.

[4] Carminati D., Tidona F, Fornasari ME, Rossetti L, Meucci A, Giraffa G. ″Biotyping of cultivable lactic acid bacteria isolated from donkey milk″. Letters in Applied Microbiol,vol. 59, pp. 299-305, 2014.

[5] Cotter PD, Ross RP, Hill C. ″Bacteriocins- A viable alternative to antibiotics″. Nat. Ref. Microbiol, vol. 11, pp. 95-105, 2013.

[6] D'Andrea M, Literacka E, Zioga A, Giani T, Baraniak A, Fiett J, Sadowy E, Tassios PT, Rossolini GM, Gniadkowski M, Miriagou V. ″Evolution and spread of a multidrug-resistant Proteus mirabilis clone with chromosomal AmpC-type cephalosporinases in Europe″. Antimicrob. Agents Chemother, vol.55, pp. 2735–2742, 2011.

[7] Israyelyan A, Karapetyan K, Tkhruni F, Arstamyan L, Balabekyan Ts. ″Sensitivity of different pathogens to biological antimicrobial agents″. European J. of Biomedical and Life Sciences, vol. 3, pp. 61-66, 2015.

[8] Israyelyan A, Tkhruni F, Arstamyan L, Balabekyan Ts, Khachatryan T, Karapetyan K. ″ Comparative characterization of endemic lactic acid bacteria isolated from several regions of Armenia and Nagorno karabakh Republics″. Biol. Journal of Armenia, special Issue, vol. V(LXVIII), pp. 50-57, 2016.

[9] Kanda T, Nishida A, Ohno M, Imaeda H, Shimada T, Inatomi O. ″Enterococcus durans TN-3 induces regulatory t cells and suppresses the development of dextran sulfate sodium (DSS)-induced experimental colitis.″PLoS One, 2016.

[10] Karapetyan K, Tkhruni F, Balabekyan Ts, Khachatryan L, Isakhanyan A. ″Low molecular mass bacteriocins from Lactobacillus rhamnosus 109″. Biol. Journal of Armenia, LXV, supplement 1, pp.84-85, 2013.

[11] Lister PD, Wolter DJ, Hanson ND. ″Antibacterial-Resistant Pseudomonas aeruginosa: Clinical Impact and Complex Regulation of Chromosomally Encoded Resistance Mechanisms″. Clin Microbiol Rev, vol. 22. No 4, pp. 582–610, 2009.

[12] Melik-Andreasyan GG, Tkhruni FN, Tsakanyan AV, Karapetyan KJ, Khanjyan GJ, Khachatryan TV. ″Evaluation of antibiotic resistance of human gut microbiota pathogens″. International scientific-practical conference "Modern problems of infectious pathology of human" October 31-November 1, Minsk, Belarus .vol. 6, pp. 208-213,2013.

[13] Moreno M RF, Callewaeri R, Devreese B. Van Beeumen J, De Vuyst L. ″ Isolation and biochemical characterization of enterocins produced by enterococci from different sources″. J. Appl. Microbiol , vol. 94, pp. 214–220, 2003.

[14] Okuda K, Zendo T, Sugimoto S, Iwase T, Tajima A, Yamada S, Sonomoto K, Mizunoe Yo. ″ Effect of bacteriocins on methicillin-resistant Staphilococcus aureus Biofilm″. Antimicrobial agents and chemotherapy. Vol.57, no. 11, pp.5572-5579, 2013.

[15] Parente E. Brienza C. Moles M. Ricciardi A. ″Comparison of methods for the measurement of bacteriocin activity″. J. Microbiol Methods, vol.22, pp. 95-108, 1995.

[16] Quigley L, O'Sullivan O, Stanton C, Beresford TP, Ross RP, Fitzgerald GF, Cotter PD. ″ The complex microbiota of raw milk″. FEMS Microbiol Rev, vol.37, pp. 664–698, 2013.

[17] Russo TA, Johnson JR. ″Medical and economic impact of extraintestinal infections due to Escherichia coli: an overlooked epidemic″. Microbes Infect , vol.5, pp.449–56.2003.

[18] Santos KMO, Silva Vieira AD, Salles HO, Silva Oliveira J, Costa Rocha CR, de Fátima Borges M, Bruno LM, de Melo Franco BGG, Todorov SD. ″Safety, beneficial and technological properties of Enterococcus faecium isolated from Brazilian cheeses″. Brazilian J. of Microbiol , vol.46, no 1, pp. 237-249, 2015.

