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Quercetin Promotes the Expression of Genes Involved in Phagocytosis in Bovine Neutrophils

[1]Suphakit Srikok, [1]Sukij Nambut, [1]Kanruethai Wongsawan and [1,2]Phongsakorn Chuammitri

[1]Department of Veterinary Biosciences and Public Health,
Faculty of Veterinary Medicine, Chiang Mai University, Chiang Mai 50100, Thailand
[2]Excellent Center in Veterinary Biosciences (ECVB), Chiang Mai University, Chiang Mai 50100, Thailand

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Corresponding Author:
Phongsakorn Chuammitri
Department of Veterinary Biosciences and Public Health, Faculty of Veterinary Medicine, Chiang Mai University, Chiang Mai 50100, Thailand
E-mail:phongsakorn@gmail.com

Abstract: This study was conducted to explore the effect of Quercetin (QH) on phagocytosis of bovine neutrophils. Neutrophils were isolated from fifteen multiparous Holstein cows. We *in vitro* treated neutrophils with PBS or 50 µM of Quercetin (QH) or Cytochalasin B (CytB), as phagocytosis inhibitor, prior to monitoring phagocytosis of *Escherichia coli* by flow cytometry and microscopic examination. Additionally, the expressions of *CORO1A*, *CYBA* (gp91^{phox}), *LAMP1*, *RAB7A*, *RAC1* and *PAK1* mRNA were analyzed by real-time PCR. In the time-course experiment, treated neutrophils were allowed to co-cultured with live bacteria for 30, 60 and 90 min before measuring gene expressions. The expression levels of *IL-1 β* and *TNF* genes in order to demonstrate the anti-inflammatory property of quercetin were assessed by conventional RT-PCR. The results of flow cytometry and microscopic examination showed that the percentage of neutrophils performing phagocytosis were significantly higher in QH group in comparison with other groups. We reported here that mRNA expressions of *CORO1A*, *CYBA*, *LAMP1*, *RAB7A*, *RAC1* and *PAK1* genes involved in phagocytosis, were significantly up-regulated in QH group. As expected, CytB group had profound down-regulation of genes with one exception in *PAK1*. From our observations, the expression levels of phagocytic process genes in the QH group were optimum at 60 min and started to decline at 90 min of incubation. The data also indicated that quercetin inhibited inflammation by reducing the expressions of both *IL-1β* and *TNF*. In conclusion, the information in our experiments conclude that quercetin has the ability to boost the expression of genes involved in phagocytosis while reduces the expression of proinflammatory genes.

Keywords: Bovine, Neutrophil, Quercetin, Phagocytosis, Gene Expression

Introduction

The rapid and efficient innate defense mechanisms to resist microbial invasion may contribute to the overall health and well-being of human and animals. In innate immunity, neutrophils are actively phagocytic cells performing host defense through a variety of effector functions, i.e., Reactive Oxygen Species (ROS) generation, phagocytosis, degranulation and Neutrophil Extracellular Traps (NETs) release (Amulic *et al*., 2012; Brinkmann *et al*., 2004; Chuammitri *et al*., 2015). Neutrophil phagocytosis is a process, in which a large particle, such as bacteria, immune complex or apoptotic cell is being intake into this cell (Esmann *et al*., 2010; Grinberg *et al*., 2008). Innate immune cells; macrophages, neutrophils and dendritic cells (DCs) possess this effector function. Phagocytosis of human Polymorphonuclear leukocytes (PMNs) was assisted by the Fc receptor and Complement Receptors (CRs) led to the expression of many involving genes in cytokine production and signal transduction (Kobayashi *et al*., 2002). The gene expression patterns differ when phagocytosis is examined during the 90 min-interval as the genes were either induced or repressed (Kobayashi *et al*., 2002). The available information stated that gene expression and cell signaling pathways involved in phagocytosis, AMP-activated Protein Kinase (AMPK), may result in anti-inflammatory in phagocytosis of *E. coli*, as well as, the changes in cytoskeletal reorganization (Bae *et al*., 2011). The AMPK signaling also stimulated *PAK1/2* genes and moreover the *RAC1* gene that helped reduce the expression of other genes

involved in the inflammatory process, such as *TNF-α* in neutrophils via the induction of Toll Like Receptor 4 (TLR4) (Bae *et al*., 2011). The microarray study of human PMN gene expression during bacterial Phagocytosis revealed the global changes of many key gene transcripts (Kobayashi *et al*., 2003). The genes in associated with the inflammatory response and apoptosis regulators i.e., *IL-1α, IL-1β*, *IL-1ε*, *IL-1RN*, *IL-8*, *IL-10*, *IL-12β*, *IL-15*, *TNFα*, Vascular Endothelial Growth Factor (*VEGF*), OncostatinM (*OSM*), *IL-6*, *GROβ* and *GROγ* were up-regulation as shown and reviewed by (Kennedy and DeLeo, 2009; Kobayashi *et al*., 2003; 2005).

At present, the role of herbs and natural substances classified as one option to help strengthen the immune system of cattle. One of spotlight herbal phenolic substances is quercetin, which is found in many edible plants, fruits and vegetables (Erlund, 2004; Formica *et al*., 1995). The bioavailability studies of quercetin and its metabolites; aglycone and rutin, in cows demonstrated that quercetin can be absorbed via digestive tracts and eventually entered the plasma (Berger *et al*., 2012; Gohlke *et al*., 2013; Maciej *et al*., 2015). Several *in vivo* studies of quercetin in neonatal calves, periparturient and lactating cows, in the aspects of bovine health and production, have been recently reported (Gohlke *et al*., 2013; Maciej *et al*., 2015; Stoldt *et al*., 2015); however, another point of view remains scarce in bovine innate immunity.

