Microbial Pathogenesis 122 (2018) 63–72

Contents lists available at ScienceDirect

Microbial Pathogenesis

journal homepage: www.elsevier.com/locate/micpath

Panax ginseng extract reduces *Staphylococcus aureus* internalization into bovine mammary epithelial cells but does not affect macrophages phagocytic activity

Camila Beccaria[a,b,1], Paula Silvestrini[a,b,1], María S. Renna[a,b], Hugo H. Ortega[a,b], Luis F. Calvinho[c], Bibiana E. Dallard[a,b], Celina Baravalle[a,b,*]

[a] Laboratorio de Biología Celular y Molecular Aplicada, Facultad de Ciencias Veterinarias, Universidad Nacional del Litoral, Argentina
[b] Instituto de Ciencias Veterinarias del Litoral (ICIVET-Litoral), Universidad Nacional del Litoral - Consejo Nacional de Investigaciones Científicas y Tecnológicas (UNL-CONICET), Argentina
[c] Estación Experimental Agropecuaria Rafaela, Instituto Nacional de Tecnología Agropecuaria (INTA), C.C. 22, 2300, Rafaela, Santa Fe, Argentina

ARTICLE INFO

Keywords:
Panax ginseng
Bovine mammary epithelial cells
Cytokines
Staphylococcus aureus
Macrophages

ABSTRACT

Panax ginseng extract (PGe) has been shown to possess immunomodulatory effects in healthy dairy cows at drying off and to trigger an adequate immune response to protect from an experimental intramammary infection (IMI) with *Staphylococcus aureus* in a murine model. *S. aureus* is one of the major pathogens isolated from bovine IMI; being capable to invade and survive within mammary epithelial cells. However, the precise mechanism by which PGe interacts with bovine mammary epithelial cells (MAC-T) and bovine macrophages in the course of a *S. aureus* infection remains unclear. We evaluated the effect of PGe on MAC-T cytokine response and on the internalization of *S. aureus* into MAC-T. In addition, we evaluated the effect of PGe on the phagocytic activity of macrophages isolated from bovine mammary secretions. Results shown that MAC-T cells TLR4 and NF-κB mRNA expression was not affected by PGe at all evaluated times. IL-6 mRNA expression and protein level and IL-4 protein level were significantly induced in MAC-T treated with 3 mg/ml of PGe. PGe at 3 mg/ml reduced significantly the internalization of two *S. aureus* strains in MAC-T. In addition, PGe did not affect the percentage of phagocytosis and the NO and ROS production of macrophages co-cultured with two strains of *S. aureus*. These results, obtained in *in vitro* models together with those obtained in *in vivo* previous studies carried out in bovines and mice can contribute to improve the understanding of the effects of PGe following inoculation in bovine mammary glands.

1. Introduction

Medicinal plants are used to treat diseases of man and animals since ancient times. *Panax ginseng* C.A. Meyer, commonly called “Ginseng” has a long history as a general tonic to promote health [1]. Ginseng contains various pharmacological components including saponins (ginsenosides), polyacetylenes, polyphenolic compounds, and acidic polysaccharides [2]. Ginseng extract (PGe) appears to induce an increase in host resistance through stimulation of the immune system [3–5]. Previous studies in noninfected cows at the end of lactation have demonstrated that PGe has immunostimulant properties and may enhance early mammary involution [6–9]. These immunomodulatory properties were associated with increased expression of toll like receptors (TLRs), pro inflammatory cytokines, and activation of the transcription factor NF-κB in bovine mammary glands [8]. Moreover, *in vivo* studies have described the potential preventive and therapeutic effects of parenterally administered PGe against *Staphylococcus aureus* intramammary infections (IMI) in lactating cows [10].

Staphylococcus aureus is one of the most frequently isolated major pathogens from bovine IMI [11,12] and most likely presents as chronic subclinical mastitis with a low cure rate after conventional antibiotic therapy [12]. The frequent failure of antibiotic treatment to clear bacterial infections and recurrence of these infections may be related, among several factors, to the ability of *S. aureus* to invade and survive within a wide variety of eukaryotic cells, such as epithelial cells of the mammary gland or immune cells [13–15]. Within this context, there is a need for alternative approaches to prevent and control *S. aureus* mastitis in dairy cows to complement current practices.

* Corresponding author. Instituto de Ciencias Veterinarias del Litoral (ICIVET-Litoral). R. P. Kreder 2805, 3080, Esperanza, Santa Fe, Argentina.
E-mail address: cbaraval@fcv.unl.edu.ar (C. Baravalle).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.micpath.2018.06.010
Received 22 August 2017; Received in revised form 5 June 2018; Accepted 6 June 2018
Available online 07 June 2018
0882-4010/

Contact between bacteria entering the mammary gland with both immune system cells present in the milk and with the lining mammary epithelial cells results in the induction of the innate immune response. A rapid acting and effective innate immune response is predicated on early recognition of pathogens [16]. It has been documented that establishment of *S. aureus* IMI is associated with an impairment of the immune response, mediated by factors of either host or *S. aureus* origin resulting in a late and low neutrophil recruitment [17,18]. In a previous study, using a mouse mastitis model, we demonstrated that PGe was able to trigger an adequate immune response protect from a *S. aureus* experimental infection partially inhibiting its multiplication within the mammary gland [19]. However, the precise mechanism by which PGe interacts with mammary epithelial cells and macrophages present in bovine mammary gland in the course of a *S. aureus* infection has not been explored. Therefore, in this study, the effect of PGe on MAC-T cells cytokine response and its effect on the internalization of *S. aureus* into MAC-T cells were evaluated. In addition, the effect of PGe on the phagocytic activity of macrophages isolated from bovine mammary secretions was evaluated.

2. Materials and methods

2.1. Panax ginseng extract and S. aureus strains

Panax ginseng extract (PGe) was provided by Indena Company (Indena® SpA, Milan, Italy). A 50 mg/ml PGe stock solution was prepared by dissolving the extract in pyrogen free 0.89% NaCl saline solution and then conveniently diluted to different working concentrations [8]. Bacteria used in this study were *S. aureus* strain Newbould 305 (ATCC 29740) and *S. aureus* 5011 strain isolated from a cow with a subclinical persistent mastitis [20].

2.2. Mammary epithelial cells culture

MAC-T cells, an immortalized epithelial cell line isolated from bovine mammary tissue [21], were cultured at 37 °C with 5% CO2 in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco BRL), insulin (5 μg/ml), hydrocortisone (1 μg/ml), penicillin (100 U/ml), and streptomycin sulfate (100 μg/ml) (Sigma Chemical Co., MO, USA).

2.3. Effects of PGe on MAC-T viability

Cell viability was determined with Cell Proliferation Kit II (XTT) assay (Roche Life Science, Basilea, Switzerland). In brief, MAC-T cells (2×10^4 cells/ml) were seeded in 96-well plates at 37 °C, 5% CO_2 and incubated with different concentrations of PGe (0.5, 1 and 3 mg/ml) or without PGe for 2, 8 and 24 h. After PGe treatment, 50 μl/well of 0.3 mg/ml of XTT labeling mixture (XTT labeling reagent: sodium 3′-[1-(phenylaminocarbonyl)- 3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate and electron-coupling reagent: PMS (*N*-methyl dibenzopyrazine methyl sulfate) were added and incubated in a humidified atmosphere for 2 h. Controls of MAC-T cells incubated in culture medium alone were included in all experiments carried out. Three independent experiments were performed in triplicate for each condition. Finally, the absorbance was measured at 450 nm on a microplate spectrophotometer SPECTROstar Nano (Life Technologies) and the results were express as optical density.

2.4. Analysis of mRNA expression of inflammatory genes

In order to analyze the effects of PGe on the modulation of mRNA expression of inflammatory response genes at 2, 6, 12 and 24 h post treatment, total RNA of MAC-T cells cultured in 24-well plates with or without different concentrations of PGe was extracted with Trizol LS reagent (Invitrogen, Life technology, CA, USA) according to the manufacturer's instructions. Two micrograms of total RNA obtained was treated with DNase I and then the reverse transcription (RT) reaction was performed as previously described [8,19]. Non-transcriptional (without the reverse transcriptase and with RNA as sample) controls were run for both sample and gene to assess complete degradation of genomic DNA. Subsequently, real time PCR (qPCR) was performed using a SYBR Green I reagent (Invitrogen, Life Technology, CA, USA) and a StepOne Real Time PCR instrument (Applied Biosystems, Life Technology, CA, USA). Primers were acquired from Invitrogen (Table 1). All primers were designed to ensure cDNA specific amplification using Primer-Quest software (http://www.idtdna.com/Primerquest/Home/Index). Primers and amplification products were verified in silico using Basic Local Alignment Search Tool (BLAST; http://www.ncbi.nlm.nih.gov/BLAST) to confirm gene specificity. The forward and reverse primers for all genes were designed when possible to produce amplification products that spanned at least two exons. An optimized protocol was used: initial denaturation at 98 °C for 3 min; 40 cycles of denaturation at 98 °C for 5 s; annealing at 58 °C for 15 s (TLR4) 62 °C for 30 s (NF-κB), 61 °C for 30 s (IL-1β), 56 °C for 15 s (IL-6), 62 °C for 25 s (TNF-α) and 60 °C for 15 s (β-actin); extension at 72 °C for 20 s; and fluorescence reading at 80 °C. All measurements for each sample were performed in duplicate. Negative DNA template controls were included in all PCR assays and yielded no consistent amplification. The specificity of amplification products was verified by melting curve analysis. Quantitative real-time PCR was performed using the comparative Ct method ($2^{-\Delta\Delta Ct}$) using β-actin as an internal control [22].

