INTERPRETIVE SUMMARY

"Menaquinone-4 (vitamin K_2) induces proliferation responses in bovine peripheral blood mononuclear cells"

Bai et al.

The aim of this study was to examine the effects of vitamin K (VK) on cow immune cells, using blood samples. We found that VK promoted the proliferation of immune cells, but did not alter the expression levels of T-cell related genes in the immune cells. This study demonstrated that VK has a positive effect on the proliferation response of cow immune cells, demonstrating the possibility of the cow-specific activation of immune cells.

SHORT COMMUNICATION: VITAMIN K2 AND BOVINE PBMCS

Title: **Menaquinone-4 (vitamin K_2) induces proliferation responses in bovine peripheral blood mononuclear cells**

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ABSTRACT

The effects of vitamin K (VK) on immune cells in ruminants are yet to be fully investigated. The objective of this study was to examine the effects of VK on peripheral blood mononuclear cells (PBMCs) in Holstein dairy cows. A cell proliferation assay was performed to evaluate the effect of menaquinone-4 (MK-4, the biologically active form of VK) on immune response of PBMCs. The proliferation of PBMCs stimulated by MK-4 was significantly higher than that of non-stimulated controls. The expression of T cell-related genes in PBMCs, stimulated with MK-4, was assessed by quantitative polymerase chain reaction. No significant changes were observed in the mRNA expression levels of both *CD4* and *CD8* as helper T cell and cytotoxic T cell markers respectively. The present study demonstrated that MK-4 positively influenced cow PBMCs proliferation and suggested the possibility of bovine-specific immune cell activation. The present study lays a foundation for understanding the physiological role of VK in cattle.

***Key words**: Cow, Menaquinone-4, Peripheral blood mononuclear cells*

Short communication

Vitamin K (**VK**) is a fat-soluble vitamin and an important factor in blood clotting (Dam and Schønheyder, 1936). The VK family consists of phylloquinones (VK_1 in plants), menaquinones (**MK**, or VK_2, produced by bacteria), and menadione (VK_3, chemically produced). Dietary phylloquinones are endogenously converted to MK-4 (Okano et al., 2008; Nakagawa et al., 2010), which is the dominant form of VK in animal tissues (Thijssen et al., 1994; Yamamoto et al., 1997).

In cattle, VK is synthesized by the rumen microbes to meet dietary requirements. VK is also found in the pasture and green roughages (NRC, 2001). Thus, most studies have been conducted on vitamins A, D, and E due to the frequent occurrences of their deficiency (Weiss, 2017), whereas studies on VK in ruminants are limited. Recently, increasing interest in the potential roles of VK has been expressed besides the regulation of blood clotting. Critical benefits of VK have been demonstrated, including positive effects on bone health, aging-related diseases, cancer, and inflammation (Xv et al., 2018; Simes et al., 2019). Several studies have shown the effects of VK on human peripheral blood mononuclear cells (**PBMCs**); VK derivatives attenuated T cell-mediated immunity in activated T cells, and increased the CD4+CD25+Foxp3+Treg cell population (Hatanaka et al., 2014). Cattle synthesize sufficient amount of VK and never present with deficiency symptoms. Nevertheless, understanding of the physiological functions of VK in cattle would be informative. To date, no study has investigated the effects of VK on bovine immune cells. Hence, the aim of this study was to assess the effects of VK on bovine PBMCs proliferation and T cell-related gene expression.

Animal care, sampling, and PBMCs preparation were performed as previously described (Bai et al., 2019). Cows were randomly selected, and no diseases were detected

before and during the experiment. All study procedures were conducted in accordance with Hokkaido University guidelines regarding the care and use of animals (Approval No. 16-0019). Blood samples were collected in 10-ml tubes containing heparin (Terumo, Tokyo, Japan) from 12 Holstein cows (3–7 years old, 1–5 parity, 24.8 ± 1.0 kg/d milk yield) through the external jugular vein between 9 and 10 AM at the Hokkaido University farm. PBMCs were prepared on the same day as sampling. PBMCs were isolated from whole-blood samples using Lymphocyte Separation Medium 1077 (TaKaRa Bio Inc., Shiga, Japan) according to the manufacturer's instructions. Blood samples were diluted with an equal volume of phosphate-buffered saline (**PBS**), layered onto a separation medium, and centrifuged at 450 ×*g* for 40 min. The erythrocytes were then carefully lysed in NH_4Cl-base lysis buffer, washed twice with PBS, and suspended in RPMI-1640 medium (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The separated viable PBMCs were counted using the trypan blue test and seeded on a 96-well plate (1.0×10^4 cells/well) for proliferation assays, or on a 6-well plate (3.0×10^5 cells/well) for RNA extraction. Cell viability were approximately 90 % after separation and 60–70 % after culturing.