Many published works have emphasized that quercetin also exerts its effects by promoting and acting as an antioxidant and anti-inflammation (Boots *et al*., 2008; Cho *et al*., 2003; Chuammitri *et al*., 2017; De Marchi *et al*., 2009; Nair *et al*., 2006). In fact, quercetin affects the mRNA gene expressions and associated proteins in the innate immune cells, such as the reduction of the IL-1β mRNA level during inflammation was documented (Valerio *et al*., 2009). We have previously demonstrated the *in vitro* effects of quercetin on bovine neutrophil phagocytosis of *Escherichia coli* (Chuammitri *et al*., 2015), but in great detail study of how gene cascades in bovine neutrophil phagocytic processes were regulated due to proposed quercetin effects is incompletely elucidated at molecular levels. To our knowledge, little is known about the action of quercetin on the dynamic of gene expressions during bacterial phagocytosis of bovine neutrophils. To continue examining the immunomodulatory action of anticipating quercetin effects, this study aims to investigate how quercetin alters the expressions of key phagocytosis genes; *CORO1A*, *CYBA* (gp91^{phox}), *LAMP1*, *RAB7A*, *RAC1* and *PAK1* during bacterial phagocytosis.

Materials and Methods

Reagent

Quercetin Hydrate (QH) cat. No 337951, Cytochalasin B (CytB), Propidium Iodide (PI), Hanks' Balanced Salt Solution without calcium and magnesium (HBSS), Citrate-Dextrose solution (ACD) and Trypan blue were purchased from Sigma-Aldrich, St. Louis, MO, USA. Fetal Bovine Serum (FBS), Gibco RPMI 1640 medium with phenol red and RNA*later* were obtained from Life Technologies, Carlsbad, CA, USA.

Ethical Approval

The animal experimental procedure was approved to be conducted under the Animal Care and Use Ethics Committee (FVM-ACUC Ref. No: S5/2557).

Animals, Blood Collection and Bovine Neutrophil Isolation

Healthy, multiparous Holstein cows during non-lactating period were used as blood donors (Jinghui *et al*., 2014; Martinez *et al*., 2014). A total number of 15 cows in 3 independent experiments, 5 cows each, were recruited in the study. All dairy cows were housed at local dairy farms of Mae On district and Mae Hia Agricultural Research, Demonstration and Training Center. Whole blood was collected by jugular venipuncture into a sterile syringe containing 1× ACD solution. Neutrophil isolation protocol was previously described (Chuammitri *et al*., 2015). Finally, cell density was adjusted to approximately 2×10^6 cells per ml in RPMI 1640 medium supplemented with 1% FBS.

Quercetin

Stock Quercetin solution at 5 mM was prepared by dissolving dry quercetin powder in 95% ethanol and filter sterile. Working solution at 50 μM concentrations was freshly prepared on the day of the experiment, protected from light and stored at room temperature until use. The 50 μM of quercetin showed no *in vitro* cytotoxicity to bovine neutrophils as previous report (Chuammitri *et al*., 2015).

Escherichia coli Propagation and Opsonization

Escherichia coli (*E. coli*) was isolated from clinical mastitis cow. The bacteria were freshly prepared by colony picking method and propagation in Luria-Bertani Broth (LB broth, Caisson Laboratories, North Logan, UT, USA).The bacterial number was adjusted to approximately 10^8 CFU/ml. Live *E. coli* was opsonized with 10% heat-inactivated normal bovine serum for 20 min at 37°C prior to use in the experiment.

Fluorescent Staining of Bacteria

Fluorescent *E. coli* was prepared by growing bacteria to log phase, heat killed (70°C, 60 min), washed with PBS and centrifuged (12,000 rpm, 5 min). Bacterial DNA pellet (10^8 CFU/ml) was stained with 5% Propidium Iodide (PI) in PBS and incubated at room temperature in the dark for 60 min (Keogh *et al*., 2011).

PI labeled *E. coli* was adjusted to 10^6 CFU/ml with PBS and stored at 4°C until use. Fluorescent *E. coli* was opsonized with 10% heat-inactivated normal bovine serum for 20 min at 37°C before using in phagocytosis assay.

Phagocytosis

The phagocytosis of PI labeled *E. coli* was assessed via flow cytometry (Della Libera *et al*., 2015). In brief, neutrophils (2×10^5 neutrophils) were seeded into duplicate wells of a 96-well, flat bottom cell culture plate. Cells were treated with 50 µM quercetin, or 10 µg/ml cytochalasin B (CytB), in order to inhibit phagocytosis (Khatua *et al*., 2012), or PBS for 30 min at 37°C, 5% CO_2. Subsequently, opsonized fluorescent *E. coli* at MOI of 10 were then added to the cells, centrifuged and incubated (37°C, 5% CO_2) for 45 min. Sample acquisitions (50,000 events) were performed by CyAn ADP High-Performance Flow Cytometer (Beckman Coulter), then analyzed by using the FlowJo 10 (Treestar, Ashland, OR, USA).

Enumeration of Phagocytosis of E. coli by Bovine Neutrophils under Light Microscope

Neutrophils (2.5×10^4 neutrophils) were seeded into duplicate wells of a 96-well, flat cell culture plate. Cells were treated as previously described in phagocytosis assay. Next, live, opsonized fluorescent *E. coli* at MOI of 10 were then added to the cells, centrifuged and incubated for 45 min. Cytospin slides were prepared from an aliquot of neutrophils after phagocytosis and seeded onto circular coverlips (15 mm diameter) that were placed into a 24-well plate. Slides were fixed with absolute ethanol, stained with Dip Quick and examined with a Zeiss Axio Scope A1 (Carl Zeiss, Thornwood, NY, USA). Percentage of phagocytosis with different treatments were manually counted from 5 random fields (100 cells per field) at 10× objective by three well-trained observers in a blinded technique (Yan *et al*., 2012).