Table 1
Quantitative real-time PCR primers. Probe sequences (5–3′).

Molecule	Primer sequence (5′to 3′)	Reference sequence
TLR4	For AGCTTCAACCGTATCATGGCCTCT Rev ACTAAGCACTGGCATGTCCTCCAT	AY634630
NF-κB	For GCTGAGTTGAGAGAGAGTAACC Rev CTTTCTGTTGTCACTGCTGC	NM001076409.1
IL-6*	For GATGCAGTCTTCAAACGAGTGGGT Rev AGGTTTCTGACCAGAGGAGGGAAT	X57317
IL-1β	For AGTCTGTCCTGTACCCTAAC Rev GAGAGGGTTTCCATTCTGAAG	NM_174093.1
TNF-α	For GCTTTACCTCATCTACTCACAG Rev CTTGATGGCAGACAGGATG	EU276079.1
β-actin	For CGGAACCGCTCATTG CC Rev ACCCACACTGTGCCCATCTA	BT030480.1

Forward (For).
Reverse (Rev).
*References: IL-6: interleukin 6; IL-1β: interleukin 1 beta; TNF-α: tumor necrosis factor-alpha; TLR4: toll-like receptor 4, NF-κB: nuclear factor κB; β-actin: beta actin.

2.5. Analysis of cytokine production

Supernatants from MAC-T cells incubated in presence or absence of different concentrations of PGe at different times (2, 6, 12 and 24 h) were subsequently employed for cytokine assays using a bovine IL-1β, IL-6 and IL-4 enzyme-linked immunosorbent assay (ELISA) kits, respectively, according to the manufacturer's instructions (Thermo Fisher Scientific, MA, USA). Absorbance was measured using a microplate spectrophotometer ($A_{450\ nm}$ minus $A_{550\ nm}$), and concentrations extrapolated from standard curves performed with recombinant bovine IL-1β, IL-6 and IL-4 provided by the kits. Data were expressed as picogram per milliliter (pg/ml).

Considering that there are no previous studies showing that MAC-T cells produce IL-4, the intracellular production of this cytokine was evaluated by flow cytometry. Briefly, MAC-T cells were incubated for 24 h at 37° C-5% CO_2 without PGe, with 3 mg/ml of PG or with 50 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) and 1 μg/ml

ionomycin (Io, Sigma-Aldrich) both as a positive control of cytokine production (this stimulus was applied only in the last 6 h of culture). The last 6 h of incubation, BD GolgiStop™ (BD) was added to the culture in order to accumulate cytokines intracellularly. MAC-T cells were treated with 0.25% trypsin 0.1% EDTA and collected for intracellular cytokine staining. Briefly, cells were fixed and permeabilized with LEUCOPERM™ (Biorad) and stained for 1 h with anti-IL-4- FITC mAb or with isotype control mAb (Biorad) on ice in the dark. Finally, cells were washed and resuspended in focusing fluid. Staining was analyzed on an Attune NxT flow cytometer using FlowJo software. The percentage of IL-4 + MAC-T cells was analyzed.

2.6. Effects of PGe on S. aureus growth

Bacteria were activated from frozen stocks by overnight culture at 37 °C on trypticase soy agar (TSA) (Britania, Buenos Aires, Argentina) under aerobic conditions. To evaluate the effect of PGe on growth of two *S. aureus* strains, Newbould 305 and 5011, 1.5×10^8 CFU (colony forming units)/ml and 1.15×10^9 CFU/ml respectively were cultured at 37 °C in Müeller-Hinton broth (MH), (Britania) pH 7.4 supplemented with different concentrations of PGe (0.5, 1 and −3 mg/ml) or without PGe. The growth of both *S. aureus* strains was monitored turbidimetrically at 630 nm every hour during 24 h using a SynergyTM HT Multi-Mode Microplate Reader (Biotek).

2.7. Effects of PGe on S. aureus internalization into MAC-T cells

The MAC-T cells were cultured in 24-well plates in DMEM supplemented with 0.5, 1 and −3 mg/ml of PGe or without PGe, antibiotics and FBS for 24 h, then MAC-T were washed three times with phosphate-buffered saline (PBS 1X; pH 7.4) and then infected with two *S. aureus* strains with a multiplicity of infection (MOI) of 100:1 bacteria per cell in the same media without antibiotics. To select the MOI, appropriate dilutions were performed to obtain varying numbers of organisms per epithelial cell (data not shown). After challenged with *S. aureus* for 2 h in 5% CO_2 at 37° C, the MAC-T cells were washed three times with PBS and treated with gentamicin (100 μg/ml, Sigma Chemical Co., St. Louis, MO) in DMEM for 2 h to eliminate extracellular bacteria. Supernatants were then collected and plated on mannitol salt agar to verify extracellular killing by gentamicin. After washing for three times the MAC-T cells were treated with 0.25% trypsin 0.1% EDTA (Gibco, BRL) and lysed with Triton X-100 (Amersham, Arlington Heights, IL, USA) at a final concentration of 0.025% (v/v) in sterile distilled water to release intracellular staphylococci. The MAC-T lysates were diluted 10-fold, plated on mannitol salt agar and incubated at 37 °C overnight. Total CFU was determined by standard colony counting technique. The internalization assay was performed in triplicate and experiments were repeated three times. Data were expressed as $\log_{10}$ of recovered CFU/ml from the MAC-T lysates.

2.8. Effects of PGe on phagocytic activity

2.8.1. Macrophages viability assay

To verify if PGe affected viability of macrophages isolated from mammary secretion an XTT assay was performed. Mammary secretions were collected from nonlactating healthy cows 10–15 days after cessation of milking and macrophages were isolated as described previously [23,24]. Approximately 25 ml of mammary secretion was diluted 1:2 with PBS and centrifuged at 400 g for 10 min at 4 °C using a high capacity centrifuge (RC6 plus, Sorvall, Thermo Fisher Scientific). Then, fat was carefully removed; the pellet was washed twice with PBS and centrifuged at 400 g for 10 min. After counting the isolated mammary secretion cells and determination of the viability with tripan blue, cells were resuspended to a concentration of 1×10^6 viable cells/ml with complete RPMI 1640 medium and 100 μl were seeded in 96-well plates at 37 °C, 5% CO_2 for 24 h. Cells were washed with PBS to remove non adherent cells, resuspended with complete RPMI 1640 medium and treated with different concentrations of PGe (0.5, 1 and 3 mg/ml) or without PGe during 24 h. Then, the same XTT assay described previously for MAC-T cells were performed. Three independent experiments were performed in triplicate.

2.8.2. Phagocytosis assay

The phagocytosis assay was performed by flow cytometry (Attune, NxT Acoustic Focusing Cytometer A24860, Life Technology) using fluorescein isothiocyanate (FITC)-labelled *S. aureus* Newbould 305 and *S. aureus* 5011 strains. One millilitre of PBS containing FITC (10 mg/ml in dimethylsulfoxide) and 1×10^9 CFU/ml of both *S. aureus* Newbould 305 and 5011 strains were incubated at room temperature for 1 h with slight shaking. Both labelled bacteria were washed four times and resuspended to initial volume with PBS. Cellular pellets from mammary secretions were obtained as previously described in macrophages viability assay. Macrophages from mammary secretion were isolated using Histopaque-1083 (Sigma) at a density of 1.083 g/ml according to Dosogne et al. [24] with modifications. Macrophages (1×10^6 cells/ml) were seeded in tubes and then treated with 3 mg/ml of PGe in complete RPMI 1640 medium, keeping untreated macrophages with culture media only as controls for 2 h in slight shaking at 37 °C. After PG treatment, cells were incubated with both Newbould 305- and 5011-FITC labelled bacteria in a MOI of 100:1 bacteria per cell at 37 °C for 30 min, respectively. To stop phagocytosis cells were incubated with cool NaCl 0.85%/EDTA 0.04%, centrifuged at 400 g for 5 min and resuspended in focusing fluid and acquired by flow cytometry.

2.8.3. Selection of macrophages population and phagocytosis analysis

Prior to analyzing the phagocytic activity the macrophage population was identified using flow cytometry based on CD14 positivity [25,26]. Briefly, one tube per sample of isolated macrophages was incubated with a labelled CD14 mAb (APC anti-human CD14, Clone M5E2, BioLegend®, CA, EEUU), for 30 min on ice and in the dark. Then, cells were centrifuged, further resuspended in focusing fluid and acquired by flow cytometry. After identifying the macrophages population positive for CD14 using FlowJo (Tree Star, Ashland, OR), cells with FITC-green fluorescence were regarded as phagocytic events, and the percentage of cells with fluorescence represented the phagocytic activity. Median fluorescence intensity (MFI) was used to estimate the number of bacteria phagocyted per positive cell [27].

