CHAPTER IV
RESULTS

4.1 Growth performance

The results of growth performance in Nile tilapia fed with experimental diets are presented in Table 4.1. After the 30-day feeding trial, no significant differences were observed in final weight, final length, weight gain, FCR, ADG, SGR, and RGR ($p \geq 0.05$). However, the PER in both the wild-type *B. subtilis* and *B. subtilis* expressing *GULO* groups was significantly different ($p < 0.05$) compared to the control and vitamin C groups. After 90 days of feeding trial, final weight, weight gain, FCR, ADG, SGR, and RGR of the *B. subtilis* expressing *GULO* group were significantly higher ($p < 0.05$) when compared to the control, vitamin C and wild-type *B. subtilis* groups.

4.2 The ascorbic acid levels in Nile tilapia serum were examined using High-performance liquid chromatography (HPLC)

The accumulation of serum ascorbic acid in Nile tilapia after 90 days of feeding with experimental diets showed that the vitamin C group had significantly higher levels than the *B. subtilis* expressing *GULO* group, both of which were significantly higher compared to the control group ($p < 0.05$).

Table 4.1 Effects of dietary supplementation with recombinant probiotic *B. subtilis* expressing L-gulonolactone oxidase on growth performance.

Diet	Initial weight (g)	Final weight (g)	Initial length (cm)	Final length (cm)	Weight gain (g)	FCR	ADG (g day^{-1})	SGR (% day^{-1})	RGR	PER
30 days										
CON	75.11±5.81	141.47±13.78	15.97±0.58	19.40±0.51	66.36±8.78	1.52±0.06	2.21±0.29	0.91±0.06	88.23±7.43	2.14±0.07^{a}
VC	73.16±2.39	138.80±6.24	15.71±0.19	19.19±0.25	65.64±4.90	1.55±0.02	2.19±0.16	0.93±0.05	89.74±6.13	2.15±0.02^{a}
BS	80.18±1.34	151.29±10.30	16.30±0.13	19.73±0.46	71.11±10.24	1.44±0.07	2.57±0.07	0.97±0.04	95.94±4.78	2.32±0.12^{b}
BS+GULO	78.56±3.50	158.64±3.55	16.21±0.45	19.99±0.09	80.09±0.50	1.41±0.01	2.67±0.02	1.02±0.03	102.09±4.56	2.37±0.02^{b}
90 days										
CON	75.11±5.81	268.75±7.19^{a}	15.97±0.58	25.15±1.74	195.72±0.73^{a}	1.60±0.07^{b}	2.17±0.01^{a}	0.63±0.03^{a}	268.99±22.79^{a}	2.23±0.09
VC	73.16±2.39	312.17±13.12^{b}	15.71±0.19	25.83±0.35	237.73±14.47^{b}	1.52±0.09^{ab}	2.64±0.16^{b}	0.69±0.03^{a}	319.60±24.91^{a}	2.20±0.13
BS	80.18±1.34	327.06±12.45^{b}	16.30±0.13	25.94±0.49	246.88±12.08^{b}	1.54±0.03^{ab}	2.74±0.13^{b}	0.68±0.02^{a}	307.93±14.78^{a}	2.17±0.05
BS+GULO	78.56±3.50	381.25±8.13^{c}	16.21±0.45	27.46±0.41	302.75±3.18^{c}	1.42±0.00^{a}	3.36±0.04^{c}	0.76±0.02^{b}	386.31±20.30^{b}	2.34±0.00

Means with a different superscript in each column differed significantly ($p < 0.05$). Values are means ± SD of ten replicates. Abbreviations: a basal diet (CON); a basal diet + vitamin C (VC); a basal diet + wild-type *B. subtilis* (BS); and a basal diet + recombinant *B. subtilis* (BS+GULO).

Table 4.2 Accumulation of serum ascorbic acid in Nile tilapia fed with experimental diets for 90 days.