MK-4 (VK_2, biologically active form of VK; Wako) was dissolved in 99.5% (v/v) ethanol (1 μg/ml, as a stock solution) and diluted with the culture medium. The collected PBMCs were cultured in RPMI-1640 medium containing 5% fetal bovine serum and an antibiotic-antimycotic solution (Thermo Fisher Scientific K.K., Yokohama, Japan), with (50 ng/ml) or without (cultured medium only, as the control or 1% ethanol only, as the vehicle control) of MK-4. The PBMCs were cultured for 72 h under a humidified atmosphere of 5% CO_2 at 38.5 °C.

Cell proliferation assays were conducted using Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. The PBMCs were treated with CCK-8 reagent (1:10, v/v) and incubated for 2 h. To estimate PBMCs proliferation, absorbance at 450 nm wavelength was measured using an auto-microplate reader (ARVO X; PerkinElmer Japan Co., Ltd., Kanagawa, Japan). All assays were performed in triplicate. The stimulated index (SI) was calculated as the ratio of the average absorbance of three wells containing stimulated cells relative to that of three wells containing non-stimulated cells.

RNA was extracted and reverse-transcribed into cDNA for use in quantitative PCR (qPCR) as previously described (Bai et al., 2019). RNA quality was verified with an Agilent 2100 Bioanalyzer and the RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, CA, USA) (Supplementary Figure. 1). All experiments were performed according to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments guidelines (Bustin et al., 2009). Target gene expression levels were determined by qPCR using LightCycler® 96 (Roche Diagnostics, Basel, Switzerland) and THUNDERBIRD™ SYBR® qPCR Mix (Toyobo, Osaka, Japan). Detailed primer information and thermal cycling conditions are shown in Table 1. The relative mRNA abundance was calculated on the based on the geometric mean of the reference gene expression levels.

The results are presented as means ± standard error of the mean (SEM). Data were analyzed using StatView statistical software (Version 5.0, SAS Institute Inc., NC, USA). One-way analysis of variance (ANOVA) followed by a Tukey-Kramer test (post-hoc analysis) for the cell proliferation assay data among three groups (Cont., Vehicle, and MK-4, n = 12). Student's *t*-test (unpaired, two tailed) was performed for comparison of

the qPCR data between two groups. Changes relative to control with *p*-values < 0.05 were considered statistically significant.

The proliferation of the MK-4 treated PBMCs significantly increased (1.36 ± 0.11) compared with those of the non-stimulated controls ($P < 0.01$, Fig. 1). ~~Post-culture cell viability was 96.6% for the vehicle treatment and 147.6% for the MK treatment relative to the non-stimulated control.~~ The MK-4 treatment was not significantly cytotoxic (Supplementary Figure. 2). To examine whether the T cell population changed with the increase in cell proliferations, the expression of T cell-related genes in PBMCs after MK-4 treatment was observed using qPCR (Fig. 2). No differences were noted in the mRNA expression of *CD4* ($P = 0.92$) and *CD8* ($P = 0.34$) with MK-4 treatment, which is a marker of the helper T and cytotoxic T cells, respectively. In addition, there was no difference in the T cell lineage (Th1, Th2, Th17, and Treg cells)-related genes or, inflammatory cytokine genes with and without MK-4 treatment (Supplementary Fig. 3).

T cell proliferation was reported to be involved in CD4 and CD8 balance in lactating cows (Mehrzad and Zhao, 2008). In addition, VK_2 was reported to alter the T cell population in mitogen-activated human PBMCs (Hatanaka et al., 2014). By contrast, we did not observe a change in the expression of *CD4* and *CD8*, and their population or producing cytokine genes. However, we only assessed the effect of single MK-4 treatment on mRNA expression levels. To fully understand the effect of VK on cow PBMCs, combined assays involving both MK-4 and mitogen are required. Furthermore, cell surface protein expression should be measured by flow cytometry.

