Short communication: Effects of moringa extract on adhesion and invasion of *Escherichia coli* O55 in bovine mammary epithelial cells

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ABSTRACT

The objective of this study was to evaluate the antibacterial activities of extract derived from moringa leaves. In particular, the effect of moringa extract (Mor) on adhesion and invasion of *Escherichia coli* O55, *Enterococcus faecalis*, *Staphylococcus simulans*, and *Serratia liquefaciens* was evaluated in bovine mammary epithelial cells (MAC-T). Broth microdilution method, minimum inhibitory concentration and minimum bactericidal concentration assays, adhesion and invasion assays, and real-time PCR were performed. The minimum inhibitory concentration and minimum bactericidal concentration of Mor ranged from 12.5 to 50 mg/mL on 18 out of 27 tested isolates. Treatment of *E. coli* O55 with Mor (100 and 200 µg/mL) inhibited the adhesion and invasion on MAC-T cells via down-regulation of adhesion factors (i.e., *papC*, *f17c-A*, and *eaeA*). Also, when MAC-T cells were pretreated with Mor (200 µg/mL, 12 h) and incubated with *E. coli* O55, *Enterococcus faecalis*, *Staphylococcus simulans*, or *Serratia liquefaciens*, both *E. coli* O55 and *Enterococcus faecalis* showed a significant decrease in adhesion and invasion. *Staphylococcus simulans* exhibited decreased adhesion and increased invasion. *Serratia liquefaciens* showed increased adhesion and decreased invasion. In addition, Mor increased mRNA expression of antioxidant enzymes (e.g., heme oxygenase-1, NAD(P)H:quinone oxidoreductase-1, and thioredoxin reductase 1) in MAC-T cells. In conclusion, 12.5 to 50 mg/mL of Mor exhibited antibacterial activity against 18 out of 27 tested isolates. Also, pretreatment of 200 µg/mL of Mor to MAC-T cells modulated adhesion and invasion of *E. coli* O55 and other mastitis-associated pathogens. Furthermore, Mor increased antioxidant capacities in MAC-T cells, but further in vivo studies are needed.

Key words: moringa, bovine mastitis, bovine mammary epithelial cells, antimicrobial effects, antioxidant effects

Short Communication

Bovine mastitis is characterized by inflammation of the mammary gland in dairy cattle (Oviedo-Boyso et al., 2007). Bovine mastitis is the most common disease in dairy cows, causing economic losses in the dairy industry worldwide due to decreased milk production, discarded milk, veterinary services, labor, product quality, and culling (Halasa et al., 2007). Numerous microorganisms have been identified as potential pathogens in mastitis with the majority of infections caused by staphylococci (e.g., *Staphylococcus aureus*), streptococci (e.g., *Streptococcus uberis*), and enterobacteria (e.g., *Escherichia coli*) (Watts, 1988). These bacteria produce many virulence factors such as microbial surface components recognizing adhesive matrix molecules, adhesin, and other substances that facilitate bacterial colonization and internalization to the host cells (Ghanbarpour and Oswald, 2010; Gogoi-Tiwari et al., 2015). The main treatment for bovine mastitis is antibiotic therapy during the dry period and lactation (Oliver and Sordillo, 1988; Erskine et al., 2003). However, due to the emergence of antibiotic-resistant microorganisms, researchers are searching for new alternative approaches, especially natural compounds, to control bovine mastitis. In contrast to antibiotics, natural compounds are economical, easily available, and affordable (Rakholiya et al., 2013). More importantly, plant-derived antibacterial substances can possibly decrease the selective pressure on development of antibiotic resistance (Lewis and Ausubel, 2006). In fact, natural products isolated from plants were reported to exert antimicrobial activities, for example, root extract of *Thalictrum minus* L. and its isolated constituents were reported to have antimicrobial effects against *Staphylococcus xylosus*, *Staphylococcus lentus*, *Staphylococcus equorum*, *Enterococcus faecalis*, and *Pantoea agglomerans* (Mushtaq et al., 2016). The combination of *Eucalyptus globulus* and *Juglans regia* was reported to have antibacterial activities against *Staphylococcus aureus* isolates through reducing their virulence factor (i.e., biofilm formations) (Gomes et al., 2019).

In addition to virulence of pathogens, the defensive role of host cells is also important in bovine mastitis.