Diet	Ascorbic Acid Level (μg mL^{-1})
CON	5.88 ± 1.21^{a}
VC	20.29 ± 2.91^{c}
BS	5.92 ± 0.66^{a}
BS+GULO	10.43 ± 1.20^{b}

Significant differences among diet groups are denoted by different letters ($p < 0.05$). Values are means ± SD of three replicates. Abbreviations: a basal diet (CON); a basal diet + vitamin C (VC); a basal diet + wild-type *B. subtilis* (BS); and a basal diet + recombinant *B. subtilis* (BS+GULO).

4.3 Examination of vitamin C by using qRT-PCR

The *GULO* mRNA expression in Nile tilapia fed a diet supplemented with recombinant *B. subtilis* for 30 and 90 days, the result showed that the level of *GULO* mRNA expression in the intestine was significantly higher at day 90 compared to day 30 ($p < 0.05$).

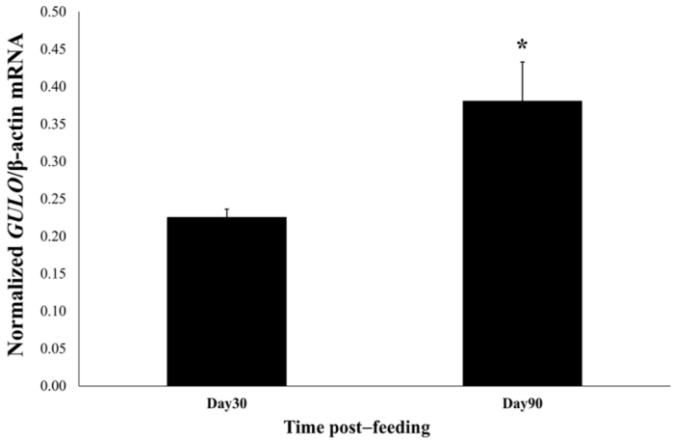
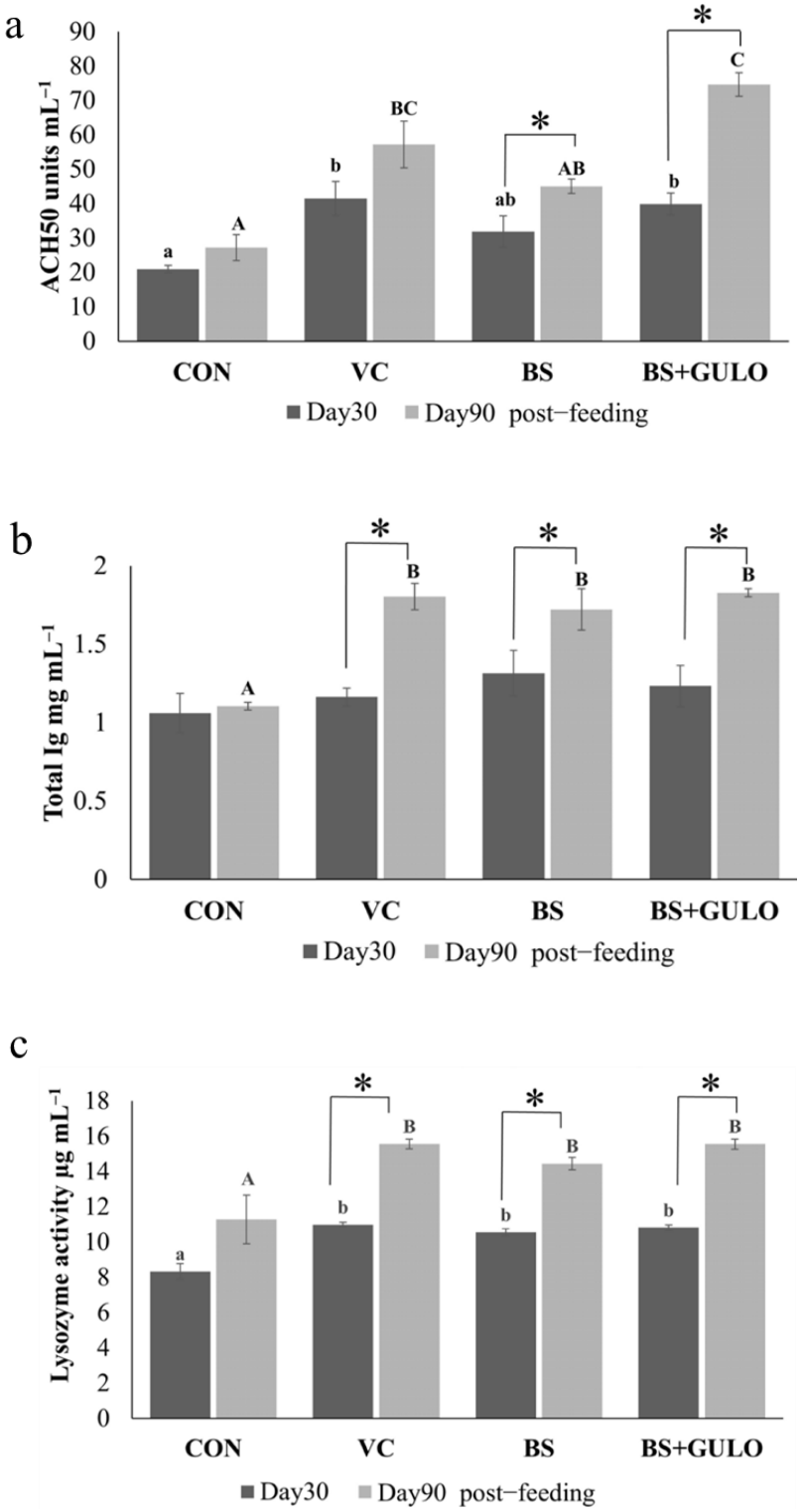