2.8.4. Determination of NO concentration and ROS production

Macrophage supernatants from the phagocytosis assay were recovered for all conditions: (Macrophages with 3 mg/ml of PGe: Basal + PGe; macrophages with culture media: Basal cells; macrophages with 3 mg/ml of PGe + Newbould 305 or 5011 *S. aureus*: Newbould 305 + PGe or 5011 + PGe; macrophages with Newbould 305 or 5011 *S. aureus* without PGe: Newbould 305 or 5011) to determine nitric oxide (NO) concentration. Briefly, supernatants were filtered through 0.22 μM membranes (Millipore) to eliminate bacteria and then nitrite concentration (NO2-) in cell-free media was measured using the Griess Reagent Kit (Thermo Fisher Scientific) as described previously (Denis et al. [28] and Gutiérrez-Barrosoa et al. [29]). Briefly, the Griess reagent was prepared by mixing equal volumes of stock solution A (10% sulfanilamide, 40% phosphoric acid) and stock solution B [1% *N*-(1-naphthyl) ethylenediamine dihydrochloride] and then incubated with the filtered supernatant for 30 min at room temperature before the measurement of nitrite. Parallel to this, a standard curve with a nitrite standard solution (provided by the kit) was performed. Total nitrite levels, indicative of the amount of NO production, were evaluated by reading the optical density of each sample at 550 nm and calculated with reference to a standard curve of sodium nitrite (0.78–100 μM).

Once completed the phagocytosis assay, macrophages were incubated with hydroethidine (HE) (1 μM) at 37 °C for 15 min in darkness

and then centrifuged and resuspended in focusing fluid to assessed reactive oxygen species (ROS) production [30]. The percentage of macrophages producing ROS was analyzed using flow cytometry. The ROS production was estimated from the HE-orange fluorescence for each treatment. The MFI of ROS production, that is a measure of the amount of ROS produced per cell, was estimated from the means of HE-fluorescence.

2.9. Statistical analysis

All statistical analyses were performed using a software package (SPSS 11.0 for Windows, SPSS Inc., Chicago, Illinois). Results were expressed as mean ± standard deviation (SD). Differences between groups in XTT, ELISA and intracellular cytokine staining for IL4, internalization and NO production from phagocytosis assays were assessed by one-way analysis of variance (ANOVA), followed by Duncan's multiple range tests as a multiple comparison test. Differences in macrophage phagocytosis assay and ROS production between groups were assessed by Student t-tests to determine differences between groups. The general linear model procedure was used for data analysis for IL-6 ELISA and PCR assays. The model tested for main effects of treatment (0, 0.5, 1 and 3 mg/ml of PGe), time (2, 6, 12 and 24 h) and the treatment * time interaction. Individual means were compared by Duncan test. The effect of PGe on *S. aureus* growth were analyzed with two-way repeated measures ANOVA (RM-ANOVA) followed for Duncan's tests. P values of < 0.05 in all tests employed were further considered significant.

3. Results

3.1. Effects of PGe on MAC-T cells viability

The potential cytotoxicity of PGe on MAC-T cells was evaluated by the XTT assay. Cells viability was not affected by the PGe at concentration of 0.5, 1 and 3 mg/ml at 2, 8 and 24 h post treatment (Fig. 1).

3.2. Effects of PGe on TLR4 and NF-kB gene expression in MAC-T cells

To evaluate if PGe could modulate the expression of TLR4 and NF-κB in MAC-T cells we analyzed the genes profiles of these molecules. The TLR4 mRNA expression in MAC-T cells was influenced by treatment ($P = 0.043$), but not by sampling time ($P = 0.490$). No interaction between treatment and sampling time was observed ($P = 0.622$). Although TLR4 mRNA peaked at 24 h at 1 mg/ml of PGe treatment, no differences were observed between groups (Fig. 2A).

The mRNA expression of NF-κB in MAC-T cells was influenced by treatment ($P = 0.001$), and by sampling time ($P = 0.001$). No interaction between treatment and sampling time was observed ($P = 0.724$). Although the transcript levels for NF-κB in MAC-T treated with 1 mg/ml of PGe were higher than the transcript levels observed for treatments at all sampling times, no differences were observed between groups (Fig. 2B.).

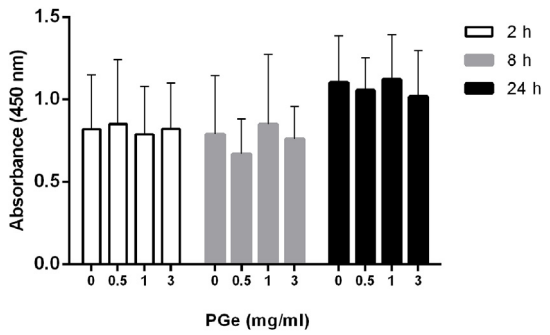

Fig. 1. Effect of different concentrations of PGe (0.5, 1 and 3 mg/ml) on MAC-T cells viability at 2, 8 and 24 h post treatment. Each bar represents the means of triplicates ± standard desviation (SD) of three independent experiments. P values of < 0.05 were considered significant.

3.3. Effects of PGe on cytokines mRNA expression and production in MAC-T cells

To analyze the effects of PGe in MAC-T cells, we determined whether PGe affected the mRNA expression and production of several cytokines by qPCR, ELISA and flow cytometry.

The mRNA expression of IL-6 in the MAC-T cells was influenced by treatment ($P = 0.007$), and by sampling time ($P < 0.001$). There was interaction between treatment and sampling time ($P = 0.038$). At 12 h transcript levels for IL-6 in MAC-T cells treated with 1 and 3 mg/ml of PGe were higher than transcript levels observed at 0.5 mg/ml of PGe treated group and controls ($P < 0.05$). At 24 h mRNA expressions of IL-6 were higher in cells treated with 1 mg/ml of PGe compared with cells treated with 0.5 and 3 mg/ml ($P < 0.05$), but not differences were observed compared with the controls ($P > 0.05$) (Fig. 3A).

All samples from all groups had weak IL-1β and TNF-α mRNA expression, and, since 75% of the samples expressed this gene outside the linear dynamic range, its relative quantification could not be performed.

Protein levels of IL-6 in the MAC-T cells was influenced by treatment ($P = 0.045$) and by sampling time ($P < 0.001$). There was interaction between treatment and sampling time ($P = 0.007$). At 12 and 24 h post treatment, production of IL-6 was significantly induced in MAC-T cells treated with 3 mg/ml of PGe compared with control cells ($P < 0.05$) (Fig. 3B).

Protein levels of IL-1β were below the lowest detectable test limit in all evaluated groups at all sampling times.

At 2, 6 and 12 h post treatment with 0.5, 1 and 3 mg/ml of PGe or without PGe, secretion of IL-4 was below the lowest detectable test limit in all evaluated groups. However, after 24 h of treatment the secretion of IL-4 was significantly induced by 3 mg/ml of PGe in MAC-T cells compared with all evaluated groups ($P < 0.01$) (Fig. 3C).

Treatment with 3 mg/ml of PGe during 24 h did not change significantly the IL-4 production by MAC-T cells compared with untreated cells (Fig. 4). Intracellular IL-4 production was significantly induced in MAC-T cells treated with PMA and ionomycin compared with cells treated with PGe and untreated control cells ($P < 0.05$; Fig. 4).

3.4. Effect of PGe on S. aureus growth

To evaluate the effect of PGe on *S. aureus* growth, two *S. aureus* strains were cultured in the presence of different concentrations of PGe (0.5, 1 and 3 mg/ml of PGe) or without PGe. The addition of PGe to the bacterial culture medium stimulated the growth of both *S. aureus* Newbould 305 and *S. aureus* 5011 strains after 24 h of culture ($P < 0.001$), exhibiting a higher effect at 3 mg/ml ($P < 0.05$) (Fig. 5A and B respectively).

3.5. PGe effect on S. aureus internalization into MAC-T cells

To evaluate the effect of PGe on *S. aureus* internalization, MAC-T cell incubated with different concentrations of PGe and then exposed to *S. aureus* Newbould 305 and *S. aureus* 5011 strains was carried out. PGe at 3 mg/ml reduced *S. aureus* Newbould 305 internalization in MAC-T compared with 1 and 0.5 mg/ml and controls ($P < 0.05$) (Fig. 6A). In addition, 3 mg/ml of PGe reduced *S. aureus* 5011 internalization in MAC-T compared with 0.5 mg/ml and control ($P < 0.05$) (Fig. 6B).

