Invited review: Mastitis Escherichia coli strains-Mastitis-associated or mammo-pathogenic?

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Invited review: Mastitis *Escherichia coli* strains—Mastitis-associated or mammo-pathogenic?

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ABSTRACT

Bovine mastitis remains a major concern for dairy farmers, mainly because of its effect on the economy of their activity and on animal welfare. Because *Escherichia coli* is considered a major mastitis pathogen, the diversity of *E. coli* strains isolated from mastitis cases has been studied for decades, with the aim to discover new ways to fight this infection. With the recent advances in whole-genome sequencing, a detailed view of the peculiarities of mastitis *E. coli* strains has emerged. This review aims to bring together the knowledge garnered over the years with the more recent results of whole-genome analyses. Whereas the concept of a mammary pathogenic *E. coli* has been proposed, because a common set of virulence genes cannot be identified among mastitis *E. coli* strains, we prefer the use of mastitis-associated *E. coli* (MAEC), with MAEC being more an "ecotype" rather than a "pathotype." Indeed, data available so far suggest that a common feature of MAEC would rather be an enrichment in fitness capabilities that makes them well-suited for survival and rapid adaptation to changing biotopes in the mammary gland, which we qualify as intramammary ecotopes.
Key words: *Escherichia coli*, mastitis, MAEC

INTRODUCTION

Escherichia coli is a very versatile bacterial species that encompasses a wide variety of isolates able to occupy various biological environments, among which a major habitat is the intestinal tract of healthy mammals and the environment.

Equipped with specific gene repertoires, some isolates have acquired the ability to cause infection that can be classified, based on the entry site and location or their ability to colonize specific anatomic sites, in 2 distinct pathogroups. The intestinal pathogenic *E. Coli* (**InPEC**) pathogroup includes strains responsible for intestinal infections and has been further divided, based on phenotypic properties, into different pathotypes, such as enterotoxigenic (**ETEC**), enteropathogenic (**EPEC**), and enterohemorrhagic (**EHEC**) strains. Strains of the second pathogroup, referred to as extraintestinal pathogenic *E. Coli* (**ExPEC**), include strains responsible for extraintestinal infections, such as urinary tract infections, newborn meningitis, septicemia, or avian colibacillosis. Peculiarities of these pathotypes, in terms of virulence armamentarium, genetic profiles, or phenotypic properties, which sometimes overlap, have been reviewed in detail in several recent publications (Denamur et al., 2021; Geurtsen et al., 2022).

Among *E. coli*-associated diseases in animals, bovine mastitis represents a major burden for dairy producers and still remains as such despite decades of research to understand the pathophysiology of this infection and how the host response can be modulated to improve the outcome of the infection.

The first description of *E. coli* mastitis dates back to the late 19th century at a time when this species was still named *Bacterium coli commune* after its discovery by Theodor Escherich in 1886 (Escherich, 1885; Lucet, 1889). Despite the hypothesis that the outcome of *E. coli* mastitis is mainly determined by host factors (Burvenich et al., 2003), with the breadth of knowledge on other *E. coli* pathotypes and recent advances in whole-genome sequencing, several studies have sought to unravel bacteria properties that determine infection.

This review aims to emphasize the specific traits of *E. coli* strains isolated from mastitis cases, to highlight properties that are shared by most, if not all, *E. coli* isolates associated with bovine mastitis, and to support the case that, rather than a mammary pathogenic *E. coli* pathotype (**MPEC**), these isolates would be better defined as mastitis-associated *E. coli*.

In a previous study, Kaipainen et al. (2002) suggested that bovine mastitis shared resemblance with "urinary

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The list of standard abbreviations for JDS is available at adsa.org/jds-abbreviations-25. Nonstandard abbreviations are available in the Notes.

tract infection in that the infection is ascending, caused by bacteria from the environment." Further elaborating on this analogy, one should be able to identify "bacterial virulence factors (are) required to fight the host's selection pressure and for the bacteria to colonize, multiply and survive in the udder" (Kaipainen et al., 2002). Indeed, the type of selection pressure—notably mediated by physicochemical conditions as well as inflammatory and immune responses—imposed on the bacteria by the mammary gland is likely to dictate the specificities required for *E. coli* to first colonize the healthy mammary gland and then survive the hostile environment of the inflamed mammary gland.

Indeed, *E. coli* mastitis could be viewed as a two-step process inflicting different selection pressures on the bacteria. Initially, *E. coli* multiplies in a medium replete in nutrients and where antibacterial defenses are diluted. As the host mounts an innate immune response, *E. coli* is then confronted with an environment where the number of newly recruited immune cells and soluble defense compounds increase significantly.

We will first summarize the epidemiology of *E. coli* mastitis, its frequency and effect on dairy cows, and the various forms the infection may take. We will then highlight the specificities of *E. coli* strains isolated from mastitis cases, both in terms of phylogenetic relatedness to other *E. coli* strains and possession of particular genetic features that could contribute to its adaptation to the mammary gland. This will allow us to discuss whether these specific traits warrant the definition of a new mammary pathogenic *E. coli* pathotype, as others suggested, or whether mastitis-associated *E. coli* better describes the diversity of strains able to cause mastitis. The host response mounted upon colonization of the udder by *E. coli* will then be explored which, in the final part of this review, will lead us to consider how this knowledge may be exploited to develop new strategies to prevent and to fight against *E. coli* mastitis.

Epidemiology

Based on clinical signs, such as modification of milk, udder inflammation, loss of appetite, prostration, or fever, mastitis can be categorized as clinical or subclinical in the absence of such signs.

For a long time, *E. coli* has been recognized as a major mastitis pathogen mainly responsible for clinical mastitis. Yet, the prevalence of *E. coli* varies depending on the type and severity of the infection. In France, whereas *Streptococcus uberis* and *Staphylococcus aureus* are responsible for most subclinical or nonsevere clinical mastitis, *E. coli* is the most frequent species isolated from severe cases of clinical mastitis (Figure 1; Poutrel et al., 2018).

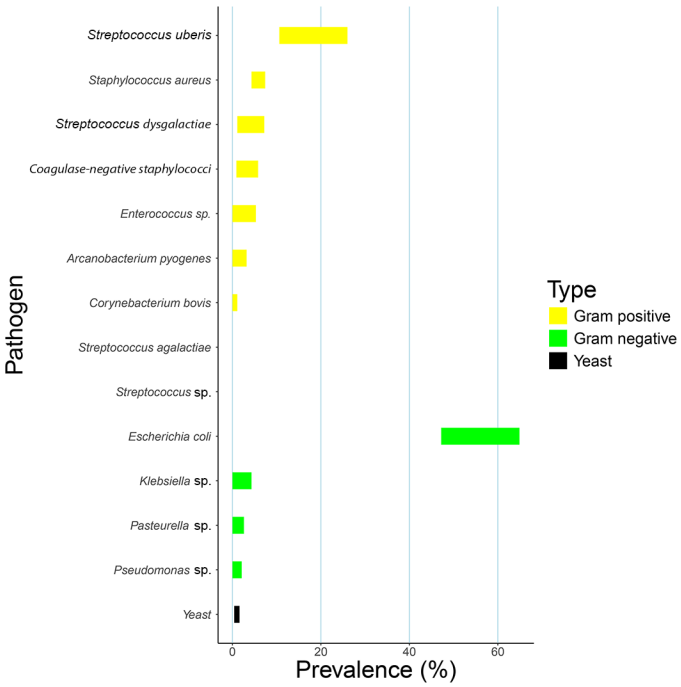

Figure 1. Proportions of mastitis pathogens responsible for severe clinical mastitis in France (based on data from Poutrel et al. 2018).

The ranking of mastitis pathogens based on the proportion of clinical mastitis cases varies from country to country. Coliforms, in particular *E. coli* and *Klebsiella pneumoniae*, are frequent causes of clinical mastitis (Barkema et al., 1998; Roberson et al., 2004). In New Zealand, *S. uberis* seems to be the major cause of clinical mastitis (**CM**), whereas *S. aureus* was found to contribute significantly to CM cases in Ireland (Douglas et al., 2000; Keane et al., 2013). In Belgium, a 2014 study found *E. coli* as the second most prevalent mastitis pathogen detected in clinical mastitis cases (Verbeke et al., 2014).

The ranking of pathogens also depends on the health status of the herd. In herds with low bulk milk SCC, CM is mostly due to *E. coli*, and the incidence of *S. aureus* increases with increased bulk milk SCC (Barkema et al., 1998)—possibly linked to farm management practices (Barkema et al., 1998; Olde Riekerink et al., 2008).

Nevertheless, most severe cases of clinical mastitis are often due to *E. coli* (Hogan et al., 1989; Oliveira et al., 2013; Salat et al., 2023). Similar situations were observed in Germany, where the most severe cases were caused by coliforms (Schmenger and Kromker, 2020), or in the United States (Oliveira et al., 2013).

One feature worth emphasizing is that the spontaneous cure rate for *E. coli* mastitis is rather high, whereas that of *S. aureus* is close to zero and remains low even when antibiotic treatment is applied (Oliveira et al., 2013; Schmenger and Kromker, 2020; Ruegg, 2021). This does

not disqualify *E. coli* as an important mastitis pathogen, in particular when one considers the possibility that *E. coli* CM cases can evolve into very severe mastitis, with an increased probability of bacteremia and fatal cases (Wenz et al., 2001; Brennecke et al., 2021).

Mastitis-Associated E. coli Isolates: Genetic Diversity

Phylogeny and Serogroups. *Escherichia coli* is a highly genetically flexible species, which is evident not only in genome composition but also in phylogenetic diversity. Recent advances in molecular typing methods allowed the identification of 8 major phylogroups, namely, A, B1, B2, C, D, E, F, and G (Denamur et al., 2021). As shown in Figure 2A, mastitis *E. coli* strains are generally clustered in phylogroups A and B1, whatever the country of origin of the strains suggesting a property shared by most *E. coli* strains. Similar to mastitis *E. coli strains*, fecal *E. coli* strains from bovine were found to mainly belong to phylogroups A and B1 (Carlos et al., 2010; Tiwari et al., 2023).

