Monitoring mRNA expression patterns in macrophages in response to two different strains of probiotics

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**Running headline:** Controlling macrophage polarization by probiotics
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Abbreviations: DEG, differentially expressed gene; FBS, fetal bovine serum; FDR, false discovery rate; IL, interleukin; pAPC, professional antigen presenting cell; PBS, phosphate-buffered saline; PM, peritoneal macrophage; PRR, pattern recognition receptor; qRT-PCR, quantitative reverse-transcriptase real-time PCR; TNF, tumor necrosis factor.

Total words in abstract: 224

Total words in text: 1685
Short Communications

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Abstract

As an initial study to elucidate the molecular mechanism of how probiotics modulate macrophage activity, we monitored mRNA expression patterns in peritoneal macrophages (PMs) treated with two different strains of probiotics. After treatment with either *Weissella cibaria* WIKIM28 or *Latilactobacillus sakei* WIKIM50, total RNAs from PMs were isolated and subjected into gene chip analyses. As controls, mRNAs from vehicle (phosphate-buffered saline, PBS)-treated PMs were also subjected to gene chip analysis. Compared to vehicle (PBS)-treated PMs, WIKIM28-treated and WIKIM50-treated PMs exhibited a total of 889 and 432 differentially expressed genes (DEGs) with expression differences of at least 4 folds, respectively. Compared to WIKIM28-treated PMs, WIKIM50-treated PMs showed 25 up-regulated genes and 21 down-regulated genes with expression differences of more than 2 folds. Interestingly, mRNA transcripts of M2 macrophage polarization marker such as *anxa1*, *mafb*, and *sepp1* were increased in WIKIM50-treated PMs comparing to those in WIKIM28-treated PMs. Reversely, mRNA transcripts of M1 macrophage polarization marker such
as *hdac*9, *ptgs*2, and *socs*3 were decreased in WIKIM50-treated PMs comparing to those in WIKIM28-treated PMs. In agreement with these observations, mRNA expression levels of tumor necrosis factor-α (TNF-α) and interleukin-1α (IL-1α) were significantly reduced in WIKIM50-treated macrophages compared to those in WIKIM28-treated macrophages. These results may indicate that probiotics can be classified as two different types depending on their ability to convert macrophages into M1 or M2 polarization.

**Keywords:** differentially expressed gene, macrophage, probiotics
Colonization of selective probiotics in gastrointestinal tract might lead to immune modulation within gut microenvironment by communications with mucosal immune cells (Belkaid and Hand, 2014). Furthermore, the crosstalk between probiotics and immune cells could affect the status of various physiological conditions of humans (Kumari et al., 2021). According to this, several connections between gut and specific organs such as gut-brain, gut-liver, and gut-lung axes have been proposed to explain the functional importance of selective probiotics (Dumas et al., 2018; Powell et al., 2017; Tripathi et al., 2018).

Probiotics can modulate immune responses by mainly interacting with pattern recognition receptors (PRRs) expressed on professional antigen presenting cells (pAPCs) or epithelial cells (Fukata and Arditi, 2013). After interacting with PRRs, specific receptor-mediated signal transduction pathway can turn on and change the phenotype of pAPCs (Fukata and Arditi, 2013). Besides dendritic cells, macrophages are a major population of pAPCs that can modulate inflammatory responses by either
triggering inflammation or suppressing inflammation for tissue repair (Lawrence and Natoli, 2011). Therefore, two types of macrophages, inflammatory macrophages (M1 macrophages) and anti-inflammatory macrophages (M2 macrophages), have been proposed depending on their functions (Lawrence and Natoli, 2011). Recently, many observations have demonstrated that M1 or M2 macrophage skewing conditions might affect several pathologic conditions in human diseases (Shapouri-Moghaddam et al., 2018).

Among probiotics, *Weissella cibaria* is considered as a good starter of Kimchi fermentation because it has many beneficial effects including anti-microbial activities, antioxidant activities and immunomodulatory effects (Lim et al., 2017; Yu et al., 2018; Zu et al., 2022). *Latilactobacillus sakei* is also used for the food fermentation or preservation. Especially, *L. sakei* is known for a commercial meat starter of meat fermentation (Leroy et al., 2006). Therefore, *W. cibaria* and *L. sakei* are good resources for food industry.