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The dairy cow body has an endogenous antioxidant defense system such as antioxidant enzymes that help to encounter the production of reactive oxygen species (ROS) during metabolisms (Sordillo and Aitken, 2009). However, during the periparturient period, production of ROS overwhelms the antioxidant defense system, resulting in increased oxidative stress and the risk of various bovine diseases including mastitis (Bernabucci et al., 2005; Sordillo and Aitken, 2009). One of the strategies to delay, prevent, or remove these oxidative damages is through supplementation of antioxidants (e.g., vitamin C and selenium) (Sordillo, 2018). Indeed, antioxidant-rich plant and plant-derived substances such as pomegranate (Safari et al., 2018) and resveratrol (Jin et al., 2016) were reported to improve the antioxidant status of the dairy cow and bovine mammary epithelial cells.

Moringa oleifera is a tree native to India that has high nutritive values. The leaves are rich in minerals, vitamins, and other bioactive components (e.g., quercetin, kaempferol derivatives, rutin, apigenin, chlorogenic acid, caffeoylquinic acid, and feruloylquinic acid) (Atawodi et al., 2010; Nouman et al., 2016) that have potential antioxidant, anti-inflammatory, and antimicrobial activity (Gopalakrishnan et al., 2016; Vergara-Jimenez et al., 2017). In our previous study, extract of *M. oleifera* leaves (Mor), was found to exhibit anti-inflammatory and antioxidative effects in bovine mammary cells (MAC-T) upon stimulation with LPS (Cheng et al., 2019). However, its effect on mastitis bacteria has yet to be investigated. Therefore, since Mor contains phytochemicals that exhibit antimicrobial and antioxidant activities, we hypothesized that (1) Mor has antimicrobial effects on bovine mastitis-associated pathogens, (2) Mor is able to modulate the adhesion and invasion of mastitis-associated pathogens, and (3) Mor can increase the antioxidant capacities of bovine mammary epithelial cells. We performed the following experiments to test our hypotheses.

The Mor was prepared according to a previous study (Cheng et al., 2019). Briefly, 20 g of moringa leaf powder (Philippine Moringa & More Corporation, Rizal, Philippine) was added to 200 mL of 80% (vol/vol) methanol, and stirred for 3 h at room temperature. The mixture was then filtered with Whatman No. 1 filter paper (GE Healthcare Life Sciences, Buckinghamshire, UK) and the remaining solvent was evaporated under reduced pressure at 50°C using a rotary evaporator (Tokyo Rikakikai Co. Ltd., Tokyo, Japan). Residues (i.e., Mor) were freeze-dried and stored at -80°C until use. The Mor was dissolved in dimethyl sulfoxide (DMSO; Amresco, Solon, OH) at 400 mg/mL and filtered with a 0.22-µm syringe filter before usage.

We determined the MIC and minimum bactericidal concentration (MBC) of Mor against 27 bacteria, *E. coli* O55 (KVCC-BA0002381) that was obtained from the Korea Veterinary Culture Collection (Gimcheon, Korea), and 10 gram-negative and 16 gram-positive bacteria that were isolated from milk samples in cows with mastitis (Gyeonggi-do, Korea). Bacteria were grown on tryptone soy agar (TSA; BD-Difco, Sparks, MD) overnight, and a single colony from TSA was inoculated in 10 mL of tryptone soy broth (TSB; BD-Difco) and incubated for overnight at 37°C with shaking (150 rpm). Then, 100 µL of incubated bacterial culture was inoculated in 10 mL of TSB and allowed to grow in an incubator at 37°C and 150 rpm, until reaching 10⁸ cfu/mL.

