

## Cellular response of the bovine mammary gland after *Weissella confusa* infusion to control *Streptococcus agalactiae*<sup>□</sup>

*Respuesta celular de la glándula mamaria bovina después de la infusión con Weissella confusa para el control de Streptococcus agalactiae*

*Resposta celular da glândula mamaria bovina após infusão com Weissella confusa para o controle de Streptococcus agalactiae*

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### Summary

**Background:** the use of lactic acid bacteria (LAB) as a potential therapeutic agent to control bovine mastitis was previously proposed. However, little is known about the cellular response of the bovine mammary gland in cattle infected with *Streptococcus agalactiae* and treated with LAB. **Objective:** to assess the cellular response by the mammary gland in lactating cows after infection with *Streptococcus agalactiae* followed by infusion with *Weissella confusa* as antibacterial treatment. **Methods:** healthy udder quarters of lactating cows were infected with *S. agalactiae* (10<sup>7</sup> cfu/mL). After 24 h of pathogen infusion, 50% of the quarters were infused with 10<sup>9</sup> cfu/mL of *W. confusa* (SW) and the remaining 50% were kept as control units (S). At days 1, 2, 3, 4, 5, 11, and 14 post-infusion of the pathogen, the clinical signs of mastitis and the degree of cellular response by the mammary gland were evaluated using the California mastitis test, somatic cell count, electrical conductivity, and differential leukocyte count in milk. **Results:** the SW quarters showed clinical inflammation of the mammary gland associated with a significant increase in somatic cell count, California mastitis test, electrical conductivity and high proportion of polymorphonuclear neutrophils. The results suggest that the infusion with *W. confusa* cells induced a higher cellular immune response in the bovine mammary gland than *S. agalactiae* alone. **Conclusions:** results indicate that *W. confusa* infusions for controlling *S. agalactiae* should not be adopted. However, the activation mechanism of somatic cells in the mammary gland needs to be elucidated.

**Key words:** bovine mastitis, lactic acid bacteria, leukocytes.

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## Resumen

**Antecedentes:** previamente se propuso el uso de bacterias ácido lácticas (LAB) como agentes terapéuticos potenciales para el control de mastitis bovina. Sin embargo, poco se ha investigado sobre la respuesta celular de la glándula mamaria bovina ante la aplicación de LAB en bovinos infectados con *Streptococcus agalactiae*. **Objetivo:** evaluar, en vacas lactantes, la respuesta celular de la glándula mamaria después de la infección con *Streptococcus agalactiae* y la infusión intrapezón con *Weissella confusa* como tratamiento. **Métodos:** cuartos sanos de ganado criollo Hartón del Valle en estado de lactancia, se infectaron con *S. agalactiae* ( $10^7$  ufc/mL). Transcurridas 24 horas post-infusión del patógeno, al 50% de los cuartos se les aplicó *W. confusa* a concentración de  $10^9$  ufc/mL (cuartos SW) y el 50% de los cuartos restantes se tomaron como unidades experimentales control (cuartos S). En los días 1, 2, 3, 4, 5, 11 y 14 pos-infusión del patógeno, se evaluaron signos clínicos de mastitis y el grado de respuesta celular de la glándula mamaria, a través de California mastitis test, recuento de células somáticas, conductividad eléctrica y diferencial de leucocitos en leche. **Resultados:** en los cuartos SW se observaron evidencias clínicas de inflamación de la glándula mamaria asociada con incremento significativo de recuento de células somáticas, California mastitis test, conductividad eléctrica, y altos porcentajes de neutrófilos polimorfonucleares. Los resultados sugieren que la infusión con células de *W. confusa* genera mayor respuesta celular en la glándula mamaria bovina que *S. agalactiae*. **Conclusiones:** los resultados indican que no puede utilizarse la infusión de células vivas de *W. confusa* como tratamiento para el control de *S. agalactiae*. Se debe investigar otro mecanismo de uso de la LAB para el tratamiento de mastitis bovina.

