



Title	Systemic and local bactericidal potentiality in late lactation Holstein-Friesian cows following a combined antibiotics and Enterococcus faecium SF68 dry-cow treatment
Author(s)	Tiantong, Attapol; Piamya, Piya; Chen, Shuen-Ei; Liu, Wen-Bor; Chang, Fang-Yu; Lin, Pei-Chi; Nagahata, Hajime; Chang, Chai-Ju
Citation	Japanese Journal of Veterinary Research, 63(3), 139-150
Issue Date	2015-08
DOI	10.14943/jjvr.63.3.139
Doc URL	http://hdl.handle.net/2115/59878
Type	bulletin (article)
File Information	JJVR63-3_p.139-150.pdf



[Instructions for use](#)

Systemic and local bactericidal potentiality in late lactation Holstein-Friesian cows following a combined antibiotics and *Enterococcus faecium* SF68 dry-cow treatment

Attapol Tiantong¹⁾, Piya Piamya¹⁾, Shuen-Ei Chen¹⁾, Wen-Bor Liu¹⁾, Fang-Yu Chang¹⁾, Pei-Chi Lin¹⁾, Hajime Nagahata²⁾ and Chai-Ju Chang^{1,*)}

¹⁾Department of Animal Science, National Chung Hsing University, Taichung 402, Taiwan

²⁾Department of Animal Health, School of Veterinary Medicine, RakunoGakuen University, Ebetsu, Hokkaido 069-8501, Japan

Received for publication, March 10, 2015; accepted, July 23, 2015

Abstract

Antibiotic dry-cow treatment contributes a major part to the total use of antibiotics in dairy herds. *Enterococcus faecium* strain SF68 (SF68) was of human origin but has been authorized in EU as probiotic feed additive. In the present study, one of the front and rear quarters of twelve late lactation Holstein-Friesian cows were infused once with a commercial antibiotic dry-cow formula (antibiotics quarter) on the first milk-stasis day (d 1), when the contralateral quarters were infused with 5×10^8 -CFU SF68 plus half-dose antibiotic dry-cow formula (SF68/antibiotics quarter) meanwhile. Gelatinase level and cellular reactive oxygen species (ROS) production capacity were measured for blood and quarter secretion. The results showed that the count of blood total leukocytes minorly decreased on d 3 only but the microscopic somatic cell count (MSCC) continuously increased up to d 7, especially in SF68/antibiotics quarters. Plasma level of gelatinase A remained similar up to d 7 but gelatinase B was not detectable in plasma throughout the study. The level of gelatinase A in quarter secretion increased up to d 7 but gelatinase B increased even more drastically, especially in SF68/antibiotics quarters. The ROS production capacity of blood leukocytes increased temporarily only on d 3, but that of milk cells continuously increased up to d 7, especially in SF68/antitiotics quarters. Overall, late lactation Holstein-Friesian cows were systemically adaptable to the combined antibiotics and SF68 dry-cow treatment, while the local bactericidal potentiality in mammary gland was actively responsive to additional SF68 intramammary treatment.

Key Words: *Enterococcus faecium* SF68, Holstein-Friesian cows, Dry-cow treatment, Gelatinase, ROS

*Corresponding author: Chai Ju Chang, Department of Animal Science, National Chung Hsing University, Taichung 402, Taiwan

Phone: +886-4-22874512. Fax: +886-4-22860265. E-mail: crchang@mail.nchu.edu.tw

doi: 10.14943/jjvr.63.3.139

Introduction

Phagocytic cells, notably neutrophils in circulation and macrophages in infected tissues, are armed with an array of highly microbicidal weapons such as proteolytic enzymes and free radicals. Microbial pathogens are taken up into the phagosome of phagocytes and into which microbicidal agents are released. Gelatinase, elastase, cathepsin G, and myeloperoxidase are contained in the azurophilic and/or specific granules of neutrophils which fuse with the phagosome to perform the intracellular degradation of microbes³. Degranulation in neutrophils accounts for the rapid elevation of gelatinase B level within 15–30 min exposure to TNF- α , IL-8, or G-CSF³⁰. Engulfed microbes could also be destroyed in phagosome by the reactive oxygen species (ROS) produced from the NADPH oxidase^{20,21,23} through reducing oxygen molecules. The bactericidal activity of ROS is related to their reactions with unsaturated carbon bonds that may lead to toxic lipid peroxidation, sulphhydryl groups that may lead to the inactivation of sulphhydryl containing enzymes, amino group and possibly peptide bonds that may lead to their breakage, as well as the destruction of nucleic acids. The capacities of degranulation and ROS production represent the most powerful efferent arms of innate immune systems for further cascades of innate and adaptive immune responses.

Probiotic bacteria could elicit moderate systemic immune responses in human and animals through gut-associated immune system^{2,11,29,37} where the probiotic bacteria or their fragments passed the epithelial cells of the Peyer's patches in a paracellular way and activated the surveillance immune cells¹⁸. The beneficial effects of probiotic bacteria on both systemic and local immune systems support their use in improving animal health and as an alternative to antibiotics in protection against infectious agents. Most of the probiotics applied to animals are members of the indigenous microbiota of host

intestine. *Enterococcus faecium* strain SF68 (SF68) is a gram-positive endospore-forming bacteria originally isolated from a healthy Swedish baby, but its dietary supplementation benefited the general immunity of pets^{6,38,40} and pigs^{4,34,35} and has been authorized as one of the probiotic feed additives in EU.

The practice of a dry period in late lactation has increased the risk of mastitis in dairy cows²¹. Consequently, systematic drying-off with antibiotics has become the major part of the total use of antibiotics in dairy herds in many areas of the world including Taiwan. The efficacy of intramammary probiotic application in the prevention and treatment of mastitis so far has been investigated for bovine udder-derived *Lactococcus* and *Lactobacillus* species^{1,8,10,16}. Despite exogenous to bovine, SF68 dry-cow treatment has expressed potential local benefits to Holstein-Friesian cows^{25,39}. The purpose of the current study was to further evaluate the systemic immune status, in addition to local immune status, of late lactation cows following a SF68 dry-cow treatment. We estimated the gelatinase level of plasma and mammary secretion and the ROS production capacity of blood leukocytes and milk cells as indicators of the general bactericidal potential of cows.