Among phylogroup A strains, ST10 or ST10-related strains (**CC10**) are frequently found in mastitis isolates (Blum and Leitner, 2013; Leimbach et al., 2017; Alawneh et al., 2020). More generally, CC10 is a very frequent lineage in the *E. coli* species, with strains belonging to CC10 being identified in samples of diverse geographic origins, in samples from humans, animals, or from the environment, and either from healthy individuals or associated with diseases. In agreement with such a high diversity, no properties specific to these CC10 strains were identified in a recent whole-genome–based study (Reid et al., 2019). Similar to observations with other serotypes (described in the following text), no association with a particular phylogroup has been observed when comparing strains from persistent or transient mastitis cases (Suojala et al., 2011; Dogan et al., 2012). Neither was any association of a particular phylogroup with cases of mild, moderate, or severe mastitis (Wenz et al., 2006).

Of note, despite an ascending infectious process similar to urinary tract infections where phylogroup B2 strain prevalence is high, phylogroup B2 strains are only rarely observed among mastitis *E. coli* isolates.

Because cattle are considered the main reservoir for Shiga toxin–producing *E. coli* (**STEC**) strains, it is not surprising that STEC are associated with bovine mastitis (Lira et al., 2004; Murinda et al., 2019). Indeed, serotype O157 strains are found in raw milk and are therefore a potential risk for human health (Stanford et al., 2005; Fremaux et al., 2006; Cízek et al., 2008).

Yet, the prevalence in mastitis *E. coli* of strains possessing O-antigen frequently found in STEC such as O157, O55, or O26 is rather low (Lohuis et al., 1990; Lira et al., 2004; Murinda et al., 2004; Dogan et al., 2012). In agreement with the low prevalence of STEC-related serotypes, a low prevalence of Shiga-toxin genes generally characterizes mastitis *E. coli* strains (Barrow and Hill, 1989; Stephan and Kuhn, 1999; Murinda et al., 2004). Nevertheless, as reviewed by Murinda et al. (2019), it should be kept in mind that the prevalence of STEC strains might be higher in some countries and therefore increase the risk to human health (Correa and Marin, 2002; Momtaz et al., 2012; Osman et al., 2012; Ahmadi et al., 2020).

Early studies using serotyping had given a hint of the diversity of serotypes of the *E. coli* species with more than 180 different O-antigen serogroups (Orskov et al., 1977; Fratamico et al., 2016).

Whereas some studies reported serotypes more frequently encountered than others in mastitis *E. coli*, such as O8, O9, or O74, mastitis-associated *E. coli* serotypes are very diverse, and none have been identified as specific for mastitis (Figure 2B; Linton et al., 1979; Sanchez-Carlo et al., 1984; Wenz et al., 2006; Dogan et al., 2012). Similar observations were deduced from whole-genome-based analyses (Leimbach et al., 2017).

In particular, there does not seem to be any difference in O-antigen distribution between environmental and mastitis isolates, although complete results were not fully reported (Blum et al., 2008). Furthermore, no clear link between serogroup and the type of mastitis, subclinical or clinical, or its severity, mild, moderate, or severe, could be established (Linton and Robinson, 1984; Wenz et al., 2006). Furthermore, no significant differences between transient and persistent strains were evidenced (Dogan et al., 2012).

Despite this high diversity, the O-antigen plays a critical role in protecting *E. coli* from the bactericidal action of serum and against recognition by antibodies targeting conserved motifs in the outer membrane (see the following text; Salamon et al., 2020; Rainard et al., 2021b).

Virulence Genes, Fitness Factors, and Associated Phenotypes. Initial studies investigated the presence of specific virulence gene subsets by gene-specific approaches. These investigations focused on well-known virulence-associated factors in other *E. coli* pathovars and considered factors contributing to the different steps of the infectious process, such as entry, growth, adhesion, invasion, stimulation of the innate host response, and resistance to serum and other host-defense mechanisms. As can be seen from Figure 2C, recognized virulence genes are rather seldom in mastitis *E. coli* strains. A potential role for the type VI secretion system in mastitis has recently been suggested based on systemic infections of mice with T6SS deficient *E. coli* isolates (Sun et al., 2021). Although T6SS have been detected in some isolates (Richards et al., 2015; Sun et al., 2021),

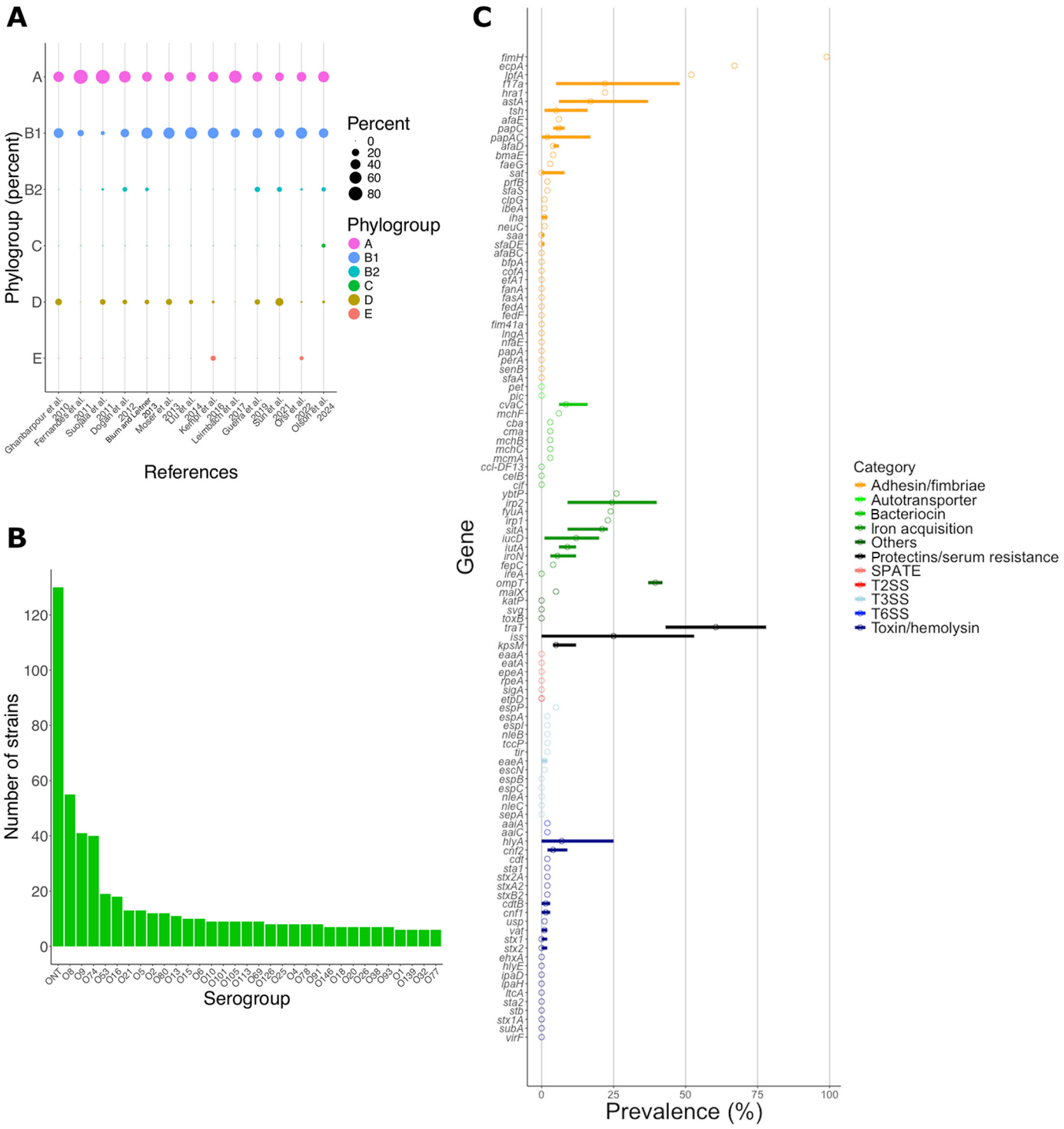

Figure 2. Phylogroup, serogroup, and repertoire of virulence genes in *Escherichia coli* mastitis isolates. (A) Prevalence of the different phylogroups of *E. coli* mastitis isolates is indicated with the corresponding study. The size of the symbols if proportional to the prevalence of the different phylogroups in each study. (B) Prevalence of O-serogroup was calculated by cumulating prevalence described in Linton et al. (1979), Linton and Robinson (1984), Sanchez-Carlo et al. (1984), Lira et al. (2004), Wenz et al. (2006), Dogan et al. (2012), Leimbach et al. (2017). ONT = O nontypable. (C) The mean prevalence of virulence genes is indicated by a circle, and the bar indicates the highest and lowest values observed in Nemeth et al. (1991), Stephan and Kuhn (1999), Ghanbarpour and Oswald (2010), Suojala et al. (2011), Blum and Leitner (2013), Moser et al. (2013), Liu et al. (2014), Fairbrother et al. (2015), Guerra et al. (2019), Orsi et al. (2023), Olson et al. (2024).

the majority of phylogroups A and B1 mastitis isolates lack a fully functional T6SS and demonstration of a role in mastitis has not been carried out so far (Kempf et al., 2015; Leimbach et al., 2017).