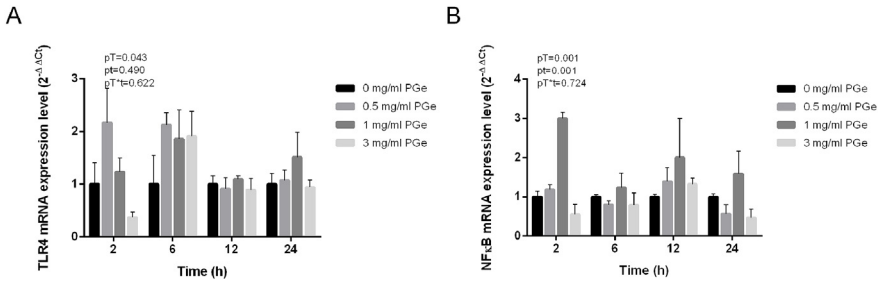

Fig. 2. Relative mRNA expression of TLR-4 (A) and NF-κB (B) in MAC-T cells treated with different concentrations of PGe (0.5, 1 and 3 mg/ml) or without PGe at 2, 6, 12 and 24 h post treatment. Each bar represents the means of triplicates ± standard desviation (SD) of three independent experiments. The main effects of treatment (T), time of sampling (t) and treatment * time interaction (T*t) are shown.

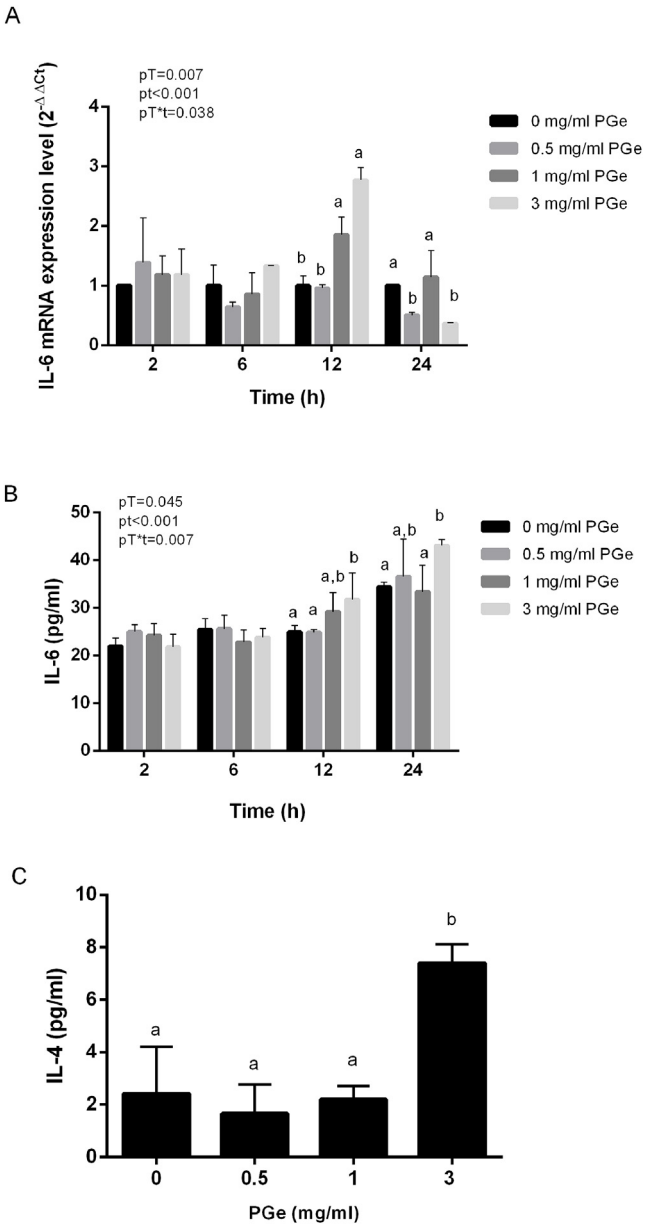

Fig. 3. Relative mRNA expression of IL-6 (A) and protein levels of IL-6 (B) in MAC-T cells treated with different concentrations of PGe (0.5, 1 and 3 mg/ml) or without PGe at 2, 6, 12 and 24 h post treatment. Protein levels of IL-4 (C) in MAC-T cells treated with different concentration of PGe (0–3 mg/ml) at 24 h of treatment. Each bar represents the means of triplicates ± standard desviation (SD) of three independent experiments. For IL-6 analysis, the main effects of treatment (T), time of sampling (t) and treatment * time interaction (T*t) are shown. At each sampling time, different letters correspond to statistically significant differences ($P < 0.05$).

3.6. Effect of PGe on the phagocytic activity, ROS and NO production

The potential cytotoxicity of PGe on macrophages isolated from secretions obtained from involuting mammary glands was evaluated by the XTT assay. PGe concentrations up to 3 mg/ml did not affect cells viability after 24 h of treatment (Fig. 7).

The percentage of CD14 positive cells in macrophages region was 38% (33–40.7%) from all analyzed mammary secretions. Then, the effect of PGe on the ability of macrophages in this region to ingest *S. aureus* was assessed. No significant differences were observed in percentage of macrophages phagocytosis and MFI from involution secretions treated with PGe and controls that were co-cultured with both *S. aureus* strains (Table 2).

PGe did not change the ROS production by macrophages during co-culture with both *S. aureus* strains ($P = 0.096$ and $P = 0.985$, respectively). No significant differences were observed in MFI of ROS production when macrophages were co-cultured with *S. aureus* 5011 with or without PGe ($P = 0.722$). However, when macrophages were co-cultured with *S. aureus* Newbould 305 and treated with PGe the MFI of ROS production was higher than in macrophages without PGe ($P = 0.046$) (Table 2).

The NO production by phagocytic macrophages was evaluated using Griess reaction. PGe did not change NO production in macrophages without *S. aureus* (basal levels). When macrophages were exposed to both *S. aureus* strains, an increase in NO production was observed compared with basal levels ($P < 0.05$), but this increase was not affected by PGe treatment (Fig. 8A and B).

4. Discussion

Mammary epithelial cells and macrophages are the first cells to be confronted with a pathogen following access to the mammary gland; being an important source of pro-inflammatory cytokines and chemokines that attract neutrophils in early infection processes [31]. In the present study, we evaluated the effects of PGe both on the internalization of *S. aureus* into MAC-T cells and phagocytic activity of macrophages isolated from mammary secretion.

First, we evaluated the effect of PGe on MAC-T cells viability and on *S. aureus* growth at a range of concentrations from 0.5 to 3 mg/ml. In previous studies *in vivo,* we observed that a concentration of 3 mg/ml proved to be safe for cows after intramammary inoculation, causing only mild inflammation [32]. In the present study, PGe showed no toxicity to MAC-T cells after 24 h of incubation. In agreement with these results, in previous studies in cows and mice we observed that following PGe inoculation, normal mammary gland architecture was not altered as indicated by maintenance of alveolar and stromal integrity, suggesting absence of adverse effects on mammary gland structure and function [19,32].

In the present study, the addition of PGe to the bacterial culture medium stimulated *S. aureus* strains growth, with a greater effect at 3 mg/ml. These results indicate that in an *in vitro* culture system, PGe at high concentrations could act as an additional nutrient for the growth of *S. aureus.* However, this may not occur in an *in vivo* system, where bacterial growth would not only be regulated by the availability of

