

CHAPTER 5

Results

5.1 Phagocytosis assay

To compare the percentages of phagocytosis against *P. marneffei* conidia of wild-type and laccase mutant strains (quadruple *lac* gene deletion strain and single *lac* gene deletion strains), the number of macrophages that ingested conidia were determined using microscopy. The results indicated a high efficiency of phagocytic activity of THP-1 macrophage cells against the *P. marneffei* conidia of wild-type and laccase mutants. There was no phagocytosis at the time zero (data not shown). The phagocytosis occurred when the conidia were incubated with the THP-1 macrophage cells for 15 minutes at 37°C. More internalized conidia in the THP-1 macrophage cells were seen after longer incubation time, and reached to the maximum after 120 minutes of incubation. From microscopic observation, the number of conidia which were phagocytosed, was about 0-8 per macrophage (Figure 5.1). The percentages of THP-1 macrophage cells phagocytosing wild-type, quadruple *lac* gene deletion strain and all single *lac* gene deletion strains were presented in Table 5.1. The percentage of phagocytosis was similar (approximately 5-6 %) for all strains at 15 and 30 minutes of incubation, and reached to the maximum of all strains of *P. marneffei* at 120 minutes of incubation. As a comparison, the conidia of quadruple *lac* gene deletion strain was phagocytosed by macrophage better than wild-type and all single *lac* gene deletion strains. Statistical analyses of the results showed no significant difference in percent

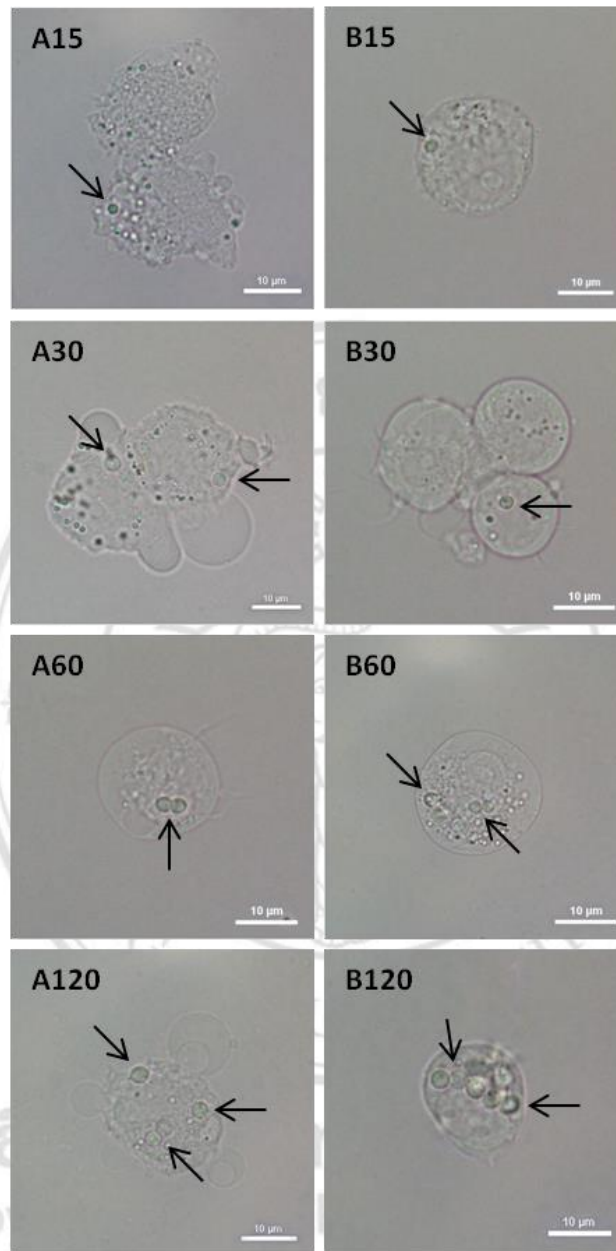


Figure 5.1 Phagocytosis of *P. marneffei* wild-type and quadruple *lac* gene deletion strain by THP-1 macrophage cells. Microscopic observation was performed at time 15, 30, 60 and 120 minutes (columns A and B represent wild-type and quadruple *lac* gene deletion strain, respectively). The arrows indicate the intracellular conidia (magnification, 1,000x).

phagocytosis of the laccase mutant strains and wild-type at 15 and 30 minutes of incubation. However, there were a significant difference between quadruple *lac* gene deletion strain and wild-type at 60 and 120 minutes of incubation ($P<0.05$) (Figure 5.2).