Broth microdilution method was performed as described by the Clinical and Laboratory Standards Institute with some modifications (CLSI, 2018). Briefly, 400 mg/mL of Mor stock was diluted with TSB to 100 mg/mL, then a 2-fold serial dilution of Mor (50, 25, 12.5, 6.25 mg/mL) was prepared. All the diluted samples contain the final concentration of 25% of DMSO in the solution. Next, 100 µL of each sample was added to a sterile polystyrene 96-well plate, followed by inoculation of 100 µL of bacterial inoculum (approximately 10⁶ cfu/mL) to reach the final concentrations of 50, 25, 12.5, 6.25, 3.125, and 0 mg/mL. The highest concentration of Mor was selected based on the study of El Sohaimy et al. (2015), where MIC of methanolic extracts of moringa leaves were reported to be 40 mg/mL against *Staphylococcus aureus*, *Staphylococcus epidermis*, *Streptococcus pyogenes*, *Escherichia coli*, *Bacillus subtilis*, and *Salmonella* Senftenberg (El Sohaimy et al., 2015). The plate was incubated for 24 h at 37°C. The MIC was defined as the lowest concentration at which no visible turbidity was observed. To determine MBC, 100 µL of medium from a growth-free well was cultured on TSA and incubated for 24 h at 37°C. Bacterial growth was determined by the formation of bacterial colonies on TSA. The lowest concentration of Mor with no growth of bacterial colonies was indicated as the MBC. Three isolates were randomly selected for identification to be used for the next experiments. The isolates were identified as *Enterococcus faecalis*, *Staphylococcus simulans*, and *Serratia liquefaciens*.

The MAC-T cells were cultured in Dulbecco's modified Eagle's medium (Welgene, Gyeongsan, Korea) supplemented with 10% fetal bovine serum (Welgene), 5 µg/mL insulin (Sigma-Aldrich, St. Louis, MO), and 1 µg/mL progesterone (Sigma-Aldrich) in 10-cm cell culture dishes in a CO₂ humidified incubator at 37°C (Jeong et al., 2017; Cheng et al., 2019). The MAC-T cells (passage 20 to 30) were seeded in 6-well plates and

allowed to grow to reach a monolayer with confluence of approximately 80 to 90% for cell treatments.

We studied the effect of Mor on adhesion and invasion of *E. coli* O55 in MAC-T cells according to a previous study with some modifications (Döpfer et al., 2000). Serogroup O55 is one of the most prevalent serotypes of *E. coli* isolated from mastitis milk samples (Correa and Marin, 2002; Lira et al., 2004; Lamey et al., 2013). The *E. coli* O55 was previously used as a bovine mastitis reference pathogen (Kang et al., 2016); therefore, it was selected in our study. Briefly, 100 μ L of overnight-incubated *E. coli* O55 culture was inoculated in 10 mL of TSB with a different concentration of Mor (100 and 200 μ g/mL) until 10^8 cfu/mL. Control group was added with 0.1% DMSO. One milliliter of bacterial culture was centrifuged at $7,800 \times g$ for 5 min at 4°C. The pellet was resuspended in 1 mL of cold PBS (Gibco, Grand Island, NY) and treated to a monolayer of MAC-T cells (80–90% confluency) at a multiplicity of infection (MOI; ratio of bacteria to MAC-T cells) of 10:1 (Döpfer et al., 2000; Bouchard et al., 2013). *Escherichia coli*-treated MAC-T cells were incubated for 2 h in a humidified CO₂ incubator at 37°C (Döpfer et al., 2001). The MAC-T cells were then washed 3 times with PBS. Cells were then detached with 200 μ L of trypsin (Gibco) and lysed with 800 μ L of 0.1% Triton X-100 (Sigma-Aldrich). Cell lysates were serially diluted, plated on TSA, and incubated overnight at 37°C; number of colonies formed on TSA were counted and expressed as colony-forming units per milliliter. For the invasion assay, a monolayer of MAC-T cells was infected with Mor-treated *E. coli* O55 at an MOI of 10:1 for 2 h. The cells were washed with PBS, and incubated with fresh Dulbecco's modified Eagle medium supplemented with 100 μ g/mL of gentamycin sulfate salt (Sigma-Aldrich). The cells were incubated for another 2 h to kill the extracellular bacteria. Next, the cells were washed and lysed, and plated on the TSA. The bacterial population was measured as described in the adhesion assay. Invasion was expressed as the total number of cfu/mL recovered per well.