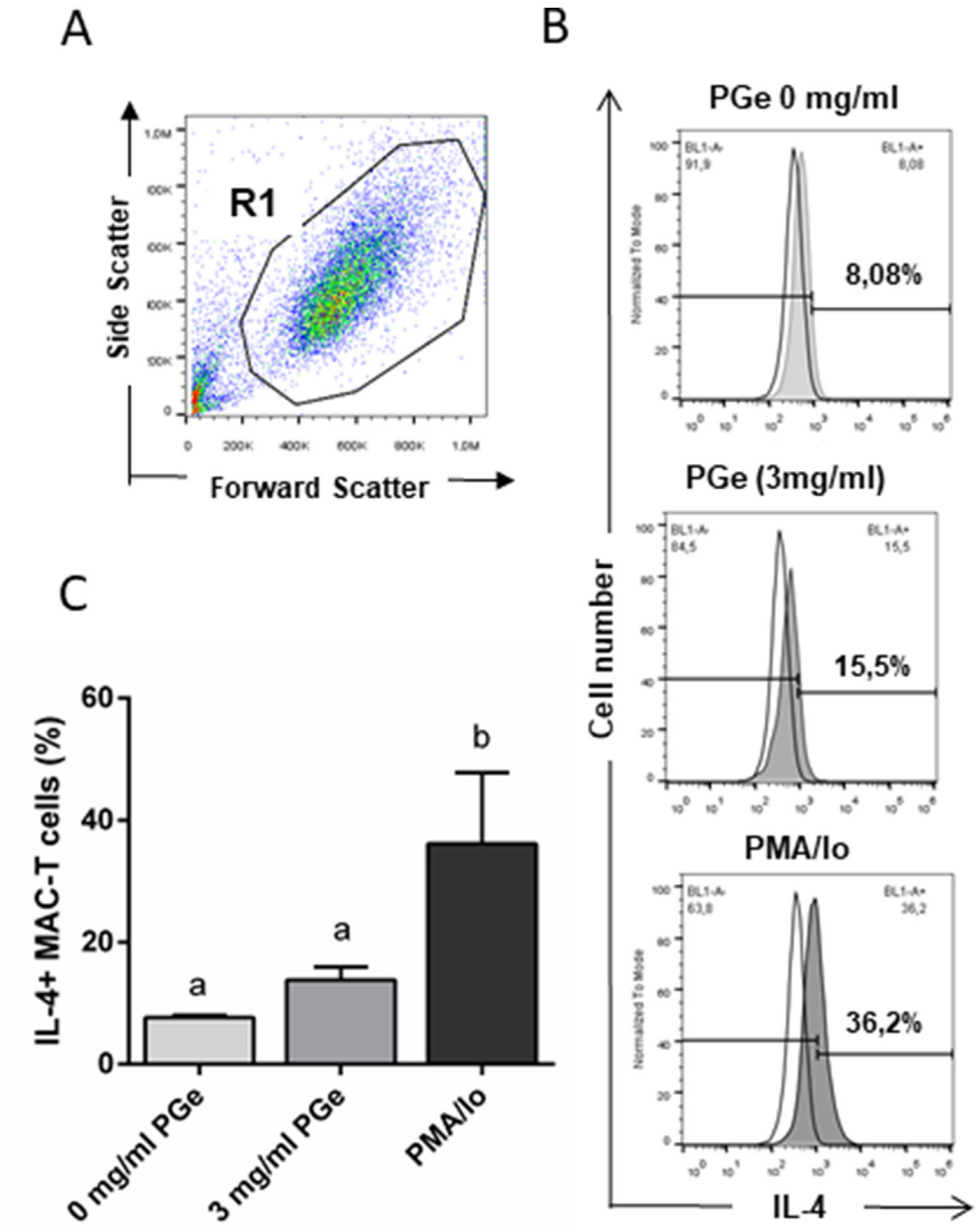

Fig. 4. Effect of PGe (3 mg/ml) on intracellular IL-4 production by MAC-T cells using flow cytometry analysis. A) Representative forward scatter versus side scatter density plots showing gate in R1 for further IL-4 analysis on MAC-T cells population. B) Representative histograms showing cells stained with isotype control (empty) or IL-4 mAb (filled). C) Percentages of IL-4+ MAC-T cells are shown. Data are expressed as mean ± SD of three independent experiments. Different letters correspond to statistically significant differences ($P < 0.05$).

nutrients but also by the host immune response. In this regard, in a previous study, we observed a significant decrease in the number of bacteria recovered from the mammary gland of mice treated with PGe and further challenged experimentally with *S. aureus* [19].

Mammary epithelial cells express specific pattern recognition receptors (PRR) on their surfaces or within the cells, such as TLRs, that recognize particular bacterial molecules called pathogen-associated molecular patterns (PAMP) [33]. Upon ligand binding, TLRs initiate signal transduction pathways that culminate in NF-κB and MAP kinase activation and induction of inflammatory cytokines, which are synthesized by infiltrating cells [34] as well as resident cells in response to infection [35]. In addition to microbial components, TLRs also recognize non-pathogenic molecules like plant derived and stress related proteins [36,37]. To determine whether TLR4 and NF-κB could be associated with PGe effect in MAC-T cells we analyzed the mRNA expression of these genes. We observed that although expression of both genes was higher in MAC-T cells treated with different PGe concentrations at most sampling times, no significant differences were found compared with controls. These results suggest that TLR4 is not the main receptor involved in the recognition of PGe by MAC-T cells or it could act indirectly in association with other receptors that were not object of study [38]; and that TLR4 gene expression observed *in vivo* [8] reflects activity of other cells within the mammary tissue.

It has been shown that the number of TLR4 molecules involved in recognition determines whether the innate immune response will be activated [39]. Besides, it has been reported that ginseng regulates immune responses through the modulation of cytokine secretion [40]. In this study, although we found higher mRNA levels of TLR4 in MAC-T

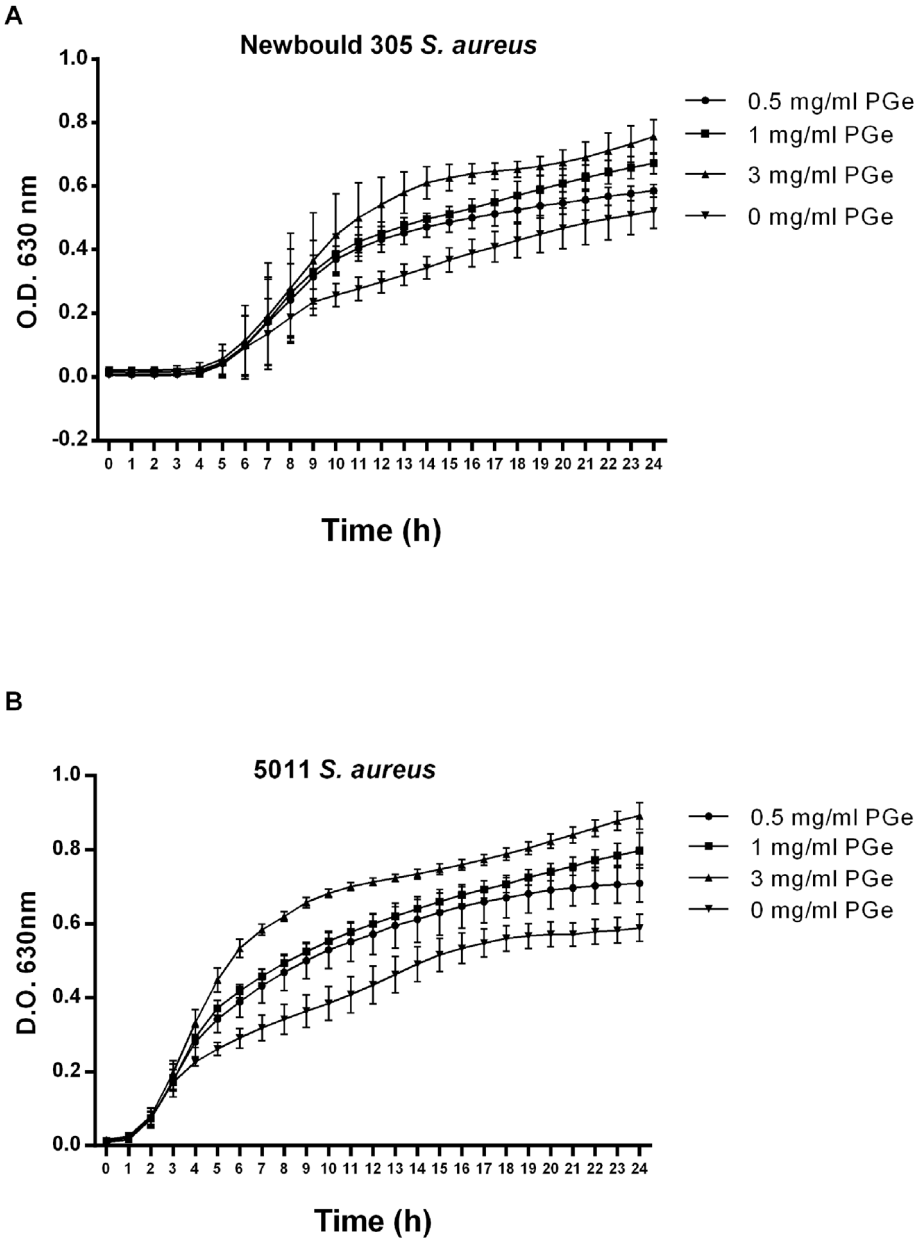

Fig. 5. Effect of different concentrations of PGe (0.5, 1 and 3 mg/ml) on growth of *S. aureus* Newbould 305 (A) and *S. aureus* 5011 (B) up to 24 h. Values represent the means of triplicates ± standard desviation (SD) of three independent experiments. Two-way repeated measures ANOVA indicated a significant effect of PGe treatment ($P < 0.001$).

treated with different PGe concentrations at most sampling times, these were not sufficient to cause significant stimulation of IL-1β and TNF-α mRNA expression in mammary epithelial cells. These results suggest that PGe does not affect TLR4 and their signalling transduction pathways in MAC-T cell model. Whether PGe stimulates other TLRs signalling pathways or associates with other receptors in mammary

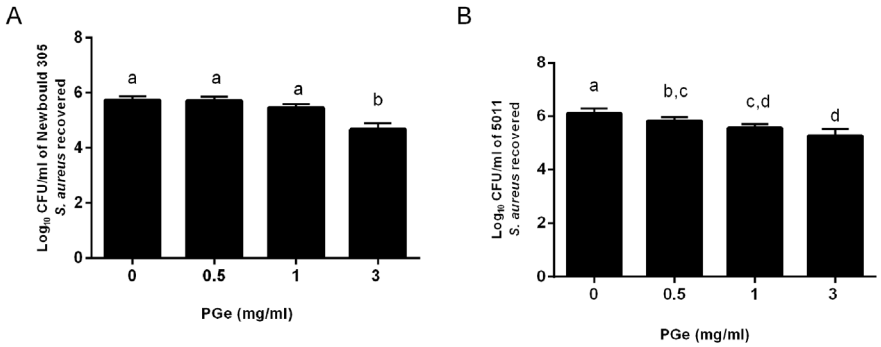

Fig. 6. Effect of different concentrations of PGe (0.5, 1 and 3 mg/ml) on *S. aureus* Newbould 305 (A) and *S. aureus* 5011 (B) internalization into MAC-T cells. Data are presented as $\log_{10}$ of CFU/ml recovered from MAC-T cells lysis. Bars show the mean of triplicates ± standard desviation (SD) of three independent experiments. Bars with different superscript letters denote significant differences ($P < 0.05$).