The phagocytic index of wild-type, quadruple *lac* gene deletion strain and all single *lac* gene deletion strains are presented in Table 5.2. The results showed that the phagocytic index of quadruple *lac* gene deletion strain at difference times during infection were significantly higher than those of wild-type and all single *lac* gene deletion strains at 15, 30, 60 and 120 minutes of incubation ($P<0.05$) (Figure 5.3).

Table 5.1 The percentage of phagocytosis of conidia from wild-type, quadruple *lac* gene deletion strain, $\Delta lac1$, $\Delta lac2$, $\Delta lac3$ and $\Delta arb2$ strain by THP-1 macrophage cells at difference incubation times

Time of incubation (minutes)	Strains of <i>P. marneffei</i>					
	Wild-type	Quadruple <i>lac</i> gene deletion	$\Delta lac1$	$\Delta lac2$	$\Delta lac3$	$\Delta arb2$
15	5.50±0.71	6.75±1.06	4.75±1.06	4.75±0.35	5.75±0.35	3.75±0.35
30	12.50±1.41	14.00±1.41	9.00 ± 2.12	12.00±1.41	12.75±1.06	8.25±1.06
60	14.00±1.41	22.25±1.77	13.25±2.47	13.50±0.71	16.00±0.71	12.00±1.41
120	25.00±1.41	31.00±2.12	21.25±1.06	22.75±3.18	23.25±1.06	20.25±1.06

