Investigación e Innovación en Nutrición Acuícola

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Interacción Bacteria Microalga en el Cultivo de Semilla del Ostión Kumamoto *Crassostrea sikamea*

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Resumen

En este estudio se evaluó *in vitro* la interacción entre bacterias probióticas del género *Bacillus* y dos especies de microalgas y su efecto posterior *in vivo* en el cultivo del ostión Kumamoto *Crassostrea sikamea*. Las cepas probióticas de *Bacillus licheniformis* (MAt32), *B. subtilis* (MAt43) y *B. subtilis subtilis* (GAtB1) se inocularon individualmente por triplicado en matraces de 250 mL conteniendo 1×10^4 unidades formadoras de colonias (UFC) mL^{-1} de bacterias y 4.5×10^4 cél mL^{-1} de microalgas (*Isochrysis galbana* o *Chaetoceros calcitrans*) para evaluar su crecimiento durante un cultivo de 7 días. Adicionalmente, se trataron por triplicado semillas de *C. sikamea* con cuatro cepas de bacilos individuales o combinadas en un cultivo de 28 días a una concentración de $1x10^6$ UFC mL^{-1} de la manera siguiente: (a) Control, sin tratamientos; (b) Combinación de dos antibióticos (10 mg L^{-1}); (c) *B. licheniformis*; (d) *B. subtilis*; (e) *B. subtilis subtilis*; (f) mezcla de bacilos. Los resultados mostraron incremento significativo ($P < 0.05$) en el crecimiento de cepas de *Bacillus* en co-cultivo con microalgas mientras que el crecimiento de *I. galbana* co-cultivado con bacterias no se redujo significativamente ($P > 0.05$) con el grupo control. La semilla de *C. sikamea* tratada con *Bacillus* mostró crecimiento y supervivencia significativo comparado con el grupo control. En este estudio, la microalga *C. calcitrans* fue susceptible a la presencia de bacterias probióticas. Sin embargo, la reducción del

crecimiento microalgal observada *in vitro* no afectó el incremento en crecimiento y supervivencia en el cultivo de semilla de *C. sikamea* expuesta a bacterias probióticas comparada con aquellas semillas cultivadas sin probióticos.

Palabras clave: *Crassostrea sikamea, Bacillus* spp., microalgas, moluscos, antibióticos

Bacteria and Microalgae Interaction on Rearing Kumamoto Oyster *Crassostrea sikamea* spat

Ángel I. Campa-Córdova[1]*, Ana C. Sánchez-Ortiz[2], Gabriel Aguirre-Guzmán[3], José M. Mazón-Suástegui[1], Ma del C. Flores-Miranda[2], Antonio Luna-González[4], Norma Ochoa-Álvarez[1]

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Abstract

This study assessed *in vitro* interaction between *Bacillus* bacteria and microalgae and their posterior *in vivo* effect on rearing Kumamoto oyster *Crassostrea sikamea*. The probiotic strains *Bacillus licheniformis* (MAt32), *B. subtilis* (MAt43), and *B. subtilis subtilis* (GAtB1) were individually inoculated in triplicate into 250 mL flasks containing 1×10^4 colony forming units (CFU) mL^{-1} of bacteria and 4.5×10^4 cell mL^{-1} of microalgae (*Isochrysis galbana* or *Chaetoceros calcitrans*) to evaluate their growth during a 7-day culture. Single cultures of microalgae or bacilli served as control. Additionally, *C. sikamea* spat was treated for 28 days with four single/combined bacillus treatments in triplicate at a concentration of $1x10^6$ CFU mL^{-1} as follows: (a) Control, without treatments; (b) Combination of two antibiotics (10 mg L^{-1}); (c) *B. licheniformis*; (d) *B. subtilis*; (e) *B. subtilis subtilis*; (f) mixed bacilli. The results showed a significantly ($P < 0.05$) increased growth of *Bacillus* strains co-cultured with microalgae while the growth of *I. galbana* co-cultured with bacteria was not reduced significantly ($P > 0.05$) compared with the control group. *C. sikamea* spat treated with *Bacillus* showed significantly ($P < 0.05$) higher growth and survival than the control group. In this study, *C. calcitrans* microalgae were susceptible to the presence of probiotic bacteria. Nonetheless, this reduction in microalgal growth observed *in vitro* increased growth and survival of *C. sikamea* spat exposed to probiotic bacteria compared to spat without probiotics.