Figure 4.1 The intestinal *GULO* mRNA expression levels in Nile tilapia fed a diet supplemented with recombinant *B. subtilis* were compared between the 30- and 90-day feeding periods. The asterisk indicates significant statistical differences ($p < 0.05$).

4.4 Effects of dietary supplementation with recombinant probiotic *B. subtilis* expressing L-gulonolactone oxidase on innate immune responses in normal Nile tilapia



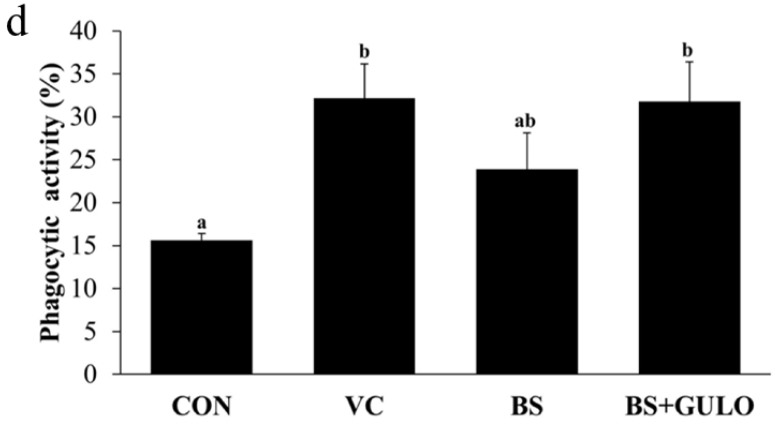


Figure 4.2 Immune parameters of Nile tilapia fed experimental diets for 30 days and 90 days of a feeding trial. ACH_{50} (a); total Ig (b); LZM (c). Bars with asterisks indicate significant differences between day 30 and day 90 of the feeding trial, whereas bars labeled with different lowercase letters denote significant differences for day 30 of the feeding trial, and bars labeled with uppercase letters indicate significant differences for day 90 of the feeding trial, respectively ($p < 0.05$). Phagocytic activity (%) of phagocytic cells in PBLs of Nile tilapia fed experimental diets for 90 days of the feeding trial (d). Bars with different letters indicate significant differences ($p < 0.05$). Abbreviations: a basal diet (CON); a basal diet + vitamin C (VC); a basal diet + wild-type *B. subtilis* (BS); and a basal diet + recombinant *B. subtilis* (BS+GULO).

