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COORDINATORE: PROF. LORENZO LEONCINI

CICLO XXXIII

Bacterial infections of the genital tract of infertile couples and *in vitro* fertilization

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CHAPTER 1 – Abstract

Infertility is estimated to affect approximately 9-12% of reproductive aged couples worldwide. The causes of infertility can be attributed to several pathological conditions affecting one or both partners. The introduction of "Assisted Reproduction Technologies" (ART) has allowed remarkable scientific and medical advances in the field of assisted reproduction. ART consists of different strategies to overcome some infertility factors, thereby improving reproduction efficacy. Infections of the urogenital tract may contribute to infertility with different sexually transmitted diseases (STDs) being directly or indirectly associated to infertility. Among the pathogens that have been associated to infertility, there is *Enterococcus faecalis*. This microorganism, previously considered just as a member of the gut microbiota of both animals and humans, is now acknowledged as an important human pathogen responsible for a variety of infections, including infections of the urogenital tract, but also life-threating infections such as sepsis and endocarditis. The presence of acquired resistance to major antibiotic classes, in addition to natural intrinsic resistance, is a relevant issue for the treatment of enterococcal infections. Aminoglycosides in combination with β-lactams is the frontline drug combination therapy for severe enterococcal infections. Of special concern is the acquisition, by horizontal transfer, of genes coding for aminoglycoside modifying enzymes (AMEs) which confer resistance to high levels of aminoglycosides (HLA) and eliminate the synergistic bactericidal activity with β-lactams. Despite genital tract infections can affect human fertility, there are no consensus guidelines available on their management (*i.e*., microbial screening, antibiotic treatment, therapy outcome) in infertile couples undergoing ART treatment.

In the present thesis, an attempt was made to better understand how infections impact on couple fertility. We have also explored the effect of hormone therapy on vaginal microbiota and reproductive outcomes of females undergoing *in vitro* fertilization (IVF). In addition, we have characterized the antibiotic susceptibility and population structure of a collection of *E. faecalis* clinical strains isolated from the genital tract of infertile couples to provide clinicians with relevant data to implement management of urogenital infections in infertile couples.

Chapter 2 is a general introduction to infertility-associated infections with a special focus on *E. faecalis.* In the first section, virulence determinants, disease pathogenesis and clinical

manifestations of *E. faecalis* are described. Then, treatment of enterococcal infections with a broad overview on action and resistance mechanisms of major antibiotic classes is provided.

Chapter 3 explores the role of urogenital infections on couple fertility starting from a collection of vaginal/endocervical swabs and semen samples from 285 infertile couples. The impact of different bacterial species on the outcome of IVF was examined. The results showed the negative impact of *E. faecalis* on sperm quality and the association of different bacterial pathogens with reduced levels of vaginal lactobacilli. Interestingly, the presence of *E. faecalis* together with *Ureaplasma urealyticum*/*Mycoplasma hominis* in genital samples of infertile couples was predictive for a negative outcome of IVF.

Chapter 4 describes the phenotypic and genotypic features of 41 "infertility-associated *E. faecalis*" (IAF) strains described in chapter 3. Antibiotic susceptibility of different drugs was carried out (Vitek, MIC and disk diffusion methods) and assessed using EUCAST guidelines. The majority of IAF isolates were susceptible to clinically relevant antibiotics, except for 8 strains that were resistant to HLA and 1 which was also resistant to fluoroquinolones. In order to characterize the IAF isolates, whole genome sequences were used to get insights into the IAF population structure and analyse the genetic bases of antimicrobial resistance. Multi-locus sequence typing (MLST) showed a high diversity of the IAF population. However, a clonal structure of HLA resistant strains was found, as 6 out of 8 resistant IAF isolates belonged to the same clonal complex (CC)/sequence type (ST) CC16/ST480.

The work described in chapter 5 evaluates the effect of treatment with exogenous gonadotropins on the vaginal microbiota of 108 infertile women undergoing controlled ovarian stimulation prior to being subjected to IVF. A significant increase of vaginal diamines was observed following hormonal treatment. Analysis of vaginal swabs revealed that a shift occurred after hormone treatment from a *Lactobacillus*-based microbiota to a microbial population mostly constituted by streptococci, enterococci, enterobacteria, staphylococci and yeasts. A highly significant association between reduced amounts of vaginal lactobacilli and the presence of above mentioned pathogens was found. Finally, IVF outcome was significantly decreased in the patients whose vaginal samples were positive for high levels of diamines and presence of bacterial pathogens, suggesting that a link may exist between vaginal microbiota dysbiosis due to hormonal treatment and IVF failure.

Chapter 6 contains a short paragraph with the main conclusions of this Ph.D. thesis.

CHAPTER 2 – Introduction

1. *ENTEROCOCCUS FAECALIS*

Enterococci are gram-positive cocci facultative anaerobes, asporigenous, catalase-negative and homofermentative. Thiercelin et *al.* has described enterococci as saprophytic cocci living in the gut but also capable of causing infection (Thiercelin and Jouhaud, 1899). Phylogenetically, enterococci were classified as group D streptococci (Sherman, 1938) until 30 years ago when they were described as a separate genus (Schleifer and Kilpper-Bälz, 1984). Nowadays, the genus *Enterococcus* (*Lactobacillales* order, *Enterococcaceae* family) is composed of many species present in the human and animal gut as well as in fermented food, dairy products, soil and water (Murray, 1990; Klein, 2003). Their presence in different environments is supported by the capability of multiply at temperatures ranging between 10 and 45°C and survive at 60°C for 30 min. Enterococci are halophile and can grow at pH of 9.6 (Tendolkar et al., 2003).

Among enterococcal species, *Enterococcus faecalis*, along with *Enterococcus faecium*, is a core member of the human microbiota, but it is also responsible for several infections in both community and hospital settings (Tendolkar et al., 2003; Kristich et al., 2014). The amount of enterococci (with *E. faecalis* being the most abundant species) in the gut is 10^4-10^6 colony forming units (CFU) per gram of wet weight, thereby representing about 1% of the human fecal flora (Tendolkar et al., 2003; Eckburg et al., 2005; Dubin and Pamer, 2014). Once considered non-virulent gut commensals, enterococci are now also associated with life-threatening infections. *E. faecalis* and *E. faecium* are amongst the most prevalent species encountered in human disease and are responsible for a large proportion of urinary tract infections, intra-abdominal infections, peritonitis, abscesses, endocarditis and bacteremia/sepsis (Solomkin et al., 2010; Mercuro et al., 2018).

1.1 Virulence factors

Virulence factors are crucial for disease onset and progression (Table 1) (Richards et al., 2000; Sava et al., 2010). This section describes the different virulence determinants produced by *Enterococcus* spp. For easier understanding, these factors have been divided into three subsections according to their localisation in the bacterial cell.

1.1.1 Secreted factors

Enterococcal cytolysin CylL contributes to enterococcal disease and it has been associated to patient mortality (Coburn and Gilmore, 2003). It is a two-peptide lytic system belonging to a family of toxins secreted by various Gram-positive bacteria. This family includes the lantibiotics and streptolysin S of *Streptococcus pyogenes*. The cytolisin operon is either located on the bacterial chromosome within a 150 kb (Chow et al., 1993) pathogenicity island (PAI) (Chow et al., 1993) or on a conjugative pheromone-responsive plasmid, which generally is pAD1 (Van Tyne et al., 2013). The toxin consists of two subunits, encoded by the genes $cylL_L$ and $cylL_S$, which form a pore in the target cell membrane (Van Tyne et al., 2013). It can lyse both red and white human blood cells *in vitro* (Jett et al., 1992; Madsen et al., 2017) and has been shown to contribute to disease in experimental models of endophthalmitis and endocarditis (Table 1) (Figure 1) (Chow et al., 1993; Madsen et al., 2017).

Gelatinase GelE is an extracellular zinc-metalloprotease that degrades host collagen, fibrinogen, fibrin and complement factors. It is one of two proteases of *E. faecalis*, the other one being the serine protease SprE, whose encoding gene *sprE* is localized downstream and co-transcribed with *gelE* (Thurlow et al., 2010; Madsen et al., 2017). GelE is involved in biofilm formation and is capable of destroying endothelin-1, bradykinin and LL-37 (Garsin et al., 2001; Thurlow et al., 2010). These virulence determinants are regulated by the Fsr system that comprises the genes *fsrA*, *fsrB* and *fsrC* localized upstream of *gelE*. Inactivation of the Fsr system blocks the production of gelatinase and prevents biofilm formation on abiotic surfaces (Figure 1) (Table 1) (Hancock and Perego, 2004).

1.1.2 Cell surface-associated factors

Cell wall components play an important role in enterococcal pathogenesis (Figure 1) (Hancock et al., 2014). Lipoteichoic acids (LTA) are key components involved in immune

recognition. It has been demonstrated that LTA can stimulate leukocytes to release inflammatory mediators. LTA are able to stimulate Toll-like receptor 2 (TLR-2) and promote TNF-α production through the NF-κB and p38 MAPK pathways in differentiated THP-1 macrophages (Baik et al., 2008; Wang et al., 2015°). Fabretti *et al.* have observed that reduced D-alanination of LTA is associated to higher susceptibility of *E. faecalis* to antimicrobial peptides and reduced ability to form biofilm and adhere to host cells (Table 1) (Fabretti et al., 2006).

The *E. faecalis* capsule is at the forefront against host immune defences. There are 4 serotypes (A, B, C and D) (Thurlow et al., 2009). The capsule locus *cps* in *E. faecalis* contains nine genes (*cpsC*, *cpsD*, *cpsI*, *cpsF*, *cpsG*, *cpsH*, *cpsI*, *cpsJ*, *cpsK*). Mutations in *cps* genes make the isolates more susceptible to opsonophagocytic killing by neutrophils (Table 1) (Hancock and Gilmore, 2002; Thurlow et al., 2009).

The polysaccharide antigen Epa is recognized by sera from patients with systemic enterococcal infection (Teng et al., 2009). The loss of Epa is associated to reduced biofilm formation (Mohamed et al., 2004) and diminished resistance to killing by neutrophils (Teng et al., 2002). Zeng *et al.* showed that an *epa* mutant was not able to translocate across a monolayer of polarized human enterocyte-like T84 cells, suggesting a role for Epa in invasion of epithelial cells (Zeng et al., 2005). These findings have been confirmed in different animal models of disease, such as experimental peritonitis and urinary tract infection (UTI) mouse models (Table 1) (Teng et al., 2009).

Aggregation substance (AS) is a group of closely related, multifunctional, surface-anchored polypeptides encoded by genes carried by pheromone-responsive conjugative plasmids (McCormick et al., 2000; Waters et al., 2003). The AS coding genes from the three most studied plasmids are *asa1* from pAD1, *asp1* from pPD1, and *asc10* from pCF10 (Waters et al., 2003). AS is involved in adhesion to intestinal and genitourinary epithelial cells (Vu and Carvalho, 2011). Asa1 promotes adherence to renal tubular cells and contributes to enterococcal survival in human macrophages (Kreft et al., 1992; Süssmuth et al., 2000). In particular, *E. faecalis*, harbouring the pheromone-responsive plasmid pAD1 that carries the AS encoding genes, was more efficient to bind to cultured pig renal tubular cells compared to plasmid-free isolates (Kreft et al., 1992). Asc10 enhances enterococcal internalization and intracellular survival in polymorphonuclear leukocytes (Olmsted et al., 1991). Both Asc10 and Asa1 have been shown to contribute to adherence to extracellular matrix proteins in a rabbit endocarditis model (Chow et

al., 1993; Muscholl-Silberhorn, 1998; Waters et al., 2003). In addition, AS proteins promote bacterial internalization into different intestinal epithelial cells *in vitro*, indicating that AS may participate in translocation of *E. faecalis* across the intestinal epithelial cells leading to systemic infection (Table 1) (Sartingen et al., 2000; Süßmuth et al., 2000).

Enterococcal surface protein Esp is a virulent factor carried by a large PAI. Different studies have shown the persistence of *E. faecalis* strains producing Esp in the human bladder. About one-third of *E. faecalis* isolates from patients with bacteremia and UTIs produce the Esp protein, while it is generally not present in faecal isolates (Shankar et al., 2001, 2002). *E. faecalis* mutants devoid of Esp showed reduced biofilm production, suggesting that the protein may play a role in biofilm formation (Tendolkar et al., 2003, 2005). On the other hand, no correlation was found between biofilm formation and Esp in clinical isolates (Table 1) (Hancock and Perego, 2004; Raad et al., 2005; Di Rosa et al., 2006; Hancock et al., 2014; Kristich et al., 2014).

The cell-wall anchored adhesins MSCRAMMs ("Microbial Surface Components Recognizing Adhesive Matrix Molecules") are involved in adhesion to the vascular tissue (Singh et al., 2010). The most studied MSCRAMM of *E. faecalis* is Ace ("Adhesin to Collagen of *E. faecalis"*), which mediates bacterial attachment to host tissues via interaction with collagen I and IV (Rich et al., 1999; Nallapareddy et al., 2000; Lebreton et al., 2009; Singh et al., 2010). The regulator Ers is part of the MSCRAMM family and acts as a repressor of *ace* (Nallapareddy et al., 2000). Recent studies suggest that Ace contributes to adhesion to immobilized collagen located on heart valves and aortic tissue in mice (Lebreton et al., 2009; Cohen et al., 2013; Madsen et al., 2017). Ace is also involved in the pathogenesis of experimental UTI in the mouse (Lebreton et al., 2009; Singh et al., 2010). Other MSCRAMMs (Fss1, Fss2, and Fss3) have been shown to bind to fibrinogen (Sillanpää et al., 2004; Sava et al., 2010). Inactivation of the gene encoding Fss2 in the reference strain OG1RF resulted in reduced fibrinogen adherence, whilst complementation of the mutant restored full adherence (Table 1) (Sillanpää et al., 2004; Sava et al., 2010).

EfbA promotes binding to collagen I, V, and fibronectin which is generally exposed if the cardiac tissue is damaged (Singh et al., 2015). It has been shown that deletion of the *efbA* gene significantly reduced enterococcal binding to immobilized fibronectin (Table 1) (Torelli et al., 2012; Singh et al., 2015). In addition, EfbA was demonstrated to play an important role in

biofilm formation and colonization of aortic valves in an experimental endocarditis rat model (Singh et al., 2015).

Finally, pili of *E. faecalis* are implicated in biofilm production and adhesion to a large variety of human cells. *E. faecalis* and *E. faecium* harbour two pilin gene clusters, designated as *ebp* ("Endocarditis and Biofilm-associated Pili") and *bee* loci ("Biofilm Enhancer in Enterococci") (Nallapareddy et al., 2006). The *bee* locus is located on a conjugative plasmid and is present in only 5% of *E. faecalis* isolates, whereas the *ebp* operon is present in the majority of the isolates (Cobo Molinos et al., 2008). Ebp pili contribute to the pathogenesis of biofilm-associated infections, such as endocarditis and UTI. Ebp pili are also important for adhesion to the urinary epithelium and urinary catheters by binding to the fibrinogen shedded into the bladder or urethra during catheterization (Table 1) (Nallapareddy et al., 2011; Nielsen et al., 2012). Furthermore, Ebp pili are antigenic in human endocarditis (Madsen et al., 2017).

1.2 Pathogenesis of enterococcal disease

The capability of *E. faecalis* to adhere, aggregate and form biofilm is a shared trait of commensal and virulent enterococcal strains, thereby contributing to both commensalism and pathogenicity. Many virulence factors, that have been shown to be involved in enterococcal disease, are often also produced by commensal isolates (Kao and Kline, 2019). This section attempts to describe how commensal *E. faecalis* can turn into a human pathogen.

1.2.1 E. faecalis *as a commensal*

Colonization of the gut by the resident microbiota can prevent infection by intestinal pathogens via different mechanisms: i) maintenance of the mucosal barrier integrity; (ii) direct antagonism; (iii) indirect inhibition through stimulation of innate immune defences (Figure 1) (Dubin and Pamer, 2014). Enterococci exploit their ability to adhere and aggregate in order to colonize various niches of the host. *E. faecalis* colonizes the gut of 60-80% of infants within the first week of life and 80% of adults (Adlerberth and Wold, 2009; Yang et al., 2015). Biofilm formation is the main strategy used by *E. faecalis* to colonize and persist in the gut mucosa (Banla et al., 2019). *E. faecalis* may also be present in other body districts, such as the oral cavity (20% of healthy individuals) (Portenier et al., 2003) and the urinary tract (Whiteside et al., 2015).

E. faecalis has some features in common with probiotics as it can interfere with colonization by food-borne pathogens (Kao and Kline, 2019). A study showed that *E. faecalis* can compete with *Listeria monocytogenes* in the adhesion to Caco 2 cells *in vitro* (Cebrián et al., 2012). The combination of *E. faecalis* with *Bifidobacterium* spp. and *Lactobacillus* spp. strengthens the intestinal epithelial barrier by promoting the production of tight junction proteins and reducing the release of proinflammatory cytokines (Shi et al., 2014). Heikkila *et al.* have shown that *E. faecalis* isolated from human breast milk can inhibit the growth of *Staphylococcus aureus in vitro* (Heikkilä and Saris, 2003).

1.2.2 From commensal to pathogen

E. faecalis is also an important human pathogen (Arias and Murray, 2012; Garsin et al., 2014). The first report of a severe enterococcal infection ("*Micrococcus zymogenes*") dated back at the end of 19th century by MacCallum *et al*. who described an "acute endocarditis of the aortic and mitral valves with infarctions of the spleen and kidneys" (MacCallum and Hastings, 1899).

Different triggering factors, including antibiotic treatment and host inflammation, can alter the gut microbiota and promote enterococcal overgrowth which may lead to bacterial translocation across the intestinal barrier (Figure 1) (Selleck et al., 2019). Enterococci are able to survive in inflamed host tissues. In the gut, a recent study reported that the amount of *E. faecalis* in the faeces of patients with ulcerative colitis (UC) and Crohn's disease (CD) was higher than that of healthy individuals (Zhou et al., 2016). Moreover, *E. faecalis* is the most prevalent species in inflammatory conditions of other body districts, such as the oral cavity, where it can cause both periodontal and endodontic lesions and persist despite antibiotic treatment (Dahlén et al., 2000; Souto and Colombo, 2008).

As above mentioned, overgrowth of *E. faecalis* in the gut increases the risk for translocation across the intestinal mucosa (Figure 1) (Kao and Kline, 2019). Bacterial translocation is promoted by GelE that degrades collagen in the basal membrane and activates host protease activated receptor-2 (PAR-2) and matrix metalloproteinase-9 (MMP-9), thus causing damage to the extracellular matrix (Maharshak et al., 2015; Shogan et al., 2015). *E. faecalis* can also directly invade the enterocytes (Figure 1). Following the invasion of the gut lamina propria, bacteria can access the blood leading to a systemic infection. Once in the bloodstream or deeper tissues, bacteria respond to the new environment by promoting the

production of virulence factors, including the capsule, the cytolysin Cyl, Epa, GelE and AS (Table 1) (McCormick et al., 2000; Hancock and Gilmore, 2002; Teng et al., 2002; Waters et al., 2003; Baik et al., 2008; Thurlow et al., 2009, 2009; Wang et al., 2015a; Madsen et al., 2017; Kao and Kline, 2019).

E. faecalis can escape from host immune defences by evading immune surveillance and/or resisting intracellular killing by phagocytes, thus enabling a successful transition from commensal to pathogen (Kao and Kline, 2019).

E. faecalis has evolved several strategies to avoid the engulfment by professional phagocytes by impairing opsonisation (Figure 1). Gaglani *et al*. showed that *E. faecalis* is generally susceptible to neutrophil-mediated killing resulting in less than 10% bacterial survival after an hour of infection. Such efficient killing is strongly dependent on opsonisation (Gaglani et al., 1997). The capsule is at the frontline defence against complement-mediated phagocytosis. Capsular serotypes C and D mask bacterial surface-bound C3 from recognition by complement receptors on macrophages *in vitro* (Thurlow et al., 2009). Moreover, the presence of C/D capsules can hinder LTA from immune recognition (Baik et al., 2008). Other crucial virulence factors involved in host immune escape are Epa and GelE. Epa provides resistance to neutrophil mediated killing by inhibiting bacterial uptake and providing resistance to antimicrobial compounds (Teng et al., 2002, 2009). GelE can also degrade C3, thereby limiting complement activation *in vivo* (Park et al., 2008). It has been also proven that some enterococcal strains can produce a modified LTA that limits the activation of the complement lectin pathway (Geiss-Liebisch et al., 2012). *E. faecalis* can also evade antibody driven immunity by secreting the endoglycosidase EndoS that degrades human IgG (Collin and Fischetti, 2004).

Another strategy to evade defences is resisting to intracellular killing. Macrophages and neutrophils are equipped with numerous antimicrobial components including lysozyme, myeloperoxidase (MPO), elastase and defensins that can efficiently kill enterococci (Harvey et al., 1992). Epa has been shown to confer high-level resistance to lysozyme and other antimicrobial molecules (Hébert et al., 2007; Teng et al., 2009; Painter et al., 2017), while AS allows survival within neutrophils by mediating resistance to MPO (Rakita et al., 1999; Vanek et al., 1999). *E. faecalis* is also resistant to superoxide stress mediated by the superoxide dismutase SOD (Verneuil et al., 2006). Finally, enterococci in biofilms are generally resistant to immune recognition and phagocytic killing (Kao and Kline, 2019).

1.3 Clinical manifestations

1.3.1 Urinary tract infections

UTIs are the most common enterococcal infections (Abat et al., 2016a). UTI is characterized by different signs and symptoms, including increase in urinary frequency, dysuria and/or suprapubic pain, costovertebral angle pain and tenderness, fever, malaise, altered sensorium (Miller et al., 2018).

Among the etiologic agents of UTI, enterococci are the second most common causative agent after *Escherichia coli* (Nicolle et al., 2019; CAUTI Guidelines | Guidelines Library | Infection Control | CDC, 2019). Enterococcal UTI frequently localize in the lower urinary tract, causing cystitis, urethritis, prostatitis and epididymitis (Figure 2). Enterococcal UTIs are generally more common in hospitalized or long-term facility patients than in the community (Hidron et al., 2008; Agudelo Higuita and Huycke, 2014; Flores-Mireles et al., 2015). UTIs caused by *Enterococcus* spp. occur at a rate of 12.9 per 1,000 hospital discharges and represent from 34% to 46% of all hospital infections in the US (Swaminathan and Alangaden, 2010).

Enterococcal colonization of the urinary tract and asymptomatic bacteriuria are common and do not generally require therapy (Hidron et al., 2008; Arias and Murray, 2012; Weiner et al., 2016). However, bacteraemia can occur after enterococcal UTI, especially in older men (Graninger and Ragette, 1992). Over 10% of catheter-associated UTIs (CAUTIs) are caused by enterococcal species, which are then considered as the most common agents of CAUTIs caused by Gram-positive bacteria (Figure 2) (Hidron et al., 2008; Agudelo Higuita and Huycke, 2014; Weiner et al., 2016). Out of 20,000 cases of enterococcal CAUTIs reported by CDC in 2015 (CDC, 2021), over 50% were caused by *E. faecalis* followed by *E. faecium* (20%) (Abat et al., 2016a). *E. faecalis* strains isolated from the urinary tract are often resistant to commonly tested antibiotics (Hooton et al., 2010; Abat et al., 2016b; Faron et al., 2016; Nicolle et al., 2019). Of note, 85% of *E. faecium* isolates from the urinary tract is vancomycin-resistant (Weiner et al., 2016).

Different studies have shown the importance of the interaction of enterococci with the uroepithelial tissue for the onset and development of UTI (Shankar et al., 2002; Hidron et al., 2008; Arias and Murray, 2012; Weiner et al., 2016). *E. faecalis* isolates from UTIs adhere more efficiently to urinary tract epithelial cells suggesting tissue tropism (Shankar et al., 2002;

Lebreton et al., 2014). As previously mentioned, uropathogenic enterococci produce several virulence factors that are crucial for adhesion to the urinary tract (see chapter 1.1).

Finally, enterococci can also cause genital tract infections. *E. faecalis* was identified in approximately 50% of semen samples from male partners of infertile couples (Mehta et al., 2016) and its presence has been associated with impaired sperm quality (Rodin et al., 2003; Qiang et al., 2007; Ruggeri et al., 2016; Ricci et al., 2018) and negative outcome of *in vitro* fertilization (IVF) (Figure 2) (Ricci et al., 2018).

1.3.2 Enteritis, peritonitis and pelvic infections

Enterococci are often identified in intra-abdominal, pelvic and soft tissue infections. Moreover, *E. faecalis* can cause peritonitis (Figure 2) (Agudelo Higuita and Huycke, 2014). Most of the infections caused by *E. faecalis* at the gastrointestinal level originate from damage of gut epithelial cells. Enterococci have also been associated with inflammatory bowel disease (IBD), CD and UC (Zhou et al., 2016; Růžičková et al., 2020). Finally, a few studies have begun to associate *E. faecalis* to colorectal cancer. The causes of this association have not been clarified yet, but it was shown that higher viable counts of *E. faecalis* in faecal samples were present in colorectal cancer patients compared to healthy individuals (Balamurugan et al., 2008; Tjalsma et al., 2012; Lucas et al., 2017).

1.3.3 Bacteraemia and endocarditis

Enterococci are the second leading cause of healthcare-associated bacteraemia and, among the enterococcal species, *E. faecalis* is the most important responsible for infective endocarditis (Figure 2) (Hidron et al., 2008). The genitourinary tract or intra-abdominal districts are the body sites from where bacteraemia originates (Agudelo Higuita and Huycke, 2014). From the bloodstream, bacteria can seed in various district including the endocardium or heart valves, causing endocarditis (Dahl et al., 2019). In the elderly, enterococcal endocarditis mostly occurs on prosthetic valves (Rice et al., 1991; Anderson et al., 2004). *E. faecalis* can also adhere directly to the endocardium by forming a biofilm which may be difficult to be cleared by host defences and antibiotics (Madsen et al., 2017). Because of the enterococcal intrinsic resistance to a wide range of antibiotics, treatment is difficult and sometimes requires removal of the infected valve (Stevens and Edmond, 2005). Clinically, enterococcal endocarditis is an infection characterized

by heart failure and associated with a mortality rate up to 15% (McDonald et al., 2005). Since the symptoms are often difficult to diagnose, the condition may be mistaken with other inflammatory conditions.

1.4 Epidemiology of enterococcal infections

Enterococci are common in the environment because they can survive in adverse environmental conditions. They can be found in the soil (both in temperate and tropical areas), water and vegetation (Murray, 1990; Klein, 2003; Byappanahalli et al., 2012). In addition, enterococci are isolated from a large variety of food, including many fermented foods made from meat and milk (Foulquié Moreno et al., 2006). Peters *et al.* have isolated 416 enterococcal strains from food samples of animal origin in Germany. Of those, 72% were *E. faecalis* and 13% *E. faecium* (Peters et al., 2003). The natural habitat of enterococci is, however, the gastro-intestinal tract of human and animals, from which they can spread to the environment (Byappanahalli et al., 2012). The distribution of *Enterococcus* species varies throughout Europe. In Italy, Spain and the UK, *E. faecalis* and *E. faecium* are the most commonly isolated species from both clinical and environmental sources (wastewater, soil, wild and domestic animal), while in Sweden there is the lowest prevalence of *E. faecium* in Europe (Kühn et al., 2003) .

Enterococcal clinical isolates are mainly represented by *E. faecalis* and *E. faecium*. *E. faecalis* is present in about 40% of healthy individuals (Mutnick et al., 2003). Nevertheless, as abovementioned, enterococci are the second and third most common causes of UTIs and bacteraemia, respectively (de Fátima Silva Lopes et al., 2005). *E. faecalis* is responsible for 80-90% of endodontic infections caused by enterococci (Peciuliene et al., 2001). Moreover, in the last decades, an increase in enterococcal infections, especially endocarditis, has occurred (Edmond et al., 1999; Agudelo Higuita and Huycke, 2014).

Of special concern are nosocomial enterococcal infections, as patients in hospitals or long-term facilities are generally more susceptible to infections (Howden et al., 2013; Dubin and Pamer, 2014). Enterococcal species can survive for prolonged periods on different abiotic surfaces, such as medical devices and bed rails (Hota, 2004; Duckro et al., 2005). Moreover, horizontal transmission by healthcare workers can directly transfer the bacteria to hospitalized patients, including immunocompromised individuals. In the latest years, hospital-acquired infections caused by antibiotic resistant enterococci have dramatically increased (Hidron et al.,

2008; Howden et al., 2013). Since the gastro-intestinal tract represents the major site colonized by (antibiotic-resistant) enterococci, it also constitutes an important source of hospital-associated infections, especially vancomycin-resistant enterococci (VRE) infections (Drees et al., 2008).

1.5 Antibiotic therapy

Treatment of enterococcal infections depends upon the: i) species, ii) antibiotic susceptibility, iii) body site and severity of infection. Uncomplicated enterococcal infections may be adequately treated with a monotherapy, whereas severe infections, such as endocarditis or sepsis, need a synergistic regimen (Hollenbeck and Rice, 2012). In addition, enterococci are intrinsically resistant to many commonly used antimicrobial agents (Figura 3). For infections caused by antibiotic susceptible isolates of *E. faecalis*, ampicillin remains the preferred therapy. In the rare case of strains producing β-lactamases, combination with a β-lactamase inhibitor improves disease outcome. When complicated infections are caused by susceptible strains, an aminoglycoside should be added to a cell wall biosynthesis inhibitor for synergistic killing (Hollenbeck and Rice, 2012; Mercuro et al., 2018). Among aminoglycosides, only gentamicin and streptomycin are used for combined therapy with β-lactams (Mercuro et al., 2018). These drug combinations overcome the intrinsic aminoglycoside resistance exhibited by enterococci resulting in a synergistic bactericidal effect. However, enterococci can acquire resistance to one or more drugs of the combination therapy. Therefore, susceptibility testing is of great importance on those enterococcal isolates responsible for infections where combination therapy is mandatory (Hollenbeck and Rice, 2012). In complicated infections resistant to high-levels of gentamicin and streptomycin, alternative therapies are represented by the combinations of ceftriaxone-ampicillin (Gavaldá et al., 2003), ceftriaxone-fosfomycin (Farina et al., 2010) or ampicillin-imipenem (Brandt et al., 1996).

UTIs usually respond to ampicillin, while vancomycin can be used in patients allergic to penicillins. Amongst alternative agents, linezolid and daptomycin should be considered as well as ciprofloxacin and nitrofurantoin (Heintz et al., 2010; Mercuro et al., 2018).

In uncomplicated *E. faecalis* bacteraemia, as an alternative to the standard combined therapy and/or in case of penicillin, aminoglycoside or glycopeptide resistance, linezolid, daptomycin or dalbavancin are also indicated especially for catheter-associated bloodstream infections (Raad et al., 2005; Foo et al., 2014). In complicated bacteraemia and endocarditis,

clinical evidences support ampicillin combinations with another antibiotic. If bacteraemia is caused by ampicillin- and/or vancomycin-resistant enterococci, daptomycin in combination with an aminoglycoside, a cephalosporin or intravenous fosfomycin should be considered (Beganovic et al., 2018; Mercuro et al., 2018).

Intra-abdominal or pelvic infections are rarely caused by a single microbial species, so treatment should include a combination of broad-spectrum antibiotics. Fluoroquinolones can also be used against enterococcal soft tissue infections (Saurina et al., 1997; Mercuro et al., 2018).

2. ANTIBIOTIC RESISTANCE IN *ENTEROCOCCUS FAECALIS*

The clinical importance of enterococci is also associated to their broad resistance to antibiotics that may result in treatment failure and negative disease outcome. Antibiotic resistance patterns of *E. faecalis* and *E. faecium* are widely different, but they can generally be classified as tolerance, intrinsic and acquired resistance (Figura 3). The first part of this section describes the three different types of resistance. The second part explains the main resistance mechanisms divided by antibiotic classes, with a specific focus on *E. faecalis*.

2.1 Tolerance, intrinsic and acquired resistance

The genus *Enterococcus* is tolerant to the activity of cell wall inhibitors, such as β-lactams and glycopeptides. Enterococci can be inhibited by clinically achievable concentrations of the antibiotic, but the bactericidal effect will be reached only at concentrations far in excess of the inhibitory concentration. Such phenomenon of tolerance can be overcome by adding aminoglycosides to peptidoglycan synthesis inhibitors (Kristich et al. 2014). Testing tolerance *in vitro* (i.e. killing curves) is not routinely used in diagnostics but has an important impact on therapy for treating enterococcal infections, especially for endocarditis that needs a bactericidal effect (Kristich et al. 2014).