Gene Expression Studies by Quantitative Real-Time Reverse Transcription (qRT-PCR) and Semi-Quantitative RT-PCR

Neutrophils (1×10^6 cells) were seeded into duplicate wells of a 24-well cell culture plate. Then, cells were treated with PBS, quercetin, or CytB for 30 min before co-cultured with 10 MOI of opsonized *E. coli* in serum-free RPMI-1640 medium for another 45 min. After incubation, cells from two wells were combined, washed and spun. Neutrophil pellet was resuspended in RNA*later*. In the time-course experiment, the assay setting was nearly identical to the above stated procedures, but with some modifications. In particular, neutrophils were allowed to co-cultured with opsonized *E. coli* for 30, 60 and 90 min at 37°C, 5% CO_2, respectively.

RNA Extraction and Complementary DNA (cDNA) Synthesis

Total RNA was extracted and purified by NucleoSpin RNA (Macherey-Nagel, Bethlehem, PA, USA) according to the provided protocols. Complementary DNA (cDNA) was reverse-transcribed from a starting amount of 2 µg of total RNA by Tetro cDNA Synthesis Kit (Bioline, Taunton, MA, USA) and stored at -20°C until further use.

Primers for qRT-PCR and Semi-Quantitative RT-PCR

Primers for real-time PCR; *CORO1A*, *CYBA*, *LAMP1*, *RAB7A*, *RAC1*, *PAK1* and *ACTB* as endogenous control (Shirasuna *et al*., 2015) were designed using Primer3 Plus. Primers for *IL1B*, *TNF* and *GAPDH* were previously reported (Chuammitri *et al*., 2015). The PCR primers were synthesized by Macrogen, Seoul, Korea. The sequence information of oligonucleotide primers used in this study is indicated in Table 1.

Quantitative Real-Time Reverse Transcription PCR (qRT-PCR)

The qRT-PCR was performed to investigate the effect of quercetin on phagocytosis related gene expressions in bovine neutrophils. The quantification of mRNAs was performed in triplicate on ABI 7300 Real-Time PCR System equipped with SDS Software v1.4 (Life Technologies). The qRT-PCR was performed on 100 ng cDNA using SensiFAST SYBR Hi-ROX Kit (Bioline) as per manufacture's instruction. Subsequently, specificity was confirmed by dissociation curve analysis (T_m). Correct product sizes were also determined by 2% agarose gel (Chuammitri *et al*., 2017). Analysis of relative gene expression was calculated from the C_t of the gene of interest (target) and *ACTB*. The expression levels (fold difference) were reported using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Semi-Quantitative Reverse Transcription PCR (RT-PCR)

Semi-quantitative RT-PCR was performed to investigate the effect of quercetin on *IL1B* and *TNF*, as pro-inflammatory cytokine gene expressions in bovine neutrophils after phagocytosis and time-course induction of phagocytosis. PCR reactions were performed using MyTaq HS Red Mix (Bioline). The annealing temperature at 59°C (*IL1B* and *GAPDH)* or 57°C (*TNF*) was set. The amplified PCR products were electrophoresed in 1.5% agarose gel. Gel band intensities were quantified by Gel Analyzer Options in ImageJ version 1.46r (Chuammitri *et al*., 2015). Expression levels are represented relative to *GAPDH* expression.

Table 1. Details of PCR primer sequences

Gene	Accession no.	Primer	Sequence	Annealing temp (°C)	Product size (bp)
CORO1A	NM_174521	Forward	TGCCGTGTTTGTATCCGATG	59	177
		Reverse	AGGTAGACGATGTTGGTGTCAG		
CYBA	NM_174034	Forward	TCAGTTCACCCAGTGGTACC	59	135
		Reverse	ACTCTGGTCAGGTACTTCTGTC		
LAMP1	NM_001075124	Forward	ACAACGTTTCTGGCAGCAAC	59	125
		Reverse	GGTCTTGTTGGGGTTGACATTG		
RAB7A	NM_001035081	Forward	ACCATGCAGATTTGGGACAC	59	126
		Reverse	AAGGGTCTTGAACGTGTTGG		
RAC1	NM_174163	Forward	TGCCAATGTCATGGTGGATG	59	193
		Reverse	ACAATGGTGTCGCACTTCAG		
PAK1	NM_001076898	Forward	AAGGAACGGCCAGAGATTTCTC	59	158
		Reverse	TGCGGGTTTTTCTTCTGCTC		
ACTB	NM_173979	Forward	TGCGGCATTCACGAAACTAC	59	146
		Reverse	AGGGCAGTGATCTCTTTCTGC		
IL1B	M35589	Forward	CGTACCTGAACCCATCAACGAAAT	59	564
		Reverse	GGCGTATCACCTTTTTTCACACAA		
TNF	Z14137	Forward	TCTCAAGCCTCAAGTAACAAGCCG	57	414
		Reverse	CAGGTAGTCCGGCAGGTTGATCTC		
GAPDH	NM_001034034	Forward	AGTTCAACGGCACAGTCAAG	59	243
		Reverse	TCACGCCCATCACAAACATG		

Heat Map of Gene Expressions

Gene expression patterns from the average expression level of each gene presented in the form of a heat map of an average linkage method with Euclidean distance. Heat map was generated by Genesis. 1.7.6 (Sturn *et al*., 2002).