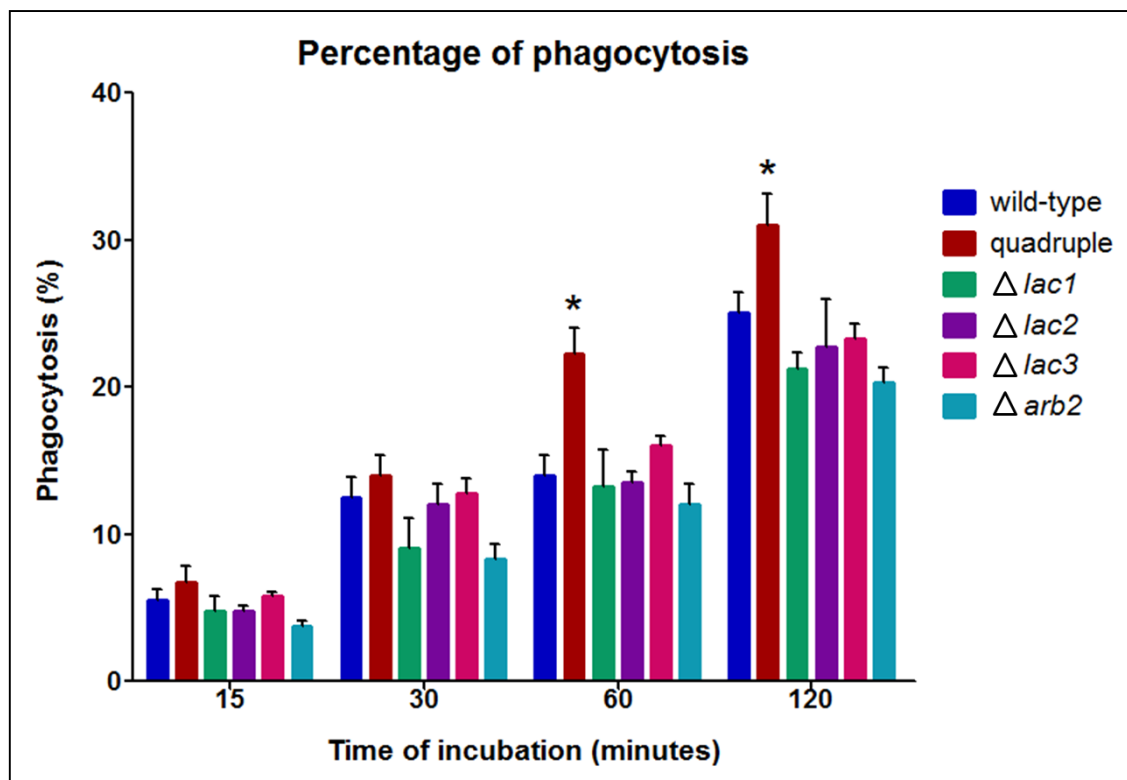


Figure 5.2 The percentage of phagocytosis of conidia from wild-type, quadruple *lac* gene deletion strain, $\Delta lac1$, $\Delta lac2$, $\Delta lac3$ and $\Delta arb2$ strain by THP-1 macrophage cells at difference incubation times. Each bar represents the mean \pm SEM of two experiments, each performed in duplicate. And above for each of the analyzed parameters; * $P < 0.05$ was compared between wild-type and laccase mutant strains. The phagocytosis occurred at 15 minutes of incubation and reached maximum at 120 minutes. There were no significant difference in percentage of phagocytosis between laccase mutant strains and wild-type at 15 and 30 minutes of incubation. However, there were a significant difference between quadruple *lac* gene deletion strain and wild-type at 60 and 120 minutes of incubation.

Table 5.2 The phagocytic index of conidia from wild-type, quadruple *lac* gene deletion strain, $\Delta lac1$, $\Delta lac2$, $\Delta lac3$ and $\Delta arb2$ strain at difference incubation times

Time of incubation (minutes)	Strains of <i>P. marneffei</i>					
	Wild-type	Quadruple <i>lac</i> gene deletion	$\Delta lac1$	$\Delta lac2$	$\Delta lac3$	$\Delta arb2$
15	1.06±0.09	1.86±0.21	1.11±0.15	1.32±0.19	1.33±0.30	1.04±0.05
30	1.18±0.16	1.81±0.13	1.07±0.02	1.27±0.12	1.25±0.06	1.45±0.10
60	1.22±0.15	1.94±0.21	1.02±0.05	1.44±0.07	1.48±0.05	1.17±0.05
120	1.23±0.16	2.28±0.07	1.23±0.12	1.38±0.30	1.61±0.06	1.18±0.16