Intrinsic resistance is encoded within the core genome of all strains of the species. Enterococci are intrinsically resistant to many commonly used antimicrobial agents (Table 2). The production of penicillin-binding proteins (PBPs) with low-affinity for beta-lactams leads to resistance to all semi-synthetic penicillins and most cephalosporins (Arbeloa et al. 2004). Enterococci are also intrinsically resistant to clindamycin, and *E. faecalis* is naturally resistant to quinupristin-dalfopristin (Singh, Weinstock, e Murray 2002). Enterococci also have intrinsic resistance to clinically achievable concentrations of aminoglycosides, which impedes their use as monotherapy (Hollenbeck e Rice 2012). Trimethoprim-sulfamethoxazole is active against enterococci only *in vitro* on folate-deficient media (Zervos e Schaberg 1985).

Enterococci are able to acquire resistance to virtually any antimicrobial agent used for clinical treatment (Table 3). Incorrect and extended use of antibiotics for the treatment of enterococcal infections has permitted to select clinical isolates resistant to different antibiotic classes. Introduction of chloramphenicol, erythromycin and tetracyclines was quickly followed

by the emergence of resistance that in some cases reached levels that precluded their empirical use (Kristich et al. 2014). The occurrence of ampicillin resistance in *E. faecalis* is rare, whereas there is broad high-level resistance to ampicillin among *E. faecium* clinical isolates. Increasing rates of high-level aminoglycoside (HLA) resistance is occurring in both *E. faecalis* and *E. faecium* strains, and can severely hamper the synergism between cell-wall active agents and aminoglycosides (Miller, Munita, e Arias 2014; Sarti et al. 2012). VRE mainly comprise *E. faecium* strains (Mercuro et al. 2018; Swenson, Facklam, e Thornsberry 1990).

The acquisition of antibiotic resistance can occur by spontaneous mutation or by gene transfer. Horizontal gene exchange among enterococci is often mediated by mobile genetic elements, such as plasmids and/or transposons (Hollenbeck e Rice 2012). Multiple plasmids and transposons can be detected in clinical strains.

Plasmids identified in *E. faecalis* belong to two different classes:

i) "Pheromone-responsive" plasmids are species-specific. They are predominantly found in *E. faecalis*, where they transfer and acquire genetic material with a highly efficient mechanism (Depardieu et al. 2004; Panesso et al. 2005). The plasmids can encode both virulence factors and determinants involved in antibiotic resistance. The most thoroughly studied plasmid is pCF10 that harbors the *tetM* conjugative transposon Tn*925*. This transposon carries resistance to tetracycline and minocycline (Christie et al. 1987). Other well-known examples are plasmids pMG2200, carrying the VanB-type vancomycin resistance cassette, and pMG2201, harbouring erythromycin resistance carried by the *erm* gene (G. Zhang, Ma, e Lu 2009).

ii) "Broad host range" plasmids are conjugative plasmids able to spread both within enterococci and also to other bacterial species (Galli, Lottspeich, e Wirth 1990; Palmer, Kos, e Gilmore 2010). The first plasmids studied are pAMβ1, which carries resistance genes to macrolides, and pIP501, which confers resistance to macrolides and chloramphenicol (Panesso et al. 2005).

Another important genetic element involved in enterococcal antibiotic resistance is the transposon, a sequence of heterologous DNA integrated into the chromosome, but capable of moving within the same bacterial cell or transferring to different bacterial species and genera (Clewell et al. 2014; Zhang e Saier 2011). Three different classes of transposons have been identified in *E. faecalis*:

i) Tn3-family transposons consist of a single insertion sequence (IS). Examples are Tn*917*, conferring macrolide, lincosamide and streptogramin B resistance (MLSB), and Tn*1546*, conferring glycopeptide resistance (Arthur et al. 1993). Tn*552*, that carries a beta-lactamase coding gene, has also been identified in *Enterococcus* spp.

ii) Conjugative transposons are able to promote their intracellular and extracellular transposition (Manganelli et al. 1995; Salyers et al. 1995). A well-known conjugative transposon is Tn*916* that confers resistance to minocycline and tetracycline (Rice 1998:916). Tn*5382* encodes the VanB glycopeptide resistance operon (Rice et al. 1998).

iii) Composite transposons consist of a central region and two lateral IS regions. Tens of composite transposons have been described (Kristich et al. 2014). Determinants of resistance are often present in the central region (Palmer et al. 2010). Tn*5281* carries resistance to all aminoglycosides except for streptomycin (Hodel-Christian e Murray 1991; Simjee, Fraise, e Gill 1999). Tn*4001* confers gentamicin resistance, while Tn*5384* confers resistance to several antibiotic classes. Tn*5384* is a 26kb-long element that carries genes which confer gentamicin, macrolide, and mercury resistance (Bonafede, Carias, e Rice 1997). Tn*5385* is a large, transferable element found in the chromosome of *E. faecalis* CH116 that confers multi-drug resistance (streptomycin, tetracycline, gentamicin, erythromycin, β-lactams). It is a 65kb-long element whose ends are composed of two direct repeats copies of IS1216. Other transposons may lie within this element, including Tn*4001* (which confers gentamicin resistance) (Lyon et al., 1984) and the above mentioned Tn*5384* (Bonafede et al. 1997).

2.2 Molecular mechanisms of antibiotic resistance in *E. faecalis*

2.2.1 Beta-lactams

Beta-lactams are the first-line of therapy for the treatment of enterococcal infections (Mercuro et al. 2018). Beta-lactams inhibit the synthesis of peptidoglycan by binding to PBPs (Table 2). PBPs are DD-transpeptidases that catalyse the transpeptidation step in the synthesis of peptidoglycan. PBPs vary in their affinity for penicillin and other β-lactams (Bycroft e Shute 1985; Kong, Schneper, e Mathee 2010). PBPs are divided in two classes: A and B. Class B differs from class A because it contains only a transpeptidase domain, but not a transglycosylase domain which is necessary for the initial polymerization of the disaccharide of the peptidoglycan

precursor. *E. faecalis* can produce three class A (PbpF, PonA and PbpZ) and three class B (PBP5, PbpA and PbpB) PBPs (Arbeloa et al. 2004). *E. faecalis* strains are generally susceptible to penicillins, while most *E. faecium* isolates are resistant. Intrinsic resistance affecting all classes of PBPs is due to the production of PBP5 (Arbeloa et al. 2004; Kristich et al. 2014; Palmer et al. 2010), which mediates intrinsic resistance to cephalosporins and also intrinsic and acquired resistance to penicillins (Sauvage et al. 2008).

Cephalosporins. *E. faecalis* and *E. faecium* are naturally resistant to cephalosporins due to chromosomal determinants. Deletion mutants that lack PBP5 result in reduced resistance to cephalosporines. In particular, Arbeloa *et al.* showed that deletion of PBP5 led to a 4,000-fold reduction in the MIC of ceftriaxone and to a 4-fold reduction in the MIC of ampicillin in *E. faecalis* JH2-2 (Arbeloa et al. 2004). *E. faecalis* PBP5 is aided by PbpF and PonA (Arbeloa et al. 2004). Recently, Djoric´ *et al.* showed that PbpA is also required for cephalosporine resistance, and ceftriaxone is bactericidal towards *pbpA* deletion mutants. PbpA and PBP5 are not functionally redundant (Djorić, Little, e Kristich 2020).

In addition, two-component systems (TCSs) are important in the response to cell wall stress to promote intrinsic resistance to cephalosporins and other beta-lactams (Table 2). These systems include the transmembrane Ser/Thr kinase IreK and the phosphatase IreP that act antagonistically leading to cephalosporin resistance (Kristich, Wells, e Dunny 2007). In *E. faecalis*, IreB, an endogenous substrate of IreK and IreP, acts as a negative regulator of the pathway (Hall et al. 2013; Madsen et al. 2017). Hall *et al.* showed that the activity of IreB is modulated by IreK-dependent phosphorylation in a signalling pathway conferring resistance to cephalosporins (Hall et al. 2013). The second TCS consists of the CroS sensor kinase and its regulator CroR. Inactivation of CroRS in *E. faecalis* makes the strains more sensitive to ceftriazone (Comenge et al. 2003; Hartmann et al. 2012). In the response to antibiotic-mediated cell wall stress, *E. faecalis* stimulates CroR-dependent transcription (Comenge et al. 2003). Three genes regulated by CroR have been identified, with *croR* being the one with a major role in cephalosporin resistance (Comenge et al. 2003; Murray 1990).

Finally *E. faecalis* produces MurAA and MurAB that are two homologs of the enzyme that catalyzes the first step in the synthesis of the peptidoglycan precursor UDP-N-acetylglucosamine 1-carboxyvinyl transferase. Deletion of *murAA* lead to increased

susceptibility to extended spectrum cephalosporins and fosfomycin (Kristich et al. 2014; Vesić e Kristich 2012).

Ampicillins. Unlike *E. faecium*, PBP5 in *E. faecalis* gives a lower intrinsic resistance to ampicillin (typical MICs are 1–4 μg/ml) compared to cephalosporins. Another mechanism of ampicillin resistance, originally described in staphylococci, depends on the presence of the *blaZ* gene (encoding a β-lactamase) which is part of an operon composed by *blaR1*, coding for a transmembrane sensor and signal transducer, and *blaI*, encoding a repressor (Table 2) (Kristich et al. 2014). When performing *in vitro* susceptibility testing, enterococci generally produce only small amounts of the enzyme resulting susceptible to ampicillin, while during infection, the bacterial load may be high enough to produce large amount of BlaZ leading to ampicillin resistance (Miller et al. 2014; Sarti et al. 2012). However, addition of the β-lactamase inhibitor sulbactam is generally sufficient for antibiotic efficacy (Miller et al. 2014).

In Europe, resistance rates to ampicillin in *E. faecalis* in 2019 varied from 0% in several countries to 9.8% in Lithuania. The rate of ampicillin resistant strains in Italy is 2%. On the other hand, ampicillin resistance in *E. faecium* exceeds 75% in all european countries (Figure 4) (Anon 2020a).

2.2.2 Glycopeptides

Glycopeptides inhibit bacterial growth by interfering with the synthesis of peptidoglycan. These molecules bind to the D-Ala-D-Ala lateral chain of the pentapeptide precursor, thereby blocking peptidoglycan synthesis (Gold 2001; Swenson et al. 1990). The biochemical basis of resistance to glycopeptides consists in a modification of the antibiotic target. Glycopeptide-resistant enterococci produce altered precursors, in which the D-Ala-D-Ala chain is modified with a D-Ala-D-Lactate or D-Ala-D-Ser. These substitutions eliminate the binding capacity of the antibiotic to the peptidoglycan precursors. Altered precursors can still serve as substrates for the enzymes involved in the biosynthesis of the bacterial cell wall and are able to generate a functional peptidoglycan, while decreasing the target affinity to glycopeptides (Swenson et al. 1990).

Nine clusters of *van* genes that confer glycopeptide resistance have been described in enterococci (Kristich et al. 2014). Eight phenotypic variants (VanA, VanB, VanD, VanE, VanG, VanL, VanM and VanN) confer acquired resistance, while one type (VanC) causes intrinsic

resistance and it is unique in *E. gallinarum* and *E. casseliflavus* (Table 3) (Courvalin 2006). The change from D-Ala-D-Ala to D-Ala-D-Lac (VanA, VanB, VanD, VanM) leads to a 1,000-fold decrease of affinity for vancomycin, whereas a change to D-Ala-D-Ser (VanC, VanE, VanG, VanL, VanN) causes a 7-fold decreased affinity for vancomycin (O'Driscoll e Crank 2015; Werner et al. 2008). VanA is present in most VRE around the world and is mostly carried by *E. faecium* isolates. The *van* determinants differ genotypically and phenotypically in: i) genetic location (mobile genetic elements or chromosome); ii) specific drug to which confer resistance; iii) level of resistance; iv) type of resistance (inducible or constitutively expressed), and v) type of peptidoglycan precursor produced (Arthur et al. 1993). The *van* gene clusters encode: i) a regulatory module consisting of a TCS that senses the presence of glycopeptides and activates the expression of the resistance genes; ii) enzymes that produce modified peptidoglycan precursors; iii) a D,D-carbossipeptidase which forms crosslinks (Kristich et al. 2014). Among the encoded enzymes there is also a ligase that joins D-Ala to D-Ser/Lac, and based on the type of ligase, the various Van clusters are denominated.

The *van* gene cluster is transcribed by two promoters. One of the promoters transcribes the regulatory apparatus that consists of VanR and VanS. VanS is a sensor kinase anchored to the cytoplasmic membrane. $VanS_A$ contains a small extracellular loop and can receive activating signals in or immediately adjacent to the membrane. $VanS_B$ has a large extracellular domain that serves as a ligand-recognition domain. The VanS sensor kinase recognizes the stimulus given by the presence of vancomycin in the environment. Once activated, VanS self-perforates a conserved residue of His in its cytoplasmic portion. The phosphorylated group is transferred to an Asp residue on VanR, leading to its dimerization and ultimately resulting in increased transcription of VanR (Woodford 2001). The phosphatasic activity of VanS is essential to keep the signaling pathway inactive in the absence of the antibiotic. Mutations that affect such phosphatasic activity lead to a constitutive expression of the genes (Foucault et al. 2010; Kristich et al. 2014). Evidence suggests that kinases encoded in the host genome may also contribute to the regulation of *van* resistance genes (Courvalin 2006; Depardieu et al. 2004; Foucault et al. 2010). This may indicate that enterococci encode endogenous TCSs with the function of monitoring the integrity of the cell wall (Depardieu et al. 2004). The most common genotypes in the clinical isolates of *Enterococcus* spp. are *vanA* and *vanB* (Arthur e Quintiliani 2001). The *vanA* determinant confers high level resistance to both vancomycin and teicoplanin and it is

typically carried by the transposon Tn*1546*. The *vanB* locus gives moderate to high level resistance to vancomycin, but not to teicoplanin, and it is usually harboured by Tn*5283* or Tn*1549* transposons.

Around the world, the rates of VRE are at their highest in North America. According to the National Health-Care Safety Network, from 2009 to 2010, more than 30% of *E. faecium* hospital-associated infections were resistant to vancomycin, ranking as the second most common cause of nosocomial infections in the US. On the other hand, only 5% of *E. faecalis* isolates is vancomycin resistant in the US (Anon 2020b; O'Driscoll e Crank 2015; Weiner et al. 2016).

In Europe, vancomycin resistant *E. faecium* is less prevalent than in the US, but it is increasing. In the EU/EEA, the population weighted mean percentage for vancomycin-resistant *E. faecium* increased from 10.5% in 2015 to 18.3% in 2019 (Figure 5) (Anon 2020a). However, the above rates vary depending on the country, with VRE ranging from less than 1% in France, Spain and Sweden to more than 20% in Italy, Greece, Ireland, Portugal and the United Kingdom (Anon 2020a; O'Driscoll e Crank 2015; Werner et al. 2008). Vancomycin resistance in *E. faecalis* is still low in most european countries reaching less than 1% (Anon 2020a).

Finally, the class of lipoglycopeptides such as telavancin, dalbavancin and oritovancin also offer alternative drugs targeting the bacterial cell wall with a spectrum of action that also includes the enterococcal isolates with glycopeptide resistance due to VanB (Mercuro et al. 2018).

2.2.3 Lipopeptides

Daptomycin is a cyclic lipopeptide with bactericidal activity against Gram-positive bacteria including enterococci (Alborn, Allen, e Preston 1991). The antibiotic alters the bacterial cell homeostasis through interactions with the phospholipids of the cytoplasmic membrane (Table 3) (Tran, Munita, e Arias 2015). Insertion of daptomycin into the cytoplasmic membrane is dependent on Ca^{2+} (Ho et al. 2008; Jung et al. 2004; Taylor e Palmer 2016). The oligomerization of daptomycin-Ca^{2+} complexes and the translocation of the oligomers into the inner layer of the cell membrane form functional ring-like pores (Muraih et al. 2012). Daptomycin pores are cation- and size-selective with high permeability to Na+, K+ and other metal cations (Zhang et al. 2014). The presence of pores in the membrane leads to ion leakage, membrane depolarization and cell death without lysis (Zhang et al. 2014).

Daptomycin resistance is generally caused by mutations in chromosomal genes and has mostly been studied in *Staphylococcus aureus* (Kristich et al. 2014). Genes involved in daptomycin resistance include those encoding regulatory proteins involved in cell wall stress response, such as LiaFSR (*liaF*) and YycFGHIJ (*yycF*), and genes encoding enzymes involved in the metabolism of phospholipids important for cell membrane homeostasis, such as glycerophosphoryl diester phosphodiesterase (*gdpD*) and cardiolipin synthetase (*cls*) (Table 3) (Arias et al. 2011; Miller, Bayer, e Arias 2016; Palmer et al. 2010; Suntharalingam et al. 2009; Tran et al. 2015). Mutations in several other genes have also been described in datomycin resistant *E. faecalis*, but further experimental studies are needed to confirm that those mutations are linked to daptomycin resistance (Bender et al. 2018).

E. faecium generally has higher rates of resistance to daptomycin than *E. faecalis*, although resistance *in vivo* has been described in both species and has been reported to be approximately 0.6% for *E. faecium* and 0.1% for *E. faecalis*. Daptomycin resistance is more common in Asia and Europe than in North America (Kelesidis et al. 2011, 2012).

2.2.4 Aminoglycosides

Aminoglycosides bind to the 16S rRNA of the 30S ribosomal subunit and interfere with protein synthesis. Enterococci are intrinsically low-level resistant to aminoglycosides, with MICs ranging from 4 μg/mL up to 256 μg/mL (Aslangul et al. 2006). Low-level resistance to all aminoglycosides is mediated by the proteins involved in electron transport that limit drug uptake (Table 2) (Aslangul et al. 2006; Leggett 2015). The 6'-N-aminoglycoside acetyltransferase (*aac(6')-Ii*) also confers low level of resistance (Costa et al. 1993). Additional aminoglycoside intrinsic resistance mechanisms in *E. faecium* have been described. Inactivation of *efmM*, that encodes the chromosomal rRNA methyltransferase EfmM, decreases the susceptibility to aminoglycosides in *E. faecium* (Galloway-Peña et al. 2012).

Despite all enterococci possess intrinsic low-level resistance to aminoglycosides, association with a cell wall active drug allows the access of aminoglycosides to the ribosomal target leading to a synergistic bactericidal activity (Leggett 2015). Co-administration of ampicillin or vancomycin with an aminoglycoside significantly increases the uptake of the aminoglycoside (Joseph W. Chow 2000; Galloway-Peña et al. 2012; Krogstad et al. 1978; Moellering e Weinberg 1971).

In contrast to intrinsic resistance mechanisms, acquired resistance lead to high-level aminoglycoside (HLA) resistance in both *E. faecium* and *E. faecalis* (Mercuro et al. 2018; Murray 1990). HLA-resistance eliminates the synergistic killing activity with β-lactams or glycopeptides. Among many known mechanisms of HLA resistance, enzymatic modification is the most prevalent in the clinical setting (Ramirez e Tolmasky 2010) and it is usually mediated by genes coding for aminoglycoside modifying enzyme (AME). AMEs catalyse the modification at different –OH or $–NH_2$ groups of the 2-deoxystreptamine nucleus or the sugar portion, and can be classified as: i) acetyltransferases (AACs), ii) phosphotransferases (APHs), or iii) nucleotidyltranferases (ANTs).

AACs use acetyl-CoA to acetylate an amino group of the antibiotic. The *aac* genes have been found in plasmids and chromosomes, and are often part of mobile genetic elements (Ramirez e Tolmasky 2010). In *E. faecalis*, AAC(6′) enzymes are generally associated with APH(2″) proteins (see below) (Table 3) (W. Zhang, Fisher, e Mobashery 2009).

APHs use ATP to phosphorylate a hydroxyl group of the antibiotic. The APH(3′)-III subclass, encoded by the *aph(3')-III* gene, confers resistance to kanamycin, neomycin, lividomycin, paromomycin, livostamycin, butirosin, amikacin and isepamicin (Vakulenko e Mobashery 2003). The APH(2″) mediates gentamicin resistance in different Gram-positive bacteria (Ramirez e Tolmasky 2010). In *E. faecalis*, APH(2″) is encoded by *aph(2'')-Ic* and confers gentamicin resistance with MIC values below the cut-off of HLA. However, *E. faecalis* isolates carrying the *aph(2'')* gene are still resistant to the synergistic activity of gentamicin plus cell-wall active antibiotics (Joseph W. Chow 2000; Mahbub Alam et al. 2005). The APH(2″)-Ia is generally found as a fusion to AAC(6′)-Ie, which is located at the N-terminal portion of the composite protein (Ferretti, Gilmore, e Courvalin 1986). APH(2″)-Ia-AAC(6′)-Ie confers resistance virtually to all aminoglycosides except for streptomycin (Joseph W. Chow 2000). Cloning both regions as separate genes resulted in two active proteins suggesting that the ancestor genes were originally separated (Ferretti et al. 1986). In *E. faecalis* and *S. aureus*, AAC(6′)-Ie is located at the amino terminal end of a bifunctional enzyme with both AAC(6′) and APH(2″) activities (Boehr, Daigle, e Wright 2004; Ferretti et al. 1986). The *aac(6')-aph(2'')* gene is usually carried by Tn*4001*-like transposons (Culebras e Martínez 1999).

ANTs use ATP to adenylylate a hydroxyl group of the antibiotic. Genes coding for enzymes with related amino acid sequences have been named as *ant(6)-Ia*, *ant6*, *ant(6)*, *str* and

aadE. They all show the same resistance pattern to streptomycin, but are not identical (Ramirez e Tolmasky 2010). The *ant(6)* gene is often found in the gene cluster *ant(6)-sat4-aph(3')-III* that confers resistance to aminoglycosides and streptothricin (Cerdá et al. 2007). The cluster is part of Tn*5405* and other Tn*5405*-like transposons, which are widely distributed among staphylococci and enterococci and are located in plasmids and chromosomes (Werner, Hildebrandt, e Witte 2003). Finally, two enzymes, ANT(9)-Ia and ANT(9)-Ib, have been described that mediate resistance to spectinomycin. The genes coding for these enzymes were denominated under several names including *ant(9)-Ia* and *ant(9)-Ib*, *spc* and *aad(9)*, thus increasing classification confusion. The amino acid sequences of ANT(9)-Ia and ANT(9)-Ib share 39% identity. ANT(9)-Ia was first described in *S. aureus* and then in *Enterococcus avium*, *E. faecium* and *E. faecalis*. In all four species the gene is carried by Tn*554* (Mahbub Alam et al. 2005; Murphy 1985).

In Europe, high-level gentamicin resistance rates significantly diminished between 2015 and 2019, with similar decreasing national trends reported by almost one quarter of the european countries. The percentage of high-level gentamicin resistance of *E. faecalis* in Europe was 26.6% in 2019, with national percentages ranging from 7.8% to 41.6% (Figure 6). The rate of high-level gentamicin resistant strains in Italy (35.2%) is among the highest in Europe together with UK, Spain, Portugal and Eastern Europe (Anon 2020a).

2.2.5 Macrolides, streptogramins and lincosamides

Macrolide-lincosamide-streptogramin (MLS) antibiotics constitute an alternative therapy for the treatment of several enterococcal infections. MLS bind to the 50S ribosomal unit and inhibit protein synthesis (Tables 2 and 3) (Gaynor e Mankin 2003; Vannuffel e Cocito 1996).

Streptogramins are composed of A and B components, which are bacteriostatic alone, but bactericidal when combined (Vannuffel e Cocito 1996). *E. faecalis* is intrinsically resistant to streptogramins. In particular, most *E. faecalis* isolates are resistant to quinopristin (streptogramin B class) and dalfopristin (streptogramin A class) (Table 2) (Deshpande et al. 2007; Mercuro et al. 2018). Despite intrinsic resistance in *E. faecalis* isolates, this class of antibiotics is approved for treatment of infections caused by vancomycin-resistant *E. faecium* and it generally used in combination therapy (McDonald 2006; Mercuro et al. 2018). The molecular basis for the difference between the two enterococcal species is the presence of the ABC-drug efflux pump Lsa in *E. faecalis*, but not in *E. faecium* (Singh et al. 2002). Deletion of the *lsa* gene in *E.*

faecalis OG1RF results in decreased MIC for quinopristin/dalfopristin and also for lincosamides (Singh et al. 2002). Twelve genes involved in streptogramin resistance have been described in enterococci. Acquired resistance has also emerged in *E. faecium* and is mediated by acquired mobile genetic elements involved in either acetylation of the antibiotic or modification of the drug target by dimethylation of 23S rRNA (Table 3) (Mercuro et al. 2018).

Dimethylation of 23S rRNA in the 50S ribosomal subunit is the most common mechanism that reduces binding affinity of MLS to the ribosome (Portillo et al. 2000; Singh et al. 2002). High-level resistance to erythromycin occurs through acquisition of either the *ermA* (phenotype MLS_A) or *ermB* (phenotype MLS_B) genes carried by broad host range plasmids like pAMβ1 (Portillo et al. 2000). Low-level macrolide resistance in *Enterococcus* spp. is instead conferred by an efflux pump encoded by the transferrable *mefA* gene (Table 3) (Chouchani et al. 2012).

E. faecalis is naturally resistant to lincosamides through two different mechanisms. In addition to the alteration of the antibiotic target site mediated by the rRNA methyltransferase encoded by *erm* genes, lincosamide resistance is also conferred by inactivation of the antibiotic through nucleotidylation by nucleotidyltransferases (Trieu-Cuot, Carlier, e Courvalin 1988). A number of nucleotidyltransferases encoded by *lnu* or *lin* have been described in various bacteria (Bozdogan et al. 1999; Zhu et al. 2017). In *E. faecalis*, the lincosamide nucleotidyltransferase gene *linG*, located on the mobile element Tn*6260*, was recently identified (Zhu et al. 2017).

2.2.6 Oxazolidons

Linezolid is an antibiotic that interferes with the bacterial growth by inhibiting protein synthesis through interaction with the translational initiation complex (Bi et al. 2018). It is approved by FDA for the treatment of infections caused by VRE isolates in synergistic combination with gentamicin (Mercuro et al. 2018; Munita e Arias 2016). The most common resistance mechanism is the presence of the nucleotide substitution G2576T in the 23S rRNA, which prevents the drug from binding to the target in the 50S subunit (Bourgeois-Nicolaos et al. 2007; Kuroda et al. 2018). The gene *cfr*, already reported in *S. aureus*, is capable of conferring resistance to different classes of antibiotics, including linezolid, but not tedizolid (Long e Vester 2012). The gene encodes a rRNA methyltransferase that methylates an adenosin in the antibiotic target site (Table 3) (Liu et al. 2012). Mutations in *rplC* and *rplD*, encoding the riboproteins L3

and L4 respectively, are predominantly linked to linezolid resistance in enterococci that lack the G2576T mutation and that do not express the multidrug resistance gene *cfr* (Greene et al. 2018; Pogue et al. 2007). In addition, a plasmid-mediated linezolid resistance has also been described. The plasmid-borne *optrA* gene confers resistance via active drug efflux in enterococci (Brenciani et al. 2016; Y. Wang et al. 2015). The novel gene *poxtA*, encoding an ABC-transporter protein subfamily (PoxtA), seems to be involved in reduced susceptibility to both linezolid and tedizolid in Gram-positive cocci, including *E. faecalis*, and it is associated with a putative mobile element that may contribute to its horizontal dissemination (Table 3) (Antonelli et al. 2018).

Linezolid resistance have been described in both vancomycin resistant and vancomycin susceptible *E. faecium* and *E. faecalis* (Gómez-Gil et al. 2009; Gonzales et al. 2001; Hegstad et al. 2014; Herrero, Issa, e Patel 2002; Ntokou et al. 2012; O'Driscoll e Crank 2015; Rahim et al. 2003). Several national and international surveillance epidemiological networks exist that monitor linezolid resistance (Bender et al. 2018). A recent review confirms the overall low prevalence (<1%) of linezolid resistance among clinical strains of *Enterococcus* spp. (Bi et al. 2018). The occurrence of resistance mechanisms was also analysed, demonstrating the presence of *cfr* in 4.7% of linezolid resistant strains (Bender et al. 2018). European reference laboratories have also reported outbreaks of linezolid resistant isolates (Bender et al. 2018). The international "Zyvox Annual Appraisal of Potency and Spectrum" (ZAAPS) program, assessing linezolid resistance in 32 countries, reported a single *E. faecium* isolate in Italy with the known G2756 T mutation in 2015 (Mendes et al. 2018; Pfaller et al. 2017). No linezolid resistance were detected in Italy between 2012 and 2014 (Stefani e Dowzicky 2016). The national italian surveillance system of antibiotic resistance AR-ISS has reported linezolid resistance in 0.6% (2015) and 0.3% (2016) of *E. faecalis* isolates from bloodstream infections (EpiCentro 2019).

2.2.7 Tetracyclines and glycylcyclines

Tetracyclines inhibit protein synthesis by interfering with the binding of the aminoacyl tRNA to the A site of the 30S subunit (Table 3) (Kristich et al. 2014; Speer, Shoemaker, e Salyers 1992). Tetracycline resistance is one of the most common phenotypes in enterococci. Clinical use of tetracycline is declining, but minocycline and glycylcyclines (*i.e.* tigecycline) are still used. Two major groups of *tet* genes have been recognized (Rice 1998). The first group includes the chromosomal resistance determinants *tetM*, *tetO* and *tetS*, which encode three

ribosomal protection proteins (RPPs) (Pepper et al. 1987). These genes confer resistance to doxycycline and minocycline as well as to tetracycline, and can be transferred by the Tn*916* transposon (Manganelli et al. 1995; Miller et al. 2014; Pepper et al. 1987). Bacteria carrying the *tet* gene produce RPPs that allow the ribosomes to continue the protein synthesis even in the presence of high intracellular concentration of tetracyclines (Chopra e Roberts 2001). The second group includes *tetK* and *tetL* plasmid genes, that encode the "Major Facilitator Superfamily" (MFS) efflux pumps that confers resistance to tetracycline but not to minocycline (Table 3) (Chopra e Roberts 2001).

Tigecycline is a glycylcycline antibiotic derived from minocycline (Pankey 2005). Its high antimicrobial activity is due to the binding to both 30S and 70S ribosomes with 5-100 fold higher affinity than tetracyclines (Bergeron et al. 1996; Olson et al. 2006). Despite low rates of tigecycline resistance, its use is limited by pharmacokinetics and tolerability (Pontikis et al. 2013). Glycylcyclines are poorly affected by the genetic determinants (MFS efflux pumps and RPPs) conferring resistance to tetracyclines (Grossman 2016; Thaker, Spanogiannopoulos, e Wright 2010). Clinically, tigecycline resistance is mainly described in MDR Gram-negative bacteria (Sun et al. 2013). However, Cattoir *et al.* have recently described *E. faecium* strains with reduced susceptibility to tigecycline (Cattoir et al. 2015).

2.2.8 Rifampicin

Rifampicin inhibits bacterial growth by binding to the β-subunit of the RNA polymerase RpoB subunit, resulting in inhibition of transcription (Wehrli et al. 1968). Mutations in the *rpoB* gene reduce affinity of rifampicin for the polymerase and are responsible of rifampicin resistance (Kristich et al. 2014). Rifampicin is a well-known antibiotic used against *Mycobacterium tuberculosis*, but it is also widely used for treating infections caused by different Gram-positive bacteria, including *Staphylococcus* spp. (Table 3) (Aubry-Damon, Soussy, e Courvalin 1998). Rifampicin has not been extensively used to treat enterococcal infections because acquired resistance is quite common since the early 2000's (Hollenbeck e Rice 2012). Old reports described that 57% of enterococcal isolates collected in Europe were rifampicin resistant (Andrews et al. 2000).

2.2.9 Quinolones

Quinolones interfere with bacterial DNA replication, specifically by binding to the type IV topoisomerase and DNA gyrase. Enterococci present low levels of intrinsic resistance, but can acquire high-level resistance through different mechanisms (Table 3) (Miller et al. 2014; Pham, Ziora, e Blaskovich 2019). Quinolone resistance occurs through mutations in the "quinolone resistance determining regions" of the genes *gyrA* and *parC* that encode subunit A of gyrase and subunit C of topoisomerase IV, respectively (Hooper e Jacoby 2015; Kanematsu et al. 1998). These mutations lead to reduced drug binding to the enzyme-DNA complex (Hooper e Jacoby 2015). Another resistance mechanism is the efflux through MDR efflux pumps. The genome of *E. faecalis* V583 encodes 34 different MDR efflux pumps (Jonas, Murray, e Weinstock 2001). Two of these pumps, EmeA and EfrAB, play a major role in quinolone resistance (Lee et al. 2003). A third mechanism of resistance, found only in *E. faecalis*, is mediated by the *qnr* gene. The presence of the Qnr protein protects the DNA gyrase by decreasing binding of the quinolone to DNA (Table 3) (Arsène e Leclercq 2007). Despite the MICs of fluoroquinolones in *E. faecalis* are close to the drug concentrations achievable in plasma, fluoroquinolones, especially ciprofloxacin, are commonly used drugs to treat enterococcal UTIs (Rattanaumpawan et al., 2011; Yasufuku et al., 2011).