Key words: *Crassostrea sikamea, Bacillus* spp., microalgae, mollusks, antibiotics

Introduction

The Kumamoto oyster *Crassostrea sikamea* is appreciated as food by local inhabitants along the Gulf of California [1]; despite its preference, few studies have been performed with this species describing distribution and abundance [1, 2], hatchery seed production [3], reproductive biology [4], diseases and parasitism [5, 6] and composition and diversity of the gut [7].

Infectious diseases are the main cause of major economic losses in the oyster industry, particularly during seed production in hatchery [4]. Most of the mortality peaks have been attributed to pathogenic bacteria (*Vibrio* spp.) that has caused high economic losses [8]. Antibiotics are commonly used to avoid the adverse effects of pathogens in aquaculture [9, 10]. However, misuse and overuse of antibiotics in bivalve aquaculture for controlling these mortalities contributes to the development of more virulent pathogenic strains, associated with a reduced microalgal density, poor organism growth, and mass mortalities that raise production costs [11, 12]. Antibiotics can remain active for long periods in water and sediments, which is harmful to the environment, contributing to the selective development of resistant bacteria, some highly pathogenic for farming organisms and even humans [13]. One alternative of natural origin, and therefore, low environmental impact, is using beneficial microorganisms to reduce the use and overuse of antibiotics [14]. Several studies have shown the benefits of bacterium administration because of its positive impact on various metabolic and immunomodulatory processes, which translates into higher host growth and survival [15, 16].

The purpose of adding microorganisms in diets of farming organisms is to generate a beneficial relationship between the microorganism and the host and between the microorganism and microflora in the digestive organs; thus, these relationships seek the following characteristics: (1) pathogen antagonism by competitive exclusion or antimicrobial production and stimulation of host immune response; (2) metabolite production; (3) nutritional substance production; (4) survival and colonisation in the host digestive tract (DT) by adhesion; (5) storage stability; (6) safety; (7) origin from animal source [12, 16]. The use of beneficial organisms or probiotics offers wide opportunities for shellfish aquaculture [17, 18] because of their antimicrobial properties [19], particularly against different species of pathogenic *Vibrio* spp. [20]. However, few studies of the interaction between bacteria and the microalgae used as food for molluscs are available [21]. Some microalgal species are more susceptible than others to the presence of bacteria [22].

Therefore, the aim of this study was to evaluate the *in vitro* growth of beneficial bacteria co-cultured with microalgae and the effect of the bacterium on rearing of Kumamoto oyster *C. sikamea* spat. The probiotic strains reported in this study were previously isolated from *Anadara tuberculosa* and evaluated *in vitro* [23] and *in vivo* in juvenile white shrimp *Litopenaeus vannamei* [23, 24].

Materials and Methods

Bacterial cultivation

Bacillus strains GAtB1 (*B. subtilis* sub. *subtilis*), MAt32 (*B. licheniformis*) and MAt43 (*B. subtilis*) were isolated from the digestive tract (DT) of *A. tuberculosa* [23]. Each strain was grown in Triptic Soy Broth (TSB, #257107, Bioxon BD Difco, Franklin Lakes, NJ, USA) with 2.5% NaCl and incubated at 35 °C for 24 h. Cultures were washed twice, centrifuged at 3000 × *g* at room temperature for 10 min (Beckman model GS-15R, Beckman Coulter Life Sciences, Indianapolis, IN, U.S.A.) and re-suspended in sterile seawater. Bacterial suspension was adjusted to an optical density of 1.0 at 600 nm (Beckman DU 600, Beckman Coulter Life Sciences, Indianapolis, IN, U.S.A.). The cell density was adjusted to 1×10^6 CFU mL^{-1}, which was confirmed by counting CFU of serial dilutions on agar plate (Triptic Soy Agar; TSA, # 211670, Bioxon BD Difco, Franklin Lakes, NJ, USA).