As shown in figure 4.2. The innate immune responses after feeding. After 30 and 90 days of the trial, the ACH_{50} levels results showed that vitamin C and recombinant *B. subtilis* expressing *GULO* groups were significantly higher ($p < 0.05$) compared to the control group. Notably, only the wild-type and recombinant *B. subtilis* groups exhibited significant increases in ACH_{50} levels between day 30 and day 90 ($p < 0.05$) (Figure 4.2a).

Total immunoglobulin of fish fed all supplemented diets after 30 days of feeding were not significantly different ($p > 0.05$). However, by day 90, the vitamin C, wild-type *B. subtilis*, and recombinant *B. subtilis* expressing *GULO* groups showed significant increased total Ig levels compared to the control group, with levels rising markedly between days 30 and 90 (Figure 4.2b).

In the case of lysozyme activity, significant increases were observed on both day 30 and day 90 in the vitamin C, wild-type *B. subtilis*, and recombinant *B. subtilis* groups compared to the control, with levels increasing significantly over time ($p < 0.05$) (Figure 4.2c).

Furthermore, the phagocytic activity of Nile tilapia fed experimental diets for 90 days of the feeding trial was significantly elevated only in vitamin C and recombinant *B. subtilis* expressing *GULO* groups compared to the control group ($p < 0.05$) (Figure 4.2d).

4.5 Effects of dietary supplementation with recombinant probiotic *B. subtilis* expressing L-gulonolactone oxidase on antioxidant activity of Nile tilapia

After 90 days of the feeding trial, antioxidant activity in the serum of Nile tilapia was assessed. The results showed that serum TAC, SOD, GSH-Px, and CAT levels were significantly higher in the vitamin C and recombinant *B. subtilis* expressing *GULO* groups compared to the control group ($p < 0.05$). Serum MDA levels in vitamin C, wild-type *B. subtilis*, and recombinant *B. subtilis* groups were significantly lower compared to the control group ($p < 0.05$).

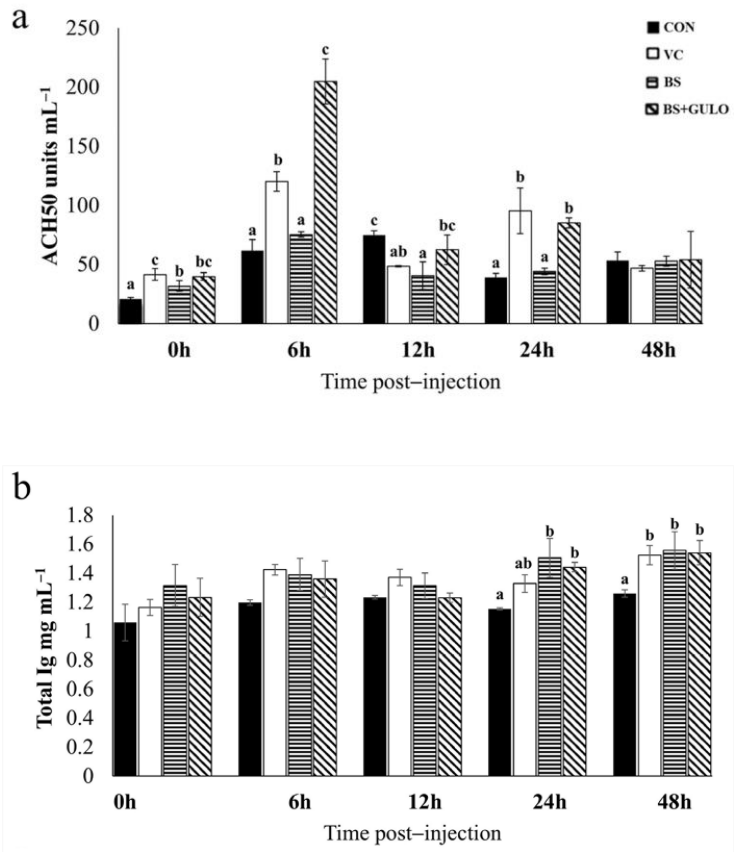
Table 4.3 Antioxidant parameters of Nile tilapia fed experimental diets for 90 days.