Materials and Methods

Experimental cows: Twelve Holstein-Friesian cows over 220 day-in-milk, between 32 and 56 mo in age, less than 10 kg/d in milk yield, and within the last two months of the second or third pregnancy were selected from the dairy farm of National Chung Hsing University (Taichung, Taiwan). All experimental cows have complete DHI production records and were free of previous mastitis or other health problems. They were maintained on a commercial dry-cow ration (3000 kcal/kg metabolic energy, 16% crude protein) (Lee Han Co. Ltd., Kao Hsiung, Taiwan) with free accessible pangola hay and water since

one wk prior to drying-off and throughout the entire dry period. The experimental treatment and sampling protocols were approved by the committee of Care and Use of Agriculture Animals of National Chung Hsing University.

Preparation of SF68 dry-cow solution: The current SF68 dosage was 2×10^8 CFU/ml with an infusion volume of 2.5 ml per quarter, equivalency of a 5×10^8 CFU SF68 per quarter. This SF68 dosage was 2.5-fold of that used by Peng *et al.*²⁵⁾ and Tiantong *et al.*³⁹⁾ with the intend to exaggerate possible effects. The SF68 working solution was diluted from the stock solution of 5×10^8 CFU/ml prepared by dissolving the commercial micro-solid SF68 product (1×10^9 CFU/g, Cylactin_ME10; Cerbios-Pharma, Barbengo, Switzerland) in 20-ml endotoxin-free PBS (Sigma-Aldrich, St. Louis MO, USA). A 20-min intermittent ultrasonication (Vibra cell, Model VC 50T, Sonics & Materials Inc.) was then applied under ice bath to the stock solution before further dilution to 50 ml with endotoxin-free PBS. Syringes (2.5 ml) were loaded with the SF68 working solution and stored under -20°C until use. Same batch of SF68 working solution was used for all experimental cows.

Dry-cow treatment: A balanced dry-cow treatment was applied across the four quarters of each experimental cow to reduce the error related to individual variation. Dry-cow treatment was performed immediately following the final morning milking. One of the front and rear quarters were randomly assigned to a full dose of commercial antibiotic dry-cow formula (antibiotics quarter), while the contralateral quarters were assigned to the SF68 dry-cow treatment supplemented with half-dose of commercial antibiotics dry-cow formula (SF68/antibiotics quarter). Quarters were firstly disinfected externally with 75% ethanol, then a blunt-end cannula (length 11/3", J-12; Jorgenson Laboratories, Inc. Loveland, CO, USA) was fitted to the teat opening, and either the Cepravin Dry

Cow (250 mg, Intramammary Suspension, Schering Plough Animal Health Ltd., Uxbridge, UK) or a syringe of SF68 working solution was infused accompanying with upward massages on the exterior of quarters. An additional half-dose of Cepravin Dry Cow was immediately supplemented to the quarters just infused with SF68 working solution.

Sampling: Blood samples (10 ml) were collected from tail vein immediately prior to dry-cow treatment (d 1 in experiment), and same time of the day on d 3 and d 7 in experiment. Meanwhile, samples (25 ml) of mammary secretion were manually collected from the individual quarters of each cow and those from the two SF68/antibiotics quarters and the two antibiotics quarters were pooled, respectively, within cows before further processing.

Sample preparation: Blood samples in heparinized tubes were centrifuged by $1000 \times g$ at 4°C for 30 min to separate the plasma and the particulate fraction. Plasma was stored in aliquots under -20°C for assays within two mo. Red blood cells in the particulate fraction was lysed (BioLegend, San Diego, Ca, USA) and the remaining leukocytes were washed with ice cold Dulbecco's PBS (DPBS, without $\text{Ca}^{+2}/\text{Mg}^{+2}$; Sigma-Aldrich), and then suspended in Hanks' balanced salt solution (HBSS, enriched with 0.25 mM $\text{Ca}^{+2}/\text{Mg}^{+2}$; Sigma-Aldrich). The HBSS leukocyte suspension was enumerated under microscope, and the volume of HBSS suspension necessary for immediate assay of ROS production capacity of blood leukocytes was calculated.

Quarter secretion was processed following that of Peng *et al.*²⁵⁾ by firstly skimming by $400 \times g$ at 4°C for 20 min to recover the clear supernatant and then the supernatant was stored in aliquots under -20°C for assays within two mo. The bottom pellet was washed three times with DPBS, reconstituted in HBSS, and enumerated under microscope. The obtained microscopic somatic cell count (MSCC) was used

to calculate the volume of HBSS suspension necessary for immediate assay of ROS production capacity of milk cells.

The protein content of plasma and skimmed supernatant of mammary secretion were determined using a dye-binding reagent (Bio-Rad Laboratories, Hercules, CA, USA) in a microplate format (Multiskan Ascent, ThermoLabsystems, Helsinki, Finland) and were used to calculate the volume necessary for gelatin zymography.

Gelatin zymography for gelatinase assay: Gelatin zymography was a derivation of native SDS-PAGE in which gelatin was incorporated into the resolving gel as a substrate. Gelatinases A and B were identified based on molecular size of the digested band after overnight gelatinolysis at 37°C^{28,30,39,42} and gelatinase level was expressed as the area of the digested band after scanning. In brief, aliquot of plasma or skimmed quarter secretion containing 20-µg protein was first mixed (1 : 1) with a sample buffer of pH 6.8 consisting 62.5 mM Tris-HCl, 25% glycerol, 4% SDS, and 0.01% bromophenol blue (Bio Rad), and then was subjected to 7.5% native SDS-PAGE (Minigel, Bio-Rad)¹⁷ containing 0.1% bovine gelatin (Sigma-Aldrich) in the resolving gel. Afterwards, the gels were incubated in the renaturing solution of 2.5% (v/v) Triton-X100 at room temperature for 30 min, fully dripped with distilled H₂O, and then incubated overnight at 37°C in 50-mM Tris-base buffer, pH 7.4, containing 200-mM NaCl, 0.02%-Brij-35, and 5-mM CaCl₂ for gelatinolysis. The next day, gels were stained for 30 min with 0.1% coomassie blue R-250 (Sigma-Aldrich) in methanol/glacial acetic acid/distilled water (400/100/500), then destained for 30 min in methanol/glacial acetic acid/distilled water (300/75/625), the destaining solution was replaced and destained for another 30 min, and finally preserved in distilled water. Gelatinases were visualized as clear bands against the blue background. The level of gelatinase was expressed as area of the band captured using Epson Stylus TX130 (Seiko Epson Corporation, Nagano, Japan) and integrated

using software Image J, version 1.45 (National Institutes of Health, 2013) (Bethesda, MA, USA). Plasma of clinically healthy volunteers was used as a reference¹⁹ and for normalization, samples collected at different experimental days from the same cow were resolved on the same gel in parallel with the reference.