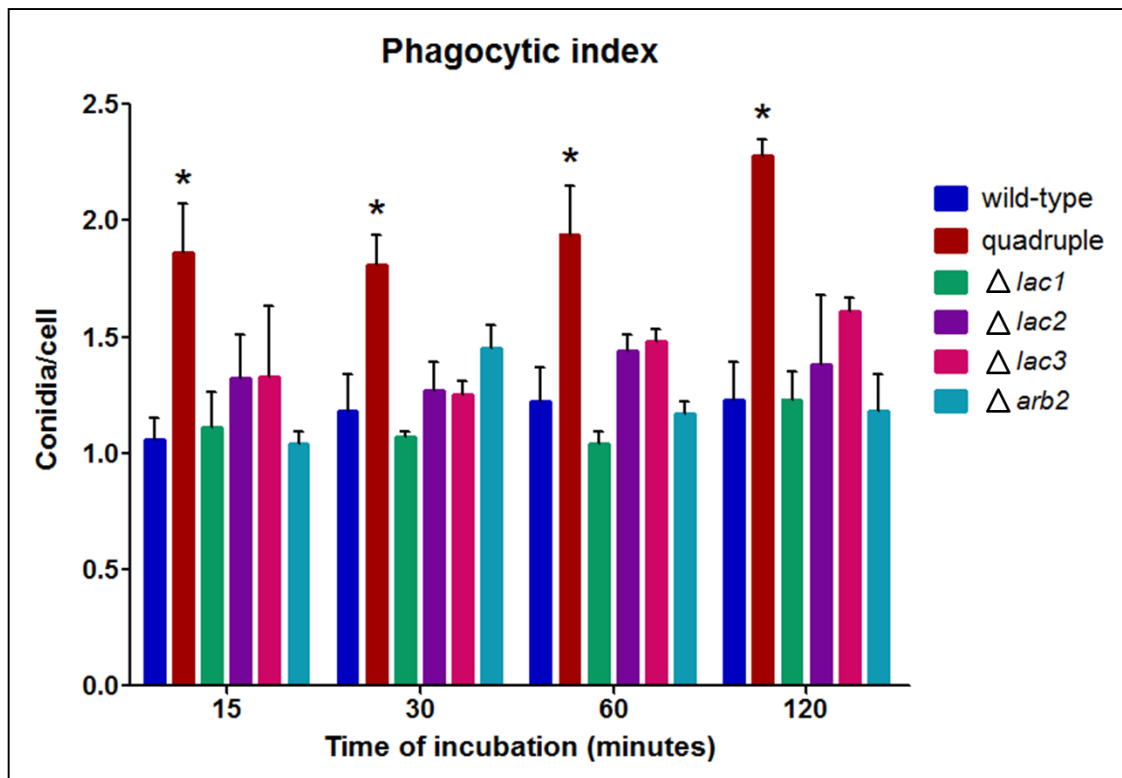


Figure 5.3 The phagocytic index of conidia from wild-type, quadruple *lac* gene deletion strain, $\Delta lac1$, $\Delta lac2$, $\Delta lac3$ and $\Delta arb2$ at difference incubation times. Each bar represents the mean \pm SEM of two experiments, each performed in duplicate. And above for each of the analyzed parameters; * $P < 0.05$ was compared between wild-type and laccase mutant strains. The graph indicated that the phagocytic index of quadruple *lac* gene deletion strain were significantly higher than those of wild-type at 15, 30, 60 and 120 minutes of incubation.

5.2 Killing assay

To compare the percentages of intracellular killing in THP-1 macrophage cells against *P. marneffei* conidia of wild-type and laccase mutant strains, the viable colony count was determined by serial dilution plating. The killing activity of THP-1 macrophage cells against the conidia of wild-type, quadruple *lac* gene deletion strain and all single *lac* gene deletion strains were presented in Table 5.3. The results showed that the killing of all strains occurred when intracellular conidia were incubated for 2 hours, and the intracellular killing in THP-1 macrophage cells were time dependent and reached to the maximum after 8 hours of incubation. The quadruple *lac* gene deletion strain was killed by THP-1 macrophage cells higher than wild-type and all single *lac* gene deletion strains at 2, 4 and 8 hours, while the killing percentages of all single *lac* gene deletion strains were similar to wild-type at difference incubation times. There were no significant difference between quadruple *lac* gene deletion strain and wild-type at 2 hours of incubation ($P>0.05$). However, statistical analyses of the results showed a significant difference the killing percentage of the quadruple *lac* gene deletion strain and wild-type after longer incubation time, 4 and 8 hours ($P<0.05$) (Figure 5.4).

Table 5.3 The percentage of intracellular killing of conidia from wild-type, quadruple *lac* gene deletion strain, $\Delta lac1$, $\Delta lac2$, $\Delta lac3$ and $\Delta arb2$ strain by THP-1 macrophage cells at difference incubation times

Time of incubation (hours)	Strains of <i>P. marneffei</i>					
	Wild-type	Quadruple <i>lac</i> gene deletion	$\Delta lac1$	$\Delta lac2$	$\Delta lac3$	$\Delta arb2$
2	10.08±7.07	17.24±7.97	11.34±4.00	14.70±6.86	14.50±9.60	13.23±6.70
4	23.80±8.71	38.56±4.44	21.04±9.73	24.82±4.64	22.76±9.60	26.54±15.42
8	41.18±8.80	55.19±4.87	40.08±10.29	41.52±9.68	39.63±11.29	40.76±8.47