Diet	TAC	SOD	MDA	GSH-Px	CAT
	μmol mL^{-1}	U mL^{-1}	nmol mL^{-1}	U mL^{-1}	nmol min^{-1} mL^{-1}
CON	28.16 ± 0.91^{a}	3.32 ± 0.10^{a}	0.36 ± 0.006^{c}	0.068 ± 0.001^{a}	10.39 ± 0.83^{a}
VC	38.85 ± 1.32^{b}	4.34 ± 0.34^{b}	0.23 ± 0.025^{a}	0.121 ± 0.013^{b}	31.27 ± 2.59^{c}
BS	32.03 ± 1.02^{ab}	4.04 ± 0.12^{ab}	0.31 ± 0.002^{b}	0.088 ± 0.007^{ab}	17.29 ± 1.32^{ab}
BS+GULO	36.01 ± 1.78^{b}	4.69 ± 0.32^{b}	0.28 ± 0.006^{b}	0.117 ± 0.016^{b}	20.34 ± 4.16^{b}

Means with a different superscript in each column differed significantly from each other ($p< 0.05$). Values are means ± SD of three replicates. Abbreviations: a basal diet (CON); a basal diet + vitamin C (VC); a basal diet + wild-type *B. subtilis* (BS); and a basal diet + recombinant *B. subtilis* (BS+GULO).

4.6 Immune parameter after *S. agalactiae* injection

Following the challenge test, ACH_{50} levels showed a rapid and significant upregulation at 6 hours post-injection in the groups supplemented with vitamin C and recombinant *B. subtilis* expressing *GULO* compared to the control group ($p < 0.05$) (Figure 4.3a). For total Ig, significant increases were observed at 24- and 48-hours post-injection in the wild-type *B. subtilis* and recombinant *B. subtilis* expressing *GULO* groups ($p < 0.05$), but these increases were not significantly different from those in the vitamin C group (Figure 4.3b). In the case of lysozyme activity, all experimental groups exhibited significantly higher levels compared to the control group at all measured time points ($p < 0.05$) (Figure 4.3c).



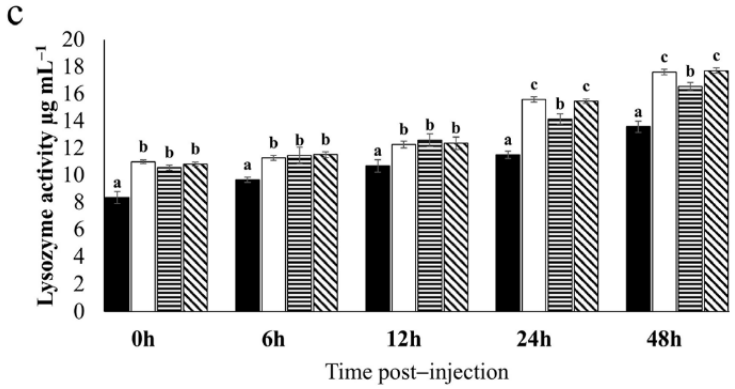


Figure 4.3 Immune parameters of Nile tilapia in response to *S. agalactiae* at different time points following the 30-day feeding trial. ACH_{50} (a); total Ig (b); LZM (c). Bars with different letters indicate significant differences ($p < 0.05$). Abbreviations: a basal diet (CON); a basal diet + vitamin C (VC); a basal diet + wild-type *B. subtilis* (BS); and a basal diet + recombinant *B. subtilis* (BS+GULO).