**Palabras clave:** bacterias ácido lácticas, leucocitos, mastitis bovina.

## Resumo

**Antecedentes:** previamente foi proposto o uso de bactérias ácido-lácticas (LAB), como agentes terapêuticos potenciais para o controle da mastite bovina. No entanto, pouca pesquisa tem sido feita sobre a resposta celular da glândula mamária de bovinos após aplicação de LAB em bovinos infectados com *Streptococcus agalactiae*. **Objetivo:** avaliar, em vacas em período de lactação, a resposta celular da glândula mamária após a infecção com *Streptococcus agalactiae* e a infusão dentro da teta de *Weissella confusa* como tratamento. **Métodos:** tetas saudáveis de gado nativo Harton del Valle em lactação foram infectados com *S. agalactiae* ( $10^7$  ufc/mL). Venti-quatro horas após a infusão do agente patogênico, a 50% dos quartos foi administrada *W. confusa* em concentração de  $10^9$  ufc/mL (quartos SW) e 50% dos quartos foram usados como unidades experimentais de controle (quartos S). Nos dias 1, 2, 3, 4, 5, 11 e 14 após a infusão do agente patogênico, foram avaliados os sinais clínicos de mastite e o grau de resposta celular da glândula mamária através do Califórnia Mastite Teste, contagem de células somáticas, condutividade elétrica e diferencial de leucócitos do leite. **Resultados:** nos quartos SW observou-se evidência clínica de inflamação da glândula mamária associado ao aumento significativo na contagem de células somáticas, Califórnia mastite teste, condutividade elétrica, e altas porcentagens de neutrófilos polimorfonucleares. Os resultados sugerem que a infusão de células de *W. confusa* gera maior resposta celular na glândula mamária bovina que o *S. agalactiae*. **Conclusões:** os resultados indicam que a infusão de células vivas de *W. confusa* como um tratamento para o controle de *S. agalactiae* não pode ser usado. Outros métodos de uso da LAB para o tratamento de mastite devem ser pesquisados.

**Palavras chave:** bactérias do ácido láctico, leucócitos, mastite bovina.

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## Introduction

Bovine mastitis is an inflammation of the mammary gland usually caused by microbial infections (Bradley, 2002). It is considered one of the most prolific diseases responsible for reducing milk production and quality in infected animals, and requires specific veterinary

treatments (Aranguren Parra *et al.*, 2009). During the development of the infection in the mammary gland, a specialized defense system is activated, recruiting polymorphonuclear neutrophils (PMNs), macrophages and lymphocyte cells (Sladek and Rysanek, 2010). The PMNs play a key role in the defense against intramammary infections in cows. They are unique leukocytes in milk capable

of producing large quantities of reactive oxygen species that destroy the invading bacteria, and also indirectly interfere in the balance among bactericidal activity, inflammatory response, and tissue damage to the mammary gland (Mehrzaad *et al.*, 2010).

Despite advances in veterinary science, nutrition, and molecular biology, mastitis is still a problem in dairy farms. Prevention strategies such as antibiotic therapy have been proposed (Pellegrino *et al.*, 2008). However, these therapies are costly and occasionally cause the elimination of innocuous or beneficial organisms due to the low specificity of antibiotics (Synnott *et al.*, 2009). Although the use of antibiotics has been effective against mastitis, it has resulted in higher pathogen resistance (Ochoa-Zarzosa *et al.*, 2008). Consequently, there is a growing need for alternative methods to control mastitis in lactating cows. In recent years, the use of lactic acid bacteria (LAB) strains for the control of bovine mastitis has interested the research community. Klostermann *et al.* (2008) reported that intramammary applications of *Lactococcus lactis* were as effective as conventional antibiotic treatments against mastitis. Crispie *et al.* (2008) reported that intramammary administration of *L. lactis* to uninfected cows prompted an immune modulation effect with significant recruitment of PMNs and lymphocytes in the udder quarter where LAB was applied. Furthermore, Beecher *et al.* (2009), using *L. lactis* to modulate the immune response in the mammary gland, suggested the potential of LAB to control mastitis without the use of antibiotics.