Chemiluminescence assay for the ROS production capacity of cells: The phorbol 12-myristate 13-acetate (PMA)-stimulated, luminol-enhanced chemiluminescence assay^{21,25,41} was an *ex vivo* assay for the ROS production capacity of cells. In brief, an aliquot of fresh HBSS cell suspension containing 2×10^6 blood leukocytes or milk cells was first maintained at 37°C for 10 min, then 300-µl of 1 mM luminol (Sigma-Aldrich) in 0.1 M DMSO/0.05 M Na₂CO₃/1.5 mM CuSO₄ was added. The chemiluminescence assay was initiated by inclusion 50 µl of the PMA working solution (1 µg/ml) diluted from the PMA stock solution (200 µg/ml DMSO) with HBSS, and then the reaction volume was made up to 1000 µl with HBSS. The emission counts per second (CPS) was recorded instantly every min for 20 min in luminometer (Triathler, type 425-014, Hidex, Turku, Finland). The ROS production capacity of cells was defined as the net response to *ex vivo* PMA stimulation which was expressed as the area under CPS-time curve (AUC) after adjusting for the starting CPS value.

Statistical analyses: Data was analyzed using the GLM procedure and Duncan's new multiple range test³³ with days in experiment and dry-cow treatments as the major factors. All results were presented as mean and SE.

Results

Clinical observation

Fever, redness, swollness, or pain was not observed in experimental cows during the one-week experimental period, except on d 3 when the

secretion from SF68/antibiotics quarters appeared slightly yellowish and colloidal in contrast to the normal whitish and watery secretion from antibiotics quarters. But on d 7, secretions from all quarters appeared normal again.

Blood total leukocyte count and MSCC

The means and SE of blood total leukocyte count (Fig. 1A) and MSCC of quarter secretion (Fig. 1B) were shown. Blood total leukocyte counts were not significantly different ($P > 0.05$) among d 1, d 3, and d 7 despite the slightly lower counts on d 3 compared to d 1 and d 7. The MSCC in secretions from both antibiotics and SF68/antibiotics quarters were significantly ($P < 0.05$) higher on d 3 than on d 1. Furthermore, the MSCC of secretions from antibiotics quarters was significantly ($P < 0.05$) higher on d 7 than on d 3, which was not observed for SF68/antibiotics quarters. On the other hand, the MSCC of secretion on d 1 were not significantly different ($P > 0.05$) between antibiotics and SF68/antibiotics quarters, but the MSCC of secretions from SF68/antibiotics quarters were significantly ($P < 0.05$) higher on both d 3 and d 7 compared to that of antibiotics quarters.

Gelatinase level in plasma and quarter secretion

Representative gelatin zymogram of plasma and skimmed quarter secretion supernatant was shown, respectively, in Fig. 2. The plasma zymogram (Fig. 2A) was absent in gelatinase B during the experimental period, whereas both gelatinases A and B were detectable in the secretion zymogram for antibiotics and SF68/antibiotics quarters despite of the low level on d 1 (Fig. 2B).

The means and SE of gelatinase levels in plasma indicated similar ($P > 0.05$) gelatinase A level and no gelatinase B during the experimental period (Fig. 2A).

The means of gelatinase A level in secretions from both antibiotics and SF68/antibiotics quarters were significantly ($P < 0.05$) higher on d 3 than on d 1 and higher on d 7 than on d 3 and d 1. On the other hand, the level of gelatinase A in

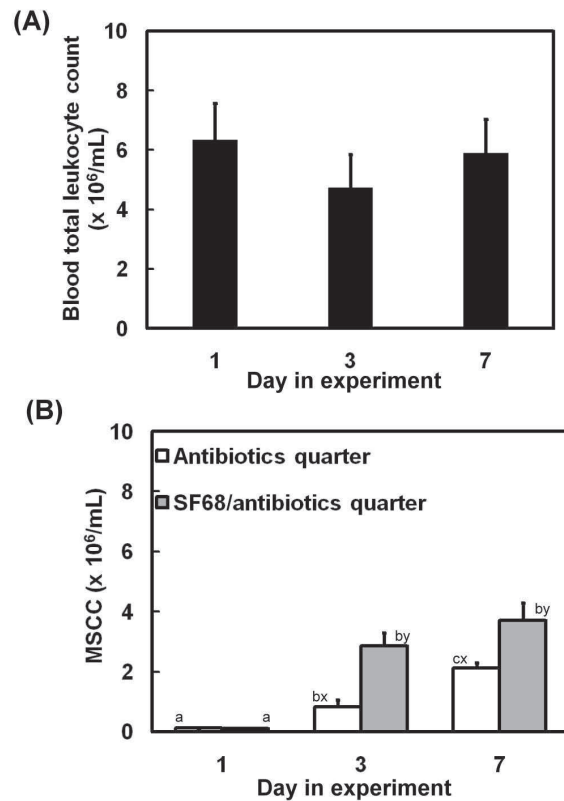


Fig. 1. The means and SE of blood total leukocyte count (A) and microscopic somatic cell count (MSCC) of quarter secretion (B) for late lactation Holstein cows received antibiotics and SF68 dry-cow treatment. Experimental cows received dry-cow treatment on the first milk stasis day (d 1) when antibiotics quarters received one dose of Cefravin Dry Cow (250 mg, Intramammary Suspension, Schering Plough Animal Health Ltd., Uxbridge, UK) and SF68/antibiotics quarter received 5×10^8 cfu-SF68 plus half-dose Cefravin Dry Cow.

^{a,b,c} Values with different superscripts differ significantly ($P < 0.05$) among days in experiment within the same quarter treatment.

