DAVID GERMANO GONÇALVES SCHWARZ

STUDIES ON THE INTERACTION BETWEEN *Mycobacterium avium* subsp. *paratuberculosis* AND BOVINE MASTITIS ASSOCIATED *Escherichia coli* IN A MAMMARY EPITHELIAL CELL MODEL AND IDENTIFICATION OF PASSIVE SHEDDING IN SMALL RUMINANTS

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Medicina Veterinária, para obtenção do título de *Doctor Scientiae.*

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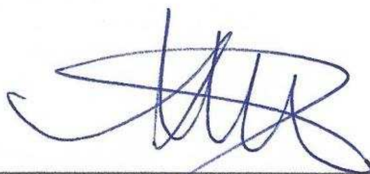

Sérgio Santos de Azevedo

Eduardo de Almeida Marques da Silva

Srinand Sreevatsan
(Coorientador)

Leandro Licursi de Oliveira
(Coorientador)

Maria Aparecida Scatamburlo Moreira
(Orientadora)

"Happy people remember the past with gratitude, rejoice in the present and face the future without fear". Epiruco

"As pessoas felizes lembram o passado com gratidão, alegram-se com o presente e encaram o futuro sem medo". Epicuro

To my parents, Adolfo and Edna, for illuminating my dreams...

Aos meus pais, Adolfo e Edna, por iluminarem meus sonhos...

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To God, for giving me life and renewing my strength.

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BIOGRAPHY

David Germano Gonçalves Schwarz, son of Adolfo Germano Schwarz and Maria Edna Vitorina Gonçalves Schwarz, was born in Campinas-SP on May 16, 1984. As a child he moved to Joinville-SC.

In March of 2004 he began to study Veterinary Medicine in Universidade do Estado de Santa Catarina- UDESC, Lages-SC, finishing in December of 2008.

In April 2009, he began the *Lato sensu* postgraduate study in Animal Health Defense, receiving the specialist title in March 2012.

He has the title of *Magister Scientiae* in the Postgraduate Program (*Strictu sensu*) in Veterinary Medicine, Universidade Federal de Viçosa, obtained in April 2008, under the supervision of Professor Maria Aparecida Scatamburlo Moreira.

Currently, he is a student of the postgraduate program in veterinary medicine, at the doctoral level. He received a fellowship of CAPES (Coordination for Improvement of Higher Education Personnel) and continues under the guidance of Professor Maria Aparecida Scatamburlo Moreira.

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ABSTRACT

SCHWARZ, David Germano Gonçalves, D.Sc., Universidade Federal de Viçosa, December, 2016. **Studies on the interaction between *Mycobacterium avium* subsp. *paratuberculosis* and bovine mastitis associated *Escherichia coli* in a mammary epithelial cell model and identification of passive shedding in small ruminants.** Adviser: Maria Aparecida Scatamburlo Moreira. Co-advisers: Abelardo Silva Junior, Mary Hellen Fabres Klein, Leandro Licursi de Oliveira and Srinand Sreevatsan.

Mastitis caused by *Escherichia coli* can intensely stimulate the immune system and rapidly trigger inflammation in the mammary gland. In contrast, *Mycobacterium avium* subsp. *paratuberculosis* (MAP), the etiological agent of paratuberculosis, characterized by chronic granulomatous enteritis, can infect the mammary gland without intensely stimulating the inflammatory response. The interaction of these two species in the mammary gland is still unknown. In some cases, both the elimination of MAP by milk and faeces may occur passively, either through ascending infection of the mammary gland or through ingestion of MAP, respectively. These animals are called passive-shedders and are important as a source of infection to susceptible animals in the herd. The aim of this study was to investigate the relationship between MAP and *E. coli* in mammary gland cells under experimental conditions and verify the presence of passive-shedder animals. The relationship between a K-10 strain of MAP and *E. coli* isolated from mastitic milk in mammary epithelial cell lines was evaluated. Cells previously infected by MAP decreased *E. coli* invasiveness during 120min experimentation. However, the efficiency of *E. coli* translocation was not compromised, nor was the viability of the MAC-T cells. In contrast, cells previously infected by *E. coli* showed increased basal-apical translocation capacity of MAP up to 30 min and decreased at 120 min postinfection. Quantification of cytokines showed that IL-1β expression at 120 min was significantly increased in cells infected by MAP + *E. coli* and *E. coli* only. Expression of MAPKp^{38} and IL-10 were not significant, regardless of time postinfection. To determine the occurrence of passive-shedders, 10 properties were previously investigated for MAP detection. Thirteen goats were positive by faeces culture and/or milk PCR. Among the positive animals, four (4/13) were evaluated by IS*900*-PCR, feces culture, milk and tissue culture and serology (ELISA). All the results were negative over a one-year period, demonstrating that the animals performed pass-through phenomenon and upward contamination of the mammary gland without becoming infected. Together, these results indicate that the presence of MAP in

mammary cells may hamper capacity of *E. coli* invasion, but when within the mammary cell, the bacteria evade more efficiently. However, when the cells are pre-infected by *E. coli*, MAP is rapidly attracted from the subepithelial region to the cell surface. IL-1β production enhances the attraction of macrophages to the site of infection, where MAP benefits by infecting them.

RESUMO

SCHWARZ, David Germano Gonçalves, D.Sc., Universidade Federal de Viçosa, dezembro de 2016. **Estudos da interação entre *Mycobacterium avium* subsp. *paratuberculosis* e *Escherichia coli* associada à mastite bovina em um modelo de células epiteliais mamárias e identificação de transmissores passivos em pequenos rumantes.** Orientadora: Maria Aparecida Scatamburlo Moreira. Coorientadores: Abelardo Silva Júnior, Mary Hellen Fabres Klein, Leandro Licursi de Oliveira e Srinand Sreevatsan.

Mastite causada por *Escherichia coli* tem a capacidade de estimular intensamente o sistema imunológico e desencadear rapida inflamação na glândula mamária. Em contraste, *Mycobacterium avium* subsp. *paratuberculosis* (MAP), agente etiológico da paratuberculose, caracterizada por enterite granulomatosa crônica, pode infectar a glândula mamária sem estimular intensamente a resposta inflamatória. A interação dessas duas bactérias na glândula mamária ainda é desconhecida. Em alguns casos, tanto a eliminação de MAP pelo leite como pelas fezes podem ocorrer de forma passiva, após a infecção ascendente da glândula mamária ou após ingestão de MAP, respectivamente. Estes animais, chamados passive-shedders, são importantes como uma fonte de infecção a animais suscetíveis no rebanho. O objetivo deste estudo foi investigar a relação entre MAP e *E. coli* em células de glândula mamária sob condições experimentais e verificar a presença de animais passive-shedders. A relação entre uma cepa K-10 de MAP e *E. coli* isolada de leite mastítico em linhagem de células epiteliais mamárias (MAC-T) foi avaliada. As células previamente infectadas com MAP diminuíram a invasão de *E. coli* durante 120 min de experimentação. Contudo, a eficiência da translocação de *E. coli* e a viabilidade das células MAC-T não foram comprometidas. Ao contrário, células previamente infectadas por *E. coli* aumentaram a capacidade de translocação baso-apical de MAP até os 30 min e diminuiu aos 120min pós-infecção. A quantificação de citocinas relevou que a expressão de IL-1β aos 120min foi significativa ($P<0.05$) para células infectadas por MAP + *E. coli* e *E. coli* apenas. As expressões de $MAPKp^{38}$ e IL-10 não foram significativas, independente do tempo pós-infecção. Para detectar a ocorrência de animais passive-shedders, 10 propriedades foram previamente investigadas para a presença de MAP. Treze cabras foram positivas por cultura de fezes e/ou PCR de leite. Dentre os animais positivos, quatro (4/13) foram adquiridas e avaliados por IS*900*-PCR, cultura de fezes, de leite e de tecido, e sorologia (ELISA). Todos os resultados foram negativos no período de um

ano, demonstrando que os animais realizaram o fenômeno pass-through e a contaminação ascendente da glândula mamária, sem tornarem-se infectados. No geral, esses resultados indicam que a presença de MAP nas células mamárias pode dificultar a capacidade de invasão de *E. coli*, mas quando no interior da célula mamária, translocam-se mais eficientemente. No entanto, quando as células são previamente infectadas por *E. coli*, MAP é rapidamente atraído da região subepitelial para a superfície celular. A produção de IL-1β intensifica a atração de macrófagos para o sítio de infecção, onde MAP se beneficia, infectando-os.

1. GENERAL INTRODUCTION

Mycobacterium avium subsp. *paratuberculosis* (MAP) is the etiological agent of paratuberculosis, a disease that is of major importance in domestic ruminants. It is a chronic granulomatous enteritis, with diarrhea, progressive loss of weight, dehydration and death. Economically, paratuberculosis inflicts heavy losses in the dairy industry, as it significantly reduces milk production, increases the early culling of affected animals and harms exports of animal products. In addition, the lesion characteristics present in some human intestinal inflammatory diseases, such as Crohn's disease (CD), and the presence of MAP in intestinal biopsies, suggest a possible relationship between this agent and CD. After ingestion, MAP has a predilection for the terminal portions of the small intestine (jejunum and ileum) which are invaded through enterocytes and/or M cells, activating the pathways responsible for signaling the immune response, until they reach the antigen-presenting cells (macrophages and dendritic cells). In these cells, MAP blocks phagosome acidification and remains within these cells without being efficiently eliminated by the host immune system. As a result, granuloma formation occurs, making it difficult for the animal to absorb nutrients. In addition to the intestinal epithelial cells, MAP is also able to invade mammary epithelial cells and establish infection. Although the presence of MAP in milk of animals with the disease is described, the relationship of MAP with the mammary gland cell or to a secondary infection has not yet been evaluated. In mammary cells, MAP internalizes in cytoplasmic vacuoles, inferring the possibility that the mammary gland is a reservoir of MAP. Thus, the presence of MAP could interfere with local inflammatory processes or the invasion of other pathogens such as *Escherichia coli* and vice versa. In the literature, MAP-positive cows are reported to have fewer new cases of subclinical mastitis and lower rates of chronic infection. *E. coli* is the main etiological agent of environmental mastitis with great adaptive capacity within the mammary gland. It has the ability to remain within mammary gland cells or to form biofilms under adverse conditions and to reinfect the mammary epithelial cells when external conditions become suitable for their process of multiplication and invasion. All these factors associated with the presence of MAP and *E. coli* in the mammary gland, causing different intensities of immune response, reinforce the interest of studies investigating the relationship in a coinfective process in bovine mammary epithelial cells.

2. LITERATURE REVIEW

2.1. Paratuberculosis - Johne`s disease

In the last decades, among a great variety of diseases that affect domestic herds, paratuberculosis, caused *by Mycobacterium avium* subsp. *paratuberculosis* (MAP), has gained significant importance in public health (Crohn's disease) and in livestock (economic losses) worldwide. First, the presence of MAP in patients with Crohn's disease (DC), ulcerative colitis and other inflammatory bowel diseases (IBD) has been verified (Carvalho et al., 2015), but the real relationship between MAP and IBD is not yet present. Economically, MAP inflicts significant losses in the dairy industry due to a decrease in milk production (Lombard et al., 2005, Smith et al., 2009) increased early culling, low value sales of animals, and infertility (Mckenna et al., 2006, Kudahl and Nielsen, 2009). These losses have contributed to developed countries investing in the implementation of paratuberculosis control and eradication programs. However, developing countries still lack this same preventive priority in their herds (Schwarz et al., 2012). In the United States of America (USA), there has been an increase in the prevalence of the disease in bovine herds and, therefore, increasing economic losses (Ott et al., 1999; USDA-APHIS, 2011). In some regions of Australia, 40% of sheep herds are infected, with annual mortality rates of 2-15% (Bush et al., 2006). In addition to these countries, Japan, the Netherlands and Denmark have implemented and re-implemented programs with the intention of controlling and eradicating paratuberculosis (Benedictus et al., 2000; Nielsen, 2009). In South America, it has been observed that there is little information regarding the prevalence of this agent in their domestic herds. In a systematic review of the prevalence of the disease in Latin America and the Caribbean, Fernández-Silva (2014) found a prevalence of 16.9% among animals and 75.8% among herds. However, recently in Colombia, 2% seroprevalence occurs between animals and 3.6% among herds, demonstrating that these indicators vary according to the region and type of test used (Correa-Valencia et al., 2016). In Brazil, despite the occurrence of the disease (Ristow et al., 2007; Mota et al., 2009; Oliveira et al., 2010) and the detection of the agent in native bovine and buffalo herds (Ristow et al., 2007; Brito et al., 2016), in goats and sheep (Oliveira et al., 2010; Souza et al., 2016), and in raw and pasteurized milk (Carvalho et al., 2009; Carvalho et al., 2012), it

is known that the methods to estimate the prevalence of paratuberculosis differ among states, impairing the total evaluation of national herds (Schwarz et al., 2012). Values between 11.4% (Costa et al., 2010) and 60.24% (Acypreste et al., 2005) have been verified, depending on the state and methodology applied. Although there are no official data quantifying the productive losses in the herds and no governmental control program is in place, it has already been observed that Brazil is currently experiencing some obstacles in the export of some products such as semen and bovine embryos to countries that require tests of negativity for MAP in these products (Moreira, MAS, personal communication).