Serna-Cock *et al.* (2010) found that *W. confusa*, a LAB isolated from the ruminal liquid of bovine females, exhibited *in vitro* antimicrobial activity against *S. agalactiae* and *Staphylococcus aureus* (main pathogens responsible for bovine mastitis). The objective of this study was to evaluate the cellular response by the mammary gland in lactating cows after induction of infection with *S. agalactiae* and intramammary infusion with *W. confusa* as the antibacterial treatment.

## Materials and methods

### *Ethical considerations*

The experiment was conducted at the Mario Gonzales Aranda Farm at the National University of Colombia in Palmira, Valle del Cauca, Colombia. This research was approved by the National University of Colombia, Palmira's Animal Experimentation Ethics Committee (record number 5, dated May 23, 2012). The animals were under the supervision of a veterinarian throughout the entire experiment. The use of antibiotics was not required at the end of the experiment. On day 14, cows showed normal clinical signs in the mammary glands (negative CMT test, somatic cell count < 199,526 cells/mL).

### *Type of study*

Healthy udder quarters of Hartón-del-Valle lactating cows (native Colombian breed) were the experimental units. Three conditions were used to diagnose healthy udders: physical appearance, a negative California Mastitis Test (CMT), and fewer than 200,000 somatic cells/mL count (SCC) in milk. The selected quarters were washed with potable water, dried with a towel, and disinfected with 70% (v/v) ethanol in water.

### *Intramammary infusion of *S. agalactiae**

A commercial strain of *S. agalactiae* (ATCC® 13813™) was used to induce mastitis in selected quarters. The commercial pathogen strain was reconstituted in nutrient broth (OXOID, England) and incubated at 37 °C for 24 h. Each selected quarter received 1 mL aqueous solution of *S. agalactiae* (10<sup>7</sup> colony-forming units: cfu/mL) via a 5 cm intramammary infusion through the nipple.

### *Intramammary infusion of *W. confusa**

A cryo-preserved *W. confusa* strain effective against *S. aureus* and *S. agalactiae* was used (Serna-Cock *et al.*, 2010). Bacterial growth was conducted in MRS broth (De Man *et al.*, 1960) using 1000 mL Erlenmeyer flasks (700 mL effective volume) without aeration, under continuous agitation in an orbital shaker (model 5000I, VWR, Radnor, PA, USA) set at 33 °C and 100 rpm for

4 h. In previous experiments under these conditions, the highest antimicrobial activity of *W. confusa* against *S. aureus* was observed (Serna-Cock *et al.*, 2010). The initial inoculum of *W. confusa* was 10% of the substrate volume after pH was adjusted to 6.0 using NaOH 4M. After incubation, the substrate was aseptically poured into 45 mL centrifuge sterile tubes and centrifuged for 31 min at 2860 x G (model 5804R CITI, Eppendorf, Germany). Then, the supernatant was removed and the precipitate from each tube was re-suspended in 1 mL physiological water (0.9% w/v NaCl), gently agitated, and centrifuged for 5 min at 2860 x g. The supernatant was again discarded and cells of *W. confusa* with a concentration of  $10^9$  cfu/mL were obtained. These concentrated cells were aseptically stored in 15 mL sterile tubes (5 mL effective volume) and lyophilized at -20 °C (0.120 mB absolute pressure, and -50 °C condenser temperature; LabConco, England) for later use.

To treat the udder quarters infected with *S. agalactiae*, lyophilized cells of *W. confusa* were first reconstituted. At the time of the application into the udder quarter, each tube of lyophilized cells was reconstituted in 5 mL of sterile distilled water and poured in sterile syringes. The intramammary LAB infusion was performed 24 h after the *S. agalactiae* infusion. Half of the infected quarters were randomly infused with 5 mL of the  $10^9$  cfu/mL LAB aqueous solution and half of the remaining quarters were not infused and represented the experimental control units.