Consistent with these numerous studies, Suojala et al. (2013) concluded that "no specific virulence determinants have been detected in *E. coli* isolated in mastitis." The advent of whole-genome sequencing analyses has allowed a more in-depth analysis of commonalities between mastitis isolates (Richards et al., 2015; Kempf et al., 2016; Leimbach et al., 2017; Alawneh et al., 2020; Olson et al., 2024). Most studies did not demonstrate any significant correlation between the presence of virulence genes and mastitis-associated *E. coli* (**MAEC**; Kempf et al., 2016; Leimbach et al., 2017; Alawneh et al., 2020; Olson et al., 2024). Enrichment in virulence genes was rather dictated by the phylogroup, meaning that the accessory genome is likely to differ among phylogroups. Therefore, different strategies to induce mastitis could be used by mastitis isolates from different phylogroups (Kempf et al., 2016; Leimbach et al., 2017). Hence, our postulate that determinants that confer initial intramammary infection may vary among MAEC provided they give sufficient capability to allow *E. coli* to establish and flourish in the early infection stages.

Because phylogroup A strains are highly prevalent among MAEC, Goldstone et al. (2016) focused on these particular mastitis *E. coli* strains. This study confirmed some earlier findings that some traits might be specific to MAEC, showing that MAEC phylogroup A strains are more closely related than would be expected by random distribution. They identified 3 genetic loci significantly more prevalent in phylogroup A mastitis *E. coli* strains and encoding the *fec* iron acquisition system, the phenylacetic acid degradation locus, and a 2-gene cluster *ymdE-ycdU* (Goldstone et al., 2016). The *fec* locus, 1 of these 3 loci, was later confirmed as essential to confer the capability for *E. coli* to cause mastitis in the bovine host (Blum et al., 2018). This preeminence of the *fec* locus was corroborated in independent studies by Olson et al. (2018) and, further, was shown to be a crucial component in the pathogenicity of conventional ExPEC/uropathogenic *E. Coli* (**UPEC**) of phylogroup B2 (Frick-Cheng et al., 2022; Zou et al., 2023).

A recent comparative genomic analysis between mastitis and bovine commensal *E. coli* strains confirmed that the *fec* operon was enriched in mastitis isolates (Olson et al., 2024). Olson et al. (2024) also detected 3 genes enriched in commensals and 22 in mastitis isolates; additional studies are necessary to explore how these 22 genes, whose prevalence in MAEC is reduced compared with the *fec* system, may contribute to mastitis.

Hence, in addition to the *fec* locus, few specific genomic factors have as yet been associated with MAEC and validated by a "molecular Koch's postulates" approach (Falkow, 1988). To summarize, one could think of a minimal arsenal, involving fitness factors and potentially virulence genes, that could be required to overcome the different hurdles that oppose colonization of the mammary gland by MAEC and that additional armamentarium, composed of various virulence genes present in some specific isolates, increases the ability to resist host defenses or cause tissue damage. One such example indicating possible modulation of infection severity by specific gene content is given in Blum et al. (2015), where the strain inducing the most severe clinical signs and tissue damage in cows was hemolytic and possessed an RTX toxin cluster (Blum et al., 2015). Whether strains causing the most severe cases of mastitis are actually equipped with increased virulence gene repertoire remains to be explored.

Antimicrobial Resistance Genes. Antibiotic resistance is a major concern for society because it threatens human and animal lives (Neculai-Valeanu et al., 2024). Monitoring antibiotic resistance in animal pathogens has been set up in some countries, and data are updated regularly and accessible, one example being the Resapath network (Mader et al., 2021; https://shiny-public.anses.fr/ENresapath2/). Apart from public databases, current knowledge on mastitis *E. coli* resistance to antibiotics relies on many studies that have reported the prevalence of antibiotic resistance.

As can be seen from Figure 3, the prevalence of antibiotic resistance in mastitis isolates is highly variable depending on strain origin. For instance, ampicillin resistance ranged from 98.4% in the United States, between 25.3% and 41.3% in China, to between 10% and 27.8% in Canada, with additional studies observing 16.6% in South Korea (Srinivasan et al., 2007; Saini et al., 2012; Fairbrother et al., 2015; Tark et al., 2017; Zhang et al., 2018; Lan et al., 2020). Tetracycline resistance was found to be 47% in China, 14.3% in Canada, and an average of 9% in the EU, the highest levels of resistance being observed in Italy, and lowest in the United Kingdom (Saini et al., 2012; Liu et al., 2014; Thomas et al., 2015). Overall, B1 strains were more frequently resistant than A strains, especially for streptomycin, kanamycin, and ampicillin (Liu et al., 2014).

It is important to observe that MAEC are not exempted from the carriage of multiple antibiotic resistances (multidrug resistance [**MDR**]), and MDR is as much a feature of MAEC as it is for other *E. coli*, irrespective of origin or pathogenicity (Dunn et al., 2019; Shoaib et al., 2023). Such MDR phenotypes are frequently the result of acquisition and concatenation of antibiotic resistance genes (**ARG**) on mobile genetic elements, such as insertion sequences, transposons, integrative conjugative elements, and plasmids which are increasingly widely distributed

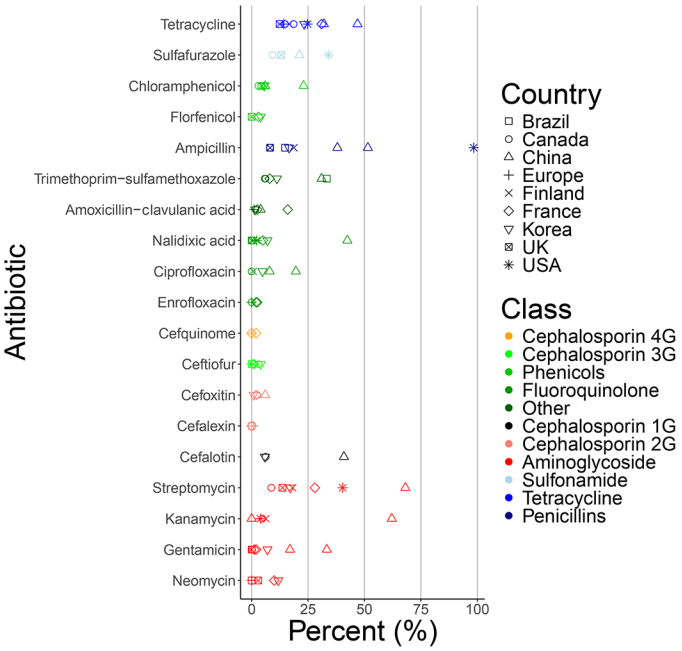

Figure 3. Prevalence of antibiotic resistance in *Escherichia coli* mastitis isolates. Values are from references (Linton et al., 1979; Srinivasan et al., 2007; Fernandes et al., 2011; Suojala et al., 2011; Saini et al., 2012; Dahmen et al., 2013; Liu et al., 2014; Fairbrother et al., 2015; Thomas et al., 2015; Tark et al., 2017; Lan et al., 2020). The prevalence is indicated by symbols corresponding to the country of the study.

among all bacterial populations including *E. coli* (Qing et al., 2024; Rana et al., 2024).

Other worthy observations include the detection of extended-spectrum β-lactamases (**ESBL**)-producing mastitis *E. coli* strains in different countries and again with a highly variable prevalence ranging from none or only a few percent in Canada, South Korea, France, or Germany (Saini et al., 2012; Dahmen et al., 2013; Tark et al., 2017, Eisenberger, et al., 2018) to much higher levels in China and Egypt (Ali et al., 2016; Ahmed et al., 2021). In most cases, ESBL-producers were often associated with multidrug resistance patterns.

Another relevant observation is the rather rare (below 3%) resistance to third- and fourth-generation cephalosporins reported in several studies so far (Srinivasan et al., 2007; Suojala et al., 2011; Saini et al., 2012; Fairbrother et al., 2015; Thomas et al., 2015; Poutrel et al. 2018). Surveillance of resistance to third- and fourth-generation cephalosporins is all the more important, as these antibiotics are among critically important antimicrobials in human and veterinary medicine (WHO, 2019; WOAH, 2024).

Currently, the consequences of antibiotic resistance on infection persistence do not seem obvious. Although Fairbrother et al. (2015) concluded that persistency was significantly associated with the number of antibiotics to which a strain was resistant, they did not clearly define a particular resistance profile in persistent isolates. Similarly, resistances to antimicrobials were not associated with the clinical signs of mastitis (Suojala et al., 2011).

Monitoring antibiotic resistance of MAEC is important to avoid the risk of ARG spreading in the environment. Although the benefit of antibiotic treatment against *E. coli* mastitis is debatable in the case of nonsevere infections (Suojala et al., 2013), AMR monitoring is also important for veterinarians to select the best antibiotics to use, keeping in mind that MIC determined based on CLSI or EUCAST recommendations do not necessarily correspond to what will be observed in milk (Thomas et al., 2015). The significance of this is exemplified in a study by Kuang et al. (2009) in which MIC for tetracycline was 4- to 32-fold greater in milk than in conventional assay conditions, whereas MIC for ampicillin was unchanged (Ganière and Denuault, 2009; Kuang et al., 2009).

Overall, evidence appears to indicate that antimicrobial resistance (**AMR**) in MAEC is a reactive process in response to antibiotic usage (whether for controlling mastitis or other infections), and there is no apparent generation of novel resistances among MAEC as yet. Nonetheless, vigilance is required as AMR is a pan-ecosystem challenge and new resistance phenotype/genotype can emerge unpredictably.

E. coli Interactions with the Mammary Gland

Typical Infectious Processes. Some studies have investigated the infection dynamics, both in terms of bacterial growth and host immune response.

Typical experimental settings start with the injection of a bacterial suspension in the teat cistern, considering that an inoculum of 10 to 10^3 is generally sufficient to initiate mammary gland colonization (Kornalijnslijper et al., 2004; Blum et al., 2017). After an initial phase of growth in milk in the absence of any host response, bacteria are recognized by host mammary epithelial cells and immune cells and then face different soluble and cellular actors of the host response. In most cases, this response leads to bacterial clearance.