The etiologic agent of paratuberculosis, *Mycobacterium avium* subsp. *paratuberculosis* (MAP), is a bacillus that, among the members of mycobacteria, is the only one that requires a siderophore to solubilize free iron from the environment. The bacteria are aerobic, catalase-positive and lack motility; they multiply inside host cells, mainly macrophages (Collins et al., 2003). Although it is structurally closer to Gram-positive, it has a cell wall rich in mycolic acid, lipids and peptides which are better stained by the Ziehl-Nilseen technique, where carbol fuchsin binds to the cell wall and resists the alcohol-acid solution, thus MAP is an acid-alcohol-resistant bacillus (Timms et al., 2011). MAP culture can be performed in liquid medium (Middlebrook 7H9-MB7H9) or in solid medium (MB7H9, *Herrold's egg yolk medium* - HEYM), but all require the addition of mycobactin *J* for its growth, taking around 12 to 16 weeks for the emergence of first colonies on solid medium. Although the temperature of 37°C is chosen for incubation of the microorganism, other studies have shown that MAP can grow at a temperature of 39°C, reducing incubation time and increasing the infectivity of MAP in macrophages (Lamont et al., 2010) or survive and multiply slowly at 5°C (Timms et al., 2016).

MAP infection results in chronic granulomatous enteritis characterized by intermittent diarrhea not responsive to antibiotic therapy, progressive weight loss, malnutrition and death (Lombard, 2011). At the onset of infection, infected animals do not have a fever or any major clinical change. Throughout the progression of infection, excretion of MAP in feces and milk occurs. In this regard, feeding from infected cows facilitates the transmission of the agent to young animals (Patel and Shah, 2011). This fact becomes even more important, since the most susceptible animals are the youngest, on average in the first six months of life (Windsor and Whittington, 2010); calves' susceptibility to infection is greater up to one year of age, when it reaches the level of

resistance equivalent to adult animals. It is not clear why the early life stages are the most important for the infection. It is suggested that the immaturity of the innate and/or adaptive immune system and the intestinal absorption capacity of intact macromolecules in young animals could be crucial for susceptibility (Sweeney 2011). Therefore, although there is a possibility of vertical transmission, the main form of transmission is horizontal, where contaminated mammary secretions and the contact of udders and dirty teats with feces of affected animals can infect calves during lactation (Sweeney et al., 1997; Clarke et al., 2000; Doré et al., 2012). In an experiment with five cattle under the same nutritional and environmental conditions, it resulted in the clinical manifestation of two animals after oral MAP inoculation. The other animals had subclinical infections. These results demonstrate that there are individual, possibly genetic, factors that influence in the age and severity of the lesions (Mortier et al., 2015).

Traditionally cattle were diagnosed as positive or negative, based on the presence of MAP in fecal cultures and categorized as low or high shedders according to the number of colonies observed (Pradhan et al., 2011). However, evidence that some infected animals could ingest the microorganism and release it through the faeces without becoming infected determined the need for subsequent evaluations to detect truly positive animals (active-shedders) and false positives (passive-shedders) (Sweeney et al., 1996; Kralik et al., 2014). According to Pradhan et al. (2011), animals that have low colon count for MAP (<10 CFU/tube) are not necessarily passive-shedders. The degree of MAP detection in faeces may be related to other factors, mainly to the amount of MAP ingested. In general, after ingestion of MAP by susceptible animals, it reaches the intestine where the intestinal epithelium serves as the first defensive barrier against the microorganism, mainly by innate mechanisms such as glycocalyx, cell junctions and antimicrobial peptides (Bannantine and Bermudez 2013). However, MAP has the ability to bypass these defenses and cause infection by passing through two cell types: M (microfold cells) and enterocytes. MAP entry into M cells is dependent on the binding between fibronectins (FN) and fibronectin binding proteins (FAPs) expressed by the microorganism. Therefore, the predilection of MAP for M cells is primarily related to binding by an FN-dependent mechanism and subsequently, the FN-opsonized ones bind to β1 integrins present on the luminal surface of M cells (Byrd et al., 1993; Secott et al., 2004). Blocking of integrin subunits revealed a decrease in MAP invasion in M cells, indicating the selective importance of the β1 subunit and the involvement of the αVβ3

integrin in this process (Secott et al., 2004). In another study, when using mouse anti-α5 β1 IgG antibody, a 37.3% reduction of MAP entry in cells was observed, showing that the microorganism activated other pathways besides FN-dependent for internalization (Bermudez et al., 2010).

Although studies have shown that MAP has a greater predilection for the M cells of Peyer's paches (Ponnusamy et al., 2013), it was observed that MAP had the same invasive behavior between intestinal segments with or without Peyer's paches in experimentally infected mice (Bermudez et al., 2010). In this study, comparing the MAP and *Mycobacterium avium* subsp. *hominissuis* (MAH), it was found that MAP was limited to sub mucosa while MAH was found in the liver and kidneys over the same evaluation period. This demonstrates the characteristic of local infection of MAP and its chronic characteristic of infection. Pott et al. (2009) verified that MAP has marked tropism of intestinal cells adhering and rapidly internalizing in m-IC_{cl2} cells with presence of MAP in the late endosome after 30 minutes in contact.

When MAP penetrates cells, there is a rapid suppression of cellular communications pathways that include the communicating junctions, gap junctions and adherent junctions. This intercellular junction complex is capable of establishing greater resistance to infection, but when there is a decrease in the expression of genes related to this pathway, it reduces the efficacy of the local immune barrier (Khare et al., 2012). This behavior may be an important factor in the maintenance of MAP within these cells. The mechanism by which MAP evades epithelial cells is not yet clearly described, but it is known that within M cells, MAP is easily displaced to the basal region and captured by dendritic cells or macrophages. On the other hand, when inside enterocytes, MAP seems to stimulate Cdc42 and RhoA GTPases proteins promoting MAP traffic to the interior of endosomes, where it becomes acidified. This process is crucial for the rapid production of IL-1β, attracting subepithelial macrophages and infecting them more efficiently (Figure 1) (Lamont et al., 2012; Bannantine and Bermudez 2013). Patel et al. (2006) found that the efficiency of invasion in bovine epithelial cells was higher when MAP was previously incubated in milk or when it has previously been passaged through mammary epithelial cells, demonstrating that there are other factors that contribute to the greater spread of the agent to the subepithelial macrophages.

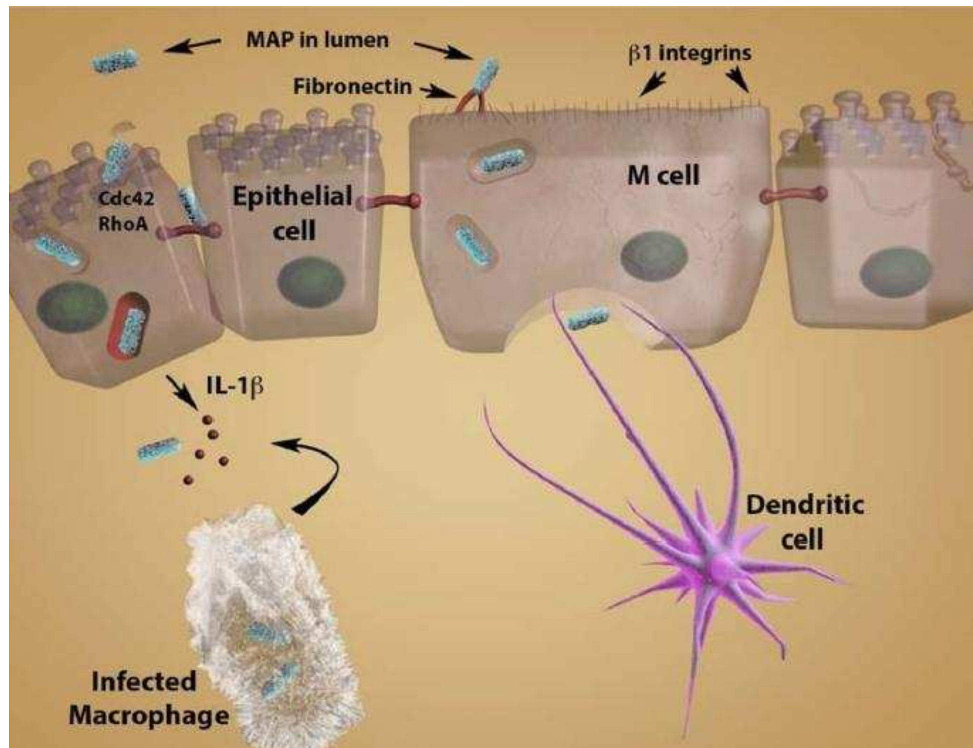

Figure 1. Mechanism of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) internalization in M cells and enterocytes. Infection process of MAP in M cells and enterocytes. MAP binds to β1 integrins via fibronectin-dependent binding and invades M cells until they are picked up by dendritic cells. MAP can also adhere to enterocyte surfaces and activate Cdc42 and RhoA proteins for activation and endosome acidification, resulting in the release of IL-1β and chemotaxis of macrophages to the site of infection (Bannatine and Bermudez 2013).

MAP can penetrate macrophages by different pathways, coupling to distinct receptors (complement receptors, immunoglobulin receptors, mannose and scavenger) and leading to different stimuli of the immune system (Guirado et al., 2013). In the interior of the macrophages, MAP is able to prevent the maturation and acidification of phagosomes, as well as the formation of phagolysomes, responsible for the activity of catabolization of foreign components. This process occurs because MAP can stimulate SapM lipid phosphatase production by eliminating phosphatidylinositol 3-phosphate (PI3P) from the phagosome membrane and preventing its maturation (Vergne et al., 2005). It has been demonstrated that the inhibition of acidification of phagosomes does not occur at the transcriptional level. In *Mycobaterium tuberculosis*, the production of protein tyrosine phosphatase (PtpA) allows it to bind to a subunit of the H + -ATPase (V-ATPase) machinery of the vacuolar macrophage preventing its luminal acidification and consequently the phagosome-lysosome fusion (Wong et al., 2011). Thus, it is suggested that this mechanism responsible for the transport of protons through the

membrane with the use of ATP can also be blocked when MAP invades subepithelial macrophages (Arsenault et al., 2014).

Detection and activation of cell pathways between microorganisms and the host occurs by binding of Pattern Recognition Receptors (PRRs), such as Toll-like receptors (TLRs) to Pathogen-associated molecular patterns (PAMPs). Different TLRs are activated by mycobacterial infection, among them TLR2, TLR4 and TLR9. Manosilated lipoarabinomanan (Man-LAM) is a cell wall lipoglycan of pathogenic mycobacteria, including MAP, capable of binding to TLR2 and activating the intracellular signaling of the MAPK-p^{38} pathway. The activation of this pathway results in the production of IL-10 responsible for suppressing the expression of proinflammatory cytokines, chemokines, IL-12 and major histocompatibility factor type II (Arsenault et al., 2014). Furthermore, IL-10 has been shown to be a mediator of MAP survival within macrophages and evidence shows that the TLR2-MAPK-p38 signaling pathway is involved in the suppression of the MAP antimicrobial response (Souza et al., 2013). Man-LAM also reduces the expression of TNF-α and IL-12 and increases the expression of SHP-1, a tyrosine phosphatase responsible for suppressing the immune response in macrophages (Knutson et al., 1998).

MAP infection results in the early production of IFN-γ, remaining for a long period during infection. It is known that IFN-ɣ is responsible for the control and elimination of intracellular agents due to binding of target cells and high affinity IFN-receptors, which activate the JAK-STAT pathway (Bach et al., 1997). In order to be able to remain inside the macrophage and resist the action of IFN-ɣ, MAP uses some strategies to activate SHP-1 and inhibit JAK/STAT signaling. Therefore, MAP is able to increase the expression of suppressors of cytokine signaling (SOCS), reducing the regulation of receptors for IFN-ɣ and decreasing the expression of receptor genes (Arsenault et al., 2014).