#### *Milk sample collection and test determinations*

Milk samples were taken during morning hours in sterile bottles at 1, 2, 3, 4, 5, 11, and 14 d post-infusion of *S. agalactiae*. The degree of cellular response of the mammary gland was assessed by analyzing the milk samples for CMT, SCC, electrical conductivity (EC), and differential leukocyte count (polymorphonuclear neutrophils, lymphocytes, and macrophage cells).

The CMT test results were grouped according to the following classification (Contreras *et al.*, 1996): (0) absence of any variation in viscosity, no thickening of the mixture; (1) distinct thickening, no gel formation; (2) gel formation, immediate

thickening; and (3) gel formed and stacked to the paddle. The SCC was measured using an automatic cell counter (DeLaval Cell counter DCC 2828, DeLaval, Sweden). The EC was conducted using a Mastitis Detector (Milk Checker N-4 L, Oriental Instruments Co. Ltd., Japan).

The differential leukocyte count test was conducted using light microscopy observations of milk smears, stained with Wright dye and Giordano buffer (MOL LABS, Colombia) as described by Lindmark-Manson (2006), with modifications. The modified differential leukocyte technique is described as follows: 20 mL of milk was mixed with 20 mL of buffer solution (1L of di-ionized water, 7.65 g NaCl, 1.994 g  $\text{Na}_2\text{HPO}_4$ , and 0.21 g  $\text{KH}_2\text{PO}_4$ ) at pH 7.45. The milk-buffer mixture was centrifuged for 10 min at 2860 x G and 4 °C. The supernatant was discarded and the pellet was re-suspended in 1 mL buffer solution and centrifuged for 10 min at 2800 x G and 4 °C. Once again, the supernatant was discarded and the pellet re-suspended in 0.6 mL soy broth. A 0.01 mL portion of the obtained suspension was spread into a plate slide forming a thin film. The film was dried at room air conditions, methanol added (MERCK, Germany) for 5 min and then removed, dried again, and stained with Wright dye and Giordano buffer for 8 min. Distilled water was used to wash the film and 70% (v/v) ethanol in water was used to remove any excess of water. Once the film dried, each cell was differentiated by light microscopy at 100x (Gargouri *et al.*, 2008).

#### *Experimental Design*

A completely randomized 2 x 7 factorial design with three replicates was used. Factors were: infusion at two levels *S. agalactiae* (S) and *S. agalactiae* + *W. confusa* (SW) and evaluation time at seven levels 1, 2, 3, 4, 5, 11 and 14 d post-infusion of *S. agalactiae*.

The response variables were degree of cellular response of the mammary gland through California Mastitis Test (CMT), somatic cell count (SCC), electrical conductivity (EC), and differentiation of leukocytes in milk (polymorphonuclear neutrophils, lymphocytes and macrophages cells). For the SCC, a logarithmic transformation ( $\text{Log}_{10}$ ) was performed before conducting the analysis of variance.

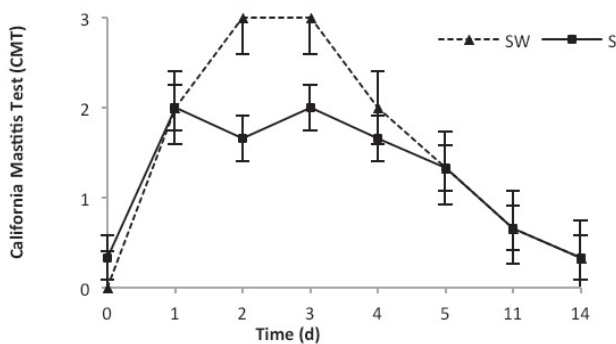


Analysis of variance tested the treatment effects. Significant differences among means were determined using the least significant difference (LSD) test. SAS software (Version 9.2; SAS Institute Inc., Cary, NC, USA) was used for statistical analysis.