In conclusion, we found that a single MK-4 treatment positively influenced bovine PBMCs proliferation but had no apparent effect on T cell-related genes expression, which is in contrast to previous results in humans (Hatanaka et al., 2014). To the best of our

knowledge, this is the first study to examine the effects of VK on bovine PBMCs. It was reported that VK-dependent protein S and co-factor activated protein C of bovines and humans display species-specific activities (He et al., 1995). Although the detailed mechanism of the effect of MK-4 on bovine PBMCs remains unclear, species-specific immune cell activation mechanism may exist. To reveal the mechanism of the immune regulatory functions of VK, further studies are required to examine the differences in T cell populations as well as gene expression differences between cattle and humans.

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Table 1. Primers for quantitative PCR.

Name (GenBank accession No.)	Sequence (5'-3')	Product length (bp)	References
T cell marker genes			
CD4	F: AGCAGAAAGTGAAACTCGTGG	88	Wang et al., 2013
(NM_001103225)	R: ACCAACTTCGGCTGATTTGAG		
CD8	F: CGCAGACTAGGTCGGTCTCT	176	Allan et al., 2015
(NM_174015)	R: GTTCCGGCGGTAGCAGAT		
Internal control			
ACTB	F: TGGACTTCGAGCAGGAGATG	222	Bai et al., 2019
(AY141970)	R: GTAGAGGTCCTTGCGGATGT		
GAPDH	F: CACCCTCAAGATTGTCAGCA	103	Bai et al., 2019
(NM_001034034)	R: GGTCATAAGTCCCTCCACGA		
H2AFZ	F: AGAGCCGGTTTGCAGTTCCCG	116	Bai et al., 2019
(NM_174809)	R: TACTCCAGGATGGCTGCGCTGT		

F: Forward, R: Reverse

Cycling conditions: One cycle at 95 °C for 30 s, followed by 50 cycles at 95 °C for 10 s, 60 °C for 15 s, and then 72 °C for 30 s.

Each run was completed with a melting curve analysis to confirm the specificity of amplification and the absence of primer dimer formation.

The concentration of RNA and cDNA was assessed using the NanoDrop 2000c spectrophotometer. RNA quality was checked using the Agilent 2100 Bioanalyzer and the RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, CA, USA).

Figure legends

Fig. 1. Measurement of the proliferation of bovine peripheral blood mononuclear cells (PBMCs) in the presence (50 ng/ml) or absence (culture medium only, as control or 1% ethanol, as the vehicle control) of MK-4. SI: stimulation index (absorbance of stimulated wells/absorbance of non-stimulated wells; the value of non-stimulated cells in each group was considered Cont. = 1.0). Asterisks indicate significant differences in absorbance levels: $^{**}P < 0.01$, One-way ANOVA with Tukey–Kramer post-hoc test (three groups; Cont., Vehicle, and MK-4, n = 12 per group).

Fig. 2. Expression of T cell subset marker genes (*CD4* and *CD8*) in bovine peripheral blood mononuclear cells (PBMCs). Data represent expression levels relative to those of three internal control genes (i.e., geometric means of *ACTB*, *GAPDH*, and *H2AFZ*). Data are presented as means ± standard error of the mean (SEM). No significant differences were observed after MK-4 treatment, both CD4 (0.99 ± 0.11, $P = 0.92$) and CD8 (0.86 ± 0.16, $P = 0.34$) expression based on the Student's *t*-test (unpaired, two tailed, n = 12, per group).

Supplemental Fig. 1. Analysis of quality of RNA extracted from bovine PBMCs.

RNA quality was checked with an Agilent 2100 Bioanalyzer and the RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, CA, USA). Gel images of RNA and bioanalyzer electropherograms for representative samples are shown (top: Control, bottom: MK-4 treatment). Average RNA integrity number (RIN) value was 6.8 ± 0.3. Note that the threshold of RIN value for real-time PCR data is > 5–6 (Fleige and Pfaffle, 2006; Schroeder et al., 2006).