2.2.10 Trimethoprim-Sulfamethoxazole

The antibiotic combination trimethoprim-sulfamethoxazole (SXT) blocks two sequential steps in the tetrahydrofolate synthesis pathway, thereby inhibiting folate synthesis (Kielhofner 1990). *Enterococcus* spp. is SXT-susceptible when *in vitro* susceptibility testing are performed in culture media without an exogenous folate source, but the treatment is ineffective in clinical practice (Mercuro et al. 2018; Zervos e Schaberg 1985). Enterococci are able to obtain folate from the environment and do not require *de novo* folate synthesis. Hence, they are intrinsically resistant to SXT (Hollenbeck e Rice 2012).

2.2.11 Others

Fosfomycin and nitrofurantoin are two drugs with *in vitro* high bactericidal activity against enterococci.

Fosfomycin is an inhibitor of the MurA enzyme, that catalyzes the first committed step in peptidoglycan synthesis, namely the reaction of UDP-N-acetylglucosamine (UDP-GlcNAc) with phosphoenolpyruvate to form UDP-GlcNAc-enoylpyruvate and inorganic phosphate (Michalopoulos, Livaditis, e Gougoutas 2011; Silver 2017). Fosfomycin resistance can be mediated by the transposon Tn*1546* that contains both the VanA operon and the *fosB* gene (Sun et al. 2017). FosB is a Mn^{2+}-dependent fosfomycin-inactivating enzyme that catalyzes nucleophilic addition of either L-cysteine (L-Cys) or bacillithiol to the antibiotic, resulting in a modified compound with no bactericidal properties (Thompson et al. 2014, 2015). Mutations in the *murA* gene have been shown to reduce its affinity for fosfomycin (Table 3) (Falagas et al. 2016).

Nitrofurantoin is converted by bacterial nitroreductases to electrophilic intermediates, which inhibit the citric acid cycle as well as the synthesis of DNA, RNA and protein (McOsker e Fitzpatrick 1994). The drug is used to treat *E. faecalis* UTI (Heintz et al. 2010). Zhanel *et al.* have shown that nitrofurantoin is active against *vanA*- and *vanB*-carrying isolates of *E. faecium* and *E. faecalis* (Zhanel, Hoban, e Karlowsky 2001). Therapy with nitrofurantoin rarely selects bacterial resistant strains because it acts on many different targets. However, there are few studies in *E. coli* describing nitrofurantoin resistance (Guay 2001; McOsker e Fitzpatrick 1994; Zhanel et al. 2001). In *E. coli*, mutation of the *nfsA* and *nfsB* genes, encoding nitroreductase enzymes, is the main mechanism leading to nitrofurantoin resistance (Shanmugam, Esak, e Narayanaswamy 2016). In addition, overexpression of OqxAB that encode an efflux pump may contribute to nitrofurantoin resistance (Zhang et al. 2018).

3. COUPLE INFERTILITY AND BACTERIAL INFECTIONS

Clinical infertility is defined by the World Health Organization (WHO) as a disease of the reproductive system caused by the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse (Zegers-Hochschild et al. 2009). Infertility is estimated to affect approximately 9-12% of reproductive aged couples worldwide with peak rates till 30% in certain geographic areas (Inhorn e Patrizio 2015).

The causes of infertility can be attributed to pathological conditions affecting one or both partners (Pellati et al. 2008). However, a large number of infertility cases still remain unexplained. In women, infertility factors are endocrine, vaginal, cervical, uterine, tubal and pelvic-peritoneal (Pellati et al. 2008). In men, reduced fertility can be caused by congenital or acquired urogenital, genetic, and/or immunological/inflammatory factors, such as infections of the male accessory glands, increased scrotal temperature, endocrine disorders, genetic abnormalities (Fraczek e Kurpisz 2015). In 40-50% of infertile couples, abnormal semen parameters are considered as the major factor associated to male infertility (Esteves e Chan 2015; Farsimadan e Motamedifar 2020) However, the causes of male infertility remain unknown in about 50% of men (Brugo-Olmedo, Chillik, e Kopelman 2001).

The introduction of Assisted Reproduction Technologies (ART) has provided an opportunity to improve basic reproductive processes (Brugo-Olmedo et al. 2001; Moragianni et al. 2019). ART is defined as the set of procedures that include *in vitro* handling of human oocytes and sperm cells or embryos to establish a pregnancy (Rubino et al. 2016). Among the *in vitro* fertilization (IVF) approaches, the intra-cytoplasmic sperm injection (ICSI) procedure represents the most commonly used ART treatment (70-80%) because it allows to overcome male factor infertility and also advanced maternal age (Rubino et al. 2016).

Infections of the urogenital tract affecting one or both partners may participate in infertility. Many sexually transmitted diseases (STDs) can be directly or indirectly associated with infertility. STDs represent a significant problem in reproductive health and are considered a potential threat to fertility (Günyeli et al. 2011; Ochsendorf 2008). Many studies have shown that infections of the reproductive tract may impair reproductive function in both men and women (Moragianni et al. 2019; Ruggeri et al. 2016). Among the pathogens that have been long

recognised to impair fertility are *Neisseria gonorrhoeae* and *Chlamydia trachomatis* (Rowley et al. 2019).

3.1. Impact of bacterial infections on female fertility

Alterations of the human microbiota play an important role in reproductive health (Bracewell-Milnes et al. 2018; Kalia, Singh, e Kaur 2020). The collection of microorganisms that live on or in the human body constitutes the microbiota that actively interacts with the human host, both beneficially as well as deleteriously (Cho e Blaser 2012). An increasing number of studies is highlighting a correlation between infertility and vaginal microbiota. It has been described that infertile women host a different microbiota in the lower and/or upper reproductive system compared to fertile women (Tomaiuolo et al. 2020).

Lactobacillus spp. has been identified as the most abundant genus throughout the female reproductive system (Franasiak e Scott 2017; Kyono et al. 2018; Miles, Hardy, e Merrell 2017; Mitchell et al. 2015; Moreno et al. 2016; Ravel et al. 2011; Smith e Ravel 2017). In particular, *Lactobacillus crispatus*, *Lactobacillus gasseri*, *Lactobacillus iners* and *Lactobacillus jensenii* are the most represented species in the vaginal tract (Ravel et al. 2011; Younes et al. 2018).

Lactobacilli contribute to the maintenance of the health status, and alterations in their quantity and quality have been associated with different gynaecological disorders (Moreno et al. 2016). Indeed, lactobacilli have been reported to exert protective effects towards pathogens invasion of the vaginal mucosa, whereas vaginal lactobacilli dysbiosis has been related to several diseases, including chronic endometritis, endometriosis, pelvic inflammatory disease and gynaecological cancers (Kyono et al. 2018; Tomaiuolo et al. 2020). Predominance of this genus seems to be age-dependent and strictly related to the reproductive age: in childhood *E. coli* and anaerobes predominate, while *Lactobacillus* spp. colonization begins in puberty throughout the female reproductive life until they decrease after menopause (Gliniewicz et al. 2019; Tomaiuolo et al. 2020). *Lactobacillus* spp. play an important role in the maintenance of vaginal homeostasis, as underlined by the fact that their depletion has been associated to several diseases (Younes et al. 2018). Lactobacilli are the main component of the vaginal microbiota that maintain homeostasis by different direct and indirect anti-pathogenic mechanisms, including the formation of microcolonies that create a physical barrier against pathogen colonization and/or immune activation against pathogens (Aldunate et al. 2015; Tachedjian, O'Hanlon, e Ravel 2018). The

production of lactic acid, by lowering the pH, is one of these protective mechanisms and is able to hamper replication of several pathogens. In addition, hydrogen peroxide has also been shown to damage vaginal pathogens and may also increase pathogen sensitivity to antibiotics (Tachedjian et al. 2018). The reduction of vaginal lactobacilli is correlated with the increase of anaerobic microorganisms. Modifications of the vaginal flora can lead to the production of amino compounds and higher vaginal pH, thus creating an environment more susceptible to pathogen infections and unhealthy conditions that may result in negative reproductive outcomes (Tomaiuolo et al. 2020). To strengthen this concept, studies showed that the presence of certain *Lactobacillus* species during ART procedures may increase implantation rates (Figura 7) (Eckert et al. 2003; Mangot-Bertrand et al. 2013; Moore et al. 2000).

Bacterial vaginosis (BV) is a vaginal microbiota disorder occurring when the normal *Lactobacillus* flora is reduced and replaced by mostly anaerobic microorganisms. BV is regarded as a dysbiosis of the vaginal microbiota and is associated to a heterogeneous cluster of pathogens rather than a single etiologic agent. The list of BV agents continues to enlarge and includes *Gardnerella vaginalis*, *Atobopium vaginae*, *Mycoplasma hominis*, and different species of *Prevotella*, *Porphyromonas*, *Mobiluncus*, *Sneathia*, *Peptoniphilus*, *Anaerococcus* and *Clostridium* (Onderdonk, Delaney, e Fichorova 2016). Recent epidemiological evidence indicate that BV may be sexually transmitted, suggesting that the male partner may serve as a reservoir for infection and reinfection (Unemo e Jensen 2017). Several studies have reported that BV is prevalent among infertile women, especially those with infertility due to tubal/pelvic factors (Haahr et al. 2016; T. Haahr et al. 2019; Thor Haahr et al. 2019; van Oostrum et al. 2013). In contrast, aerobic vaginitis (AV) has been described as an inflammatory condition in which a *Lactobacillus*-based microbiota shifts to a microbiota dominated by enterobacteria, staphylococci, streptococci and enterococci (Donders et al. 2017). Both disorders of the normal vaginal microbiota have been associated to increased risk of miscarriage and preterm birth (Haahr et al. 2016; van Oostrum et al. 2013; Ralph, Rutherford, e Wilson 1999).

Infections of the female genital tract may affect the vagina, the cervix, the uterus, or the tubal/pelvic area. Ascending infections are considered the most relevant for infertility as they can cause pelvic inflammatory disease and salpingitis which can eventually lead to tissue adhesions and tubal damage (Pellati et al. 2008). The impact on fertility of *C. trachomatis*, *N. gonorrhoeae* and *Ureaplasma urealyticum* has been well established (Akande et al. 2010; Casari et al. 2010;

Chemaitelly et al. 2020; Fenkci, Yilmazer, e Aktepe 2002; Imudia et al. 2008; Piscopo et al. 2020; TSEVAT et al. 2017; Walker e Sweet 2011). In women, *C. trachomatis* infection may cause pelvic inflammatory disease (PID), tubal occlusion and extra uterine pregnancy. *C. trachomatis* causes 25% of total tubal infertility, it is responsible for about 60% of acute salpingitis in young women (Paavonen e Eggert-Kruse 1999) and can cause chronic pelvic pain and ectopic pregnancies (Zanetti et al. 2007). *N. gonorrhoeae* and *G. vaginalis* can also cause PID (Mastromarino et al. 2014; Ruggeri et al. 2016). Kaur *et al*. suggested that the presence of *S. aureus* and *E. coli* in Balb/c mouse vaginal tract may play a negative role in fertility outcome (Kaur e Prabha 2014). *E. coli* may contribute to infertility, as its clearance from vagina resulted in fertility (Kaur e Prabha 2014). Furthermore, the presence of bacterial pathogens in the vagina is often accompanied with the release of proinflammatory cytokines, and a correlation between elevated IL-1 beta, IL-8 and idiopathic infertility has been suggested (Mastromarino et al. 2014; Ruggeri et al. 2016). Recent evidence indicate that the inflammation induced by symptomatic (and possibly asymptomatic) vaginal infections disturb the reproductive functions (Bracewell-Milnes et al. 2018; Peymani e DeCherney 2016; Ricci et al. 2018).

3.2 Impact of bacterial infections on male fertility

Abnormal semen parameters are found in approximately 50% of male partners subjected to ART treatment (Altmäe, Franasiak, e Mändar 2019; Zhang, Dai, e Chen 2021). As in the case of the female genital microbiota, also in the male, fertility requires a healthy urogenital tract for adequate sperm production and function. Male infections concern about 15% of total male infertility (Ko, Sabanegh, e Agarwal 2014; Kumar e Singh 2015; Pellati et al. 2008). Several studies indicated that the presence of bacteria in semen should not be considered as commensal flora, but instead as a sign of an urogenital infection (Altmäe et al. 2019; Jarvi et al. 1996; Kiessling et al. 2008). On the contrary, other studies suggest that the microorganisms harvested from semen are just contaminants from the urethral microbiota (Hou et al. 2013; Monteiro et al. 2018; Weng et al. 2014). Some studies reported that bacteriospermia is negatively associated with fertility (Fraczek e Kurpisz 2015; Ricci et al. 2018). In contrast, Rodin *et al*. described that bacteria are also present in semen of fertile individuals with normal sperm parameters (Rodin et al. 2003).

Inflammatory processes and bacterial infections have been associated with male infertility, but the exact mechanisms behind remain not totally understood. A chronic infection with mild inflammation may have more negative consequence on sperm function and spermatogenesis than acute infections. Pathogens that chronically colonize the male urogenital tract could affect semen concentration or motility and induce sperm apoptosis (Baud et al. 2019; Kaur e Prabha 2014; Stojanov et al. 2018). Different bacteria have been isolated from the genital tract of fertile and infertile men. *C. trachomatis*, *U. urealyticum*, *Ureaplasma parvum*, *Mycoplasma genitalium*, *M. hominis*, *E. coli*, E. *faecalis*, *S. aureus*, *Helicobacter pylori*, *Streptococcus agalactiae*, *G. vaginalis*, *Anaerococcus* spp., *N. gonorrhoeae*, and *Pseudomonas aeruginosa* are among the most commonly isolated bacteria, which can affect semen quality and quantity, thereby interfering with male fertility (Beeton, Payne, e Jones 2019; Farsimadan e Motamedifar 2020; Ochsendorf 2008; Qing et al. 2017). *C. trachomatis* and *E. coli* are the most prevalent species. *C. trachomatis*, the most common agent of non-gonococcal urethritis, may cause testicular atrophy, epididymitis, orchitis and ejaculatory ductal obstruction (Figure 8) (Cunningham e Beagley 2008; Stojanov et al. 2018).

Finally, bacteria in semen can also contaminate the female genital tract during ejaculation causing gynaecological diseases, such as vaginitis, endometritis, cervicitis, ectopic pregnancy and also miscarriages (Enwurua et al. 2016; Oghbaei et al. 2020).

3.3 Role of *Enterococcus faecalis* in infertility

Bacteria of the gut microbiota, such as *E. coli* and *E. faecalis*, may be involved in the pathogenesis of different urogenital disorders (Oghbaei et al. 2020). *E. faecalis* can cause urogenital infections that may be associated to male infertility. Despite the role of Gram-positive bacteria in sperm damage has been poorly investigated, *E. faecalis* has been associated with poor semen quality (Qiang et al. 2007). The presence of *E. faecalis* in semen has been associated with reduced sperm motility and concentration, and also altered sperm morphology (Mehta et al. 2016; Moretti et al. 2009; Ricci et al. 2018). The haemolysin produced by *E. faecalis* has been shown to damage the membrane of human sperm head, neck and midpiece (Qiang et al. 2007; Rodin et al. 2003), (Figure 8). On the contrary, Vilvanathan *et al.* did not show any detrimental effect of asymptomatic infection with *E. faecalis* on sperm parameters, despite it was the most commonly isolated microorganism from semen samples (Farsimadan e Motamedifar 2020;

Vilvanathan et al. 2016). A large body of evidence has shown that *E. faecalis* was frequently isolated from semen samples of infertile patients (Filipiak et al. 2015; Lackner et al. 2006; Parida 2019; Vilvanathan et al. 2016; Zeyad et al. 2018), suggesting that it may represent a contributing cause of infertility (Rodin et al. 2003; Ruggeri et al. 2016).

There is no direct evidence of the influence of *E. faecalis* on female fertility, although *E. faecalis* belongs to the microbial species involved in AV (Donders et al. 2017; García-Velasco, Menabrito, e Catalán 2017). Jahić *et al.* reported that *E. faecalis* was often associated with the presence of all the three signs of bacterial vaginosis, namely pH > 4.0, changed colour of vaginal secretions and positive amino smell test (Jahić, Nurkić, e Fatusić 2006).

4. TABLES AND FIGURES

Table 1. Virulence factors and their role in disease pathogenesis by *E. faecalis*

Virulence factor [a]	Gene	Function	Reference
Secreted			
Cyl	*cylL*$_{L}$, *cylL*$_{S}$	Cell membrane damage	van Tyne *et al*., 2013
GelE	*gelE*	Biofilm formation	Thurlow *et al.*, 2010
Fsr	*fsrABC*	Regulation of *gelE*	Hancock *et al.*, 2004
Cell surface associated			
LTA		Stimulation of inflammation	Baik *et al.*, 2008
Capsule	*cps*	Resistance opsonophagocytosis	Hangcock and Gilmore, 2002
Epa	*epa*	Biofilm formation	Mohamed *et al.*, 2004
		Resistance to neutrophil killing	Zeng *et al.*, 2004
		Invasion	
AS	*asa1*	Adherence to renal tubular cells	KreftB *et al.*, 1992
		Survival in macrophages	Sussmuth *et al.*, 2000
	asp1	Adherence to extracellular matrix	Shilievert *et al.*, 1998
	asc10	Invasion and survival in neutrophils	McCornick *et al.*, 2000
		Adherence to extracellular matrix	
Esp	*esp*	Biofilm formation	Tendolkar *et al.*, 2005
MSCRAMM	*ace*	Collagen (type I, IV) binding	Rich *et al.*, 1999
	ers	Repressor of *ace*	Nallapareddy *et al.*, 2011
	fss1, *fss2*, *fss3*	Fibrinogen binding	Sava *et al.*, 2010
EfbA	*efbA*	Collagen (type I, IV) and fibronectin binding	Singh *et al.*, 2015
		Biofilm formation	
Pili	*ebp, bee*	Biofilm formation	Madsen *et al.*, 2017

[a] LTA, Lipoteichoic acids; MSCRAMM, "Microbial Surface Components Recognizing Adhesive Matrix Molecules"

Table 2. Intrinsic antibiotic resistance in *E. faecalis*

Antibiotic	Mechanism of action	Mechanism of resistance[a]	Genetic bases of resistance		Reference
			Gene[b]	Function	
β-lactams					
Penicillins Cephalosporins Monobactams	Inhibition of peptidoglycan synthesis	Drug target modification	*pbp5 (+pbpA)*	Transglycosylase	Arbeloa *et al.*, 2004; Djoric´ *et al.*, 2019
Cephalosporins	Inhibition of peptidoglycan synthesis	Reduced drug entry	*ireB*	Ser/Thr kinase substrate	Hall *et al.*, 2013; Kristich *et al.*, 2007
			croRS	Sensor kinase and regulator	Hartmann *et al.*, 2010; Comenge *et al.*, 2003
Aminoglycosides	Inhibition of protein synthesis	Reduced drug entry (cell low permeability)	-	-	Aslangul *et al.*, 2006
MSL					
Macrolides Streptogramins	Inhibition of protein synthesis	Drug efflux	*msrA*	Efflux pump	Ross *et al.*, 1990
Lincosamides Streptogramins type a	Inhibition of protein synthesis	Drug efflux	*lsa*	Efflux pump	Singh *et al.*, 2002
Trimethoprim-Sulfametoxazole	Inhibition of folate synthesis	Use of exogenous folate	-	-	Hollenbeck *et al.*, 2012

[a] Low-level antibiotic resistance.
[b] Genes conferring resistance or being involved in regulation of antibiotic resistance.

Table 3. Acquired antibiotic resistance in *E. faecalis*

Antibiotic	Mechanism of action	Mechanism of resistance	Genetic bases of resistance		Reference
			Gene[a]	Function	
β-lactams					
Penicillins	Inhibition of peptidoglycan synthesis	Drug inactivation	*blaZ*	β-lactamase	Hackbarth, 1993
		Decreased affinity for PBPs	*pbp5*	Transglycosylase	Arbeloa *et al.*, 2004;
			(+pbpA)		Djoric *et al.*, 2019
Cephalosporins		Drug target modification	*murA*	UDP-N-acetylglucosamine enolpyruvyl transferase	Kristich *et al.*, 2012
Glycopeptides					
Vancomicin	Inhibition of peptidoglycan synthesis	Drug target modification	*vanB*, *vanG*	Ligase	O'Driscoll *et al*., 2015
Vancomicin Teicoplanin	Inhibition of peptidoglycan synthesis	Drug target modification	*vanA*, v*anD*, *vanE*, *vanL*	Ligase	O'Driscoll *et al*., 2015
Lipopeptides					
Daptomicin	Membrane depolarization	Drug efflux/drug target modification	*liaFSR*	Regulatory system	Suntharalingam *et al*. 2009
			yycF	Regulatory system	Palmer *et al.*, 2011
			cls	Cardiolipin synthetase	Palmer *et al*, 2011
			gdpD	Glycerophosphoryl diester phosphodiesterase	Arias *et al*., 2011
Aminoglycosides[b]					
Gentamicin	Inhibition of protein synthesis	Drug target modification	*aac(6')-aph(2')*	Aminoglycoside N-acetyltransferases/O-phosphotransferases	Chow *et al*., 2000
Gentamicin	Inhibition of protein synthesis	Drug target modification	*aph(2'')-Ic*	Aminoglycoside O-phosphotransferase	Chow *et al.*, 2000
Streptomycin	Inhibition of protein synthesis	Drug target modification	*ant(6')-Ia*	Aminoglycoside O-nucleotidyltransferase	Hollinshead *et al.*, 1985
Kanamycin	Inhibition of protein synthesis	Drug target modification	*rpsL*	Ribosomal protein S12	Finken *et al*, 1993
		Drug target modification	*aph(3'')-III*	Aminoglycoside O-phosphotransferase	Vakulenko and Mobashery, 2003
MSL					
Macrolides Lincosamides	Inhibition of protein synthesis	Drug target modification	*ermA/ermB*	23S rRNA methylase	Portillo *et al.*, 2000

Streptogramins					
Macrolides		Drug efflux	*mefA*	Efflux pump	Clancy *et al.*, 1996;
Lincosamides		Drug target modification	*lnuB*	Nucleotidyltransferase	Zhu *et al.*, 2016
Quinolones					
Ciprofloxacin	Inhibition of DNA replication	Drug target modification	*gyrA*	Gyrase A	Hooper and Jacoby, 2015
		Drug target modification	*parC*	Topoisomerase IV	Hooper and Jacoby, 2015
		Drug target modification	*qnr*	pentapeptide repeat protein	Arsène and Leclercq, 2007
Oxazolidons					
Linezolid	Inhibition of protein synthesis	Drug target modification	23S rRNA (G2576T, G2505A)	Ribosomal RNA	Bourgeois-Nicolaos *et al.*, 2007
		Drug target modification	*rplC*/*rplD*	Ribosomal protein	Chen *et al.*, 2013
		Drug target modification	*cfr*	rRNA methyltransferase	Long *et al.*, 2006
		Drug efflux	*optrA*	ABC-F transporter	Long et *al.*, 2006
			poxtA	ABC-F transporter	Antonelli *et al.*, 2018
Tetracyclines					
Tetracycline Doxycycline Minocycline	Inhibition of protein synthesis	Drug target modification	*tetM, tetO, tetS*	Ribosomal protection proteins	Pepper *et al.*, 1987; Miller *et al.* 2014
Tetracycline	Inhibition of protein synthesis	Drug efflux	*tetK, tetL*	Efflux pump	McMurry *et al.* 1987
Rifampicin	Inhibition of transcription	Drug target modification	*rpoB*	RNA polymerase β-subunit	Brian *et al.*, 2012
Fosfomycin	Inhibition of peptidoglycan synthesis	Drug target modification	*murA*	UDP-N-acetylglucosamine enolpyruvyl transferase	Falagas *et al.*, 2016
		Drug target inactivation	*fosB*	Metallothiol transferase	Thompson *et al.*, 2014

[a] Genes conferring resistance or being involved in regulation of antibiotic resistance.
[b] High-level resistance; resistance to gentamycin mediated by *aac(6')-aph(2')* also includes resistance to tobramycin, amikacin, kanamycin, netilmicin and dibekacin. Resistance to kanamycin mediated by *aph(3')-III* includes resistance to neomycin, lividomycin, paromomycin, livostamycin, butirosin, amikacin and isepamicin.

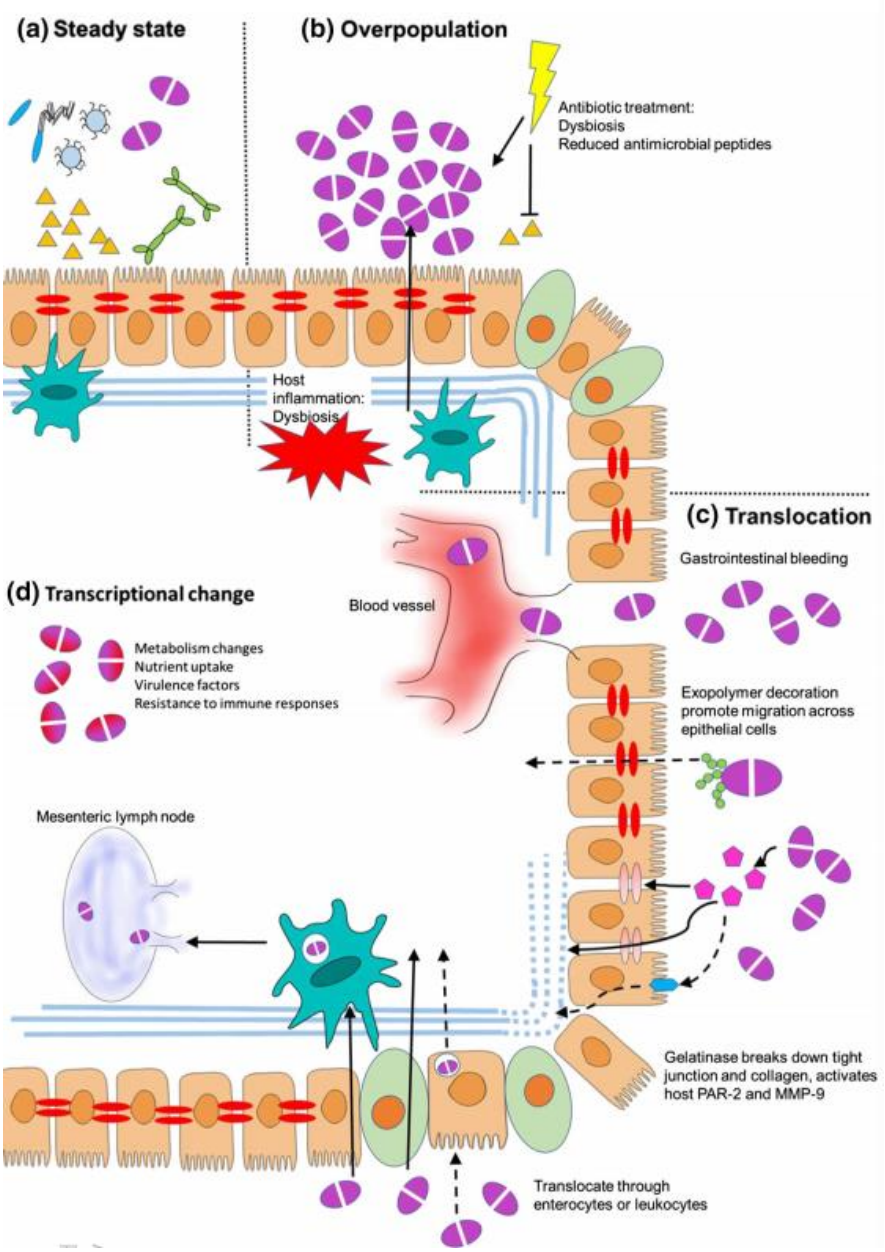

Figure 1. *E. faecalis* transition from commensal to pathogen. *E. faecalis* initially colonizes the human gut. Biofilm formation is crucial to colonize and persist in the gut mucosa (**a**). Different triggering factors, including antibiotic treatment and host inflammation, can alter the gut microbiota and promote enterococcal overgrowth (**b**) which may increase the risk for translocation across the intestinal barrier (**c**). Bacterial translocation is promoted by GelE, that degrades collagen in the basal membrane and activates host protease activated receptor-2 (PAR-2) and matrix metalloproteinase-9 (MMP-9) causing damage to the mucosa. *E. faecalis* can also directly invade the enterocytes. Following the invasion of the gut lamina propria, bacteria can access the blood leading to a systemic infection. Once in the bloodstream or deeper tissues, bacteria promote the production of virulence factors, including the capsule, Epa, GelE, cytolysin and AS (**d**). Figure modified from Kao and Kline, J Mol Biol 2019.

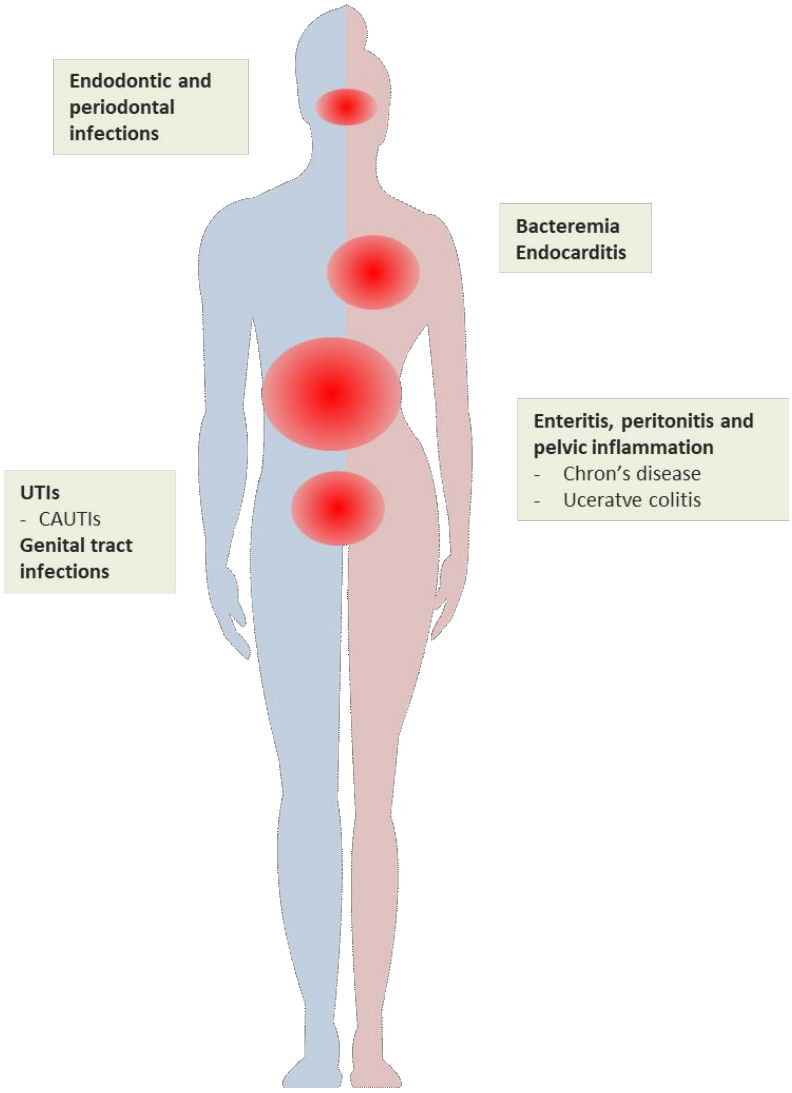

Figure 2. Clinical manifestations of *E. faecalis*. *E. faecalis* is involved in different inflammatory conditions of several body districts. The most common infections are the urinary tract infections (UTIs), in particular cystitis and prostatitis in the lower urinary tract and pyelonephritis in the upper urinary tract. *E. faecalis* can also cause genital infections and has been associated to couple infertility. Bacteremia and endocarditis are the most serious infections caused by *E. faecalis*. The most common route of entry into the blood is from the urinary and/or gastrointestinal tract. *E. faecalis* is also responsible of intra-abdominal, pelvic, and soft tissue infections. Moreover, *E. faecalis* can cause peritonitis and has also been associated with inflammatory ulcerative colitis and Chron's disease. In the oral cavity, *E. faecalis* is responsible of periodontal and endodontic infections. CAUTI, catheter-associated UTIs.