^{x,y} Values with different superscripts differ significantly ($P < 0.05$) between antibiotics and SF68/antibiotics quarters within the same day in experiment.

secretions were not significantly ($P > 0.05$) different between antibiotics and SF68/antibiotics quarters during the experimental period (left panel, Fig. 2B)

The means of gelatinase B level in secretions from both antibiotics and SF68/antibiotics quarters were significantly ($P < 0.05$) higher on d 3 than on d 1, but only in antibiotics quarters was the level of gelatinase B in secretion higher ($P < 0.05$) on d 7 than on d 3 and d 1. On the other hand,

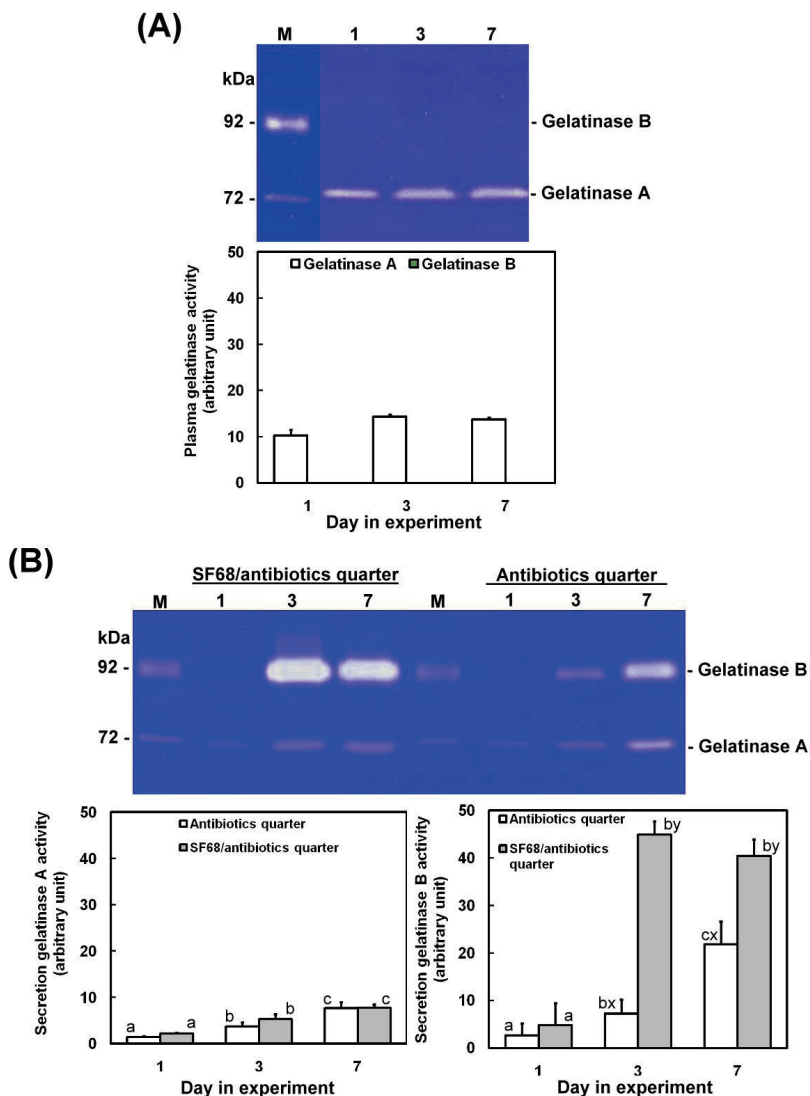


Fig. 2. Typical gelatin zymogram and the means and SE of gelatinase level in plasma (A) and quarter secretion (B) for late lactation Holstein cows following antibiotics and SF68 dry-cow treatment. Experimental cows received dry-cow treatment on the first milk stasis day (d 1) when antibiotics quarters received one dose of Cephadrine Dry Cow (250 mg, Intramammary Suspension, Schering Plough Animal Health Ltd., Uxbridge, UK) and SF68/antibiotics quarter received 5×10^8 cfu-SF68 plus half-dose Cephadrine Dry Cow.

^{a,b,c} Values with different superscripts differ significantly ($P < 0.05$) among days in experiment within the same quarter treatment.

^{x,y} Values with different superscripts differ significantly ($P < 0.05$) between antibiotics and SF68/antibiotics quarters within the same day in experiment.

the level of gelatinase B in secretions from SF68/antibiotics quarters were significantly ($P < 0.05$) higher on d 3 and d 7 compared to antibiotics quarters (right panel, Fig. 2B).

ROS production capacity of blood leukocytes and milk cells

The means and SE of the CPS time curves of

2-million blood leukocytes and milk cells were displayed in Fig. 3A. Different CPS scales were used for blood leukocytes and milks cells due to the much greater peak CPS value of blood leukocytes on d 3, compared to the relatively lower peak CPS value of milk cells during the experimental period. The CPS value at 1 min recorded for milk cells from SF68/antibiotics

