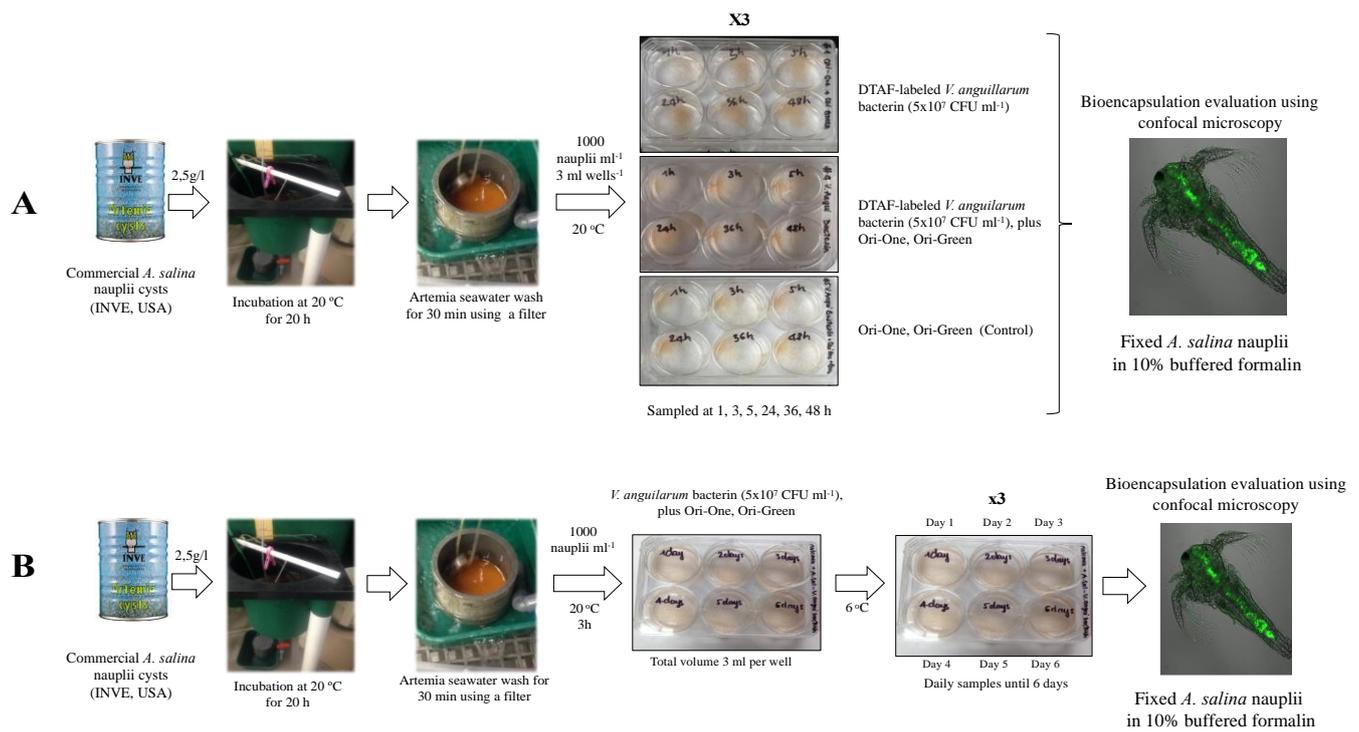


Supplementary figures

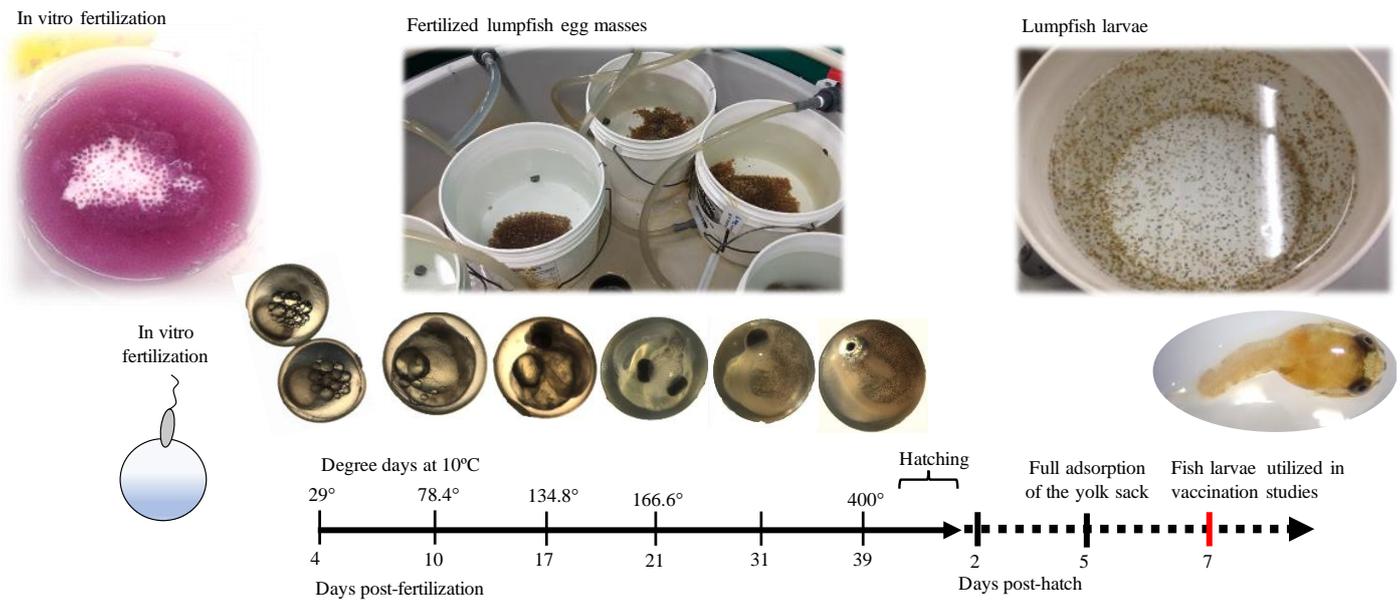
# Oral Immunization of Larvae and Juvenile of Lumpfish (*Cyclopterus lumpus*) against *Vibrio anguillarum* Does Not Influence Systemic Immunity

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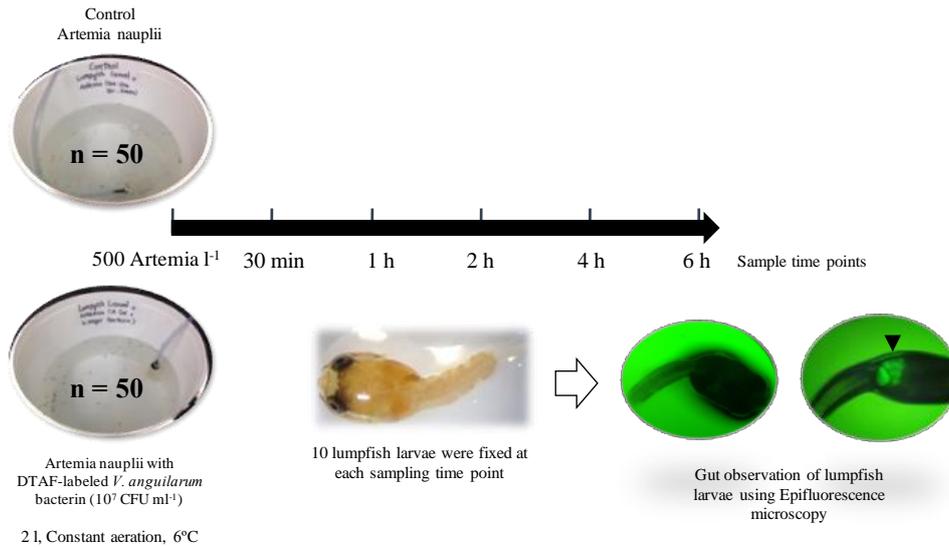
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**Figure S1. Experimental design for optimization of *V. anguillarum* bacterin bio-encapsulation in *A. salina* nauplii.** (A) **Optimization of bio-encapsulation conditions and time.** The effects of supplementation with Ori-One and Ori-Green on *V. anguillarum* bacterin bio-encapsulation in *A. salina* were assessed. In addition, to determine the optimal incubation time for bio-encapsulation to occur, incubations were performed at 20 °C for either 1, 3, 5, 24, 36 or 48 h post-inoculation. The presence of the *V. anguillarum* bacterin in the *A. salina* intestine was then assessed using confocal microscopy. (B) **The stability of the *V. anguillarum* bacterin in the intestine of *A. salina* nauplii post bio-encapsulation.** Once the bio-encapsulation process was completed under the optimal conditions determined in (A), *A. salina* containing the *V. anguillarum* bacterin were stored at 6 °C. The presence of the *V. anguillarum* bacterin in the *A. salina* intestine was then assessed daily for 6 days using confocal microscopy.