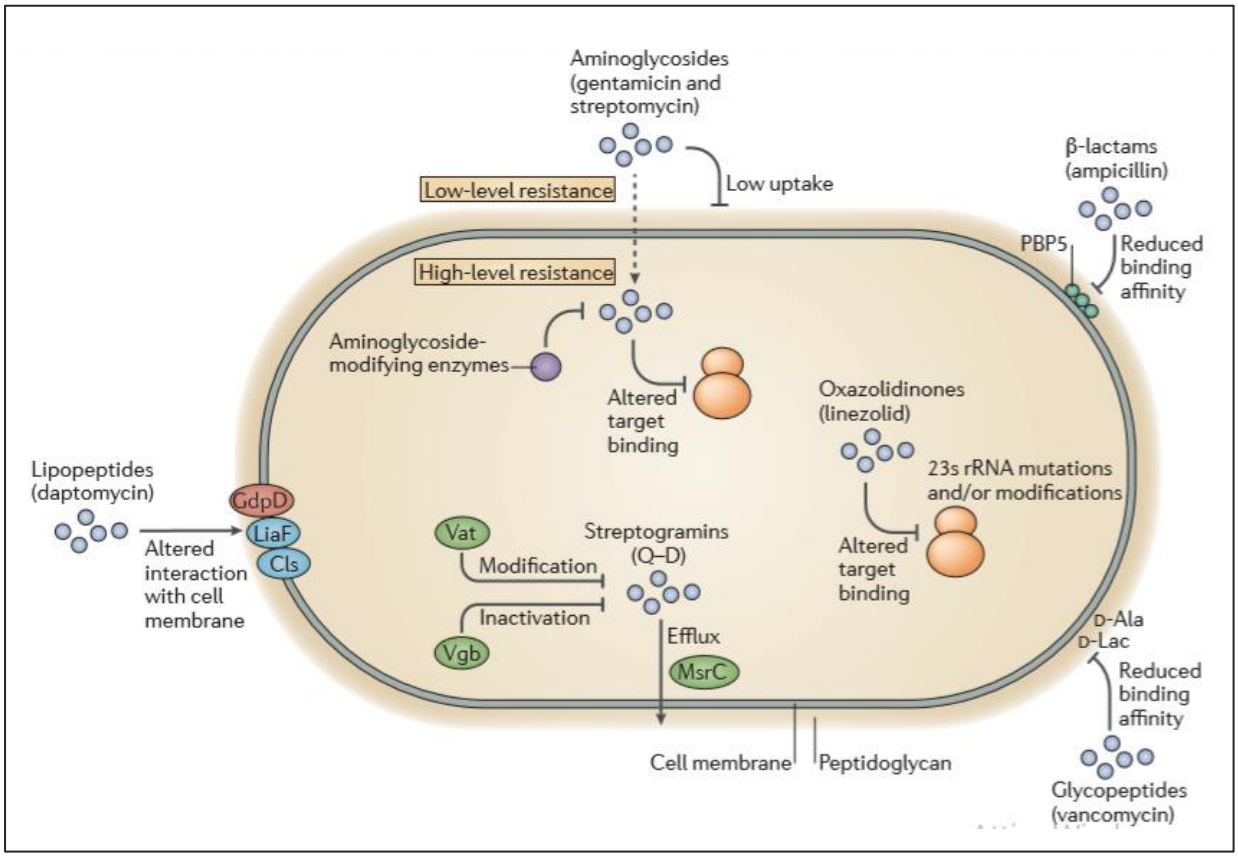

Figure 3. Main mechanisms of antibiotic resistance in enterococci. Several mechanisms of antibiotic resistance are present in *E. faecalis*: i) inhibition of peptidoglycan synthesis by reducing affinity binding to PBPs or peptidoglycan precursors (β-lactams, glicopeptides); ii) reduced uptake of antibiotic (low-level aminoglycoside resistance); iii) altered interaction with the cell membrane (lipopeptides); iv) drug modification/inactivation (streptogramins, β-lactams); v) drug target modification (high-level aminogycosides, oxazolidones, macrolides, tetracyclines); and vi) drug efflux pumps (macrolides, streptogramins, quinolones, oxazolidons, tetracyclines). Figure modified from Arias *et al.,* Nature 2012.

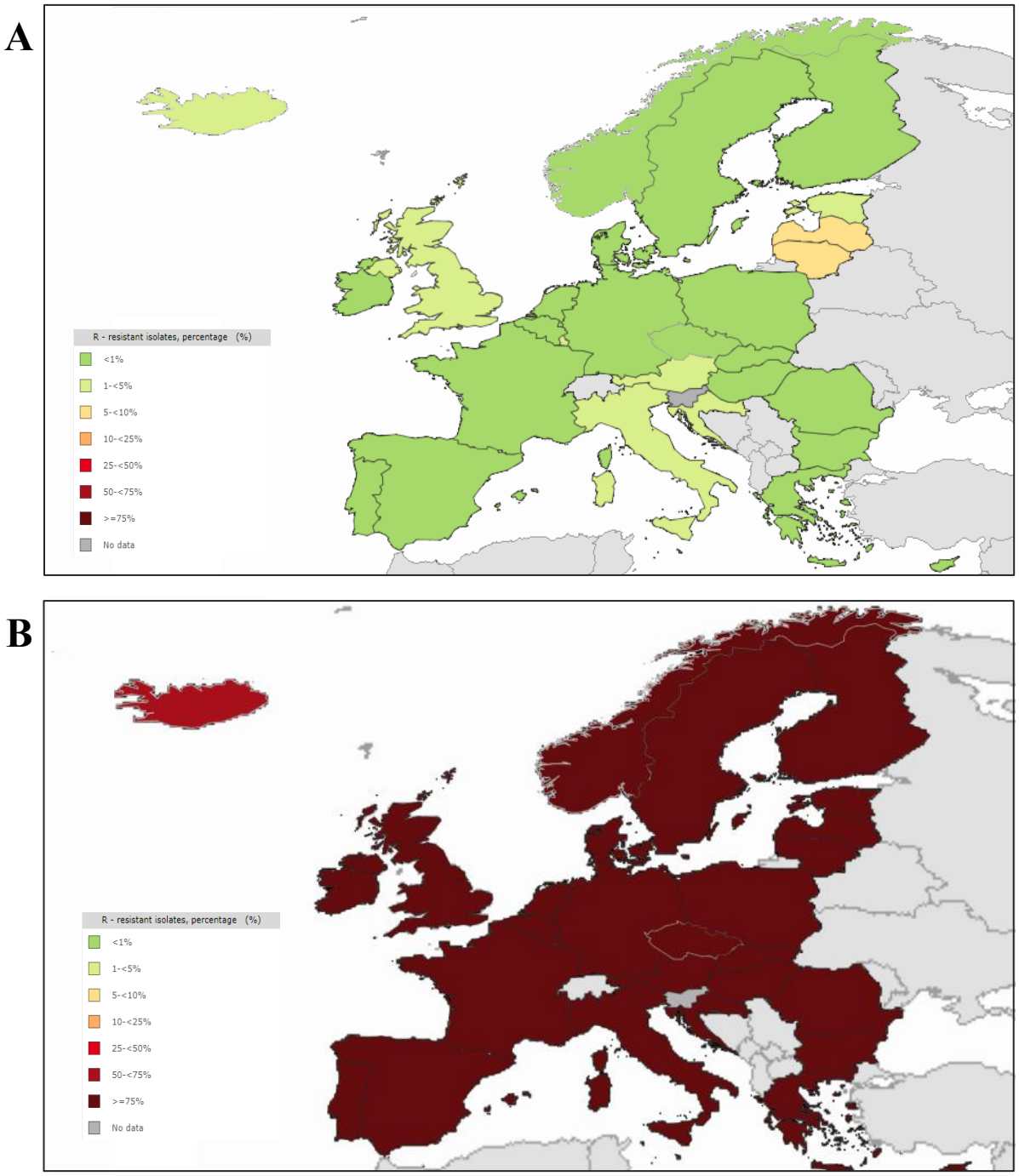

Figure 4. Incidence of ampicillin resistant *E. faecalis* and *E. faecium* in Europe in 2019. The rate of ampicillin-resistant *E. faecalis* strains in Italy is 2%. Lithuania is the country with the highest rate (9,8%) of ampicillin resistance in Europe. Ampicillin resistance in *E. faecium* (**B**) exceeds 75% in all countries. Maps modified from ECDC Surveillance Atlas of Infectious Diseases (https://www.ecdc.europa.eu/en/antimicrobial-resistance/surveillance-and-disease-data/data-ecdc).

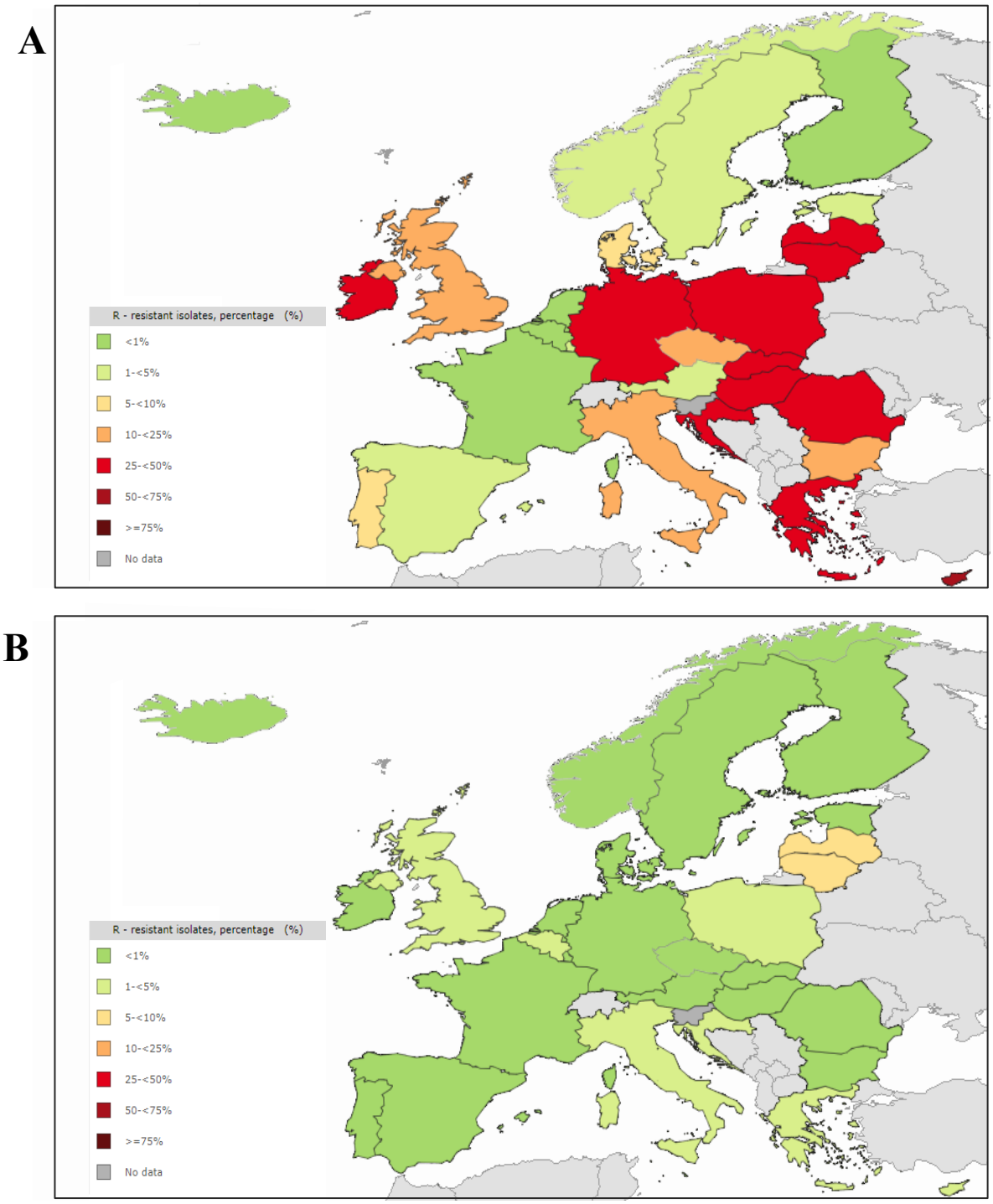

Figure 5. Incidence of vancomycin resistant *E. faecium* and *E. faecalis* in Europe in 2019. The EU/EEA population-weighted mean percentage of vancomycin resistance in *E. faecalis* (**B**) is low (0-2%) in most countries. In *E. faecium* isolates (**A**), vancomycin resistance is 18.3%. National percentages vary depending on the country, with VRE ranging from less than 1% in France, Spain, and Sweden to more than 20% in Italy, Greece, Ireland, Portugal and the UK. Maps modified from ECDC Surveillance Atlas of Infectious Diseases (https://www.ecdc.europa.eu/en/antimicrobial-resistance/surveillance-and-disease-data/data-ecdc).

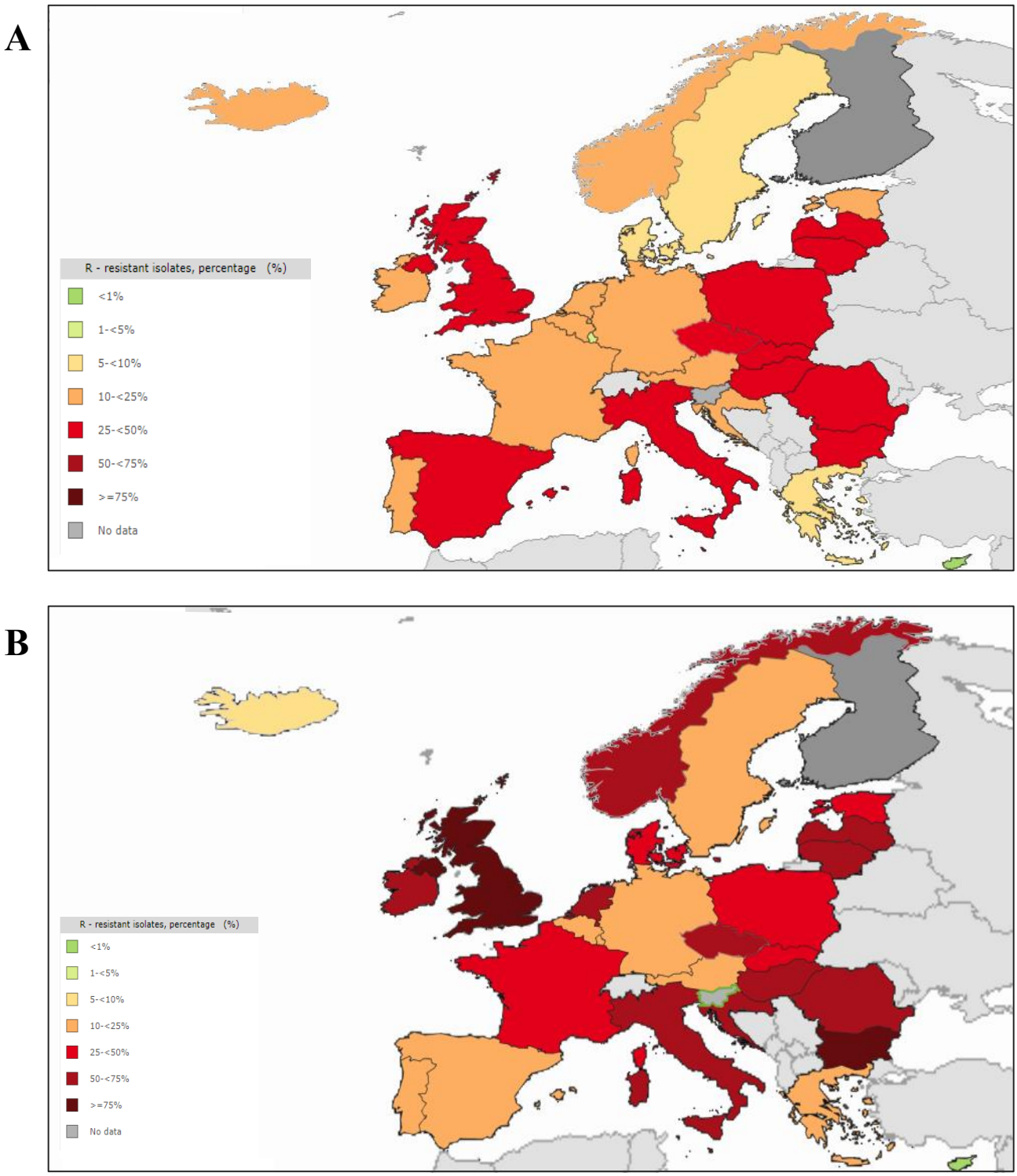

Figure 6. Incidence of high-level gentamicin resistant *E. faecalis* and *E. faecium* in Europe in 2019. The percentage of high-level gentamicin resistance in *E. faecalis* (**A**) was 26.6%, with national percentages ranging from 7.8% to 41.6%. In Italy, resistance (35.2%) is among the highest in Europe together with UK, Spain, Portugal and Eastern Europe. National percentages of high-level gentamicin resistance in *E. faecium* were higher than for *E. faecalis* (**B**). Maps modified from ECDC Surveillance Atlas of Infectious Diseases (https://www.ecdc.europa.eu/en/antimicrobial-resistance/surveillance-and-disease-data/data-ecdc).

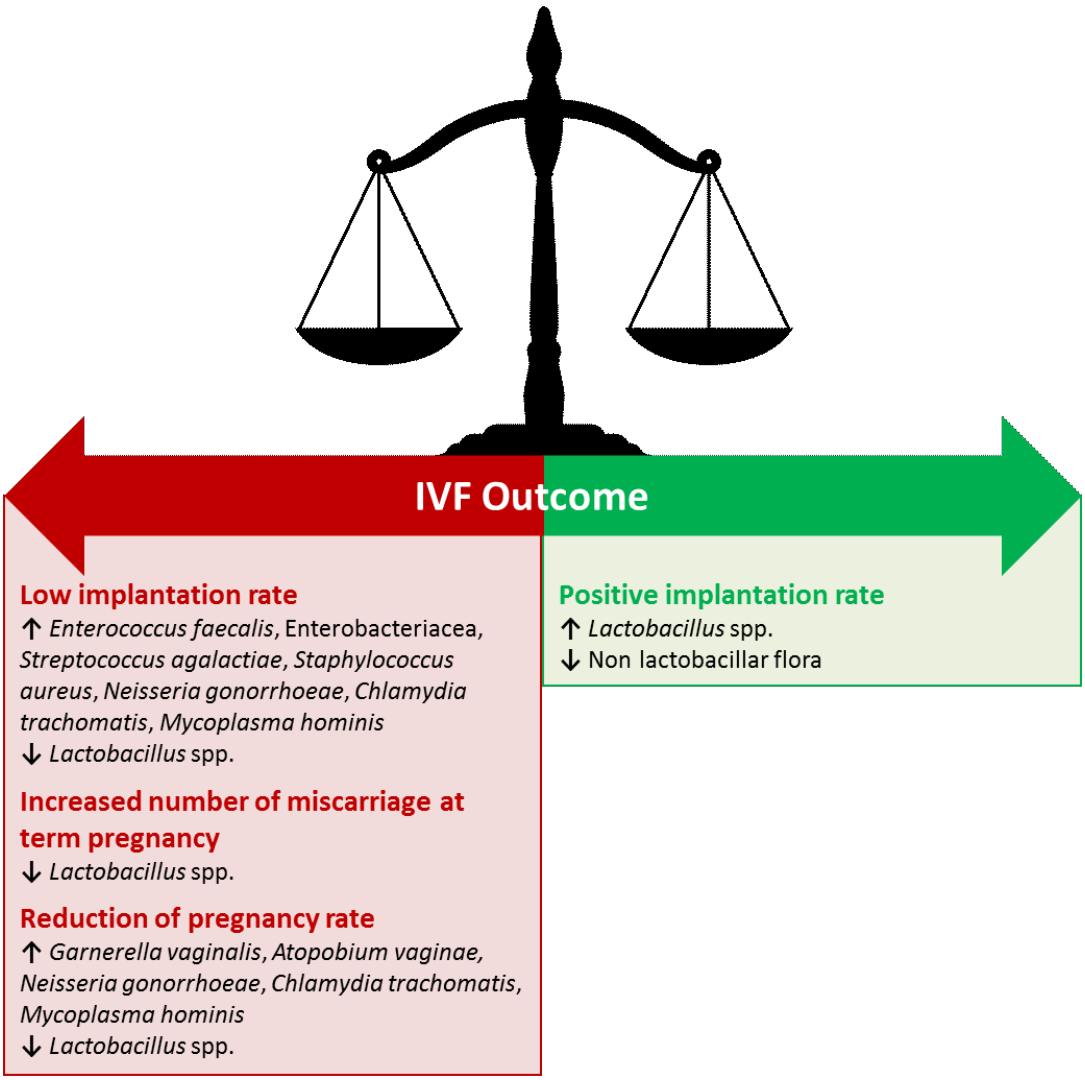

Figure 7. Protective and risk factors for the outcome of *in vitro* fertilization (IVF). The status of the female microbiota is important for IVF outcome. Disbyosis of the vaginal microbiota, bacterial vaginosis and/or aerobic vaginitis are considered as risk factors for a negative IVF outcome. Low implantation rates, increased numbers of miscarriages, and reduced pregnancy rates have been associated with the presence of different bacterial species (red arrow). *E. faecalis* has been shown to negatively impact on implantation rates after IVF. *Lactobacillus* spp. exerts beneficial effects and is regarded as a protective factor (green arrow). The figure is modified from Tomaiuolo *et al*., High-Throughput 2020.

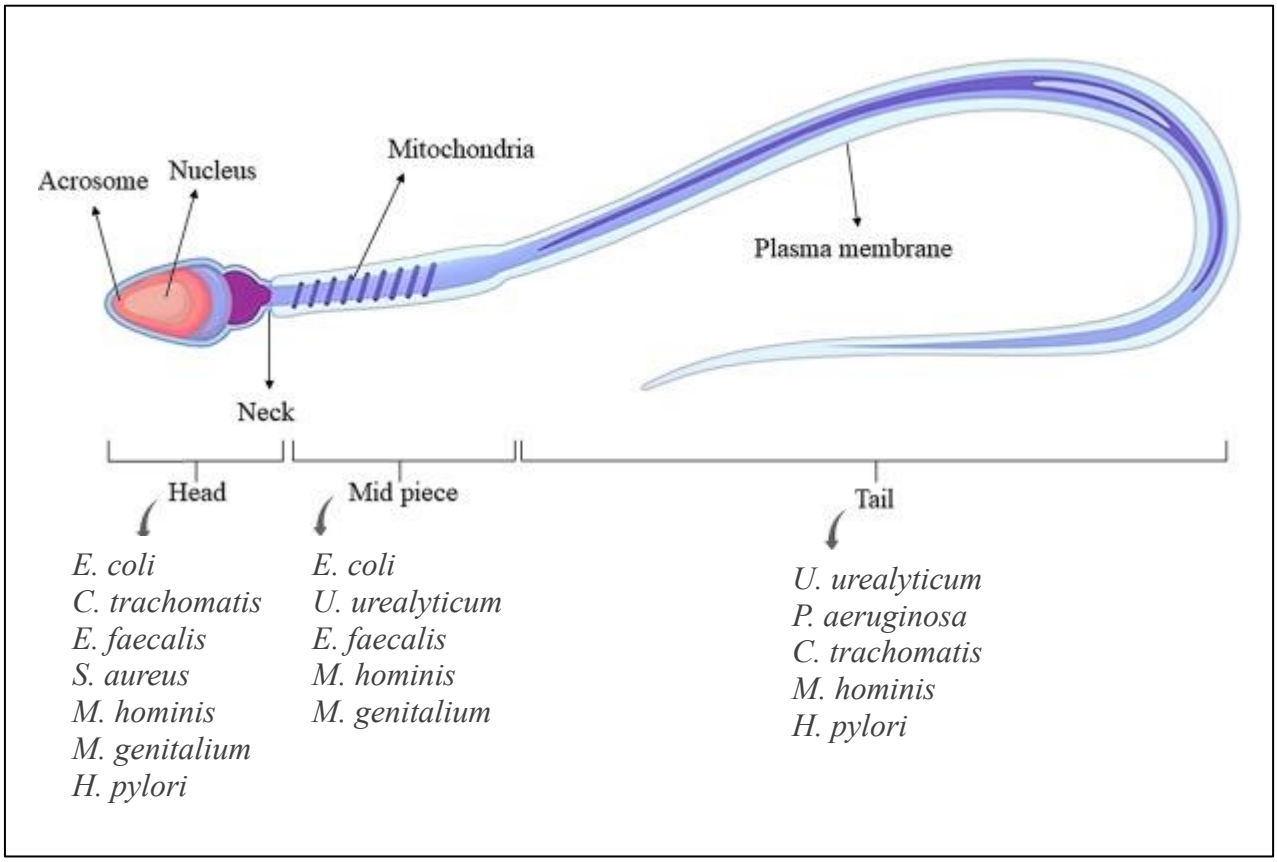

Figure 8. Impact of bacterial infections on the sperm cell. Different parts of the sperm cell are the target for bacterial infection. Several bacterial species that can preferentially damage the head, mid-piece and tail are indicated. *E. faecalis* has been shown to target the head and mid-piece of spermatozoa. The figure is modified from Farsimadan *et al.*, J Reprod Immunol 2020.

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CHAPTER 3

Impact of Asymptomatic Genital Tract Infections on *In Vitro* Fertilization (IVF) outcome

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RESEARCH ARTICLE

Impact of asymptomatic genital tract infections on *in vitro* Fertilization (IVF) outcome

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Abstract

Background

Infertility is estimated to affect approximately 9–30% of reproductive-aged couples. Several conditions involving one or both partners may contribute to infertility. The aim of this study is to evaluate the role of asymptomatic genital tract infections in the outcome of *In Vitro* Fertilization (IVF) in couples with infertility.

Methods

A total of 285 infertile couples were enrolled in the study. Vaginal/endocervical swabs and semen samples were collected and subjected to microbiological analysis. Spermiograms were carried out on semen specimens, and lactobacilli were quantified in vaginal swabs. Data were associated with IVF results and analysed by using non parametric tests and multivariate analysis.

Results

Microbiological analysis showed that 46.3% of couples presented with an asymptomatic genital tract infection. Spermiogram results showed a significantly diminished motility of sperm cells in samples positive to microbiological testing compared to negative specimens. *Enterococcus faecalis* was the most prevalent species (11.6%) in positive semen samples and was found to negatively affect both sperm morphology ($p = 0.026$) and motility ($p = 0.003$). Analysis of genital swabs from females showed that the presence of *E. faecalis* ($p<0.0001$), *Escherichia coli* ($p = 0.0123$), *Streptococcus agalactiae* ($p<0.0001$), and *Gardnerella vaginalis* ($p = 0.0003$) was significantly associated to reduced levels of vaginal lactobacilli. Association of microbiological data with IVF outcome showed that 85.7% of IVF+ couples was microbiologically negative, while IVF was successful in just 7.5% of couples infected with *E. faecalis* and/or *U. urealyticum* and/or *M. hominis* ($p = 0.02$).

Conclusions

The results show the negative impact of *E. faecalis* on sperm quality and the association of definite bacterial pathogens with reduced levels of vaginal lactobacilli. The presence of *E. faecalis* and/or *U. urealyticum* and/or *M. hominis* in genital samples of infertile couples is predictive for a negative outcome of IVF.

Introduction

Infertility is a medical condition that is appraised to affect between 9% to 30% of reproductive-aged couples worldwide [1]. In 2009, the International Committee for Monitoring Assisted Reproductive Technology (ICMART) and the World Health Organization (WHO) have defined 'infertility' as the failure to achieve a clinical pregnancy after 12 months of regular unprotected sexual intercourse [2]. Different pathological conditions affecting one or both partners may participate in infertility, including infections of the urogenital tract. Chronic or not appropriately treated infections are generally regarded as more critical for infertility compared to acute infections. Infections of the female genital tract may concern the vagina, the cervix, the uterus, or the tubal/pelvic area. Ascending infections are considered the most relevant for infertility as they can cause pelvic inflammatory disease and salpingitis which can eventually lead to tissue adhesions and tubal damage [3]. Male infections account for about 15% of total male infertility [3,4], leading to qualitative and quantitative sperm alterations [5]. Microbial pathogens present in semen can directly and indirectly impact on sperm quality and function [5]. Bacteriospermia may be accompanied by leukocytospermia, although its clinical relevance in male infertility is controversial [6–8].

Various genital pathogens have been implicated in infertility with different degrees of statistical significance. Infections caused by *Neisseria gonorrohoeae*, *Chlamydia trachomatis*, *Treponema pallidum*, human immunodeficiency virus (HIV), and mumps are relevant for infertility. In particular, *N. gonorrohoeae* can influence both male and female fertility, while *C. trachomatis* can affect sperm motility and viability, but it is particularly dangerous for the female where it can lead to tubal infertility [3,4,9]. Both Gram-positive and Gram-negative bacteria can alter sperm function. *Enterococcus faecalis* has been associated with oligozoospermia and teratozoospermia [5], whereas *Escherichia coli* has been shown to induce apoptosis in sperm cells and reduce their motility [5,10]. *Ureaplasma urealyticum* and *Mycoplasma hominis* are more commonly isolated from the genital tract of women and are considered as relevant for female infertility [3,11,12]. Nonetheless, both species were found to affect sperm motility and vitality [5,10,13]; additionally, *M. hominis* has been associated with abnormal sperm morphology [14] and *U. urealyticum* has been shown to damage nuclear chromatin with possible implications for embryo development [15]. On the contrary, neither *Mycoplasma genitalium* nor *Ureaplasma parvum* could be correlated to male infertility based on a recent meta-analysis [16], while *M. genitalium* has lately been appraised in relation to cervicitis and pelvic inflammatory disease [17].

The health status of the female genital tract is largely related to the presence of a normal vaginal microbiota [18]. The composition of the vaginal microbiota can profoundly influence all stages of female reproduction, starting from conception, throughout pregnancy until birth. Bacterial vaginosis (BV) is a vaginal microbiota disorder occurring when the normal flora, primarily composed of *Lactobacillus* spp., is reduced and replaced by mostly anaerobic microorganisms. BV is regarded as a dysbiosis of the vaginal microbiota and is associated to a heterogeneous cluster of pathogens rather than a single etiologic agent. The list of BV agents

continues to enlarge and includes *Gardnerella vaginalis*, *Atobopium vaginae*, *M. hominis*, and different species of *Prevotella*, *Porphyromonas*, *Mobiluncus*, *Sneathia*, *Peptoniphilus*, *Anaerococcus* and *Clostridium* [19]. Recent epidemiological evidence indicate that BV may be sexually transmitted, suggesting that the male partner may serve as a reservoir for infection and re-infection [20]. Several studies have reported that BV is prevalent among infertile women, especially those with infertility due to tubal/pelvic factors [21]. In contrast, aerobic vaginitis (AV) has been described as an inflammatory condition in which a *Lactobacillus*-based microbiota shifts to a microbiota dominated by enterobacteria, staphylococci, streptococci, and enterococci [22]. These disorders of the normal vaginal microbiota have been associated to increased risk of miscarriage and preterm birth [21,23–25].

Assisted Reproductive Technology (ART) consists of all procedures that include *in vitro* handling of human oocytes and sperm cells or embryos with the purpose of establishing a pregnancy [2]. Among the *in vitro* fertilization (IVF) approaches, introduction of the intracytoplasmic sperm injection (ICSI) procedure in the early 1990s represented a major breakthrough in reproductive medicine. ICSI, initially preferred to other techniques to overcome male factor infertility, is now also employed for advanced maternal age and idiopathic infertility. Currently, ICSI reckons for approximately 70–80% of total ART cycles, thus representing the most commonly used ART treatment [26].

Despite the fact that genital tract infections are recognized to affect human fertility, there are still no consensus guidelines available on the microbiological management of infertile couples undergoing IVF treatment. In the present study, 285 infertile couples were tested for the presence of asymptomatic infections of the genital tract before being subjected to IVF, and results were associated with the outcome of IVF.

Materials and methods

Patients

A total of 285 couples, consecutively attending for 3 years the Centre for Diagnosis and Treatment of Couple Sterility at Siena University Hospital, were enrolled in the study. All couples presented with fertility disorders and were subjected to different medical examinations prior to undergoing IVF procedures. Among the medical tests, the presence of genital tract pathogens in semen and vaginal/endocervical swabs and the quality of semen were evaluated. None of the couples had signs or symptoms of genital infection. Written informed consent was obtained from each patient. The local ethical committee CEAVSE (Comitato Etico Area Vasta Sud Est) approved conduction of the study.

Samples

Semen and vaginal/endocervical specimens were obtained about two months before IVF procedure. Semen was collected by masturbation after 3–5 days of sexual abstinence and subjected to spermiogram according to WHO guidelines [27]. Male patients were given instructions to perform semen collection after accurate genital hygiene and discard of urine first void. For each female patient, both a vaginal and an endocervical swab were collected using sterile cotton swabs (FL Medical, Padova, Italy). All samples were sent to the laboratory of clinical microbiology for analysis.