Supplemental Fig. 2. Cytotoxic effect of MK-4 treatment on bovine PBMCs. Cytotoxic effect of MK-4 was evaluated using a Cytotoxicity LDH Assay Kit-WST (Dojindo Laboratories). PBMCs were cultured either in the presence (50 ng/ml) or absence (culture medium only, as control or 1% ethanol, as vehicle control) of MK-4. Absorbance at 490 nm wavelength was measured using an auto-microplate reader (ARVO X; PerkinElmer Japan Co., Ltd.). LDH activity did not significantly change after MK-4 treatment according to the One-way ANOVA (three groups; Cont., Vehicle, and MK-4, n = 10 per group).

Supplemental Fig. 3. Expression levels of (A) Th1- (*IFNG*), (B) Th2- (*IL4*), (C) Th17- (*IL17*) (D) Treg (*CD25*, *IL10*) cell related genes, and (E) inflammatory cytokine genes (*IL2* and *IL6*) in PBMCs. The values represent the mRNA expression levels relative to those of three internal control genes (i.e., geometric means of *ACTB*, *GAPDH*, and *H2AFZ*). The data are presented as mean ± SEM. No significant differences or tendency were observed, based on the Student's *t*-test ($P > 0.1$, n = 10).

The primers for qPCR are listed in Supplemental Table 1.

The concentration of RNA and cDNA was assessed using the NanoDrop 2000c spectrophotometer. RNA quality was checked using the Agilent 2100 Bioanalyzer and the RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, CA, USA).

316 Bai et al., Figure1.

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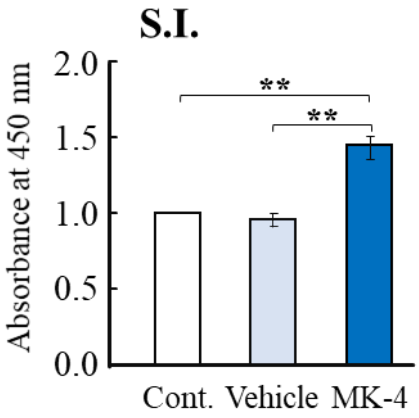

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323 Bai et al., Figure2.

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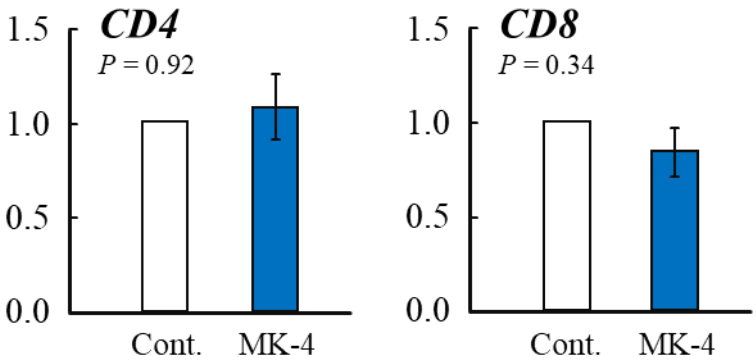