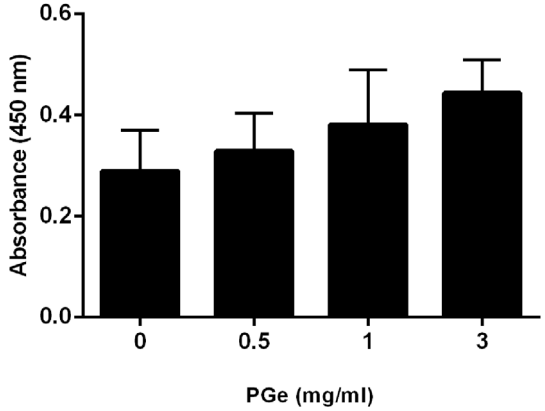

Fig. 7. Effect of different concentrations of PGe (0–3 mg/ml) on macrophages from mammary involution secretions viability at 24 h of treatment. Each bar represents the means of triplicates ± standard desviation (SD) of three independent experiments. P values of < 0.05 were considered significant.

epithelial cells remains unclear and needs further investigation.

IL-6 is expressed by a variety of cells, including lymphocytes, monocytes, macrophages, neutrophils, endothelial cells, epithelial cells, and fibroblasts, and its expression is induced both by bacteria and viruses [41]; having both pro and anti-inflammatory properties [42]. In this study, we demonstrated the mRNA expression and secretion of IL-6 in MAC-T cells in response to PGe. In fact, the higher levels of mRNA were found at 12 h of PGe treatment, followed by an increase in protein release at 24 h of PGe treatment. Interestingly, the significant increase in the production of IL-4, in MAC-T cells treated with PGe at 24 h was in parallel with the increase in the production of IL-6. The latter cytokine possesses both pro and antiinflammatory properties [42], and is expressed by various cell types following TLR-induced inflammatory cascade. Both results, those found for IL-6 and IL-4 would suggest that PGe has the ability to modulate the immune response, either promoting or suppressing it, and these effects can be dependent on PGe concentration. Moreover, in the present study we demonstrated for the first time that bovine mammary epithelial cells have de ability to produce IL-4 against different stimulus.

The capacity of *S. aureus* to invade and survive within the mammary epithelial cells, which confers protection against the host immune system and the antibiotic treatment, is among the factors that contribute to persistence of this pathogen [43,44], leading to chronic IMI [45]. Recent studies showed that PGe partially inhibited *S. aureus* multiplication within the murine mammary gland and can thus play a protective role against IMI [19]. To determine if there is a relationship between the protective role postulated for PGe in a mouse mastitis model and the capacity of PGe to inhibit early interactions between the organism and mammary epithelial cells, we performed internalization assays in MAC-T cells with two *S. aureus* strains. Interestingly, a concentration of 3 mg/ml of PGe significantly reduced both Newbould 305 and 5011 *S. aureus* strains internalization on MAC-T cells. Considering that PGe does not have an inhibitory effect on *S. aureus*, a direct interaction of PGe with MAC-T is most likely to be causing interference with bacteria internalization. It has been determined that internalization of *S. aureus* by nonprofessional phagocytes involves an interaction between fibronectin (Fn) binding protein (FnBP) and the host cell, resulting in signal transduction, tyrosine kinase activity, and cytoskeletal rearrangement [46]. Interestingly, it has been reported that ginsenoside Rg1 from *Panax notoginseng* decreased the protein levels of collagen I and fibronectin in a dose-dependent manner in rat renal tubular epithelial cells [47]. On the basis of these observations, we hypothesised that molecular interactions between PGe and MAC-T cells surface proteins may occur, and this could prevent the correct adhesion and subsequent internalization of *S. aureus* to the mammary epithelial cells. As far as we know, this is the first report about properties of PGe on inhibiting *S. aureus* internalization in MAC-T cells. Nevertheless, the mechanism leading to reduction of PGe on *S. aureus* internalization remains to be elucidated.

When activated by various stimuli, macrophages enhance their phagocytic activity, increase their ability to kill ingested pathogens, and produce many cytokines and inflammatory mediators such as nitric oxide and H_2O_2 [48]. Several reports have shown that ginseng extract enhanced phagocytic activity of macrophages. Shin et al. [49] have shown that peritoneal murine macrophages treated with red ginseng acidic polysaccharides (RGAPs, ginsan) stimulated the phagocytosis of zymosan, a preparation of cell wall from *Saccharomyces cerevisiae*. In addition, treatment of murine macrophages with a combination of RGAPs and IFN-γ has shown to potentiate the activation of macrophages, and a substantial improvement in the NO production [50]. In the present study, the percentage of macrophages phagocytosis and the MFI phagocytosis cells in the PGe-treated group did not differ from the control group for both Newbould 305 and 5011 *S. aureus* strains. Direct comparisons between previous research [50] and the present study cannot be carried out due to the different chemical nature of the PGe employed as well as the distinct origin of cells evaluated. During active mammary involution, the indiscriminate ingestion of milk components by macrophages, render these cells less effective for phagocytosis [51]. However, macrophages have receptors for IgG1 and IgG2 [52] and those obtained at 10–15 days after cessation of milking showed enhanced *S. aureus* uptake when incubated with specific antibodies [53].

In this study, low levels of NO in macrophage basal cell culture medium (without bacteria) was detected and significantly higher levels were induced upon infection with Newbould 305 and 5011 *S. aureus* strains; however, this increase was not affected by PGe treatment. In contrast, another study has demonstrated that NO production increased in macrophages from RGAP-treated mice [54]. Discrepancies between studies may be due to the PGe used and the source of macrophages employed in our study.

The reactive oxidants play a critical role in host defense and other

Table 2
Effect of PGe on the phagocytic activity of macrophages from involution mammary secretions.

Variable	PGe treatment	Control	P (value)
Newbould 305 *S. aureus* phagocytosis (%)	84.40 ± 2.52[a]	83.43 ± 2.40[a]	0.656
Intensity of Newbould 305 *S. aureus* phagocytosis (MFI)	112500.33 ± 22432.70[a]	88834.00 ± 25289.31[a]	0.985
5011 *S. aureus* phagocytosis (%)	87.97 ± 1.46[a]	88.90 ± 3.001[a]	0.654
Intensity of 5011 *S. aureus* phagocytosis (MFI)	106092.00 ± 2455.65[a]	121818 ± 9939.61[a]	0.056
Newbould 305 *S. aureus* ROS production	12.00 ± 1.21[a]	16.07 ± 3.01[a]	0.096
Newbould 305 *S. aureus* intensity of ROS production (MFI)	647.00 ± 95.52[a]	488.33 ± 11.37[b]	0.046
5011 *S. aureus* ROS production	11.48 ± 1.42[a]	11.46 ± 2.85[a]	0.985
5011 *S. aureus* intensity of ROS production (MFI)	355.00 ± 12.86[a]	387.33 ± 103.54[a]	0.722

The results are shown as the mean of the values ± standard deviation (SD). MFI: median fluorescence intensity; ROS: reactive oxygen species. Different superscripted letters denote significant differences (P < 0.05) between the values.