2.2. Mammary gland - *Mycobacterium avium* subsp. *paratuberculosis* and *Escherichia coli*

Although an experimental study by Larsen and Miller (1978) has reported that apparently MAP does not cause bovine mammary gland inflammation, findings from

subsequent studies have demonstrated the presence of MAP within mammary glands and in the milk of symptomatic or asymptomatic animals (Barrington et al., 2003; Streeter et al., 1995). These observations infer the possibility that the mammary gland is a reservoir of MAP, contributing to the increase of local inflammation of the tissue or the alteration of the permeability of the mammary cells. Within mammary gland cells, MAP is found inside cytoplasmic vacuoles, similarly observed when infected in macrophages (Patel et al., 2006). Experimentally, MAP has been shown to invade bovine mammary epithelial cell lines (MAC-T) both by the apical route and by the basolateral route with similar efficiency. This finding demonstrates that MAP can translocate in mammary cells both through the ascending and systemic pathways (Patel et al., 2006). A study by Lamont et al. (2012) demonstrated by coculture of MAC-T cells and macrophages derived from bovine monocytes that MAP is dependent on extracellular calcium for the acidification of phagosomes and IL-1β production – host factors necessary for bacterial translocation to macrophages. The study of cytokines in MAP-infected mammary cells is poorly understood. However, by the study of Lamont et al. (2012), it was possible to understand that MAP uses a mechanism different from other mycobacteria, causing acidification of phagosomes and stimulation of IL-1β to attract macrophages to local mammary epithelial cells, subsequently infecting them. Although mammary infection can occur by several infectious agents, the mutual contribution of other bacteria in the action of MAP under the mammary gland is not established. Mastitis is known to represent the most common infectious inflammatory phenomena of the mammalian mammary gland (Bradley, 2002). It is a multifactorial disease that has the interrelations between the host, the environment and the infectious agents for its occurrence (Leblanc et al., 2006). Bovine mastitis is divided into contagious and environmental mastitis (Andrews et al., 2008). In contagious mastitis, the predominant pathogens are *Staphylococcus aureus*, *Streptococcus agalactiae*, *Corynebacterium bovis*, *Streptococcus dysgalactiae* and *Mycoplasma* spp. In environmental mastitis, the most frequently isolated microorganisms are Gram-negative bacteria, such as *E. coli*, *Klebsiella* spp., *Enterobacter* spp., *Pseudomonas* spp. and *Proteus* spp. (Oliver et al., 2011). *E. coli* is the most representative pathogen isolated from environmental mastitis cases (Wenz et al., 2006; Oliver et al., 2011). The *E. coli* isolates from animals with mastitis are quite similar to those found in faeces (Burvenich et al., 2003), with large varieties of serotypes. However, the central regions of the cell wall and lipid A from LPS are common to all serotypes and the Enterobacteriaceae

family in general (Burvenich et al., 2003). Among these, *E. coli* produces several virulence factors involved in the pathophysiology of bovine mastitis, which are necessary to colonize and infect the mammary gland, and protect the microorganism against the animal's defense mechanisms (Burvenich et al. 2003; Naves et al., 2008). These factors include toxins, adhesins, proteins secreted in animal cells, polysaccharide capsule, O-antigen and biofilms (Dyer et al., 2007). In general, these factors trigger important inflammatory reactions in the mammary gland and are responsible for recurrence of the infection.

Although *E. coli* is one of the main etiological agents of clinical mastitis, its isolates are considered opportunistic pathogens with different virulence factors, indicating that there are no specific pathotypes that cause mastitis (Fernandes et al., 2011). Also, *E. coli* associated with persistent intramammary infection present greater ability of adherence, invasion, survival and multiplication in mammary epithelial cells. Possibly the cytoskeletal rearrangement of host cells and the signaling of cascades mediated by phosphorylation facilitate the invasion and permanence of this microorganism within mammary epithelial cells (Dogan et al., 2006). Therefore, *E. coli* is extremely adaptable to the invasion process, being able to induce the formation of biofilms inside the mammary gland under adverse conditions, such as administration of antimicrobials commonly used in the treatment of mastitis (Silva et al., 2014). Its characteristic of escaping the immune system, remaining in the mammary gland and causing recurrent mastitis, instigates the study of its behavior against a process of co-infection in mammary epithelial cells.

3. OBJECTIVES

3.1. General objective

To determine the relationship between *Mycobacterium avium* subsp. *paratuberculosis* (MAP) and *Escherichia coli* in bovine mammary epithelial cells under experimental conditions and detection of passive-shedders goats in Brazil.

3.2. Specific objectives

A) To assess the ability of baso-apical MAP translocation in bovine mammary epithelial cell lines (MAC-T) previously infected with *E. coli*.

B) Evaluate the inflammatory factores produced by MAP and *E. coli* coinfection in MAC-T cells by cytokine quantification.

C) Evaluate the ability of MAP to influence the process of *E. coli* infection in MAC-T cells.

D) Verify the occurrence of pass-through and contamination in the mammary gland of lactating goats.

4. CHAPTER I.

INHIBITION OF *Escherichia coli* INVASION INTO BOVINE MAMMARY EPITHELIAL CELLS PREVIOUSLY INFECTED BY *Mycobacterium avium* subsp. *paratuberculosis*

Schwarz, D. G. G., Oliveira, L. L., Moreira, M. A. S.

ABSTRACT

Mastitis and paratuberculosis are diseases that affect all ruminants, but have special importance in dairy herds. *Escherichia coli* is the main etiological agent of environmental mastitis presenting an acute inflammatory character with intense stimulation of the immune response. *Mycobacterium avium* subsp. *paratuberculosis* (MAP), the etiologic agent of paratuberculosis, leads to a chronic granulomatous enteritis that can release the microorganism through feces or milk. Although MAP has not been reported to cause mastitis, its contribution to the subsequent *E. coli* infection process in mammary epithelial cells is unknown. In this study, the ability of MAP to interfere with the invasion and translocation process of *E. coli* in MAC-T cells was evaluated. Previous MAP infection in MAC-T cells inhibited *E. coli* invasion at 10 min, 30 min and 120 min. In contrast, there was no significant interference of MAP in the course of *E. coli* through the cells as tested in a translocation assay. Similar efficiency in translocation of *E. coli* between MAP-pre-infected cells and MAP-uninfected cells was observed. The MTT assay revealed that both MAP and *E. coli* did not decrease the viability of MAC-T cells for 2h. Furthermore, *E. coli* growth was optimized when 75% of MAP culture supernatant was included in Luria-Bertani medium. Overall, these findings suggest that cows positive for MAP and presenting the microorganism in mammary epithelial cells could be more resistant to *E. coli* infection, reducing the invasive and persistent capacity of this agent.

Key-words: Coinfection, mastitis, paratuberculosis, epithelial cells, mamary gland

INTRODUCTION

Escherichia coli is the most abundant facultative anaerobic and opportunistic pathogen that can infect humans and animals in worldwide (Jensen et al. 2013; Conte et al., 2016). In animals, mastitis is a major disease that affects a large part of the world's dairy herds and inflicts great economic losses in the dairy industry (Bradley 2002). Among more than 150 different etiologic agents, *E. coli* is the main bacteria isolated from clinical mastitis. This infection causes an intense immune response which elicits bacterial clearance in a short time. Although the matitis caused by *E. coli* is considered a transient infection, it can advance to a clonal persistent intramammary infection, suggesting an adaptation of this pathogen to the bovine udder environment (Bradley and Green 2003; Dogan et al., 2006),

In order for an infection to be established, the pathogen must successfully interact with the host cells through microbial adherence to the host, pathogen uptake, and bacterial survival and multiplication within mammalian cells (Finlay and Cossart, 1997). *E. coli* associated with persistent intramammary infection has a better ability to adhere to, invade, survive and replicate in mammary epithelial cells than *E. coli* associated with transient infection (Dopfer et al., 2000; Dogan et al., 2006; Dogan et al., 2012). Invasion of *E. coli* into mammary epithelial cells is not fully understood and there are currently conflicting hypotheses that seek to explain this process. Dogan et al. (2006) verified that *E. coli* can use the rearrangement of the cytoskeleton and phosphorylation-mediated signaling cascades to facilite this process. However, Passey et al. (2008) identified that the invasion process occured though a membrane-bound endocytic vacuole.

Mycobacterium avium subsp. *paratuberculosis* (MAP) is the ethiologic agent of paratuberculosis, or Johne´s disease. The disease causes a chronic and incurable granulomatous enteritis in ruminants. It is characterized by the reduction in milk production, diarrhea, progressive weight loss, malnutrition and death (Collins et al., 2003; Lombard, 2011). Once ingested, MAP enters the organism's digestive tract through Peyer's patches by fibronectin-dependent mechanisms or by endocytosis in the enterocytes. It can then reach the subepithelial macrophages, the bloodstream and multiply in satellite lymph nodes (Bermudez et al., 2010). In this way, MAP can be released by faeces or milk from affected animals continuously or intermittently. The infectious capacity of MAP in mammary epithelial cells has been proven (Patel et al.,

2006; Lamont et al., 2012). However, there is no evidence that MAP is associated with mastitis (Larsen and Miller, 1978).

Few studies have demonstrated the influence of coinfection between pathogenic bacteria in the mammary gland invasion process. Recent studies have shown that probiotics (*Lactobacillus casei* and *Lactococcus lactis* V7) were able to inhibit the adhesion and invasion of *Staphylococcus aureus* and *E. coli* in mammary cells (Bouchard et al., 2013; Assis et al., 2015). Similarly, the presence of cell wall components from *Saccharomyces cerevisiae* were effective in inhibiting MAP adhesion to mammary epithelial cells (MAC-T) and bovine primary epithelial cells (Li et al., 2016).

In this work, we hypothesize that MAC-T cells previously infected by MAP could interfere with a subsequent infection by *E. coli* strain isolated from bovine mastitic milk.

MATERIALS AND METHODS

Ethics Statement

All experiments were carried out in accordance with the Ethics Committee on Animal Use (CEUA) at the Universidade Federal de Viçosa, Brazil, under approval protocol 36/2014.

Bacterial strains and culture

Mycobacterium avium subsp. *paratuberculosis* (MAP) strain K-10 (cattle isolate) and an *Escherichia coli* strain isolated from mastitis bovine milk, kindly provided by Dr. Srinand Sreevatsan, College of Veterinary Medicine, University of Minnesota, USA, were used in this experiment. The MAP strain was grown in Middlebrook (MB) 7H9 broth containing 0.2% glycerol, 10% oleic acid dextrose-catalase (OADC) and mycobactin *J* (2 mg/L) (Allied Monitor, Inc. Fayette, MO), at 37°C for six weeks until reaching the optical density (OD_{600}) of 0.5 (equivalent to 10^6 CFU/mL). The culture was determined free of contaminating microorganisms by the absence of colonies in Brain Heart Infusion (BHI) medium. *E. coli* isolate was grown on Luria-Bertani (LB) agar overnight and one colony was incubated for 4 h (to mid-log

phase) at 37°C in 5 ml BHI broth without shaking to an optical density at 600 nm [OD_{600}] = 0.3 (equivalent to 10^8 CFU/mL).

Mammary epithelial cells and culture conditions

Bovine mammary epithelial cells (MAC-T) line have been widely used in adhesion/invasion assays (Li et al., 2016) and thus was in this experiment. MAC-T cells were cultured in T25 cell culture flasks (TPP Techno Plastic, St. Louis, MO, USA) at 37°C with 5% CO_2, containing Dulbecco`s Modified Eagle Medium (DMEM; Gibco-BRL, Grand Island, NY, USA) supplemented with 10% heat inactivated fetal bovine serum (FBS; Sigma-Aldrich, St. Louis MO, USA) and 1% penicillin/streptomycin (100µg/ml) up to the confluence of the cells. After visualizing the monolayer on a Nikon phase contrast microscope (Nippon Kogaku K. K., Tokyo, Japan), cells were washed with PBS (pH 7.2), resuspended by the addition of 0.25% trypsin (Sigma-Aldrich, St. Louis, MO, USA) and subsequently used in the experiments.