Detection and identification of cultivable pathogens

Standard bacteriological culture methods were used to detect genital pathogens from semen specimens and vaginal swabs. Semen was used directly upon arrival at the laboratory, while

vaginal swabs were immersed in 1 ml of saline solution (0.9% NaCl) for 15 min prior to testing. Vaginal swabs were employed to assess for the presence of vaginal pathogens along with lactobacilli (see below). Selective and differential solid media (Oxoid, Milan, Italy) were used for cultivable microorganisms, including gram-positive cocci, gram-negative bacteria, lactobacilli, anaerobes, and fungi. Species identification was carried out by using Matrix Assisted Laser Desorption Ionisation-Time Of Flight (MALDI-TOF) VITEK MS (Biomérieux Italia S.p.A., Florence, Italy) coupled with the Myla software v2.0 with a cut-off identification $\geq$ 99%. Semen samples were considered positive if bacterial concentrations were $\geq 5x10^3$ cfu/ml according to WHO guidelines recommending 10^3 cfu/ml as the cut-off value for 'significant bacteriospermia' [27,28]. Vaginal swabs were regarded as positive at viable counts $\geq 10^5$ cfu/swabs.

Vaginal lactobacilli

Quantitative analysis of lactobacilli in vaginal swabs was carried out on selective media for lactobacilli (Rogosa agar, Oxoid) and anaerobes (Schaedler agar, Oxoid). Presence of lactobacilli was regarded as 'normal' when cfu counts were $\geq 10^4$ cfu/swab, and 'low' at values $< 10^4$ cfu/swab. Assay detection limit was 10^2 cfu/swab.

Detection and identification of non-cultivable pathogens

Genital tract pathogens with fastidious growth requirements or non-cultivable, including *C. trachomatis*, *U. urealyticum*, *M. hominis*, *N. gonorrhoeae*, *Trichomonas vaginalis* and HSV, were searched out both in semen and vaginal/endocervical swabs by Real-Time PCR. Endocervical swabs were the specimen of choice for searching *U. urealyticum*, *C. trachomatis* and *N. gonorrhoeae*, whereas vaginal swabs were assessed for the presence of *M. hominis*, *T. vaginalis* and HSV. As abovementioned, swabs were immersed in 1 ml of saline solution (0.9% NaCl) for 15 min prior to analysis. All genital samples were heat-inactivated (85˚C for 10 min) and subjected to automated DNA extraction using the MagNA Pure LC DNA Isolation Kit III (Roche Diagnostics GmbH, Mannheim, Germany) and the MagNA Pure LC machinery (Roche Diagnostics) according to the manufacturer's instructions. For PCR reactions, 2 μl of DNA, 10 pmol of each primer (S1 Table) and 5 pmol of TaqMan probe (Roche Diagnostics) were used. Samples were transferred into a 96-multiwell plate (Roche Diagnostics), placed in the Light Cycler 480 (Roche Diagnostics) and programmed for 40 cycles of amplification (denaturation at 95˚C for 15 sec, annealing and extension at 60˚C for 1 min). Samples were considered positive with an average C_T value of 38. The initial target copy number in clinical samples was determined based on external standard curves specific for each microorganism. Standard curves generated by 10-fold dilutions of control DNA were linear over a range of 5 log units with an efficiency of 1.747 and a slope of -4.129. Detection limits were between 10 and 10^3 target copies/μl of sample, depending on the efficiency of primer pairs and Taqman probe and the quality of biological samples.

Semen analysis

Specimens were analysed according to WHO guidelines [27]. After liquefaction of the ejaculate, sperm concentration (number of sperm cells/ml), progressive and total motility were determined. Eosin Y test was used to detect necrotic sperm cells. Morphological examination of the specimens was carried out by counting 200 spermatozoa/sample and evaluating morphological abnormalities of sperm organelles (nucleus, acrosome, tail). Values of concentration, motility and morphology of sperm cells were considered altered if placed below the fifth percentile of the reference population [27]. Samples were evaluated as leukocytospermic from counts of 10^6 leukocytes/ml of semen, according to WHO [28]. Leukocytes in semen

specimens were counted by using the method of Politch [29]. Briefly, H_20_2 (0.0375%) was added to 4 ml of benzidine stock solution (0.0125% in 50% ethanol, w/v; Sigma-Aldrich, Milano, Italy). Ten μl of seminal fluid were mixed with 20 μl of freshly prepared benzidine-H_2O_2 solution. After 5 min, 160 μl of phosphate buffered saline (PBS) were added, and peroxidase-positive (round brown cells) and peroxidase-negative (unstained) cells were counted using a Mackler chamber and a phase-contrast microscope.

IVF procedure

Multiple follicle growth was obtained by using recombinant Follicle Stimulating Hormone (rFSH) at a dose of 150–300 IU based on the ovarian response as evaluated by hormone serum levels and ultrasound examination. As soon as the dominant follicle reached 14 mm in diameter, a Gonadotropin Releasing Hormone (GnRH) antagonist was administered daily. Ovulation was induced by injection of human chorionic gonadotropin (hCG) when at least three follicles of size > 16 mm were present in the ovaries. Oocyte pick-up was scheduled 34–36 h after hCG injection. Oocytes were fertilized by the ICSI procedure, and embryo transfer (ET) was performed 3–5 days after IVF. Serum levels of hCG were determined at day 14 post-ET, and the presence of a gestational sac was evaluated by transvaginal ultrasound examination at week 7 of gestation.

Statistical analysis

Statistical analysis was performed using the software GraphPad Prism 5.0. Analysis of microbiological results was carried out by evaluating each infectious agent as a distinct unit, except for *Candida* species and *Enterobacteriales* other than *E. coli* which were analysed as two microbial groups. Results of spermiogram and microbiological analysis of semen samples were analysed by using the Mann-Whitney test. The Chi square (χ^2) test (with Yates' correction or Fisher exact test) was employed to assess the association between the presence of genital pathogens and vaginal lactobacilli in vaginal/endocervical swabs. The association of genital tract pathogens in infertile couples with the outcome of IVF was analysed by using the χ^2 test (with Yates' correction or Fisher test). Multivariate analysis was chosen to identify single infectious agents or groups associated as independent risk factors to IVF outcome. A p value lower than 0.05 was regarded as significant.

Results

In this study, 285 infertile couples were tested for the presence of genital tract pathogens prior to undergoing IVF treatment. Microbiological analysis was conducted on a total of 855 genital samples, of which 285 semen specimens, 285 vaginal swabs and 285 endocervical swabs. A total of 195 clinical strains belonging to 25 different microbial species was detected in the samples tested (Table 1). *E. faecalis* represented the most common finding with a prevalence of 24.1% (47/195). Other frequently identified microbial species included *S. agalactiae* (15.9%), *E. coli* (15.4%), *M. hominis* (10.8%), *Candida* spp. (8.2%), and *U. urealyticum* (5.1%) (Table 1). Co-presence of two different pathogens was detected in 14 semen specimens and in 16 vaginal/endocervical swabs, while the simultaneous presence of 3 pathogens was observed in 3 samples. It should be noted that neither *C. trachomatis* nor *N. gonorrhoeae* were found in genital tract samples.

Prevalence and aetiology of genital tract infections in infertile couples

Microbiological results showed that 29.1% (83/285) of males and 26.3% (75/285) of females were found positive to at least one genital pathogen. A total of 132 couples (46.3%) was positive

Table 1. Prevalence of microbial species in genital tract samples from infertile couples.

Microbial species [a]	Genital tract samples [b]		
	Semen	Vaginal/Endocervical swab	Total
E. faecalis	33	14	47
S. agalactiae	13	18	31
E. coli	19	11	30
M. hominis	3	18	21
Candida spp.	5	11	16
U. urealyticum	6	4	10
Other *Enterobacteriales*	5	4	9
S. aureus	2	4	6
S. haemolyticus	6	0	6
Group F *Streptococcus*	5	1	6
G. vaginalis	0	5	5
T. vaginalis	1	3	4
P. asaccharolyticus	2	0	2
P. aeruginosa	1	0	1
HSV	1	0	1
Chlamydia trachomatis	0	0	0
Neisseria gonorrhoeae	0	0	0
Total	102	93	195

[a] *Candida* spp., *C. albicans*, *C. parapsilosis*, *C. glabrata* and *C. krusei*; other *Enterobacteriales*, *K. pneumoniae*, *K. oxytoca*, *P. mirabilis*, *M. morganii*, *E. aerogenes*, and *C. kroserii*.

[b] A total of 855 genital samples (285 semen specimens, 285 vaginal and 285 endocervical swabs) from 285 males and 285 females were subjected to microbiological analysis. Both a vaginal and an endocervical swab were collected from each female patient. Endocervical swabs were used to search for *U. urealyticum*, *C. trachomatis* and *N. gonorrhoeae*, whereas vaginal swabs were employed to test for all other pathogens.

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for at least one pathogen in at least one of the two partners. Of the 132 positive couples, the male partner only was positive in 57 cases, the female only was positive in 49, and both partners were positive in 26 couples (Fig 1). Out of these 26 couples, 16 shared at least one genital pathogen between partners. The most prevalent microbial pathogens found in infected couples included *E. faecalis* (32.6%), *E. coli* (22%), *S. agalactiae* (20.5%), *M. hominis* (13.6%), *Candida* spp. (9.8%), *Enterobacteriales* other than *E. coli* (6.8%), and *U. urealyticum* (6.8%) (Fig 2). *M. hominis* and *T. vaginalis* were more prevalent in females, *G. vaginalis* was not found in males, and *S. haemolyticus* was not detected in females (Fig 2).

Impact of genital tract pathogens on semen quality

A total of 285 semen specimens were analysed, of which 12 samples were azoospermic. Out of 273 samples, 72 (26.4%) had oligozoospermia (concentration $< 15x10^6$/ml), 65 (23.8%) showed asthenozoospermia (total motility $< 40\%$), 62 (22.7%) exhibited leukocytospermia (leukocyte counts $\geq 10^6$ cell/ml) and 28 (10.3%) presented with teratozoospermia (typical morphology $< 4\%$). Percent sperm motility in samples with leukocytospermia (total motility = 46±17; progressive motility = 43±18) was significantly lower compared to that of specimens with normal counts of seminal leukocytes (total = 56±17; progressive = 53±18), ($p = 0.0003$).

Spermiogram results showed that both total ($p = 0.012$) and progressive ($p = 0.0098$) motility were significantly diminished in samples positive to microbiological testing compared to negative specimens (Table 2). Out of all pathogens identified, only *E. faecalis* was found to significantly alter semen parameters. Concentration, motility, and typical morphology of sperm

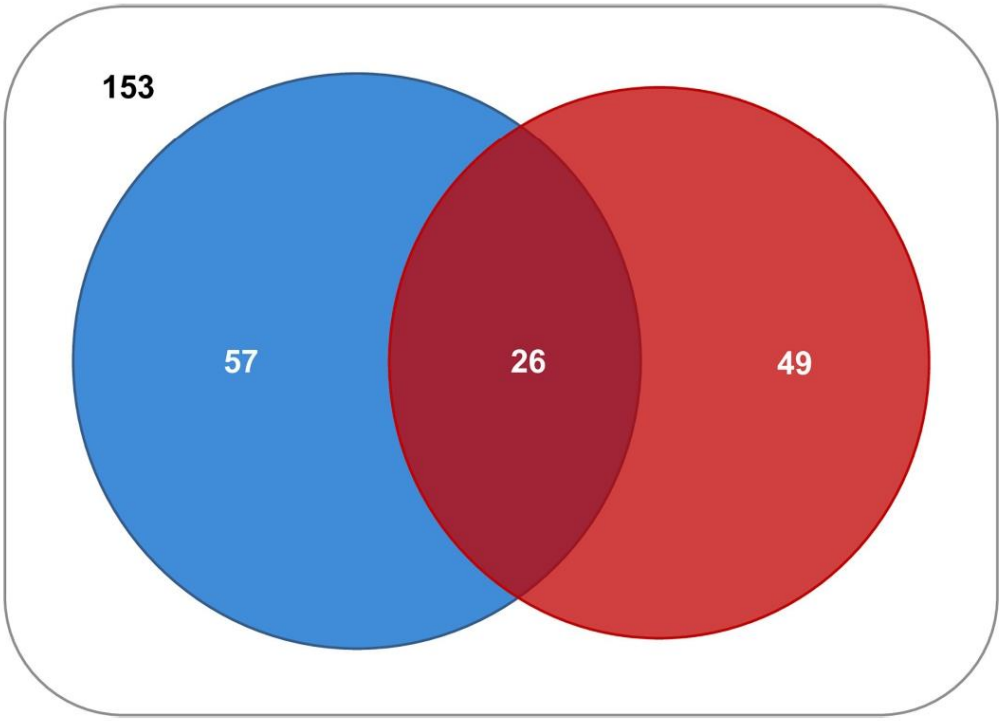

Fig 1. Venn diagram of genital tract infections in infertile couples. A total of 285 infertile couples were enrolled in the study and subjected to microbiological analysis to search for genital tract pathogens in semen specimens and vaginal/endocervical swabs prior to IVF. One hundred and thirty-two (46.3%) couples were positive for at least one pathogen in one or both partners. The male was positive in 57 cases (20%; blue) and the female in 49 cases (17.2%; red). The intersection of blue and red regions represents the couples (n = 26; 9.1%) where both partners were positive for at least one genital tract pathogen. The external set comprises all the couples included in the study.

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cells were reduced in *E. faecalis*-positive compared to *E. faecalis*-negative samples (Table 2). Differences in total ($p = 0.005$) and progressive ($p = 0.003$) motility and in typical morphology ($p = 0.026$) of sperm cells between semen cultures positive and negative for *E. faecalis* were statistically significant (Table 2). Out of 62 samples with leukocytospermia, 49 (79%) were microbiologically negative (Table 3). Although leukocytospermia was higher in negative (25.8%) than in positive (15.7%) semen samples, differences were not significant (Table 3).

The presence of *E. faecalis*, *E. coli*, *S. agalactiae*, and *G. vaginalis* is associated to reduced levels of vaginal lactobacilli

Genital swabs from 285 females were assessed for the presence of both genital tract pathogens and vaginal lactobacilli. A highly significant association ($p<0.0001$) between reduced amounts of vaginal lactobacilli ($< 10^4$ cfu/swab) and the presence of genital tract pathogens was found in the female study population (Fig 3A). When analysis was applied to each individual pathogen, a statistically significant association was observed between reduced levels of lactobacilli

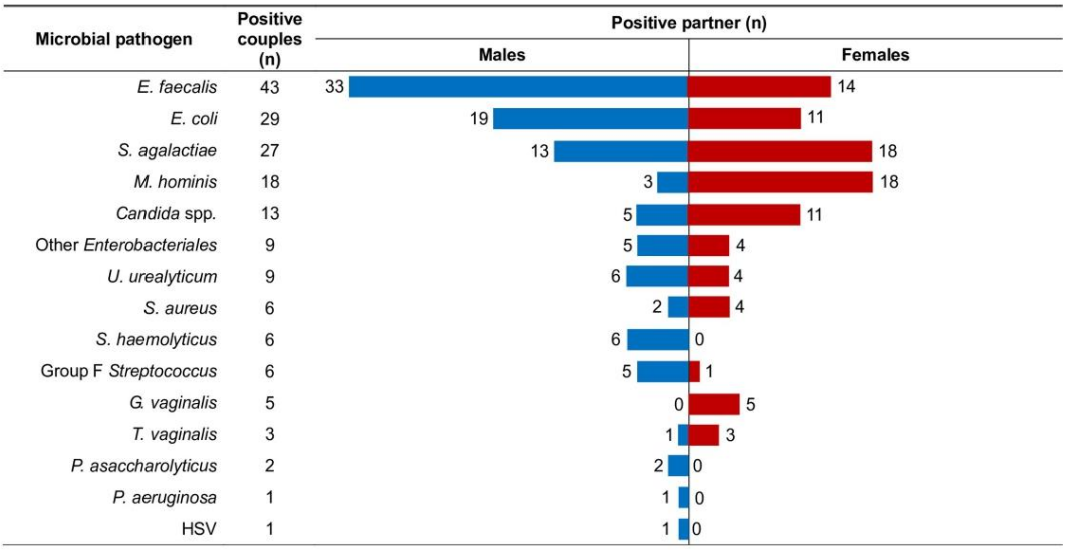

Fig 2. Prevalence of genital tract pathogens in infertile couples. Tornado graph showing the number of clinical strains of different microbial pathogens found in genital tract samples from male (blue bars) and female (red bars) patients. For each pathogen, the number of positive couples is also shown. Pathogens are listed according to their prevalence in infected couples. *Enterobacteriales* other than *E. coli* included *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Proteus mirabilis*, *Morganella morganii*, *Enterobacter aerogenes*, and *Citrobacter kroserii*. *Candida* spp. included *C. albicans*, *C. parapsilosis*, *C. glabrata*, and *C. krusei*.

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and the presence of *E. faecalis* ($p<0.0001$), *E. coli* ($p = 0.0123$), *S. agalactiae* ($p<0.0001$), and *G. vaginalis* ($p = 0.0003$), (Fig 3B). It is worth noting that *G. vaginalis*, a key bacterial species of BV, was detected only in swabs with decreased load of vaginal lactobacilli.

Infection with *E. faecalis* and/or *U. urealyticum* and/or and *M. hominis* is predictive of a negative IVF outcome in infertile couples

To investigate whether specific genital tract pathogens could be associated to IVF failure, microbiological results were correlated to IVF outcomes. IVF success was slightly higher in non-infected than in infected couples. Microbiological data indicated that specific pathogens (*E. faecalis*, *U. urealyticum*, *M. hominis*, *G. vaginalis*, *E. coli*) were more prevalent in unsuccessful (IVF-) than successful (IVF+) couples, however, no significant differences were calculated when each pathogen was tentatively associated with IVF outcome. Therefore, analysis was performed by examining couples positive for groups of genital tract pathogens after sequential exclusion of the pathogens that seemed not to affect IVF outcome. The microbial group constituted of *E. faecalis*, *U. urealyticum*, *M. hominis*, *G. vaginalis*, and *T. vaginalis* was more prevalent in IVF- than IVF+ couples, but differences were not significant ($p > 0.05$, χ^2 test with Yates' correction). Elimination of *T. vaginalis* showed that prevalence of the microbial group was significantly higher in IVF- (36.3%) compared to IVF+ (16.7%) couples ($p = 0.03$, χ^2 test with Yates' correction). Finally, by further excluding *G. vaginalis*, the smallest infectious group significantly associated with IVF failure included *E. faecalis* and/or *U. urealyticum* and/or *M. hominis* (Table 4). Analysis of the IVF+ couples showed that 30/35 (85.7%) were negative to microbiological testing, whereas out of the couples infected with *E. faecalis* and/or *U.*

Table 2. Correlation of genital tract pathogens with semen parameters in infertile males [a].

Microbial pathogen (n) [b]	Concentration (sperm cells/ml)	Motility (%) [c]		Typical morphology (%)
		Total	Progressive	
E. faecalis (33)	$4.03 \times 10^7 \pm 7.43 \times 10^6$	**44.9 ± 3.1** **	**40.3 ± 3.3** **	**11.5 ± 1.4***
E. coli (19)	$5.01 \times 10^7 \pm 1.32 \times 10^7$	49.5 ± 4.0	44.8 ± 4.5	16.2 ± 2.0
S. agalactiae (13)	$5.19 \times 10^7 \pm 9.01 \times 10^6$	46.9 ± 6.1	44.4 ± 6.3	12.0 ± 2.1
M. hominis (3)	$9.03 \times 10^7 \pm 1.05 \times 10^7$	54.3 ± 8.8	53 ± 8.6	15.9 ± 1.7
Candida spp. (5)	$1.97 \times 10^7 \pm 1.31 \times 10^7$	45.4 ± 5.6	41.4 ± 8.2	11.8 ± 4.1
Other *Enterobacteriales* (5)	$4.23 \times 10^7 \pm 1.53 \times 10^7$	41.0 ± 8.4	38.8 ± 8.2	12.1 ± 3.3
U. urealyticum (6)	$9.13 \times 10^7 \pm 2.02 \times 10^7$	60.5 ± 6.9	59.8 ± 6.5	20.4 ± 5.1
S. aureus (2)	$5.15 \times 10^7 \pm 9.50 \times 10^6$	55.5 ± 0.5	54.5 ± 0.5	14.0 ± 7.0
S. haemolyticus (6)	$2.39 \times 10^7 \pm 1.39 \times 10^7$	39.5 ± 9.1	37.5 ± 7.8	11.3 ± 1.7
Group F *Streptococcus* (5)	$2.28 \times 10^7 \pm 9.81 \times 10^6$	40.2 ± 9.1	35.6 ± 9.7	11.5 ± 3.4
T. vaginalis (1)	5.00×10^7	86.0	81.0	14.0
P. asaccharolyticus (2)	$6.80 \times 10^7 \pm 3.80 \times 10^7$	66.5 ± 18.5	66.5 ± 18.5	22.5 ± 1.5
P. aeruginosa (1)	7.30×10^7	61.0	55.0	10.5
HSV (1)	3.00×10^7	54.0	50.0	12.6
Total Positive (83)	$4.44 \times 10^7 \pm 4.84 \times 10^6$	**47.6 ± 1.3** *	**44.2 ± 1.4** **	13.6 ± 0.8
Total Negative (190)	$5.49 \times 10^7 \pm 3.73 \times 10^6$	53.4 ± 1.28	50.4 ± 1.4	14.9 ± 0.6

[a] All data are represented as mean ± SEM.

[b] Numbers (in brackets) of genital tract pathogens identified in 273 semen specimens. Twelve patients were excluded because azoospermic. Total positive (n = 83), number of males positive for at least one pathogen. Total negative (n = 190), number of males negative to microbiological testing.

[c] Total motility, sperm cells moving in all directions; progressive motility, sperm cells moving along a straight line.

* Statistical analysis (Mann-Whitney test) was performed against the group of negative males. Significant differences are in bold: *, $p < 0.05$; **, $p < 0.01$.

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urealyticum and/or *M. hominis*, just 5/67 (7.5%) obtained a successful IVF ($p = 0.02$, χ^2 test with Yates' correction; Table 4). Interestingly, among the IVF- couples positive for this microbial group, *E. faecalis* and *U. urealyticum* were found in approximately 90% of cases, whereas *M. hominis* was detected in all the couples with a poor IVF outcome.

Discussion

Infertility is an ongoing challenge throughout the world and is increasingly being considered not only as a private matter but also as a public health burden. ART has allowed to overcome certain issues, however, rates of conception are still low [30]. Several factors can participate in infertility, including genital tract infections. However, no consensus guidelines are available yet on microbiological evaluation of infertile couples prior to undergoing IVF.

Table 3. Association between the presence or absence of genital tract pathogens and leukocytospermia in semen samples.

Pathogens [a]	Semen samples [b]			*p* value [c]
	Absence of leukocytospermia (%)	Presence of leukocytospermia (%)	Total	
Absence	141 (74.2)	49 (25.8)	190	0.09
Presence	70 (84.3)	13 (15.7)	83	
Total	211	62	273	

[a] Microbial pathogens were searched in a total of 273 semen samples after exclusion of 12 specimens from azoospermic males.

[b] Semen samples were considered as leukocytospermic when leukocyte counts were $\geq 10^6$/ml.

[c] χ^2 test with Yates' correction.

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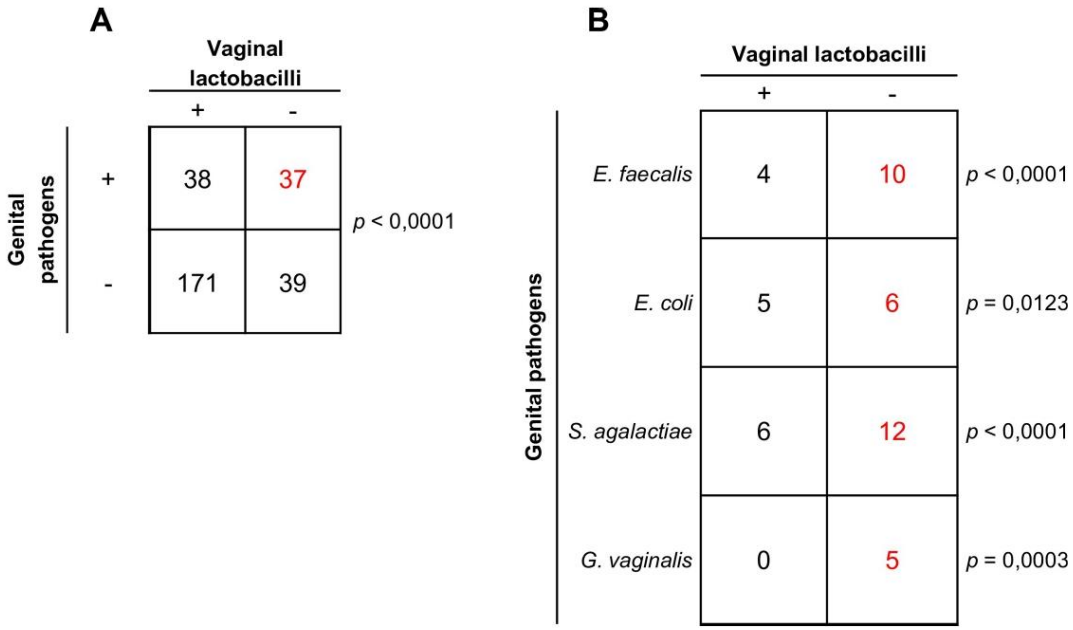

Fig 3. Association between genital tract pathogens and vaginal lactobacilli in infertile females. Both a vaginal and an endocervical swab were collected from each female patient enrolled in the study. Endocervical swabs (n = 285) were used to search for *U. urealyticum*, *C. trachomatis* and *N. gonorrhoeae*, while vaginal swabs (n = 285) were simultaneously tested for all other pathogens and vaginal lactobacilli. Lactobacilli were quantified on selective solid media, and counts $< 10^4$ cfu/swab were regarded as reduced levels of lactobacilli. **A.** Contingency table reporting the number of swabs negative (-) or positive (+) for at least one genital tract pathogen in relation to the number of swabs with normal (+) or reduced (-) levels of lactobacilli. **B.** Contingency table showing the number of swabs positive for *E. faecalis*, *E. coli*, *S. agalactiae*, and *G. vaginalis* that presented with either normal (+) or low (-) levels of lactobacilli. The χ^2 test with Yates' correction was used for all cases except for *G. vaginalis* (Fisher exact test). For each of the above pathogen, statistical analysis was performed against the negative samples with normal (n = 171) or reduced (n = 39) levels of vaginal lactobacilli.

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The present study focused on the impact of asymptomatic genital tract infections on couple fertility. Main findings are: (i) approximately half of the couples was diagnosed with a genital tract infection, (ii) *E. faecalis* had a significantly negative impact on sperm motility and morphology, (iii) the presence of *E. faecalis*, *E. coli*, *S. agalactiae*, and *G. vaginalis* in females was

Table 4. Association between the presence of *E. faecalis* and/or *U. urealitycum* and/or *M. hominis* and IVF outcome in infertile couples.

Pathogens	IVF outcome			*p* value [b]
	IVF- couples (%)	IVF+ couples (%)	Total	
Absence [a]	111 (78.7)	30 (21.3)	141	0.02
E. faecalis, and/or *U. urealitycum*, and/or *M. hominis*	62 (92.5)	5 (7.5)	67	
Total	173	35	208	

[a] Results refer to all the couples negative to microbial testing (n = 153) after exclusion of 12 couples where the male partner was azoospermic.

[b] χ^2 test with Yates' correction.

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significantly associated to reduced levels of vaginal lactobacilli, (iv) the presence of the group *E. faecalis*, *U. urealyticum*, and *M. hominis* in infertile couples was significantly associated to IVF negative outcome.

All the infertile couples enrolled in the study were asymptomatic for genital tract infections, but nearly half (46.3%) resulted positive to microbiological testing. As to our knowledge no data are available in the literature on the prevalence of asymptomatic genital tract infections in naturally fertile couples, it is difficult to estimate the impact of asymptomatic infections on fertility. The microbial species identified in our study are mostly colonizers of the male anterior urethra and coronal sulcus or the vaginal milieu [31,32], but are also responsible of urinary tract (UTI), genital and systemic infections. The prevalence of pathogens was different between males and females, with certain microbial species showing a clear predominance in males (*E. faecalis*, *S. haemolyticus*, Group F *Streptococcus*) or females (*M. hominis*, *Candida* spp., *G. vaginalis*, *T. vaginalis*). *E. faecalis* and *M. hominis*/*S. agalactiae* were the most frequently detected microbes in males and females, respectively. *M. hominis* is a common endosymbiont of *T. vaginalis*, which acts not only as a protective niche but also as a 'Trojan horse' to transmit the bacteria to the human genital tract [33]. In our case, although the prevalence of the protozoan in infected females was low (4%), 66.7% of the *T. vaginalis*-positive vaginal swabs also contained *M. hominis*. Out of all pathogens detected in infected couples, *E. faecalis* was the most common (32.6%) and was shown to adversely affect couple fertility.

Genital infection and inflammation can impact on male fertility in several ways, including deterioration of spermatogenesis, impairment of sperm functions, generation of ROS leading to DNA fragmentation or oxidative protein modification, production of sperm antibodies, and obstruction of the seminal tract [4]. The relationship between bacteriospermia, leukocytospermia and semen parameters in infertile men is still debated. Some studies showed a detrimental influence of microbial pathogens or/and leukocytes on semen quality [5,7,10,34–37], while others did not observe any effect [6,38,39]. In this study, 29.1% of semen specimens was positive to microbiological analysis, which is in accordance with literature data reporting that rates of bacteriospermia in infertile men can fluctuate from 15 to 60% [5–7,36,39]. The most prevalent species was *E. faecalis*, which was significantly associated to reduced motility and altered morphology of spermatozoa, suggesting that enterococci may have a direct negative influence on semen quality as previously published [5,6,36]. In contrast, significant sperm abnormalities were not observed in *E. coli*- and *S. agalactiae*-positive samples, the second and third most commonly isolated species. Some authors have shown that *E. coli* induces alterations in human spermatozoa, resulting in reduced motility, altered acrosomal function, and decreased vitality [5,35,40]. Nonetheless, the fact that those data mostly originate from *in vitro* studies based on large bacterial concentrations that will unlikely be reached during *in vivo* infection, may explain our negative result on *E. coli*. Leukocytospermia is generally considered as a marker of inflammation with a poor diagnostic value for genital tract infections [6,7,41]. Several factors independent of infectious challenges have been associated to elevated seminal leukocytes, including ageing, medications, smoking, alcohol and drug abuse [7,8]. In our study, leukocytospermia was not significantly associated with the presence of microbial pathogens in semen ($p = 0.09$), as evidenced by the fact that 79% of leukocytospermic samples were microbiologically negative as reported before [42]. This finding, which is not unexpected [6,7,41,42,43], probably indicates that the presence of potential pathogens in semen does not necessarily lead to a full-fledged inflammatory response with substantial leukocyte recruitment. The hypothesis may be especially valid in asymptomatic patients as those enrolled in the present study. Moreover, the time of semen collection could be another relevant factor to explain our data since bacteria and leukocytes may not be present simultaneously in semen samples as described [44].

BV, AV and abnormal vaginal flora (decrease/absence of lactobacilli) have been reported to affect pregnancy rate and outcome [24]. A recent systematic review and meta-analysis showed that BV is associated with female infertility, preclinical pregnancy loss and preterm birth, although it does not seem to impact on conception rates [21]. Prevalence of BV in infertile women varies considerably from 10% to 45% depending on the study population and diagnostic criteria [21,25,45,46]. Diagnosis of BV can be performed based on clinical, microscopic, and (cultural and/or molecular) microbiological criteria [19]. The Nugent scoring system was the first standardized method founded on classification and enumeration of 'morphotypes' in Gram-stained vaginal smears [47]. Despite being long recognized as the gold standard for BV diagnosis, the Nugent system has several limitations [19]. In the current study, we chose a combined culture- and molecular-based diagnostic approach for detection and quantification of both pathogens and lactobacilli in genital swabs. Microbiological analysis showed that the presence of vaginal lactobacilli was generally associated to absence of genital pathogens; conversely, detection of *E. faecalis*, *E. coli*, *S. agalactiae*, and *G. vaginalis* was significantly associated with reduced levels of vaginal lactobacilli. *G. vaginalis* is strongly linked to BV [19], whereas *E. coli*, *S. agalactiae*, and *E. faecalis* are not typical BV-defining microorganisms but are instead common agents of UTI and AV. Absence of lactobacilli, besides being a key feature of BV, has also been correlated to AV [22,48] and recurrent UTI by *E. coli* [49] and other uropathogens [50], underlining the protective function exerted by lactobacilli against urogenital infections. In contrast, the presence of *M. hominis*, another recurrent species in BV, was not significantly associated to decreased numbers of vaginal lactobacilli in this study population. It should also be noted that a small percentage (18.6%) of females negative to microbiological testing had low amounts of lactobacilli, indicative of either a healthy microbiota not dominated by *Lactobacillus* spp. [18] or a transitional stage in the dynamic shifts of the vaginal microbial ecosystem [51]. The outcome of IVF has been associated with the composition of the vaginal microbiota on the day of ET [52], and a microbiota exclusively composed by lactobacilli is considered the most promising scenario for successful IVF [53]. In our case, only 12.8% of patients with reduced quantity of lactobacilli belonged to the IVF+ couples, yet again emphasizing the key role played by a *Lactobacillus*-dominated microbiota for a positive outcome of IVF.