Data Analysis

The data were first screened for potential outlier by robust Regression and Outlier Removal (ROUT) methods. The normality test was done by the D'Agostino-Pearson omnibus test prior to performing the unpaired two-tailed Student *t* test, one-way ANOVA or Kruskal-Wallis Test and two-way ANOVA. The statistical analysis was considered significant when $p<0.05$. The results are reported as mean with standard error (mean ± SE). Information obtained from the statistical analyses is represented as graphs generated by GraphPad Prism (GraphPad software, San Diego, CA, USA).

Results

Neutrophil Phagocytosis of E. coli by Flow Cytometry

The ability of neutrophil phagocytosis under the supplementation of quercetin and phagocytosis inhibitor can be reflected by the amounts of fluorescently labelled bacteria being ingested or Median Fluorescence Intensity (MFI) as determined by flow cytometry. Neutrophils treated with quercetin was found to have the highest MFI when compared with PBS and CytB group (Fig. 1B and 1C, $p = 0.018$), with an average MFI in PBS (20.77±1.82), quercetin (28.54±2.65) and CytB (21.35±1.44), respectively.

Phagocytosis under Light Microscope

The mean percentages of phagocytosis as depicted in Fig. 2A-2B by neutrophils received PBS, quercetin and CytB revealed under light microscope that the ability of phagocytosis can be enhanced when supplementation of quercetin in the condition other than with PBS or an inhibitor (CytB). The average percentages of phagocytosis in PBS, quercetin and CytB experiment group was equivalent to 42.03±4.11, 58.43±4.17 and 39.94±3.13, respectively Fig. 2B, ($p<0.005$).

Gene Expressions by Real-Time PCR

We have previously reported that bovine neutrophils responded well for phagocytosis when supplemented with 50 µM quercetin (Chuammitri *et al*., 2015). Here we further explored the effects of quercetin on the levels of gene expressions during and at the end of phagocytosis of *E. coli*. The results suggested that the expression of genes involved in that process after neutrophils has been supplemented with PBS, quercetin and CytB for 30 min and cells were allowed to intake opsonized bacteria for another 45 min. The measurable gene expression after 45 min-interval on real-time PCR showed the expression of all tested genes; *CORO1A* ($p<0.0001$), *CYBA* ($p <0.0001$), *LAMP1* ($p = 0.0002$), *RAB7A* ($p = 0.0001$), *RAC1* ($p<0.0001$), *PAK1* ($p = 0.4464$), in the quercetin group had higher levels than that of either control (PBS) or CytB supplemented groups. Although the expression level of *PAK1* in the quercetin group was higher when compared to the control group, but the expression of this gene in CytB has increased as well, Fig. 3A. Unsupervised hierarchical clustering confirmed that the gene expression profile of quercetin-treated neutrophils helps increase the phagocytic activity Fig. 3B.

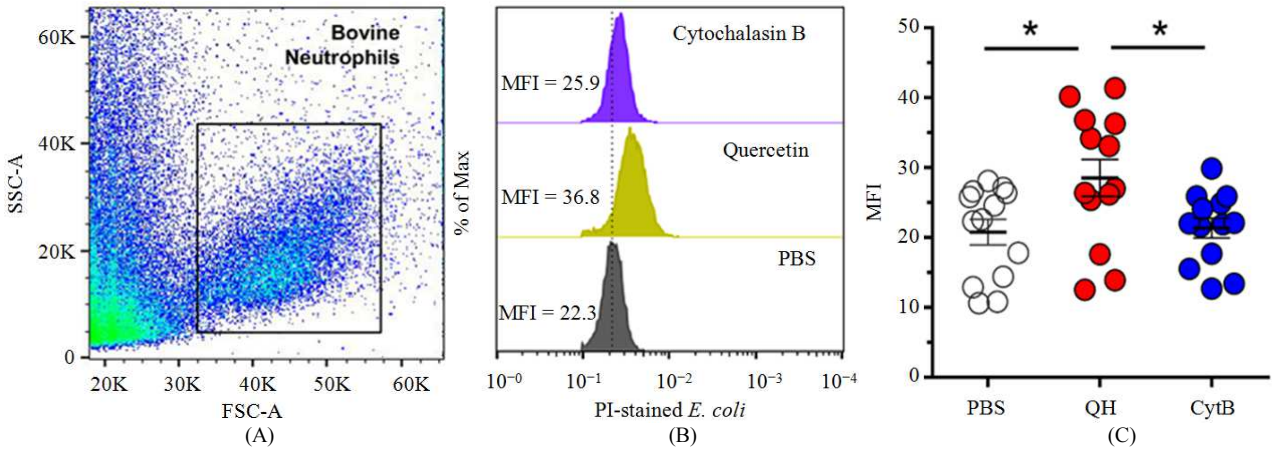

Fig. 1. Representative flow cytometry results of phagocytosis of PI-stained *E. coli* in bovine neutrophils. (A) Gating strategy of bovine neutrophils (B) Histograms shows Median Fluorescence Intensity (MFI) of each treatment (PBS, Quercetin (QH) and cytochalasin B (CytB) as inhibitor). (C) Each data point in scatter plots correspond to an individual cow (n = 13 each group). Data are mean ± SE, one-way ANOVA, * $p<0.05$