Internalization assay

Internalization assay was performed following the procedure described previously (Bouchard et al., 2013) with modifications. Briefly, confluent monolayers of MAC-T cells, prepared as described above, were washed twice with PBS, dislodged with 0.25% trypsin for 5 min, and counted with 0.4% trypan blue (Invitrogen) using a hemocytometer chamber slide. MAC-T cells were seeded ($3x10^5$ cells) into 24-well polystyrene plates within cell culture medium (DMEM, 10%FBS, without antibiotics) and incubated in a humidified chamber at 37°C with 5% CO_2 for 24h. A 21 gauge syringe needle was used for breaking the MAP clumps and upper three-fourths of it were used for the challenge. After 90-95% of monolayer confluence, cells were washed twice with warmed PBS and challenged with MAP (1.0 x 10^6 CFU) for 2h. Cells were washed three times with warmed PBS and challenged with *E. coli* (1.0 x 10^8 CFU/ml) for 10min, 30min and 120min, following an additional 2h incubation step with DMEM supplemented with gentamicin (100 µg/mL). This step ensured that any extracellular bacteria (adhered or planktonic) did not remain viable due to the bactericidal action of the antimicrobial. Subsequently, culture supernatants were removed and monolayers were washed four times with PBS and lysed with 50µL of 0.25% (wt/vol) trypsin (Sigma-Aldrich, St. Louis, MO) and 0.1% (vol/vol) Triton X-100 (Amersham, Arlington Heights, IL) in PBS for 10 min. Lysates were serially diluted and plated on

LB agar, and bacterial concentrations were determined from the colony counts after incubation at 37°C for 24h. Positive control-wells (*E. coli*) and negative control-wells (MAC-T cells) were cultivated as described above in triplicate. All assays were reapeated three times.

Transepithelial bacterial translocation assay

Approximately 3.0 x 10^5 MAC-T were seeded onto the apical side of 3.0-µm-pore-size Transwell®-Clear inserts (Corning, Lowell, MA) in DMEM containing 10% FBS and incubated at 37°C in a humidified chamber containing 5% CO_2 up to the confluence of the monolayer (~ 2 days). Integrity of the monolayers was determined by the following methods: (i) phase contrast microscopy observation, (ii) staining of monolayer by crystal violet, and (iii) trypan blue (0.25%) permeability assay (optical density at 540 nm). Trypan blue was added to the monolayer and 1h later the supernatant of the lower chamber was obtained for spectrophotometric reading, as previously described by Patel et al. (2006). MAP culture (OD_{600} = 0.5) was pelleted at 12000 x *g* for 15 min, washed two times in PBS and resuspended in DMEM containing 10% FBS. MAP supension (1.0 x 10^6 CFU) was then passed through a sterile 21-gauge needle 20 times for breaking the bacterial clumps and upper three-fourths of solution were used for infection. MAP was applied to the upper chamber and allowed to infect the MAC-T cells for 2h at 37°C in a humidified chamber containing 5% CO_2 followed by three whashings with PBS. Then, *E. coli* (1.0 x 10^8 CFU) was applied to the apical chamber and incubated under the same conditions described above for 10, 30 and 120 min. After these times, the supernatant from the lower chamber was centrifuged at 12,000 x *g* for 5 min, resuspended in 100 µL of 0.9% saline solution, serially diluted, and plated onto LB agar for colony count. The negative control was unchallenged MAC-T cells, and positive control was MAC-T cells challenged with *E. coli* only. All assays were carried out in triplicate and repeated three times.

MTT cell viability assays

For the viability assay, MAC-T cells (1.0 x 10^5 cells) were cultivated in 96-well polystyrene plates, at the same conditions described above, and challenged with MAP (2.0 x 10^5 CFU) for 2h. After washing three times with PBS, the MAC-T cells were challenged with *E. coli* (2.0 x 10^7 CFU) for 10, 30 and 120 min, as described above. After incubation steps, cells were washed four times and incubated in 0.5 mg/mL (3-

(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) (Sigma-Aldrich, St. Louis, EUA) in PBS for 2h at 37°C in 5% CO_2. The supernatant was removed and 200µL of Dimethyl Sulfoxide (DMSO) was added for 20 min. The purple color thus formed was measured at 550 nm in spectrophotometer. Uninfected were used as negative control (100% viability), and cells treated with 0.1% (vol/vol) Triton X-100 served as a positive control of mortality (0% viability). Relative viability was expressed with regard to uninfected cells. The assays were carried out in triplicate and reapeated three times.

Bacterial growth curve assay

MAP culture (OD_{600}=1.0) was centrifuged at 12,000 x *g* for 5 min and the supernatant was reserved. Aliquots of 1 mL (1.0 x 10^7 CFU) of *E. coli* culture (OD_{600}=0.1) were centrifuged at 12,000 x *g* for 5 min and the supernatant was discarded. The remaining pellets were resuspended in different amounts of MAP supernatants (100%, 75%, 50%, 25% and 0%) and the proportion of each amount completed with LB broth to 1mL, and homogenized. Subsequently, 250 µL of each concentration was added in 96 wells plates and every 30 min during 24h, the bacterial growth rates were evaluated by optical density 600 nm (λ) in automatic spectrophotometer (Multiskan GO, Thermo Scientific, Waltham, USA). For the negative control, MAP supernatant at different concentrations as described above was used without the presence of *E. coli*. For the positive control, different amounts of MB7H9 broth (100%, 75%, 50%, 25% and 0%) were similarly homogenized with LB broth to a final volume equal at 1 mL with *E. coli*. The results were obtained by subtracting the values from the experiment using the MAP supernatant and the positive and negative controls. The pH of the supernatants was measured by the PH meter mPA 210 (Tecnopon). All the experiments were carried out in triplicate and repeated three times.

Statistical analysis

The results obtained were analyzed by two-way analysis of variance (ANOVA) with the Tukey test in Graphpad Prism software (Software, La Jolla, CA). *P* values of less than 0.05 were considered statistically significant.

RESULTS

Influence of MAP on the internalization of *E. coli* in MAC-T cells

We tested *in vitro* the internalization ability of *E. coli* isolated from bovine mastitic milk in MAC-T cells previously infected by MAP K-10. In figure 1A, it is observed a significant (*P*<0.05) lower number of *E. coli* colonies in cells previously infected by MAP in relation to those without prior infection regardless of the challenge time. The efficiencies of internalization at 10 min, 30 min and 120 min time points were comparable; however, no significant differences were found between 10 min and 30 min post infection, but there was a significant difference (*P*<0.01) at 120 min post infection (Fig. 1B).

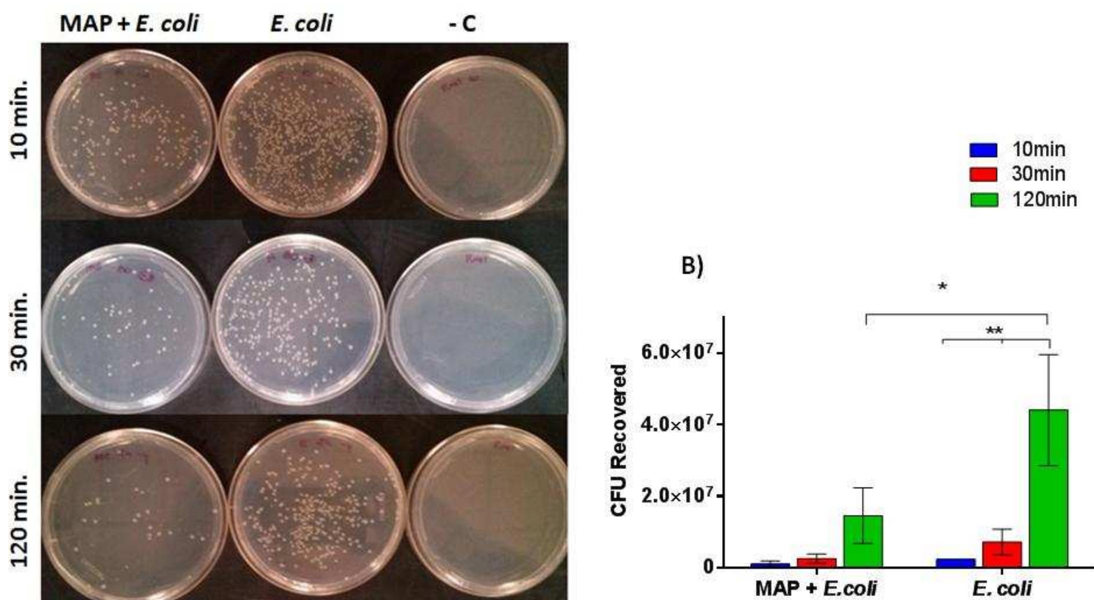

FIG. 1. *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in mammary epithelial cell line (MAC-T) inhibits *E. coli* internalization. A) Plaqueation in Luria-Bertani medium (LB) of MAC-T cell lysates challenged by MAP+*E. coli*; *E. coli* and negative control at different times post-infection (p.i.). B) Mean and standard deviation of Colony Forming Units (CFU/mL) in different treatments, according to the time p.i. Differences between treatments (MAP+ *E. coli* and *E. coli*) was statistically significant (*P< 0.05). At 120 min, it was statistically significant (**P <0.01) in relation to the other times analyzed. The assay was carried out in triplicate and reapeated three times.

In contrast to the internalization assay, there was no statistical difference between the treatments for the 10 and 30 minute timepoints. However, *E. coli* invaded more efficiently at 120 minutes (*P*<0.001) when the MAC-T cells weren't previously

treated with MAP, regardless of the time p.i. or the treatment evaluated. (Fig. 2A,B). (Fig. 2A,B).

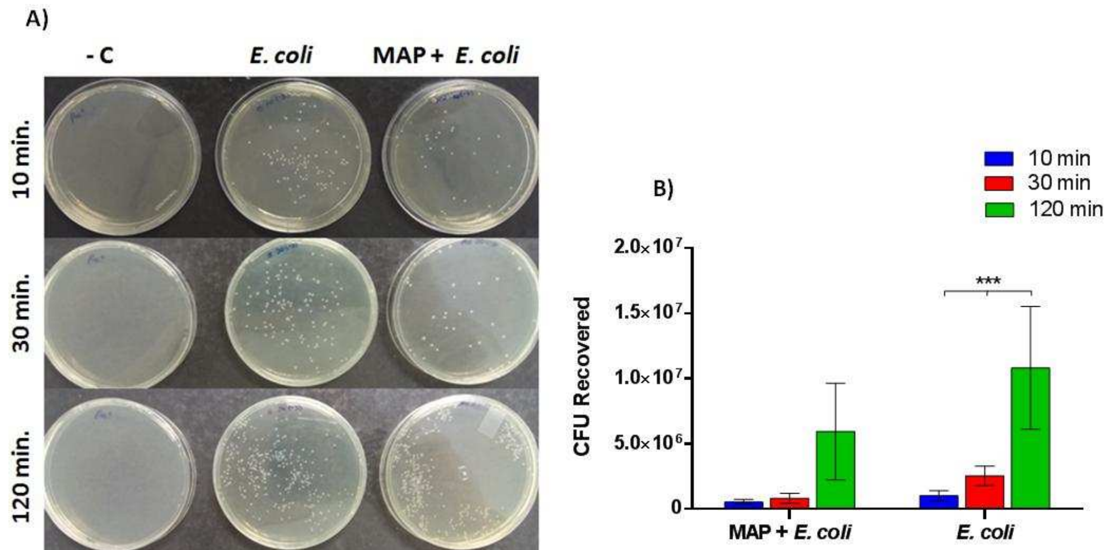

FIG.2. Presence of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in mammary epithelial cells did not alter the *E. coli* translocation. A) Plaqueation in Luria-Bertani medium (LB) of MAC-T cell lysates challenged by MAP+*E. coli*; *E. coli* and negative control at different time post-infection (p.i). B)) Mean and standard deviation of Colony Forming Units (CFU) recovered in different treatments, acording to the time p.i. No difference between the treatments (MAP+ *E. coli* and *E. coli*) was observed. In *E. coli* treatment, at 120 min, the translocation was significant (***P<0.001), regardless of the time p.i. The assay was carried out in triplicate and reapeated three times.

Viability of MAC-T cells when infected by MAP and/or *E. coli*

We showed that the prior infection by MAP significantly reduces the internalization of *E. coli* at 120 min in MAC-T cells (Fig 1B). Additional tests were done to investigate the effect of MAP, *E. coli* or MAP + *E. coli* on cell viability (Fig.3). The cell mortality assessed by MTT assay revealed no difference between treatments, i.e., the presence of MAP or *E. coli* or MAP + *E. coli* does not interfere in the viability of MAC-T cells during 2h of evaluation.