Despite reflecting the dynamics of the majority of *E. coli* mastitis cases, some exceptions deviate from the norm. In the case of severe mastitis, which can lead to lethality, some reports have described the development of bacteremia (Wenz et al., 2001; Suojala et al., 2011).

Alternatively, subclinical *E. coli* mastitis cases can evolve into chronicity (Hill and Shears, 1979; Döpfer et al., 1999; Srinivasan et al., 2007; Suojala et al., 2011). As we will discuss in the following text, no characteristics have been identified so far as determinants of the kind of infection after mammary gland colonization by *E. coli*.

The mammary gland, defined by the resources, the physical, and biotic factors it encompasses (Krausman and Morrison, 2016), could be considered as a habitat, although transient in most instances, for mastitis *E. coli*. During colonization of this habitat, which begins after crossing the teat canal and continues with dissemination within the gland, the bacteria will be confronted with different environments that define particular intramammary ecotopes, an ecotope being defined as a portion of a habitat with particular conditions (Tansley, 1939). In addition, bacteria will adapt so that the *E. coli* that enter the mammary lumen are not the same phenotypically as the *E. coli* that are confronted with the immune response that they have elicited (Figure 4).

Passing the Teat Canal. *Escherichia coli* is generally considered an environmental pathogen with contamination occurring through contact with feces or contaminated material. Colonization of the teat skin by *E. coli* is affected by different external parameters, including bedding, temperature-humidity index, and integrity of the teat rosette (Hohmann et al., 2020).

To colonize the mammary gland, lumen bacteria first have to overcome the passive defenses of the teat canal. This process can be conceptually decomposed into multiple individual steps, each requiring specific properties from the bacteria to resist ejection flux during milking: potential adhesion to the keratinized epithelium, resistance to local antibacterial compounds, local multiplication and competition with teat apex microbiota, and mobility to reach the teat cisterna and deeper localizations of the milk ducts.

Colonization of the teat canal by different mastitis pathogens has been described (Zecconi et al., 1992; Paduch et al., 2012) reaching, in the case of *E. coli*, colonization in the range of 1.5 $\log_{10}$ cfu per swab (Paduch et al., 2013).

Escherichia coli also needs to resist local antimicrobial defenses: a link between mastitis resistance and the lipid composition of the keratinized epithelium has been suggested (Paulrud, 2005). Yet, *E. coli* was not affected by long-chain fatty acids, the predominant keratin-associated lipids, indicating that antibacterial activity against *E. coli* is limited, contrary to other mastitis pathogens (Hogan et al., 1988).

In addition to keratin, proteins with antimicrobial properties have been identified from the teat canal (Hibbitt et al., 1969). More recently, S100 calcium-binding proteins were shown to be present in the teat canal lining in high abundance and could contribute to a hostile environment to which *E. coli* should adapt before colonizing the mammary gland (Smolenski et al., 2015).

Finally, flagella-mediated motility might help *E. coli* to gain access to the teat cisterna. Interestingly, comparing the proteome and motility of transient and persistent isolates, Lippolis et al. (2014) showed that persistent strains express higher levels of proteins related to motility, such as flagellar proteins FliF, FliG, and FliM or the chemotaxis protein CheA, and are better swimmers, suggesting that motility might play a role in persistence. Similarly, strains with greater motility induced more severe cases (Guerra et al., 2019).

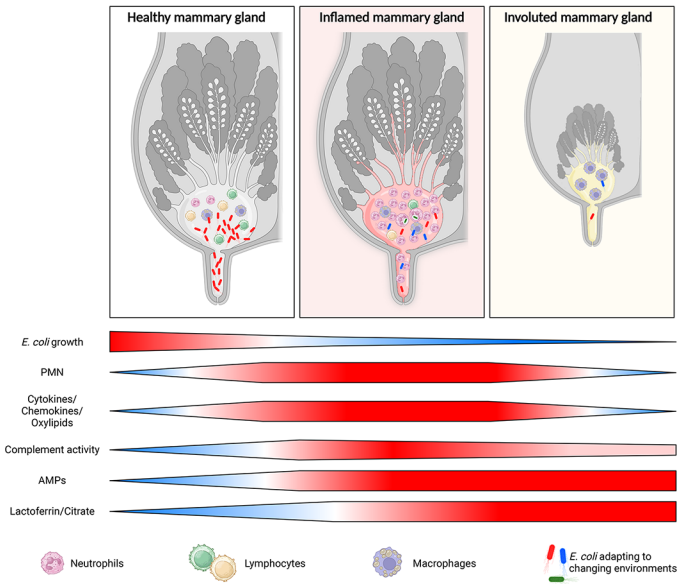

Figure 4. *Escherichia coli* faces variable environments when infecting the mammary gland. Once *E. coli* enters the lactating mammary gland, it thrives in milk, a nutrient rich medium with only limited immune defenses. Upon recognition of the pathogen, the host initiates an innate response that leads to production of cytokines, chemokines, and oxylipids, which triggers the recruitment of immune cells, in particular, neutrophils. In the involuted mammary gland, *E. coli* encounters a hostile environment where the lactoferrin:citrate ratio is high and less prone to bacterial multiplication. The major cell population present is made of macrophages. The red-blue gradient is a schematic representation of the value of each parameter, red representing the highest values, and blue the lowest. Figure was created using BioRender.com under the agreement KV27ZQM1ZC granted by BioRender (BioRender.com/i93p333).

Growing in Healthy Preinflammatory Mammary Secretions. During infection of the mammary gland, *E. coli* will face 3 different environments which we will consider in the following text: healthy mammary gland (lactating and dry) and inflamed mammary gland (**MG**).

Preinflammation Mammary Gland Ecotope. The first ecotope is the milk secreted by the healthy mammary gland. Milk is a complex matrix containing proteins, carbohydrates, lipids, and other minor constituents, such as minerals and vitamins (O'Mahony and Fox, 2013). The reader is referred to important reviews for more details on milk composition (Walstra et al., 1984; O'Mahony and Fox, 2013). The concentration of proteins and lipids is ~32 to 33 g/L, whereas lactose is the main carbohydrate with a concentration of 45 to 50 g/L (Haug et al., 2007). Contrary to lactose, minerals and vitamins, which are in

the soluble fraction of milk, lipids are mostly found as milk fat globules. The pH of milk is ~6.7 at 20°C (Walstra et al., 1984) and depends on lactation status, with higher values during involution (Sordillo et al., 1987).

Milk proteins are found either in the soluble fraction, as is the case for the whey proteins β-LG and α-LA, or, in the case of caseins, as micelles (Martin et al., 2013). Caseins, of which 4 forms (α_{s1}-, α_{s2}-, β-, and κ-caseins) are present in bovine milk as colloidal aggregates or micelles, are the major proteins of milk, making almost 80% of milk protein content (Farrell et al., 2004).

Other proteins of interest but in lower amounts include enzymes, such as lactoperoxidase and lysozyme, complement proteins, as well as Ig (Farrell et al., 2004). Among Ig, whose total amount is ~0.7 g/L, IgG1 constitute almost 70% to 80% of all isotypes, the rest being, by decreasing amount, IgM, IgG2, and IgA (Farrell et al., 2004). Such a low proportion of IgA among milk Ig typifies ruminant milk. Complement is present at low concentrations in the milk of healthy udders. It plays an important role as a bactericidal agent, in opsonization of bacterial agents, and recruitment of leucocytes through C5a generation (Rainard, 2003). Cytokines and chemokines, such as IL1β, IL6, or CXCL8, are also present in milk at different concentrations depending on the inflammatory status of the MG. Yet, CXCL3, another chemokine, is present in significant concentrations in milk, even in the absence of inflammation (Rainard et al., 2008).

Lipids, which include, in particular, triacylglycerol, diacylglycerol, glycerophospholipids, and nonesterified fatty acids, are the third major component of milk. They are secreted as 2 to 5 μm milk fat globules with a triacylglyceride core surrounded by a triple-layer phospholipid membrane (Liu et al., 2018; Thum et al., 2023). Milk lipids can act as precursors for the synthesis of oxylipids, which are of importance for the onset and regulation of the inflammatory response which characterizes mastitis (Sordillo, 2018).

Milk minerals play a key role in milk's physicochemical properties (Gaucheron, 2005). Milk is rich in ionized calcium, which is essential for the stabilization of casein micelles. Citrate is a cation-binding compound of interest for this review. Citrate concentration is ~9 m*M* in milk and is a hallmark of lactation, as it is mainly formed within the mammary epithelial cells during milk secretion. Citrate is either free or complexed with calcium as calcium citrate (Faulkner and Peaker, 1982). An important function of citrate is its ability to act as an iron scavenger which bacteria can use to their advantage to capture the iron needed for growth (Reiter, 1978).

Capacity to Grow in Milk. The capacity to multiply in mammary secretions is a prerequisite for bacteria to cause an infection of the MG, and controlling the proliferation of bacteria in milk is a key element in the MG defense against *E. coli* (Roussel et al., 2017). The rate of MG infection is not constant across the lactation cycle, and part of this variation can be related to the *E. coli* growth capacity in MG secretions. During lactation, mastitis isolates have a doubling time of 20 to 25 min in vivo, and bacterial growth in the early phase of infection conditions the infection bacterial load (Kornalijnslijper et al., 2004). Bacteria can reach concentrations exceeding 10^7 cfu/mL in milk within 12 h following introduction in the lumen of the lactating MG (Hogan et al., 1992).