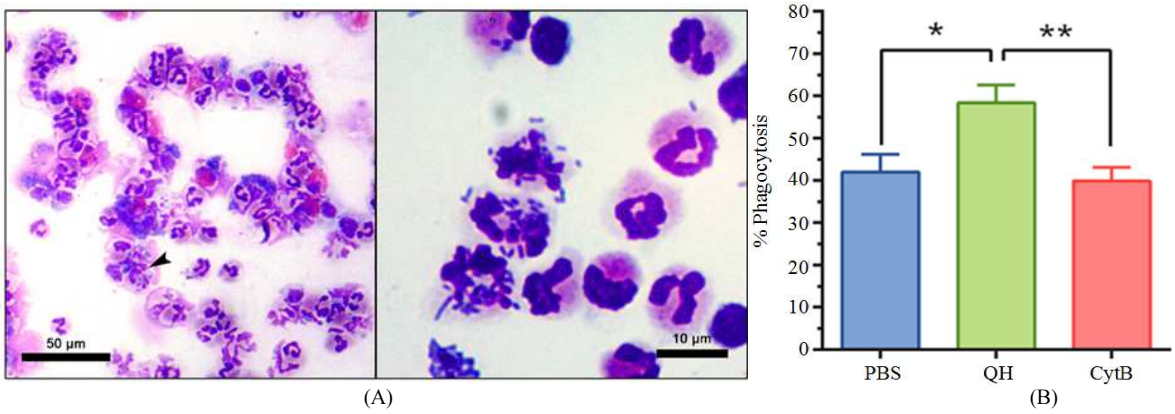

Fig. 2. Representative microscopic images of bovine phagocytosis of live *E. coli* under supplementation of quercetin. (A) Arrowhead points to phagocytosing neutrophil, 100× (left) and 200× magnification (right) (B) The inclusive data show the percentage of phagocytosis in PBS, Quercetin (QH) and cytochalasin B (CytB) groups. Data are mean ± SE of three independent experiments (n = 11-13 each group), one-way ANOVA, * $p<0.05$, ** $p<0.01$

Time-Course of Gene Expressions

As reported earlier in this section that quercetin enhanced genes the expression of the phagocytic process at the determining time (45 min), we asked that the dynamic of genes involved in that process may depend on contact time with quercetin treatment. Neutrophils were fortified with PBS or quercetin for 30, 60 and 90 min of allotted time. The expression of all studied genes after receiving quercetin have changed dramatically in quercetin as measured by real-time PCR Fig. 4. The data show that cells in the quercetin group had higher expression levels of *CORO1A*, *LAMP1*, *RAB7A* and *RAC1* compared to the control group at 30 and 60 min of phagocytosis with the highest levels at 60-min Fig. 4A. The expression levels of aforementioned genes in the quercetin group were declined significantly in 90-min time. The gene expression data of *CYBA* and *PAK1* from cell treated with quercetin demonstrated the different patterns. The maximum expression of these two genes was found to be significantly increased at 30 min of incubation, but the levels were gradually decreased at 60 and 90 min-time, respectively Fig. 4A. A heat map of gene expression in quercetin groups at 30 to 60 min-time also depicted the increased in phagocytic activity, but later declined at 90 min-time Fig. 4B.

Pro-Inflammatory Gene Expressions

Effect of quercetin to reduce pro-inflammatory gene expressions in neutrophils being phagocytose *E. coli* was measured by reverse-transcription PCR. The expression of *IL1B* and *TNF* revealed a significant reduction of both *IL1B* ($p = 0.0004$) and *TNF* ($p = 0.026$) by quercetin compared with control Fig. 5.

