Occurrence, molecular and antimicrobial resistance of *Enterococcus* spp. isolated from raw cow's milk trade by street trading in Meknes city, Morocco

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Abstract

Background ***Enterococcus*** **spp. belongs to a group of pathogens which are responsible for serious infections. This study aims at highlighting the raw milk microbiological contamination and at providing data for prevalence and antimicrobial resistance of** ***Enterococcus*** **spp. isolated from raw cow's milk marketed (without any pasteurization) by street traders.**

Methods During the period of May 2015 through April 2016, 150 cow's raw milk samples were collected from street traders in Meknes city. They were examined for the identification of ***Enterococcus*** **spp. using biochemical tests and 16S rRNA gene sequencing. The antimicrobial susceptibility of the isolates was determined.**

Results The results showed that 11.3% (17/150) of samples were positive for the presence of ***Enterococcus*** **spp., of which 64.7% were identified as** ***Enterococcus faecalis*****, 17.6% as** ***Enterococcus faecium*****, 11.8% as** ***Enterococcus durans*** **and 5.9% as** ***Enterococcus hirae*****. The antimicrobial susceptibility showed that all** ***Enterococcus*** **spp. were resistant to ampicillin. The species** ***E. faecalis*****,** ***E. faecium*****,** ***E. durans*** **and** ***E. hirae*** **were resistant to streptomycin, with percentages of 52.9% (9/17), 11.8% (2/17), 11.8% (2/17), and 5.9% (1/17) respectively. All isolated strains of** ***E. faecalis*** **and** ***E. faecium*** **were resistant to tetracycline. The multiple antibiotic resistance index was elevated in the majority of** ***Enterococcus*** **spp., reaching values higher than 0.5, indicating a risk for public health.**

Conclusion This study shows that the raw milk consumed by the population is contaminated with strains of ***Enterococcus*** **resistant to antibiotics used in breeding for prophylactic purposes. This requires raising the awareness of those involved in the production and marketing of milk, so as to take measures to apply good hygienic practices and rationalize the use of zootechnical antibiotics.**

Keywords Antimicrobial resistance, MAR (index), ***Enterococcus*** **spp., raw cow's milk, 16S rRNA.**

Introduction

Bacteria belonging to *Enterococcus* spp. are Gram-positive, catalase negative, non-spore forming, and facultatively anaerobic. They optimally grow at 35 °C, but tolerate temperatures between 10-45 °C.[1] They are commensals of the gastrointestinal tract of animals and humans. They are responsible for serious infections, such as surgical infection, aortic valve endocarditis, meningitis and bacteremia.[2] While *Enterococcus faecalis* and *Enterococcus faecium* are most commonly recovered from humans, *Enterococcus hirae* is more frequently identified in animal infections.[3] *Enterococcus durans* is found in the intestines of animals and is responsible for very rare cases of endocarditis in humans.[4]

Received: 15 December 2017; revised: 07 March 2018; accepted: 16 March 2018.

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In Morocco, the consumption level of milk and dairy has considerably increased in recent years.[5] However, milk and milk products delivery from the farm to consumer involves two types of marketing channels. The first one is referred to as "organized" and involves industrial collection centers with production plants. The second is known as "street trading" and is based on the sale of milk by the persons who collect it from the farms and sell it to traditional dairies (Mahlabates), coffee houses and urban households, using motorcycles as transportation means. The informally marketed milk can sometimes reach an amount of 30% of the total milk consumed in towns and in cities.[6] The milk marketed by street traders confronts several constraints such as contamination by utensils or by personnel involved in its supply chain and the absence of a cold storage chain. Also, street traders work in illegal conditions without control and without regulatory legislation and are unaware of good hygienic practices. It should be mentioned that the milk marketed by street traders is generally consumed directly by consumers.

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Article downloaded from www.germs.ro
Published June 2018
© GERMS 2018
ISSN 2248 – 2997
ISSN – L = 2248 – 2997

The aim of this study was to evaluate the microbiological quality of raw milk and its contamination with *Enterococcus* spp. in order to determine the infectious risks associated to its consumption. The originality of this work lies in the particularity that this study was performed in the city of Meknes and the demonstration of the different species of *Enterococcus* spp. identified as well as their profile of resistance to antibiotics.