Compared with fecal or environmental isolates, mastitis isolates grow faster in normal milk (Blum et al., 2008) and even more easily in mastitis milk (Nemeth et al., 1994), more specifically, under growth in near anaerobic conditions with anaerobic fermentation of lactose. Adonitol fermentation was also observed as more prevalent in mastitis isolates (Sanchez-Carlo et al., 1984; Nemeth et al., 1994). Another inferred phenotype/genotype for mastitis isolates was the capacity to use uric acid as a nitrogen source and metabolize D-serine (Blum et al., 2015). The latter ability is conferred by the *dsdCXA* locus which is highly prevalent among phylogroups A and B2 strains but rare among other phylogroups (Connolly et al., 2015). However, no direct investigation of *dsdCXA* on intramammary pathogenicity of MAEC has been carried out to date.

Given the limited access to free iron in milk essentially because of the high citrate concentration, iron acquisition in milk is of particular interest when considering the importance iron plays in *E. coli* growth and the virulence of other *E. coli* pathovars. Among the different iron acquisition systems, some are part of the core genome (e.g., *fhuABCD*, *fepABCDEG*), whereas the others are part of the accessory genome with variable prevalence. The *fepABCDEG* locus along with others confers the production of enterobactin, which is a "core" capability of *E. coli*. The deletion of an intact enterobactin system is nonessential for the ability to grow in milk but reduces the capacity to colonize the MG in a murine mastitis infection model (Vander Elst et al., 2020).

Among the accessory iron acquisition systems, the *fec* stands out as being more prevalent in mastitis-associated strains compared with other *E. coli* strains. In addition, genetic studies have shown that the *fec* operon is essential for the prototypical mastitis *E. coli* strain P4 to cause mastitis (Blum et al., 2018). In addition, it is interesting to note that the *fec* operon is generally absent in O157 serogroup strains, and these strains are only rarely found responsible for mastitis (Nemeth et al., 1994; Blum et al., 2008). The *fec* system was also shown to be important for growth in unpasteurized milk and to cause mastitis in a mouse model (Olson et al., 2021).

Yet, what remains a paradoxical observation is that, although iron seems important for mastitis develop-

ment, the prevalence of several iron acquisition systems remains low in mastitis-associated *E. coli* strains (Nemeth et al., 1994; Lehtolainen et al., 2003; Ghanbarpour and Oswald, 2010; Fairbrother et al., 2015). The *irp2*, a gene associated with the high-pathogenicity island that encodes for yersiniabactin, was found in up to 26% of strains (Suojala et al., 2011; Liu et al., 2014). The aerobactin system (*iuc-iut* genes) was identified as the second most prevalent virulence gene in a collection of mastitis *E. coli* strains from Iran (Ghanbarpour and Oswald, 2010). Interestingly, the presence of *sitA*, *iroN*, and yersiniabactin in isolates is associated with higher odds in persistent isolates (Blum et al., 2015; Fairbrother et al., 2015), although few isolates were available for study. If siderophore-mediated iron acquisition was of such importance, as is the case for classical ExPEC (principally phylogroup B2) strains, one would expect these iron acquisition systems to confer a selective advantage to the strains and thus be highly prevalent in MAEC. Overall, carriage of siderophore accessory systems (aerobactin, salmochelin, yersiniabactin) does not appear to be a significant feature among MAEC, as the presence of core siderophore enterobactin system and the accessory citrate (*fec*) system are sufficient to facilitate florid intramammary growth.

Another possibility is that the *fec* system serves as a sensor-regulator of gene transcription in milk through the FecIR regulation pathway (Braun and Braun, 2002): not only could iron acquisition be necessary for mastitis but it can also be a key signal for the onset of specific adaptation mechanisms. For example, iron presence or absence through chelation was shown to deeply modulate the motility of mastitis isolates (Lippolis et al., 2016). Iron availability was also shown to effect phagocytosis by neutrophils, growth in iron-depleted conditions yielding lower phagocytosis (Wise et al., 2002).

Together, the preceding observations strongly implicate metabolic capabilities as significant determinants in MAEC infectivity (as in ExPEC), whether influencing metabolism directly (and, hence replication), or whether indirectly effecting virulence via yet-to-be-defined physiological or regulatory processes. In MAEC, metabolic capabilities and their interplay with virulence require further exploration.

Capacity to Grow in Nonlactating Gland. When the infection takes place during the dry period, the environment is significantly different from the healthy lactating gland. Dry secretions are enriched in many proteins, in particular, lactoferrin, haptoglobin, and LPS-binding protein (**LBP**; Reinhardt and Lippolis, 2020), whereas the amount of proteins related to lactation, such as β-LG and α-LA, are reduced. Reinhardt and Lippolis (2020) found that "many of these upregulated proteins, during the first 3 weeks of the dry period, were similar to proteins upregulated in mild experimental mastitis." Alteration of the protein composition shows increased expression of proteins related to immune function or stress-related proteins (Reinhardt and Lippolis, 2020).

After cessation of lactation, the rate of infection by *E. coli* is rather elevated during the first 2 wk of the dry period (involution), very low in the fully involuted gland, and highest during the last 2 wk before parturition (colostrogenesis; Smith et al., 1985). The involuted MG tends to be refractory to *E. coli* infection, contrary to the lactating gland (Bramley, 1976). These observations can be paralleled with the growth of bacteria in mammary secretions. The pattern of infection rate coincides with the changing growth inhibitory properties of mammary secretion across the dry period. Growth inhibition increases as involution progresses during the first 2 wk postdrying off (Todhunter et al., 1990). Secretions from fully involuted MGs do not readily support the growth and multiplication of *E. coli* (Dutt et al., 1986; Oliver and Bushe, 1987). The variations in multiplication during the lactation cycle correlated with the concentrations of lactoferrin and citrate in the secretions, and iron availability.

Lactoferrin is considered a resistance factor to infection of the bovine MG (Smith and Schanbacher, 1977). This iron-chelating protein is a major contributor to nutritional immunity in different secretions, including breast milk (Hood and Skaar, 2012). As evoked earlier, among trace metals, Fe plays a prominent role (Haschka et al., 2021). The need for iron by both host and pathogenic bacteria has led to fierce competition at the molecular and cellular levels for this metal in all environments where pathogens develop (Murdoch and Skaar, 2022). Lactoferrin is a glycoprotein secreted by epithelial cells in most exocrine fluids, including milk (Masson and Heremans, 1971). Lactoferrin can bind 2 molecules of ferric iron (Fe^{+++}), each with 1 molecule of bicarbonate (HCO_3-; Masson and Heremans, 1968). Milk lactoferrin is synthesized by mammary epithelial cells (**MEC**). Lactoferrin concentration in bovine milk is low (<0.2 mg/mL) but increases dramatically during the first 3 to 4 wk after cessation of milking (up to 10 to 100 mg/mL), although with wide variations between animals, then concentrations decrease markedly during colostrogenesis (Welty et al., 1976). Secretions of the fully involuted MG are bacteriostatic for *E. coli* with high concentration and high bicarbonate/citrate ratio (Todhunter et al., 1990). Normal milk has no bacteriostatic activity due to high citrate but low lactoferrin concentrations (Reiter et al., 1975). The rare occurrence of clinical mastitis during the dry period when the MG is fully involuted could not relate exclusively to the bacteriostatic activity of lactoferrin, because this protein exerts other antibacterial and immunomodulatory effects (Vorland, 1999).

Apo-lactoferrin destabilizes the outer membrane of gram-negative bacteria, releasing LPS from *E. coli* (Ellison et al., 1988). This effect could be linked to the chelation of Ca^{2+} and Mg^{2+}, ions that stabilize the LPS layer (Ellison et al., 1990), and the interaction of lactoferrin with the lipid A part of LPS (Appelmelk et al., 1994). The high affinity of lactoferrin for the lipid A allows it to compete with LBP (Elass-Rochard et al., 1998), leading to antiinflammatory effects by reducing the response of immune cells (production of proinflammatory cytokines) to *E. coli* endotoxin (Legrand, 2012). Whether the antiinflammatory activity of lactoferrin participates in the low reactivity of the dry mammary gland to LPS remains to be documented.

Two other antibacterial elements could interfere with the growth of *E. coli* in milk. The lactoperoxidase thiocyanate-hydrogen peroxide system ($LP\text{-}SCN^{-}\text{-}H_2O_2$) is bactericidal in vitro to *E. coli* in milk when some H_2O_2 is provided (Reiter et al., 1976). Optimal conditions for this activity are usually not met in milk, notably during mastitis due to very low oxygen tension. The other antibacterial defense is mediated by xanthine oxidoreductase, an enzyme of the membrane of MEC and milk fat globules, as well as free in milk (Silanikove et al., 2007), which catalyzes the formation of nitric oxide from inorganic nitrite, leading under low oxygen tension to the generation of peroxynitrite, a powerful bactericidal agent. Thus, the xanthine oxidoreductase, which is an essential protein for milk fat droplet secretion, is a component of the innate immune system (Vorbach et al., 2003). It is likely to be activated during *E. coli* mastitis (Silanikove et al., 2007). By converting nitrate into nitrite, xanthine oxidase provides a substrate to lactoperoxidase which in turn produces nitric dioxide, a potent antibacterial oxidative radical, events that could occur during inflammation or involution of the MG (Silanikove et al., 2005).

Adhesion and Invasion. Although adhesion to mammary gland epithelia has not been observed in situ during mastitis, some authors have shown that mastitis isolates can adhere in vitro to MEC (Frost, 1975; Opdebeeck et al., 1988; Thomas et al., 1992; Lammers et al., 2001). Although adhesion to the mammary gland epithelial cells has been described in vitro, it is strongly inhibited by milk, questioning the relevance of adhesion in vivo (Harper et al., 1978).