The adhesion of *E. coli* is usually regulated by fimbria, the thin and short filamentous proteinaceous structures that extend from the bacterial cell membrane (Le Bouguénec, 2005). We assumed that fimbriae might play a role in adhesion and invasion of *E. coli* O55 to MAC-T cells; therefore, we investigated the effect of Mor on the gene expressions of adhesion factors [type 1 fimbria D-mannose specific adhesin (*fimH*), F17c fimbriae-A (*f17c-A*), P fimbrial usher protein PapC (*papC*), and intimin (*eaeA*)] in *E. coli* O55 (Nagy and Fekete, 1999; Wang et al., 2002; Le Bouguénec, 2005). For quantification of mRNA expression of these genes, 100 μ L of *E. coli* O55 was incubated with 200 μ g/mL

of Mor in 10 mL of TSB for 4 h. The control group was added with 0.1% DMSO, which is the amount of Mor added in the treatment group. One mL of bacterial pellet was obtained via centrifugation at $7,800 \times g$ for 5 min at 4°C. The pellet was lysed with 500 μ L of TRIzol reagent (Life Technologies, Eugene, OR), and RNA was extracted. The cDNA was synthesized using the TOPscript RT DryMIX kit (Enzynomics, Daejeon, Korea). The mRNA expression of genes was analyzed by Real-Time PCR (Roche LightCycler 96 System, Basel, Switzerland) using the Real-Time PCR mix (SolGent, Daejeon, Korea). The PCR conditions were as follows: 95°C for 15 min (denaturation) and 50 cycles of amplification at 95°C for 20 s, followed by respective annealing temperature and finally held at 4°C. The primer sequences (Bionics, Seoul, Korea) used are listed in Table 1. Data analysis was performed via relative quantification method ($\Delta\Delta C_q$), in which the relative mRNA expression of target mRNA (i.e., *fimH*, *f17c-A*, *papC*, and *eaeA*) was compared with that of a constitutively expressed gene (i.e., *E. coli* 16S rRNA, *E16S*).

Next, we investigated the effect of Mor in MAC-T cells toward adhesion and invasion of *E. coli* O55, *Enterococcus faecalis*, *Staphylococcus simulans*, and *Serratia liquefaciens*. Briefly, a monolayer of MAC-T cells was pretreated with 200 μ g/mL of Mor for 12 h (treatment group; with Mor). Concentration and incubation time of Mor were selected based on a previous study (Cheng et al., 2019). The control group (without Mor) was treated with 0.1% DMSO. Both treatment group and control group were exposed with 10^8 bacterial inoculum at an MOI of 10:1 and 100:1. The adhesion and invasion assays were conducted.

In addition, we investigated whether Mor can improve the antioxidant capacity of MAC-T cells. The mRNA expression levels of antioxidant genes [heme oxygenase-1 (*HO-1*), NAD(P)H:quinone oxidoreductase-1 (*NQO1*), and thioredoxin reductase 1 (*TXNRD1*)] were measured in MAC-T cells. In the treatment group (with Mor), MAC-T cells were pretreated with 200 μ g/mL of Mor for 12 h, whereas the control group (without Mor) was treated with 0.1% DMSO, followed by the addition of *E. coli* O55 to the cells at an MOI of 10:1 and 100:1 for 2 h. The MAC-T cells without *E. coli* O55 exposure served as controls. The MAC-T cells were collected with 500 μ L of TRIzol reagent. Same procedures were conducted for RNA extraction, cDNA synthesis, and real-time PCR, as described above. The primers used are shown in Table 1. Data were analyzed using the relative quantification method ($\Delta\Delta C_q$), in which the relative expression of target mRNA (i.e., *HO-1*, *NQO1*, and *TXNRD1*) was compared with that of a constitutively expressed gene (i.e., *GAPDH*).

Table 1. The base sequence and annealing temperatures of target gene primers (F = forward; R = reverse)