In the present study, we profiled mRNA expression patterns in macrophages after treatment with two different strains of probiotics isolated from Kimchi,
Weissella cibaria WIKIM28 and Latilactobacillus sakei WIKIM50 (Lim et al., 2017). Results of this study might provide valuable data to understand the molecular mechanisms of macrophage polarization triggered by different types of probiotics.
Materials and methods

Probiotics

Isolation and species identification of *Weissella cibaria* WIKIM28 from Gat Kimchi have been described previously (Lim et al., 2017). WIKIM50 was isolated from Baechu Kimchi and identified as *Latilactobacillus sakei* based on 16S rRNA gene sequencing according to a previously established method (Lim et al., 2017). WIKIM28 and WIKIM50 were sub-cultured more than 10 times before treating PMs for RNA-Seq data analyses.

Stimulation of PMs by probiotics

PMs were isolated according to a previously established method (Pineda-Torra et al., 2015). Briefly, 3% thioglycolate (Becton Dickinson, Sparks, MD, USA) in phosphate-buffered saline (PBS) was intraperitoneally injected into C57BL/6 mice
Orient Bio, Seongnam-si, Korea) at 1 mL per mouse. On day 3, thioglycolate-injected mice were sacrificed to harvest peritoneal lavages. Cell pellets were then acquired after centrifuging peritoneal lavages at 1,500 rpm for 5 min at 4 °C and resuspended in DME/F-12 (Thermo Fisher Scientific, Waltham, MA, USA) medium supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% penicillin/streptomycin (DMEM/F-12-10). After resuspension, cells were plated into tissue culture plates and incubated in a humidified 5% CO₂ atmosphere at 37 °C. After overnight culture, adherent cells were harvested and counted. The purity of peritoneal macrophages was validated by flow cytometry analyses using anti-mouse CD11b (eBioscience, San Diego, CA, USA) and anti-mouse F4/80 (BioLegend, San Diego, CA, USA) antibodies. More than 90% of cells were CD11b⁺F4/80⁺ cells (data not shown). After isolation, PMs (2 × 10⁶ cell/well) were seeded into a 12-well plate in 1 mL of DMEM/F-12-10. Heat killed WIKIM28 or WIKIM50 (2 × 10⁷ CFU/mL) was co-cultured with PMs in a humidified 5% CO₂ atmosphere at 37 °C for 24 hours. As controls, vehicle (PBS) was used to treat PMs without incubation with probiotics.
Gene chip analyses

Total RNAs were isolated from vehicle (PBS)-, WIKIM28-, or WIKIM50-treated PMs using TRIzol (Thermo Fisher Scientific) according to the manufacturer's protocol. The quality and quantity of each RNA sample were measured using an ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, USA) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Gene chip analyses were then performed using Agilent SurePrint G3 Mouse gene expression V2 microarrays (8 × 60K) according to the manufacturer’s protocol (Agilent Technology, V 6.5, 2010).

Briefly, total RNAs from each sample were linearly amplified and labeled with Cy3-dCTP. These labeled cRNAs were then purified with an RNAeasy mini kit (Qiagen, Hilden, Germany). The concentration and specific activity of the labeled cRNAs (pmol Cy3/μg cRNA) were measured with an ND-1000 spectrophotometer. For hybridization of microarray, each labeled cRNA was fragmented by adding 5 μl of 10 × blocking agent and 1 μl of 25 × fragmentation buffer and then heated at 60 °C for 30 min. After heating, 25 μl of 2 × hybridization buffer was added to dilute the labeled cRNA.
Subsequently, 40 μl of hybridization solution was loaded into the gasket slide and assembled to microarray slides (Agilent Technologies). After assembling, microarray slides were incubated at 65 °C in an Agilent hybridization oven (Agilent Technologies) for 17 hours. After incubation, microarray slides were washed at room temperature using washing buffer provided by manufacturer (Agilent Technologies). The hybridized array was then immediately scanned with an Agilent Microarray Scanner D (Agilent Technologies).