In the current study, IVF negative outcome was slightly more elevated in infected than non-infected couples, but differences were not significant. However, specific pathogens were more frequently found in IVF- couples, and hence a definite infectious group (*E. faecalis* and/or *U. urealyticum* and/or *M. hominis*) was identified that significantly correlated with IVF failure ($p = 0.02$). It should be noted that *G. vaginalis* was initially included in the group ($p = 0.031$), in accordance with a recent prospective study reporting the association of *G. vaginalis* and *A. vaginae* with low pregnancy rates in IVF patients [25]. Identification of a group of infectious agents, rather than a single microbial species, is not unexpected, as the above pathogens can be found as agents of polymicrobial genital tract infections [19,50,54]. *M. hominis* has been shown to synergistically cooperate with *G. vaginalis* in BV [19,55] and is a frequent symbiont of *T. vaginalis* in trichomoniasis and BV [33,56], while *E. faecalis* can be detected in polymicrobial urogenital and biofilm-based infections [50]. Whether these microbes act as either independent or bystander pathogens in genital tract infections is not clear yet, however, increasing evidence is accumulating on their role in infertility, adverse pregnancy outcome, and post-partum complications [11,13,19,21,22,24,25,54,57]. Lower rates of fertilization, implantation and clinical pregnancy were observed in couples undergoing ICSI that were positive to *E. coli*, *E. faecalis*, *S. agalactiae*, and *Staphylococcus* spp. [52,58]. Detection of genital *Mollicutes* in couples undergoing IVF was also associated to reduced pregnancy rates [59,60] and increased miscarriages [61]. To this regard, it is interesting to note that all the couples of this study infected by *M. hominis* had a negative IVF outcome. The present work shed light on

three specific genital tract pathogens as predictive infectious markers for poor outcome of IVF, emphasizing the importance of microbiological testing of infertile couples for *E. faecalis*, *U. urealyticum*, and *M. hominis* prior to IVF procedures.

In conclusion, infertility is a multi-factorial and multi-faceted clinical condition which poses a profound economic and psychological burden on affected couples and high costs on the healthcare system. In this study, we have identified an infectious group that significantly correlated as an independent risk factor to infertility and negative outcome of IVF. However, the causes of IVF failure often remain unknown. A joint effort between clinical microbiologists, infectious diseases and reproductive medicine specialists is desirable to produce consensus guidelines on testing for pathogens associated to infertility and assessment of both microbiological and clinical outcomes before ART treatment. Improved management of genital tract infections in infertile couples may be helpful to increase pregnancy rates, reduce the total number of treatment cycles and possibly enhance first level fertility approaches with beneficial effects on couple well-being and healthcare costs.

Supporting information

S1 Table. Primers used for Real-Time PCR. List of primers and probes used to identify non-cultivable pathogens in genital tract samples.
(DOCX)

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CHAPTER 4

Sequence typing and antimicrobial susceptibility testing of infertility-associated *Enterococcus faecalis* reveals clonality of aminoglycoside resistant strains

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Running title: Infertility-associated *Enterococcus faecalis*

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The Tree of Life, Stoclet Frieze
Gustav Klimt, 1909

Abstract

Background. *Enterococcus faecalis* is a member of the gut microbiota in humans and also a pathogen responsible of different infections, such as urinary tract infections, endocarditis and sepsis. Recently, we have shown that the presence of *E. faecalis* in genital samples of infertile couples was predictive for a negative outcome of *In Vitro* Fertilization (IVF). The aim of this study is to characterize the antibiotic susceptility and population structure of 41 'infertility-associated *E. faecalis*' (IAF) strains isolated from genital tract infections of infertile couples.

Methods. Antibiotic susceptibility testing included VITEK 2, MIC and disk-diffusion assays, and results were evaluated according to EUCAST guidelines. Whole genome sequences were obtained by Oxford Nanopore and Illumina sequencing and used for hybrid genome assemblies. Genomes were employed for multilocus sequence typing (MLST) and identification of antimicrobial resistance genes.

Results. All 41 strains were susceptible to β-lactams, glycopeptides, tigecyclin, linezolid and nitrofurantoin, whereas 8/41 isolates (19.5%) were resistant to at least one antimicrobial agent. All the 8 resistant strains showed resistance to high-level aminoglycosides (HLA), of which 7 were resistant to high-level gentamicin. Only one strain was resistant to gentamycin, streptomycin, ciprofloxacin and levofloxacin. Simpson's index of diversity (D=0.889, $CI_{95\%}$=0.83-0.95) indicated a highly divergent IAF population. A total of 17 different sequence types (STs) were identified through MLST analysis of the enterococcal genomes, with ST40, ST81 and ST179 being the most prevalent. Blast with *E. faecalis* MLST database clustered the 17 STs into 3 distinct clonal complexes (CCs) and 14 singletons. CC40 was the most predominant (12/41 isolates), followed by ST81(7/41) and CC16 (6/41). Interestingly, 85.7% (6/7 strains) of the isolates resistant to high-level gentamicin clustered in CC16/ST480. Searching of aminoglycoside modifying enzyme (AME) genes in the 8 HLA resistant strains showed the presence of the *aac(6')-aph(2'')* gene in the 7 gentamicin-resistant strains, whereas the *ant(6)-Ia* gene was found in the 3 isolates with resistance to streptomycin. The AME genes were located either in the chromosome (6/8 strains) or on plasmids (2/8). Resistance to ciprofloxacin and levofloxacin was mediated by two well-known point mutations in the genes *gyrA* and *parC*.

Conclusions. The *E. faecalis* isolates showed susceptility to the majority of clinically relevant antimicrobial agents. However, HLA resistance was found in almost 20% of the population, and most of the gentamicin resistant strains clustered in CC16/ST480, suggesting clonality of infertility-associated *E. faecalis* isolates resistant to high-level aminoglycosides.

Introduction

Infertility is estimated to affect 9 to 12% of reproductive-aged couples all over the world (Inhorn and Patrizio, 2015). Amongst the several causes of couple infertility, infections of the urogenital tract are considered a relevant factor. Infections by *Neisseria gonorrohoeae*, *Chlamydia trachomatis*, *Treponema pallidum*, human immunodeficiency virus (HIV), and mumps have long been recognised as important for infertility (Pellati et al., 2008; Garrido-Gimenez and Alijotas-Reig, 2015). Latest meta-analyses demonstrated that bacteriospermia can negatively impact on sperm quality, and different bacterial species were linked to altered sperm parameters (Pergialiotis et al., 2018; Farahani et al., 2021). *Enterococcus faecalis* was associated to reduced concentration (oligozoospermia), motility (asthenozoospermia) and abnormal morphology (teratozoospermia) of spermatozoa (Mehta et al., 2002; Rodin et al., 2003; Moretti et al., 2009). Recently, our group has also reported that *E. faecalis* was significantly associated with asthenozoospermia, teratozoospermia, and lower levels of vaginal lactobacilli (Ricci et al., 2018). Moreover, the presence of *E. faecalis* (together with *Mycoplasma hominis* and *Ureaplasma urealyticum*) in genital samples of infertile couples was predictive of a negative outcome of *In Vitro Fertilization* (Ricci et al., 2018).

Enterococci, previously considered just as members of the gut microbiota of both animals and humans, are now acknowledged as important human pathogens responsible for a variety of infections, including urinary tract infections (UTI), sepsis, endocarditis, peritonitis, abdominal/pelvic and soft tissue infections (Agudelo Higuita and Huycke, 2014). The most frequent clinical manifestation is UTI, of which *E. faecalis* is the second most common agent worldwide after *Escherichia coli* (Flores-Mireles et al., 2015). *E. faecalis* is also the leading pathogen among Gram-positive bacteria of cathether-associated UTI (CAUTI) in healthcare settings (Peng et al., 2018). Ascending UTI and intra-abdominal infections can lead to bacteremia and endocarditis. Both *Enterococcus faecium* and *E. faecalis* have a remarkable tropism for the endocardium and/or the heart valves, but *E. faecalis* alone accounts for about 97% of all cases of infective endocarditis, especially in risk groups (Baddour and Prendergast, 2018).

Treatment of asymptomatic bacteriuria is not recommended by most recent guidelines (Nicolle et al., 2019). Uncomplicated enterococcal UTI are generally treated with nitrofurantoin, fosfomycin or fluoroquinolones, while drugs for complicated UTI and pyelonephritis include

daptomycin, linezolid and quinipristin-dalfopristin (Swaminathan and Alangaden, 2010). Treatment of enterococcal endocarditis and deep-tissue infections require the synergic combination of penicillins or glycopeptides with aminoglycosides, typically gentamicin (Mercuro et al., 2018). Despite *E. faecalis* is the most common uropathogen harvested from semen samples of infertile men (Pergialiotis et al., 2018; Farahani et al., 2021), to our knowledge, there are no shared guidelines among reproductive health clinicians for the treatment of enterococcal bacteriospermia.

Antimicrobial therapy of enterococcal infections is complicated by their intrinsic resistance to several antibiotic classes, including cephalosporins, sulphonamides and low concentrations of aminoglycosides (Kristich et al., 2014). In addition, acquired antibiotic resistance may limit the number of therapeutic options especially for severe infections. Of special concern is the acquisition by horizontal transfer of genes coding for aminoglycoside modifying enzymes (AMEs) and conferring resistance to high concentrations of aminoglycosides (Chow, 2000; Kristich et al., 2014). As a result, the synergistic bactericidal effect between beta-lactams and aminoglycosides is eliminated. According to the latest european surveillance report, the population-weighted mean percentage of high-level aminoglycoside (HLA) resistance in *E. faecalis* was 26.6 (Antimicrobial resistance in the EU/EEA (EARS-Net) - Annual Epidemiological Report for 2019, 2020). In Italy, national data on *E. faecalis* chemoresistance in 2019 described 35.2% resistance to high-level gentamicin, whereas resistance to aminopenicillins and vancomycin were 2% and 1.7%, respectively (Antimicrobial resistance in the EU/EEA (EARS-Net) - Annual Epidemiological Report for 2019, 2020).

Multilocus sequencing typing (MLST) was developed to determine genetic lineages in different bacterial species, including *Enterococcus faecium* and *E. faecalis*, and has successfully differentiated globally distributed population structures of isolates from different sources (Maiden et al., 1998). MLST of enterococci has shown the emergence of specific genetic clonal complexes (CCs) of isolates resistant to antibiotics and/or responsible for hospital and community outbreaks (Ruiz-Garbajosa et al., 2006; Kawalec et al., 2007; Sun et al., 2009; Solheim et al., 2011; Kuch et al., 2012). Unlike *Enterococcus faecium*, *E. faecalis* generally has an overall non-clonal population structure due to high level of genetic recombination (Ruiz-Garbajosa et al., 2006; Sparo et al., 2018).

In the present work, MLST and antibiotic susceptibility testing were used to define the population structure and resistance to clinically relevant antibiotics in a collection of 41 clinical strains of *E. faecalis* isolated from the genital tract of infertile couples.

Materials and Methods

Clinical isolates

A total of 41 clinical isolates of *E. faecalis* from 285 couples attending the Centre for Diagnosis and Treatment of Couple Sterility at Siena University Hospital were analyzed. All couples were asymptomatic for genital tract infections. *E. faecalis* strains included 28 isolates from semen samples and 13 from vaginal swabs from infertile couples as described before (Ricci et al., 2018).

Bacterial growth conditions

Each *E. faecalis* isolate was growth on solid BHI medium (Oxoid, Milan, Italy) enriched with 5% defibrinated horse blood (Liofilchem, Teramo, Italy) at 37°C overnight (o. n.). Four to six isolated bacterial colonies were suspended in 10 ml of liquid BHI medium (Oxoid) and incubated at 37°C until they reached the optical density at 590 nm (OD_{590}) of 0.5. Cultures were aliquoted, added with 10% glycerol (Baker, Bridgend, England) and stored at -80°C until use.

Antimicrobial susceptibility testing

For each isolate, antimicrobial susceptibility testing (AST) was initially performed using Vitek-2 (Biomerieux Italia S.p.A., Florence, Italy) with the AST-P658 card (Biomerieux) covering the antibiotics recommended for enterococci by EUCAST (The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters, version 11.0, 2021).

To confirm and implement the Vitek data, both Minimal Inhibitory Concentration (MIC) and disk diffusion (Kirby-Bauer and E-test) assays were carried out. *E. faecalis* OG1RF (Bourgogne et al., 2008) was used as reference strain. Each isolate was cultured o. n. on blood-agar BHI plates, and then few isolated colonies were suspended in dH_2O to reach the turbidity of 0.5 McFarland. For MIC test of gentamicin (GEN) and streptomycin (STR), bacterial suspensions were diluted (1:700) in liquid Mueller Hinton medium (Biomérieux) and distributed into the wells (50 µl/well) of a custom microtiter plate for gram-positive bacteria (Sensititre GPN3F; Thermo Fisher Scientific, Milano, Italy). The plate was incubated at 37°C o. n., and MICs were determined by manual reading. Kirby-Bauer assays were used to test susceptibility to

all antibiotics (Oxoid S.p.A., Milano, Italy), while E-test was employed for linezolid (LZD). Results were assessed based on EUCAST breakpoints (The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters, version 11.0, 2021).

Genomic DNA preparation

Genomic DNA extraction was carried out by combining two previously described methods (Pozzi et al., 1990; Wilson, 2001) with some modifications. Briefly, bacterial frozen stocks were diluted (1:100) in 40 ml of BHI and grown until an OD_{590} of 2.0. Cultures were centrifuged, washed in phosphate buffered saline (PBS, EuroImmune, Padova, Italy), and resuspended in 3 ml of protoplasting buffer [5 mg/ml lysozyme (Sigma-Aldrich, Milan, Italy), 20% raffinose (Sigma-Aldrich), 50 mM Tris-HCl, 5 mM EDTA]. After incubation at 37°C for 90 min, samples were centrifuged (6500x*g*, 10 min) and resuspended in 4 ml of ddH_2O containing 0.5% SDS (VWR International Srl, Milan, Italy) and 100 µg/ml of proteinase K (Sigma-Aldrich). Samples were incubated at 37°C for 30 min and then added with 0.5 M NaCl and 0.5 M CTAB/NaCl (Wilson, 2001). After two extractions with phenol:chloroform:isoamyl alcohol (25:24:1) (Sigma-Aldrich) and chloroform:isoamyl alcohol (24:1) (Sigma-Aldrich), DNA was precipitated o.n. at -20°C with ice-cold 96% ethanol (Carlo Erba S.r.l., Milan, Italy). Samples were centrifuged (6500*x*g, 30 min), washed with 70% ethanol, air-dried at room temperature for 30 min, and resuspended in 0.9% NaCl. Samples were analysed by gel electrophoresis on 0.5% agarose gel (Promega Italia S.r.l., Milan) in TBE running buffer 0.5x. DNA was quantified with the Qubit 2.0 Fluorometer (Invitrogen, Life Technologies, Carlsbad, CA, United States) using the Qubit dsDNA BR assay kit (Thermo Fisher Scientific), and DNA quality was evaluated by spectrophotometer measurement at the wavelength of 260 and 280 nm (Implen GmbH, Munich, Germany).

Oxford Nanopore Sequencing

Sequencing reactions were carried out in 1.5 ml LoBind tubes (Sarstedt, Verona, Italy) using wide bore (Ø 1.2 mm) tips in order to reduce DNA shearing. DNA size selection of genomic DNA was obtained with 0.5 vol of AMPure XP beads (Beckman Coulter s.r.l., Milano, Italy) according to the manufacturer's instructions. A total of 2.5 µg of size-selected DNA was

employed for library construction by using the Nanopore sequencing kit SQK-LSK 108 (Oxford Nanopore Technologies, Oxford, United Kingdom). Pooling of multiple samples was obtained by the Nanopore 'Native Barcoding Expansion 1-12 kit' (Oxford Nanopore Technologies). Library preparation was performed following the manufacturer's protocol with the following modifications: (i) incubation on a rotator mix for 15 min; (ii) the 'Library Loading Beads' (LLB) were not employed. Finally, the DNA library (at least 200 ng) was loaded onto a R9.4 MinION flow cell (Oxford Nanopore Technologies). Sequencing run was performed on the GridION5X device (Oxford Nanopore Technologies) until a 100x genome coverage for each sample was reached (approximately 8-12 h). Real time base calling (quality cutoff >Q7) and demultiplexing of nanopore reads were performed using Guppy v4.0.11 (https://github.com/nanoporetech/pyguppyclient). Base called reads were analysed by NanoPlot v1.18.2 (De Coster et al., 2018). Nanopore read features are reported in Supplementary Table S1.

Illumina sequencing

Illumina sequencing was performed at Microbes NG (University of Birmingham, Birmingham, UK), using the Nextera library preparation kit (Illumina Inc., San Diego, USA) followed by HiSeq2500 sequencing (Illumina Inc.) (2x250 bp paired-end sequencing). Illumina reads were trimmed using Trimmomatic v0.32 17 (Bolger et al., 2014) and analysed by NanoPlot v1.18.2. (De Coster et al., 2018). Illumina read features are reported in Supplementary Table S2.

Genome assembly, annotation and analysis

Nanopore and Illumina reads of all 41 strains were assembled using the Unicycler v0.4.7 tool with default parameters (Wick et al., 2019; https://github.com/rrwick/Unicycler.git). For the strains of which a fully contiguous single chromosome assembly was not obtained, assembly was repeated as follows: nanopore reads were initially filtered using the Filtlong v0.2.0 software (https://github.com/rrwick/Filtlong) and assembled with Unicycler v0.4.7, and then two polishing rounds were carried out using the Pilon v1.22 tool (Walker et al., 2014) with the Illumina reads. The presence of putative erroneous indels in the assembly was assessed by Ideel (https://github.com/mw55309/ideel), and genomes were automatically annotated using Prokka v1.14.5 (Seemann, 2014; https://github.com/tseemann/prokka). Genome analysis was carried out using: (i) Artemis and Artemis Comparison Tool (ACT) v17.0.1 (Carver et al., 2008); (ii) Blast

(https://blast.ncbi.nlm.nih.gov/Blast.cgi); (iii) PlasmidFinder v2.0.1 (Carattoli et al., 2014). *E. faecalis* OG1RF complete genome was obtained from the NCBI Microbial Genome Database (https://www.ncbi.nlm.nih.gov/genome/808?genome_assembly_id=168518, CP002621.1) and used as a reference strain.

Statistical analyses

Starting from the sequenced genomes, sequence type (ST) of each IAF isolate was assigned based on the 7 housekeeping genes *gdh*, *gyd*, *pstS*, *gki*, *aroE*, *xpt*, and *yqiL used for enterococcal typing* (Jolley et al., 2018). For each locus, a distinct allele number was assigned in accordance with the *E. faecalis* MLST database (https://pubmlst.org/organisms/enterococcus-faecalis/). The relatedness amongst different STs was investigated by the UPGMA agglomerative hierarchical clustering method using the PHYLOViZ v2.0 (Francisco et al., 2012). Simpson's index of diversity (D) with 95% confidence interval ($CI_{95\%}$) was calculated ($0 \leq D \leq 1$, with values near zero corresponding to high diversity and values near one corresponding to more homogeneous populations). Clusters of related STs differing in ≤ 2 allelic loci and descending from a common ancestor were grouped into clonal complexes (CCs) by using goeBURST (Francisco et al., 2012). A singleton was defined as a ST not grouped in a CC. The UPGMA method was used to construct a dendrogram from the matrix of pairwise allelic differences between the STs. The nearest two clusters were joined into a higher level cluster, and the distance (Hamming distance) between any two clusters is the mean distance between elements of each cluster (Michener and Sokal, 1957; Mohammadi-Kambs et al., 2017).

Antimicrobial resistance genes (ARGs) were identified using ABRicate v1.0.1 (Seemann T, *Abricate*, https://github.com/tseemann/abricate) on the following different databases ARG-ANNOT (Antibiotic Resistance Gene-ANNOTation) (Gupta et al., 2014), CARD (Comprehensive Antibiotic Resistance Database) (Jia et al., 2017), MEGARES 2.00 (Doster et al., 2020) and ResFinder (Zankari et al., 2012).

Results

Antimicrobial susceptibility of infertility-associated *E. faecalis*

Susceptibility of 41 *E. faecalis* strains to 14 clinically relevant antimicrobial drugs was tested according to the EUCAST guidelines. Results obtained with the VITEK 2 automated system were confirmed by both disk diffusion and broth microdilution MIC methods. All of the strains tested (41/41) were found to be susceptible to β-lactams, glycopeptides, tigecyclin, linezolid and nitrofurantoin, whereas 8/41 (19.5%) were resistant to at least one antimicrobial agent (Fig. 1). High level aminoglycoside (HLA) resistance was found in all resistant isolates (8/8), whereas only one strain (1/8) was resistant to the fluoroquinolones ciprofloxacin (CIP) and levofloxacin (LVX). A total of 4 different phenotypic antimicrobial resistance patterns were defined (Table 1). Five isolates were resistant only to GEN, one only to STR, one to both GEN e STR, and finally one to GEN STR CIP LVX (Table 1).

Sequence types and identification of clonal complexes

Complete genomes of infertility-associated *E. faecalis* were obtained by both MinION and Illumina sequencing, followed by hybrid assembly (De Giorgi *et al*., in preparation). Whole genome sequences were used to perform multilocus sequence typing (MLST) on the 7 genes employed for *E. faecalis* typing. MLST allowed to assign 17 different sequence types (STs) (Fig. 2). All accounted STs were present in the *E. faecalis* database. The most frequently found types were ST40 (11/41 isolates), ST81 (7/41) and ST179 (5/41) (Fig. 2 and Supplementary Table 1). The other 14 STs were identified in ≤3 isolates (Fig 2 and Table S1). Calculation of the Simpson's index of diversity (D=0.889, $CI_{95\%}$=0.83-0.95) showed a high level of diversity of the 41 IAF strains.

The goeBURST algorithm was then used to group the STs with allelic variants in one or two loci into clonal complexes (CCs). Analysis resolved 3 groups and 11 singletons with single- and double-locus variants. Blast with *E. faecalis* MLST database clustered the 17 STs of IAF isolates into 14 distinct CCs, of which 3 (CC40, CC16, and CC21) comprised strains belonging to at least two different STs and 11 were singletons. The most prevalent cluster was CC40 (12/41 isolates), followed by ST81 (7/41) and CC16 (6/41), (Table S1). CC40 comprised ST40 and the

single-locus variant ST268, CC16 covered ST16 and the single-locus variant ST179, while CC21 comprised ST21 and ST117 (Table S1).

Phylogenetic relatedness of isolates resistant to high-level aminoglycosides

Analysis of the distribution of aminoglycoside resistance among the STs showed that 6 out of the 8 HLA resistant isolates were closely related (Fig. 2). In particular, 4 isolates (strains 5245, 2819, 4638 and 5034) belonged to ST179, while the other 2 were part of ST16 (strain 5410) and ST480 (strain 4774). The remaining 2 isolates belonged to the more distant ST211 (strain 4153) and ST40 (strain 4953) (Fig. 2). Construction of the minimum spanning tree containing the allelic variants in just 1 locus (n-1, n=7) indicated that ST179 and ST16 belonged to the same CC, of which ST16 is the group founder (CC16) (Fig. 3). Further inclusion of allelic variants in 4 gene loci (n-4) allowed to comprise also ST480 in the group (Fig. 3). Interestingly, 6 out the 7 isolates (85.7%) resistant to high-level GEN clustered in CC16/ST480, suggesting clonality of high-level aminoglycoside resistant strains. In contrast, strains belonging to ST211 and ST40 presented allelic variants in 6 and 7 loci, respectively, indicating high phylogenetic distance to the other strains (Fig. 3).

Identification of high-level aminoglycoside resistance genes

The complete genomes of the 8 resistant strains were searched for the presence of genes encoding aminonoglicoside modifying enzymes (AME) and conferring high-level resistance to GEN and STR, using ABRicate (Seemann T, *Abricate*, https://github.com/tseemann/abricate). All of the 7 GEN resistant strains were found to carry one copy of the *aac(6')-aph(2'')* gene coding for the bifunctional 6'-aminoglycoside acetyltransferase- 2''-aminoglycoside phosphotransferase enzyme, whose presence in gram-positive bacteria is known to confer resistance to GEN and most other aminoglycosides, except for STR (Chow, 2000) (Fig. 4). A single copy of the *ant(6)-Ia* gene, conferring high-level STR resistance, was found in all the 3 STR resistant strains. The ANT(6)-Ia enzyme is an aminoglycoside O-nucleotidyltransferases with streptomycin as a unique substrate (Hormeño et al., 2018) (Fig. 4). Strain 4774 harbored both *aac(6')-aph(2'')* and *ant(6)-Ia* genes (Fig. 4). Analysis of the genomic location of AME genes showed that they were located in the *E. faecalis* chromosome in 6 out of 8 isolates, whereas in two cases (strains 4953 and 4153) they were carried by plasmids (Fig. 4).

Resistance to fluoroquinolones is due to mutations

Strain 4774 was the only isolate resistant to both CIP and LVX. The genome of 4774 was searched both for the presence of acquired resistance genes and point-mutations in chromosomal genes *gyrA* and *parC*. The strain was found to carry two point mutations that conferred fluoroquinolone resistance in both *gyrA* (Ser83Tyr) and *parC* (Ser80Ile), as also confirmed by MIC results (4 μg/ml for both CIP and LVX).

Discussion

Infertility is a multifactorial clinical condition affecting more than 180 million people worldwide (Inhorn and Patrizio, 2015), and urogenital tract infections are an important contributing factor (Pellati et al., 2008). Chronic and/or incorrectly treated infections are considered to be highly relevant for infertility (Ochsendorf, 2008), as low-grade and prolonged inflammation can negatively impact on human reproductive efficiency. Specifically, inflammatory mediators such as cytokines, chemokines and reactive oxygen species can impair the funtions of Sertoli cells resulting in reduced spermatogenesis and failed acrosome reaction (Pergialiotis et al., 2018; Altmäe et al., 2019; Farahani et al., 2021). Inflammation can also disturb the health status of vaginal microbiome leading to microbial dysbiosis, vaginitis and also bacterial vaginosis (van Oostrum et al., 2013; Onderdonk et al., 2016; Donders et al., 2017; Kalia et al., 2020; Tomaiuolo et al., 2020). Moreover, as these infections may remain asymptomatic, they can be transmitted to the uninfected partner further contributing to fertilization and pregnancy failure. Yet, as urogenital infections are potentially treatable causes of infertility, an improved effort should be done to identify and successfully treat infections caused by microbial pathogens associated to couple infertility.

In the present study, we have characterised a collection of *E. faecalis* clinical strains previously isolated from genital samples of infertile couples (Ricci et al., 2018) and thereby here denominated as infertility-associated *E. faecalis* (IAF). Although *E. faecalis* did not cause an overt infection in either partner, it negatively affected sperm parameters and levels of vaginal lactobacilli, likely contributing to the observed IVF failure (Ricci et al., 2018). Starting from these findings, we have here investigated the antibiotic susceptibility and population structure of 41 IAF clinical isolates for an improved management of antibiotic treatment of urogenital infections in infertile couples.

Several epidemiological studies reported the prevalence of HLA resistant *E. faecalis* strains isolated from different body sites (Kawalec et al., 2007; Quiñones et al., 2009; Butcu et al., 2011; Solheim et al., 2011; Jabbari Shiadeh et al., 2019), however, to our knowledge, no previous work has specifically described the antibiotic resistance profile of IAF isolates. The majority of IAF isolates (33/41) here characterised were susceptible to antibiotics routinely used in clinical practice (Fig. 1). The 8 remaining strains were resistant to high-level aminoglycosides (HLA), and 1 of those (strain 4774) was also resistant to fluoroquinolones (Table 1). Distribution

of HLA resistant *E. faecalis* clinical strains varies based on geographic areas and source of isolate (Agudelo Higuita and Huycke, 2014). In this study, the prevalence of high-level gentamicin and streptomycin resistance in IAF isolates was 17.1% and 7.3%, respectively. The 2 strains displaying high-level resistance to both gentamicin and streptomycin were the vaginal isolates (5034 and 4774), in accordance with a report by Quinones *et al.* describing a MDR phenotype associated to community-acquired vaginal isolates over a 5 year-period in Cuba (Quiñones et al., 2009). Compared to the most recent european surveillance report (Antimicrobial resistance in the EU/EEA (EARS-Net) - Annual Epidemiological Report for 2019, 2020), high-level gentamicin resistance rate in IAF strains was significantly lower than both the european (26.6) and italian (38.2) mean rate percentages, which however, did not specifically refer to UTI or genital infections.

MLST analysis showed a highly divergent IAF population. However, a homogeneous group was found to be constituted by 5 out the 8 HLA resistant strains which all belong to CC16 (Figs 2 and 3), which is widely spread among both hospital and community isolates throughout Europe (Ruiz-Garbajosa et al., 2006; Kuch et al., 2012). Moreover, the 4774 vaginal strain also belonged to the closely related ST480, indicating that resistance of IAF strains to clinically relevant drugs mostly clustered within CC16 and ST480. Consistent with our data, a recent genomic analysis on *E. faecalis* strains collected from 16 middle-east and african countries showed that CC16 was the most predominant complex and almost all the ST16/ST480 strains originated from UTI and CAUTI and were resistant to high-level gentamicin (Farman et al., 2019). Association of high-level gentamicin resistance with ST16 was also observed in european community-acquired isolates (Kuch et al., 2012), suggesting that high-level gentamicin resistant *E. faecalis* strains causing urogenital infections may cluster in ST16 (CC16) and ST480. In constrast, our results showed that mono-resistance to streptomycin (strain 4953, CC40) was clonally distant from CC16/ST480. Interestingly, an *E. faecalis* epidemic clone circulating in Poland over a 10 years-period, was enriched with MDR virulent strains mostly belonging to ST40 (CC40) (Kawalec et al., 2007). In our case, CC40 was indeed the largest CC of the IAF population gathering 26.8% of the isolates, but did not exhibited resistance to antibiotics used in clinical practise. None of the 8 HLA resistant strains belonged to the high-risk enterococcal clonal complexes (HiRECCs) CC6 (previously referred as CC2) and CC9, which are well

adapted to the hospital environment and are capable of global dissemination (Ruiz-Garbajosa et al., 2006; Kawalec et al., 2007; Kuch et al., 2012; Sparo et al., 2018).

HLA resistance in enterococci in the clinical setting is generally mediated by enzymatic drug modification by AMEs, including phosphotransferases (APH), acetyltransferases (AAC), and nucleotidiltransferases (ANT) (Chow, 2000; Ramirez and Tolmasky, 2010). The most common gene is *aac(6')-Ie-aph(2'')-Ia*, encoding the bifunctional enzyme AAC(6')-APH(2''), that confers resistance to virtually all clinically available aminoglicosydes except for streptomycin (Leclercq et al., 1992) and partially to arbekacin (Chow, 2000). Also in our case, 7 out of 8 HLA resistant isolates carried the *aac(6')Ie-aph(2'')Ia* gene, were resistant to high-level gentamicin (MIC ≥1024 μg/ml), and showed a clear clonality within CC16/ST480 (Figs. 3 and 4). On the other hand, the *ant(6)-Ia* gene was identified in the 2 vaginal isolates (strains 4774 and 5034) and in strain 4953 which all showed resistance to high-level streptomycin (MIC ≥2048 μg/ml) (Fig. 4). As previously described (Werner et al., 2001), the *ant(6)-Ia* gene was found to be part of the known gene cluster *ant(6)-sat4A-aph(3')-III*, which mediates resistance to all aminoglycosides (except for gentamicin) and streptothricin (De Giorgi, in preparation). Indeed, the 3 high-level streptomycin resistant isolates were also resistant to kanamycin and streptothricin (De Giorgi, in preparation). In addition, strain 4774 was also resistant to ciprofloxacin and levofloxacin due to 2 previously described point-mutations in both *parC* (S83Y) and *gyrA* (S80I) (Kanematsu et al., 1998; Hooper and Jacoby, 2015), suggesting it as an MDR (Gm^R Sm^R Cip^R Lvx^R) isolate probably generated by antibiotic selective pressure.Whole genome sequencing analysis of HLA resistant isolates showed that the resistance genes were located either on the chromosome (6/8 strains) or on plasmids (2/8 strains) (De Giorgi, in preparation). Interestingly, IAF isolates resistant to clinically relevant antibiotics showed a strong clonal structure, as all the chromosomally-located HLA resistance genes and the fluoroquinolone-conferring resistance mutations clustered within CC16/ST480.