Target gene	Sequence 5'-3'	Annealing temperature (°C)
<i>fimH</i> (Zhang et al., 2018)	(F) TGCAGAACGGATAAGCCGTGG (R) GCAGTCACCTGCCCTCCGGTA	60
<i>fl7c-A</i> (Ghanbarpour and Oswald, 2010)	(F) CTGATAAGCGATGGTGTAATTAAC (R) GCAGGAACCGCTCCCTTGCC	52
<i>papC</i> (Ghanbarpour and Oswald, 2010)	(F) GACGGCTGTACTGCAGGGTGTGGCG (R) ATATCCTTTCTGCAGGGATGCAATA	55
<i>eaeA</i> (Momtaz et al., 2012)	(F) TCGGCGACAACAGGCGGCGA (R) CGGTGCGCCGACCAGGATTC	60
<i>E16S</i> (Wang et al., 2002)	(F) CCCCCTGGACGAAGACTGAC (R) ACCGCTGGCAACAAAGGATA	55
<i>HO-1</i> (Cheng et al., 2019)	(F) AGGATTTGTCAGAGGCCCTGAA (R) CAAAGACGCCATCACCAGCTTA	60
<i>NQO1</i> (Cheng et al., 2019)	(F) GGTGCTCATAGGGGAGTTCG (R) GGGAGTGTGCCAATGCTAT	60
<i>TXNRD1</i> (Cheng et al., 2019)	(F) CGGTATTGCTGGCAATAGGAAGAG (R) GGCATAGATGTAAGGCACGTTGGT	60
<i>GAPDH</i> (Cheng et al., 2019)	(F) ATGATTCCACCCACGGCAAGTT (R) ACCACATACTCAGCACCAGCAT	60

For all the experiments in this study, data were statistically analyzed using IBM SPSS Statistics for Macintosh, Version 23 (IBM, Armonk, NY) by Student's *t*-test. Data were expressed as mean $\pm$ standard error of the mean. *P*-values <0.05 were considered statistically significant. Each experiment consisted of 3 replicates and was repeated 3 times.

Table 2 presents the MIC and MBC values of Mor against 27 bacteria. The MIC and MBC of Mor against the 11 gram-negative isolates ranged from 12.5 to 50 mg/mL. On the other hand, MIC of Mor against gram-positive bacteria were either 12.5 to 25 mg/mL (7 out of 16 isolates) or 50 mg/mL (7 out of 16 isolates). The Mor showed no bactericidal effect on 9 out of 16 gram-positive isolates (MBC >50 mg/mL). The bacteriostatic and bactericidal effect of Mor might be attributed to the presence of antimicrobial phytochemical constituents in moringa leaves such as alkaloids, saponins, anthraquinones, tannins, flavonoids, and phenols (Kasolo et al., 2010; Sankhalkar and Vernekar, 2016). These compounds bind to the bacterial cell wall and inhibit microbial growth depending on their type

and chemical structure (El Sohaimey et al., 2015). For instance, chlorogenic acid was reported to bind to the outer membrane of *Staphylococcus aureus*, *Shigella dysenteriae*, and *Streptococcus pneumoniae*, and that disrupted the membrane, exhausted the intracellular potential, released cytoplasm macromolecules, and eventually led to bacterial death (Lou et al., 2011; Li et al., 2014). In addition, flavonoids, especially kaempferol and quercetin, are known to inhibit *E. coli* DNA gyrase involved in bacterial replication (Wu et al., 2013).

The varying degrees of MIC and MBC of Mor against the 27 tested isolates might be attributed to the complexity of the bacterial cell walls. The cell wall of gram-negative bacteria is constructed of 2 layers, a peptidoglycan inner membrane and an outer membrane formed by lipoproteins and LPS that prevents penetration of high molecular weight antibiotics and hydrophilic molecules (Stefanović, 2018). In contrast, the cell wall of gram-positive bacteria, even though it is composed of several layers of peptidoglycan, is mostly permeable to various compounds (Stefanović, 2018). In our study, 7 out of 16 gram-positive isolates had lower

Table 2. Determination of MIC and minimum bactericidal concentration (MBC) of moringa extract (Mor) against 26 isolated bacterial strains and the reference strain *Escherichia coli* O55

Mor (mg/mL)		Number of isolated bacteria	
MIC	MBC	Gram-positive	Gram-negative
12.5	12.5	6 (including <i>Staphylococcus simulans</i>)	2 (including <i>Serratia liquefaciens</i>)
12.5	25	1	1
25	25	0	5 (including <i>E. coli</i> O55)
25	50	0	1
50	50	0	2
50	>50	7 (including <i>Enterococcus faecalis</i>)	0
>50	>50	2	0

MIC (≤ 50 mg/mL Mor) than 11 isolates in gram-negative bacteria, which may be due to the differences in cell walls. Our results are comparable with a previous study by Alzoreky and Nakahara (2003) where methanolic extracts of 26 species of plants were evaluated for inhibitory effects on bacteria growth. The MIC of most plant extracts against gram-positive bacteria (*Bacillus cereus*, *Staphylococcus aureus*, and *Listeria monocytogenes*) ranged from 165 to 2,640 mg/mL; however, only *Cinnamomum cassia* extract had an antibacterial effect on gram-negative bacteria (*E. coli* and *Salmonella infantis*) with MIC of 2,460 mg/mL.