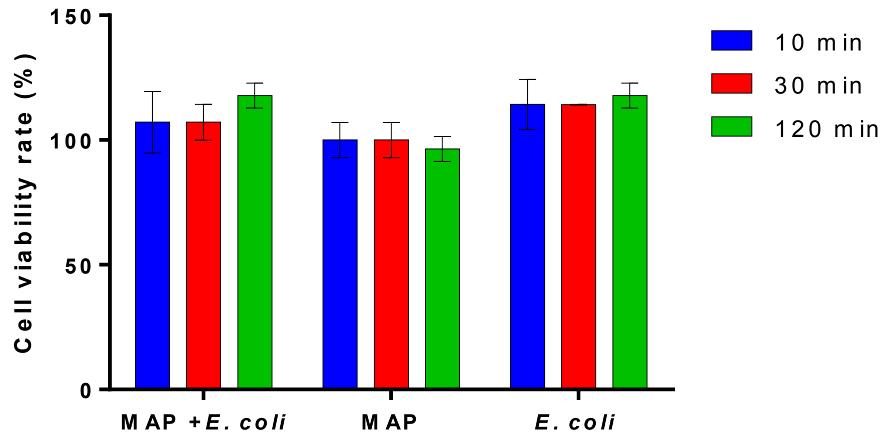

FIG.3. MAP and/or *E. coli* infection did not alter the viability of MAC-T cells. Cell viability was assessed in 10 min, 30 min and 120 min post-infection in different treatments (MAP+*E. coli*; *E. coli* and MAP) by MTT assay. MAC-T cells alone was used as a positive control (100% viability, not shown) and cells treated with 0.01% triton as a negative control (0% viability at each time point, not shown). The assay was carried out in triplicate and reapeated three times.

MAP supernatant alters the growth of *E. coli*

To ascertain whether the MAP culture supernatant could interfere with the bacterial growth of *E. coli*, decreasing amounts of the MAP supernatant were homogenized with increasing amounts of LB and the results relativized with the positive control. Although 100% of the supernatant allowed considerable growth of *E. coli* during the 24h of analysis, only with 75% was there a significant difference ($P<0.05$) in relation to the other proportions (Fig.4). In addition, a higher log-phase of *E. coli* was observed in 75% supernatant compared with other supernatants' concentrations. There were no large variations in pH, remaining around 7.2. This demonstrates that pH did not interfere in this experiment.

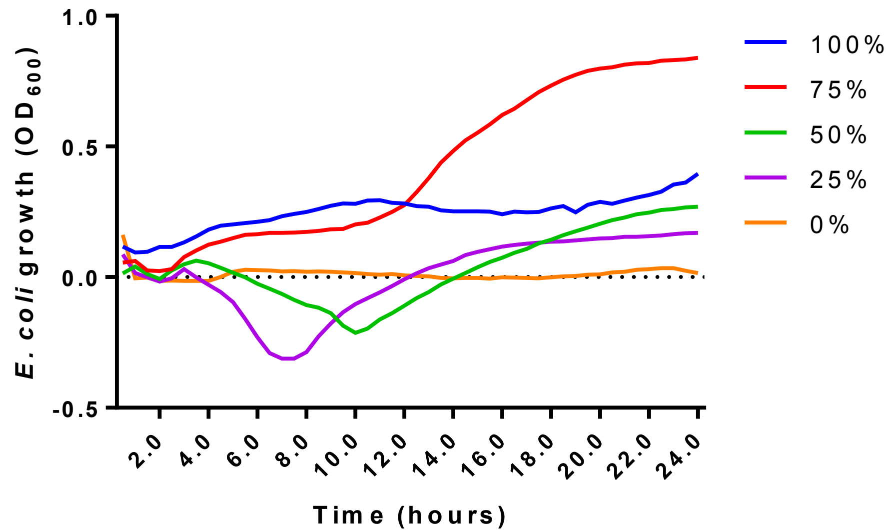

FIG.4. The proportion of 75% MAP supernatant revealed significant increase of *E. coli* growth. Relation between *E. coli* growth rate, detected by spectrophotometry (OD_{600}), and the time. The results presented are the subtraction of *E. coli* growth in medium with different percentages of the MAP supernatant and the respective positive controls (Middlebrook 7H9). The assay was carried out in triplicate and reapeated three times.

DISCUSSION

Among the bacteria that infect the mammary gland, *E. coli* is well known for causing rapid clinical manifestation with an intense immune response (Bradley and Green 2001). On the other hand, some bacteria, e.g., *Mycobacterium avium* subsp. *paratuberculosis* (MAP) even being released by milk, appear to poorly stimulate the local inflammatory response and are not considered as the ethiologic agent of classic mastitis (Larsen and Miller, 1978). The mechanisms of how a nonmastitis pathogen could infect the mammary gland and interfere in a subsequent infection by another mastitis pathogen are still unknown. We hypothesized that prior MAP infection in bovine mammary epithelial cells could alter the subsequent ability of *E. coli* to invade these cells. Our results demonstrated that MAP was able to rapidly inhibit the invasion of *E. coli* in MAC-T cells. Immediately at 10 min, a reduction in the number of *E. coli* colonies in MAC-T cells previously treated with MAP was observed when compared to cells infected only by *E. coli* (Fig. 1A). This pattern of infection was observed at all time-points within a 2h period. Regardless of the time evaluated, rates of internalization to MAC-T cells were 2-to-3-fold lower for cells infected by MAP + *E. coli* than for

infected by *E. coli* only, demonstrating that the presence of MAP inhibited this invasion process. Similarly, it was found that *Lactococcus lactis* V7 was able to inhibit the internalization of *Staphylococcus aureus* and *E. coli* in MAC-T cells, ranging from 45% to 88% inhibition (Assis et al., 2015). Besides the presence of bacteria, it was verified that other factors may influence the process of invasion in mammary cells. For example, Silva et al. (2014) verified that the subinhibitory concentrations of antimicrobials in the medium increased the internalization capacity of *E. coli* isolated from bovine mastitis in MAC-T cells, but did not alter the adhesion. This fact suggests that the internalization process is not necessarily associated with the adherence ability.

In the present study, to evaluate whether the presence of MAP inhibits not only the invasion but also interferes with *E. coli* translocation through the cells, a transepithelial assay was performed. Interestingly, the number of translocated colonies of the apical-basal region were statistically the same, regardless of the treatment (Fig 1A,B). Since MAP inhibited the internalization of *E. coli* but maintained the number of bacteria evaded from the cells when compared to its positive control (*E. coli* only), it could be inferred that MAP enabled higher efficiency in translocating *E. coli*. This event could provide a distinct survival advantage that allows the pathogens that have invaded cells previously infected by another microorganism to escape rapidly from the cells in order to avoid possible changes caused by this agent in the cellular microenvironment. It has been reported that in non-phagocytic cells, both pathogenic (García-Pérez et al., 2003) and non-pathogenic (García-Pérez et al., 2008) mycobacteria use macropinocitosis with membrane ruffle formation to internalize. Thus, it is suggested that in mammary epithelial cells, MAP could use this mechanism to be internalized in the endosomes. Interestingly, contrary to what is observed in other mycobacteria, when invading mammary epithelial cells, MAP is able to induce phagosome acidification between 10 and 30 min post infection and IL-1β production in order to attract subepithelial macrophages at the site of infection (Lamont et al., 2012). Instead, Passey et al. (2008) have verified that *E. coli* can invade mammary cells without resulting in a visible rearrangement of the cytoskeleton and may activate its intracellular traffic avoiding mechanisms of acidification of phagosomes. Possibly the process of induction and control of intracellular acidification triggered by MAP may have been crucial for the ability of *E. coli* to internalize and/or evade the cells.

A potential concern about the preceeding observations was that the reduction of *E. coli* internalization to epithelial cells was due to decreased cell viability. Li et al.

(2016) demonstred that the yeast cell wall components reduced MAP binding to mammary epithelial cells, but this fact was partially attributed to decreased cell viability. Although some authors have worked with the MAP invasion process in MAC-T cells (Lamont et al., 2012; Li et al., 2016), it is known that MAP is capable of inducing apoptosis in macrophages by a caspase-dependent or caspase-indepentent mechanism and damage mitochondrial (Periasamy et al., 2013). In the present study, regardless of the time of exposure and the treatment (MAP and *E.coli*, MAP or *E. coli*) there was no reduction in viability of MAC-T cells. These results demonstrate that cell viability did not influence the reduction of *E. coli* internalization.

We futher investigated if the inhibition of *E. coli* internalization in MAP-infected cells could be related to some MAP-released factor (e.g., proteins) that could decrease *E. coli* growth or metabolism. The higher efficiency of *E. coli* growth in the proportions of 75% and 100% of MAP supernatant suggested that MAP can release substances capable of stimulating the growth rate of *E. coli* isolated from bovine mastitic milk. It has been reported that high concentrations of reactive oxygen species (ROS) impair *E. coli* growth due to irreversible damage to cellular components (Imlay 2008; Baez and Shiloach, 2013). To control the deleterious performance of ROS, some bacteria are able to produce enzymes called superoxide dismutase (SOD) that metabolize O_2 and avoid the harmful cascade of ROS (Imlay 2008). In the culture supernatant of *Mycobacterium tuberculosis,* SOD was identified in high quantity demonstrating its importance in oxidative control (Andersen et al., 1991). Similarly to *M. tuberculosis*, MAP is able to secrete SOD into the culture supernatant, suggesting that pathogenic mycobacteria exhibit this property (Liu et al., 2001). Furthermore, Gram-negative and Gram-positive bacteria have been reported to release ATP to the culture supernatant and their levels are regulated by the growth phase in all bacterial species. Live bacteria appear to deplete extracellular ATP, mainly in the log phase, hydrolyzing or degrading extracellular ATP on the cell surface. Possibly the extracellular ATP produced by one group of bacteria could provide energy for the growth of another group of bacteria (Mempin et al., 2013). Thus, contrary to what we hypothesize, MAP supernatant stimulates *E. coli* growth and does not interfere with the invasion process. Although the focus of the present study was not research between SOD or extracellular ATP produced by MAP and the growth capacity of *E. coli*, future studies should be performed to clarify this correlation.

The results from the present study demonstrate that the internalization of *E. coli* to bovine mammary epithelial cells previously infected by MAP was significantly reduced and the translocation through the cells did not alter its efficiency. In general, it may be suggested that the persistence of *E. coli* inside of the mammary gland would be less frequent in MAP-infected cows, either by inhibition of invasion or by rapid translocation to the subepithelial layer. In practical terms, the presence of MAP within mammary epithelial cells could prevent recurrent mastitis by *E. coli* infection. For the authors, this is the first study relating the influence of MAP upon *E. coli* invasion on mammary epithelial cells.

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5. CHAPTER II.

RAPID BASO-APICAL TRANSLOCATION OF *Mycobacterium avium* subsp. *paratuberculosis* IN MAMMARY EPITHELIAL CELLS IN THE PRESENCE OF *Escherichia coli*

Schwarz, D. G. G., Shoyama, F. M., Oliveira, L. L., Sreevatsan, S., Moreira, M. A.S.

ABSTRACT

Infection of bacterial pathogens in mammary gland cells begins with adhesion, invasion, and progresses to their permanence within the cells or systemic distribution. Some bacteria, e.g. *Escherichia coli*, are known to activate an innate immune response of the mammary gland, leading to acute mastitis. Others, although lactated through milk, poorly stimulate the immune response of the mammary gland, e.g., *Mycobacterium avium* subsp. *paratuberculosis* (MAP), the etiological agent of paratubeculosis. Interactions between these pathogens in mammary epithelial cells is not yet known. The aim of the present study was to verify if *E. coli*-infected mammary epithelial cells would be able to translocate MAP from subepithelial to apical region under experimental conditions, and to evaluate the production of cytokines during this process. We show that the presence of *E. coli* in mammary epithelial cell lines (MAC-T) increased basal-apical translocation of MAP at 30 min p.i. and decreased at 120 min p.i. Cells previously infected by *E. coli* + MAP and *E. coli* only demonstrated a significant increase ($P < 0.05$) in IL-1β mRNA expression at 120min. There was no significant expression of MAPKp^{38} and IL-10, regardless of the treatment. Thus, presence of *E. coli* in MAC-T cells alters the translocations of MAP through epithelial cells, enabling its rapid translocation to the celular surface. Expression of IL-1β was demonstrated to influence the apical-basal translocation of MAP at 120 min. This is the first report of MAP translocation in MAC-T cells influenced by the presence of *E. coli* isolated from mastitic milk.