Raw data were extracted using a Feature Extraction Software (v11.0.1.1) provided by Agilent (Agilent Technologies). Comparative analysis between test sample and control sample was carried out using paired t-test. False discovery rate (FDR) was controlled by adjusting p-value using Benjamini-Hochberg algorithm (Benjamini and Hochberg, 1995). For a differentially expressed genes (DEG) set, hierarchical cluster analysis was performed using complete linkage and Euclidean distance as a measure of similarity.
Transcriptional analysis by quantitative reverse-transcriptase real-time PCR (qRT-PCR)

To confirm gene chip analyses, qRT-PCR were conducted as described previously (Choi et al., 2018). Briefly, total RNAs from vehicle (PBS)-, WIKIM28-, or WIKIM50-treated PMs were isolated for synthesizing cDNAs using a First-Strand cDNA Synthesis Kit with oligo-dT primers (SuperScript RT; Thermo Fisher Scientific). Subsequently, quantitative real-time PCR was performed using one μg of each total RNA with QGreen™ 2X qPCR Master Mix (GenDEPOT, Katy, TX, USA) on a Bio-Rad CFX96 Real-Time Detection System (Bio-Rad, Hercules, CA, USA). Expression of each mRNA transcript was compared to the expression level of mouse Gapdh to obtain relative gene expression. Primer sequences for mouse gapdh were 5′-

ATGGTGAAGGTCGGTGTGAA-3′ (sense) and 5′-

GGTCGTTAGGGCAACAATCTC-3′ (anti-sense), resulting in a 100 bp product (Kang et al., 2022). PCR primers of M1 or M2 marker genes used in this study are listed in Table 1.
Table 1. Primer sets of M1 or M2 macrophage signature genes analyzed by quantitative real-time reverse transcriptase-PCR (qRT-PCR)

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Classification</th>
<th>Primer sequence (5′→ 3′)</th>
<th>Tm (℃)</th>
<th>Product size</th>
<th>Genbank accession</th>
</tr>
</thead>
<tbody>
<tr>
<td>anxa1</td>
<td>M2 macrophage marker</td>
<td>F: AGCTCTGGATCTGGAACTGA</td>
<td>55.4</td>
<td>103</td>
<td>NM_010730.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: TTCGTACAGCTTTCTCGGCAA</td>
<td>55.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>M2 macrophage marker</td>
<td>F: GCGAGCAACTACCAGCAGAT</td>
<td>57.4</td>
<td>125</td>
<td>NM_010658</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: GCACGGAAGCCGTCGAA</td>
<td>59.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>M2 macrophage marker</td>
<td>F: GAGCATCTTGCGACGAGTAA</td>
<td>55.4</td>
<td>140</td>
<td>NM_001042614</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: GTGGTGTCCTAGCTCTCTAA</td>
<td>55.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>M1 macrophage marker</td>
<td>F: ATCGCTAGTGGACGTGAA</td>
<td>55.4</td>
<td>103</td>
<td>NM_024124</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: GATCCCACAGGCATCATC</td>
<td>57.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>M1 macrophage marker</td>
<td>F: ATCGGTCAATGGTGAGAGG</td>
<td>58.2</td>
<td>137</td>
<td>NM_011198</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: CATCAGACCAGGCACAGA</td>
<td>57.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>M1 macrophage marker</td>
<td>F: CCTCAAGACCTTCAGCTCCAA</td>
<td>58.2</td>
<td>82</td>
<td>NM_007707</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: CCAGTAGAATCCGCTCTCTT</td>
<td>57.4</td>
<td></td>
<td></td>
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</table>

Statistical analyses

Statistical significance of independent variable values was evaluated by Student’s t-test. Significant differences at a confidence level of 95%, 99%, 99.9% are labeled on each graph and indicated by asterisks ‘*’, ‘**’, and ‘***’, respectively.
Results and Discussion

DEGs in PMs treated with either WIKIM28 or WIKIM50

Compared with vehicle (PBS)-treated PMs, PMs treated with WIKIM28 and WIKIM50 showed a total of 889 and 432 DEGs with expression differences of at least 4 folds, respectively. Among them, 652 from WIKIM28-treated cells and 375 genes from WIKIM50-treated cells were up-regulated compared to those in vehicle (PBS)-treated PMs. Contrarily, 237 genes from WIKIM28-treated cells and 57 genes from WIKIM50-treated cells were down-regulated compared to those in vehicle (PBS)-treated cells.