## Results

### Assessment of CMT, SCC, and EC

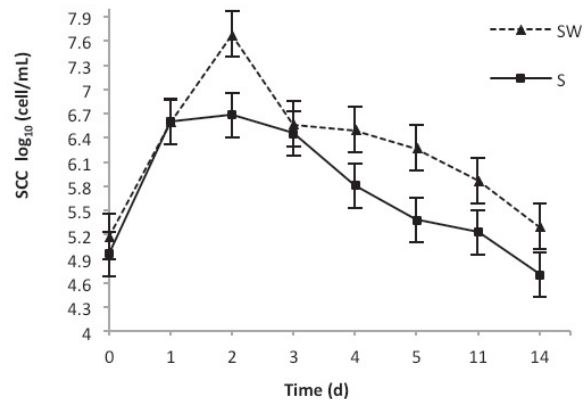
Figures 1, 2 and 3 show CMT, SCC, and EC test results of milk from quarters inoculated with *S. agalactiae* and then with *W. confusa*, respectively. Significant statistical differences were found ( $p < 0.05$ ) due to evaluation time and treatments (S vs. SW). The CMT in milk samples from the control quarters (S) showed subclinical mastitis grade 2 at 24 h post-infusion of the pathogen (Figure 1). After day 2 post-infusion, the presence of subclinical mastitis in the S quarters started decreasing until average values were 0.33 at day 14. Milk samples from the SW-treated quarters, showed mastitis grade 3 at 24 h and 48 h post-infusion of *W. confusa* (i.e., at days 2 and 3 post-infusion with *S. agalactiae*), with apparent clinical signs of inflammation. Severe induration was observed in addition to temperature changes, suggesting that *W. confusa* intensified the inflammatory response of the mammary gland. At days 4, 5, 11, and 14, this response gradually decreased (Figure 1).



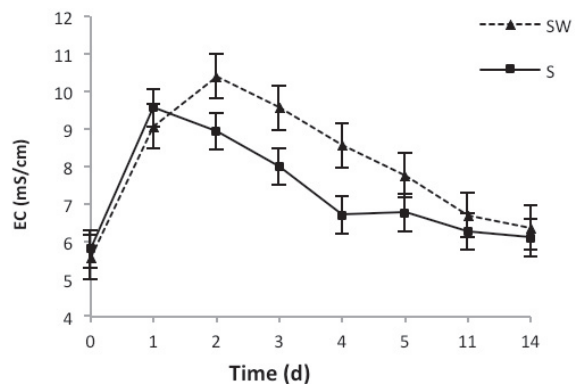
**Figure 1.** California mastitis test (CMT) of milk from bovine udder quarters treated with *S. agalactiae* (S) and *S. agalactiae* + *W. confusa* (SW). Day 0, *S. agalactiae* infusion; day 1, *W. confusa* infusion. Bars are the standard errors of the means.

After 24 h of the pathogen infusion, SCC and EC evidenced high values ( $p < 0.05$ ), indicating that the

presence of *S. agalactiae* increased cell counts and altered the normal ion composition of milk (Figures 2 and 3). EC values in milk from the S treated quarters started decreasing at day 2 post-infusion, while SCC values remained high until day 3 post-infusion. A significant reduction in cell counts was observed at day 4 ( $p < 0.05$ ), reaching normal  $\text{Log}_{10}$  SCC values of 4.7 (i.e.,  $5.0 \times 10^4$  cells/mL) at day 14 (Figure 2).



**Figure 2.** Average values of Somatic cells count (SCC). Udder bovine quarters infused with *S. agalactiae* (S) and with *S. agalactiae* + *W. confusa* (SW). Quarters with SCC values  $< 5.3$  ( $< 199,526$  cells/mL) are considered healthy and infection-free. Day 0, *S. agalactiae* infusion; day 1, *W. confusa* infusion. Bars are the standard errors of the means.