Individual *E. coli* strains possess many fimbrial and nonfimbrial adhesins which may bind to distinct receptor ligands. Adhesins that could contribute to MAEC might be different. Type 1 fimbriae, maybe the most prevalent fimbriae found in the *E. coli* species, are frequently found in MAEC (Vandemaele et al., 2004; Fernandes et al., 2011). The F17 fimbriae, frequently encountered in ETEC strains, have only been found in a limited number of MAEC, reaching 20% of F17A-positive strains in some studies (Mainil et al., 2000; Ghanbarpour and Oswald, 2010; Suojala et al., 2011; Liu et al., 2014).

Curli, present on the surface of bacteria as thin fibrous structures, were originally discovered by screening a collection of mastitis isolates for binding to fibronectin and were detected in half of the screened mastitis isolates (Olsén et al., 1989). Whereas a similar prevalence was observed in later studies, the proportion of curli-producing strains among environmental isolates is similar to that of mastitis isolates (Olsén et al., 1989; Dyer et al., 2007). Other adhesins such as the P fimbriae, afimbrial adhesin Afa, and S fimbriae are not frequently found (Ghanbarpour and Oswald, 2010; Suojala et al., 2011).

Long polar fimbriae (**LPF**) were also considered as potential virulence factors of MAEC. Several variants of LPF have been identified in a variety of pathovars. Yet, most recent studies failed to demonstrate a significant role in mastitis, with at most only a slight reduction in virulence in a mouse model (Olson et al., 2018; Zhou et al., 2021).

Adhesion of strains to epithelial cells could sometimes be the initial step of intracellular invasion of these same cells. Although adhesion has never been documented in vivo, it is worth noting that invasion might play a role in certain cases of mastitis, as some authors have reported different invasion abilities depending on the type of mastitis, as well as the possibility of forming intracellular bacterial communities, as observed with UPEC isolates (Döpfer et al., 2000; Hunstad and Justice, 2010).

In general, adhesion is the initial step in biofilm formation. Similar to the lack of evidence for adhesion in vivo, biofilm formation in vivo has not been documented. Biofilm formation by mastitis *E. coli* isolates has only been documented in vitro (Costa et al., 2014; Silva et al., 2014; Milanov et al., 2015).

Capacity to Withstand the Inflammatory Response

Setting Up Inflammation. Experimental *E. coli* mastitis can be induced with as little as 15 cfu (Shuster et al., 1996; Kornalijnslijper et al., 2004; Vangroenweghe, 2004; Blum et al., 2017). Multiplication of the bacteria, with such low inocula or with higher doses, is observed as early as 4 h postinoculation, whereas inflammation, the first sign being the increase of somatic cells in milk, is only detected 12 h postinoculation (Vandeputte-Van Messom et al., 1993; Vangroenweghe et al., 2004; Herry et al., 2017).

The onset of the inflammatory response relies on the efficient recognition of the bacteria by host cells, such as MEC and macrophages. The contribution of these cell types to the recognition of *E. coli* has been recently described in detail (Rainard et al., 2022). Several *E. coli* membrane components contribute to the recognition

of bacteria by host cells, the most important one being LPS. An LPS molecule contains 3 regions: the lipid A, the core oligosaccharide, further divided into outer and inner parts, and the O-antigen polysaccharide. Lipid A is the minimal active component (endotoxin) required to induce an immune response (Rietschel et al., 1993), and most of the biological activities have been associated with this lipid moiety (Huber et al., 2006). Whereas lipid A acylation degree varies among gram-negative species, *E. coli* lipid A carries 6 acyl chains that are essential for the efficient recognition of LPS by the TLR4/MD-2 complex, in association with the membrane-bound or soluble form of the CD14 coreceptor (Raetz and Whitfield, 2002; Beutler and Rietschel, 2003; Jiang et al., 2005; Raetz et al., 2007; Whitfield and Trent, 2014; VanOtterloo and Trent, 2024).

Bovine MEC are essential sentinels for the recognition of LPS: They express the TLR4/MD-2 complex and have recently been shown to produce soluble CD14, which is required for an efficient recognition of LPS by MEC (Sauter et al., 2007; Ibeagha-Awemu et al., 2008; Porcherie et al., 2012; Roussel et al., 2015; Tsugami et al., 2021).

Events downstream of the TLR4/MD-2 receptor follow 2 different pathways: the MyD88-dependent pathway involving TIRAP and the TRIF-TRAM pathway. In non-myeloid cells, such as MEC, full activation of the latter by LPS possessing an O-antigen requires the presence of the soluble coreceptor CD14 in the external milieu (Védrine et al., 2018).

Because this recognition involves specific interactions between TLR4/MD-2 and the lipid A moiety, any modification of the lipid A structure could disturb this process: yet, based on a study of a limited set of strains, enzymes involved in lipid A modification are not expressed by mastitis *E. coli* strains (Needham and Trent, 2013; Kempf et al., 2016). Variations in the structure of the LPS core have also been described. Five different LPS core types exist in the *E. coli* species and have been detected in various mastitis *E. coli* isolates, but their effect on the LPS/TLR4/MD-2 interaction remains to be elucidated (Heinrichs et al., 1998; Kempf et al., 2016). Finally, because the presence of the O-antigen dictates the requirement of sCD14 for LPS recognition, it is possible that different O-antigens differently modulate the response to *E. coli*, but this remains to be demonstrated.

Aside from LPS, other motifs that can be recognized include membrane lipoproteins, mostly triacylated in *E. coli*, as well as peptidoglycan subunits (El Rayes et al., 2021). Triacylated lipoproteins are recognized by TLR1/TLR2 heterodimer expressed on the surface of MEC (Takeuchi et al., 2002; Ibeagha-Awemu et al., 2008; Petzl et al., 2008; Porcherie et al., 2012). Peptidoglycan fragments can be detected by the cytosolic sensors NOD1 and NOD2, but their contribution to the onset of an efficient immune response to *E. coli* invasion is debatable. Although NOD are expressed by MEC and potentially other cell types of the mammary gland (Whelehan et al., 2011; Porcherie et al., 2012), because their engagement requires entry of bacteria or transport of bacterial components into the cytosol, they would likely come into play later than the TLR4/MD-2 complex during the infection process.

Surprisingly flagellin, although it is a well-known *E. coli* motif recognized by innate immunity receptors, does not contribute to the recognition of *E. coli* by the bovine mammary gland: contrary to LPS injection, direct injection of flagellin into the MG does not induce inflammation. Bovine MEC do not express the TLR5 receptor, and bovine macrophages only weakly respond to flagellin (Porcherie et al., 2012; Metcalfe et al., 2014).

Resisting Innate Immunity Host-Defense Responses. The direct consequence of *E. coli* recognition by the mammary gland immune system is the profound modification of the bacterial environment with, in particular, the production of antimicrobial peptides, the secretion of various cytokines, chemokines, and oxylipids, resulting in the recruitment of neutrophils and the influx of complement subunits. We will refer the reader to a recent review for a detailed description of the events downstream of the recognition of *E. coli* (Rainard et al., 2022).

Capacity to Grow in the Inflamed MG. As mentioned previously, mastitis can easily be reproduced experimentally by the injection of as few as 15 organisms into the teat cistern via the teat duct, indicating that milk is devoid of strong inhibiting activities against MAEC strains (Shuster et al., 1996; Kornalijnslijper et al., 2004; Vangroenweghe, 2004; Blum et al., 2017). Nevertheless, several milk components, such as lactoferrin, AMP, lactoperoxidase, or complement factor, have been shown to hamper *E. coli* growth, as already mentioned.

During *E. coli* mastitis, lactoferrin concentration increases markedly (Harmon et al., 1976), and bacteriostatic activity can appear in mastitic milk due to increased pH and reduction in the citrate/lactoferrin ratio (Rainard, 1987). Several gram-negative pathogens can acquire iron from lactoferrin through surface receptor proteins, but there is no report of a specific lactoferrin receptor expressed by *E. coli* (Schryvers, 2022). Bovine lactoferrin is not bactericidal to MAEC strains at low to moderate concentrations but could be for some strains at high concentrations (20 mg/mL; Bishop et al., 1976). Bovine lactoferricin, a proteolytic split fragment (comprising a cationic stretch) of the N-terminal end, is bactericidal in vitro against various bacteria including *E. coli* (Bellamy et al., 1992). However, the bactericidal activity is influenced by the medium components: metal ions (Ca^{2+} and Mg2+) and citrate markedly augment the minimal

active concentrations (Bellamy et al., 1992). There is no published evidence that lactoferricin plays a role in vivo during infection of the MG.

Resistance to Serum and Bactericidal Activity of Complement in Mammary Secretions. The ability to survive the bactericidal activity of the complement system is advantageous to bacteria that induce inflammation at epithelial barriers, where they are confronted with local innate defense proteins and exudation of blood plasma. The deposition of the C5b-9 membrane attack complex (**MAC**) onto the outer membrane of *E. coli* is a prerequisite for serum-mediated killing, leading to the degradation of cytoplasmic membrane phospholipids (Taylor and Kroll, 1984). With resistant strains, there is less deposition of MAC components and the cytoplasmic membrane is not degraded. Whether mastitis-causing isolates are complement susceptible or resistant has generated much interest and sometimes confusing data.

In principle, resistance to complement is defined by the capacity of a bacterial strain to survive when exposed to blood, serum, or plasma. Many variables determine the degree of resistance to the bactericidal activity of complement. Culture conditions, such as culture medium, culture phase (bacteria in exponential phase are less resistant), treatment of bacteria after culture (washing, thermal shock), presence of antibodies (IgM and IgG) and serum concentration in the assay, size of the inoculum, buffers and diluents (pH, ionic strength, concentrations of cationic ions Mg2+ and Ca^{2+}), and duration of exposure to serum, all these parameters have marked effects on the final result (Taylor, 1983). "Unfortunately, no single technique has emerged as a readily acceptable and widely applicable standard assay for estimation of serum activity, and the reader is confronted by a bewildering array of assays that frequently make meaningful comparisons between studies difficult" (Taylor, 1983). Moreover, interpretation of results may also differ among researchers. A key feature that emerges from most studies is that serum resistance is not an all-or-nothing trait but a property that exhibits a continuum within a sample of strains and variations within a given strain, depending on the test conditions. Supplemental Table S1 (see Notes) summarizes the results of studies that have evaluated the ability of mastitis isolates or strains from the bovine feces or environment to resist killing by serum complement.