On the other hand, factors such as biofilm, bacterial efflux pumps, and quorum sensing are also important factors for determining MIC and MBC (Winn et al., 2010; Savoia, 2012). In particular, biofilm is known to protect bacteria from environmental stress such as antibiotics and the immune system (Jacques et al., 2010). Biofilm played a role in recurrent infections and antimicrobial agents/host immune defense system resistance; hence, biofilm formers are a difficult target in the control and treatment of bovine mastitis (Gomes et al., 2016). Among 2 identified gram-positive isolates in our study, *Staphylococcus simulans* (MIC and MBC: 12.5 mg/mL Mor) has been reported as a non-biofilm or weak biofilm former (Tremblay et al., 2013; Szczuka et al., 2016; Phophi et al., 2019); and *Enterococcus faecalis* (MIC and MBC: 50 and >50 mg/mL Mor) is a common biofilm producer (Toledo-Arana et al., 2001; Elhadidy and Elsayyad, 2013; Anderson et al., 2016). Therefore, biofilm formation may possibly protect bacteria from Mor and contribute to the high MIC and MBC of Mor, at least in part, in 9 out of 16 gram-positive isolates. However, further studies are necessary to identify the underlying mechanisms.

Figure 1A showed that treatment of *E. coli* O55 with 100 and 200 μ g/mL of Mor significantly decreased the percentage of bacterial adhesion to cells to 72 to 76%, compared with untreated controls ($P < 0.05$). The percentage of invasion was also decreased ($P < 0.05$) to 83% and 89% with 100 and 200 μ g/mL of Mor-treated *E. coli* O55, respectively (Figure 1B). Results of real-time PCR showed that Mor treatment (200 μ g/mL) decreased the mRNA expression levels of *f17c-A*, *papC*, and *eaeA* ($P < 0.05$), but not *fimH*, in *E. coli* O55 (Figure 1C to 1F). These data suggest that Mor decreased adhesion and invasion of *E. coli* O55 in MAC-T cells by downregulating the fimbrial adhesion factors.

Adhesion of *E. coli* O55, *Enterococcus faecalis*, and *Staphylococcus simulans* to 200 μ g/mL Mor-pretreated MAC-T cells was significantly lower than that of the corresponding control (Figure 2A, 2C, and 2E). The invasion of *E. coli* O55, *Enterococcus faecalis*, and

Serratia liquefaciens in Mor-pretreated cells was significantly lower than that of the corresponding control (Figure 2B, 2D, and 2H). However, invasion of *Staphylococcus simulans* in MAC-T cells was higher than that of the control (Figure 2F). The adhesion of *Serratia liquefaciens* in MAC-T cells was higher than that of the control (Figure 2G). Different adhesion and invasion patterns were observed in the tested bacteria; however, the underlying mechanisms are unclear. One possible speculation is that adhesion and internalization of different bacteria are dependent on different mechanisms; in particular, different bacteria bind to different receptors on host cells. Pretreatment of Mor with MAC-T cells might have affected these receptors. A past study reported that quercetin prevented *E. coli* O157:H7 adhesion to human colonic epithelial cell line Caco-2 through attenuated expression of integrin $\beta 1$ of Caco-2 cells (Xue et al., 2019). It is also possible that Mor altered only some of the surface receptors of MAC-T cells, which may take part in the adhesions of *E. coli* O55, *Enterococcus faecalis*, and *Staphylococcus simulans* but not *Serratia liquefaciens*. Further studies are necessary to identify the differential effects of Mor on adhesion and invasion of bacteria and the underlying mechanism.

