Characterization of an Enterococcus faecium strain in a murine mastitis model

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Abstract
Aim: The aim of the study was to characterize phenotypically and genotypically Enterococcus faecium strains collected from bovine mastitis milk and to evaluate one of them for its virulence in a murine mastitis model.

Methods and Results: A total of five E. faecium isolates were collected from cows with subclinical mastitis. EF-7A showed resistance to antibiotics tested, it presented alpha haemolysin and did not present gelatinase activity. It yielded cyA, efafm and gelE1 genes and it could be characterized as a moderate biofilm producer. It was able to internalize in MAC-T cells and 1.910^8 colony forming unit ml^-1 was able to establish an intramammary infection in mice. The strain could be recovered from liver, kidney and blood samples. RAPD profiles showed different bands with respect to the inoculated strain. Histopathology analyses showed different grades of polymorphonuclear neutrophils infiltration in mammary glands.

Conclusion: This is the first report that studied E. faecium strain in a lactating mouse model of mastitis and showed that the experimental inoculation was able to stimulate an inflammatory response resulting in mastitis. Results contribute to a better understanding of intramammary infections caused by E. faecium.

Significance and Impact of the Study: This investigation shows that mice represent a valuable model for the study of the mastitis pathogenesis caused by E. faecium considering the high costs of using cows for mastitis research.

Introduction
Bovine mastitis is a disease that produces significant economic losses to the dairy industry around the world (Hogeveen et al. 2011). Mastitis is caused by a wide variety of agents that affect milk quality and yield. Different efforts have been made to control the disease and vaccines have had little success because numerous agents are able to cause the disease. Control programmes, as vaccines, should be able to decrease the frequency of new infections (Bradley et al. 2015).

Among Gram-positive bacteria, streptococci and enterococci are important agents of intramammary infection in dairy herds, being the major pathogens responsible for high bulk tank somatic cell counts in appropriate managed herds (Carrillo-Casas and Miranda-Morales 2012). The primary source of environmental mastitis pathogens is the cow surroundings since infections usually take place during the milking practice.

Enterococcus spp. are part of the normal microbiota of gastrointestinal and urogenital systems of mammals. Due to their high tolerance to adverse conditions, they occupy a great variety of niches being part of the microbiota of the soil, natural waters and plants (Lebreton et al. 2014). Methods to control these agents sometimes are inadequate (Smith and Hogan 1993). Mastitis can be caused by both the contact of the teat with the enterococcal pathogen and the host immune resistance to infection (Smith and Hogan 1993).
2003). *E. faecium* and *E. faecalis* can cause bovine mastitis (Carrillo-Casas and Miranda-Morales 2012). *E. faecium* secretes several molecules that are putative virulence factors, including enterococcal surface protein gene (*esp*), aggregation substances (*agg*), cell wall adhesins (*efaAfm*), gelatinase (*gelE*) and cytolysin (*cyl*) (Eaton and Gasson 2001). Different virulence factors have also been described in enterococci isolated from food of animal origin (Valenzuela et al. 2009). Furthermore, the capacity to form biofilm is also considered as a virulence factor for enterococcal infections contributing to antibiotic resistance (Eaton and Gasson 2001; Tendolkar et al. 2004). Additionally, the *Enterococcus* group is well-known for its ability to transfer conjugative plasmids (Clewell 1990), increasing the virulence and leading to antibiotic resistance. This fact represents an important problem for therapeutic treatment.

To the best of our knowledge, there is limited information available on *E. faecium* as mastitis agent (Pettersson-Wolfe et al. 2009). Furthermore, different studies have been assayed using murine models. Among them, Sillanpää et al. (2010) reported a study of virulence in a murine model of urinary tract infection. Leendertse et al. (2009) studied the role of the enterococcal surface protein *Esp* in the ability to adhere to uroepithelial cells and the role that it plays in urinary tract infection and peritonitis using *Esp*-expressing *E. faecium* (E1162) and its isogenic *Esp*-deficient mutant (E1162Δ*esp*) in a murine model. Similarly, Panesso et al. 2011 studied the *hyl* *Efm* gene, which has been considered as a virulence determinant of hospital-associated *E. faecium*. They constructed mutants of the *hyl* *Efm*-region and they evaluated their effect on virulence using a murine peritonitis model. However, no study reports the use of a murine model for *E. faecium* as a mastitis pathogen. The development of experimental intramammary infection models facilitate the study of the mastitis pathogenesis.