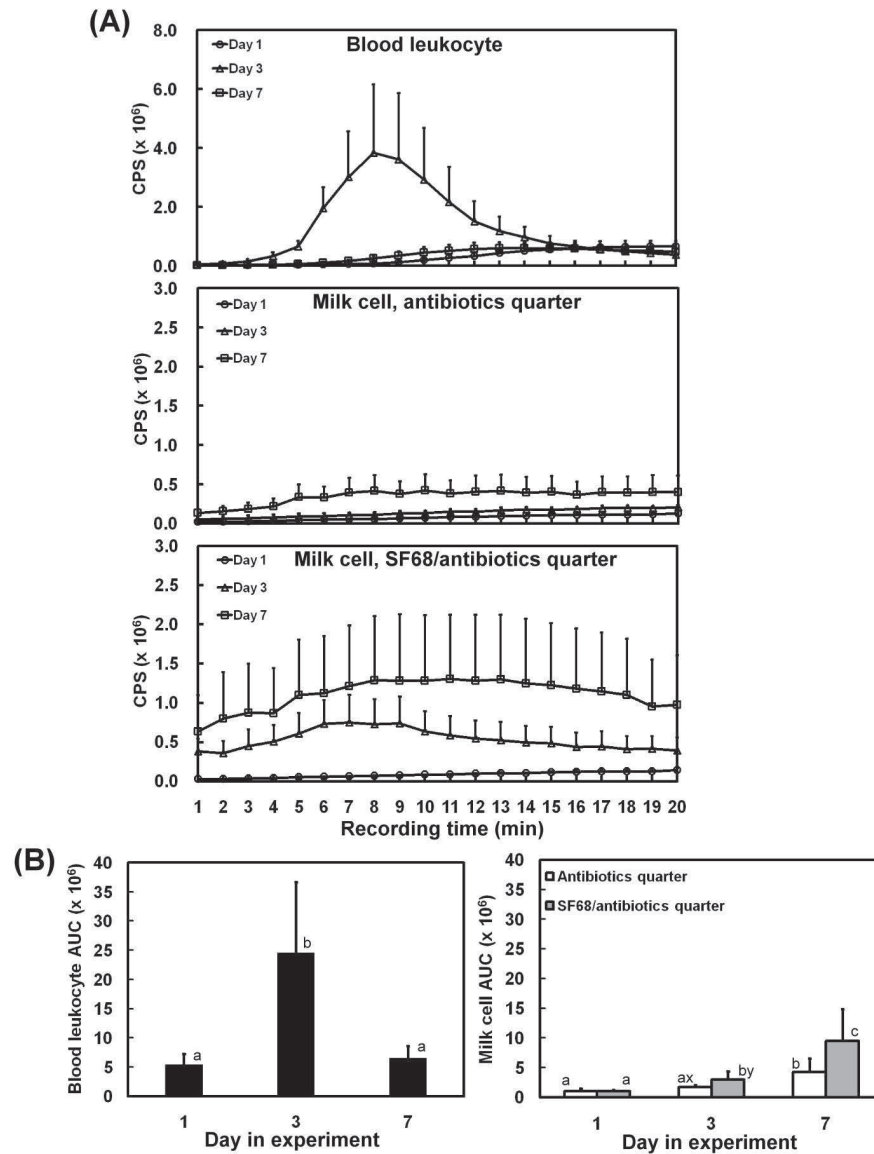


Fig. 3. The means with SE of counts per second (CPS) time curves (A) and the corresponding area under the CPS time curve (AUC) after adjusting for the different starting CPS value at 1 min (B) for blood leukocytes and milk cells from quarters of late lactation Holstein cows following antibiotics and SF68 dry-cow treatment. Experimental cows received dry-cow treatment on the first milk stasis day (d 1) when antibiotics quarters received one dose of Cepravin Dry Cow (250 mg, Intramammary Suspension, Schering Plough Animal Health Ltd., Uxbridge, UK) and SF68/antibiotics quarter received 5×10^8 cfu-SF68 plus half-dose Cepravin Dry Cow.

^{a,b,c} Values with different superscripts differ significantly ($P < 0.05$) among days in experiment within the same quarter treatment.

^{x,y} Values with different superscripts differ significantly ($P < 0.05$) between antibiotics and SF68/antibiotics quarters within the same day in experiment.

quarters tended to be higher on d 3 and d 7 than on d 1, but less so for blood leukocytes or milk cells from antibiotics quarters.

The means of adjusted AUC for blood leukocytes were significantly ($P < 0.05$) greater on

d 3 than on d 1 and d 7 but that on d 7 was not significantly ($P > 0.05$) different from d 1 (left panel, Fig. 3B). The means of adjusted AUC for milk cells were significantly ($P < 0.05$) greater on d 7 than on d 3 in both antibiotics/SF68/

antibiotics quarters, but the means of adjusted AUC for milk cells were significantly ($P < 0.05$) greater on d 3 than on d 1 only in SF68/antibiotics quarters. On the other hand, the means of adjusted AUC for milk cells were not different ($P > 0.05$) on d 1 and d 7 between antibiotics and SF68/antibiotics quarters, but the means of adjusted AUC for milk cells was significantly greater ($P < 0.05$) on d 3 for SF68/antibiotics quarters than for antibiotics quarters.

Discussion

We have previously reported potential benefits of SF68 dry-cow treatment in local innate immune function²⁵⁾ and the involution of dairy cows³⁹⁾. The current study further examined the systemic immune status, in addition to local immune status, of late lactation Holstein-Friesian cows during the week following a combined antibiotics and SF68 dry-cow treatment. Our study was the first to examine the overall tolerance of dairy cows towards intramammary probiotics treatment and we found that intramammary probiotics treatment seemed elicit differential bactericidal potentialities in systemic and local systems and there were some likely mutual interactions.

We firstly noticed a seemingly reversal change in cell counts between blood leukocytes and milk cells (Fig. 1) and similar observation has been reported for the model of LPS-challenged cows²⁰⁾. Mehrzad *et al.*²⁰⁾ interperlated this phenomenon as the result of massive influx of circulating leukocytes into mammary gland. It is generally aware that the production and destruction of circulatory leukocytes is tightly regulated so that their counts in blood could be maintained in a relatively narrow range. The blood total leukocyte counts of healthy adult bovine range from 4.5 to 10/ml, more than 60% being neutrophils^{20,21)}. The mean total leukocyte count of our experimental cows was within this normal range, although that on d 3 was at the

lower edge (Fig. 1A). Neutrophils are the most actively extravasated phagocytic cells^{5,21,24)} due to its multilobulated nucleus that could line up in a thin line, permitting rapid migration between endothelial cells. Other leukocytes, on the other hand, are more difficult to migrate between endothelial cells because of the large, horseshoe shaped nucleus. Neutrophils also dynamically diapedesis across the blood-milk barrier²⁷⁾ and the prevalence of neutrophils in somatic cells of dairy cows increased faster than other cell types during mammary gland inflammation and early dry period^{26,41)}. Similar mechanism might have occurred following the current intramammary SF68 infusion. Chemotactic factors, endogenous or exogenous, are, at least partly, responsible to the continuous influx of immune cells into mammary gland. Nevertheless, although the presence of chemotactic factors in inflammatory mammary gland is indisputable²⁷⁾, the presence of strong chemotactic factors in non-inflamed udders, such as in our study, is still disputable.

Secondly, stable but low level of plasma gelatinase A was observed throughout the current experimental period (Fig. 2A), in contrast to the extremely high gelatinase B level in SF68/antibiotics quarters (Fig. 2B). Systemic and local gelatinase level has been used as a supportive diagnostic tool in human diseases³⁰⁾, so as in horse arthritis¹⁵⁾ and cow mastitis³²⁾. Plasma gelatinases A is recognized mainly derived from stromal cells such as fibroblasts, endothelial cells, and osteoblasts¹²⁾ and plasma gelatinase B mainly from polymorphonuclear leukocytes⁹⁾. As for the source of milk gelatinase, results of immunohistochemical methodology has found high expression of gelatinase A, besides MMP-1, MMP-14, MMP-19, TIMP-1 and TIMP-2, in involuting mammary gland compared to lactating mammary gland, while no expression of gelatinase B was found in mammary gland at either stage³¹⁾. The observed high gelatinase B level in the mammary secretions of our study was apparently not synthesized by mammary tissue. We have previously reported a close association between

milk gelatinase B level and MSCC^{7,39)} and suggested that the high gelatinase B level in dry secretion was not because of the concentrating effect associated with yield reduction but was mainly related to increased neutrophil influx and degranulation. Much higher gelatinase B level in SF68/antibiotics quarters compared to antibiotics quarters is presumably related to the faster neutrophil influx and, consequently, degranulation following SF68 infusion. We considered this higher milk gelatinase level advantageous for involution cows in terms of both defense and remodeling of mammary gland.