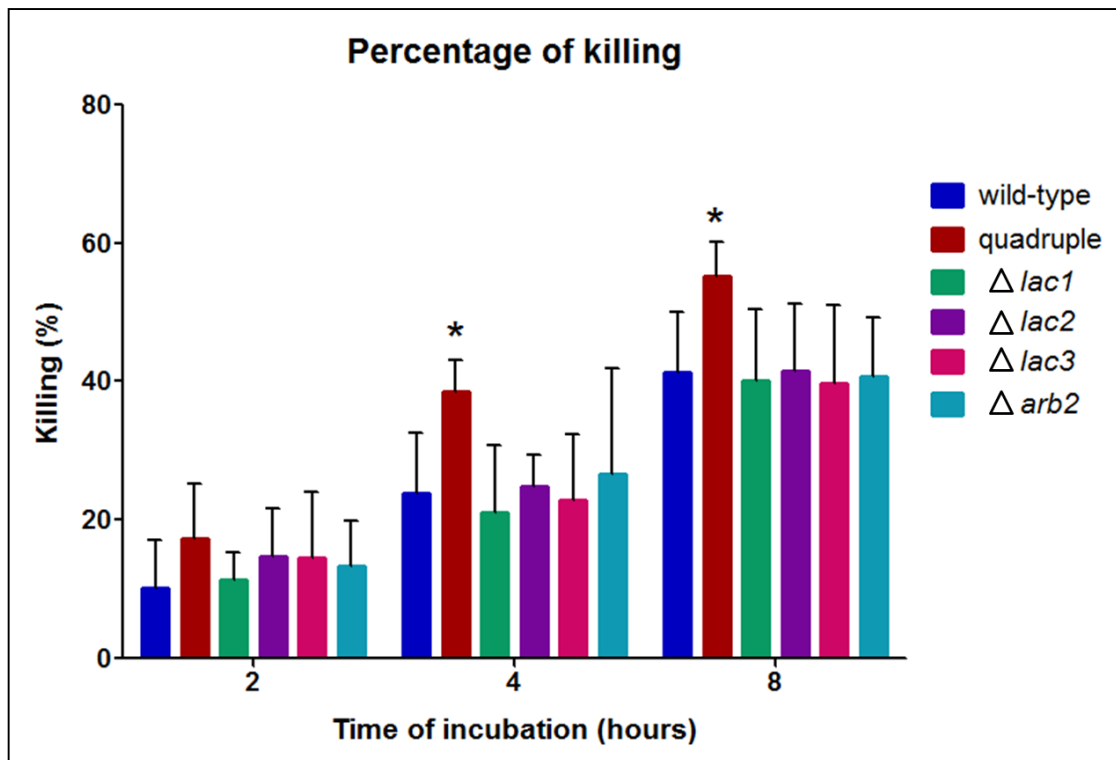
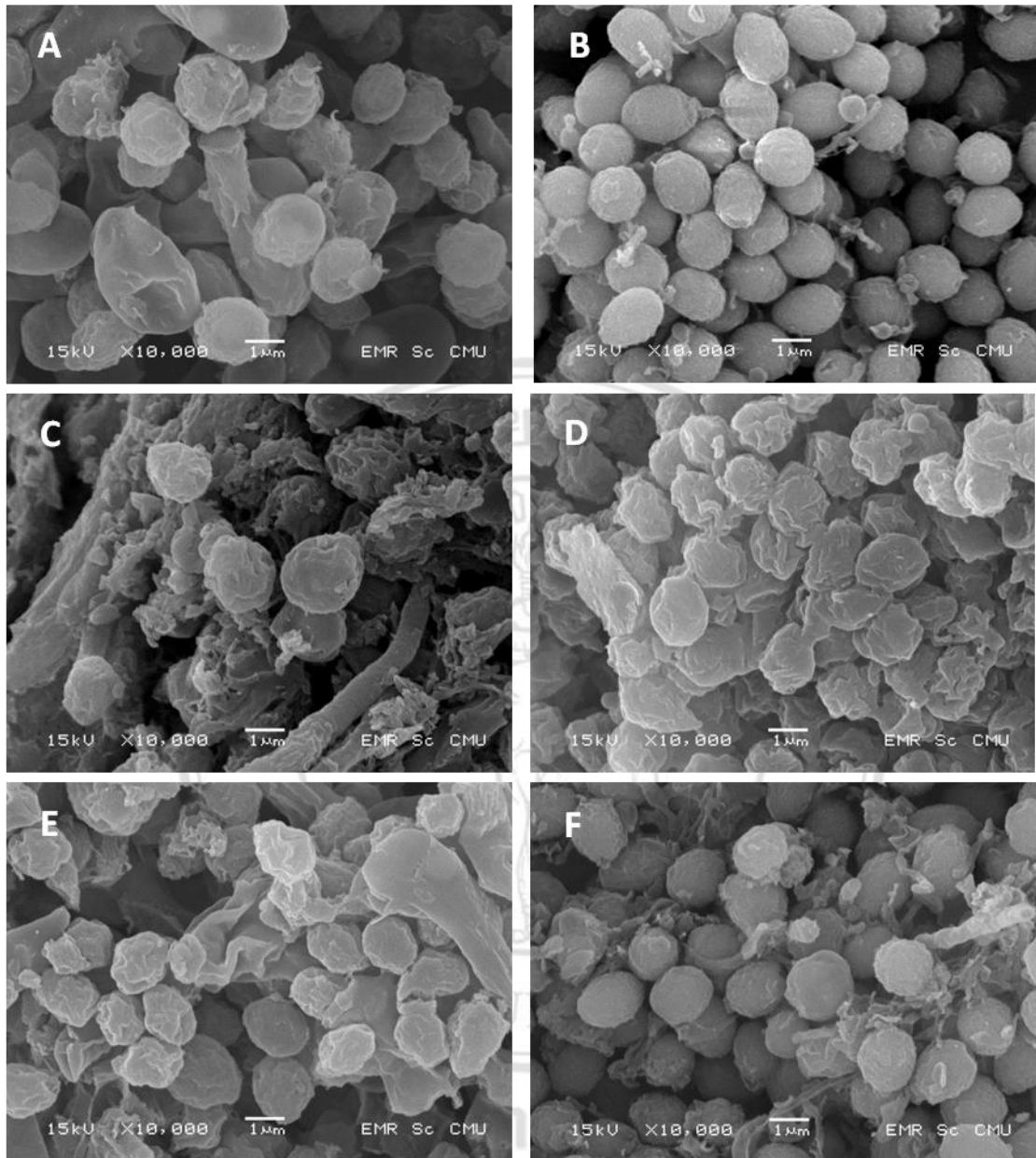