The aim of this study was to characterize phenotypically and genotypically *E. faecium* strains collected from bovine mastitis milk and to evaluate one of them for its virulence in a murine mastitis model.

**Materials and methods**

**Bacterial isolates**

Twelve milk samples were collected from cows, with subclinical mastitis. Each milk sample was collected from an individual cow. Mastitis diagnosis was obtained from field veterinarians and subclinical disease was diagnosed based on the SCC values that exceeded 250 000 cells/ml⁻¹ (Ruegg 2010). Milk samples were kept on ice until processing.

Bacterial isolates were identified as streptococci or enterococci according to conventional bacteriological methods (Teixeira et al., 2011) by colony appearance, Gram stain reaction and catalase test (Shankar et al., 1999) and later confirmed by 16 rRNA sequencing and additionally by matrix-assisted laser desorption/ionisation—time of flight mass spectrometry (MALDI-TOF MS system—Bruker Daltonik MALDI Biotyper). 16 rRNA sequencing was carried out by CERELA-CONICET sequencing service and MALDI-TOF analysis by Microbiology Laboratory of the Hospital Privado Universitario de Córdoba service. Bacterial isolates were maintained in a frozen state at a temperature of −20°C in Todd-Hewitt broth (THB Sigma-Aldrich Co.) containing 20% glycerol, for further characterization.

**Phenotypic characterization**

All bacterial isolates were subcultured from storage media onto THB for phenotypic characterization.

**Antibiotic susceptibility testing**

Antibiotic susceptibility was determined by the standardized agar diffusion test on Müller-Hinton agar (MH, Britannia, Argentina) according to the recommendations of Clinical and Laboratory Standards Institute (CLSI - Clinical Laboratory Standards Institute, 2013). The following antibiotic disks currently applied on mastitis treatment were used: ampicillin (10 µg ml⁻¹), gentamicin (10 µg ml⁻¹), streptomycin (15 µg ml⁻¹), chloramphenicol (30 µg ml⁻¹), teicoplanin (30 µg ml⁻¹), erythromycin (15 µg ml⁻¹) and vancomycin (30 µg ml⁻¹) (Britannia, Argentina). The isolates were categorized as susceptible and resistant. *E. faecalis* ATCC 29212 was used as quality control.

**Enterococcal virulence factors**

The production of gelatinase and haemolysin was determined (Eaton and Gasson 2001). Gelatinase was assayed in tubes with 3 ml⁻¹ of THB with the addition of gelatin (4%). After 24 h of incubation at 37°C, the tubes were cooled for 35 min at 4°C and results were observed. Haemolysin production was determined on defibrinated horse blood agar plates. The isolates were grown during 24 h at 37°C and production of haemolysins was determined as clearer areas around bacterial colonies. Assays were repeated three times in different occasions.

**Biofilm assay**

The ability of *E. faecium* strains to form biofilm in vitro, on an abiotic surface, was determined using a sterile 96-well flat bottom polystyrene plate as described previously (Moliva et al. 2017). Briefly, bacteria were grown on polystyrene
Adherence and internalization assay

Adherence assays were carried out as described by Matthews et al. (1994) with modifications. MAC-T cells were grown in Dulbecco’s modified Eagle’s medium (DMEM, Gibco, Grand Island, NY) supplemented with addition of heat-inactivated fetal bovine serum (10%, Gibco), hydrocortisone (4 μg ml⁻¹) and antibiotics (Antibiotic Mixture: ATM (100×), Gibco). Prior to each assay, the cells were diluted at 5 × 10⁶ cells/well in 96-well culture plates and incubated during 24 h at 37°C in 5% CO₂ to 100% confluence growth. The cells were washed with PBS and co-cultured with bacterial inoculum at a multiplicity of infection (MOI, ratio of E. faecium to cells) of 10:1 for 2 h at 37°C in 5% CO₂. Afterward, the cells were washed with PBS, lysed with Triton 0.025% and MAC-T lysates were diluted 10-fold. Then, they were plated on Trypticase Soy Agar (TSA, Britannia, Argentina) and incubated overnight at 37°C. Colony forming units per mL (CFU ml⁻¹) of the bacteria adhered to MAC-T cells were determined by standard colony counting technique.