Methods

Samples collections

The choice of sampling sites is based on the geographical location, with significant population density, and poor hygienic conditions. These sites were in a traditional market (souk) and overcrowded popular neighborhoods (site 1 and site 2 respectively) located in the city of Meknes, Morocco.

During the period of May 2015 through April 2016, a total of 150 raw cow's milk samples were randomly collected from different street traders with twelve samples per month. Each milk sample consisted 1 L of raw milk collected aseptically into a sterile container. Samples were brought to the Microbiology Laboratory at the Faculty of Science in Meknes, in cool boxes and kept at 4 °C. The analyses were performed the same day.

Isolation and identification of *Enterococcus* spp.

A quantity of 25 g of each milk sample was aseptically taken, placed in 225 mL of buffered peptone water (Oxoid, Cheshire, UK), and then homogenized through a Stomacher 400 Circulator (Seward, West Sussex, UK) for 3 min at 260 rpm. From this primary enrichment, 1 mL was streaked to Slanetz and Bartely Agar (Oxoid) and incubated for 24±2 h at 37±1 °C. *Enterococcus* spp. suspected colonies that had a maximum diameter of 1 mm, a dark red and a narrow white border were transferred to Tryptone Soya Agar (Biokar, Beauvais, France) and incubated for 24±2 h at 37±1 °C. Isolates were identified to be *Enterococcus* spp. using the following biochemical analyses: Gram staining, mobility, pigmentation, hydrolysis of esculin in

the presence of bile, absence of catalase and growth in 6.5% NaCl.[6]

PCR amplification and sequencing of bacterial 16S rRNA

Seventeen isolates showed the same phenotypical characteristics for *Enterococcus* spp. as described by Castillo et al. in 2013.[6] To confirm this result, the DNA was extracted from each strain using the Isolate II Genomic DNA kit (Bioline, Taunton, MA, USA), following the manufacturer instructions. The quality of the DNA was controlled by a 1% agarose gel; and its quantification was determined by the Nanodrop 8000 (ThermoFisher Scientific, Waltham, MA, USA). The 16S rRNA for each strain was amplified using FD1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and RP2 (5′-ACGGCTACCTTGTTACGACTT-3′).[7] PCR was performed in 25 µL reaction mixture containing 15.3 µL of ddH_2O, 2.5 µL of buffer (10×) (KAPA Biosystems, Wilmington, MA, USA), 0.75 µL of 50 mM $MgCl_2$, 2 µL of template DNA (50 ng/µL^{-1}), 0.45 µL of Taq DNA polymerase (5 U/µL KAPA Biosystems), 0.5 µL of 10 Mm dNTPs mix (KAPA Biosystems) and 1.75 µL of 50 pmol from each primer.

The PCR temperature profile (Verity Machin) comprises an initial denaturation at 95 °C for 4 min, 35 cycles of denaturation at 95 °C for 30 s, annealing at 50 °C for 1 min and extension at 72 °C for 1 min, with a final extension at 72 °C for 7 min. The PCR products were separated by gel electrophoresis using 1.5 % (w/v) agarose (FMC, Products, Mississauga, Canada) in 0.5 TBE buffer at 100V; the gel was stained with ethidium bromide and visualized under UV light. All PCR products were sequenced by the platform of molecular biology and functional genomic in the National Center for Scientific and Technical Research (CNRST) of Rabat city (Morocco), using Analyzer ABI-3130 XL (ThermoFisher Scientific). The sequences were trimmed, analyzed through the National Center of Biotechnology Information (NCBI) server using Blast N. The sequences were deposited in GenBank under accession numbers: MF185235-MF185251.

Antimicrobial susceptibility testing

Enterococcus spp. isolates were tested for susceptibility to 14 antimicrobial compounds, which were selected on the basis of their use against infections and for prophylactic purposes. The disk diffusion method described by the Clinical and Laboratory Standards Institute was used.[8] The antimicrobial disks examined in this study are presented in Table 1. The measured inhibition zones were interpreted as sensitive, intermediate and resistant according to the CLSI guidelines. *Enterococcus faecalis* 2921 was used as a reference strain.