Figure 5.4 The percentage of killing of conidia from wild-type, quadruple *lac* gene deletion strain, $\Delta lac1$, $\Delta lac2$, $\Delta lac3$ and $\Delta arb2$ strain by THP-1 macrophage cells at different incubation times. Each bar represents the mean \pm SEM of two experiments, each performed in duplicate. And above for each of the analyzed parameters; * $P < 0.05$ was compared between wild-type and laccase mutant strains. The killing activity of THP-1 macrophage cells against the conidia of quadruple *lac* gene deletion strain was significantly higher than wild-type after longer incubation time, 4 and 8 hours.

5.3 Scanning electron microscopy

Scanning electron microscopic studies showed that the conidia of all strains are roughened and verrucose when viewed in high magnification of 10,000X (Figure 5.5). Conidia of quadruple *lac* gene deletion strain and all single *lac* gene deletion strains were similar in size and shape to the wild-type. Conidia were broadly ellipsoidal to ovoid, about 1-2 μm in diameter.





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Figure 5.5 Scanning electron micrographs of conidia of wild-type (A), quadruple *lac* gene deletion strain (B), $\Delta lac1$ (C), $\Delta lac2$ (D), $\Delta lac3$ (E) and $\Delta arb2$ (F). Bars represent 1 μ m.

5.4 Measurement of cytokines

To determine whether laccase affected cytokine productions in the infected macrophages, cytokine secretion by THP-1 macrophage cells were measured by ELISA at 2, 8 and 24 hours. As shown in Figure 5.6, the quadruple *lac* gene deletion strain and all single *lac* gene deletion strains slightly increased TNF- α levels versus wild-type after 2 hours of incubation. In addition, the quadruple *lac* gene deletion strain stimulated a significantly higher production of TNF- α cytokine in THP-1 macrophage cells than wild-type and all single *lac* gene deletion strains at 8 and 24 hours of incubation ($P<0.05$).

The IL-6 cytokine production induced by quadruple *lac* gene deletion strain and all single *lac* gene deletion strains at 2 hours of incubation was similar to wild-type. However, the THP-1 macrophage cells stimulated with quadruple *lac* gene deletion strain produced higher amounts of IL-6 than wild-type at 8 and 24 hours of incubation. Statistical analyses of the results showed a significant difference between the quadruple *lac* gene deletion strain and wild-type at 24 hours of incubation ($P<0.05$) (Figure 5.7).

Likewise, the IL-1 β response induced by quadruple *lac* gene deletion strain and all single *lac* gene deletion strains were similar to wild-type at 2 and 8 hours of incubation. IL-1 β levels were significantly elevated in quadruple *lac* gene deletion strain compared to wild-type at 24 hours of incubation ($P<0.05$) (Figure 5.8).