Chemiluminescence assay has indicated decreasing functionality of bovine blood leukocytes during transition from pregnancy to lactation¹⁴⁾ and during coliform mastitis¹³⁾. Also, neutrophils isolated from infected cow quarters had lower chemiluminescence response than that from uninfected quarters²⁰⁾. Therefore, PMA-stimulated chemiluminescence assay is a reliable tool that could estimate in real-time the defense function of phagocytes. In the current study, we noticed transitory, compared to the LPS-challenged cow model²⁰⁾, elevation of the ROS production capacity in blood leukocytes on d 3 (Fig. 3). We suggest this observation evident of physiological response compared to the more pathological response in cows received LPS challenge. Based on the AUC results, we were able to confirm that milk neutrophils were less efficient in ROS production than their blood counterparts, besides other phagocytic activity^{20,22)}. For milk cells, we noticed apparently higher CPS value at 1 min on d 3 and d 7 in relative to d 1, especially for SF68/antibiotics quarters. Although no statistical test was applied to individual recording time, we suspect that milk cells in SF68/antibiotics quarter might have been pre-activated by SF68 before the *ex vivo* PMA-stimulated luminol-enhanced ROS production measurement. Therefore, in our AUC calculation, the different CPS starting values were adjusted by zeroing and thus obtained adjusted AUC represented only the part of ROS production that was stimulated by PMA.

We found generally greater ROS production by milk cells from SF68/antibiotics quarter than those from antibiotics quarters (Fig. 3B), suggesting that SF68 treatment might have primed the milk cells. On the other hand, as the ROS production by milks cells from antibiotics quarters also slightly increased during the experimental period, it is likely that milk somatic cells might be spontaneously activated during dry period, likely by the presence of milk components. We also noticed apparently greater variations in the ROS production of milk cells from SF68/antibiotics quarters than antibiotics quarters and we regard this a confirmation that there is great individual difference in the immune responses to foreign objects.

The blind alveolar architecture of mammary gland is different from the tube-like intestines. We propose that, following milk stasis and intramammary SF68 infusion, the transmigration of circulatory neutrophils into mammary lumen is facilitated by the leakyness of barrier resulting from both pressure build-up and gelatinase B degradation. As neutrophil influx and gelatinase B degradation perpetuate, cytokines are also released, and some probiotic bacteria and their fragments, together with cytokines, pass through the paracellular space. The neighboring surveillance plasma cells are eventually activated, thereby, more cytokines are released, and they are presumably the biological signals that modify the systemic immune status (Fig. 4).

In conclusion, we used the level of gelatinase in plasma and quarter secretion and the ROS production capacity of blood leukocytes and milk cells as a measure of the bactericidal potentialities of systemic and local mammary gland in responsive to SF68 dry-cow treatment. Our observations suggest that SF68 dry-cow treatment seems to be systemically tolerable to late lactation Holstein-Friesian cows when the local mammary gland were relatively more responsive to it in terms of bactericidal potentiality.

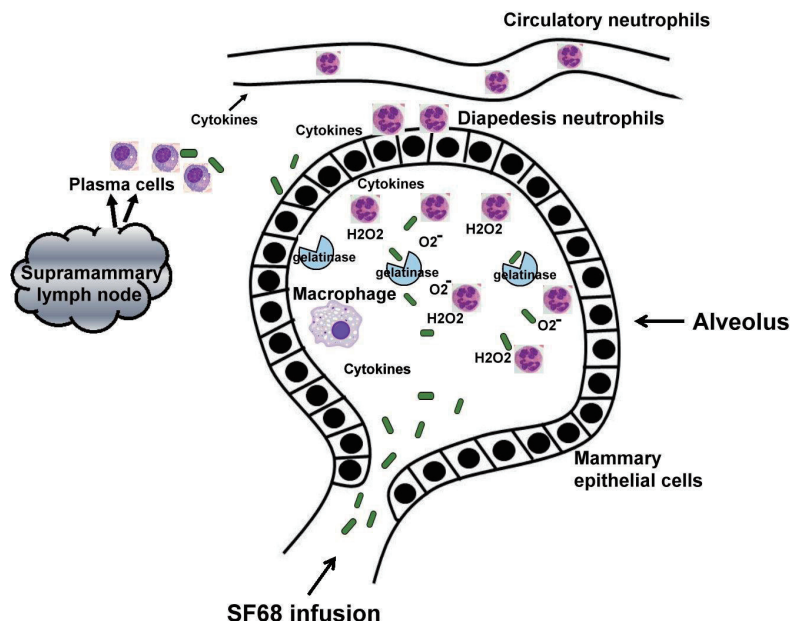


Fig. 4. The proposed pathways for SF68 dry-cow treatment-mediated local and systemic immune reactions. Following milk stasis and intramammary SF68 infusion, the transmigration of circulatory neutrophils into mammary lumen is facilitated by the leakiness of barrier resulting from both pressure build-up and gelatinase B degradation. As neutrophil influx and degradation perpetuate, cytokines are released, and some probiotic bacteria and their fragments, together with cytokines, pass through the paracellular space. The neighboring surveillance plasma cells are eventually activated, thereby, more cytokines are released, and they are presumably the biological signals that modify the systemic immune status.

Acknowledgements

The authors are thankful to National Chung Hsing University for funding this study and to the personnel in dairy farm of National Chung Hsing University for helping collect the samples that made this study possible. The authors owe to Dr. Stanley Guan for his devoted English proofreading.