Key-words: Mammary gland, mastitis, paratuberculosis, cytokine

INTRODUCTION

Mycobacterium avium subsp. *paratuberculosis* (MAP) is the etiological agent of paratuberculosis, or Johne's disease, that mainly affects ruminants. The disease is characterized by an incurable chronic granulomatous enteritis, resulting in diarrhea, progressive thinning, dehydration and death (Lombard, 2011). Infected animals may excrete MAP by faeces and/or milk. Thus, milk intake from infected cows facilitates the transmission of MAP to calves, which are the most susceptible to infection (Patel and Shah, 2011). Although it has been inferred that MAP possibly does not cause inflammation in mammary gland (Larsen and Miller, 1978), the microorganism has been found inside the cytoplasmic vacuoles, as it is observed in infected macrophages (Patel et al., 2006). Still, its interactions with other microorganisms have been poorly studied. Recently, dead yeast and yeast cell wall components from *Saccharomyces cerevisiae* have been found to decrease the MAP binding effectiveness in bovine mammary epithelial cells under experimental conditions (Li et al., 2016). This suggests that these components could contribute to the reduction of the risk of MAP infection in the mammary gland.

Another disease that affects a large part of the world's dairy herd and inflicts great economic losses in the dairy industry (Bradley, 2002) is known as mastitis, an inflammation of the mammary gland. Mastistis caused by *Escherichia coli* usually results from environmental contamination and is characterized by an intense immune response with acute and limiting clinical manifestations (Bradley and Green, 2001). *E. coli* strains from persistent bovine mastitis can invade more efficiently mammary epithelial cells than strains from transient mastitis, resulting in recurrent mastitis due to the presence of *E. coli* inside these cells or in biofilms (Dogan et al., 2006; Costa et al., 2012; Silva et al., 2014).

In mamary epithelial cells, MAP can use phagosome acidification and IL-1β production as a strategy to chemoattract macrophages to be infected (Lamont et al., 2012). Within macrophages, MAP has the ability to prevent phagosomal acidification by inhibition of activation of the vacuolar H^+-ATPase (Arsenault et al., 2014). In addition, after membrane binding by TLR-2, MAP activates signaling pathways capable of producing cytokines that modulate the immune response. Mitogen activated protein kinases (MAPK) are stress activated kinases which are activated by TLR-2 signaling. Among them, $MAPKp^{38}$ has been shown to play an important role in the suppression of

the antimicrobial response in macrophages (Weiss et al., 2008). It also induces the production of an anti-inflammatory cytokine, IL-10. Together, both MAPKp[38] and IL-10 act to suppress the antimicrobial response and immune response, leading to the persistence of MAP within macrophages (Souza et al. 2006; Bannantine et al. 2015). In Gram-negative bacteria, TLR-4 recognizes LPS that activates the NF-κB pathway by the MyD88-dependent and results in the transcription of genes related to innate and inflammatory responses (Asehnoune et al., 2004).

In 1993, a study by Wilson et al. found that new cases of subclinical mastitis and chronic infection were lower in MAP-positive cows, and also demonstrated that *Staphylococcus aureus* and *Serratia* sp. infection rate was reduced in cows with paratuberculosis. Although these results suggested a possible interaction between MAP and bacteria isolated from bovine mastitis, this relationship has not been studied.

The objectives of this work were to determinate whether *E. coli* within mammary epithelial cells is able to attract MAP of the basal-apical region and to evaluate the cytokines responsible for the signaling pathway in host cells.

MATERIALS AND METHODS

Ethics statment

The experiment was approved by the Ethics Committee on Animal Use (CEUA) at the Universidade Federal de Viçosa, Brazil, under protocol number 26/2014.

Bacterial strains and culture conditions

Mycobacterium avium subsp. *paratuberculosis* (MAP) strain K-10 and *Escherichia coli* isolated from bovine mastitic milk were used in this experiment. MAP strain was maintained in Middlebrook (MB) 7H9 broth containing 0.2% glycerol, 10% oleic acid dextrose-catalase (OADC) and mycobactin *J* (2mg/L) (Allied Monitor, Inc. Fayette, MO) at 37°C for six weeks at 200rpm until reaching the optical density (OD_{600}) of 0.5 (equivalent to 1.0 x 10^6 CFU/mL). MAP culture was determined to be free of contaminating organisms by the absence of growth of colonies on plaques in Brain Heart Infusion (BHI) medium. The culture was tested for purity using the standard PCR of IS*900*. A single colony of *E. coli* grown on plaque with Luria-Bertani medium (LB) was subcultured in LB broth at 37°C at 200rpm reaching OD_{600} = 0.3 (1.0 x 10^8 CFU/ml).

Bovine mammary epithelial cells culture

The bovine mammary epithelial cell line (MAC-T), widely used in infection experiments (Bouchard et. al., 2013; Assis et al., 2015), was cultured in T75 culture flask (TPP Techno Plastic, St. Louis, MO, USA) in Dulbecco's Modified Eagle Medium (DMEM) medium (Gibco-BRL, Grand Island, NY, USA) supplemented with 10% Bovine Fetal Serum (FBS) and 1% penicillin (5,000 U/ml)-streptomycin (5,000 µL/ml) (PenStrep®, Gibco, Life Technologies, Carlsbad, California, USA). MAC-T cells were incubated in humidified chamber at 37°C in 5% CO_2 until monolayer confluency. Confluency was assessed by visualization under phase-contrast microscopy.

Translocation assay

After confluence of MAC-T cells in T75 cell culture flask, the supernatant was discarded and 3 mL TrypLE™ Express (Gibco, Life Technologies, Carlsbad, California, USA) was incubated at 37°C for 10 min for resuspension of adhered cells. Approximately 3.0 x 10^5 MAC-T cells were added to the top of the permeable membrane with 3µm pore size of Transwell® inserts (Corning, Lowell, MA) and incubated in DMEM containing 10% Bovine Fetal Serum (FBS) and 1% penicillin (5000 U/mL)-streptomycin (5,000 µL/mL) (PenStrep®, Gibco, Life Technologies, Carlsbad, California, USA). The evaluation of cell monolayer integrity was verified by the following methods: i) visualization by phase microscopy, ii) measurement of transepithelial resistance (above 132 ohm) by Trans Epithelial Electric Resistance (EVON-World Precision Instruments, Inc.), iii) monolayer crystal violet staining and iv) trypan blue (0.25%) permeability assay (optical density at 540 nm) as described by Patel et al. (2006).

Prior to the coinfection experiment, the confluent cells were washed three times with preheated (37°C) PBS (pH 7.2) (D-PBS- Dulbecco's Phosphate Buffered Saline 1X -Gibco, Life Technologies, Carlsbad, Calif., USA). For the infection, 1mL of *E. coli* cultured in LB broth (1.0 x 10^8 CFU) was centrifuged at 13,000 x *g* for 5 min. The pellet was washed twice with PBS, resuspended in 200µL DMEM (10% FBS, without antimicrobials), added to the upper chamber and incubated at 37°C in a humidified chamber with 5% CO_2 for 30min. After, the compartment was washed twice with PBS and 200µl of DMEM (10% FBS without antimicrobials) was added. For the lower chamber, 1mL of MAP K-10 culture (DO_{600} = 0.5, equivalent to 1 x 10^6 CFU/mL) was

centrifuged at 13,000 x g for 5 min and washed twice with PBS. Subsequently, the pellet was resuspended in 750 µL DMEM (10% FBS, antimicrobial-free), and vortexed for 5 min. MAP suspension was then passed through a sterile 21-gauge needle 20 times for breaking the bacterial clumps and after 5 min of sedimentation, the upper three-fourths of solution were used for infection. The supernatant with suspended MAP was placed in the lower chamber and incubated at 37°C in a humidified chamber for 10 min, 30 min and 120 min. Then, the supernatant from the upper chamber was colleced for culture and molecular biology procedures. Positive controls were cells infected only by *E. coli* and cells infected only by MAP. Negative control was uninfected cells. To evaluate the cell stimulatory capacity and its influence on MAP translocation, the experiment was carried out by adding lipopolysaccharide (LPS). Controls were cells treated only with LPS and unchallenged cells. All assays were carried on in triplicate and reapeated three times.

MAP culture

Initially, antibiotics and their appropriate concentration were standardized to be added in the MAP culture medium capable of inhibiting the growth of *E. coli*, but not MAP. Two antimicrobials widely used in the treatment of bovine mastitis, nalidixic acid and ampicillin, in different concentrations (10 µg/mL, 50 µg/mL and 100 µg/mL) were included in MB7H9 agar medium containing 0.2% glycerol, 10% oleic acid dextrose-catalase (OADC) and mycobactin *J* (2mg/L). It was found that ampicillin at 50 µg/ml was inhibitory for *E. coli*, but tolerated by MAP. Thus, previously collected supernatants from the upper chamber were centrifuged at 12,000 x *g* for 5 min, resuspended in 100 µL of 0.9% saline solution, serially diluted, and plated onto MB7H9 agar with ampicilin (50 µg/mL) for colony count.

DNA extraction

To obtain DNA from the supernatant of the upper chamber, samples were first centrifuged at 12,000 x *g* for 5 min and washed twice with PBS (pH 7.2). For the DNA extraction, the DNeasy blood and tissue kit (Qiagen, Valencia, CA-USA) was used following the manufacturer's recommendations, with modifications. Briefly, after centrifugation, samples were treated with 200 mL of 2% lysozyme, 20mL of proteinase K (Promega) and for MAP cell wall rupture, the samples were treated with zirconia/silica beads (0.1mm) at 7000rpm for 50s in the MagNaLyser (Roche, Life

Science, USA) for three times. After, the samples were centrifuged at 13000 rpm for 5 min and added 200 µL of ice-cold ethanol. The remaining procedures were in accordance with the protocol of the kit manufacturer.

RNA extraction

Infected MAC-T cells and controls adhered on the transwell membrane were washed with PBS, homogenized with 500µL of TRIzol® reagent (Invitrogen, Carlsbad, CA) for cell disruption and maintenance of RNA integrity, and stored at -80°C for later use. All materials and surface were previously treated with RNase Away (Molecular Bioproducts, San Diego, CA, USA). For the RNA extraction, the Direct-zol™ RNA MiniPrep Kit (ZymoResearch, Irvine, CA, USA) was used, following the manufacturer's recommendations. RNA was treated directly into the extraction column spin with DNase I reaction Mix (ZymoResearch, Irvine, CA, USA) as described by the kit manufacturer. The RNA quality and concentration was determined by measuring the 260/280 ratio on a NanoDrop ND 1000 spectrophotometer (Nanodrop Products, Wilmington, DE, USA).

Synthesis of cDNA

The cDNA synthesis was performed using the SuperScript® III First-Strand Synthesis SuperMix for qRT-PCR kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations. A final concentration of 400ng cDNA in a total of 20µL was obtained.

Quantitative real-time PCR

Absolute quantification was performed using LightCycler® 480 SYBR Green I Master (Roche) in LightCycler 480II (Roche, Madison, WI) with its respective software. The primer set, secA F (5´-GCGCAAGGTGATCTACGC-3`) and secA R (5´-GCGCAAGGTGATCTACGC-3`) was previously constructed for amplification of a 63 bp fragment (Janagama et al., 2010). The total PCR reaction was 20 µL, with 10 µL of Mastermix (2X), 1 µL of each primer (10 µM), 3 µL of water nuclease free and 5 µL of DNA (template). For quantification, a standard curve was constructed with MAP genomic DNA sequentially diluted 10-fold and determining the number of copies per dilution (10^8, 10^7, 10^6, 10^5, 10^4, 10^3, 10^2, 10^1 copies). The mass of a MAP-DNA copy was calculated using the following equation: $m = (n)\ x\ (1{,}096\ x\ 10^{-21} g/bp)$ where *n* is

the size of the genome and *m* is the mass of the genome. For each reaction, a standard curve was constructed to relate the Ct values to the number of copies.

Relative quantification was performed using LightCycler® 480 SYBR Green I Master (Roche) on LightCycler 480II (Roche, Madison, WI) with its respective software. For the reaction, 10µL of master mix (2X), 1µL of each primer (10µM) (table 1), 1µL cDNA (20ng) and free nuclease water were used for a final volume of 20µL. The following program was used: denaturation at 95°C for 5 min and PCR at 95°C for 10s, 60°C for 10s, 72°C for 10s for 40 cycles. For negative control, IFN-ץ was used, a cytokine not produced by epithelial cells. All target expressions were normalized by the constitutively expressed β-actin gene. The results of the mean values of the Cts obtained by triplicates were used to calculate the relative gene expression of the target genes by the comparative method using the formula $2^{-\Delta\Delta Ct}$.