[19] Scharek L, Guth J, Reiter K, Weyrauch KD, Taras D, Schwerk P, Schierack P, Schmidt MFG, Wieler LH, Tedin K. ″Influence of a probiotic Enterococcus faecium strain on development of the immune system of sows and piglets″. Vet Immunol Immunopathol, vol. 105, pp. 151–61, 2005.

[20] Vuist L, Neysens P. ″The sourdough microflora: biodiversity and metabolic interactions″. Trends in food Science technology, vol. 16, no 1–3, pp. 43– 56, 2005.

[21] World Health Organization (WHO). World health statistics. [Electronic Book] France: WHO Press; 2012 [cited 17 July 2012]; Available from: http://www.who.int/healthinfo/EN_WHS. 2012_Full.pdf.

[22] Weisburg WG, Barns SM, Pelletier DA, Lane DJ. ″ 16S ribosomal DNA amplification for phylogenetic study″. J. Bacteriol , vol.173, no 2, pp. 697-703, 1991.

[23] Yermolenko E, Chernish A, Aleshina G. ″Antagonistic activity of Enterococcus faecium L3 against different groups of pathogenic streptococci″. Streptococci New Insights Into and Old Enemy. May, pp. 363–366, 2006.

[24] Zeyner A, Boldt E. ″Effects of a probiotic Enterococcus faecium strain supplemented from birth to weaning on diarrhoea patterns and per-formance of piglets″. J Anim Physiol Anim Nutr (Berl), vol. 90, pp. 25–31, 2006.

Table 1. *Comparative antimicrobial activity of AMP (200 AU/ml) from CL of different strains of Enterococcus genus against antibiotic resistant bacteria (%)*

Antibiotic resistant strains	**N**	**LAB isolation source (dairy products from different domestic animals milk)**									
		Cow				**Goat**		**Donkey**	**Buffalo**	**Sheep**	
		Ent.faecium M22	**Ent.durans M42**	**Ent.durans M 44**	**Ent.durans P13**	**Ent. faecium KAP-1**	**Ent. faecium KA 3**	**Ent. faecium AE 225-9**	**Ent. durans AG 76**	**Ent. faecalis AV 222**	**Ent. faecium KV15-1**
Staph. aureus sp.	28	0.0	0.0	0.0	25.0	50.0	50.0	0.0	50.0	50.0	0.0
Ps. aeruginosa	21	50.0	0.0	50.0	58.0	100.0	100.0	100.0	50.0	50.0	50.0
Pr. mirabilis	23	50.0	20.0	50.0	29.0	50.0	100.0	100.0	0.0	0.0	50.0
Klebsiella sp.	8	0.0	50.0	0.0	0.0	100.0	100.0	100.0	100.0	100.0	100.0
Pr. vulgaris	7	0.0	0.0	0.0	0.0	33.0	66.0	66.0	33.0	33.0	66.0
E.coli sp.	15	50.0	40.0	50.0	15.0	100.0	100.0	33.0	33.0	33.0	33.0

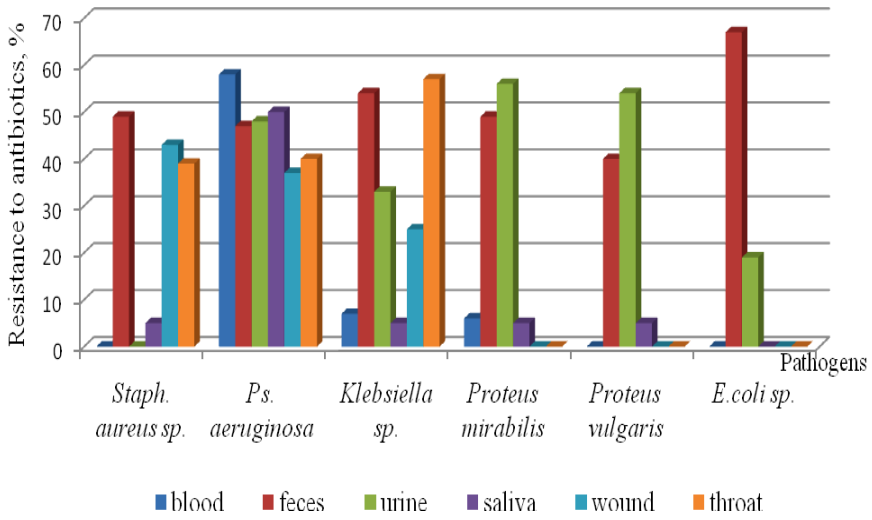

Fig.1 *Dependence of sensitivity of pathogens to antibiotic from isolation sources*

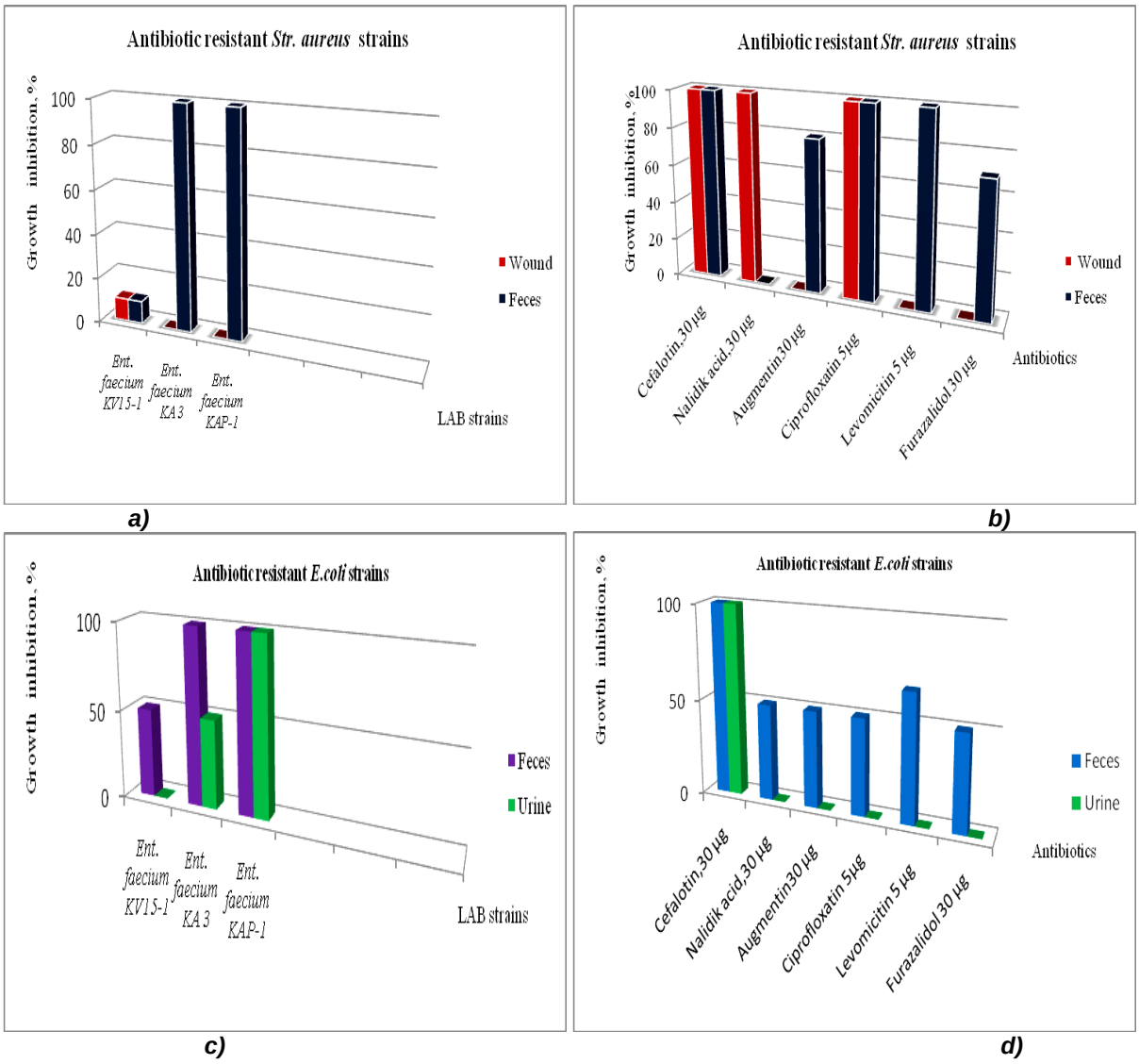

Fig. 2 *(a, b ,c, d) Comparative antimicrobial activity of antibiotics and AMP from CL different strains of Enterococcus genus*