An assay to determine the ability of the bacteria to internalize was performed. After incubation with bacteria, the cells were treated with gentamicin (100 mg ml⁻¹) for 1 h to kill extracellular bacteria. Finally, MAC-T monolayers were washed, lysed again with Triton 0.025% and diluted 10-fold, plated on TSA and incubated overnight at 37°C. CFU ml⁻¹ of E. faecium internalized in MAC-T cells were determined.

An Escherichia coli, non-invasive strain, was used as a negative control for internalization assay (0% of invasion). Experiments were conducted in triplicate and repeated three times.

Genotypic characterization

**Amplification of virulence-associated genes**

The presence of gelE, esp, agg, cyLA and efaAfm genes were analysed by PCR assays using oligonucleotide primers derived from the published sequences reported by Eaton and Gasson 2001. The oligonucleotides were synthesized by Promega Corporation. A negative control with water instead of chromosomal DNA was included in each run. In addition, a characterized chromosomal DNA (E. faecium ATCC 29212) was included as positive control for efa, cyLA and efaAfm genes.

**Plasmid isolation**

Plasmid extraction was performed according to Al-Doori et al. (2001), using lysozyme (10 mg ml⁻¹) and NaOH (0.2 N)/SDS (1%). Plasmids were visualized by electrophoresis in 0.8% agarose.

**Experimental animals**

Balb/c female lactating mice, aged 6–8-weeks, (weighing 18–20 g), supplied from Bioterio Central of the National University of Río Cuarto, were maintained in a temperature and humidity room controlled with 12 h light-dark cycles and were allowed food and water ad libitum. All experimental procedures were conducted according to the Guide for the Care and Use of Laboratory Animals 2011 (ILAR, 2011) and approved by Ethics Committee of Scientific Research (CoEdI), Universidad Nacional de Río Cuarto under resolution 121/2015 CoEdI—UNRC.

An E. faecium strain was used for murine model assay. The strain was grown for 18 h on TSB and its optical density (OD₆₆₀) values were adjusted to the appropriate bacterial concentrations. Animals were injected by intramammary route at 10 and 15 days of lactation, according to the protocol described by Chandler (1970). Mice were separated into 10 groups (2 mice per group and per time) and were inoculated in the left and right fourth mammary glands (L4 and R4 respectively) as follows:

- Groups 1-5: received 100 µl of phosphate buffer saline pH 7.4 (PBS) (control groups).
- Groups 6-10: received 100 µl of a suspension containing different concentrations of E. faecium EF-7A strain (1 × 10⁴, 1 × 10⁵, 1 × 10⁶ and 1 × 10⁷ CFU ml⁻¹).

Prior to bacterial inoculation of the mammary glands, mice were separated from their pups according to Trigo et al. (2009), with modifications. Mice were anaesthetized with 87 mg kg⁻¹ of ketamine (Vetanarcol® König, Buenos Aires, Argentina) and 13 mg kg⁻¹ of xylazine (Rompun M.R. Bayer, Buenos Aires, Argentina) (Brouillette et al. 2004). The mothers were returned to the pups 8 h after the inoculations.

After 24, 48, 72, 96 and 360 h (15 days) post-inoculation the mice were sacrificed by cervical dislocation and the mammary glands, kidneys, heart and liver were removed aseptically.
**Bacteria growth curve**

Five colonies of the *E. faecium* strain grown on blood agar at 37°C for 18 h were transferred to 5 ml of TSB. The pre-culture was incubated at 37°C with agitation for 16 h. Then, 80 ml of TSB was inoculated with 0.4 ml of the pre-culture (dilution 1/200) and incubated at 37°C. Samples were taken from the culture every 2 h, during a period of 10 h. The number of total cells per ml was measured by OD₆₆₀ and the number of viable cells per ml CFU ml⁻¹ was determined. The assay was repeated in two independent experiences.