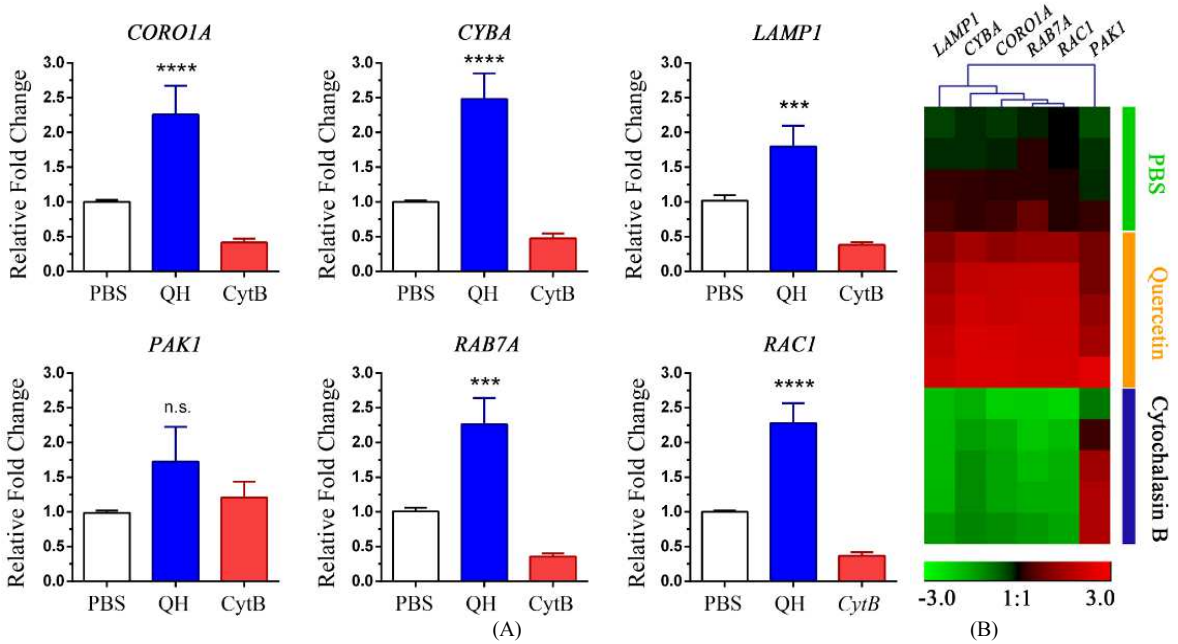

Fig. 3. Real-time RT-PCR analyses of bovine neutrophil genes involved in phagocytosis of *E. coli*. *Top panel* shows relative *CORO1A*, *CYBA*, *LAMP1* expression levels after normalization to *β-actin* expression in PBS, Quercetin (QH) and cytochalasin B (CytB) groups after incubation with live *E. coli* for 45 min. *Bottom panel* shows relative gene expressions of *PAK1*, *RAB7A* and *RAC1*. The results are inclusive of three independent experiments represented as mean ± SE (n = 7-8 each group), one-way ANOVA, *** p<0.001, **** p<0.0001, n.s = no significance

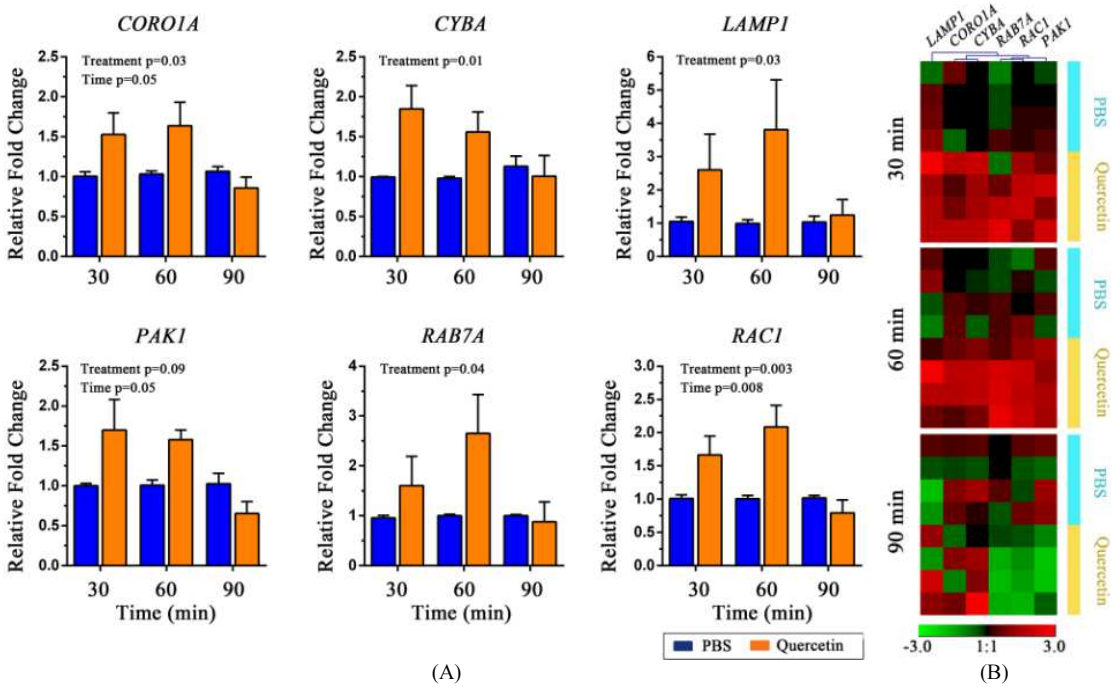

Fig. 4. Time-course differential gene expression levels in bovine phagocytosis by qPCR analyses. *Top panel* shows relative *CORO1A*, *CYBA*, *LAMP1* expression levels after normalization to *β-actin* expression in PBS and quercetin groups after incubation with live *E. coli* for 30, 60 and 90 min. *Bottom panel* shows relative gene expressions of *PAK1*, *RAB7A* and *RAC1*. The results are inclusive of three independent experiments represented as mean ± SE (n = 9-12 each group), Two-way ANOVA (treatment and time effect)

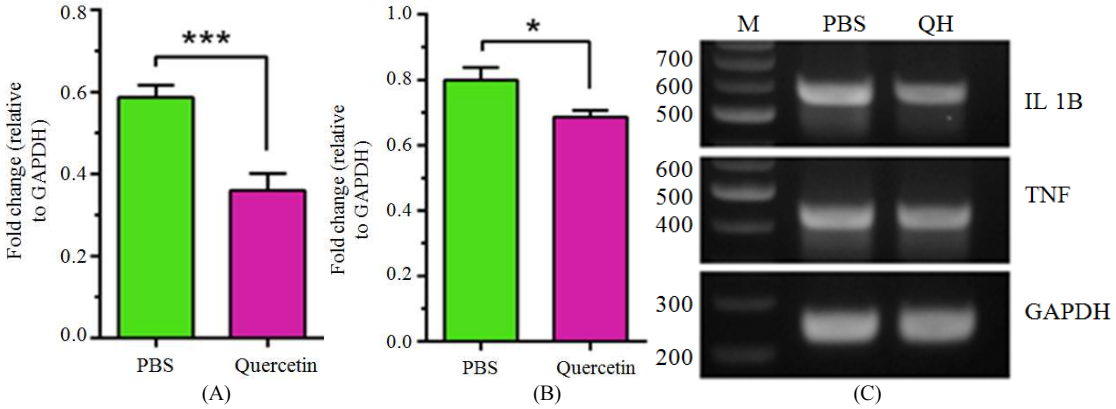

Fig. 5. Quercetin down-regulated the expression of *IL-1β* and *TNF* genes in phagocytosis. (A-B) Relative densitometric analysis of *IL-1β* (A) and *TNF* (B) mRNA expression level compared to *GAPDH*. (C) Representative RT-PCR bands from PBS or quercetin treated neutrophils. Quantification of band intensities was performed using ImageJ. Data are expressed as mean ± SE of two independent experiments (n = 6-9 per group). *p<0.05, ***p<0.001 versus PBS by unpaired t-test