**Figure S2. Lumpfish culture conditions and embryonic development.** Fertilized lumpfish egg masses were maintained in 5 l buckets (see section 1.7 for details) during embryonic development: **(A)** Segmentation and compression of yolk lipids; **(B)** Embryo with oocysts and more developed eyes; **(C)** Eye pigmentation and otoliths; **(D)** Skin pigmentation; **(E)** The embryo's body; **(F)** Large embryo ready to hatch. At 7 dph, the larvae were subjected to vaccination studies.



**Figure S3. Oral immunization of lumpfish larvae with the DTAF-labeled *V. anguillarum* bacterin bio-encapsulated in *Artemia* nauplii.** Lumpfish larvae (7 dph) were either fed *Artemia* nauplii with the bio-encapsulated DTAF-labeled *V. anguillarum* bacterin or control *Artemia* nauplii that had been inoculated with seawater, and maintained at 6 °C for 12 h. The presence of *V. anguillarum* bacterin in the gut of lumpfish larvae compared to non-orally immunized fish was then assessed at 0, 0.5, 1, 2, 4, and 6 h post-oral immunization by fluorescence microscopy. The arrow indicates the presence of green-fluorescent *V. anguillarum* bacterin.



Bio-encapsulated *V. anguillarum* bacterin  
 $6.3 \times 10^8$  cells dose<sup>-1</sup> week<sup>-1</sup> *A. salina* nauplii  
at 20 °C for 3h

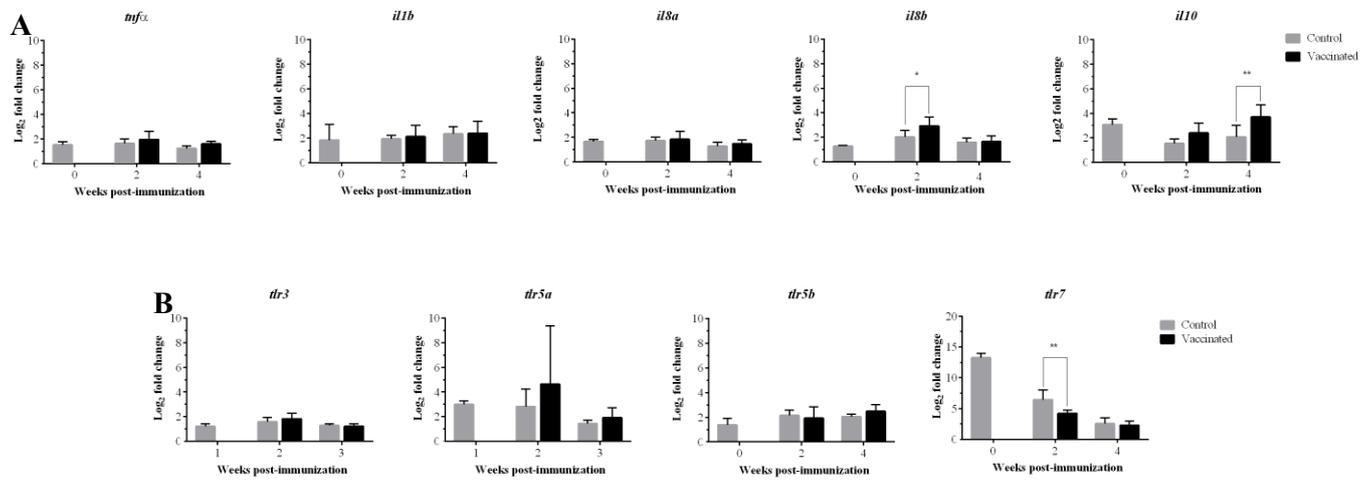


Bio-encapsulated *V. anguillarum* bacterin  
 $6.3 \times 10^8$  cells dose<sup>-1</sup> *A. salina* nauplii at 6 °C  
for feeding larvae