**Figure 3.** Electrical conductivity (EC) in milk. Udder bovine quarters infused with *S. agalactiae* (S) and with *S. agalactiae* + *W. confusa* (SW). Quarters with EC values  $< 6.5$  mS/cm are considered healthy and infection-free. Day 0, *S. agalactiae* infusion; day 1, *W. confusa* infusion. Bars are the standard errors of the means.

Significant differences ( $p < 0.05$ ) were found in SCC between S and SW treatments at 24 h after infusion of *W. confusa* (day 2). The highest  $\text{Log}_{10}$  SCC value of 7.68 (i.e.,  $4.7 \times 10^7$  cells/mL) corresponded to milk taken from the SW treated quarters. At day 3, no significant differences

between S and SW treated quarters were found ( $p = 0.1216$ ). However, at days 4, 5, and 11 SCC were significantly high in those quarters treated with SW ( $p = 0.0185$ ), ( $p = 0.0054$ ), and ( $p = 0.0309$ ), respectively (Figure 2). At day 14,  $\text{Log}_{10}$  SCC values of 5.3 (i.e.,  $1.9 \times 10^5$  cells/mL) were obtained in the SW treated quarters.

Significant differences ( $p < 0.05$ ) were found in EC between the S and SW treatments at 2, 3, 4, 5, and 11 days, with higher EC values in quarters treated with *W. confusa*, which suggests that milk from these quarters had a greater alteration

of sodium and chlorine ions due to the cellular response in the mammary gland.

#### Differential Leukocyte Count Determination

Table 1 shows the differential leukocyte count for the S and SW treatments. After 24 h post-infusion of *S. agalactiae*, more than 90% PMNs were found on the udder nipples. No significant differences were found in the proportions of PNMs ( $p = 0.3067$ ), lymphocytes ( $p = 0.1002$ ), and macrophage cells ( $p = 0.3422$ ) between the SW and S treated quarters.

**Table 1.** Differential leukocyte count (mean and standard deviation) in milk from bovine udder quarters treated with *S. agalactiae* (S) and *S. agalactiae* plus *W. confusa* (SW).

Day	Polymorphonuclear neutrophils		Lymphocytes		Macrophages	
	SW	S	SW	S	SW	S
0	34.0 <sup>a</sup> ± 10.1	35.7 <sup>a</sup> ± 15.0	25.0 <sup>a</sup> ± 10.1	28.0 <sup>a</sup> ± 15.0	41.0 <sup>a</sup> ± 3.6	36.3 <sup>a</sup> ± 10.0
1	93.7 <sup>b</sup> ± 3.2	90.7 <sup>b</sup> ± 3.7	1.7 <sup>b</sup> ± 0.6	2.3 <sup>b</sup> ± 2.3	4.7 <sup>b</sup> ± 3.0	7.0 <sup>b</sup> ± 3.0
2	90.7 <sup>b</sup> ± 2.0	88.0 <sup>b</sup> ± 8.1	3.3 <sup>b</sup> ± 1.1	4.7 <sup>bc</sup> ± 4.7	5.3 <sup>b</sup> ± 2.5	7.3 <sup>b</sup> ± 7.5
3	81.0 <sup>c</sup> ± 1.7	87.0 <sup>b</sup> ± 1.0	7.7 <sup>bc</sup> ± 3.2	6.3 <sup>bc</sup> ± 4.1	11.3 <sup>c</sup> ± 1.5	6.7 <sup>b</sup> ± 4.5
4	73.0 <sup>cd</sup> ± 5.5	72.7 <sup>c</sup> ± 7.5	15.0 <sup>c</sup> ± 4.9	6.7 <sup>bc</sup> ± 2.8	12.0 <sup>c</sup> ± 2.0	20.7 <sup>a</sup> ± 4.6
5	72.0 <sup>d</sup> ± 0.5	70.7 <sup>c</sup> ± 10.0	15.0 <sup>c</sup> ± 4.9	12.7 <sup>c</sup> ± 8.5	12.0 <sup>c</sup> ± 2.0	15.7 <sup>a</sup> ± 4.6
11	55.3 <sup>e</sup> ± 7.2	58.3 <sup>d</sup> ± 2.8	24.3 <sup>a</sup> ± 4.5	22.7 <sup>a</sup> ± 4.1	20.3 <sup>d</sup> ± 4.0	19.0 <sup>a</sup> ± 3.0
14	42.7 <sup>f</sup> ± 7.7	65.7 <sup>e</sup> ± 1.2	28.7 <sup>a</sup> ± 2.5	16.7 <sup>a</sup> ± 9.0	28.7 <sup>e</sup> ± 2.8	17.7 <sup>a</sup> ± 7.5