References

- 1) Beecher, C., Daly, M., Berry D. P., Klostermann, K., Flynn, J. and Meaney, W. 2009. Administration of a live culture of *Lactococcus lactis* DPC 3147 into the bovine mammary gland stimulates the local host immune response, particularly IL-1 β and IL-8 gene expression. *J. Dairy Res.*, **76**: 340-348.
- 2) Braat, H., van den Brande, J., van Tol, E., Hommes, D., Peppelenbosch, M. and van Deventer, S. 2004. *Lactobacillus rhamnosus* induces peripheral hyporesponsiveness in stimulated CD4⁺ T cells via modulation of dendritic cell function. *Am. J. Clin. Nutr.*, **80**: 1618-1625.
- 3) Borregaard, N. and Cowland, J. B. 1997. Granules of the human neutrophilic polymorphonuclear leukocyte. *Blood*, **89**: 3503-3521.
- 4) Broom, L. J., Miller, H. M., Kerr, K. G. and Knapp, J. S. 2006. Effects of zinc oxide and *Enterococcus faecium* Sf68 dietary supplementation on the performance, intestinal microbiota and immune status of weaned piglets. *Res. Vet. Sci.*, **80**: 45-54.
- 5) Burvenich, C., van Merris, V., Mehrzad, J., Diez-Fraile, A. and Duchateau, L. 2003. Severity of *E. Coli* mastitis is mainly determined by cow factors. *Vet. Res.*, **4**: 521-564.
- 6) Bybee, S. N., Scorza, A. V. and Lappin, M. R. 2011. Effect of the probiotic *Enterococcus faecium* SF68 on presence of diarrhea in cats and dogs housed in an animal shelter. *J. Vet. Intern. Med.*, **25**: 856-860.
- 7) Chen, W. Y., Weng, M. H., Chen, S. E., Peh, H. C., Liu, W. B. and Yu, T. C. 2007. Profile of gelatinolytic capacity in raw goat milk and the implication for milk quality. *J. Dairy*

- Sci.*, **90**: 4954-4965.
- 8) Crispie, F., Alonso-Gomez, M., O'Loughlin, C., Klostermann, K., Flynn, J. and Arkins, S. 2008. Intramammary infusion of a live culture for treatment of bovine mastitis: effect of live *Lactococcus* on the mammary immune response. *J. Dairy Res.*, **75**: 374-384.
 - 9) Faurschou, M. and Borregaard, N. 2003. Neutrophil granules and secretory vesicles in inflammation. *Microbes. Infect.*, **5**: 1317-1327.
 - 10) Frola, I. D., Pellegrino, M. S., Espeche, M. C., Giraud, J. A., Nader-Macias, M. E. F. and Bogni, C. I., 2012. Effects of intramammary inoculation of *Lactobacillus perolens* CRL 1724 in Lactating cows' udders. *J. Dairy Res.*, **79**: 84-92.
 - 11) Gill, H. S., Rutherford, K. J. and Cross, M. L. 2001. Dietary probiotic supplementation enhances natural killer cell activity in the elderly: an investigation of age-related immunological changes. *J. Clin. Immunol.*, **21**: 264-271.
 - 12) He, Y., Liu, X., Chen, Z., Zhu, J., Xiong, Y., Li, K., Dong, J. H. and Li, X. 2007. Interaction between cancer cells and stromal fibroblasts is required for activation of the uPAR-uPA-MMP-2 cascade in pancreatic cancer metastasis. *Clin. Cancer. Res.*, **13**: 3115-3124.
 - 13) Heyneman, R., Burvenich, C. and Vercauteren, R. 1990. Interaction between the respiratory burst activity of neutrophil leucocytes and experimentally induced *E. coli* mastitis in cows. *J. Dairy Sci.*, **73**: 985-994.
 - 14) Hoeben, D., Monfardini, E., Burvenich, C. and Hamann, J. 2000. Treatment of acute *Escherichia coli* mastitis in cows with enrofloxacin: effect on clinical signs and chemiluminescence of circulating neutrophils. *J. Dairy Sci.*, **67**: 485-502.
 - 15) Jouglin, M., Valette, R., Gavard, F., Quintin-Colonna, F. and Denoix, J. 2000. Metalloproteinases and tumor necrosis factor-alpha activities in synovial fluids of horses: correlation with articular cartilage alterations. *Vet. Res.*, **31**: 507-515.
 - 16) Klostermann, K., Crispie, F., Flynn, J., Ross, R. P., Hill, C. and Meaney, W. 2008. Intramammary infusion of a live culture of *Lactococcus lactis* for treatment of bovine mastitis: comparison with antibiotic treatment in field trials. *J. Dairy Res.*, **75**: 365-373.
 - 17) Laemmli, U. K. 1970. Cleavage of structural proteins during assembly of the head bacteriophage T4. *Nature*, **227**: 680-685.
 - 18) Maldonado Galdeano, C., de Moreno de LeBlanc, A., Vinderola, G., Bibas Bonet, M. E. and Perdigon, G. 2007. Proposed model: mechanisms of immunomodulation induced by probiotic bacteria. *Clin. Vaccine Immunol.*, **14**: 485-492.
 - 19) Makowski G. S. and Ramsby, M. L. 2003. Zymographic analysis of latent and activated forms of matrix metalloproteinase-2 and -9 in synovial fluid: correlation to polymorphonuclear leukocyte infiltration and in response to infection. *Clin. Chim. Acta.*, **329**: 77-81.
 - 20) Mehrzad, J., Dosogne, H., Meyer, E. and Burvenich, C. 2001. Local and systemic effects of endotoxin mastitis on the chemiluminescence of milk and blood neutrophils in dairy cows. *Vet. Res.*, **32**: 131-144.
 - 21) Mehrzad, J., Dosogne, H., Meyer, E. and Burvenich, C. 2002. Blood and milk neutrophil chemiluminescence and viability in primiparous and pluriparous dairy cows during late pregnancy, around parturition and early lactation. *J. Dairy Sci.*, **85**: 131-144.
 - 22) Mehrzad, J., Duchateau, L. and Burvenich, C. 2009. Phagocytic and bactericidal activity of blood and milk-resident neutrophils against *Staphylococcus aureus* in primiparous and multiparous cows during early lactation. *Vet. Microbiol.*, **134**: 106-112.
 - 23) Mehrzad, J. 2012. Molecular aspects of neutrophils as pivotal circulating cellular innate immune systems to protect mammary gland from pathogens. In: *Recent Advances in Immunology to Target Cancer, Inflammation and Infections*. pp. 383-422, Kanwar, J. R. ed., InTech Europe, Rijeka, Croatia.
 - 24) Paape, M. J., Shafer-Weaver, K., Capuco, A. V., Van Oostveldt, K. and Burvenich, C. 2000. Immune surveillance of mammary tissue by phagocytic cell. *Avexp. Exp. Med. biol.*, **480**: 259-277.
 - 25) Peng, H. Y., Tiantong, A., Chen, S. E., Piamya, P., Liu, W. B., Peh, H. C., Lee, J. W., Chen, M. T., Nagahata, H. and Chang, C. J. 2013. Ultrasonicated *Enterococcus faecium* SF68 enhances neutrophil free radical production and udder innate immunity of drying-off dairy cows. *J. Dairy Res.*, **80**: 349-359.
 - 26) Pezeshki, A., Collier, R. J. and Burvenich, C. 2010. An integrated view on how the management of the dry period length of lactating cows could affect mammary biology and defense. *J. Anim. Physiol. Anim. Nutr.*, **94**: 7-30.
 - 27) Pezeshki, A., Stordeur, P., Wallemacq, H.,