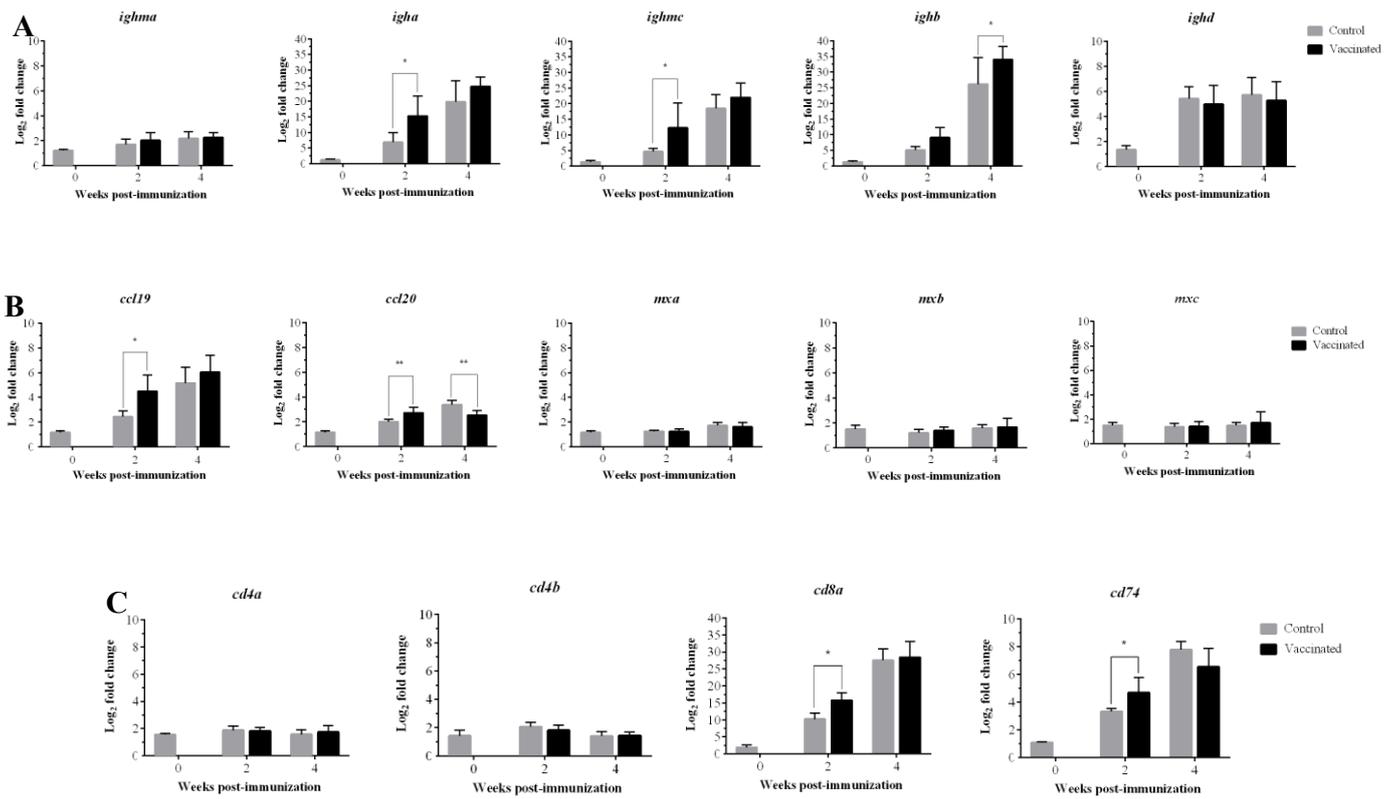


Week 1: 42 million *A. salina* nauplii  
Week 2: 84 million *A. salina* nauplii  
Week 3: 84 million *A. salina* nauplii  
Week 4: 100 million *A. salina* nauplii