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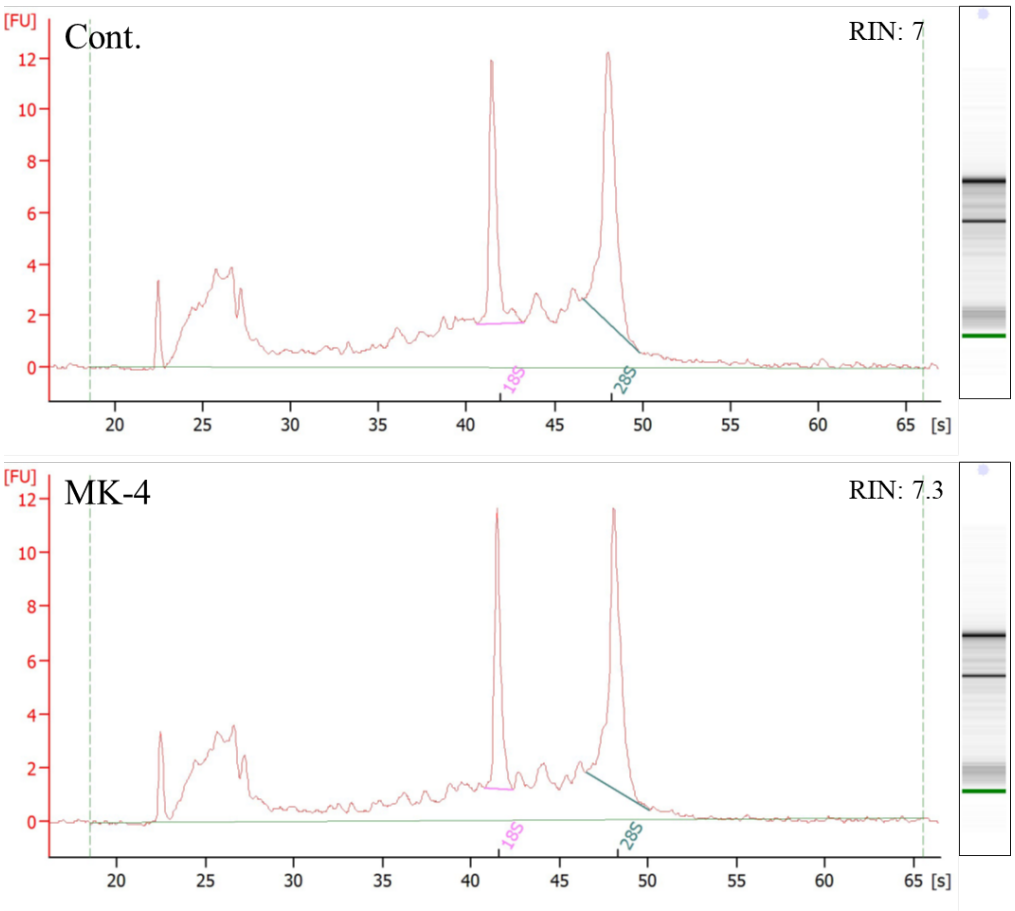
328 Bai et al., Supplemental Figure1.



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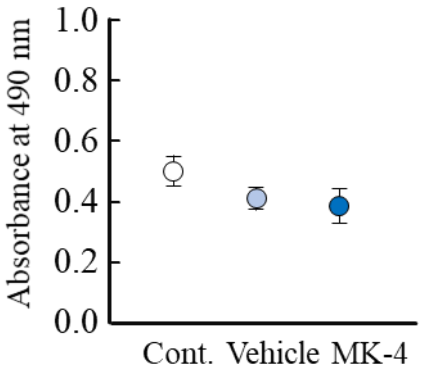
Bai et al., Supplemental Figure2.



335 Bai et al., Supplemental Figure3.

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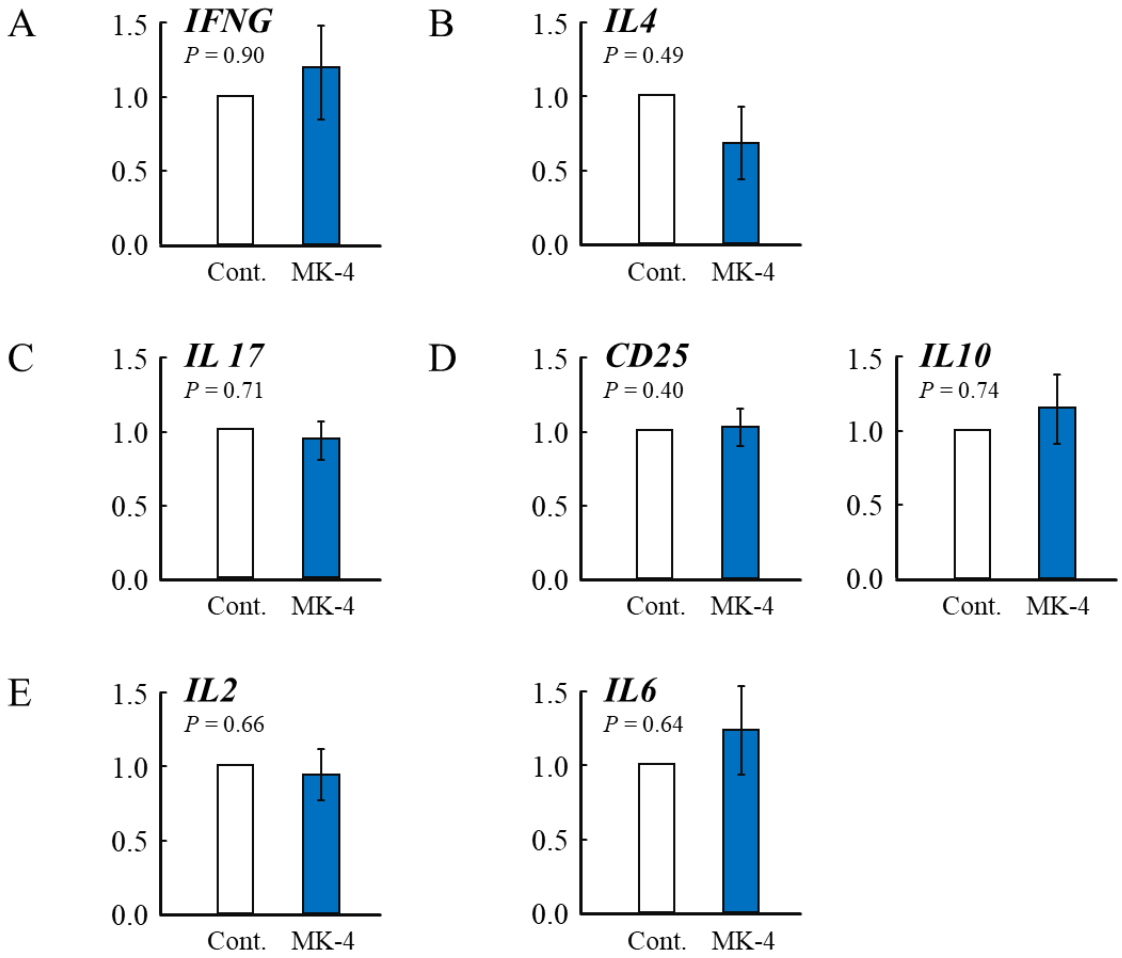