Table 1. Antimicrobial agents and the range of concentrations tested

Antimicrobial agent	Code	Disk concentration
Ampicillin	AMP	10 µg
Penicillin G	P	10 µg
Gentamicin	GEN	500 µg
Streptomycin	STR	500 µg
Vancomycin	VA	30 µg
Enrofloxacin	ENR	5 µg
Ciprofloxacin	CIP	5 µg
Ofloxacin	OFX	5 µg
Tetracycline	TE	30 µg
Erythromycin	E	15 µg
Nitrofurantoin	F	300 µg
Fosfomycin	FOT	50 µg
Chloramphenicol	C	30 µg
Imipenem	IPM	10 µg

Phylogenetic analysis

The phylogenetic and molecular evolutionary analyses were conducted using MEGA 7 of our sequences and the references *Enterococcus* spp. obtained from the NCBI databases. The 16S rRNA sequences were first aligned using the Clustal W.[9] The evolutionary trees for the dataset were inferred using the neighbor joining method.[9,10] The stability of the relationships was assessed by 1000 bootstrap replications. *Escherichia coli* ATCC8739 (NC_010468.1) was used as outgroup.

Statistical analysis

Data were analyzed to assess the existence of statistical differences between the parameters evaluated by season and site regarding the prevalence and antimicrobial resistances of

Enterococcus spp. The study of the effect of season and site of sampling was made by ANOVA with repeated measures using software (SPSS version 20, IBM Corp, Armonk, NY, USA). Differences were considered statistically significant at $p < 0.05$.

Results

Isolation and identification of *Enterococcus* spp.

The prevalence of *Enterococcus* spp. isolated from 150 raw cow's milk samples traded in Meknes city was calculated from May 2015 until April 2016. *Enterococcus* spp. were found in 17 (11.3%) of the samples. This prevalence was significantly different by season (p=0.001) and it was not significant among the sites sampled (p=0.281). The highest prevalence of *Enterococcus* spp. was recorded during the summer (p=0.013) and the lowest in winter (p=0.273). Indeed, 64% of contamination was observed in summer and 5.8% in winter. The most frequently identified species of *Enterococcus* were *E. faecalis* 11 (64.7%), *E. faecium* 3 (17.6%), *E. durans* 2 (11.8%) and *E. hirae* 1 (5.9%).

Antimicrobial susceptibility testing

The susceptibility of the isolates to different antibiotics showed that 100% of *Enterococcus* spp. isolates (17/17) were resistant to at least one antimicrobial, 82.3% (14/17) were resistant to two or more antimicrobials and 17.6% (3/17) were resistant to three or more antimicrobials (Table 2).

Furthermore, all *Enterococcus* spp. were resistant to ampicillin. *E. faecalis*, *E. faecium*, *E. durans* and *E. hirae* were resistant to streptomycin, with percentages of 52.9 % (9/17), 11.8% (2/17), 11.8% (2/17), and 5.9% (1/17) respectively. All *E. faecalis* and *E. faecium* isolates were resistant to tetracycline. A high percentage of resistance to antibiotics was recorded during summer, as follows: ampicillin (75.0%), streptomycin (50.0%) and tetracycline (50.0%), while during winter this value was lower for ampicillin (8.3%), streptomycin (16.6%) and tetracycline (12.9%). Moreover, this resistance was more pronounced in site 2 for streptomycin (77.8%), tetracycline (50.0%) and ampicillin (46.6%). All isolates were sensitive to vancomycin, nitrofurantoin and gentamicin. The resistance profile established for the different *Enterococcus* isolates showed 11 different strains of *Enterococcus faecalis*, 3 strains of *Enterococcus faecium*, 2 strains of *Enterococcus durans* and 1 strain of *Enterococcus hirae* (Tables 2 and 3).

Sequencing of the 16S rRNA genes

Recently, new species of *Enterococcus* have been discovered. In order to understand the phylogenetic relationships within the genus *Enterococcus*, we constructed a phylogenetic tree based on the 16S rRNA sequences. Results showed two branches, each with a high diversity of *Enterococcus* spp. isolated from raw cow's milk samples. The results of the determination of the disparity degree between the studied isolates and their classification according to the phylogenetic tree are illustrated in Figure 1. The first group contains 11 strains of *Enterococcus faecalis* and 1 *Enterococcus faecium*. The second group includes 2 *Enterococcus faecium*, 1 *Enterococcus hirae* and 2 *Enterococcus durans* (Figure 1).