**Bacteriological examination**

Mammary glands, kidneys and liver were homogenized in 1 ml of PBS. Tenfold dilutions of the mammary gland homogenates were done and the number of CFU ml⁻¹ was determined by plating logarithmic dilutions of the tissue samples on TSA by duplicate and incubated at 37°C for 24 h. Plates were examined for bacterial growth and isolated colonies were identified according to standard procedures. Samples of kidneys and liver homogenates were cultured in TSA plates. Blood samples from heart were also cultured. *E. faecium* EF-7A colonies were identified according to standard procedures (Teixeira et al., 2011).

**Random Amplified Polymorphic DNA (RAPD-PCR)**

RAPD-PCR assays were carried out according to Reinoso et al. (2004) in order to compare the genetic profiles of the strains recovered from the different organs with the inoculated strain. The oligonucleotide OLP13 (5'-ACCGCCTGCT-3') was used for DNA amplification. Negative and positive controls were included in each run. The assay was repeated three times in different occasions. RAPD-PCR products were separated by electrophoresis in a 1.5% agarose gel (Promega) in 0.5 X TBE buffer at a constant voltage of 4 V/cm⁻¹ stained with GelGreen™ and photographed under UV light with MiniBisPRO gel documentation (BioAmerica, USA). A 100 bp DNA marker (Promega) was used as a DNA molecular size standard. DNA fingerprints of the isolates were compared for similarity by visual inspection of the band profiles.

**Histopathological analysis**

Samples of mammary glands were fixed in neutral buffer with 4% formaldehyde, paraffin-embedded, and sections (5 μm thick) were cut and stained with haematoxylin and eosin (H/E) or were Gram stained. Sections were examined by light microscopy Zeiss Axiostar plus and pictures were obtained using a Canon G5 Camera System. The evaluation of polymorphonuclear neutrophils (PMNs) infiltration in the tissue was performed by microscopic visualization in 10 randomly selected fields per slide and a score was assigned according to Boulanger et al. (2007). The score 0 represented no PMNs infiltration; 1 a weak PMNs infiltration; 2 a moderate one and 3 a strong one with severe changes. All slides were read in a blind trial, by a veterinary pathologist.

**Statistical analysis**

All the values obtained in the assays were expressed as averages with standard deviations. The data obtained were evaluated using GraphPad Prism version 5.00.288 (San Diego, USA, 2007), compared with one-way analysis of variance (ANOVA) and the Tukey multiple comparison test. Differences were considered statistically significant at p < 0.05.

**Results**

**Phenotypic and genotypic characterization**

In this study, bacterial isolates were identified as streptococci or enterococci. Five isolates were identified as enterococci. To obtain a bacterial identification, sequence analysis of the 16S rRNA genes were performed and examined by Chromas version 2.6.4. The five isolates were identified as *E. faecium* (max score 971, total score 971, 88% identity). Subsequently, the samples were analyzed by MALDI-TOF MS, which confirmed the isolates as *E. faecium* (value score of 2.324).

The five strains, named as EF-2, EF-3, EF-4, EF-5 and EF-7A were phenotypically and genotypically characterized. Antibiotic susceptibility testing showed that all the strains were resistant to streptomycin while no resistance to teicoplanin was observed. Only EF-7A strain showed resistance to six of the seven antibiotics tested (ampicillin, gentamicin, streptomycin, chloramphenicol, erythromycin and vancomycin). The results obtained are presented in Table 1.

All the strains assayed produced haemolysin and gelatinase activity.