Overall, most mastitis isolates have been considered resistant or moderately resistant. However, serum resistance is not a sufficient criterion to distinguish MAEC strains from environmental strains. Both *E. coli* isolated from mastitic udders and environmental *E. coli* show similar percentages of serum resistance (Carroll and Jasper, 1977; Nemeth et al., 1991; Nemeth et al., 1994). This concurs with the idea that most *E. coli* mastitis cases have an environmental origin but does not settle whether or not complement resistance is necessary to induce mastitis. One must consider which *E. coli* strains are involved in terms of complement activity in healthy and inflamed MG, knowing that complement resistance has different degrees.

In mammary secretions from healthy glands, complement-dependent bactericidal activity is present, but its activity is limited and variable during the lactation cycle. In the mammary secretions of dry cows, complement-titers augment during the first weeks after cessation of milking, so that secretions can inactivate *E. coli* that are very susceptible to complement (Poutrel and Rainard, 1986). In the first days postpartum, colostrum is bactericidal to serum-susceptible *E. coli* only after dilution, due to a prozone effect by high concentrations of IgG_1 (Reiter and Brock, 1975). In postcolostral milk, bactericidal activity is usually undetectable (Reiter and Oram, 1967). A serum-susceptible strain (B41) can grow in milk from uninflamed glands but is killed in milk from inflamed glands, whereas serum-resistant strains can grow in milk from both inflamed or uninflamed glands (Hill et al., 1978; Rainard, 1983a). These observations can be explained if we consider the complement concentrations in milk. In milk from healthy glands, the classical pathway of complement does not operate due to a very low concentration of C1q, and the activity of the alternate pathway corresponds to 2.5% of serum activity (Rainard and Poutrel, 1995). In mastitic milk, complement activity increases proportionally to the magnitude of the inflammatory reaction (Rainard et al., 1984). Consequently, very susceptible strains are killed in normal milk, and moderately susceptible strains can grow but are eliminated when inflammation develops. Experimental evidence supports the inability of very susceptible strains to cause mastitis: A serum-susceptible strain which caused mild mastitis in the mouse was avirulent in the cow, unable to cause clinical signs, and was not recovered in milk after the inoculation of 250 cfu (Anderson et al., 1977); when 500 cfu of *E. coli* were inoculated into lactating healthy MGs, the 2 serum-susceptible strains tested were not recovered in milk, contrary to serum-resistant strains (Barrow and Hill, 1989).

Whereas some argue that serum resistance of MAEC is not essential and even considered spurious, one should somehow remember that some level of serum resistance is necessary (Hogan and Larry Smith, 2003). Although this remains to be verified, it can be expected that, in a collection of mastitis isolates, an increase in serum resistance will correlate with increasing virulence and could, for instance, be associated with most severe mastitis cases associated with bacteremia.

Complement resistance is a multifactorial property involving O-antigen, capsule, and even other less

well-defined contributors, such as TraT (Miajlovic and Smith, 2014).

O-antigen contribution to serum resistance was demonstrated with *E. coli* mutants deleted of O-antigen synthesis genes: such mutants became serum-susceptible and were significantly less virulent in a mouse model (Roussel et al., 2017; Védrine et al., 2018; Salamon et al., 2020). Interestingly, failure to synthesize a full-length LPS was also associated with an increased capacity to stimulate the innate immune system (Védrine et al., 2018).

In addition to the O-antigen, the different types of capsules covering the surface of *E. coli* have various protective roles (Whitfield, 2006). Such capsules, made of high-molecular-weight polysaccharides, can prevent the activation of the complement pathway or interfere with the phagocytosis by neutrophils (Hill et al., 1983b; Rick and Silver, 1996). Results on the prevalence of capsule production are to be taken cautiously because of the diversity of methods used and the diversity of capsule types and biosynthetic pathways described. Phenotypic characterization reported up to 75% of tested isolates showing visible capsules, with few strains with thick layers (>2 µm; Barrow and Hill, 1989; Fernandes et al., 2011). Other reports found only a limited number of isolates showing capsule production (Kaipainen et al., 2002; Wise et al., 2002). Detection of K1 and K5 capsule biosynthesis genes showed only a limited number of positive strains (Kaipainen et al., 2002; Fairbrother et al., 2015). The *wca* colanic acid capsule synthesis genes have been detected in some mastitis isolates and seem more prevalent in persistent isolates (Lippolis et al., 2018). Nonetheless, even when present, their role in mammary gland colonization in a mouse model remains limited (Olson et al., 2018).

The *traT* and *iss* genes were shown in other pathotypes to contribute to serum resistance. The TraT is a plasmid-encoded protein and, when present, is a major lipoprotein of the outer membrane of *E. coli* (Taylor et al., 1990). The presence of *traT* has been detected in many mastitis isolates (Kaipainen et al., 2002; Campos et al., 2022) but does not seem to be associated with serum resistance (Nemeth et al., 1991). The *iss* gene was originally described as responsible for *i*ncreased *s*erum *s*urvival and was detected in environmental and mastitis *E. coli* isolates at similar frequencies, which raises the question of its contribution to the infectious mastitis process (Blum and Leitner, 2013; Keane, 2016).

Resistance to Phagocytosis by Neutrophils. Because the initial phase of infection develops in the gland lumen, phagocytosis occurs in suspension in a liquid phase. Consequently, the minimum concentration of phagocytic cells should be high enough to ensure a good probability of encounters between phagocytes and bacteria. Moreover, opsonization is required to make the contacts productive, that is, followed by ingestion. More than 3 to 5 $\times 10^5$ neutrophils/mL are necessary to cause a significant decrease in bacterial numbers (Leijh et al., 1979; Li et al., 2002). In a healthy gland, the concentration of cells is less than 10^5/mL, thus the phagocytic efficiency is low. Consequently, bacteria are not required to be specifically equipped to resist opsonization and phagocytosis to proliferate within a healthy lactating MG. Another consequence is that owing to the short doubling time of *E. coli* in milk, the speed of recruitment of phagocytes is crucial: a delay of a few hours means acute, possibly fatal mastitis (Hill et al., 1979). Polymorphonuclear neutrophils are the first phagocytes that migrate to the MG lumen. They arrive with antibodies and complement brought by the concomitant inflammatory plasma exudation.

The LPS O-antigen of smooth MAEC strains shields most of the outer membrane antigens, except fibrillar antigens such as fimbriae that protrude from the bacterial surface (Rainard et al., 2021b). The efficient opsonic antibodies are directed to the O-antigen or capsular polysaccharides (Dijk et al., 1981; Greisman and Johnston, 1997). In the nonimmune cow, IgM are the most important isotype among opsonic antibodies (Williams and Hill, 1982). Bovine neutrophils express an IgM receptor so that IgM are opsonic without complement (Grewal et al., 1978; Williams and Hill, 1982; Gilbert and Rainard, 2024). Natural antibodies of the IgM isotype, endowed with polyspecific antibody activity, effectively opsonize *E. coli*, so that most cows possess enough naturally acquired opsonic antibodies to cope with the wide range of O serotypes of MAEC strains. Strains that can produce a capsule (K antigen) have been reported to cause more severe mastitis cases or to have a fitness advantage over nonencapsulated strains (Hill, 1981; Olson et al., 2018). It has been calculated that encapsulated strains of *E. coli* require about 100 times more opsonic antibodies than nonencapsulated strains (Hill et al., 1983b). After infection by an encapsulated strain, antibodies to the K antigen in the IgM and IgG_2 isotypes are induced, allowing neutrophils to phagocytose strains of antigenically similar capsules (Hill et al., 1983b). Of note, the proportion of encapsulated MAEC strains seems to be low. Indeed, antibodies are usually not the limiting factor of phagocytic efficiency, as shown by the high rate of severe *E. coli* mastitis postpartum when colostrum is particularly rich in antibodies (Burvenich et al., 2007). Complement in unheated bovine serum enhances opsonic activity and improves phagocytosis of MAEC by bovine neutrophils, but it is not essential (Williams and Hill, 1982). The main role of complement may be through the generation of C5a, which activates the bactericidal activity of human and bovine neutrophils (Mollnes et al., 2002; Rainard et al., 2021b). Although some ExPEC strains can survive within neutrophils (Nazareth et al., 2007), most MAEC

Table 1. Knowledge gaps in our understanding of *Escherichia coli* mastitis isolates[1]

Section	Knowledge gap
Species diversity	Are MAEC a subset of gut/commensal bovine *Escherichia coli* strains? Do MAEC and gut/commensal *E. coli* strains possess consistent genotypic or phenotypic differences, or both?
	Is there a link between increased shedding of *E. coli* strains in the gut and prevalence of mastitis in dairy herds?
	Are the strains causing very severe mastitis and responsible for animal death any different from less severe clinical mastitis isolates?
	Do persistent strains have clear specificities that allow them to resist the immune response of the host?
Colonization	Is the colonization of the teat canal by MAEC long-lasting?
	Are any recognized specific adhesins (e.g., fimbrial types) crucial to colonization or is adherence genotype subordinate to adherence phenotype?
	Is the colonization of the teat canal imposing a selective pressure that constrains the subset of MAEC that colonizes?
	Can the local teat microbiota be modified to prevent colonization by MAEC?
Virulence	Is the fec system only involved in iron acquisition? Role in adaptation to the mammary gland ecotope?
	To want extent do other eco-adaptive loci (e.g., *dsd*) contribute to mastitis causation?
	Are classical ExPEC (phylogroup B2 or D) able to cause mastitis?
	What is the genetic program (transcriptome) activated by the bacteria upon colonization of the mammary gland?
Host response	Is it possible to activate a local immune response (by vaccination, for example) without detrimental effects on the gut microbiome and gut mucosa (risk of overt inflammation)?
	Could priming/optimization of local innate immune effectors be effective in minimizing mastitis?