Discussion

This study indicates that *Enterococcus* spp. were found in 11.3% of samples of raw milk. These rates are lower than those found in Austria (96%),[11] and than those found in France (8%) in fresh milk cheese.[12] A study carried out in Australia reported a high percentage of *E. faecalis* (74.3%) and a low percentage of *E. hirae* (8.5%), *E. faecium* (8.6%) and *E. durans* (2.9%).[12] In Egypt, the overall prevalence of *Enterococcus* spp. identified in raw milk cheese is reported at 90%.[13]

Table 2. Antimicrobial resistance profiles of *Enterococcus* spp. isolated from raw cow's milk

Strains	*Enterococcus* spp.	Antimicrobial resistance profile*	MAR index
A13	*Enterococcus faecalis*	AMP, TE, S, P, ENR, OFX, CIP	0.5
A17	*Enterococcus faecalis*	AMP, TE, E, S, ENR, OFX, CIP	0.5
A18	*Enterococcus faecalis*	AMP, TE, E	0.2
A20	*Enterococcus faecalis*	AMP, TE, E, S, P, ENR, OFX, CIP	0.6
A6	*Enterococcus faecalis*	AMP, TE, E, S,	0.3
A9	*Enterococcus faecalis*	AMP, TE, E, S, P	0.4
S1	*Enterococcus faecalis*	AMP, TE, E, S, ENR, OFX, CIP, F	0.6
S10	*Enterococcus faecalis*	AMP, TE, E, ENR, OFX, CIP	0.4
S11	*Enterococcus faecalis*	AMP, TE, S, F, FOT	0.4
S5	*Enterococcus faecalis*	AMP, TE, E, S	0.3
S6	*Enterococcus faecalis*	AMP, TE, S	0.2
A1	*Enterococcus faecium*	AMP, E, S, P, ENR, OFX, CIP, FOT	0.6
A21	*Enterococcus faecium*	AMP, TE, E, S, ENR, OFX, CIP	0.5
S7	*Enterococcus faecium*	AMP, TE, E, S, ENR, OFX, CIP	0.5
S3	*Enterococcus durans*	AMP, S	0.1
S12	*Enterococcus durans*	AMP, S	0.1
S13	*Enterococcus hirae*	AMP, S	0.1

AMP – ampicillin; **CIP** – ciprofloxacin; **E** – erythromycin; **ENR** – enrofloxacin; **F** – nitrofurantoin; **FOT** – fosfomycin; **MAR** – multiple antimicrobial resistance; **OFX** – ofloxacin; **P** – penicillin; **STR** – streptomycin; **TE** – tetracycline.

*Intermediately resistant isolates are considered resistant. MAR index is the ratio of antimicrobial resistance profile to the total number of antibiotics used.

Table 3. Antimicrobial resistance percentages of *Enterococcus* spp. isolated from raw cow's milk

Antimicrobial agent	No. of *Enterococcus* spp. isolates (n=17)		
	S	I	R
Ampicillin (10 µg)	0 (0)	3 (17.6)	14 (82.4)
Penicillin (10 µg)	13 (76.5)	1 (5.9)	3 (17.6)
Gentamicin (500 µg)	17 (100)	0 (0)	0 (0)
Streptomycin (500 µg)	1 (5.9)	4 (23.5)	12 (70.6)
Vancomycin (30 µg)	17 (100)	0 (0)	0 (0)
Enrofloxacin (5 µg)	9 (52.9)	4 (23.6)	4 (23.5)
Ciprofloxacin (5 µg)	9 (52.9)	7 (41.2)	1 (5.9)
Ofloxacin (5 µg)	9 (52.9)	7 (41.2)	1 (5.9)
Tetracycline (30 µg)	5 (29.4)	0 (0)	12 (70.6)
Erythromycin (15 µg)	5 (29.4)	8 (47.1)	4 (23.5)
Nitrofurantoin (300 µg)	17 (100)	0 (0)	0 (0)
Fosfomycin (50 µg)	15 (88.2)	1 (5.9)	1 (5.9)
Chloramphenicol (30 µg)	16 (94.1)	0 (0)	1 (5.9)
Imipenem (10 µg)	16 (94.1)	1 (5.9)	0 (0)