4.7 Pro-inflammatory gene expression after *S. agalactiae* injection

After the challenge test, the vitamin C, wild-type *B. subtilis*, and recombinant *B. subtilis* expressing *GULO* groups exhibited rapid and significant upregulation of CC chemokine mRNA levels in only the spleen at 6 hours compared to the control group. However, CC chemokine mRNA expression in the recombinant *B. subtilis* expressing *GULO* group peaked at 12 hours in both the liver and spleen, followed by a decline at 48 hours across all experimental diet groups (Figure 4.4).

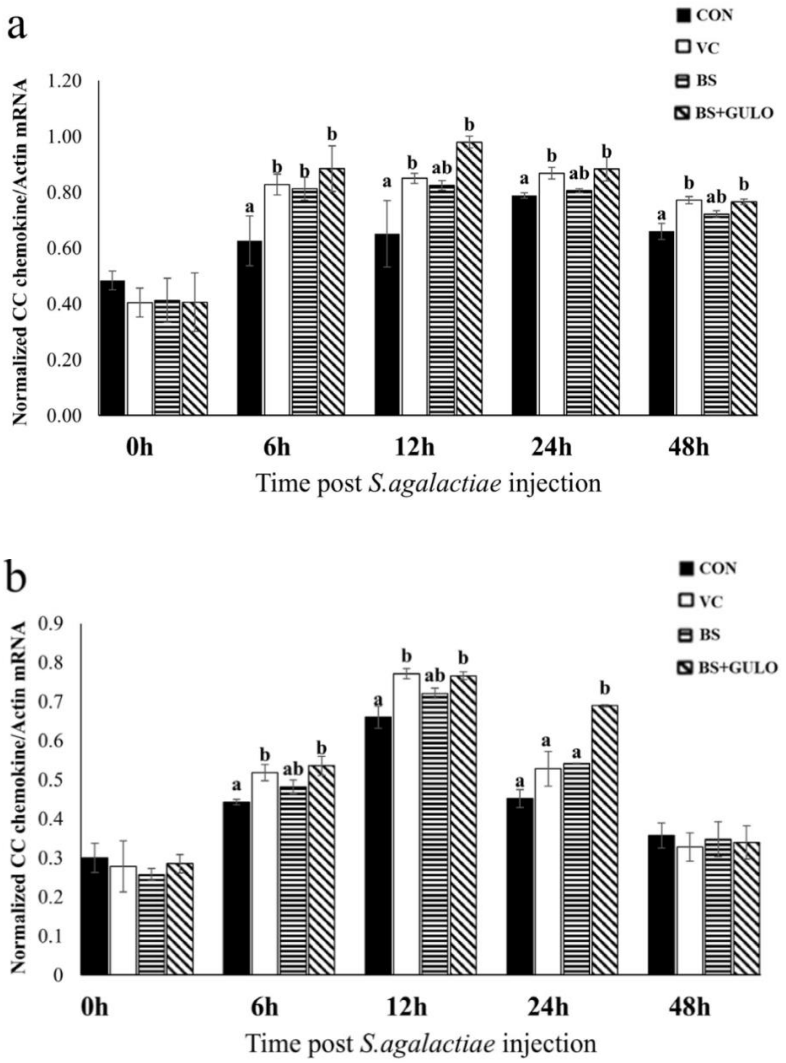


Figure 4.4 Quantitative real-time PCR analysis of CC chemokine expression in the spleen (a) and liver (b) of Nile tilapia in response to *S. agalactiae* at different time points following the 30-day feeding trial. The different letters on each bar indicate significant differences at $p < 0.05$. Abbreviations: a basal diet (CON); a basal diet + vitamin C (VC); a basal diet + wild-type *B. subtilis* (BS); and a basal diet + recombinant *B. subtilis* (BS+GULO).