Discussion

Bovine neutrophils ingest bacterial pathogens by a process known as phagocytosis and the ingested microbes are destroyed by the combination of armors equipped within these cells. The present work has emphasized bovine neutrophil phagocytosis of *E. coli* under quercetin supplementation. Our results demonstrate that quercetin mitigates the expression levels of genes associated with inflammation, including *IL-1β* and *TNF*. Those results are consistent with the report that quercetin lowers the level of pro-inflammatory gene expression as a consequence of oxidative stress and different stimuli (Chuammitri *et al*., 2015; Valerio *et al*., 2009). Accordingly, the anti-inflammatory action of quercetin on TNF-α is dose-dependent manner (Nair *et al*., 2006).

Based on the results of recent publication by Chuammitri *et al*. (2017), it could be considered that quercetin might exert its anti-inflammatory effects of mitigating of either proinflammatory genes (*IL1B*, *IL6*, *TNF*) or microRNAs (*MIR24-2*, *MIR146A* and *MIR181C*). Our results in a reduction of IL1β and TNF expressions by action of quercetin agreed with aforementioned study. Even though the two experiment settings used quercetin at 50 μM final concentration, the course of inflammatory processes might originate from diverse pathways. In fact, both bacterial cells (in the current study) and cell wall component (LPS) have one thing in common to initiate inflammatory signals, nevertheless, quercetin could possibly lessen proinflammatory gene expressions via inhibition of key transcription factors (Boesch-Saadatmandi *et al*., 2011). These phenomena could emphasize the important property of quercetin in specifically targeted of transcription factors.

Fundamentally, the transcription factor, NF-κB has played a pivotal role in mediation of genes related to the inflammatory process, such as *IL-1β*, *IL-6* or *iNOS* (Bliss *et al*., 2005; Kobayashi *et al*., 2002). Furthermore, the increased expressions of many genes involved in phagocytosis are observed during the first few hours after induction (Kobayashi *et al*., 2002). This phenomenon may be controlled by the transcription factor NF-κB. Many reports have mentioned that quercetin exerts its modulatory effects by reduction of pro-inflammatory genes through NF-κB inactivation (Boesch-Saadatmandi *et al*., 2011; Cho *et al*., 2003; Nair *et al*., 2006). From previous studies, quercetin inhibits the production of many proinflammatory cytokines, including IL-1β and TNF, the findings from the present work suggest that quercetin may be able to modulate the immune response via the NF-κB pathway.

In the experimental mouse model, supplementation of quercetin and exploratory studies on mRNA expressions of *IL-6*, C-Reactive Protein (*CRP*), Monocyte Chemoattractant Protein 1 (*MCP-1*) and Acyloxyacyl Hydrolase (*AOAH*) were irrelevant between the amount of quercetin intake and the expression levels of genes (Boesch-Saadatmandi *et al*., 2012). This study intends to demonstrate the effects of quercetin on both the influence of phagocytosis and phagosome formation. In order to select the representative genes involved in the process of phagocytosis to be monitored, we have used the KEGG pathway analysis as a searching tool. We have focused on *CORO1A* (coronin), *CYBA* (gp91^{phox}), *LAMP1*, *PAK1*, *RAB7A* and *RAC1,* which are illustrated as our candidate genes. The effects of quercetin have securely confirmed our prediction that quercetin is truly induced those genes during the phagocytosis in bovine neutrophils of live *E. coli* bacteria.

Many published works indicate the actual quercetin effects on gene expression are linked to the exposure time (Cho *et al*., 2003; Liu *et al*., 2005). Our results also show that most gene expression levels increase from 30 to 60 min of the phagocytic period by quercetin actions.

Thus, our results are consistent with a report in the macrophage cells (J774.A1) in which the changes in expression levels of gene transcription and related proteins (Hoffmann *et al*., 2010). In that particular study, the increase production of gene transcriptions and protein productions i.e., *Lamp1/2*, *Rab7* were intensified during 30 to 60 min of incubation (Hoffmann *et al*., 2010; Zhang *et al*., 2005). In this study, the data provide the information that the expression of *CORO1A*, *LAMP*, *RAB7A* and *RAC1* are all obviously increased during phagocytosis. Therefore, it may be concluded that quercetin might affect both the initial and terminal stage of phagocytosis of bovine neutrophils. In addition to the above-mentioned genes, there were some known correlations of activated genes in phagocytosis; *Cdc42*, *Rac1* and *Rho*, that potentially triggering and may have been associated with exposure time (Zhang *et al*., 2005). The F-actin polymerization, as part of internalization and the formation of the phagosome, is controlled by *PAK* gene. This structure could be disrupted by cytochalasin B treatment in which actin polymerization are seized (Bao *et al*., 2012; Berends *et al*., 2010; Zhang *et al*., 2005). In this study, when bovine neutrophils were applied with cytochalasin B, the phagocytic activities of this cell were retarded as measurement by flow cytometry, microscopy or even with the gene expression profiling. The observed data are in accordance with earlier studies (Bao *et al*., 2012; Berends *et al*., 2010).