Biofilm assay showed that all strains were able to produce biofilm and could be characterized as moderate biofilm producers, according to the categorization reported by Moliva et al. (2017). Furthermore, *E. faecium* strains were investigated for various virulence associate genes. The five strains and *E. faecium* control strain yielded *cylA* and *efaAfAm* genes. PCR amplification of *gelE* gene yielded an amplicon size of approximately 400 bp for EF-7A, EF-3, EF-4 strains and *E. faecium* control strain. *agg* and *esp* genes could not be amplified in any strain.

In addition, it was found that the five *E. faecium* strains carried plasmids (Fig 1).

The ability to adhere and internalize in host cells of *E. faecium* strains was evaluated by the epithelial MAC-T
cell invasion assay. It was observed that the strains had different levels of adhesion to MAC-T cells expressed as CFU ml⁻¹. Our results showed different levels of invasion among strains. However, EF-7A strain showed a high adhesion capacity compared to the other strains. In addition, EF-7A strain showed ability to internalize in MAC-T cells. Differences in invasion capacity were observed between EF-7A and E. coli (p < 0.05) (Fig 2). No viable internalized E. coli was detected.

Murine mastitis model

According to phenotypic and genotypic results, EF-7A strain was selected for intramammary inoculation assays. The E. faecium EF-7A strain growth curve showed an initial phase of latency of 2 h, followed by an exponential growth phase between 2 and 4 h. The maximum cell growth was determined at 6 h with 1·2 × 10⁹ CFU ml⁻¹ (Fig 3).

Mice were infected intramammary with several concentrations of E. faecium EF-7A, but only 1×10⁸ CFU ml⁻¹ was able to establish an infection in mammary glands and no animal died. The mean viable counts decreased from 8.34 log CFU ml⁻¹ at time 0 to 3.3 log CFU ml⁻¹ at 360 h in mammary gland samples. Fig 4 shows the logarithms of total number of CFU recovered from mammary glands, inoculated with 1×10⁸ CFU ml⁻¹, over the time. Although, bacterial counts were sharply decreasing until 48 h (p < 0.001 respect to initial inoculum). A slight increase in the bacterial load was observed at 72 h. A slow decrease was followed up to 360 h (15 days) and no clearance of bacteria was observed during the assay.

EF-7A strain could be isolated from liver and kidney homogenates of inoculated mice at 24, 48, 72 and 96 h post-inoculation. Bacterial counts were 2×10³ CFU ml⁻¹ in kidneys and 1×10⁴ CFU ml⁻¹ in liver at 24 h. Nevertheless, bacterial counts decreased one log at 48 h and could be recovered at 72 h and 96 h. In addition, EF-7A strain could also be cultured from blood samples at different times (24, 48, 72, 96 h). No bacteria could be recovered from negative control.

Bacteria recovered from liver and kidneys were identified as E. faecium according to standard procedures. Furthermore, RAPD-PCR assays were performed in order to compare the genetic profile of the inoculated strain with bacteria recovered from liver and kidneys. The primer used in this study was able to amplify several polymorphic DNA fragments from all samples and reproducible RAPD band profiles were generated. Fig 5 presents the RAPD profiles obtained. The genetic profiles of the recovered strains at different times (24, 48, 72, 96 h, 15 days) showed some different bands with respect to the inoculated strain (line 2). However, all the strains (inoculated strain as recovered strains) shared most of the RAPD bands. Furthermore, identical profiles were obtained within each time.

No macroscopic alteration in the mammary gland inoculated with PBS (control group) along the different times assayed were observed. Mammary glands of the inoculated group showed both discoloration and increase in their weight compared with the control group.

Histopathology changes were evaluated microscopically with H/E staining. Figs 6 and 7 show microphotographs

| ISOLATE | AMP | CHL | ERY | GEN | STR | TEC | VAN |
|--------|-----|-----|-----|-----|-----|-----|-----|
| EF-7A  | R   | R   | R   | R   | R   | S   | R   |
| EF-2   | S   | R   | S   | S   | R   | S   | S   |
| EF-3   | S   | S   | S   | R   | R   | S   | S   |
| EF-4   | S   | S   | R   | S   | R   | S   | S   |
| EF-5   | S   | S   | R   | S   | R   | S   | S   |

Abbreviations: AMP, ampicillin; CHL, chloramphenicol; ERY, erythromycin; GEN, gentamicin; STR, streptomycin; TEC, teicoplanin; VAN, vancomycin; R, resistant; S, sensitive.