DEGs with at least 2-fold expression difference in WIKIM50-treated cells compared to those in WIKIM28-treated cells are listed in Table 2. Among them, 25 genes were up-regulated, and 21 genes were down-regulated in WIKIM50-treated cells compared to those in WIKIM28-treated cells. Interestingly, several M2 macrophage marker genes such as anxa1, mafb, and sepp1 were up-regulated in WIKIM50-treated cells, whereas several M1 macrophage marker genes such as hdac9, ptgs2, and socs3
were down-regulated in WIKIM50-treated cells compared with those in WIKIM28-treated cells (Arnold et al., 2014; Barrett et al., 2015; Kim, 2017; Liu et al., 2021; Lu et al., 2017; Moraes et al., 2017). These results indicate that WIKIM28-treated macrophages are more likely to be polarized into M1 phenotype whereas WIKIM50-treated macrophages might exhibit M2 phenotype. Supporting this idea, mRNA transcripts of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and IL-1α were significantly decreased in WIKIM50-treated macrophages compared to those in WIKIM28-treated macrophages (Table 2).

Besides genes involved in macrophage polarization, genes encoding several cell surface proteins including PRRs, chemokine receptors, cytokine receptors were also identified as DEGs (Table 2). However, there was no clear physiological relevance in these DEGs.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene description</th>
<th>Fold increase</th>
<th>Gene Bank access</th>
</tr>
</thead>
<tbody>
<tr>
<td>cd55</td>
<td>CD55 antigen</td>
<td>4.04</td>
<td>NM_010016</td>
</tr>
<tr>
<td>card11</td>
<td>Caspase recruitment domain family, member 11</td>
<td>3.97</td>
<td>NM_175362</td>
</tr>
<tr>
<td>rgs2</td>
<td>Regulator of G-protein signaling 2</td>
<td>3.64</td>
<td>NM_009061</td>
</tr>
<tr>
<td>cxcr3</td>
<td>Chemokine (C-X-C motif) receptor 3</td>
<td>3.35</td>
<td>NM_009910</td>
</tr>
</tbody>
</table>
### mafb
v-maf musculoaponeurotic fibrosarcoma oncogene family, protein B (avian) 2.99 NM_010658

### tlr12
Toll-like receptor 12 2.79 NM_205823

### nlrp1b
NLR family, pyrin domain containing 1B 2.78 NM_001040696

### mrc1
Mannose receptor, C type 1 2.74 NM_008625

### nfatc2
Nuclear factor of activated T cells, cytoplasmic, calcineurin dependent 2 2.63 NM_001136073

### cd93
CD93 antigen 2.62 NM_010740

### tlr12
Toll-like receptor 12 2.79 NM_010559

### mrc1
Mannose receptor, C type 1 2.47 NM_011580

### st6gal1
Beta galactoside alpha 2,6 sialyltransferase 1 2.45 NM_145933

### pparg
Peroxisome proliferator activated receptor gamma 2.36 NM_0011146

### ccl6
Chemokine (C-C motif) ligand 6 2.34 NM_009139

### c3ar1
Complement component 3a receptor 1 2.33 NM_009779

### sepp1
Selenoprotein P, plasma, 1 2.22 NM_001042614

### anxa1
Annexin A1 2.12 NM_010730

### ccr5
Chemokine (C-C motif) receptor 5 2.12 NM_009917

### msp1
Mannan-binding lectin serine peptidase 1 2.07 XM_006521828

### iqgap3
IQ motif containing GTPase activating protein 3 2.04 NM_001033484

### cd300lb
CD300 antigen like family member B 2.02 NM_199221

### alox5
Arachidonate 5-lipoxygenase 2.01 NM_009662

### celf2
CUGBP, Elav-like family member 2 2.01 NM_010160

### clec9a
C-type lectin domain family 9, member a 2.00 NM_001205363

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**Down-regulated genes in WIKIM50-treated macrophages compared to those in WIKIM28**