Antioxidant enzymes, HO-1, NQO1, and TXNRD1, are reported to play protective roles in mammary cells of the Holstein dairy cow during the postpartum period to maintain proper redox homeostasis of the cells (Aitken et al., 2009; Han et al., 2018). Hence, these enzymes have been used as indicators of antioxidant capacities in MAC-T cells (Jin et al., 2016; Wang et al., 2016). The gene expression of HO-1 was increased by *E. coli* O55 alone in MAC-T cells (Figure 2I). These results showed that HO-1 is more likely to respond to stimulation of *E. coli* O55 compared with NQO1 and TXNRD1. Indeed, HO-1 is a phase II antioxidant or detoxifying enzyme that is activated by several physiological and pathological stimuli such as ROS, cytokines, and bacterial components (Paine et al., 2010). Because HO-1 was reported to play a role in protecting RAW264.7 macrophage against oxidative stress (Cheng et al., 2015), the observed upregulation of HO-1 is probably due to the defensive role of MAC-T cells. In contrast, the expression of NQO1 and TXNRD1 in MAC-T cells was decreased by *E. coli* O55 (Figure 2J and K). Despite the differential expression of these genes in *E. coli* O55-treated MAC-T cells, pretreatment of MAC-T cells with Mor significantly increased ($P < 0.05$) the level of these genes when compared with untreated groups. These findings indicate the upregulation of antioxidant capacities of MAC-T by Mor. In fact, Mor increased the expression of HO-1, NQO1, and

TXNRD1 in MAC-T cells challenged by LPS (Cheng et al., 2019). These antioxidant effects are attributed to the rich polyphenol content of *M. oleifera* including flavonoids and phenolic acids, such as quercetin and

kaempferol, and these compounds were reported to increase HO-1 protein expression in rat aorta smooth muscle cells and BV-2 microglia cells (Lin et al., 2004; Velagapudi et al., 2019).

Figure 1. Effect of moringa extract (Mor) on adhesion (A) and invasion (B) of *Escherichia coli* O55 in bovine mammary epithelial (MAC-T) cells. Activated *E. coli* O55 was treated with 0, 100, and 200 µg/mL of Mor until it resulted in 10^8 cfu/mL. The *E. coli* O55 was then added to the MAC-T monolayer at a multiplicity of infection (MOI; ratio of bacteria to cells) of 10:1 for 2 h. Adhesion and invasion are presented as a percentage of the control. For the measurement of gene expression, *E. coli* O55 was treated with 0 and 200 µg/mL Mor for 4 h. The mRNA expression levels of *fimH* (C), *papC* (D), *f17c-A* (E), and *eaeA* (F) were measured using real-time (RT) PCR. Details of adhesion, invasion, and RT-PCR are described in the main text of the article. Values represent means $\pm$ SEM (n = 3). *Significant difference versus control (Mor = 0 µg/mL; $P < 0.05$).

Figure 2. Effect of moringa extract (Mor) on adhesion and invasion by *Escherichia coli* O55 (A and B), *Enterococcus faecalis* (C and D), *Staphylococcus simulans* (E and F), and *Serratia liquefaciens* (G and H) in bovine mammary epithelial (MAC-T) cells. The MAC-T cells were pretreated with 0 and 200 $\mu\text{g/mL}$ followed by stimulation with activated bacteria at a multiplicity of infection (MOI; ratio of bacteria to cells) of 10:1 and 100:1. Adhesion and invasion are presented as a percentage of the control. The mRNA expression of *HO-1* (I), *NQO1* (J), and *TXNRD1* (K) was measured using real-time (RT) PCR. Details about adhesion, invasion, and RT-PCR are described in the main text of the article. Values represent means $\pm$ SEM ($n = 3$). *Significant difference versus control (MOI = 0; $P < 0.05$). #Significant difference between the 2 treatment groups (without Mor and with Mor; $P < 0.05$).

In conclusion, our data provide evidence that Mor exhibits antipathogenic effects at 12.5 to 50 mg/mL against 18 out of 27 tested bacterial isolates. Pretreatment of Mor at 200 $\mu\text{g/mL}$ to *E. coli* O55 decreased its adhesion and invasion in MAC-T cells by modulating the adhesion factors of *E. coli* O55. In addition, pretreatment of MAC-T cells with Mor (200 $\mu\text{g/mL}$, 12 h) decreased the adhesion of *E. coli* O55, *Enterococcus faecalis*, and *Staphylococcus simulans* on the cells. Furthermore, pretreatment of MAC-T cells with Mor increased the antioxidant capacity of MAC-T cells through increased expression of antioxidant genes. Although further animal studies are needed, our data suggest that *M. oleifera* can be potentially supplemented to dairy cow feed to protect the mammary glands against IMI.

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