References: Enterococcus faecium strains named as EF-7A, EF-2, EF-3, EF-4 and EF-5.
of histopathological analyses and the score of PMNs infiltration in mammary glands of female Balb/c lactating mice inoculated with $1 \times 10^8$ CFU ml$^{-1}$ of E. faecium EF-7A respectively. Histopathology analyses showed that tissue structures were intact in the control group. Likewise, satisfactory milk secretion was observed. Mammary glands of the inoculated group showed a moderate PMNs infiltration at 24 h without loss of glandular architecture tissue. A strong PMNs infiltration and loss of alveolar integrity was observed at 48 h. Finally, a moderate to strong PMNs infiltration was observed at 72 and 96 h, together with a thickening of the walls of the alveoli and a strong hyperaemia. It is important to note that PMNs infiltration occurred in the inoculated group at 96 h simultaneously with the presence of macrophages and lymphocytes. In addition, histopathological analysis at 15 days showed a weak infiltration. Furthermore, a gradual increase of adipocytes was observed in control groups and in groups which received EF-7A $1 \times 10^8$ CFU ml$^{-1}$. However, the inoculated group with E. faecium strain showed a higher increase of adipocytes (Fig 7).

Gram-positive cocci could be visualized in the mammary tissue of the inoculated groups by Gram staining. A considerable number of bacteria in the lumen of alveoli, mammary interstitium or phagocytosed by PMNs as well as associated with or internalized by the mammary cells were observed (Fig 8).

**Discussion**

Five E. faecium strains were phenotypically and genotypically characterized. The strains had different antibiotic resistance patterns and EF-7A showed multiple antibiotic resistance included vancomycin. Similarly, Wu et al. (2006) reported that pathogenic enterococci isolated from mastitic cows were frequently resistant to penicillin and sporadically to ampicillin and vancomycin. Different reports on Enterococcus spp. susceptibilities to antibiotics were reported in the literature. Nam et al. (2010) did not find any enterococci resistant to vancomycin, but Kuyucuoğlu (2011) found that 4.3% of the strains were resistant. Detection of vancomycin resistance is important due to the ability to transfer resistance. It has been known that enterococci group exchanges both antibiotic resistance and virulence genes among members of their genus and of other genera as well as Gram-negative and Gram-positive bacteria. These gene transfer mechanisms are efficient and important due to association with antibiotic resistance (Mundy et al., 2000; Herráez 2008).
So, detection of vancomycin resistance in enterococci collected from mastitic milk needs special attention. 

*E. faecium* has been reported of being capable to produce biofilm. In this study, biofilm assay showed that *E. faecium* strains could be characterized as moderate biofilm producers. Soares et al. (2014) studied 19 *E. faecium* strains and reported that two isolates were able to form biofilm, but they were considered as weak adherents. The importance of biofilm formation is that biofilm is considered as a virulence factor and contributes to antibiotic resistance (Eaton and Gasson 2001).

Different virulence factors contribute to mammary gland infection. Gelatinase encoded by *gelE* is involved in biofilm formation and hydrolyses collagen, casein and haemoglobin (Shankar et al. 1999; Hancock and Perego 2004). Break down of proteins could be important for invasion and dissemination in the mammary gland. *gelE* gene could be detected in *E. faecium* EF-7A, EF-3 and EF-4 strains and they were able to produce this enzyme *in vitro*. This result is in contrast with those of other reports which indicated that the occurrence of *gelE* gene is not necessarily associated with gelatinase activity (Marra et al. 2007; Comerlato et al. 2013).