Table 1. Sequences of forward and reverse primers used in real-time PCR

Gene and direction	Sequence (5-3`)	Reference
MAPK-p^{38}, F	TGCTTGTCCTGTTCTCTTCG	Lamont et al., 2010
MAPK-p^{38}, R	GGAAGGTCTTCACTGGCAAA	Lamont et al., 2010
IL–1β, F	ATCTTCGAAACGTCCTCCGAC	Buza et al., 2003
IL–1β, R	CCTCTCCTTGCACAAAGCTCA	Buza et al., 2003
IL-10, F	GTGATGCCACAGGCTGAGAA	Buza et al., 2004
IL-10, R	CGCCTTGCTCTTGTTTTCG	Buza et al., 2004
IFN-ץ, F*	TGGAGGACTTCAAAAGCTGATT	Pang et al., 2009
IFN-ץ, R*	TTTATGGCTTTGCGCTGGAT	Pang et al., 2009
β -actin, F**	CGCCATGGATGATGATATTGC	Okumu et al., 2010
β-actin, R**	AAGCCGGCCTTGCACAT	Okumu et al., 2010

*Reaction control/**housekeeping gene

Statistic analysis

The results were analyzed by the ANOVA means test and the interaction between the variables were verified by the Tukey test, using Graphpad Prism software (Sftware, La Jolla, CA) with 5% of statistical significance.

RESULTS AND DISCUSSION

Infection of mammary gland cells by MAP can potentially occur both through the systemic route and the ascending pathway (Patel et al., 2006). Thus, we hypothesized that previous *E. coli* infection in mammary epithelial cells could attract MAP from the subepithelial (basal) region to the apical region and be released by the

cell with greater effectiveness. To prove this hypothesis, a translocation assay was performed and the amount of MAP in the supernatant from the upper chamber was assayed by qRT-PCR. All standard curves were linear in eight orders of magnitude, with correlation coefficient around 0.99. In figure 1, the number of copies of MAP in relation to different times is demonstrated.

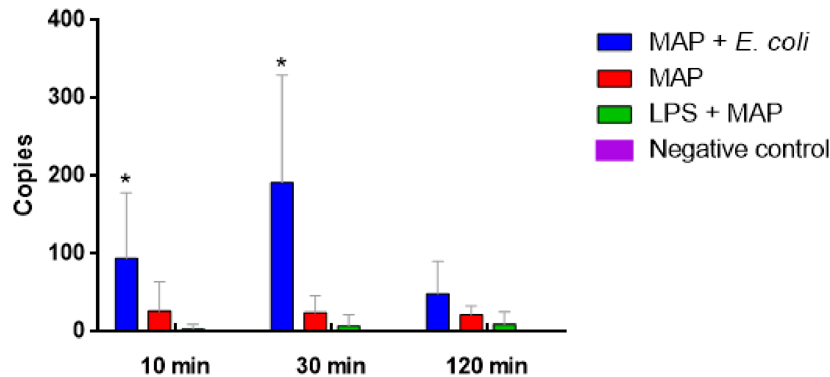

FIG.1. Previous *Escherichia coli* infection in bovine mammary epithelial cell line (MAC-T) induces rapid baso-apical *Mycobacterium avium* subsp. *paratuberculosis* (MAP) translocation. Detection of the number of MAP copies from the supernatant of the upper chamber of the transwell relative to post-infection times (10, 30 and 120 min) in different treatments. Controls used were: MAC-T cells not infected by *E. coli* (red); Cells challenged with lipopolysaccharides (LPS) and MAP (LPS + MAP) (green); and non-challenged cells (Negative Control) (purple).

It is observed that in MAC-T cells previously infected by *E. coli,* passage of MAP through the cells significantly increased ($p < 0.05$) compared to cells not infected by *E. coli*. At 10 and 30 min post infection, there was a significant increase in MAP translocation when compared to 120 min in the presence of *E. coli*. This behavior indicates that *E. coli* could alter the mechanism of natural infection in epithelial cells of the mammary gland, attracting MAP from its basal region to the apical region. These results corroborated previous studies showing that the process of MAP infection within the mammary gland can be influenced by the extracellular environment (milk) and the presence of coinfections. Li et al. (2016) described that the presence of *Saccharomyces cerevisiae* cell wall components interfered in MAP binding to MAC-T cells and to bovine intestinal epithelial cells reducing their adhesion capacity. Futhermore, Patel et al. (2006) found that when MAP was incubated in raw milk at 37°C for 24h, it increased its capacity for internalization in MAC-T cells.

The decrease in MAP detection in cells previously infected by *E. coli* at 120 min (Fig.1) shows possibly that between 30-120 min post-infection, MAP bound to the apical surface of these cells and reinternalized, demonstrating that until 30 min post-infection was crucial for rapid transepithelial migration. In part, these results corroborate with those found by Lamont et al. (2012), where between 10-30 min there was rapid MAP internalization and acidification of the phagosome. Regardless of the time of infection, no influence of LPS on MAP transepithelial migration was observed. Thus, it is not entirely clear how *E. coli* could be acting in this invasion process. Future studies should intensify these investigations in order to identify which factors (e.g., proteins) are interacting in this process.

We also performed culture of the supernatant from the upper chamber for future MAP colony count, however no colonies were observed, regardless of the time of infection. This can be explained since the amount of MAP translocated from the lower to the upper chamber, although significant, was relatively low. Although cultivation on solid medium (i.e. *Herrold's egg yolk medium* - HEYM or Middlebrook) is the gold standard for MAP detection (Collins et al., 1993), it has been inferred that the plate colony counts have limited sensitivity and low bacterial loads cannot be detected (Mitchell et al., 2015).

In addition, the physical mechanisms that MAP possibly performs for its internalization in non-phagocytic cells (e.g., macropinocitosis), and the identification of cytokines that assist in this process, are important to evaluate for their contribution to the roles of epithelial cells (first cell barrier that MAP needs to overcome) in the infective process and inflammatory response. For this, the expressions of cytokines (IL-1β, MAPK-p^{38} and IL-10) involved in MAP internalization in epithelial cells were quantified at different infection times. Expression of IL-1β was significantly higher when *E. coli* was present in the MAC-T cells (Fig 2A). Cells infected only by *E. coli* showed an up-regulation from 30 min to 120 min. When the cells were infected by *E. coli* and MAP, this increase was significant at 120 min p.i. Infection of the mammary gland by *E. coli* triggers an acute inflammatory reaction with recruitment of neutrophils from the bloodstream to the site of infection, in order to control the infection. This control mainly occurs through the production of proinflammatory cytokines such as TNF-α, IL-8, C5a and IL-1β (Oviedo-Boyso et al., 2007). In the present study, the results suggest that MAC-T cells previously infected by *E. coli* and subsequently by MAP lead to increased IL-1β expression.

One of the main outcomes of stimulation of the MAPKp^{38} pathway is increased IL-10 gene expression. As previously mentioned, IL-10 plays a key role in suppressing the antimicrobial activity of macrophages, mainly due to the inhibition of the TH-1 immune response by the downregulation of IL-12 (Theus et al., 2005). In figure 2B, an upregulatory trend in IL-10 mRNA expression is shown from 30 min and than a decrease in expression at 120 min. Although the results were not statistically significant, it is important to note that, unlike the other treatments, MAP-infected cells apparently increased their expression of IL-10 from 30 min p.i.

In the present study, it was verified that IL-1β was significantly expressed in MAC-T cells, unlike MAPKp^{38} and IL-10 gene expression (Fig 2 B,C). Knowing that IL-10 expression is MAPKp^{38}-dependent, the non-significant expression of MAPKp^{38} resulted in the same behavior for IL-10. For the control of the amplification, the evaluation of the expression of IFN-ɣ mRNA was used. IFN-ɣ is a cytokine produced predominantly by CD4^{+} T helper cell type 1 (Th1) lymphocytes, CD8^{+} cytotoxic lymphocytes, and NK cells (Schroder et al., 2003). Thus, its non-amplification ensures the accuracy of our amplification results.

In the intestine, MAP can be translocated through the Peyer's patches or enterocytes. It has been plausibly hypothesized that entry through Peyer's paches results in rapid interaction with phagocytic cells and inflammatory response. When interacted with enterocytes, it results in a slow inflammatory response and wide systemic spread (Bermudez et al., 2010). This fact could explain in part our findings, where both MAPKp^{38} and IL-10 gene expression were not significant in MAC-T cells infected by both *E. coli* and MAP, in vitro.

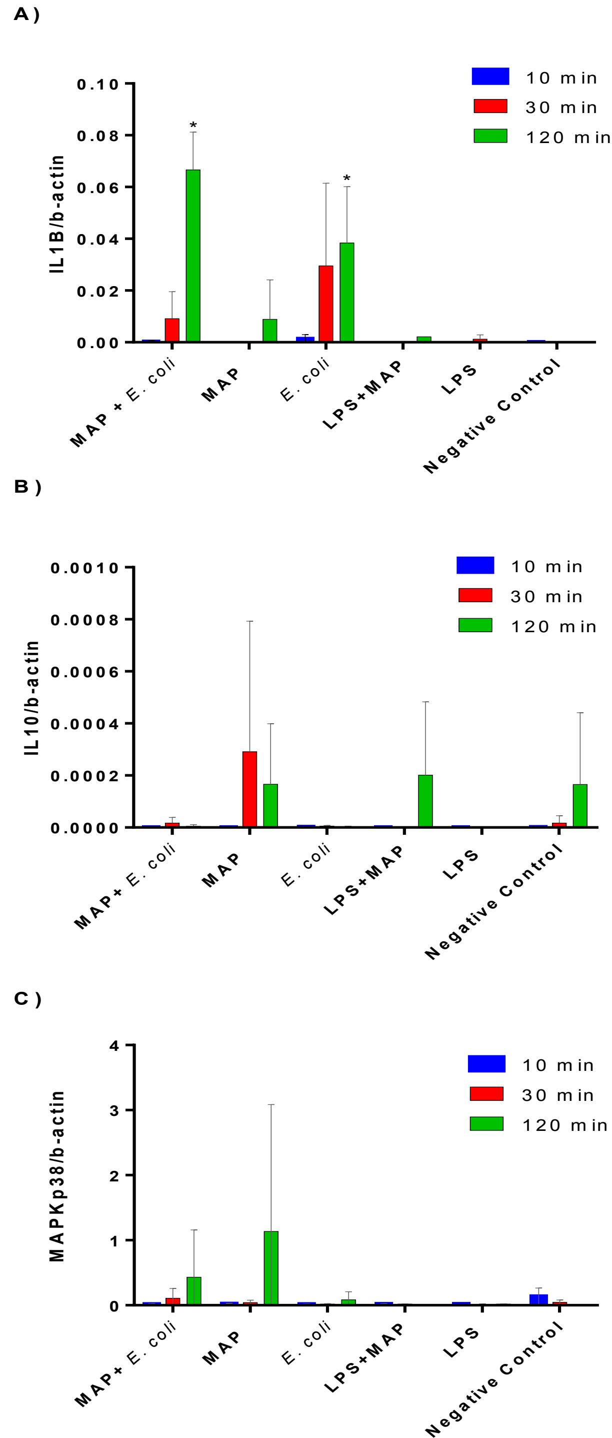

FIG.2. Expression of cytokines in mammary epithelial cells (MAC-T). Cells were previously infected with *E. coli* and subsequently infected by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) (MAP + *E. coli*) at 10, 30 and 120 min. Controls were cells infected only by *E. coli* or MAP and uninfected (Negative control). Treatments were also performed using lipopolysaccharide (LPS) with MAP (LPS + MAP) and LPS only. All target expressions were relativized by the constitutive β-actin gene. A) IL-1β mRNA expression; B) MAPKp^{38} mRNA expression; C) IL-10 mRNA expression.* $P<0.05$.

Overall, these data suggest that the presence of *E. coli* in MAC-T cells is able to facilitate MAP translocation from the basal region to the apical region of mammary epithelial cells up to 30 min p.i. This migration process associated with the presence of intracellular *E. coli* triggers increased IL-1β transcription that is responsible for attraction of macrophages to the site of infection but could also attract MAP from the apical region to the basal region. This behavior should be further studied in order to exclude other variables. Collectivelly, these findings support the hypothesis that when cows infected with MAP are co-infected with environmental mastitis by *E. coli*, they would be able to attract MAP from distant regions such as lamina propria or supramammary lymph nodes and increase their release into the glandular alveolus. This is the first report demonstrating the influence of *E. coli* under MAP infection in mammary epithelial cells under experimental conditions.

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6. CHAPTER III.