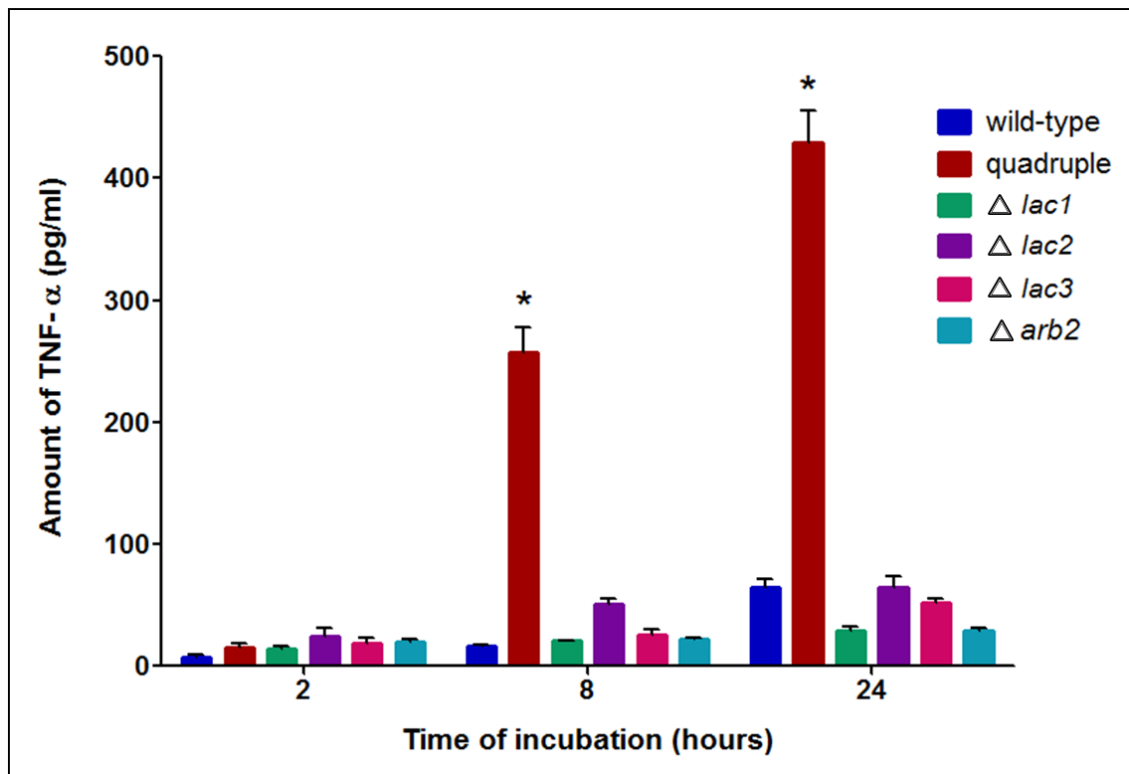


Figure 5.6 Comparison of TNF- α cytokine production by THP-1 macrophage cells induced by conidia of wild-type, quadruple *lac* gene deletion strain, $\Delta lac1$, $\Delta lac2$, $\Delta lac3$ and $\Delta arb2$. Each bar represents the mean \pm SEM of two experiments, each performed in triplicate. And above for each of the analyzed parameters; * $P < 0.05$ was compared between wild-type and laccase mutant strains.