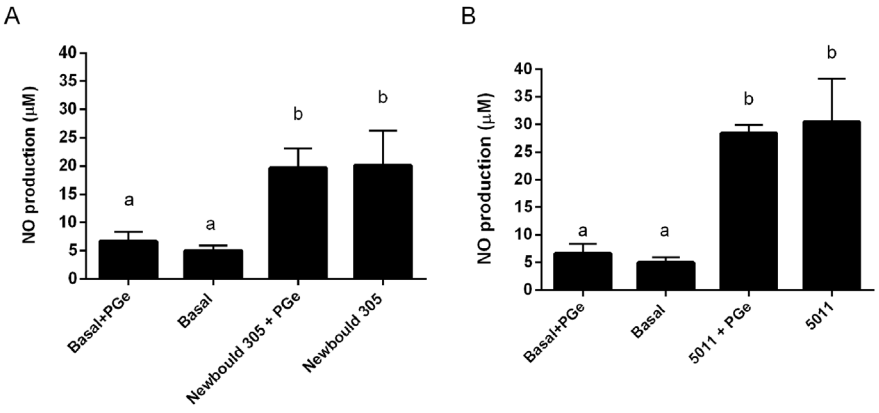

Fig. 8. Effect of PGe (3 mg/ml) on NO production into supernatant of macrophages from the phagocytic assay with Newbould 305 (A) and 5011 (B) *S. aureus* strains. Basal values represent macrophages without *S. aureus* contact. Each bar represents the means of the values ± standard desviation (SD). Bars with different superscript letters denote significant differences ($P < 0.05$).

physiological processes and are also involved in the regulation of apoptosis and immune homeostasis [55,56]. Results obtained in this study shown that PGe neither stimulated macrophages co-cultured with both *S. aureus* strain to produce intracellular ROS nor suppressed its production. Suppressive effects of non-fermented (WG) and fermented (FWG) extracts of cultured wild ginseng Root were observed on LPS-induced ROS production in RAW264.7 macrophages [57]. In addition, a mixture of three medicinal plants *Angelica gigas Nakai* (AGN), *Panax ginseng* (PG), *Rhus verniciflua Stokes* RVS (APR) suppressed intracellular ROS levels induced by LPS in RAW264.7 cells when these were stimulated with LPS for 12 h (Choi. et al., 2014). Differences found between results of our study and those of Choi et al. [58] can rely on the sampling period evaluated in the present study (only 2 h with PGe).

In conclusion, PGe reduced *S. aureus* internalization into MAC-T cells potentially by interfering early *S. aureus*-cell interaction and did not affect macrophages phagocytic activity when these were *in vitro* co-cultured with *S. aureus*. These results, obtained in *in vitro* models can contribute to improve the understanding of the effects observed following PGe in bovine mammary glands and put into perspective the complex interactions between this compound and the mammary gland immune defense.

Acknowledgments

This work was supported by National Scientific and Technical Research Council - Argentina (CONICET) (PIP 11220150100238CO/2015).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.micpath.2018.06.010.

References

[1] M. Pannacci, V. Lucini, F. Colleoni, C. Martucci, S. Grosso, P. Sacerdote, F. Scaglione, *Panax ginseng* C.A. Mayer G115 modulates pro-inflammatory cytokine production in mice throughout the increase of macrophage toll-like receptor 4 expression during physical stress, Brain Behav. Immun. 20 (2006) 546–551.
[2] M.K. Kim, J.W. Lee, K.Y. Lee, D.C. Yang, Microbial conversion of major ginsenoside Rb1 to pharmaceutically active minor ginsenoside Rd, J. Microbiol. 43 (2005) 456–462.
[3] F. Scaglione, F. Ferrara, S. Dugnani, M. Falchi, G. Santoro, F. Fraschini, Immunomodulatory effects of two extracts of *Panax ginseng* C.A. Meyer, Drugs Exp. Clin. Res. 16 (1990) 537–542.
[4] F. Scaglione, G. Cattaneo, M. Alessandria, R. Cogo, Efficacy and safety of the standardised Ginseng extract G115 for potentiating vaccination against the influenza syndrome and protection against the common cold, Drugs Exp. Clin. Res. 22 (1996) 65–72.
[5] Z.J. Song, H.K. Johansen, V. Faber, N. Høiby, Ginseng treatment enhances bacterial clearance and decreases lung pathology in athymic rats with chronic *P. aeruginosa* pneumonia, APMIS 105 (1997) 438–444.
[6] C. Baravalle, B.E. Dallard, H.H. Ortega, V.E. Neder, V.R. Canavesio, L.F. Calvinho, Effect of *Panax ginseng* on cytokine expression in bovine mammary glands at drying off, Vet. Immunol. Immunopathol. 138 (2010) 224–230.
[7] C. Baravalle, B.E. Dallard, M.C. Cadoche, E.A. Pereyra, V.E. Neder, H.H. Ortega, L.F. Calvinho, Proinflammatory cytokines and CD14 expression in mammary tissue of cows following intramammary inoculation of *Panax ginseng* at drying off, Vet. Immunol. Immunopathol. 144 (2011) 52–60.
[8] C. Baravalle, P. Silvestrini, M.C. Cadoche, C. Beccaria, C.S. Andreotti, M.S. Renna, E.A.L. Pereyra, H.H. Ortega, L.F. Calvinho, Dallard BE Intramammary infusion of *Panax ginseng* extract in bovine mammary gland at cessation of milking induces changes in the expression of toll-like receptors, MyD88 and NF-kB during early involution, Res. Vet. Sci. 100 (2015) 52–60.
[9] B.E. Dallard, S.A. Pujato, C. Baravalle, E.A. Pereyra, F. Rey, M.S. Renna, L.F. Calvinho, Intramammary infusion of *Panax ginseng* extract in the bovine mammary gland at cessation of milking modifies components of the insulin-like growth factor system during involution, Res. Vet. Sci. 94 (2013) 462–470.
[10] S. Hu, C. Concha, A. Jahannisson, G. Meglia, K.P. Waller, Effect of subcutaneous injection of Ginseng on cows with subclinical *Staphylococcus aureus* mastitis, J. Vet. Med. B 48 (2001) 519–528.
[11] Y. Persson, A. Nyman, U. Grönlund-Andersson, Etiology and antimicrobial susceptibility of udder pathogens from cases of subclinical mastitis in dairy cows in Sweden, Acta Vet. Scand. 53 (2011) 36–43.
[12] A. Zecconi, L. Calvinho, L. Fox, *Staphylococcus aureus* intramammary infections, Bull. Int. Dairy Fed. 0250-5118, 408 (2006) 39 FIL-IDF.
[13] R.A. Almeida, K.R. Matthews, E. Cifrian, A.J. Guidry, S.P. Oliver, *Staphylococcus aureus* invasion of bovine mammary epithelial cells, J. Dairy Sci. 79 (1996) 1021–1026.
[14] A. Hébert, K. Sayasith, S. Senechal, P. Dubreuil, J. Lagace, Demonstration of intracellular *Staphylococcus aureus* in bovine mastitis alveolar cells and macrophages isolated from naturally infected cow milk, FEMS Microbiol. Lett. 193 (2000) 57–62.
[15] H.W. Barkema, Y.H. Schukken, R.N. Zadoks, Invited review: the role of cow, pathogen, and treatment regimen in the therapeutic success of bovine *Staphylococcus aureus* mastitis, J. Dairy Sci. 89 (2006) 1877–1895.
[16] O. Wellnitz, R.M. Bruckmaier, The innate immune response of the bovine mammary gland to bacterial infection, Vet. J. 192 (2012) 148–152.
[17] C. Riollet, P. Rainard, B. Poutrel, Differential induction of complement fragment C5a and inflammatory cytokines during intramammary infections with *Escherichia coli* and *Staphylococcus aureus*, Clin. Diagn. Lab. Immunol. 7 (2000) 161–167.
[18] D.D. Bannerman, M.J. Paape, J.W. Lee, X. Zhao, J.C. Hope, P. Rainard, *Escherichia coli* and *Staphylococcus aureus* elicit differential innate immune responses following intramammary infection, Clin. Diagn. Lab. Immunol. 11 (2004) 463–472.
[19] P. Silvestrini, C. Beccaria, E.A.L. Pereyra, M.S. Renna, H.H. Ortega, L.F. Calvinho, B.E. Dallard, C. Baravalle, Intramammary inoculation of *Panax ginseng* plays an immunoprotective role in Staphylococcus aureus infection in a murine model, Res. Vet. Sci. 115 (2017) 211–220.
[20] E.A. Pereyra, F. Picech, M.S. Renna, C. Baravalle, C.S. Andreotti, R. Russi, L.F. Calvinho, C. Diez, B.E. Dallard, Detection of *Staphylococcus aureus* adhesion and biofilm-producing genes and their expression during internalization in bovine mammary epithelial cells, Vet. Microbiol. 183 (2016) 69–77.
[21] H.T. Huynh, Robitaille G y Turner JD. Establishment of bovine mammary epithelial cells (MAC-T): an in vitro model for bovine lactation, Cell Res. 197 (1991) 191–199.
[22] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method, Methods 25 (2001) 402–408.
[23] M. Denis, Interferon-gamma-treated murine macrophages inhibit growth of tubercle bacilli via the generation of reactive nitrogen intermediates, Cell. Immunol. 132 (1991) 150–157.
[24] H. Dosogne, F. Vangroenweghe, B. Barrio, P. Rainard, C. Burvenich, Decreased number and bactericidal activity against *Staphylococcus aureus* of the resident cells in milk of dairy cows during early lactation, J. Dairy Res. 68 (2001) 539–549.
[25] M.J. Paape, E.-M. Liluis, P.A. Wiitanen, R.H. Kontio, R.H. Miller, Intramamary defense against infections induced by *Escherichia coli* in cows, Am. J. Vet. Res. 57 (1996) 477–482.
[26] Z. Sladek, D. Rysanek, M. Faldyna, Activation of phagocytes during initiation and resolution of mammary gland injury induced by lipopolysaccharide in heifers, Vet. Res. (Paris) 33 (2002) 191–204.
[27] A. Zetterlund, P.H. Larsson, C. Müller-Suur, L. Palmberg, K. Larsson, Budesonide but