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene description</th>
<th>Fold increase</th>
<th>Gene Bank access</th>
</tr>
</thead>
<tbody>
<tr>
<td>cxcl2</td>
<td>Chemokine (C-X-C motif) ligand 2</td>
<td>-22.80</td>
<td>NM_009140</td>
</tr>
<tr>
<td>cxcl1</td>
<td>Chemokine (C-X-C motif) ligand 1</td>
<td>-17.74</td>
<td>NM_008176</td>
</tr>
<tr>
<td>ptgs2</td>
<td>Prostaglandin-endoperoxide synthase 2</td>
<td>-11.32</td>
<td>NM_011198</td>
</tr>
<tr>
<td>cav1</td>
<td>Caveolin 1, caveolae protein</td>
<td>-9.92</td>
<td>NM_001243064</td>
</tr>
<tr>
<td>cxcl5</td>
<td>Chemokine (C-X-C motif) ligand 5</td>
<td>-5.33</td>
<td>NM_009141</td>
</tr>
<tr>
<td>tnf</td>
<td>Tumor necrosis factor</td>
<td>-5.25</td>
<td>NM_013693</td>
</tr>
<tr>
<td>il1a</td>
<td>Interleukin 1 alpha</td>
<td>-4.74</td>
<td>NM_010554</td>
</tr>
<tr>
<td>hdac9</td>
<td>Histone deacetylase 9</td>
<td>-4.64</td>
<td>NM_024124</td>
</tr>
<tr>
<td>dnmt3l</td>
<td>DNA (cytosine-5-)-methyltransferase 3-like</td>
<td>-4.51</td>
<td>NM_019448</td>
</tr>
<tr>
<td>nlrp1a</td>
<td>NLR family, pyrin domain containing 1A</td>
<td>-3.35</td>
<td>NM_001004142</td>
</tr>
<tr>
<td>maff</td>
<td>v-maf musculoaponeurotic fibrosarcoma oncogene family, protein F (avian)</td>
<td>-3.30</td>
<td>NM_010755</td>
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<tr>
<td>cxcl10</td>
<td>Chemokine (C-X-C motif) ligand 10</td>
<td>-3.23</td>
<td>NM_021274</td>
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**Table 1**: Gene expression changes in M2-polarized macrophages compared to M2-unpolarized macrophages.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Expression Change</th>
<th>Gene ID</th>
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<tr>
<td>il20rb</td>
<td>Interleukin 20 receptor beta</td>
<td>-3.21</td>
<td>NM_001033543</td>
</tr>
<tr>
<td>ccl3</td>
<td>Chemokine (C-C motif) ligand 3</td>
<td>-3.10</td>
<td>NM_011337</td>
</tr>
<tr>
<td>cd40</td>
<td>CD40 antigen</td>
<td>-3.00</td>
<td>NM_011611</td>
</tr>
<tr>
<td>ccl7</td>
<td>Chemokine (C-C motif) ligand 7</td>
<td>-2.96</td>
<td>NM_013654</td>
</tr>
<tr>
<td>il10</td>
<td>Interleukin 10</td>
<td>-2.94</td>
<td>NM_010548</td>
</tr>
<tr>
<td>sox1</td>
<td>Suppressor of cytokine signaling 1</td>
<td>-2.80</td>
<td>NM_009896</td>
</tr>
<tr>
<td>sox3</td>
<td>Suppressor of cytokine signaling 3</td>
<td>-2.72</td>
<td>NM_007707</td>
</tr>
<tr>
<td>rgs16</td>
<td>Regulator of G-protein signaling 16</td>
<td>-2.57</td>
<td>NM_011267</td>
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<tr>
<td>cd38</td>
<td>CD38 antigen</td>
<td>-2.40</td>
<td>NM_007646</td>
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</table>

Note: DEGs with at least 2 folds.