Short Communication: PASSIVE-SHEDDING OF *Mycobacterium avium* subsp. p*aratuberculosis* IN COMMERCIAL DAIRY GOATS IN BRAZIL

Schwarz, D. G. G., Lima, M. C., Barros, M., Scatamburlo, T. M., Rosado, N., Oliveira, C.T.S.A.M., Oliveira, L. L., Moreira, M.A.S.

ABSTRACT

Goat farming is a low-cost alternative to dairy production in developing countries. In Brazil, goat production has increased in recent years due in part to the implementation of programs encouraging this activity. *Mycobacterium avium* subsp. *paratuberculosis* (MAP) is the causative agent of paratuberculosis, a disease that causes chronic granulomatous enteritis in ruminants, but its transmission dynamics are still poorly understood in goats. The aim of this study was to investigate the presence of passive-shedding phenomenon in Brazilian dairy goats. Ten dairy goat farms (467 animals) from Minas Gerais state were analyzed for MAP detection. Two fecal culture and 11 milk conventional PCR were positive for MAP. Since no clinical signs were observed for 1 year of monitoring, 4 positive goats (4/13) were purchased and samples of feces, milk, tissues and blood were collected for evaluation by culture of MAP, IS*900* PCR and ELISA 2 times within a 3-month interval. Subsequent results were negative for MAP detection. At necropsy, there were no macroscopic lesions related to paratuberculosis. These animals were characterized as passive shedders with upward contamination of teat canal by MAP. This is the first report of passive-shedding phenomenon in goats in Brazil and warns of the importance in the quality of dairy products.

Short Communication

During the last decade goat production has increased globally, mainly in developing countries due to low cost and goat milk serving as an alternative for the consumption of dairy foods for people with allergy to cow's milk (Haenlein 2004). Goat milk differs from cow milk in having smaller size of the fat globules, less lactose, more calcium, phosphorous and potassium. Besides that, it has better digestibility since it forms a finer curd which mimics the stomach conditions (Silanikove et al. 2010).

In 2013, there where 207.008.068 dairy goats in the world and 6.799.000 in South America. This region was also important since it had the largest milk production in the American continent, around 220.000,00 tonnes (FAOSTAT, 2016). Due the increasing dairy goats farming, Brazil is the leading producer of goat's milk in the Americas, with 69,2% of the total milk production in the continent (FAOSTAT, 2016). In this scenario, the growing production and the possibility for export of live animals

and milk products from goats encouraged the investigation and control of diseases in these herds. The health of the dairy herd should be important to any producer, since there can be no quality products without healthy goats, and no healthy goats without adequate hygienic control (Ribeiro and Ribeiro, 2010).

An important mycobacteriosis that affects ruminant herds around the world is paratuberculosis or Johne's disease, caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). The infection results in a chronic granulomatous enteritis, diarrhea, progressive weight loss, malnutrition, decrease in milk productivity and death (Lombard, 2011). The progression of the infection results in MAP excretion in feces and/or milk. Therefore, milk feeding from infected dams facilitates the transmission of the agent in young animals (Patel and Shah, 2011).

Most animals infected by MAP show a low and intermitent shedding pattern and only a few of them become high shedders (Mitchell et al., 2015). Frequently, the shedding patterns of natural MAP infection can be termed as passive shedders, where the animal swallows MAP organisms but it is not infected (also denoted as pass-through phenomenon); or active shedders, where MAP infects the intestinal epithelium and reaches the sub-epithelial macrophages (Kralik et al., 2014).

Considering the implications to Brazilian dairy goat farming and the quality of their products, this longitudinal study aimed to detect the presence of passive shedders goats in dairy herds from Brazil.

In a previous study from our research group, 10 dairy goat farms from mesoregion Zona of Mata, Minas Gerais state, the main regional producer of goat milk in southeastern Brazil, were evaluated for the detection of MAP. Among 467 animals, 2 were positive by fecal culture and 11 positive by conventional IS*900* PCR from milk samples. For details of study design, sample colletion and preliminary data processing, see previously published work (Souza et al., 2016). During one year, it was verified that these animals did not present any clinical signs suggestive of paratuberculosis, such as progressive weight loss or reduction of milk production. Therefore, after 1 year, 4 positive goats (1 positive from feces and milk samples and 3 from milk samples) and 4 controls (no MAP detection) were purchased and maintained in separate stalls at the Universidade Federal de Viçosa (UFV), Brazil. All procedures in the animals followed the rules of the ethics committee on animal use - CEUA/UFV, protocol number 26/2014. Feces and milk samples were collected in two times, with an interval of 3 months between them. At the last time point, blood samples were collected and after the

animals had been sacrificed, fragments of colon, ileum and mesenteric lymph nodes were collected for tissue culture and histopathology. In the present study, all methodologies used for the previous MAP detection were repeated and other tests were added. The processing of the feces samples followed the method described by Stabel (1997) and Souza et al. (2016). Briefly, 2g of feces were placed in 50 ml tubes containing 20 ml of autoclaved distilled water and shaked at 110 rpm for 60 min and then kept at rest for 45 min at room temperature for sedimentation. For decontamination, 5 mL of the supernatant were replaced in 50 ml tubes containing 20 ml of 0.9% hexadecylpyridinium chloride (HPC) (Sigma-Aldrich, St. Louis, MO, USA) at room temperature overnight. After, the tubes were centrifuged at 1700 x g for 20 min and the supernatant was discarted and the pellet was resuspended in 1mL of antimicrobial solution containing nalidixic acid (50 mg/L), vancomycin hydrochloride (50 mg/L) and amphotericin B (150 mg/L). A 150µL aliquot was inoculated onto 2 slanted tubes of Middlebrook (MB) 7H11 with mycobactin *J* (2mg/L) and 2 without the siderophore, incubated at 37°C and examined for MAP growth up to 12 months.

Milk samples were collected following strictly the hygiene standards. For MAP culture, the milk was processed as Pillai and Jayarao (2002). Briefly, milk samples were centrifuged at 2000 x *g* for 15min at 4°C, the supernatant was discarded and the pellet was resuspended in 15 ml of 0.75% of HPC at room temperature for 5h. Subsequently, samples were centrifuged 2000 x g for 15 minutes and the supernatant was discarded. Finally, the remaining pellet was resuspended in 600µL of antimicrobial solution and a 150µL aliquot were inoculated onto MB7H11 slopes with or without mycobactin *J* (2mg/L) and were incubated in duplicate at 37°C for up to 12 months. For molecular analysis, the milk samples were centrifuged 2000 x g for 15 min at 4°C and after discarding the supernatant, the pellet was resuspended in 200µL of 1% PBS, pH 7.2, and stored for molecular analysis. Extraction of the genomic DNA from milk samples was performed using the Wizard Genomic DNA purification Kit (Promega, Madison, MI, USA), according to the manufacture's instructions. The DNA was resuspended in 30µL of DNA rehydration solution and its concentration was quantified by NanoDrop Lite spectophotometer (Thermo Scientific, Walthen, MA, USA). Conventional IS*900* PCR reactions were performed using primers BN1 (5'-GTTATTAACGACGACGCGGAGC-3') and BN2 (5'-ACGATGCTGTGTTGGGCGTTAG-3') (Sivakumar et al., 2005) and GAPDH gene foward (5'-GGCGTGAACCACGAGAAGTA-3') and reverse (5'-

GGCGTGGACAGTGGTCATAA-3’) as internal control target for PCR reaction. Briefly, 12,5µl of mix; 1µl of each oligonucleotide, 6,5µl of MiliQ water and 4µl DNA extracted at a concentration of 200 ng/µl were used to a final volume of 25µl. The PCR were performed with initial denaturation at 94°C/4 min, 30 cycles of 94°C/1 min, 60°C/1min and 72°C/1 min and final extension at 72°C/4 min. The amplified fragments were visualized by 1% agarose gel electrophoresis (Invitrogen, Washington, DC, USA) in Tris-Borate-EDTA buffer (TBE) stained with GelRed™ Nucleic Acid Gel Stain (Biotium, Hayward, CA, USA) using an EagleEye II transilluminator (Stratagene, La Jolla, CA). MAP K-10 strain was used as positive control and ultra pure water as negative control. A 100-bp DNA ladder (Promega) was used as a molecular weight standard.

To evaluate the serology of the goats, serum of each animal was sent to Universidade Federal de Campina Grande, Brazil, to perform ELISA tests with IDEXX MAP Ab Test Kit (Maine Westbrook ME, USA) following the manufacturer’s instructions.

For tissue culture, the methodology followed as described by Greig et al. (1999) with modifications. Approximately 4g tissues were macerated in a mortar and pestle and homogenized with 2mL 0.9% saline. Then, the solution was transferred to 50 ml tubes, treated with 0.9% HPC and inoculated in MB7H11 slope tubes with and without mycobactin *J* as described above.

Histopathological analyzes were performed using established methodology whereby tissues were fixed in 10% formalin buffer solution for 24h for further processing to obtain paraffin wax blocks. All sections were cut at 4µm and stained with hematoxylin and eosin for lesion analysis and Ziehl-Nielsen (ZN) staining for MAP detection.

All analyzed samples of feces and milk previously positive for MAP and confirmed by sequencing to be of 94–99% similarity with MAP K-10 strain were negative in the current analyzes. No characteristic colonies of MAP on MB7H11 were observerd from all samples collected. Recently, Mitchell et al. (2015) verified that most naturally infected cows had a pattern of low and intermitent shedding and rarely progressed to high shedding. To disqualify the possibility that the goats were low shedders or intermintent shedders, it PCR and ELISA tests were performed at 2 time points with an interval of 3 months. Conventional IS*900*-PCR was performed and an internal control was used to ensure there was no inhibition of the PCR reaction. No

inhibition in the PCR reactions was observed, but no amplified fragments of a similar size (626bp) to that expected from the MAP gene were observed either. Similarly, the immune assay did not show any positive reaction to MAP in all serum analysed. Macroscopic analyzes of the organs do not show any characteristic alterations of paratuberculosis. Similarly, no histological changes were observed in the analyzed organs, nor the presence of MAP in samples stained by ZN.

These findings are quite important for Brazilian dairy production properties since they demonstrate for the first time that goats could spread MAP in the environment by the feces and/or infect susceptible animals through milk passively. Furthermore, the prior MAP detection in milk (PCR) and subsequent negativity suggests that MAP could enter from the environment into the teat canal, contaminating the milk but not infecting the animal. To avoid the premature culling of false positive animals, it is suggested to carry out consecutive exams to detect animals who are passive shedders. Al-Majali et al., (2008) verified that the inclusion of new animals in the herds and the joint raising of goats and sheep also increased the risk of paratuberculosis in the herds. Moloney and Whittington (2008) verified that when 1774 cattle were raised with a MAP positive sheep flock, only 1 cow became positive for the sheep strain (S), being identified as a passive-shedder. Similarly, Souza et al. (2016) demonstrated by restriction enzyme analysis that the positive goats in our study were identified as bearing the cattle strain (C). There is a lack of information in the literature regarding the relationship between the host and the types of strains ingested. However, the MAP ingestion of non-species-specific strains could intensify the occurrence of passive shedders.

In the same properties evaluated in the present study, previous investigation conducted by our research group identified MAP in 50% of the samples of water for goat consumption and in 30% of the samples of water for human consumption (unpublished data). These data associated with intensive goat production increase the risk of ingestion of contaminated feed and occurrence of this passive shedding phenomenon. MAP has been detected in pasteurized cow's milk and retail artisanal Coalho cheese for human consumption (Carvalho et al., 2012b; Faria et al., 2014). Although the zoonotic potential of MAP is not yet clear, it has a historical association with Crohn’s disease (CD) due the similarities of lesion in animals and humans, as well as the detection of the microorganism in patients with CD (Abubakar et al., 2008; Carvalho et al., 2012a).

The present study describes for the first time in Brazil cases of passive shedders and upward contamination of teat canal by MAP in dairy goats from Zona of Mata, Minas Gerais state, and highlight the importance of the identification of these animals to control programs and to ensure the quality of dairy products.

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7. GENERAL CONCLUSION

Mammary epithelial cells (MAC-T) previously infected by MAP inhibited the *E. coli* invasion process but did not alter the translocation capacity.

MAC-T cells previously infected by *E. coli* increased the basal-apical translocation capacity of MAP at the begining of the infection. The number of copies of MAP (qRT-PCR) in the supernatant was lower in late infection, coinciding with increased expression of IL-1β mRNA.

There are passive-shedders goats in dairy farms in the Zona da Mata of Minas Gerais, Brazil.

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