not terbutaline decreases phagocytosis in alveolar macrophages, Respir. Med. 92 (1998) 162–166.
[28] M. Denis, N.A. Parlane, S.J. Lacy-Hulbert, E.L. Summers, Buddle BM y Wedlock DN. Bactericidal activity of macrophages against *Streptococcus uberis* is different in mammary gland secretions of lactating and drying off cows, Vet. Immunol. Immunopathol. 114 (2006) 111–120.
[29] A. Gutiérrez-Barrosoa, J.L. Anaya-Lópeza, L. Lara-Záratea, P.D. Loeza-Larab, J.E. López-Mezaa, A. Ochoa-Zarzosaa, Prolactin stimulates the internalization of *Staphylococcus aureus* and modulates the expression of inflammatory response genes in bovine mammary epithelial cells, Vet. Immunol. Immunopathol. 121 (2008) 113–122.
[30] Y.W. Yang, S.S. Shen, Enhanced antigen delivery via cell death induced by the vaccine adjuvants, Vaccine 25 (2007) 7763–7772.
[31] K. Thompson-Crispi, H. Atalla, F. Miglior, B.A. Mallard, Bovine mastitis: frontiers in immunogenetics, Front. Immunol. 5 (2014) 493.
[32] B.E. Dallard, C. Baravalle, C. Andreotti, H.H. Ortega, V. Neder, L.F. Calvinho, Intramammary inoculation of *Panax ginseng* extract in cows at drying off enhances early mammary involution, J. Dairy Res. 78 (2011) 63–71.
[33] H. Yang, W. Zerbe, R.M. Petzl, J. Brunner, C. Günther, S. Draing, H.J. von-Aulock, H.J. Schuberth, H.M. Seyfert, Bovine TLR2 and TLR4 properly transduce signals from *Staphylococcus aureus* and *E. coli*, but *S. aureus* fails to both activate NF-κB in mammary epithelial cells and to quickly induce TNFα and interleukin-8 (CXCL8) expression in the udder, Mol. Immunol. 45 (2008) 1385–1397.
[34] Jai-Wei Lee, Douglas D. Bannerman, Max J. Paape, Ming-Kuei Huang, Xin Zhao, Characterization of cytokine expression in milk somatic cells during intramammary infections with *Escherichia coli* or *Staphylococcus aureus* by real-time PCR, Vet. Res. (Paris) 37 (2006) 219–229.
[35] Lahouassa Hichem, Moussay Etienne, Pascal Rainard, Riollet Céline, Differential cytokine and chemokine responses of bovine mammary epithelial cells to *Staphylococcus aureus* and *Escherichia coli*, Cytokine 38 (2007) 12–21.
[36] A. Asea, M. Rehli, E. Kabingu, J.A. Boch, O. Bare, P.E. Auron, M.A. Stevenson, S.K. Calderwood, Novel signal transduction pathway utilized by extracellular HSP70: role of toll-like receptor (TLR) 2 and TLR4, J. Biol. Chem. 277 (2002) 15028–15034.
[37] T.A. Nakaya, M. Kita, H. Kuriyama, Y. Iwakura, J. Imanishi, *Panax ginseng* induces production of proinflammatory cytokines via toll-like receptor, J. Interferon Cytokine Res. 24 (2004) 93–100.
[38] C.R. Stewart, L.M. Stuart, K. Wilkinson, J.M. van Gils, J. Deng, A. Halle, K.J. Rayner, L. Boyer, R. Zhong, W.A. Frazier, A. Lacy-Hulbert, J. El Khoury, D.T. Golenbock, K.J. Moore, CD36 ligands promote sterile inflammation through assembly of a Toll-like receptor 4 and 6 heterodimer, Nat. Immunol. 11 (2010) 155–161.
[39] M. Di Gioia, I. Zanoni, Toll-like receptor co-receptors as master regulators of the immune response, Mol. Immunol. 63 (2015) 143–152.
[40] S. Kang, H. Min, Ginseng, the 'Immunity Boost': the effects of *Panax ginseng* on immune system, J. Ginseng Res. 36 (4) (2012) 354–368.
[41] W.L. Biffl, E.E. Moore, F.A. Moore, V.M. Peterson, Interleukin-6 in the injured patient. Marker of injury or mediator of inflammation? Ann. Surg. 224 (1996) 647–664.
[42] D.D. Bannerman, Pathogen-dependent induction of cytokines and other soluble inflammatory mediators during intramammary infection of dairy cows, J. Anim. Sci. 87 (2009) 10–25.
[43] R.A. Almeida, S.P. Oliver, Interaction of coagulase-negative *Staphylococcus species* with bovine mammary epithelial cells, Microb. Pathog. 31 (2001) 205–212.
[44] J. Oviedo-Boyso, J.G. Barriga-Rivera, J.J. Valdez-Alarcon, A. Bravo-Patino, A. Carabez-Trejo, M. Cajero-Juarez, et al., Internalization of *Staphylococcus aureus* by bovine endothelial cells is associated with the activity state of NF-kappa B and modulated by the pro-inflammatory cytokines TNF-alpha and IL-1 beta, Scand. J. Immunol. 67 (2008) 169–176.
[45] O. Kerro Dego, J. van Dijk, Nederbragt factors involved in the early pathogenesis of bovine *Staphylococcus aureus* mastitis with emphasis on bacterial adhesion and invasion, Vet. Q. H. 24 (2002) 181–198.
[46] K. Dziewanowska, A.R. Carson, J.M. Patti, C.F. Deobald, K.W. Bayles, G.A. Bohach, Staphylococcal fibronectin binding protein interacts with heat shock protein 60 and integrins: role in internalization by epithelial cells, Infect. Immun. 68 (2000) 6321–6328.
[47] X. Xie, M. Yang, H.C. Liu, C. Zuo, H.J. Li, J.M. Fana, Rg1 Ginsenoside, A major active component isolated from *Panax notoginseng*, restrains tubular epithelial to myofibroblast transition in vitro, J. Ethnopharmacol. 122 (2009) 35–41.
[48] T.J. Guzik, R. Korbut, T. Adamek-Guzik, Nitric oxide and superoxide in inflammation and immune regulation, J. Physiol. Pharmacol. 54 (2003) 469–487.
[49] J.Y. Shin, J.Y. Song, Y.S. Yun, H.O. Yang, D.K. Rhee, S. Pyo, Immunostimulating effects of acidic polysaccharides extract of *Panax ginseng* on macrophage function, Immunopharmacol. Immunotoxicol. 24 (2002) 469–482.
[50] H.S. Choi, K.H. Kim, E. Sohn, J.D. Park, B.O. Kim, E.Y. Moon, D.K. Rhee, S. Pyo, Red ginseng acidic polysaccharide (RGAP) in combination with IFN-gamma results in enhanced macrophage function through activation of the NF-kappaB pathway, Biosci. Biotechnol. Biochem. 72 (2008) 1817–1825.
[51] L.M. Sordillo, L.A. Babiukd, Modulation of mammary neutrophil function during the periparturient period following *in vitro* exposure to recombinant bovine interferon gamma, Vet. Immunol. Immunopathol. 27 (1991) 393.
[52] J.V. Desiderio, S.G. Campbell, Bovine mammary gland macrophage: isolation, morphologic features, and cytophilic immunoglobulins, Am. J. Vet. Res. 41 (1980) 1595–1599.
[53] M.S. Renna, E.A. Pereyra, C. Baravalle, C.M. Camussone, B.E. Dallard, I.S. Marcipar, L.F. Calvinho, Functional role of antibodies generated in heifers through immunization with *Staphylococcus aureus* vaccines in invasion and phagocytosis assays, FEMS Microbiol. Lett. 360 (2014) 62–69.
[54] K.M. Park, Y.S. Kim, T.C. Jeong, C.O. Joe, H.J. Shin, Y.H. Lee, K.Y. Nam, J.D. Park, Nitric oxide is involved in the immunomodulating activities of acidic polysaccharide from *Panax ginseng*, Planta Med. 67 (2001) 122–126.
[55] C.N. Serhan, J. Savill, Resolution if inflammation: the beginning programs the end, Nat. Immunol. 6 (2005) 1191–1197.
[56] M.T. Quinn, M.C. Ammons, F.R. Deleo, The expanding role of NADPH oxidases in health and disease: no longer just agents of death and destruction, Clin. Sci. (Lond.) 111 (2006) 1–20.
[57] B.G. Park, H.J. Jung, Y.W. Cho, H.W. Lim, C.J. Lim, Potentiation of antioxidative and anti-inflammatory properties of cultured wild ginseng root extract through probiotic fermentation, J. Pharm. Pharmacol. 65 (2013) 457–464.
[58] H.S. Choi, H.S. Seo, S.R. Kim, Y.K. Choi, Y.C. Shin, S.G. Ko, Anti-inflammatory and anti-proliferative effect of herbal medicines (apr) in raw264.7 cells, Mol. Med. Rep. 9 (2014) 1569–1574.