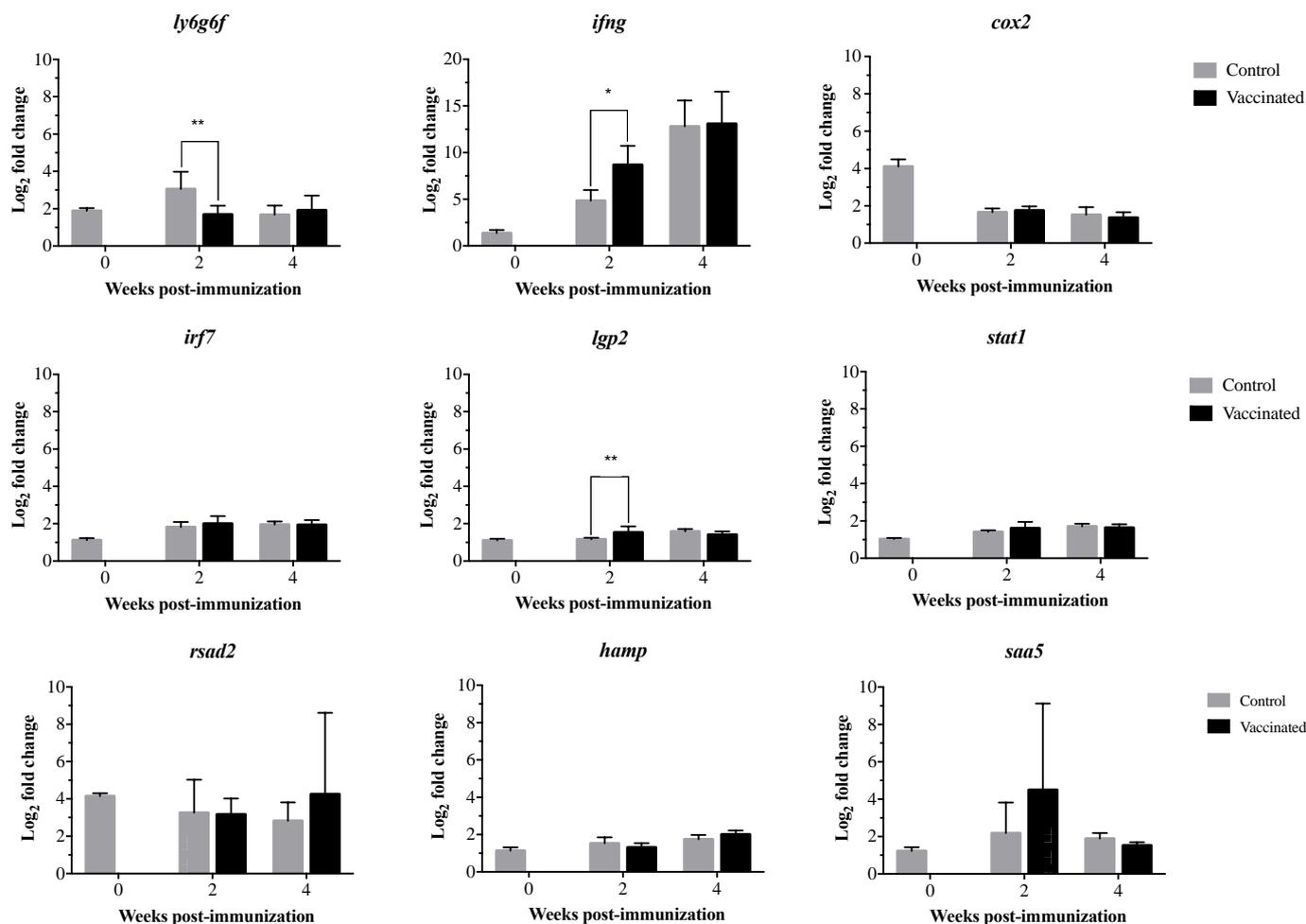
**Figure S4.** *V. anguillarum* bacterin bio-encapsulation in *A. salina* nauplii for industrial application.



**Figure S5.** Transcript expression levels of cytokines (A) and toll-like receptors (B) in lumpfish larvae orally immunized with the *V. anguillarum* bacterin bio-encapsulated in *A. salina* nauplii. Transcript expression levels were assessed pre-immunization (n= 3 pools of 10 larvae each), and at 2 weeks (n= 3 pools of 10 larvae each) and 4 weeks (n= 3 pools of 5 larvae each) post-immunization in both mock-orally (control) and orally immunized (vaccinated) larvae. Relative expression was calculated using the RQ values. A two-way ANOVA test, followed by Sidak multiple comparisons post hoc test was used to assess significant differences between the treatments (control and vaccinated) at each time point. Asterisks (\*) represent significant differences (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ).



**Figure S6.** Transcript expression levels of immunoglobulin heavy locus genes (A), cytokine CC genes and interferon-induced GTP-binding proteins (B) and cluster of differentiation genes (C) in lumpfish larvae orally immunized with the *V. anguillarum* bacterin bio-encapsulated in *A. salina* nauplii. Transcript expression levels were assessed pre-immunization (n= 3 pools of 10 larvae each), and at 2 weeks (n= 3 pools of 10 larvae each) and 4 weeks (n= 3 pools of 5 larvae each) post-immunization in both mock-orally (control) and orally immunized (vaccinated) larvae. Relative expression was calculated using the RQ values. A two-way ANOVA test, followed by Sidak multiple comparisons post hoc test was used to assess significant differences between the treatments (control and vaccinated) at each time point. Asterisks (\*) represent significant differences (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ).



**Figure S7.** Transcript expression levels of other immune-related genes in lumpfish larvae orally immunized with the *V. anguillarum* bacterin bio-encapsulated in *A. salina* nauplii. Transcript expression levels were assessed (n= 3 pools of 10 larvae each), and at 2 weeks (n= 3 pools of 10 larvae each) and 4 weeks (n= 3 pools of 5 larvae each) post-immunization in both mock-orally (control) and orally immunized (vaccinated) larvae. Relative expression was calculated using the RQ values. A two-way ANOVA test, followed by Sidak multiple comparisons post hoc test was used to assess significant differences between the treatments (control and vaccinated) at each time point. Asterisks (\*) represent significant differences (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ).