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Full Length Research Paper

Antibiotic resistance of *Enterococci* isolated from raw camel milk in the South West of Algeria

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Little information is available on the diversity and distribution of resistance and virulence factors in Enterococci isolated from camel milk. In this study, 33 samples of camel's milk collected from the south west region of Algeria were analyzed for the presence of *Enterococcus* spp. Twenty three (23) enterococcal isolates were recovered. These strains were identified by the API 20 STREP and the sodium dodecyl sulphate-polyacramide gel electrophoresis (SDS-PAGE) of whole cell protein at the species level: *Enterococcus faecalis* (n = 11), *Enterococcus faecium* (n = 8), *Enterococcus avium* (n = 2), *Lactococcus lactis* ssp *lactis* (n = 1) and *Streptococcus uberis* (n = 1). Fifteen (15) of the 23 isolates exhibited resistance to at least one of the tested antibiotics and six (6) of these 23 isolates were resistant to two antibiotics. None of the isolates were resistant to penicillin, ampicillin, or gentamicin. Resistance to vancomycin (VAN) was found in three (3) isolates which represent (13%), two *E. faecalis*, and one *E. faecium*. Six (26%) of *Enterococci* isolates were resistant to one of these antibiotics: erythromycin (ERI), tetracycline (TET) and rifampin (RIF). In conclusion, this is the first study to underline the importance of camel milk as a reservoir of *Enterococcus* spp. carrying resistance to vancomycin.

Key words: Camel milk, *Enterococcus*, sodium dodecyl sulphate-polyacramide gel electrophoresis (SDS-PAGE), antibiotic resistance.

INTRODUCTION

Enterococci are important members of gut communities in many animals and opportunistic pathogens that cause millions of infections annually. They are most frequently used as fecal indicator bacteria, or general indicators of fecal contamination, but they are also used as surrogates for pathogens and/or health effects in risk assessment and other modelling applications. These bacteria are widely distributed in a variety of environmental habitats, even when there is little or no input from human and/or animal fecal sources (Byappanahalli et al., 2012). In

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addition, they are considered as lactic acid bacteria found in traditional fermented foods and in the dairy products (Sukontasing et al., 2007).

The prevalence of enterococci in dairy products is a result of unhygienic conditions during the production and processing of milk. *Enterococci* may enter the milk either directly from human or animal feces or indirectly from contaminated water sources, exterior of the animal and/or from the milking equipment and bulk storage tank. Different species of enterococci are found in dairy products, but *Enterococcus faecalis* and *Enterococcus faecium* remain the species of greatest importance (Giraffa, 2003).

Data on the microbial diversity of raw camel milk are generally scarce compared with bovine milk. The microflora of raw and fermented camel milk products has been reported as a mix of different species of typical dairy bacteria (Christoph et al., 2012). This microflora needs to be further investigated. The environmental conditions where the milk is produced by camels and its physicochemical properties determine the group of microorganisms that can survive in such conditions. In this case, these conditions (temperature, pH, concentration of salt...) are ideal for the growth and proliferation of enterococci. The objectives of this study were to identify the species and describe the antimicrobial resistance features of *Enterococci* isolated from camels' milk.

MATERIALS AND METHODS

Sample collection

A total of 33 samples of camel's milk were collected from free range camel herd (*Camelus dromedarius*), in good health, living in the South West of Algeria (Bechar area). The milk was collected during the period of February, March and April, 2014 in sterile bottles, transported to the laboratory in an icebox and stored at +4 to +6°C before analysis. This work was performed in the biological laboratories, Department of Biology at the University of Bechar, Algeria.

Physiological and biochemical characterization of *Enterococcus* strains

Enterococcal isolates were obtained from camel's milk. Growth characteristics were tested in de Man-Rogosa-Sharpe agar (MRS), Citrate Azide agar (CA) and Citrate Azide Tween Carbonate medium (CATC) (Domig, 2003). These isolates were first phenotypically described by using conventional growth and physiological tests, according to Devriese et al. (2006). All cultures were examined for ability to grow on potassium tellurite 0.04%, for hydrolysis of esculin and for gelatine liquefaction. Production of hemolysis was determined by plating actively growing cells of the strains onto Columbia blood agar (Oxoid) supplemented with 5% (v/v) human blood. Plates were incubated at 37°C in an anaerobic atmosphere. Results were recorded at 24 and 72 h. A clear zone of β-hemolysis on blood agar plates was considered as positive result.

The type strain for *E. faecalis* ATCC 29212 was obtained from the American Type Culture Collection. Stock cultures were maintained on MRS broth supplemented with 30% glycerol and stored at -20°C. After that, all isolated strains were tested with API 20 STREP galleries according to the manufacturer's instructions (BioMérieux), and identified using the analytical profile index. Physiological and biochemical characteristics were coded as 0 for negative and 1 for positive and analyzed by the software package BioNumerics version 7.5 (Applied Maths, Kortrijk, Belgium). Agglomerative clustering was performed by the unweighted pair group method with arithmetic mean (UPGMA).

Analysis of whole-cell protein profiles by SDS-PAGE

Preparation of samples and analysis of whole-cell protein profiles by conventional one-dimensional sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) were performed as described by Merquior et al. (1994), with slight modification where the strains used for protein extraction were first plated onto CATC medium, then they were grown on brain heart infusion broth instead of Columbia blood agar. Coefficients of similarity or dice indices between isolates and the Enterococcal reference strain were determined for each isolate by using the BioNumerics version 7.5 software package (Applied Maths, Kortrijk, Belgium), and a dendrogram was constructed from the similarity matrix by the unweighted pair group method with arithmetic mean (UPGMA). The whole-cell protein extract of *E. faecalis* ATCC 29212 was used as reference profile.

Antibiotic susceptibility test

All isolates were tested for their antibiotic susceptibility by a disc diffusion method on Mueller-Hinton agar. Seven antibiotics were used: penicillin 10 U (PEN), ampicillin 10 µg (AMP), vancomycin 30 µg (VAN), erythromycin 15 µg (ERI), tetracycline 30 µg (TET), rifampin 5 µg (RIF) and gentamicin 120 µg (GEN). The diameter of inhibition zones were measured after incubation for 24 h at 35°C. Sensitivity and resistance were evaluated according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2014). Antibiotic resistance data expressed in millimeters (mm) of inhibition zone were first converted to categories (S for susceptible, I for intermediate, and R for resistant), then a dendrogram was constructed from the similarity matrix by the unweighted pair group method with arithmetic mean (UPGMA) using the BioNumerics version 7.5 software package (Applied Maths, Kortrijk, Belgium).

RESULTS

Isolation of *Enterococcus* strains

A total of 23 isolates of Enterococcal strains was isolated from camel's milk. As CATC medium is selective for enterococci, all Gram-positive, catalase-negative cocci isolated from this medium were presumptively identified as *Enterococcus* spp. The presumptive identification showed that all isolates were morphologically homogeneous, they were spherical or ovoid cells occurring in pairs or short chains, non-motile, and they were gram positive catalase negative.

Physiological and biochemical identification

All isolated strains showed the same physiological characteristics, they grew in MRS broth containing 6.5%

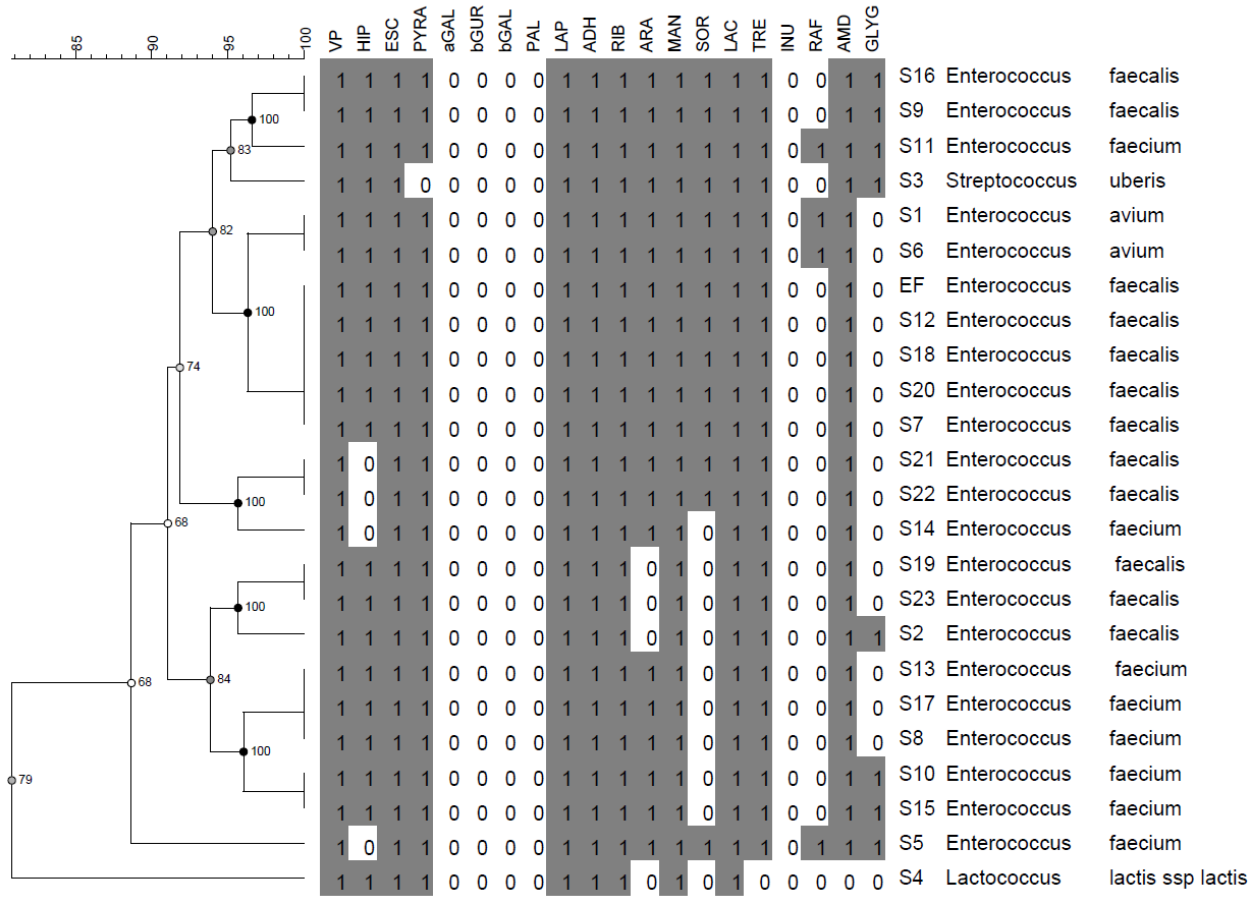

Figure 1. Results of the API 20 STREP tests represented as a clusters of biochemical profiles of the camel's milk enterococci isolates. (EF indicate reference strain).

NaCl, at pH 9.6 which is in accordance with the genus *Enterococcus*, and they grew at 10 and 45°C and resists 30 min at 63°C. They are positive for hydrolysis of esculin and negative for hydrolysis of gelatin and they do not show any tolerance for potassium tellurite. For hemolysis on blood agar they showed negative results.

API 20 strep system identification

All of the isolates were Vogues-Proskauer (VP), hippurate (HIP) (except for isolates: S5, S14, S21, and S22), esculin (ESC), pyrrolidonylarylamidase (PYRA) (except for isolate S3), leucine arylamidase (LAP), and arginine dihydrolase (ADH) positives, but negatives for alkaline phosphatase (PAL), α-galactosidase (α-GAL), β-glucuronidase (β-GUR), and β-galactosidase (β-GAL). With the exception of S4 which was unable to use trehalose and starch, all isolates were able to produce acid from ribose, mannitol, lactose, trehalose and starch by fermentation, but they were unable to produce acid from inulin. All the other tests were strain-dependent. These results were coded as 0 for negative and 1 for positive and analysed by the software package BioNumerics version 7.5 (Applied Maths, Kortrijk, Belgium), and clusters for species identification were depicted taking into account the clustering pattern of reference strain (Figure 1).

Whole-cell protein profiles identification

Whole-cell protein profiles of the isolates were compared with a type strain profile. Figure 2 shows a dendrogram that was obtained after UPGMA linkage cluster analysis of all the isolates and the type strains of *Enterococcus faecalis* ATCC 29212. Numerical analysis of the electrophoretic whole-cell protein profiles of the 23 camel's milk isolates and reference strain (*E. faecalis* ATCC 29212) by the determination of the dice correlation coefficient and UPGMA clustering, revealed that at the 65% similarity (S) level, the 23 isolates formed three distinct clusters as shown in the dendrogram (Figure 2). Cluster 1 with (71% r-value) grouped 8 isolates, five

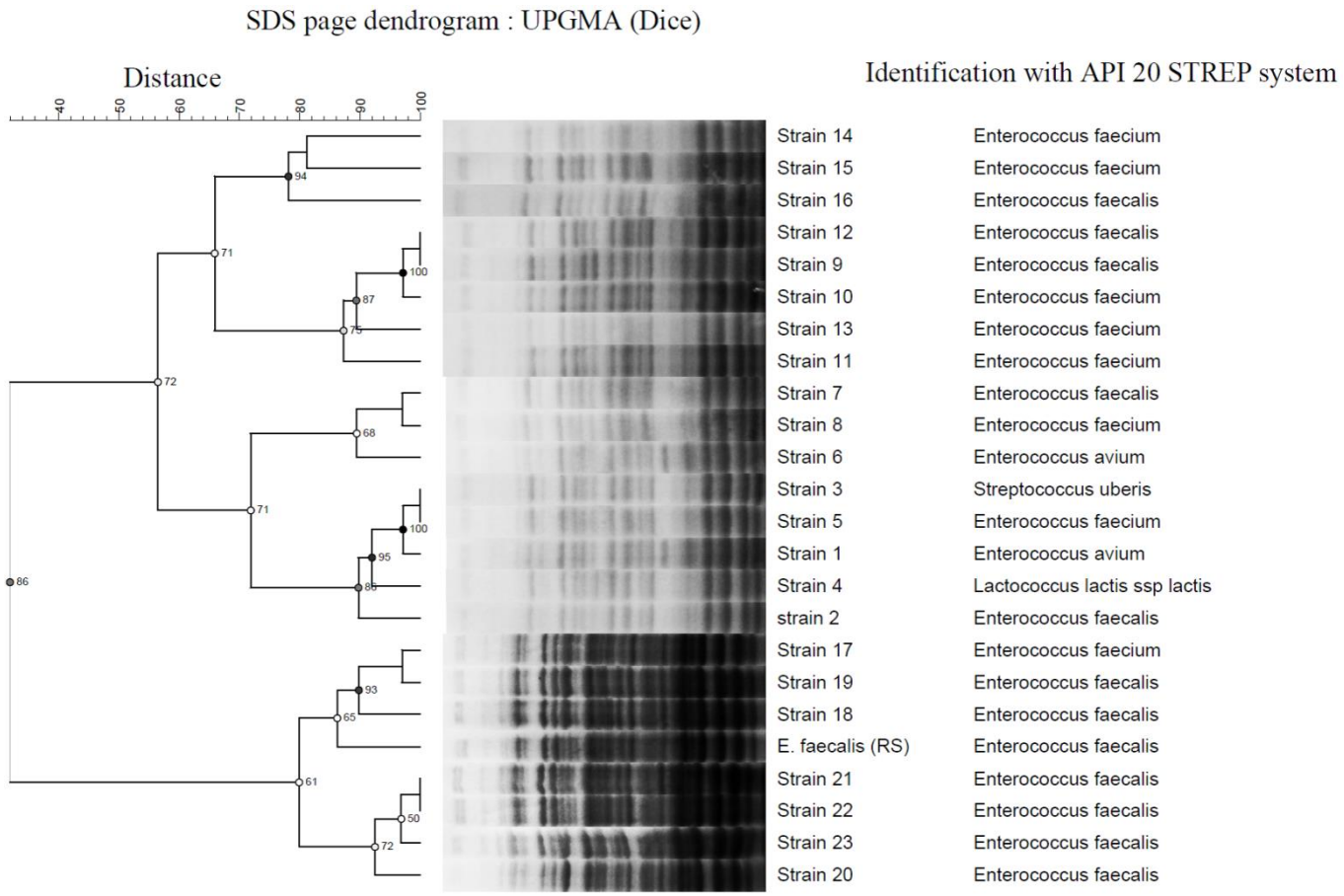

Figure 2. Electrophoretic banding patterns of whole cell protein of *Enterococcus* isolates; the mean correlation coefficient (r), represented as a dendrogram, and calculated by the unweighted average pair grouping method for some of the camel milk isolates compared with the reference strain. (RS indicates reference strain).

(S10, S11, S13, S14, and S15) were identified by the API 20 STREP system as *Enterococcus faecium*, and three (S9, S12, S16) were identified by the same system as *E. faecalis*. Cluster 2 with (71% r-value) grouped 8 isolates, two (S2, S7) were identified as *E. faecalis*, two (S5, S8) were identified as *E. faecium*, another two isolates (S1, S6) were identified as *E. avium*, one isolate (S3) was identified as *Streptococcus uberis*, and the last one (S4) was identified as *Lactococcus lactis ssp lactis*. Cluster 3 with 61% r-value also grouped 7 isolates with the reference strain, six isolates (S18, S19, S20, S21, S22, and S23) were identified as *E. faecalis*, and one isolate (S17) was identified as *E. faecium*.

Antibiotic susceptibility

Analysis of the antibiotic susceptibility of the isolates revealed that 15 of the 23 isolates exhibited resistance to at least one of tested antibiotics and 06 of these 23 isolates were resistant to two antibiotics. None of the isolates were resistant to penicillin, ampicillin, or gentamicin. Resistance to vancomycin was found in three isolates, two (S2, S19) were identified as *E faecalis*, and the other one (S5) was identified as *E. faecium*. Six isolates were resistant to erythromycin, two were identified as *E. faecalis* (S21, S23), two were identified as *E. faecium* (S10, S17), one was identified as *Streptococcus uberis*, and the last one isolate was identified as *L. lactis ssp lactis*. Six isolates were resistant to rifampin, three were identified as *E. faecalis* (S2, S18, S22), and the remaining three were identified as *E. faecium* (S11, S13, S17). Six isolates were found to be resistant to tetracycline, four were identified as *E. faecalis* (S16, S18, S19, and S22), one as *E. faecium* (S10), and the other one as *E. avium* (S1). It was found that only 34.78% of the isolates were susceptible to all tested antibiotics. The susceptibility results and patterns of all isolates tested are shown in (Table 1 and Figure 3).

DISCUSSION

Camel milk is a valuable product for the nomads in the

Table 1. Antibiotic susceptibility of enterococcal strains isolated from Camels' milk (n=23).

Antibiotics	Resistant (%)	Intermediate (%)	Sensitive
Ampicillin (10 µg)	0 (0)	4 (17)	19 (83)
Erythromycin (15 µg)	6 (26)	16 (70)	1 (4)
Gentamicin (120 µg)	0 (0)	0 (0)	23 (100)
Penicillin (10 U)	0 (0)	6 (26)	17 (74)
Rifampin (5 µg)	6 (26)	9 (39)	8 (35)
Tetracycline (30 µg)	6 (26)	3 (13)	14 (61)
Vancomycin (30 µg)	3 (13)	9 (39)	11 (48)

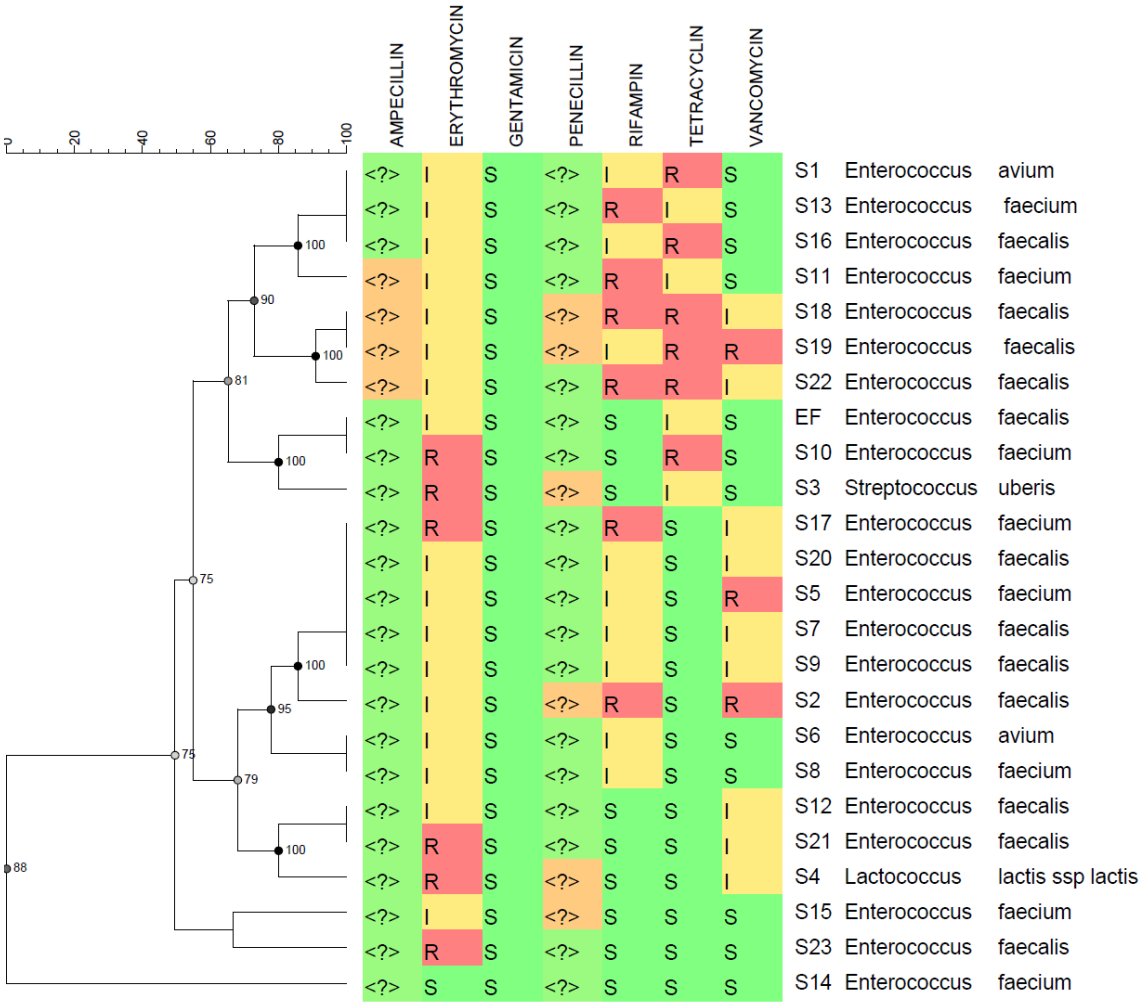

Figure 3. Antibiotic resistance profiling; *Enterococci* isolates are typically clustered based on their resistance categories using a categorical coefficient, which treats different values as different states. The colors in the comparison window correspond to the color of each antibiotic category (susceptible, intermediate or resistant). (EF indicate reference strain).

hot regions and arid countries, consumed as fresh and soured milk, this product has long been ignored and under-estimated, and it has not had its share of chance in the scientific research, when comparing it with the cow's milk that has been widely studied. The research done so far on the camel milk do not cover all aspects, studies carried out between 1997 and 2009 have been mainly concentrated on the composition, characteristics and

functionality of the camel milk (Al haj and Al Kanhal, 2010). Nevertheless, the data on the microbial diversity of camel milk are insufficient. For this purpose, an attempt was made in the current study to identify the enterococci present in camel's milk at species level and to investigate some potential pathogenic factors of these bacteria, such as hemolysis on human blood, and antibiotic resistance. The choice of enterococci was established based on their capacity to withstand harsh conditions such as drying, heat stress, and UV irradiation prevailing in the regions from where camel's milk samples were taken.

Analysis of results from physiological and biochemical tests was performed to determine characteristics that are usually considered as typical for the genus *Enterococcus*, such as growth at 45°C, 10°C, pH 9.6 and with 6.5% NaCl, and to allow a preliminary characterization of the isolates. Our isolates have given the same results of physiological tests which allow to classify them in the genus *Enterococcus* without exception, while the results of biochemical tests obtained from the API 20 STREP system have shown some differentiation between isolates, in which 11 (48%) species were identified as *E. faecalis*, 8 (35%) species as *E. faecium*, 2 (9%) species as *E. avium*, 1 (4%) species as *L. lactis ssp lactis*, and 1 (4%) species as *S. uberis*.

A number of studies on the API 20 Strep method showed that the majority of *E. faecalis*, *E. faecium*, *E. avium* and *E. durans* strains isolated of a clinical origin are correctly identified (Winston et al., 2004). However, because this system was developed prior to the recent taxonomy changes, some identifications may be in error, especially for species other than *E. faecalis* and for "*Enterococcus*-like" strains (Maria et al., 2002).

Clearly, a reliable identification of enterococci to the species and strain level by physiological and biochemical tests often appears difficult. Besides being very time consuming, this type of work yielded results that, in terms of a taxonomic identification, did not always match the results obtained by other methods. However, given the variability in the biochemical and phenotypic traits of enterococci, molecular based methods are essential for reliable and fast identification. SDS-PAGE analysis of whole-cell protein patterns is useful for clearly discriminating a multitude of species of lactic acid bacteria (Descheemaeker et al., 2000). It is equally possible to differentiate and identify *Enterococcus* species (Merquior et al., 1994). To clarify the identification of our isolates, electrophoretic analysis of the whole-cell protein profiles was performed. The profiles generated are shown in Figure 2. The results obtained show some discrepancies between results obtained by conventional phenotypic, API 20 STREP and SDS page profiling. Merquior et al. (1994) used and evaluated SDS-PAGE to identify reference, human, animal and environmental strains of *Enterococcus* species. They reported that each *Enterococcus* species had a unique and distinguishable profile. However, the limit of SDS-PAGE of the whole-cell protein profiles is that it requires several type of strains to clearly identify all isolates, our study is limited to one type strain *E. faecalis* ATCC 29212. Despite this it was found that 58% of isolates identified by API 20 STREP as *E. faecalis* were confirmed by whole-cell protein profiling that they belong to this species. The remaining 42% of isolates requires the use of other reference strains. Application of the whole-cell protein profiles analysis for enterococcal characterization requires standardization of reference banding patterns. In addition, a data bank of reference protein profiles could be constructed with which the protein profile of any unknown isolate could be compared. Whole-cell protein electrophoresis has widely been documented in numerous taxonomic and identification studies to be a reference method for species delineation because a high degree of similarity in whole-cell protein content is a reflection of a high degree of DNA homology, and therefore species identity (Vandamme et al., 1996).

The precise differentiation of enterococcal species has taken on additional importance because of the acquisition of resistance traits among strains, especially resistance to glycopeptides. To the best of our knowledge, this study provides the first detailed analysis about the ecology of antibiotic resistance and virulence in a variety of enterococci isolated from fresh raw camel milk in North Africa. The antibiotic susceptibility testing was performed according to standard disc diffusion method (Kirby–Bauer disc diffusion method) recommended by the Clinical and Laboratory Standards Institute (CLSI, 2014). Because of the limitation of techniques used to evaluate the antibiotic susceptibility, some studies have been conducted by Lee and Chung (2015), Edelmann et al. (2007) and Dickert et al. (1981) to determine the most appropriate method for antibiotic susceptibility testing, and they concluded that disk diffusion is still a valid technique and gives results that are closely similar to other techniques. It is important to develop an easy-to perform methodology that can be routinely used in the laboratory, but careful consideration regarding not only accuracy, but also cost and labor intensiveness is required.

Enterococci are known to acquire antibiotic resistance to most antibiotics used in clinical practice with relative ease and capable of spreading those resistance genes to other species (Kaçmaz and Aksoy, 2005). The occurrence of antibiotic resistance among dairy isolates seems to vary somewhat between studies, and is often strain- and region-dependent (Čanžek et al., 2005), or may differ according to the isolation method (Klein, 2003).

Our results of antibiotic susceptibility are summarized in Table 1 and Figure 3. They showed that 26% of enterococci isolates were resistant to one of these antibiotics erythromycin (ERI), tetracycline (TET), and rifampin (RIF). Teuber et al. (1999) found 64, 45 and 32% of resistance to chloramphenicol, tetracycline and

erythromycin, respectively, and they concluded that these antibiotics are a major concern for dairy *E. faecalis* isolates. Resistance to erythromycin as a representative of the macrolide antibiotics is a matter of concern. Although de Fatima Silva Lopes et al. (2005) have determined that a high percentage of *E. faecalis* strains (74%) were intermediary or resistant to erythromycin. In Poland, Wioleta et al. (2012) reported a similar prevalence of resistance to tetracycline (28.3% of isolated strains) to our study.

The most widespread resistance among dairy enterococci was tetracycline which was detected in 30.8% of the strains, this may be attributed to the widespread use of these antibiotics in veterinary practices (Pieniz et al., 2015). Huys et al. (2004) also showed that a significant proportion of tetracycline isolates exhibited co-resistance to erythromicin and/or chloramphenicol, suggesting that the selection of tetracycline genotypes may provide a suitable molecular basis for the further selection of multiple resistances. However, it should be noted that resistance to tetracycline has little clinical importance as it is not a drug of choice for the treatment of enterococcal infection. A major concern is the emergence of vancomycin resistant *Enterococci* (VRE). Vancomycin is considered as the last resort antibiotic to treat serious infections due to resistant Gram-positive bacteria, and given exclusively in a clinical environment, when all others fail. (Naoual et al., 2010).

Several studies showed the occurrence of vancomycin-resistant enterococci in food of animal origin, mainly in *E. faecalis* and *E. faecium* species, although the isolation frequency seems to be lower than in clinical samples (Klein, 2003). In our case three (3) vancomycin resistant enterococci (VRE) were found which represent 13% of all isolated strains, two (S2, S19) were identified as *E. faecalis*, and one (S5) was identified as *E. faecium*. According to Morandi et al. (2006), testing antibiotic susceptibility to vancomycin by disc diffusion method provide similar results as growth in MRS broth containing vancomycin.

Vancomycin resistance within dairy enterococci remains controversial, though several papers indicate very low or no presence of *vanA* and *vanB* resistance genes in enterococci isolated from cheese (Jurkovic et al., 2006). In another paper *vanA* gene was found in 37% of the dairy enterococci examined which, however, were all susceptible to vancomycin (Ribeiro et al., 2007). For the first time in Egypt, *E. faecalis* and *E. faecium* vancomycin-resistant strains were reported from food of animal origin by Hammad et al. (2015), which is in agreement with our finding.

The emergence of enterococci resistance to glycopeptides, including vancomycin and teicoplanin, in many of developed countries is attributed to a dual development that included clinical overuse and cross-resistance, following the use of avoparcin as an animal growth promoter (Koluman et al., 2009). Although enterococci are generally regarded as being intrinsically resistant to low levels of gentamicin, a high-level gentamicin resistance was detected in many dairy isolates (Giraffa, 2003; Hummel et al., 2007). All strains isolated in our study are susceptible to gentamicin. There are a few studies that investigate the spread of antibiotic resistance genes in camel's milk and it is very surprising to find vancomycin resistant *Enterococci* (VRE) in camel milk, knowing that this animal survives in areas far from the urban area. Camel pastoralists are nomadic, a matter which may explain the presence of enterococci isolates resistant to vancomycin, it is possible that these pastoralists carry strains resistant to vancomycin, as they are the only contact between urban centers and grazing areas where camels located. Or enterococci isolated from camel milk are intrinsically resistant to vancomycin. It is a hypothesis that needs to be verified. A hypothesis that could be confirmed by means of the additional molecular studies that are under way at our laboratory.

Conclusion

Camel milk is a very rich ecosystem that needs to be investigated. However, it has been long neglected, and has not had the opportunity to be a subject of large scale research. The results of the present study show that analysis of soluble whole-cell proteins can be used to discriminate between species of *Enterococcus* isolated from camel milk that are usually hard to differentiate by physiologic tests. Also there have been very few systematic studies to investigate acquired antibiotic resistance in enterococci of Camel's milk origin. We were surprised to discover in the camel milk the presence of vancomycin resistant enterococci, something that is scary. Fortunately, the incidence of penicillin, ampicillin and gentamicin resistance for all isolated strains was low, indicating that most of the strains tested did not acquire resistance determinants for these antibiotics. These results are about to be verified and validated by molecular techniques.

Conflict of Interests

The authors have not declared any conflict of interest.

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