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**Validation of gene expression patterns by qRT-PCR**

To validate gene chip data, mRNA expression levels of DEGs related to macrophage polarization were monitored by qRT-PCR (Fig. 2). Results of qRT-PCR analyses clearly showed that M2 macrophage marker genes such as *anxa1*, *mafb*, and *sepp1* were significantly decreased in WIKIM28-treated cells, whereas M1 macrophage marker genes such as *hdac9*, *ptgs2*, and *sox3* were significantly increased in WIKIM28-treated PMs compared with those in WIKIM50-treated PMs. Similar to our study, previous reports have demonstrated modulation of macrophage polarization by several different probiotics (Wang et al., 2020). Therefore, consumption of particular probiotics which shifts macrophage into M1 or M2 phenotype...
might help alleviate specific pathologic conditions of human diseases. For example, oral
uptake of several different strains of probiotics can ameliorate colitis or hepatic steatosis
by inducing M2 macrophage polarization in a mouse model (Jang et al., 2014; Sohn et
al., 2015). However, not many studies have attempted to dissect the molecular
mechanism of how certain strains can induce macrophage polarization. Here, we
provide evidence indicating that signature genes of macrophage polarization are
changed depending on probiotic strains. These results might be useful for finding the
molecular clue of macrophage polarization caused by probiotics. Further studies will be
required to define molecular signaling pathways involved in WIKIM28- or WIKIM50-
mediated macrophage polarizations.
Conclusion

In conclusion, our gene chip data suggest that macrophage polarization might be modulated by treatment with different probiotic strains. Therefore, oral uptake of specific strains of probiotics might change human physiology by shifting macrophage phenotypes into either M1 or M2 polarity.
None of authors have any conflict of interests.
Acknowledgements

This research was supported by grants from the World Institute of Kimchi (KE1501-2 and KE2301-2).
**Author Contributions**

Conceptualization: Chun T, Choi HJ. Data curation: Choi SP, Park SW. Formal analysis: Choi SP, Park SW. Methodology: Choi SP, Park SW, Kang SJ, Lim SK, Kwon MS. Software: Kang SJ. Validation: Choi SP, Park SW. Investigation: Chun T, Choi HJ. Writing - original draft: Choi SP, Park SW. Writing - review & editing: Choi SP, Park SW, Kang SJ, Lim SK, Kwon MS, Choi HJ, Chun T.
Ethics Approval

A protocol of animal study was approved by the Institutional Animal Care and Use Committee (IACUC) of Korea University (protocol numbers: KUIACUC-2015-0430).


Zhu XK, Yang BT, Hao ZP, Li HZ, Cong W, Kang YH. 2022. Dietary supplementation with *Weissella cibaria* C-10 and *Bacillus amyloliquefaciens* T-5 enhance immunity against *Aeromonas veronii* infection in crucian carp (*Carassiu auratus*). Microb Patho 167:105559.
**Figure Legends**

**Fig. 1.** WIKIM50-treated macrophages increase mRNA transcripts of M2 signature genes and decrease mRNA transcripts of M1 signature genes compared to WIKIM28-treated macrophages. Peritoneal macrophages (PMs) (2 × 10^6 cell/well) were co-cultured with heat killed WIKIM28 or WIKIM50 (2 × 10^7 CFU/mL) for 24 hours. Subsequently, quantitative reverse-transcriptase real-time PCRs (qRT-PCRs) were performed using total RNA from each sample as a template to determine relative mRNA expression levels of M1 or M2 macrophage signature genes. The relative quantitation of each gene expression was normalized to expression level of mouse *gapdh*. All results are shown as means ± standard errors. * p < 0.05; ** p < 0.01; *** p < 0.001 (n = 3). Vehicle, phosphate-buffered saline (PBS)-treated macrophages; WIKIM28, WIKIM28-treated macrophages, WIKIM50, WIKIM50-treated macrophages.