The five *E. faecium* strains yielded *cylA* gene and *efaAfm* gene. *cylA* encodes a cytolysin that can cause damage to cell membranes, enabling infection. Otherwise, *efaAfm* gene is involved in the adhesion of the bacteria to biotic and abiotic surfaces favouring biofilm formation (Pérez-Pulido et al. 2006). The five *E. faecium* strains yielded *efaAfm* gene and were able to produce biofilm *in vitro*. In addition, *efaAfm* gene has been showed to be involved in evasion of the immune response (Pérez-Pulido et al. 2006) and to have a crucial role in infective endocarditis caused by *Enterococcus* (Mohamed et al. 2004). The presence of *cylA* and *efaAfm* genes in *E. faecium* strains may improve colonization ability and their high resistance to different antibiotics could lead to increase virulence.

The *E. faecium* strains, assayed in this work, were able to adhere and internalize MAC-T cells. Similar results were reported in *E. faecalis* (Elhadidy and Zarhan 2013). Adhesion and internalization to the mammary epithelium...
are important facts for the development of the infection (Opdebeeck et al. 1988). However, according to our data, adhesion level and percentage of internalization did not seem to be related to each other.

According to the results obtained, *E. faecium* EF-7A strain was selected to evaluate its virulence in a murine mastitis model. The strain showed a typical growth curve, with a decrease in the cell values after 8 h due to a possible lysis effect.

Mice were infected by intramammary route with several concentrations of *E. faecium* EF-7A, but only $1 \times 10^8$ CFU ml$^{-1}$ was able to establish an infection in mammary glands. The bacteria could be recovered from mammary glands over the time. Silvestrini et al. (2007) reported that the number of *Staphylococcus aureus* strain

**Figure 6** Histopathology of mammary glands of female lactating Balb/c mice. (a) Inoculated groups with PBS (Control groups) at 96 h post-inoculation and (b), (c), (d), (e) and (f) Inoculated groups with *E. faecium* EF-7A $1 \times 10^8$ CFU ml$^{-1}$ at: 24 h, 48 h, 72 h, 96 h and 360 h post-inoculation, respectively. Arrows indicate the presence of PMNs, stars indicate lacteal secretion, arrowheads indicate alveoli and triangle indicate adipocytes. A gradual increase of adipocytes was observed in control groups and group inoculated with EF-7A $1 \times 10^8$ CFU ml$^{-1}$. A higher increased was observed in group inoculated with the bacteria. [Colour figure can be viewed at wileyonlinelibrary.com]

**Figure 7** PMNs infiltration in mammary glands of female lactating Balb/c mice, inoculated with *Enterococcus faecium* EF-7A $1 \times 10^8$ CFU ml$^{-1}$ at different times. Presence of PMNs in mammary tissue was assigned a score. 0: no PMNs infiltration, 1: weak PMNs infiltration, 2: moderate PMNs infiltration, 3: strong PMNs infiltration with severe changes. ***$p < 0.001$ significant statistical difference compared to the infiltration observed in the mammary glands inoculated with PBS (control group). □ and □.

**Figure 8** Gram stain section of mammary glands of female lactating Balb/c mice inoculated with *Enterococcus faecium* EF-7A strain $1 \times 10^8$ CFU ml$^{-1}$. The arrows indicate the presence of Gram positive cocci in the lumen of alveoli. The arrowhead indicates Gram positive cocci inside PMN. Magnification 100X. [Colour figure can be viewed at wileyonlinelibrary.com]
Newbould 305 (ATCC 29740) recovered from the mammary gland increased at 24 h post-inoculation and a strong decrease in the bacterial load was reported at 48 h, followed by a slight increase at 72 h post-inoculation. Rowson et al. (2010) and Forsberg et al. (2012) developed a murine mastitis model of Streptococcus uberis using CD-1 mice and reported that the pathogen was able to colonize the mammary gland after the intramammary inoculation with 50 CFU ml\textsuperscript{-1} at 48 h post-infection. Similarly, Zhao et al. (2015) using a S. aureus strain isolated from goats, reported that the strain was not completely removed from the mammary gland. These results agree partly with those observed in this study suggesting that E. faecium EF-7A, as other mastitis agents, may persist on mammary gland after intramammary inoculation.