The engaging genes of phagocytosis in quercetin treated neutrophils tend to maximize most of its expression levels at 60-min interval. After reaching the maximum, it begins to decline after 90 min. This observation is inconsistent with previous study (Kobayashi *et al*., 2002). In that report, the expression studies on receptor-mediated phagocytosis of human PMNs were reached the highest levels in 90 min-time point and began to decline. Our experimental setting and that of previous report are not identical, but the expression levels of our studied genes during 60 min-time period may have shared some important aspects with that study (Kobayashi *et al*., 2002). Although, our studies were not focused on gene expression that involved in ROS molecules or respiratory burst, one of our studied genes in NADPH oxidase (*CYBA*) dictates a partial involvement in phagosome maturation. Our data show that the expression of *CYBA* was apparently increased during the first 30 min of phagocytosis Fig. 4A.

The increasing global concerns over the use of antimicrobial drugs in farmed animals urge us to search for a potential herbal bioactive agent like quercetin, as a feasibility of alternatives to synthesized drugs. From immunological stand point, diets rich in flavonoids, specifically quercetin, have positive health benefits, i.e., reduce infection risk, affect immunity and inflammation. Additionally, solid evidence also suggests that quercetin ultimately benefits in promoting growth and performance and improved feed efficiency in livestock (Gohlke *et al*., 2013; Maciej *et al*., 2015; Stoldt *et al*., 2015).

We speculate that quercetin, in some extents, plays an important role in the regulations at many cellular and molecular levels of bovine neutrophils. Our data herein raises a possibility of the beneficial outcomes of quercetin related to enhancement of bovine neutrophil phagocytosis in combating with pathogenic bacteria. Furthermore, the data in the present study suggest that quercetin further diminishes some proinflammatory cytokines. Our observations are only a starting point for more future research which could result in broadening knowledge of quercetin in bovine neutrophil biology.

Conclusion

This study provides useful information regarding the use of plant derived flavonoid, quercetin, in two important aspects on bovine neutrophils and could be extended to other innate cell types. Our first finding demonstrates that the use of quercetin during the onset of bacterial infections may enhance the ability of neutrophils to phagocytose invading bacteria. The second, supplementation of quercetin would aid in the anti-inflammation property by attenuating the inflammatory mediators as a consequence of the neutrophil functional response to bacteria. The information in our experiments is worth concluded that quercetin has the ability to boost the expression of genes involved in phagocytosis of bovine neutrophils while suppresses the expression of pro-inflammatory genes.

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Author's Contributions

Suphakit Srikok and Sukij Nambut: Execute the study and involve in data acquisition, as well as, analyze and interpret the data. Read and approve the final manuscript.

Kanruethai Wongsawan: Conceive and design the study, read and approve the final manuscript.

Phongsakorn Chuammitri: Conceive and design the study, execute the study and involve in data acquisition, as well as, analyze and interpret the data. Draft and revise the manuscript. Read and approve the final manuscript.

Disclosure Statement

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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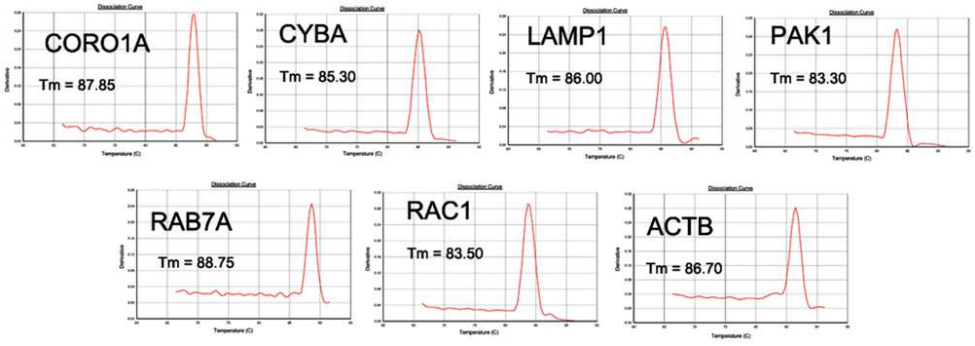
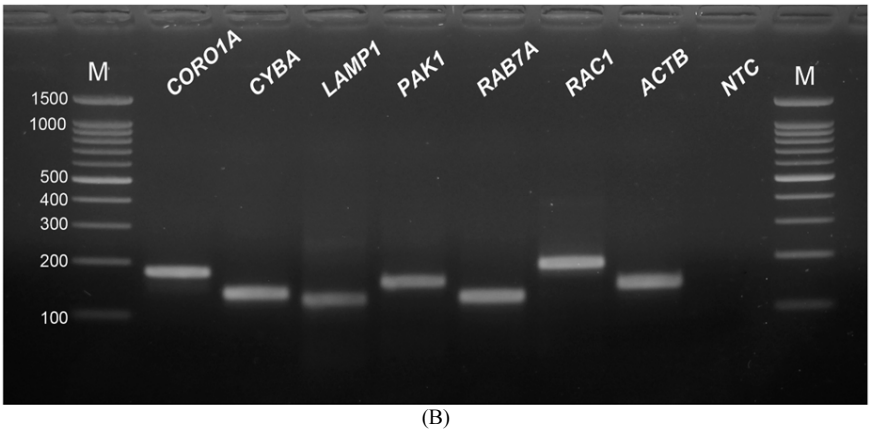


(A)



(B)

Suppl. Fig. 1. (A) Representatives of peak in melting profile (dissociation curves) of the real-time PCR reactions. (B) Representative real-time PCR products of expressed genes in bovine neutrophils on agarose gel electrophoresis and EtBr staining. All PCR products had the correct size. *ACTB* was used as a reference gene. NTC = no template control, M = 100 bp DNA marker