The presence of *Enterococcus* spp. in animal-derived food may be an indicator of contamination or poor hygienic conditions. In Morocco, the raw milk is mainly produced under conditions of food safety. However, the presence of *Enterococcus* spp. in food in general and particularly in raw milk consumed increases the risks to public health. Infants, the elderly and patients with immunodeficiency are the populations most vulnerable to poisoning.[14] In addition, antimicrobial resistance poses a major public health problem and can result in an

increase of morbidity, disease burden and mortality of the population, especially when these isolated strains are multidrug-resistant. Various studies have shown that the *Enterococcus* spp. have become resistant to a wide range of antibiotics,[15] and are widespread in dairy products, meat product and ready-to-eat foods.[16]

In our study, the resistance rate was very high for ampicillin, streptomycin, tetracycline, erythromycin and ciprofloxacin and moderate for penicillin (Tables 2 and 3). It is therefore important to report that these acquired resistances reflect the frequency and importance of antibiotics used in veterinary medicine and animal breeding.[13] Several antibiotics are banned for use in zootechny (for example vancomycin),[13] but are still used clandestinely and haphazardly.

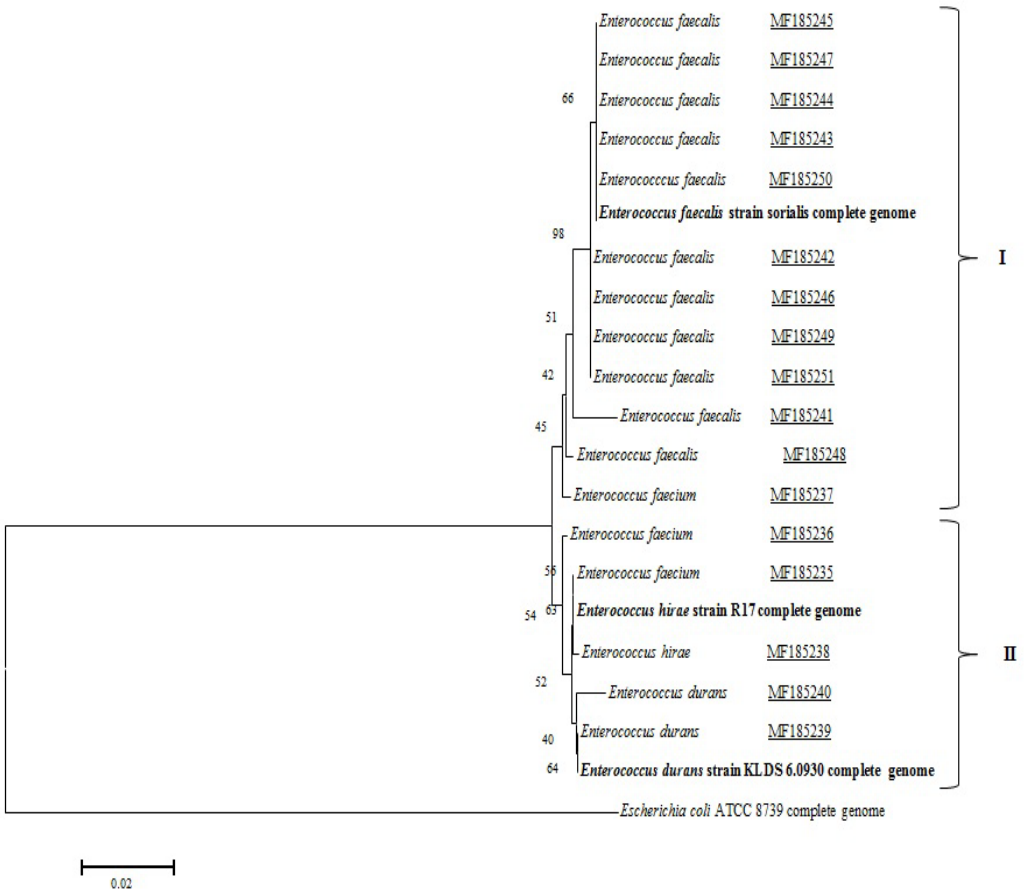


Figure 1. Phylogenetic tree of 17 *Enterococcus* isolates based on 16S rRNA sequences