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Supplemental Table 1. Primers for quantitative PCR.

Name (GenBank accession No.)	Sequence (5'-3')	Product length (bp)	References
T cell related genes			
CD25	F: GCAGGGACCACAAATTTCCA	76	Seo et al., 2007
(NM174358)	R: GGTACTCAGTGGTAAATATGAACGTATCC		
IFNG	F: TTGAATGGCAGCTCTGAGAAAC	15	Bai et al., 2019
(FJ263670)	R: TCTCTTCCGCTTTCTGAGGTTAGA		
IL4	F: TTGGAATTGAGCTTAGGCGTAT	186	Wu et al_2007
(EU276069)	R: CCAAGAGGTCTTTCAGCGTACT		
IL10	F: ATGCGAGCACCCTGTCTGAC	124	Holmgren et al,
(NM_174088)	R: TGCAGTTGGTCCTTCATTGAAAG		2014
IL17	F: CACAGCATGTGAGGGTCAAC	83	Talukder et al.,
(AF412040)	R: GGTGGAGCGCTTGTGATAAT		2018
Inflammatory cytokine genes			
IL2	F: ACATTTGACTTTTACGTGCCCAAG	307	Yasuda et al.,
(NM_180997)	R: AATGAGAGGCACTTAGTGATC		2009
IL6	F: TAAGCGCATGGTCGACAAAA	150	Bai et al., 2019
(EU276071)	R: TTGAACCCAGATTGGAAGCAT		
Internal control			
ACTB	F: TGGACTTCGAGCAGGAGATG	222	Bai et al., 2019
(AY141970)	R: GTAGAGGTCCTTGCGGATGT		
GAPDH	F: CACCCTCAAGATTGTCAGCA	103	Bai et al., 2019
(NM_001034034)	R: GGTCATAAGTCCCTCCACGA		
H2AFZ	F: AGAGCCGGTTTGCAGTTCCCG	116	Bai et al., 2019
(NM_174809)	R: TACTCCAGGATGGCTGCGCTGT		

F: Forward, R: Reverse

Cycling conditions: One cycle at 95 °C for 30 s, followed by 50 cycles at 95 °C for 10 s, 60 °C for 15 s, and then 72 °C for 30 s.

Each run was completed with a melting curve analysis to confirm the specificity of amplification and the absence of primer dimer formation.

The concentration of RNA and cDNA was assessed using the NanoDrop 2000c spectrophotometer. RNA quality was checked using the Agilent 2100 Bioanalyzer and the RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, CA, USA).