Day 0, *S. agalactiae* infusion; day 1, *W. confusa* infusion. Means within each column with different letters are significantly different ( $p < 0.05$ ).

## Discussion

Previous *in vitro* studies demonstrated the effectiveness of LAB against *S. aureus* and *S. agalactiae*. Based on CMT, SCC, and EC results, it is suggested that *W. confusa* generates a high and rapid cellular response in the mammary gland. The quarters infused with *W. confusa* showed greater changes when compared to those infected with *S. agalactiae*. The results also showed evidence of clinical mastitis (severe induration, local fever, and clot formations in milk).

Greene *et al.* (1991) compared the effects of treating subclinical mastitis with intramammary infusions of *Lactobacillus* or an antibiotic preparation on cure rates and milk SCC. They

warned that the administration of *Lactobacillus* or antibiotic treatment to all quarters based on elevated composite SCC should not be adopted. *Lactobacillus* increased SCC with no effect on the infection rate. Similar results (i.e., increase in SCC) were found in this study with LAB *W. confusa*.

According to our PMNs, lymphocytes, and macrophages results, the cellular response was the same in both S and SW quarters. More studies are needed to better understand cellular response in the mammary gland after infusing *W. confusa*. It is important to identify and differentiate how cell activation occurs, or to identify the type of pro-inflammatory cytokines that are activated in the mammary gland in the presence of *W. confusa*.

Studies with LAB *Lactococcus lactis* DPC 3147 on immune response after mammary gland infusion with a probiotic culture showed that the greatest increase in immune gene expression was observed in interleukin (IL)-1 $\beta$  and IL-8, which corresponded with peaks in somatic cell counts (Beecher *et al.*, 2009). In this study, proportions of neutrophils, lymphocytes and macrophages were assessed in milk; therefore, more studies are needed to identify and differentiate the specific type of lymphocytes present in both S and SW quarters.

The mammary gland response is specific for each bacterial pathogen. Soltys and Quinn (1999) reported that there was a difference in the type of cells present in milk depending on the specific mastitis-causing pathogen. These authors found that *S. aureus* prompted a strong T CD4<sup>+</sup> lymphocyte response, whereas *Streptococcus* spp. prompted a CD4<sup>+</sup> and CD8<sup>+</sup> lymphocyte response.

According to these results, further research should be conducted to elucidate (a) the ability of *W. confusa* to adhere to the epithelial cells of the teat canal, (b) the period of time the strain remains in the teat canal, (c) canal and cistern histology post inoculation, and (d) the activation mechanism of somatic cells in the mammary gland post inoculation. These further studies will determine whether *W. confusa* should be included or excluded in non-antibiotic formulations for the prevention and treatment of bovine mastitis.

Infusion of LAB *W. confusa* in bovine mammary gland previously infected with *S. agalactiae* produced further inflammation, high CMT, EC, and high SCC as a result of a high proportion of PMNs when compared to mammary glands where only the pathogen was present. Results indicate that *W. confusa* infusion, as a treatment for controlling *S. agalactiae*, should not be adopted. However, the activation mechanism of somatic cells in the mammary gland needs to be elucidated.

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