The 16S rRNA of 17 *Enterococcus* isolates was used to construct the tree with *Enterococcus faecalis* strain sorialis (NZ_CP015883.1), *Enterococcus hirae* strain R17 (NZ_CP015516.1), *Enterococcus durans* strain KLDS60930 (NZ_CP012384.1) and *E. coli* ATCC 8739 (NC_010468.1) as an out-group. The tree was constructed using MEGA7 with 1000 bootstrap

Comparing these results with the literature, we find that they are higher than those found in Egypt for tetracycline (65%), chloramphenicol (1.6%),[13] penicillin (0%)[17] and lower than those found for streptomycin (78%), erythromycin (40%), ciprofloxacin (15%), gentamicin (55%), and vancomycin (5%) in raw milk cheese.[13] Other studies in Morocco have shown that the following *Enterococcus* spp. were also isolated from milk and dairy products: *E. faecalis, E. faecium*,[18] *E. durans*,[19] *E. hirae*,[20] *E. sulfureus, E.*

italicus and *E. bulliens*, which were identified in fresh camel milk.[21] *Enterococcus* spp. have been isolated all over the world, for example in China *E. faecalis, E. faecium* and *E. hirae* were isolated in fresh goat milk.[22] In Tunisia, *Enterococcus faecium, Enterococcus faecalis, Enterococcus casseliflavus, E. durans and E. mundtii* were isolated in fermented dairy products (Lben, Jben and Rayeb),[23] and in Italy *E. italicus* was found in raw milk.[24]

The antibiotic resistance pattern of *Enterococcus* isolates in this study showed 11 different phenotypes for *E. faecalis*, 3 for *E. faecium*, 2 *E. durans* and 1 *E. hirae* (Figure 1). The ratio of antimicrobial resistance profile to total number of antibiotics (multiple antibiotic resistance – MAR – index) is high for both of the first species cited. The ratio varied between values of 0.2 and 0.6; more than 65% of the strains had a MAR index greater than or equal to 0.4 (Table 3), indicating a high risk of contamination for the consumer. This MAR index level is lower than that found in Brazil (from 0.25 to 0.87).[25] For this reason, a set of measures and recommendations are needed to reinforce the application of good practices for the production of raw milk, at the primary collection stage. Awareness raising campaigns for hygiene measures are needed to improve the quality of raw milk and organize the activity of street vendors. A framework for tightening up the regulations governing the activity of traditional dairies as well as imposing traceability rules are essential to fight against this informal problem.

Conclusion

The results of this study can be considered as an alert to the dangers of contamination by multidrug-resistant enterococci isolated from consumed raw milk. Awareness of hygiene and the application of good production practices are necessary to control and prevent the resulting health and socio-economic damage.

Authors' contributions statement: AB prepared the manuscript, conducted the PCR amplification and analyzed the 16S rRNA sequences. FRF edited the manuscript. SO analyzed the 16S rRNA sequences. AE conducted experiment. FB and MM approved the manuscript. KS and JA conducted the PCR amplification and read the manuscript. AEA conducted statistical analysis. BO read the manuscript. All authors read and approved the final version of the manuscript.

Conflicts of interest: All authors – none to disclose.

Funding: None to declare.

Acknowledgement: The authors would like to acknowledge the responsible of molecular biology and functional genomics platform (National Center for Scientific and Technical Research, Morocco), Dr. El Fahim M., for his great contribution, also the professor Ayoujil Said for reading this manuscript.

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Please cite this article as:
Bouymajane A, Rhazi Filali F, Oulghazi S, Ed-dra A, Benhallam F, El Allaoui A, Anissi J, Sendide K, Ouhmidou B, Moumni M. Occurrence, molecular and antimicrobial resistance of *Enterococcus* spp. isolated from raw cow's milk trade by street trading in Meknes city, Morocco. GERMS 2018;8(2):77-84. doi: 10.18683/germs.2018.1134.