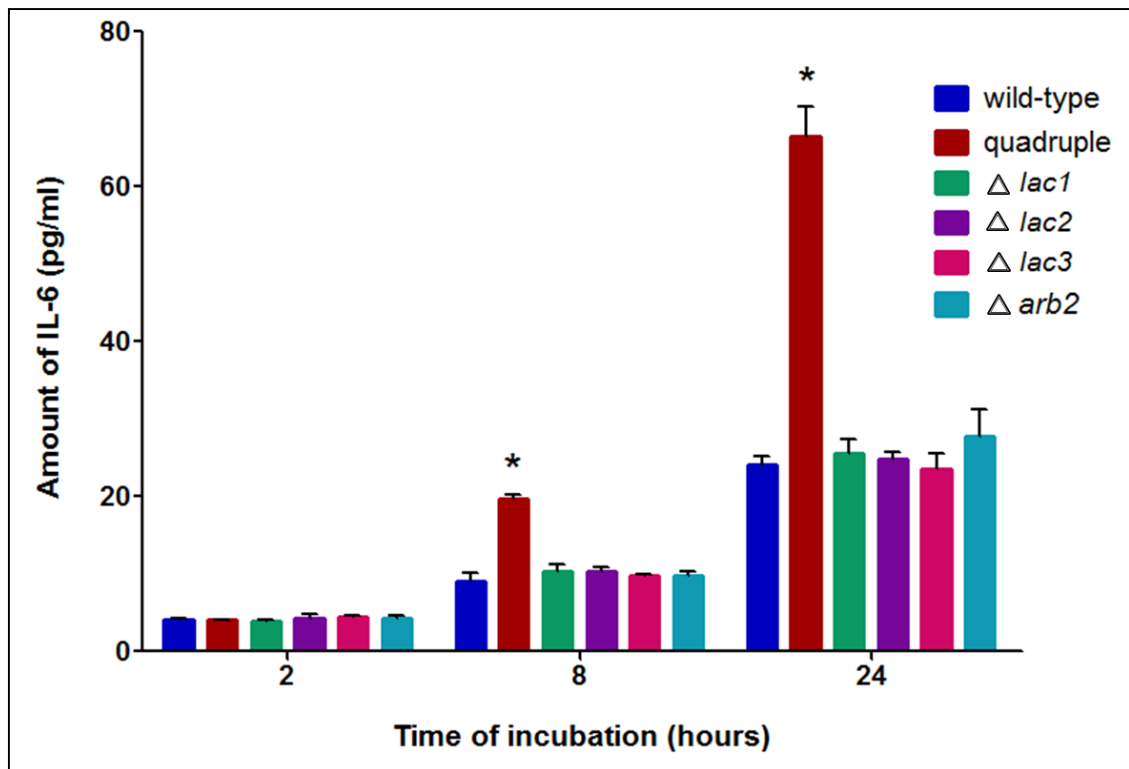


Figure 5.7 Comparison of IL-6 cytokine production by THP-1 macrophage cells induced by conidia of wild-type, quadruple *lac* gene deletion strain, $\Delta lac1$, $\Delta lac2$, $\Delta lac3$ and $\Delta arb2$. Each bar represents the mean \pm SEM of two experiments, each performed in triplicate. And above for each of the analyzed parameters; * $P < 0.05$ was compared between wild-type and laccase mutant strains.

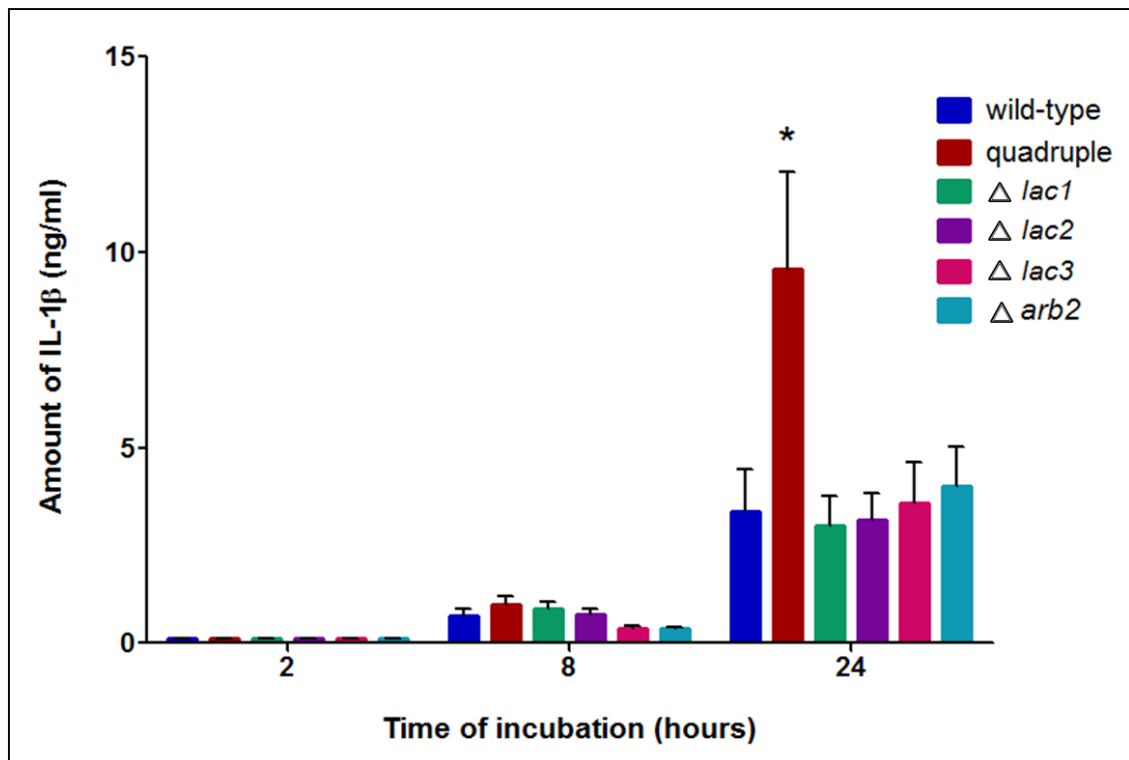


Figure 5.8 Comparison of IL-1 β cytokine production by THP-1 macrophage cells induced by conidia of wild-type, quadruple *lac* gene deletion strain, $\Delta lac1$, $\Delta lac2$, $\Delta lac3$ and $\Delta arb2$. Each bar represents the mean \pm SEM of two experiments, each performed in triplicate. And above for each of the analyzed parameters; * $P < 0.05$ was compared between wild-type and laccase mutant strains.