In conclusion, subclinical urogenital infections may seriously threaten human reproduction efficacy. Therefore, antibiotic treatment should be considered even in asymptomatic couples to improve semen features and reduce couple infertility rates. In this study, most IAF clinical isolates were susceptibile to antibiotics commonly employed to treat *E. faecalis* UTI. Nevertheless, antibiotic resistance to HLA and fluoroquinolones was still observed in 20% of the isolates, which showed clonality in CC16/ST480. HLA resistance, a key marker of

enterococcal antibiotic resistance worldwide, needs to be carefully monitored as it impedes the synergistic effect of aminoglicosides with cell-wall active agents which is the frontline therapy for enterococcal complicated UTI, endocarditis and other lifethreating systemic infections. Therefore, understanding the molecular epidemiology as well as the antimicrobial resistance of *E. faecalis* isolated from the genital tract is important not only to prevent UTI and possibile subsequent systemic infections, but also to provide a tailored-therapy to infertile couples prior to undergoing long-lasting, expensive and sometimes unsuccessful assisted reproductive technology (ART) treatments.

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Tables and Figures

Table 1. Antibiotic resistance patterns in Infertility-Associated *E. faecalis* (IAF)

Isolates (n)	Resistance pattern [a]
5	Gm^R
1	Gm^R Sm^R Cip^R Lvx^R
1	Gm^R Sm^R
1	Sm^R
8	**Total**

[a] Gm^R, high-level gentamicin resistance; Sm^R, high-level streptomycin resistance; Cip^R, ciprofloxacin resistance; Lvx^R, levofloxacin resistance.

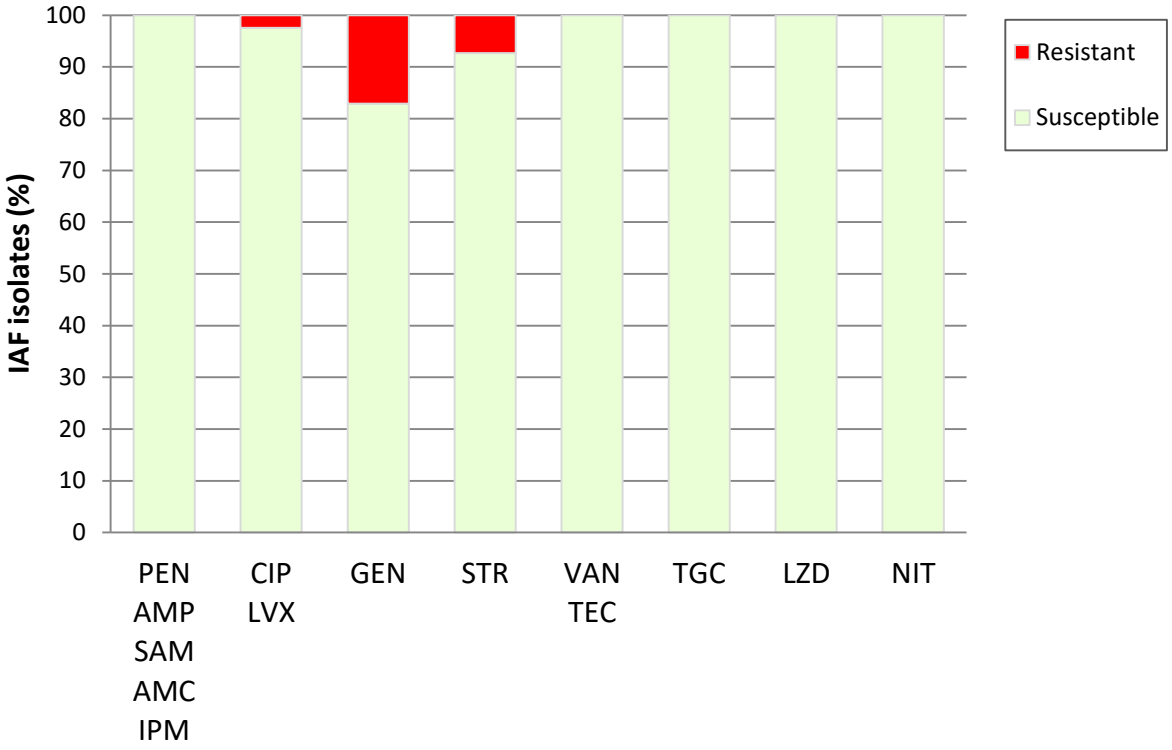

Figure 1. Antibiotic susceptibility profile of infertility-associated *E. faecalis*. A total of 41 *E. faecalis* clinical isolates were tested for their susceptibility to β-lactams (PEN, AMP, SAM, AMC, IPM), quinolones (CIP, LVX), high-level aminoglycosides (GEN, STR), glycopeptides (VAN, TEC), tetracyclines (TGC), oxazolidones (LZD) and nitrofurans (NIT). Results were obtained by VITEK-2 and confirmed by both MIC (Sensititre GPN3F plate) and diffusion-disk methods. Antibiotic susceptibility testing was performed according to EUCAST guidelines. Resistant (red), Susceptible (light green).

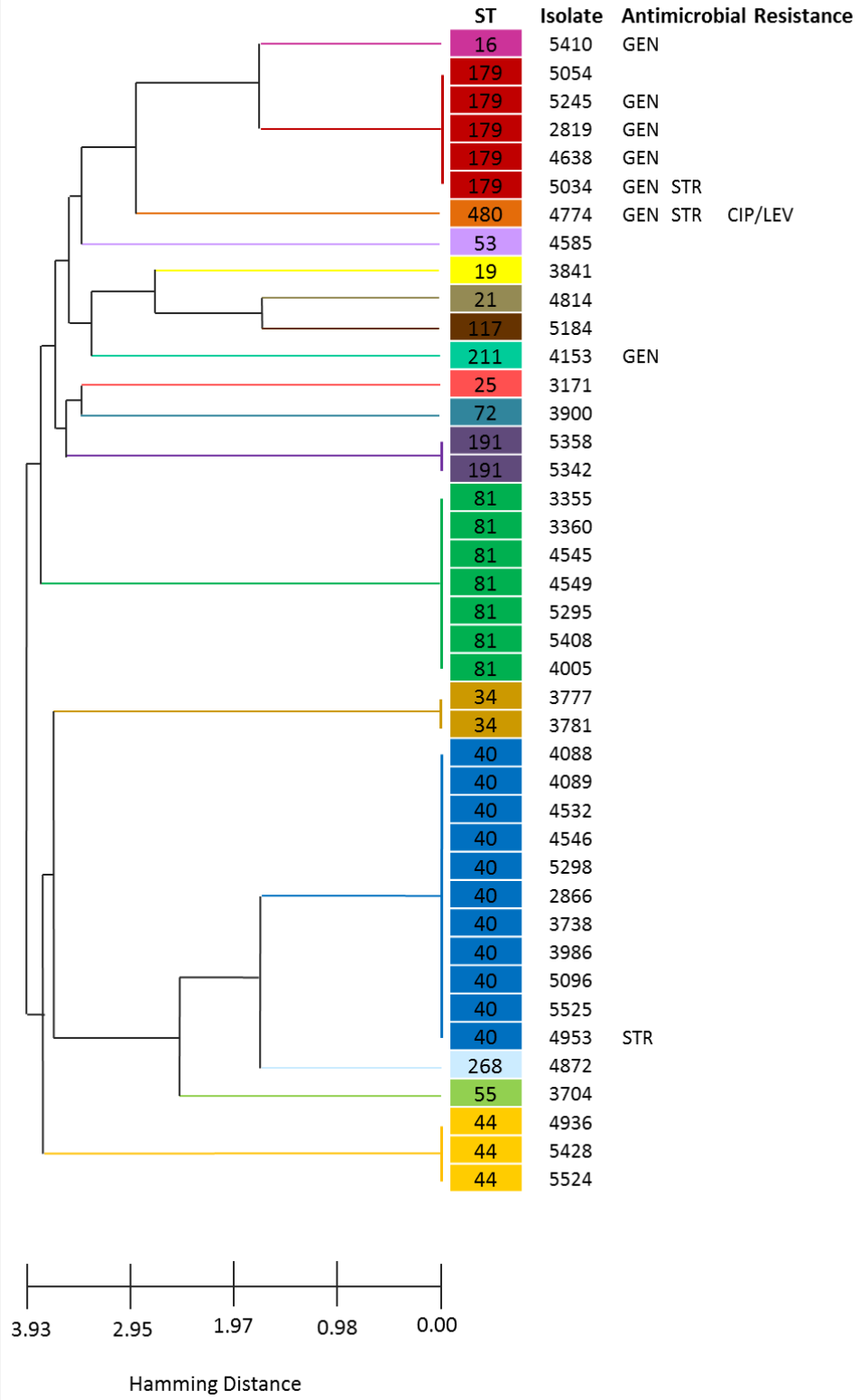

Figure 2. Unweighted pair group method with arithmetic mean (UPGMA) dendrogram of infertility-associated *E. faecalis* isolates based on sequence type (ST). The UPGMA dendogram was constructed starting from the matrix of pairwise allelic differences of the 7 loci defining the ST of *E. faecalis* by using PHYLOViZ v2.0 (Francisco et al., 2012). The two groups with the lowest number of allelic differences were combined into a higher level cluster, and the process was reiterated until the most distant groups were linked (Michener and Sokal, 1957). The mean distance between any two clusters was measured by the Hamming Distance, defined as the number of positions at which two aligned sequences differ (Mohammadi-Kambs et al., 2017). For each ST, isolate number and antimicrobial resistance according to EUCAST guidelines are shown. GEN, gentamicin; STR, streptomycin; CIP, ciprofloxacin; LEV, levofloxacin.

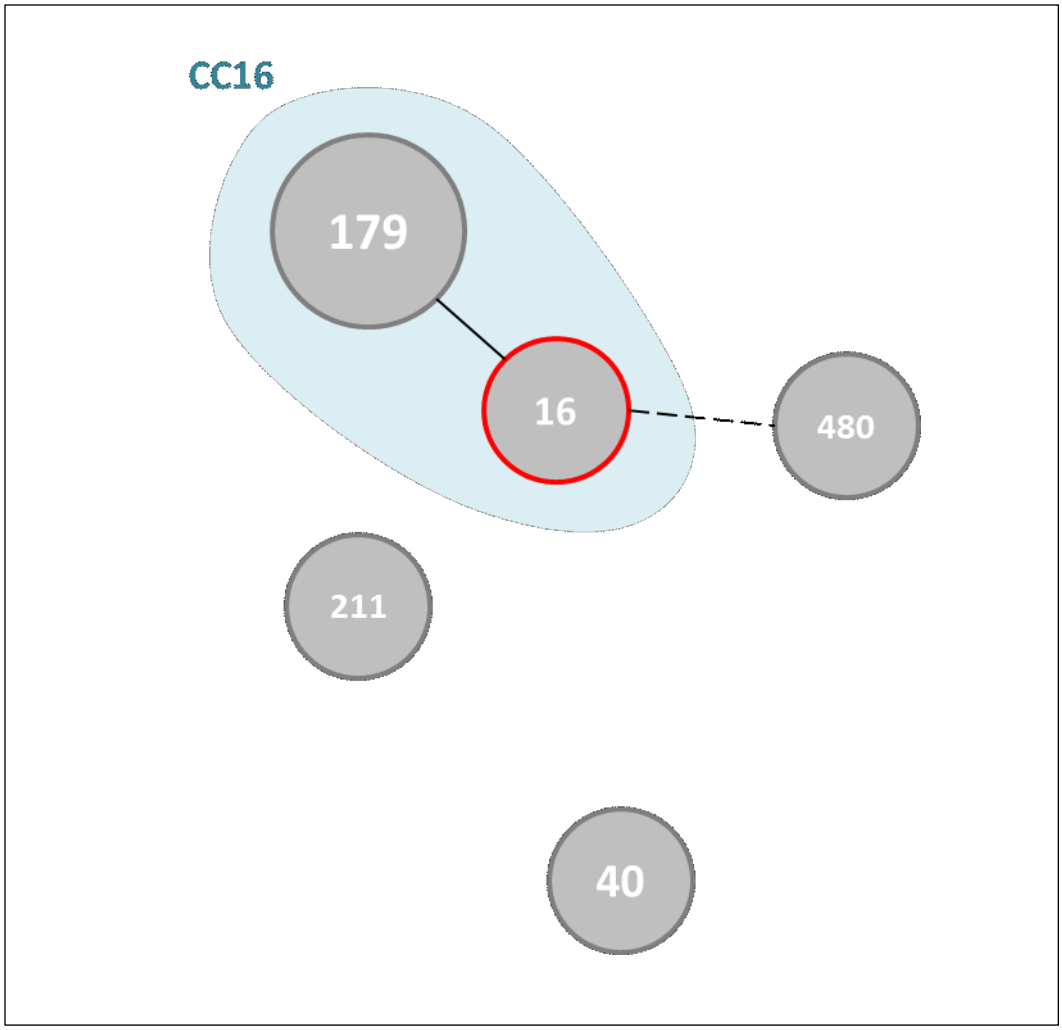

Figure 3. Minimum spanning tree of infertility-associated *E. faecalis* strains resistant to high-level aminoglycosides. The minimum spanning tree was constructed using PHYLOViZ v2.0 (Francisco et al., 2012) based on the goeBURST algorithm, which is a refinement of eBURST by Feil *et al.* (Feil et al., 2004). goeBURST divides large MLST data into nonoverlapping groups of related STs or CCs and then distinguishes the most parsimonious groups of isolate descendants within each CC from the predicted founder. In *E. faecalis*, as the number of loci (n) defining ST is 7, the tree structure can be drawn at 7 levels of relatedness. The present diagram includes the 8 enterococcal HLA resistant isolates which are grouped into 5 different STs. Each ST is represented as a node whose size varies based on the number of isolates. ST179 contains 4 isolates (all Gm^R, except for one strain which is Gm^R Sm^R), while ST16 (Gm^R), ST480 (Gm^R Sm^R), ST211 (Gm^R) and ST40 (Sm^R) comprise 1 isolate each. ST16 and ST179 belong to the same CC with ST16 being the CC16 founder (red circle). The level of relatedness between ST16 and ST79 (n-1, solid line) and between ST16 and ST480 (n-4, dotted line) are also shown. ST211 and ST40 are more distantly related (>n-4, no connecting lines).

Strain	CC [a]	MIC (µg/mL)		Aminoglycosides modifying enzyme (AME) genes		
		GEN	STR	GEN	STR	Genomic location
				aac(6')-aph(2'')	*ant(6)-Ia*	
2819	16	1024	64			Chromosome
4638	16	8192	64			Chromosome
5245	16	4096	64			Chromosome
5410	16	2048	16			Chromosome
5034	16	8192	8192			Chromosome
4774	480	8192	2048			Chromosome
4153	211	8192	64			Plasmid
4953	40	16	4096			Plasmid

Figure 4. Genetic bases of resistance to aminoglycosides in infertility-associated *E. faecalis*. For each strain, isolate number, CC, MICs of gentamicin (GEN), streptomycin (STR), and related aminoglycoside modifying enzyme (AME) genes are shown. Antibiotics recommended by EUCAST are GEN and STR. MIC values ≥1024 µg/ml were regarded as high-level aminoglycoside (HLA) resistance (red boxes). Resistance to GEN by *aac(6')-aph(2'')* covers resistance to virtually all aminoglicosides, including tobramycin, amikacin, kanamycin, netilmicin and dibekacin. Search of AME genes conferring resistance to aminoglycosides was performed on the genomes of the 8 HLA strains by using the ABRicate tool on ARG-ANNOT, CARD, Megares and Resfinder databases. All the AME genes with a coverage and identity ≥99% are shown (light blue boxes). Gene nomenclature is according to Shaw *et al*. (Shaw *et al*., 1993). MIC values of *E. faecalis* reference strain OG1RF was 1 µg/ml and 512 µg/ml for GEN and STR, respectively.

[a] CC, clonal complex. ST, sequence type. Strain 4774 (ST480) and 4153 (ST211) are singletons. Singletons are defined as STs differing at two or more alleles from every other ST in the population (Feil et al., 2004).

Supplementary material

Table S1. Sequence type (ST) and clonal complex (CC) distribution in infertility-associated *E. faecalis*.

ST	Frequency (%)	CC [a]
40	11 (26.8)	40
81	7 (17.1)	81*
179	5 (12.2)	16
44	3 (7.3)	44*
34	2 (4.9)	34*
191	2 (2.9)	191*
16	1 (2.4)	16
19	1 (2.4)	19*
21	1 (2.4)	21
25	1 (2.4)	25*
53	1 (2.4)	53*
55	1 (2.4)	55*
72	1 (2.4)	72*
117	1 (2.4)	21
211	1 (2.4)	211*
268	1 (2.4)	40
480	1 (2.4)	480*

[a] CCs were obtained by blasting the IAF isolates with the *E. faecalis* MLST database (https://pubmlst.org/organisms/enterococcus-faecalis) using goeBURST. Different STs were grouped into the same CC when they differed in 1 or 2 out of the 7 gene loci used for typing *E. faecalis* strains.

*, IAF singletons. Singletons are defined as STs differing at two or more alleles from every other ST in the population (Feil et al., 2004).

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CHAPTER 5

Effect of hormone therapy on the vaginal microbiota of women undergoing *in vitro* fertilization

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Running title: Vaginal microbiota and hormone treatment in IVF

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Henry Fold Hospital
Frida Kahlo, 1932

Abstract

Background. A healthy vaginal microbiota is generally dominated by *Lactobacillus* species, and alterations of this population correlates with increased risk of genital infections and poor reproductive outcomes. The vaginal microbiota is a dynamic ecosystem whose composition and functions are influenced by various factors, including hormones. As therapy with gonadotropins is a key step in controlled ovarian hyper-stimulation (COH) prior to *in vitro* fertilization (IVF) and embryo transfer (ET), we have here evaluated the effect of hormone therapy on the vaginal microbiota of females undergoing IVF-ET.

Methods. A cohort of 108 infertile female patients subjected to hormone therapy were prospectively enrolled in the study. Two vaginal swabs, before and after therapy, were collected from each patient. Vaginal swabs were analysed by standard culture methods for the presence of genital tract pathogens and lactobacilli. Changes in the vaginal microbiota were assessed by using a vaginal diamine assay (VADA) for the quantification of diamines, which are considered as biomarkers of the catabolic activity of bacteria involved in bacterial vaginosis (BV).

Results. Concentration of diamines in vaginal fluids was significantly higher in patients treated with gonadotropins compared to pre-treatment controls (mean increase=35.33 μM) ($p<0.0001$). Analysis of samples post-treatment showed that the vaginal microbiota shifted towards a population mainly constituted by streptococci, enterococci, enterobacteria, staphylococci and fungi, which are prevalent microorganisms in aerobic vaginitis (AV). A highly significant association between the presence of the vaginal pathogens and reduced levels of lactobacilli ($<10^4$ cfu/swab) was also found ($p\leq0.0001$), and hormone therapy further altered the composition of the vaginal microbiota by fostering the overgrowth of pathogens at the expenses of lactobacilli ($p<0.0001$). Decreased levels of lactobacilli was also significantly associated to high concentrations of diamines (>25 μM) ($p<0.0001$). Almost 95% of patients with diamine levels ≤25 μM had normal amounts of lactobacilli ($\geq10^4$ cfu/swab), suggesting the value of 25 μM as a threshold for a healthy microbiota. Finally, 48.2% of the patients with both vaginal pathogens and diamine levels >25 μM had a failed embryo implantation following IVF-ET.

Conclusions. This study shows that, upon hormone therapy, an increase of vaginal diamines as well as a transition from a *Lactobacillus*-dominated to an aerobic/facultative-anaerobic population occurred. The presence of both markers was predictive of an adverse reproductive outcome.

Introduction

About 30 years after the birth of the first 'test tube' baby, *in vitro* fertilization (IVF) and embryo transfer (ET) has become a widely available treatment for several causes of infertility. In order to enhance the efficiency of IVF procedures, high doses of exogenous gonadotropins are administered to stimulate the development of multiple oocytes in a single cycle. Ovarian hyper-stimulation (COH) with gonadotropins promotes the growth of multiple follicles in the pre-ovulatory stage by interfering with the physiological mechanisms which normally ensure the selection of a single dominant follicle. The use of such ovarian stimulation protocols may enable the selection of one or more embryos for transfer, while supernumerary embryos can be cryopreserved for later cycles (Macklon et al., 2006). However, despite ongoing advances in assisted reproductive technologies (ART), delivery rates remain stable around 24% (de Mouzon et al., 2020).

The health status of the female genital tract is largely related to the presence of a normal vaginal microbiota (Ravel et al., 2011; Smith and Ravel, 2017). The vaginal environment has long been considered to be controlled by estrogens (Amabebe and Anumba, 2018). Although microorganisms are quite adaptable to changes in their environment, they are quantitatively and qualitatively affected by changes in the substrate on which they grow. Throughout the menstrual cycle, during pregnancy and also in menopause, hormonal and other physiological changes occur that are accompanied by alterations in the vaginal microbiota (Smith and Ravel, 2017). Perturbations of the vaginal microbiota may lead to microbial 'dysbiosis', including bacterial vaginosis (BV) and aerobic vaginitis (AV) (Kaambo et al., 2018; Kroon et al., 2018). BV is a vaginal flora disorder which occurs in up to 30% of women of child-bearing age (Livengood, 2009). In BV, the normal vaginal microbiota, mainly composed of *Lactobacillus* spp., decreases and is mostly replaced by anaerobic microorganisms (Leitich et al., 2003; Fredricks et al., 2005; Onderdonk et al., 2016). BV is prevalent among infertile women due to tubal/pelvic factors (van Oostrum et al., 2013) and has been linked to low pregnancy rates in patients subjected to IVF treatments (Haahr et al., 2019). However, BV is also present in a variable percentage (6–63.7%) of pregnant women (Guaschino et al., 2003; Hogan et al., 2007), but it has been associated to increased risk of miscarriage and pre-term delivery (Ralph et al., 1999; van Oostrum et al., 2013; Donders et al., 2017). AV is an inflammatory condition where the normal vaginal microbiota

shifts to a population dominated by enterobacteria, staphylococci, streptococci and enterococci (Donders et al., 2002, 2017). AV differs from BV in some features, but they both share the displacement of the normal *Lactobacillus* flora towards genital pathogens (Kaambo et al., 2018) and the association with negative reproductive outcomes (*i.e.* pre-term birth) (Donders et al., 2011).

In contrast to AV, a hallmark of BV is the positive reaction to the "whiff" test (fishy amine odor) originally described by Pheifer *et al.* (Pheifer et al., 1978) and still used in clinical practice. The test based on the detection of amines by their typical odor upon addition of potassium hydroxide to the vaginal fluid. Diamines are small aliphatic hydrocarbon molecules produced by anaerobic bacteria of the human vaginal microbiota (Matthies et al., 1989; Arora, 2015). Recent studies have shown that the production of diamines, such as putrescine and cadaverine, is typical of BV, while vaginal samples from healthy women do not contain diamines (Delaney and Onderdonk, 2001; Wolrath et al., 2002; Cox et al., 2015; Chen et al., 2018). To evaluate the feasibility of using DA as biomarkers of vaginal health/disease status, the enzymatic test 'vaginal diamine assay' (VADA) has been developed, which detects and quantifies diamines in clinical samples (Mendonca et al., 2015). Its simplicity and low cost makes it applicable in routine diagnostic tests.

The aim of the present study is to evaluate the effect of treatment with exogenous gonadotropins on the vaginal microbiota of women undergoing CHO prior to IVF and embryo transfer (ET) by simultaneously assessing the levels of vaginal diamines by VADA and the composition of the bacterial flora by traditional bacteriological techniques. Data were then associated to the outcome of IVF-EF procedures.

Materials and Methods

Patients

A cohort of 108 infertile patients, aged >39 years undergoing COH for IVF at the Centre for Diagnosis and Treatment of Couple Sterility were enrolled over a 6 month-period. All patients were unable to conceive naturally for at least 1 year before entering the study. Exclusion criteria were antibiotic therapy a month before the beginning of the study and collection of vaginal samples >1 month before IVF-ET (exclusion just from the reproductive outcome analysis). All participants signed a written informed consent, and the study protocol was approved by the ethic regional commission (CEAVSE, Comitato Etico Regione Toscana Area Vasta Sud-Est).

Ovulation induction

Ovarian stimulation was performed by administering recombinant gonadotropins (Pergoveris, Merck-Serono, Rome, Italy) at a dose of 150-200 IU per day from the first or second day of spontaneous or induced menstruation. The dose of gonadotropins was adjusted according to ovarian response evaluated by ultrasound examination. As soon as the dominant follicle reached 14 mm in diameter, a gonadotropin-releasing hormone (GnRH) antagonist was administered daily (Orgalutran, MSD and Cetrotide, Merck Serono) until ovulation was induced. Ovulation triggering was performed by injection of human chorionic gonadotrophin (Gonasi 10,000 IU, IBSA, Italy or Ovitrelle, Merck Serono, Italy) when at least three follicles had reached 18 mm in diameter. The oocyte pick-up was performed 34-36 hours after ovulation triggering.

Reproductive outcome analysis

Reproductive outcome was evaluated as implantation rate (number of gestational sacs divided by the number of embryos transferred) and clinical pregnancy rate (ultrasound proven foetal heartbeat after 7 weeks of gestation).

Samples

Two vaginal swabs were collected from each patient. The first sample was obtained during the screening visit one month before hormone therapy, while the second swab at the time of oocyte

collection one month after hormone therapy. All samples were sent to the laboratory of clinical microbiology for analysis.

Detection and identification of cultivable bacteria

Standard bacteriological culture methods were used to identify genital pathogens from vaginal swabs. The rayon swabs (FL Medical, Padova, Italy) were resuspended in 1 ml of sterile 0.9% NaCl solution with 10% glycerol. The wet swab was directly used for plating onto agar plates, while the saline liquid was employed for assessing the concentration of diamines (VADA, see below). Selective and differential solid media, including Columbia Blood, Columbia CNA, Gardnerella, Mannitol Salt, MacConkey, Sabouraud and Schaedler agar plates (all from Oxoid, Milan, Italy) were used to detect cultivable gram-positive bacteria, gram-negative bacteria, lactobacilli, anaerobes and fungi. Microbial identification was carried out by using Matrix Assisted Laser Desorption Ionisation-Time Of Flight (MALDI-TOF) VITEK MS (Biomérieux Italia S.p.A., Florence, Italy) coupled with the Myla software v2.0 with a cut-off identification ≥99%. Vaginal swabs were regarded as positive to genital pathogens at viable counts $\geq 10^5$ CFU/swabs.

Quantitative analysis of lactobacilli in vaginal swabs was carried out on Rogosa agar (Oxoid). Presence of lactobacilli was regarded as 'normal' when CFU counts were $\geq 10^4$ CFU/swab, and 'low' at values $< 10^4$ CFU/swab. Assay detection limit was 10^2 CFU/swab.

Vaginal diamine assay (VADA)

The diamine content in vaginal swabs was quantified by using 100 µl of the samples by using the VADA. VADA is an enzymatic assay based on diamine oxidase (DAO) which catalyzes the production of H_2O_2 upon reaction with diamines such as putrescine and cadaverine, as previously described (Mendonca et al., 2015). H_2O_2 reacts with horseradish peroxidase in the presence of 4-aminoantipyrine and phenol to produce the chromogen quinone imine, which is detected by spectrophotometer measurement at the wavelength of 510 nm (peak of maximum absorbance of quinone imine). Clinical samples were tested both with and without addition of DAO to check for the presence of H_2O_2 physiologically produced by vaginal lactobacilli or some human cells, including vaginal macrophages. For each sample, the optical density at 510 nm (OD_{510}) obtained without addition of DAO was subtracted from that obtained with DAO. The resulting OD_{510} was

used to calculate the concentration of diamines using a reference standard curve obtained with a mixture of cadaverine and putrescine at 1:1 molar ratio (range 4-256 mM). Results were expressed as μM of diamine per sample.

Statistical Analyses

Statistical analyses were performed on the microbiological and diamine data pre-HT and post-HT by using the software GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA). Statistical comparisons between diamine concentrations pre-HT and post-HT were performed by the Kolmogorov-Smirnov test ($p<0.05$). The Chi square test (with Yates' correction; $p < 0.05$) was employed to assess, in both pre-HT and post-HT vaginal samples, the relation between the presence of genital pathogens and vaginal lactobacilli, and between diamine concentrations and vaginal lactobacilli. The Fisher's exact test ($p<0.05$) was performed to associate the IVF-ET outcome (evaluated as implantation rate) with diamine concentrations and amount of vaginal lactobacilli.

Results

In this study, 108 infertile women were evaluated for the presence of genital tract pathogens and for the production of diamines in vaginal samples before and after therapy with exogenous gonadotropins prior to being subjected to IVF-ET. Microbiological analysis was conducted on 216 vaginal swabs, of which 108 collected before (pre-HT) and 108 after (post-HT) hormone therapy at the time of oocyte collection. A total of 165 clinical strains belonging to 17 different microbial species was detected in the samples tested (Table 1). *Streptococcus* spp. (Group A, C, G and F *Streptococcus*) represented the most common finding with a total (pre-HT+post-HT) prevalence of 23.6% (39/165), followed by *Streptococcus agalactiae* (17%), *Candida* spp. (*C. albicans* and *C. glabrata*; 15.2%), *Escherichia coli* (13.3%) and *Enterococcus faecalis* (12.7%), (Table 1).

Prevalence and etiology of genital tract infections in female patients undergoing IVF-ET

Microbiological analysis of vaginal swabs from 108 women undergoing IVF showed that 34.3% (37/108) of pre-HT samples were positive for at least one genital tract pathogen, whereas the number of positive samples post-HT rose to 78.7% (85/108) (p=0.0007). In particular, the microbial species/group that showed the highest increase after treatment was *Streptococcus* spp. (5.5-fold increase), followed by *E. faecalis* (2.5-fold increase) and *Candida* spp. (1.8-fold increase) (Table 1). Of note, enterobacteria other than *E. coli* (19/108 swabs) and *Staphylococcus* spp. (9/108 swabs) were detected only in post-HT samples (Table 1). The data suggest that therapy with exogenous gonadotropins promote the onset of vaginal infections caused by microrganisms belonging to streptococci, enterococci, enterobacteria, staphylococci and fungi.

Impact of hormone therapy on the vaginal microbiota

The vaginal microbiota of patients pre-HT and post-HT was analyzed by determining the simultaneous presence of genital tract pathogens and lactobacilli in vaginal swabs. A highly significant association ($p \leq 0.0001$) between genital pathogens and reduced amounts of vaginal lactobacilli ($<10^4$ cfu/swab) was found in the study population (Figure 1). The number of patients with altered vaginal microbiota was 15/108 (13.9%) pre-HT (Figure 1A) and it increased by 2.6

folds to 39/108 (36.1%) post-HT (Figure 1B), suggesting that hormone treatment negatively impacts on the *Lactobacillus* flora thus favoring the overgrowth of genital pathogens.

Hormone therapy correlates with increased concentration of diamines and is associated to reduced levels of lactobacilli

Diamines were quantified in the vaginal fluids of all 108 females pre-HT and post-HT. Hormone therapy significantly impacted on diamine levels. The pre-HT group had low diamine concentrations (6.13±16.22 μM), whereas the post-HT group showed higher levels of diamines (41.46±48.08 μM), with a significant increase in diamines in vaginal fluids (35.33±4.88 μM) following hormonal treatment (Kolmogorov-Smirnov test; p <0.0001) (Figure 2). Almost 95% (102/108) of pre-HT females had a diamine concentration lower than 25 μM, while the percentage of patients with low level diamines (≤25 μM) dropped to 43.5% (47/108) after treatment (p<0.0001, Figure 3A). In order assess a possible association between increased diamine concentration and reduced vaginal lactobacilli, analysis was performed just on the patients with low levels of diamines (≤25 μM) before hormone treatment (n=102). The frequency of patients with low amounts of lactobacilli (<10^4 CFU/swab) raised from 17.6% (18/102) pre-HT to 43.1% (44/102) post-HT (p<0.0001, Figure 3B). The above results allowed to split the patient population into two groups. The first was characterised by a diamine concentration ≤25 μM and a normal lactobacilli flora, while the second presented a diamine concentration >25 μM and a vaginal microbiota with reduced quantity of lactobacilli.