In addition, EF-7A strain could be isolated from liver and kidney homogenates of inoculated mice at different times assayed post-inoculation. This result revealed that EF-7A strain could diffuse from the mammary gland and had the capacity to invade other organs as liver and kidney. Brouillette et al. (2004) reported that S. aureus Newbould 305 (ATCC 29740) was able to disseminate from mammary glands to kidneys after 12 h post-intramammary inoculation of different bacterial concentrations. Even though the study was carried out with S. aureus strains, the results agree with those obtained in the present study, where E. faecium EF-7A strain was recovered from kidneys after intramammary inoculation. Trigo et al. (2009) reported that by inoculating 1 × 10\textsuperscript{6} CFU ml\textsuperscript{-1} of a Streptococcus agalactiae using Balb/c mice observed an increase in the bacterial load recovered from kidneys and liver at 12 and 24 h post-inoculation respectively.

Different molecular methods, as RAPD-PCR, REP-PCR and PFGE have been applied to evaluate the genetic diversity of mastitis pathogens. In this work, RAPD-PCR was chosen because it is a rapid, simple, easy and less expensive technique applicable to any genome. The primer used was previously employed for RAPD typing of S. aureus mastitis bacterial strains (Reinoso et al. 2004). The presence of different RAPD profiles, according to the time of isolation, suggests that genetic changes of the strain may occur in vivo during the infection. To the best of our knowledge, no studies reported about genotypic characterization of bacteria recovered from the mammary gland of mice inoculated with E. faecium strains. Kha- zandi et al. (2015) working with S. uberis on a cow mammary challenge infection model, showed that the RAPD profiles of the recovered strains were identical to RAPD profiles of the inoculated strain. Similarly, Quesnell et al. (2012) reported that the RAPD profile of an E. coli strain inoculated by intramammary route did not change its genetic profile at least 48 to 96 h post-challenge. These results contrast with those obtained in our study, since RAPD profiles of the recovered strains presented different bands along the time assayed (24, 48, 72, 96 and 360 h post-inoculation). According to the presence of plasmids in the strains assayed, our results may be related with the fact that Enterococcus genera is characterized by its high capacity to acquire external genetic material by conjugation or by transference of plasmids or transposons in vivo (Johnston and Jaykus 2004; Albornoz et al., 2005).

Data obtained in the present study are in agreement with different works which reported that the inoculation of a pathogenic strain in a mouse mastitis model caused mastitis symptoms. The histology of inoculated mammary glands can be compared with bovine mammary glands infected (Akers and Nickerson 2011). The results of the histopathology analysis agreed with the bacterial growth in the mammary gland inoculated with E. faecium EF-7A 1 × 10\textsuperscript{6} UFC ml\textsuperscript{-1}. The intensity of the inflammation observed in the mammary glands of inoculated groups may be due to the presence of a constant number of bacteria from 48 h to 15 days. In addition, the presence of the bacteria in the mammary gland during all times assayed, showed that E. faecium EF-7A could not be removed by effector cells of the innate immune system, as PMNs and macrophages. This fact induced the activation of the defense mechanisms of adaptive immunity evidenced by the presence of lymphocytes in the mammary tissue at 96 h after infection. Our results agree with Rowson et al. (2010) who observed a massive infiltration of PMNs together with a loss of breast tissue integrity 48 h after 50 CFU ml\textsuperscript{-1} of S. uberis inoculation.

The dissimilarities found in the different studies could be attributed to the pathogen studied, its virulence factors and the number of bacteria inoculated as well. It is important to note that the experimental infection degree of the environmental pathogens such as streptococci and enterococci is considered highly variable and lower than contagious pathogens such as staphylococci. Furthermore, mice strains used may be slightly susceptible to infection by streptococci (Chandler 1970).

This is the first report that studied E. faecium strain in a lactating mouse model of mastitis. Results showed the capacity of E. faecium EF-7A strain to produce intramammary infection in mice. This investigation shows that mice represent a valuable model for the study of the mastitis pathogenesis caused by E. faecium considering the high costs of using cows for mastitis research.

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Conflict of Interest

The authors have no conflict of interest to declare.

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