- Schynts, F., Stevens, M., Boutet, P., Peelman, L. J., De Spiegeleer, B., Duchateau, L., Bureau, F. and Burvenich, C. 2011. Variation of inflammatory dynamics and mediators in primiparous cows after intramammary challenge with *Escherichia coli*. *Vet. Res.*, **42**: 15-25.
- 28) Piamya, P., Tiantong, A., Chen, S. E., Liu, W. B., Yu, C., Nagahata, H. and Chang, C. J. 2015. Fingerprinting of gelatinase subtypes for different topographic regions on non-retaining placenta of Holstein cows. *Animal*, **9**: 490-499.
- 29) Pohjavuori, E., Viljanen, M., Korpela, R., Kuitunen, M., Tiittane, M., Vaarala, O. and Savilahti, E. 2004. Lactobacillus GG effect in increasing IFN-gamma production in infants with cow's milk allergy. *J. Allergy Clin. Immunol.*, **114**: 131-136.
- 30) Pugin, J., Widmer, M. C., Kossodo, S., Liang, C. M., Preas, H. H. L. and Suffredini, A. F. 1999. Human neutrophils secrete gelatinase B in vitro and in vivo in response to endotoxin and proinflammatory mediators. *Am. J. Respir. Cell Mol. Biol.*, **20**: 458-464.
- 31) Rabot, A., Sinowatz, F., Berisha, B., Meyer, H. H. D. and Schams, D. 2007. Expression and localization of extracellular matrix-degrading proteinases and their inhibitors in the bovine mammary gland during development, function, and involution. *J. Dairy Sci.*, **90**: 740-748.
- 32) Raulo, S. M., Sorsa, T., Tervahartiala, T., Latvanen, T., Pirila, E., Hirvonen, J. and Maisi, P. 2002. Increase in milk metalloproteinase activity and vascular permeability in bovine endotoxin-induced and naturally occurring *Escherichia coli* mastitis. *Vet. Immunol. Immunopathol.*, **85**: 137-145.
- 33) SAS Institute, 2008. SAS/STAT user's guide. Release 9.2. SASInstitute Inc., Cary, NC
- 34) Scharek, L., Guth, J., Reiter, K., Weyrauch, K. D., Taras, D., Schwerk, P., Schierack, P., Schmidt, M. F., Wieler, L. H. and Tedin, K. 2005. Influence of a probiotic *Enterococcus faecium* strain on development of the immune system of sows and piglets. *Vet. Immunol. Immunopathol.*, **105**: 151-161.
- 35) Scharek, L., Guth, J., Filter, M. and Schmidt, M. F. G. 2007. Impact of the probiotic bacteria *Enterococcus faecium* NCIMB 10415 (SF68) and *Bacillus cereus* var. toyoi NCIMB 40112 on the development of serum IgG and faecal IgA of sows and their piglets. *Arch. Anim. Nutr.*, **61**: 223-234.
- 36) Schierack, P., Wieler, L. H., Taras, D., Herwig, V., Tachu, B., Hlinak, A., Schmidt, M. F. and Scharek, L. 2007. *Bacillus cereus* var. toyoi enhanced systemic immune response in piglets. *Vet. Immunol. Immunopathol.*, **118**: 1-11.
- 37) Schultz, M., Linde, H. J., Lehn, N., Zimmermann, K., Grossmann, J., Falk, W. and Schölmerich, J. 2003. Immunomodulatory consequences of oral administration of *Lactobacillus rhamnosus* strain GG in healthy volunteers. *J. Dairy Res.*, **70**: 165-173.
- 38) Simpson, K. W., Rishniw, M., Bellosa, M., Liotta, J., Lucio, A., Baumgart, M., Czarnecki-Maulden, G., Benyacoub, J. and Bowman, D. 2009. Influence of *Enterococcus faecium* SF68 probiotics on Giardiasis in Dogs. *J. Vet. Intern. Med.*, **23**: 476-481.
- 39) Tiantong, A., Peng, H. Y., Chen, S. E., Piamya, P., Liu, W. B., Chen, M. T., Yu, C., Nagahata, H. and Chang, C. J. 2015. Intramammary infusion of an *Enterococcus faecium* SF68 preparation promoted the involution of drying off Holstein cows partly related to neutrophil-associated matrix metalloproteinase 9. *Anim. Sci. J.*, **86**: 111-119.
- 40) Veir, J. K., Knorr, R., Cavadini, C., Sherrill, S. J., Benyacoub, J., Satyaraj, E. and Lappin, M. R. 2007. Effect of supplementation with *Enterococcus faecium* (SF68) on immune functions in cats. *Vet. Therapy*, **8**: 229-240.
- 41) Yu, T. C., Chang, C. J., Ho, C. H., Peh, H. C., Chen, S. E., Liu, W. B., Peng, H. Y., Piamya, P., Chen, M. T. and Nagahata, H. 2011. Modifications of the defense and remodeling functionalities of bovine neutrophils inside the mammary gland of milk stasis cows received a commercial dry-cow treatment. *Vet. Immunol. Immunopathol.*, **144**: 210-219.
- 42) Yu, T. C., Chen, S. E., Ho, T. H., Peh, H. C., Liu, W. B., Tiantong, A., Nagahata, H. and Chang, C. J. 2012. Involvement of TNF- α and MAPK pathway in the intramammary MMP-9 release via degranulation of cow neutrophils during acute mammary gland involution. *Vet. Immunol. Immunopathol.*, **147**: 161-169.