[1]MAEC = mastitis-associated *E. coli*.

strains are readily killed by bovine neutrophils, as 10% heated (56°C, 30 min) bovine serum allows neutrophils to kill (<1% survival) MAEC (207 strains tested; Hill et al., 1983b). This efficiency may contribute to the high rate of spontaneous cure of *E. coli* mastitis.

Overall, MAEC strains appear to be susceptible to the phagocytic defense of the MG. However, phagocytosis by neutrophils may be hampered under certain circumstances. Early postpartum clinical mastitis may be facilitated by the inhibition of phagocytosis by colostrum, in relation to the blocking of Fc receptors by Ig immune complexes (Targowski and Niemialtowski, 1986). Hyperketonemia (ketosis), related to the negative energy balance that occurs in high-producing cows in early lactation, may also inhibit phagocytosis of *E. coli* and formation of neutrophil extracellular traps by bovine neutrophils through the production of β-hydroxybutyrate (Grinberg et al., 2008). Also, *E. coli* strains may adapt to the inflamed MG ecotope by changing their phenotype: It has been reported that iron deprivation of MAEC strains decreases their susceptibility to phagocytosis by neutrophils (Wise et al., 2002, 2003). Furthermore, the MAEC strain P4 can grow in milk at low oxygen tension, and incubation with bovine neutrophils resulted ultimately in neutrophil lysis (Goldberg et al., 1995). These phagocytosis impairments may explain why some MG infections by *E. coli* take several days to resolve by bacterial clearance, although the causative strain appears quite susceptible to phagocytosis in typical phagocytic assays.

E. coli Facing the Adaptive Response. The *E. coli* mammary infections elicit specific antibodies. However, recurrent clinical infections are not uncommon (Hill and Shears, 1979; Döpfer et al., 1999), even by the same strain (Hill et al., 1983a). This suggests weak induction of protective adaptive immunity. Notwithstanding, several attempts have been made to improve the immune response to *E. coli* mastitis through vaccination.

The proliferation of *E. coli* in the lumen of the MG induces a strong innate immune response mainly provoked by the LPS endotoxin. Due to the strong inflammatory reaction to *E. coli* endotoxin, it is of prime importance to stop the proliferation of bacteria before they can produce a toxic amount of endotoxin overwhelming the host detoxification mechanisms of the affected cow. Thus, if colonization cannot be avoided, the next strategy to prevent *E. coli* mastitis would be to interfere with the growth of *E. coli* in the MG. Thus, the importance of iron acquisition systems as fitness attributes of MAEC strains designates them as vaccine targets. Two attempts have been reported, one using the ferric enterobactin receptor FepA, the other the ferric citrate receptor FecA, as antigens (Lin et al., 1999; Takemura et al., 2002). Antibodies to FepA hampered the growth of *E. coli* in combination with lactoferrin, suggesting that they could be useful during the dry period. Cows immunized with FecA did not fare better than control unimmunized cows when challenged with *E. coli* despite increased serum antibody titers. A major issue with neutralizing antibodies is maintaining active titers in milk (Wolf et al., 2004).

Assuming the proliferation of *E. coli* in a healthy lactating MG cannot be prevented, the recourse is phagocytosis by neutrophils. Opsonins are necessary for the efficient killing of *E. coli* by phagocytic cells. Despite the diversity of O-antigen specificities, natural or naturally acquired antibodies, mainly in the IgM isotype, are sufficient to opsonize most MAEC strains (Hill et al., 1983b). Encapsulated strains are more resistant to phago-

cytosis and killing by neutrophils, requiring more opsonins than nonencapsulated strains. However, there are enough antibodies to opsonize even encapsulated strains in early lactation milk, a time when the most severe *E. coli* mastitis occurs, suggesting that opsonic deficiency is not an issue (Hill et al., 1983b). The immune defense element that is lacking in the healthy MG is the phagocyte. Indeed, the speed at which neutrophils are recruited into the gland and milk is crucial for the severity of mastitis (Hill, 1981). Even a few hours delay in the influx of neutrophils can lead to fatal mastitis. Because immunization could increase the initial recruitment of neutrophils to the infected MG (Rainard, 1983b), research has been carried out to understand the mechanisms and implement them through vaccination. Neutrophilic inflammation is under the control of a particular class of immune response, type 3 immunity, which is characterized by the production of the cytokine IL-17A by Th17 helper T cells (Annunziato et al., 2015). A case has been made for the mobilization of type 3 immunity in the MG to improve resistance to mastitis (Rainard et al., 2020). The IL-17A is induced in the MG during *E. coli* mastitis, and this cytokine can act synergistically with LPS to increase the defense responses of MEC (Roussel et al., 2015). Local (intramammary) immunization with *E. coli* as reported by Herry et al. (2017) has elicited Th17 CD4 T cells in the mammary tissue associated with faster healing and shorter mastitis duration than unimmunized controls (Cebron et al., 2020). These observations suggest a new orientation for vaccine development. The immune response induced by the current vaccines against *E. coli* mastitis, mainly based on the use of the rough strain J5, has recently been extensively discussed (Rainard et al., 2021a). They do not improve the phagocytic efficiency or the rapidity of the neutrophilic inflammation induced by infection. There remains much room for improvement in *E. coli* mastitis vaccines.

CONCLUSION: A CASE FOR MAEC-SPECIFIC TRAITS

Although much has been performed to better understand how *E. coli* adapts to the mammary gland and to unravel the specific properties that are required for the colonization of the mammary gland, there are still several knowledge gaps that are worth mentioning and that deserve further investigation (Table 1).

With all this in mind, the MPEC pathotype remains to be defined, if any is possible. Can we really define an MPEC pathotype? As defined by Kaper et al. (2004), a pathotype is "a group of strains of a single species that cause a common disease using a common set of virulence factors." Are we then able to define such a common set of genes? From what has been exposed previously, it rather seems that mastitis *E. coli* isolates are characterized by the lack of a common set of specific virulence factors.

For such reasons, we favor the use of the term MAEC strains, with specificities that are not necessarily linked to the possession of a common set of virulence factors. Nevertheless, despite what seems to be a lack of specificities of MAEC strains, a few reports suggest that *E. coli* strains involved in mastitis cases may share some common features.

The seldom identification among mastitis isolates of strains with certain characteristics, such as O157 serotype strains or phylogroup B2 strains, is another argument that suggests that there might be some specific traits which are lacking in these isolates yet which are vital to MAEC.

As described previously, the mammary ecotope is a changing environment and colonization could be seen as a 2-step process, with early steps requiring growth in normal milk, with limited antibacterial defenses, followed by growth in a more hostile milieu as mastitic milk (Figure 4). Not only did growth in normal milk seem improved in MAEC, but the ability to grow in mastitic whey, which is required for a strain to persist at least until a strong neutrophilic response is triggered, seems to indicate selection for this trait among MAEC (Nemeth et al., 1994). More specifically, mechanisms that are required for adaptation to this changing ecotope include lactose fermentation, iron acquisition by citrate, as well as the ability to resist serum, in part through the presence of an O-antigen.

The existence of driving forces responsible for udder adaptation of *E. coli* strains has been put forward in different reports analyzing recurrent strains (Döpfer et al., 1999; Bradley and Green, 2001). Such driving forces remain to be identified to explain the emergence of MAEC in the different phylogroups. Data obtained by Goldstone et al. (2016) suggest a selective process that restricts the colonization of the udder to certain strains possessing specific genes/properties. Such a subset would result from a selection process that occurs in the bovine intestinal tract or in the environment, and thus, MAEC are recruited from the normal intestinal bovine microbiota and environment, indicating that, similar to what has been suggested for ExPEC, ability of MAEC to colonize the udder and cause mastitis is a byproduct of their adaptation to their environment (Le Gall et al., 2007). This adaptation is likely to involve multiple gene exchanges, for which strains of phylogroups A and B1 are more efficient than strains of other phylogroups, and to occur outside of the MG, for instance, in the bovine gut or in the bovine environment, because mastitis can be considered an evolutionary dead end (Touchon et al., 2020).

Hence, rather than a "pathotype," MAEC may be considered more as an "ecotype," defined as the product resulting from genotypic adaptations to a particular habitat

(Gregor, 1944), whose key defining features would be characteristics suited for survival and rapid adaptation to the environmental composition in (udder cistern) milk followed by rapid growth before the induced heightening activation of innate defenses.

NOTES

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Nonstandard abbreviations used: AMR = antimicrobial resistance; ARG = antibiotic resistance genes; CC10 = ST10-related strains; CM = clinical mastitis; EHEC = enterohemorrhagic *E. Coli*; EPEC = enteropathogenic *E. Coli*; ESBL = extended-spectrum β-lactamases; ETEC = enterotoxigenic; ExPEC = extraintestinal pathogenic *E. Coli*; InPEc = ; intestinal pathogenic *E. Coli*; LBP = LPS-binding protein; LPF = long polar fimbriae; MAEC = mastitis-associated *E. coli*; MAC = membrane attack complex; MDR = multidrug resistance; MEC = mammary epithelial cells; MG = mammary gland; MPEC = mammary pathogenic *E. coli*; STEC = Shiga toxin–producing *E. coli*; UPEC = uropathogenic *E. Coli*.

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