In the case of TNF**α**, significant mRNA upregulation was observed in the vitamin C, wild-type *B. subtilis*, and recombinant *B. subtilis* expressing *GULO* groups at 6 hours only in the liver compared to the control group, persisting until 48 hours post-injection. In the spleen, significant increases in TNF**α** mRNA levels were detected at 12 hours

post-injection only in the recombinant *B. subtilis* expressing *GULO* group compared to the control group, followed by gradual decreases at 24- and 48-hours (Figure 4.5).

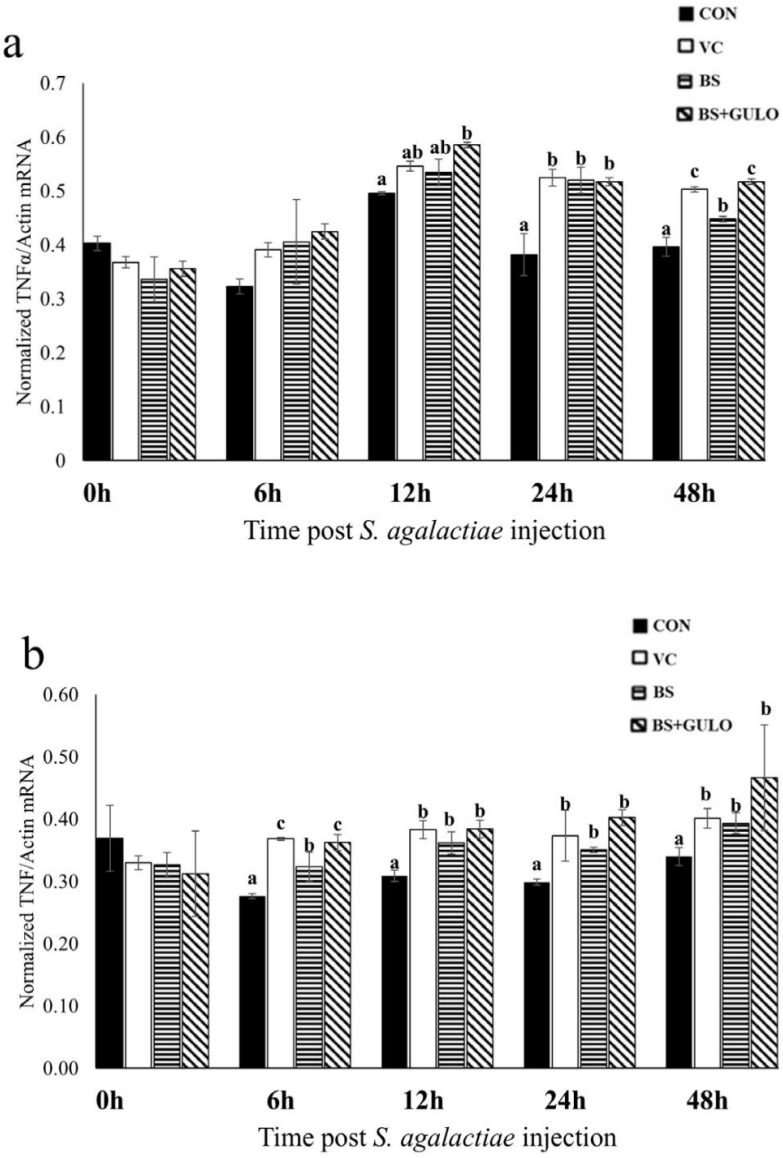


Figure 4.5 Quantitative real-time PCR analysis of tumor necrosis factor **α** expression in the spleen (a) and liver (b) of Nile tilapia in response to *S. agalactiae* at different time points following the 30-day feeding trial (n = 3). The different letters on each bar indicate significant differences at $p < 0.05$. Abbreviations: a basal diet (CON); a basal diet + vitamin C (VC); a basal diet + wild-type *B. subtilis* (BS); and a basal diet + recombinant *B. subtilis* (BS+GULO).