Vaginal microbiota dysbiosis and high diamine concentrations after hormone therapy are associated with low implantation rates

Finally, the outcome of implantation after IVF-ET was assessed in the study population. Out of 108 enrolled patients, 12 did not complete the IVF-ET cycle and 10 did not reach the ET stage due to failed fertilization. Thus, a total of 86 women were included in the analysis of reproductive results. The relationship between the presence of genital tract pathogens, concentration of diamines >25 μM, and IVF-ET outcome was examined (Fig. 4). Embryo implantation failed in 71 patients (82.6%). Of these, 41 females (47.7%) were positive for both the presence of vaginal pathogens and high level diamines, 12 were positive only for genital pathogens and 12 only for elevated concentrations of diamines (Fig. 4). The negative outcome

was significantly higher in women positive for both genital pathogens and high level diamines (41/46, 89,1%) compared to patients presenting with just one of the above risk factors (24/34, 70.6%) (p=0,0454). To be noted that 6 females devoid of both risk factors still had a failed implantation. A total of 15 patients (15/86, 17.4%), although positive for vaginal pathogens and/or high level diamines, had a successful implantation (Fig. 4).

Discussion

The composition of the vaginal microbiota differs between women and can also fluctuate throughout a woman's life (Ravel et al., 2011; Smith and Ravel, 2017). It is now acknowledged that the microbial species and their relative abundance in the vagina can greatly affect female health and disease, conception, pregnancy and reproductive outcomes. The vaginal microbiota, in turn, may be altered by exogenous and endogenous factors, including hormones (Amabebe and Anumba, 2018). Despite ongoing advances in ART procedures and broad availability of IVF-ET treatments, pregnancy rates still remain low (de Mouzon et al., 2020). Several biological, environmental and clinical factors can influence the outcome of IVF-ET (Baker et al., 2010). As COH is a key step of IVF, we have investigated the effects of therapy with exogenous gonadotropins on the vaginal microbiota and embryo implantation outcome of a cohort of female patients undergoing IVF-ET. The present results demonstrate that, following hormone therapy, both a transition from a *Lactobacillus*-dominated to a pathogen-enriched microbial population and a rise in vaginal diamines occurred and were predictive of failed embryo implantation in the patients.

High-throughput 16S rRNA sequencing studies on the composition of the vaginal microbiota in reproductive-age women evidenced the existence of at least five 'community state types' (CSTs) of microbiota, of which four (CST I, II, III and V) are dominated by *Lactobacillus* species. In contrast, CST-IV lacks lactobacilli while presenting large numbers of anaerobic bacteria (Ravel et al., 2011; Gajer et al., 2012). CST-IV is microbiologically similar to BV, which is also characterized by the presence of strict and facultative anaerobes, including *Gardnerella vaginalis*, *Atopobium vaginae*, *Prevotella* spp., *Mobiluncus* spp., *Peptostreptococcus* spp., *Mycoplasma* spp. and other species (Onderdonk et al., 2016). On the other hand, AV has been described by the 'aerobic' corresponding condition of BV (Donders et al., 2002). BV and AV are vaginal disorders that differ under several aspects, including the bacterial species involved, the lack *vs.* presence of local inflammation, the type of vaginal discharge and other clinical signs/symptoms (Kaambo et al., 2018). Though, a major shared trait consists in the displacement of the normal *Lactobacillus* flora with genital tract pathogens. An increasing body of evidence supports the association between dysbiosis of the vaginal microbiota and higher susceptibility to genital tract infections, strengthening the protective role of *Lactobacillus* spp. Our data showed that, upon hormone treatment, a shift in the vaginal

microbiota occurred from a *Lactobacillus*-based flora to a population composed by streptococci, enterococci, enterobacteria, staphylococci and yeasts (Table 1), which are common microbial pathogens retrieved from women with AV (Donders et al., 2017). The bacterial genus found to be the most abundant after hormone treatment was *Streptococcus* spp. (5.5-fold increase compared to pre-treatment) immediately followed by *Enterococcus faecalis* (formerly *Streptococcus faecalis*) (2.5-fold increase). In agreement with these data, we have also recently reported that *E. faecalis* retrieved from vaginal swabs of infertile females undergoing IVF was significantly associated to reduced levels of vaginal lactobacilli (Ricci et al., 2018).

BV, the most common vaginal disorder of women in reproductive age (van de Wijgert and Jespers, 2017), may have high recurrence rates and is strongly associated with premature rupture of membranes, preterm labor and birth, amnionitis and post-operative abortive infections (Schwebke et al., 1996; Egbase et al., 1999; Haahr et al., 2019). Therefore, we decided to indirectly assess the presence of BV-associated microorganisms by quantifying the levels of vaginal diamines, which are typical catabolites of BV species (Matthies et al., 1989; Arora, 2015), by using a recently developed enzymatic assay. The vaginal diamine assay (VADA) was validated on clinical samples by Mendonca *et al.* (Mendonca et al., 2015). The comparison between the Nugent score, considered the gold standard for the diagnosis of BV, and the VADA showed a sensitivity of 85.71% and a specificity of 100%, proving to be a valid method for dosing diamines in vaginal swabs. Interestingly, hormone treatment affected the production of diamines, as demonstrated by significantly increased concentrations of cadaverine and putrescine in post- compared to pre-treatment patients (Fig. 2). Hence, the above two findings (Table 1 and Fig. 2) appear as incoherent since the production of diamines ('Whiff test') is considered a hallmark of BV rather than AV, as originally described in the Amsel criteria (Amsel et al., 1983). Except for two samples positive for the leading cause of BV, *G. vaginalis* (Table 1), vaginal swabs were subjected to culture-based identification methods rather than molecular approaches which may have underestimated the amount of anaerobes. Nonetheless, whether the genital pathogens identified in the study population were involved in AV or BV or even belonged to the *Lactobacillus*-devoid CST-IV, their occurrence was linked to a dysbiosis of the vaginal microbiota, as shown by the fact that the presence of vaginal pathogens was significantly associated with reduced quantity of lactobacilli (Fig. 1). Interestingly, 82.3% of patients with low concentrations of vaginal diamines (≤25 μM) also had normal amounts of vaginal lactobacilli

(Fig. 3), suggesting that levels of *Lactobacillus* spp. are inversely associated with diamine concentrations and presence of vaginal pathogens. Based on this result, the value of 25 μM was regarded as the diamine threshold to define the 'healthy state' of the vaginal microbioma of female patients undergoing IVF.

In contrast to the microbiota of other human body sites, the vaginal microbiota is characterized by exceptionally low bacterial diversity and high predominance of lactobacilli. Such lack of diversity is even more pronounced during pregnancy, when the vaginal microbial community maintains a stable, homogenous and *Lactobacillus*-dominated state (Romero et al., 2014; Kroon et al., 2018; Greenbaum et al., 2019). Several studies have supported the hypothesis that the vaginal and endometrial microbiota on the day of embryo transfer affects pregnancy outcome (Fanchin et al., 1998; García-Velasco et al., 2017; Tsonis et al., 2021). A microbiota mainly constituted by lactobacilli with a large abundance of *Lactobacillus crispatus*, is regarded as the most favorable condition for predicting higher rates of reproductive success (Hyman et al., 2012; Koedooder et al., 2019). Consistently with the above reports, almost half (41/86) of the patients presenting after hormone therapy with both a vaginal dysbiosis and high diamine levels (>25 μM) had an adverse IVF-EF outcome, compared to just 5.8% (5/86) of females who obtained a successful embryo implantation despite having both the abovementioned risk factors. Interestingly, the combination of both risk factors was significantly associated to failed implantation (Fig. 4). The involvement of streptococci in vaginal dysbiosis associated with infertility was previously described by Moore *et al.* (Moore et al., 2000), who reported a significant decrease in the rate of live births when *Streptococcus viridans* contaminated the catheter used for embryo transfer. Recently, we have also shown that the presence of *E. faecalis* together with *Mycoplasma hominis* and *Ureaplasma urealyticum* in genital samples of infertile couples undergoing IVF-ET was predictive of poor reproductive outcomes (Ricci et al., 2018), further highlighting the role of *E. faecalis* as an emerging bacterial pathogen associated to couple infertility.

High estrogen levels, as occurring in puberty and pregnancy, are crucial to establish and maintain the vaginal microenvironment in an eubiotic state with a predominance of *Lactobacillus* spp. (Amabebe and Anumba, 2018). On the other hand, estrogen excess may disrupt the balance in the vaginal milieu by altering the resident microbiota. It has been reported that vaginal estrogenization in post-menopausal women favors the infection by *Candida spp*. (Dennerstein

and Ellis, 2001). Likewise, few studies have described that the hyperestrogenism associated to COH in IVF treatments may affect the composition of the vaginal microbiota with adverse effects on reproduction outcomes (Jakobsson and Forsum, 2008; Hyman et al., 2012). Here, the vaginal dysbiosis observed following hormone therapy and subsequent low implantation rates reinforces the link between an eubiotic vaginal microbiota and successful reproductive outcomes in ART procedures. Antibiotic prophylaxis prior to embryo transfer may be employed (and it is used in some ART protocols) to control vaginal colonization by genital pathogens and diminish the risk of ascending infections. However, conflicting results on its effectiveness on pregnancy rates have been reported to date (Egbase et al., 1999; Moore et al., 2000; Kroon et al., 2018). As a preferential selective influence of antibiotics was shown on certain species of lactobacilli at the expenses of others (Melkumyan et al., 2015), caution should be used when considering prophylactic antibiotic therapy in ART procedures.

In conclusion, the present study shows that a shift of the *Lactobacillus*-based vaginal microbiota towards a pathogen-enriched population occurs after hormone treatment, thereby promoting the onset and establishment of vaginal dysbiosis, which in turn may negatively affect the implantation outcome after IVF-ET.

Tables and figures

Table 1. Prevalence of microbial species in vaginal swabs from women prior to IVF.

Microbial species [a]	Hormone treatment [b]		
	Pre	**Post**	**Total**
Streptococcus spp.	6	33	39
Streptococcus agalactiae	12	16	28
Candida spp.	9	16	25
Escherichia coli	10	12	22
Enterococcus faecalis	6	15	21
Other *Enterobacteriales*	0	19	19
Staphylococcus spp.	0	9	9
Gardnerella vaginalis	2	0	2
Total	**45**	**120**	**165**

[a] *Streptococcus* spp. includes streptococci of different groups except for *S. agalactiae* (group B *Streptococcus*) and *E. faecalis* (group D *Streptococcus*); other *Enterobacteriales* comprise *Klebsiella pneumoniae, Klebsiella oxytoca, Proteus mirabilis* and *Citrobacter koserii*; *Staphylococcus* spp. includes *Staphylococcus aureus, Staphylococcus epidermidis* and other coagulase-negative staphylococci*; Candida* spp. comprises *Candida albicans* and *Candida glabrata.*

[b] A total of 216 (108 pre- and 108 post-hormone treatment) vaginal swabs were collected from 108 patients scheduled for IVF and subjected to microbiological analysis.

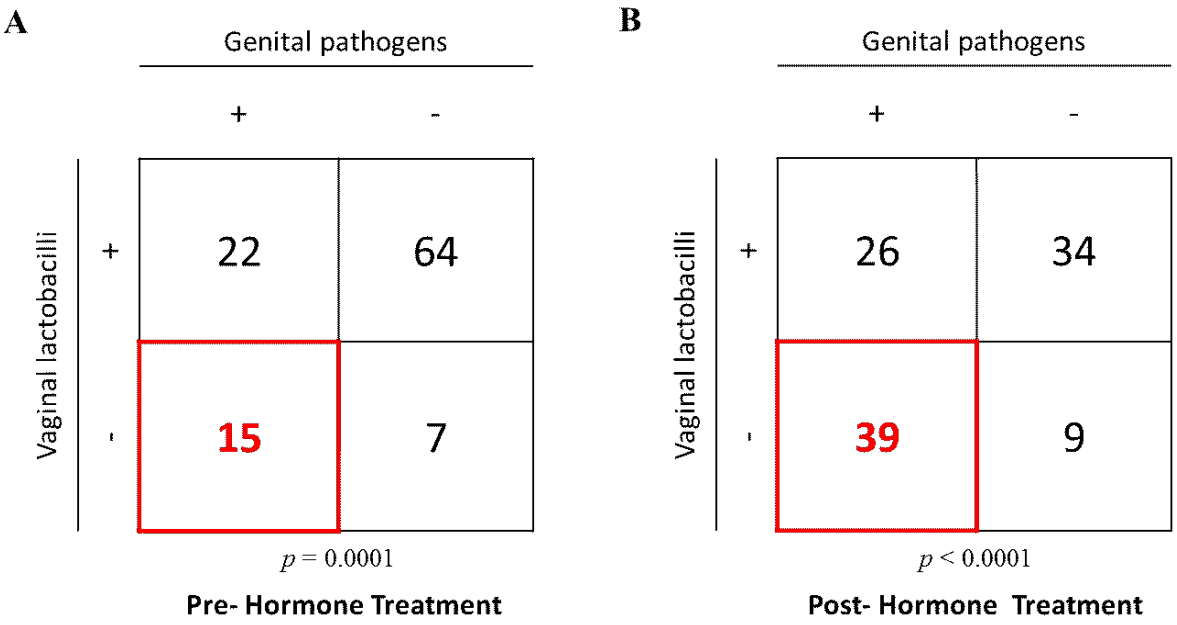

Figure 1. Association between genital tract pathogens and vaginal lactobacilli in female patients before and after hormone treatment prior to IVF. Two vaginal swabs were collected from each patient (n=108). The first sample was retrieved one month before hormone treatment (pre-HT), and the second at the time of oocyte collection one month after HT (post-HT). All the samples were simultaneously tested for the presence of genital tract pathogens and vaginal lactobacilli. **A**. Pre-HT contingency table reporting the number of swabs negative (-) or positive (+) for at least one genital pathogen in relation to the numbers of swabs with normal (+, $\geq 10^4$ CFU/swab) or reduced (-, $<10^4$ CFU/swab) levels of lactobacilli (p=0.0001). **B.** Post-HT contingency table reporting the number of swabs negative (-) or positive (+) for at least one genital pathogen in relation to the numbers of swabs with normal (+) or reduced (-) levels of lactobacilli (p<0.0001). Data were analysed by the χ^2 test with Yates' correction. The number of vaginal samples with genital pathogens and low amount of lactobacilli pre-HT (n=15) and post-HT (n=39) is shown in red.

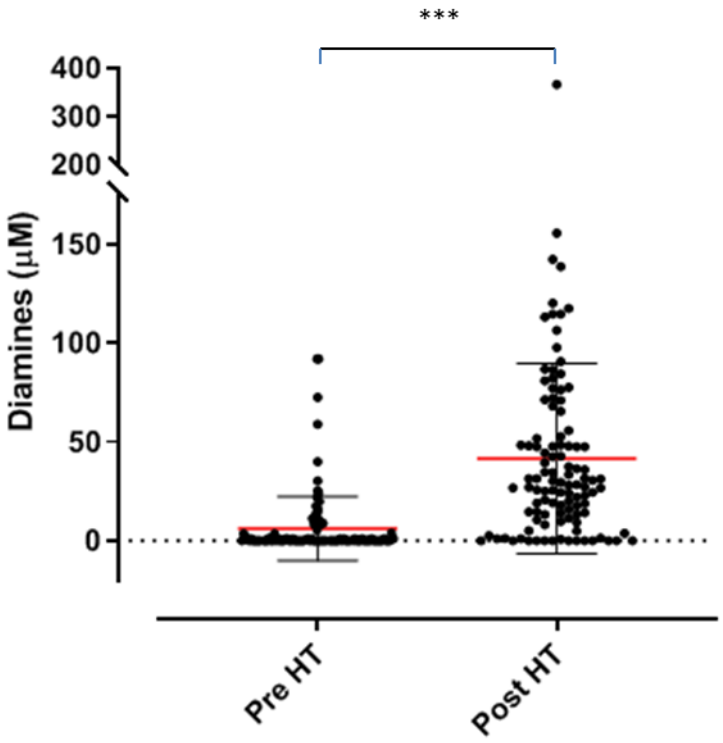

Figure 2. Concentration of diamines in vaginal fluids of patients before and after hormonal treatment. A total of 216 vaginal swabs were collected from patients scheduled for IVF: 108 samples were harvested before (pre-HT) and 108 after (post-HT) hormone treatment. The diamine concentration was determined in each vaginal swab by VADA (vaginal diamine assay) as described by Mendonca *et al*. The mean concentration of pre-HT diamines was 6.13±16.22 µM, while it raised to 41.46±48.08 µM following treatment (post-HT) (p<0.0001; Kolmogorov-Smirnov test). Each dot represents a single vaginal swab.

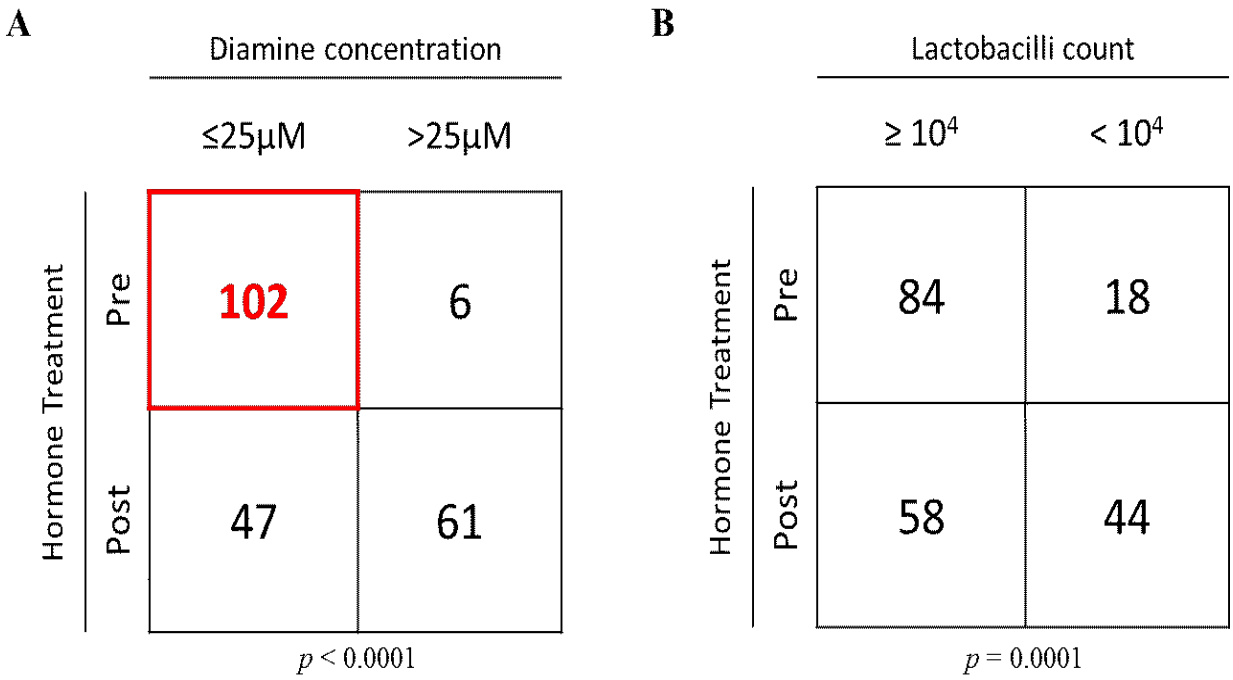

Figure 3. Effect of hormone treatment on diamine concentration and lactobacilli counts in vaginal swabs of patients undergoing IVF. A total of 216 vaginal swabs were collected from female patients scheduled for IVF, of which 108 samples were harvested before hormone treatment (pre-HT) and 108 after hormone treatment (post-HT). The diamine concentration by VADA (vaginal diamine assay) and viable counts of vaginal lactobacilli were assessed in each swab. **A.** Contingency table reporting the number of patients with low (≤25 µM) and high (>25 µM) diamine concentrations pre-HT and post-HT. The number of women with low diamine concentration pre-HT (n=102) is represented in red. **B.** Contingency table describing the effect of HT on lactobacilli viable counts (normal, $\geq 10^4$ CFU/swab; low, $<10^4$ CFU/swab) in the 102 patients shown in red in panel A. Data were analyzed by the χ^2 test with Yates' correction.

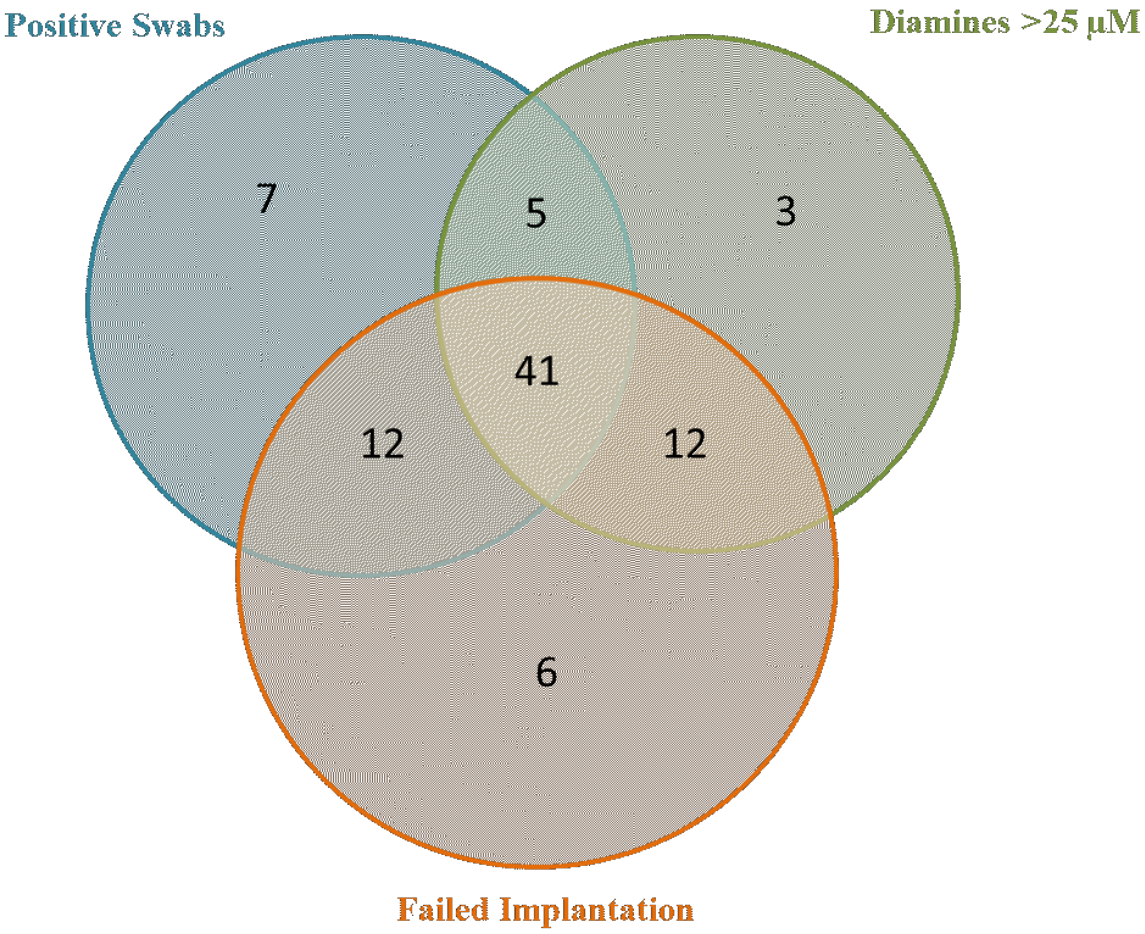

Figure 4. Effect of hormone treatment on the vaginal microbiota, diamine concentration and implantation rates in female patients undergoing IVF-ET. Venn diagram showing the intersections between the presence of genital tract pathogens in vaginal swabs (positive swabs), high concentration of diamines (>25 µM) and the outcome of IVF-ET (failed implantation) in patients treated with high doses of exogenous gonadotropins. A total of 86 women were included in the analysis. Seventy-one patients had a failed implantation (orange circle), of which 65 were positive for one or more genital pathogens (n=53, blue circle) and/or positive for high concentration of diamines (n=53, green circle). Forty-one females positive for both vaginal pathogens and high diamine levels had a failed implantation. Only 6 females with a failed implantation had association with neither genital pathogens nor high level diamines, while 15 patients, although positive for genital pathogens and/or high diamine concentrations, had a successful implantation.

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CHAPTER 6 – General conclusions

Infertility is a global problem that affects approximately 9-12% of reproductive aged couples. Amongst the many factors participating in couple infertility, urogenital tract infections can directly or indirectly impact on human reproduction efficiency.

In this thesis, specific pathogens were associated to altered sperm parameters and unhealthy vaginal microbiota. In particular, *Enterococcus faecalis* had a negative impact on sperm quality, while *E. faecalis*, *Escherichia coli*, *Streptococcus agalactiae* and *Gardnerella vaginalis* were significantly associated to reduced levels of vaginal lactobacilli (chapter 3). A dysbiosis of the *Lactobacillus*-based microbiota was also observed in women following hormonal treatment with gonadotropins prior to *in vitro* fertilization (IVF), with a shift towards a microbial population constituted by streptococci, enterococci, enterobacteria, staphylococci and yeasts (chapter 5). Interestingly, the presence of the microbial group constituted by *E. faecalis*, *Ureaplasma urealyticum* and *Mycoplasma hominis* in genital samples of infertile couples was predictive for a negative outcome of IVF (chapter 3), suggesting that *E. faecalis* may play an important role in infertility. Thereby, infertility-associated *E. faecalis* (IAF) previously isolated were further investigated in chapter 4. Most IAF clinical isolates were susceptible to antibiotics commonly employed to treat *E. faecalis* uncomplicated urinary tract infections. However, about 20% of the IAF strains were resistant to high-level aminoglycosides (HLA), and the majority of those was found to cluster in the same clonal complex (CC), suggesting clonality of HLA resistant IAF clinical isolates (chapter 4). Finally, reduced implantation rates after IVF was also associated with both increased levels of vaginal diamines and the presence of the abovementioned bacterial pathogens in women subjected to hormonal treatment. Of note, a highly significant association between vaginal microbiota dysbiosis following hormone treatment and IVF failure was found (chapter 5).

In conclusion, a better knowledge on antibiotic susceptibility profiles as well population structure of microbial species associated to couple infertility is crucial to fight pathogens causing urogenital infections. In this context, implementation of microbial screening, therapeutic management and clinical outcome of genital tract infections in infertile couples may increase natural conception rates, thereby reducing IVF strategies with beneficial effects on couple well-being and sanitary expenses.

CHAPTER 7 – Scientific *Curriculum Vitae*

EDUCATION

October 2017 – Present

Ph.D. student in Medical Biotechnologies (Cycle XXXIII)
Department of Medical Biotechnologies, University of Siena - Laboratory of Molecular Microbiology and Biotechnology (LA.M.M.B.), Policlinico Le Scotte, Siena, Italy.
Main areas of interest: clinical microbiology, antibiotic resistance, epidemiology of bacterial infections, bacterial genomics (Next Generation Sequencing).

July 27, 2017

Specialty in Microbiology and Virology
University of Pisa – Medical and Surgery Faculty
Mark: 110/110 *cum laude*
Thesis: "*High Resolution Melting Real-Time PCR* per l'identificazione e la caratterizzazione di resistenze ai farmaci di prima linea in isolati clinici di *Mycobacterium tuberculosis*"
Terzo ciclo / Level 8 EQF

March 2015 – May 2015

LIF.E. – Life Science coaching course
University of Siena
An 80-hour course, including individual coaching, with the aim of providing the skills to structure the management business plan of an innovative company with focus on life sciences.

July 2012

License and enrolment in the National Register of Biologists ('abilitazione alla professione di Biologo')
Exam to be enrolled in the National Register of Biologists (D.P.R. n. 328-2001) – University of Siena.

November 11, 2011

Master Degree in Health Biology ('Laurea Magistrale in Biologia Sanitaria' – Classe LM-6)
University of Siena – Science Faculty
Mark: 110/110 *cum laude*

Thesis: “Analisi e identificazione di specie microbiche potenzialmente correlate alla sterilità di coppia”
Secondo ciclo / Level 7 EQF

November 6, 2009
Bachelor Degree (‘Laurea in Scienze Biologiche’ – Classe L-13)
University of Siena – Science Faculty
Mark: 110/110
Thesis: “Construzione e analisi fenotipica di mutanti dell’operone degli amminozuccheri in *Streptococcus pneumoniae*”
Primo ciclo / Level 6 EQF

2001 – 2006
High School Certificate (‘Diploma di Maturità Scientifica’)
Liceo Scientifico “A. Vallone” di Galatina (Lecce, Italy)
Mark: 100/100

WORK EXPERIENCE

June 10, 2019 - July 10, 2019
U.O.C. Batteriologia – Policlinico Le Scotte, Siena, Azienda Ospedaliera Universitaria Senese
Scientific manager: Prof. Gianni Pozzi.
Occasional collaboration contract on: “*Mycobacterium chimaera* genomics”

June 1, 2016 - May 31, 2017
Department of Medical Biotechnologies, University of Siena - Laboratory of Molecular Microbiology and Biotechnology (LA.M.M.B.), Policlinico Le Scotte, Siena, Italy
Research Fellowship: “Sviluppo di modelli di infezione batterica e studio della risposta alla vaccinazione”.

April 1, 2014 - March 31, 2016
Department of Medical Biotechnologies, University of Siena - Laboratory of Molecular Microbiology and Biotechnology (LA.M.M.B.), Policlinico Le Scotte, Siena, Italy
Research Fellowship: “Studio di nuovi vettori batterici per vaccini”.

February 15, 2013 - February 14, 2014
Department of Medical Biotechnologies, University of Siena - Laboratory of Molecular Microbiology and Biotechnology (LAMMB), Policlinico Le Scotte, Siena, Italy
Research Fellowship: “Identificazione di sistemi di *uptake* ed efflusso di farmaci antimicrobici”.

February 15, 2012 – February 14, 2013
Department of Medical Biotechnologies, University of Siena - Laboratory of Molecular Microbiology and Biotechnology (LA.M.M.B.), Policlinico Le Scotte, Siena, Italy

Research fellowship: "Valutazione della suscettibilità ai biocidi identificazione di sistemi di uptake ed efflusso di farmaci antimicrobici".

LANGUAGES

ITALIAN: native
ENGLISH: Good knowledge of the English language (written and spoken)
Level acquired: ISE II Integrated Skills in English with **Merit** – Trinity (nternational examination board)
JAPANESE: Basic knowledge of the Japanese language (written and spoken)

TECHNICAL SKILLS

Excellent knowledge of molecular biology techniques applied in microbiological diagnostics:

- DNA, RNA and protein extraction from tissue and cells
- DNA cloning
- Construction of target-specific primers and TaqMan probes
- PCR, RT-PCR, Real-time PCR, Nested-PCR
- Western blotting, ELISA
- Next Generation Sequencing (MinION technology and basic knowledge of Illumina technology)
- Use of software for genomic assembly and analysis, consultation of the main biological databases useful for computational analysis

Excellent knowledge of classical bacteriology techniques with a special focus on clinical microbiology:

- Preparation of culture media (solid and liquid), sterilization techniques
- Preparation of bacterial cultures, isolation and identification of bacterial colonies
- Staining techniques and use of the optical microscope
- Antibiotic MIC and MBC assays

Excellent knowledge and expertise in mycobacteriology:

- Treatment of clinical and environmental samples
- Species identification with focus on MOTT (mycobacteria other then tuberculosis)
- Microscopy for mycobacterial detection (Ziehl–Neelsen and Kinyoun staining)
- Execution and interpretation of automatic and manual AST.

COMPUTER SKILLS

Excellent knowledge of the operating systems Windows, Microsoft Office package, in particular Excel and Power Point. Basic knowledge of Linux operating system.
ECDL European Computer Driving License

Excellent knowledge of statistics with particular focus on the analysis of biological samples using specific software such as GraphPrism 8, SPPS Statistic, R Software and MEGA.

ASSOCIATIONS

Registration in the **Ordine Nazionale dei Biologi (**AA_083531) from September 26, 2019.

Member of **ESCMID (European Society of Clinical Microbiology and Infectious Diseases)** since 2017.
Member of **SIM (Italian Society of Microbiology)** since 2016.
Ordinary member of **AMCLI (Italian Association of Italian Clinical Microbiologists)** since 2017.

Member of the cultural association **"Corte de Miracoli"** of Siena.
II level Federal Swimming Instructor (FIN) in Tuscany from 2010.

PARTECIPATION TO NATIONAL AND INTERNATIONAL MEETINGS

Refreshing course: "Focus on: Bacterial vaginosis and aerobic vaginitis" organized by AMCLI Florence, April 17, 2018.

27th ECCMID Conference
April 22, 2017 - April 25, 2017, Vienna (Austria).

44 th SIM Congress
Pisa, 25 September – 28 September 2016, Pisa (Italy).

Training course: "*Legionella* prevention and control for healthcare professionals"
October 2019, AOUS Policlinico Le Scotte, Siena.

PUBLICATIONS

Ricci S, De Giorgi S, Lazzeri E, Luddi A, Rossi S, *et al.* (2018)
Impact of asymptomatic genital tract infections on *in vitro* Fertilization (IVF) outcome.
PLOS ONE 13(11): e0207684. https://doi.org/10.1371/journal.pone.0207684