Microalgal cultivation

Isochrysis galbana (v. *aff galbana*, code UTEX LB 2307; origin: Centro de Investigaciones Biologicas del Noroeste (CIBNOR collection) and *Chaetoceros calcitrans* (origin: L'Institut Français de Recherche pour l'Exploitation de la Mer (IFREMER collection) were cultivated in sterilised glass flasks with a final volume of 700 mL of sterile seawater (35 psu) enriched with 750 µL of Guillard F/2 medium, pH 8 [25]. The strains were incubated at 28 ± 1 °C with continuous flow of filtered air and daylight-type lighting from fluorescent lamps 5940-5370 lux 50 cm away, photoperiod 16: 8 h. Cell density was determined by triplicate, samples were taken from each flask, diluted 1:10, and counted in a Neubauer Cell Chamber under an optical microscope (Olympus BH-2). Cell density was reported as cell·mL^{-1}.

Bacterium-microalga interaction

The *in vitro* interaction between two microalgae and three probiotic bacteria were evaluated as follows: (1) Is: *I. galbana*; (2) Ch: *C. calcitrans*; (3) Bl: *B. licheniformis*; (4) Bs: *B. subtillis*; (5) Bss: *B. subtilis* sub. *subtillis*; (6) Mix 1: Is+ Bl; (7) Mix 2: Ch+Bl; (8) Mix 3: Is+Bs; (9) Mix 4: Ch+Bs; (10) Mix 5: Is+Bss; (11) Mix 6: Ch+Bss. The growth of a single and mixed cultures was evaluated by triplicate inoculating flasks with 4.5×10^4 cell·mL^{-1} of microalgae and/or 1.0×10^4 CFU·mL^{-1} of bacteria and incubated at the conditions described for microalgal culture. Samples for microalgae and bacteria count were taken at days 3, 5, and 7.

Culture conditions of juvenile oysters

Kumamoto spat, *C. sikamea* (5 ± 0.5 mm mean shell length) were donated by commercial hatchery Acuacultura Robles, in La Paz, Baja California Sur, Mexico. Oysters were previously acclimated in 80-L fiberglass tanks filled with 60-L of filtered (2µm) and Ultraviolet-sterilised seawater at 25 °C ± 1°C and 36 ± 1 g.L^{-1} of salinity for one week. For the experimental bioassay, the seeds were counted, measured, weighed, and placed in 4-L plastic containers (N = 60) filled with 2-L of filtered (2 µm) and UV sterilised seawater (25 °C ± 1 °C, 36 ± 1 ups) with continuous aeration. Juveniles were fed with mixed *I. galbana* and *C. calcitrans* (1:1 proportion) microalgae, at a density of 1.7×10^3 cell·mL^{-1}·day^{-1}, divided into four portions. Containers were drained, washed, and refilled with clean, and UV-sterilised seawater every 48 h.

Exposure of juveniles to bacteria

Triplicate groups of 60 spat oysters were exposed to four bacterial treatments at a final concentration of 1 x 10^6 CFU mL^{-1} and combination of two commercial antibiotics [26] used as positive control at a concentration of 10 mg.L^{-1} [9,27,28]. The treatments were added after every seawater change for 28 days: **T1** = Antibiotic (ampicillin + streptomycin; 1:1 proportion); **T2** = *B. licheniformis*; **T3** = *B. subtilis*; **T4** = *B. subtilis* subsp. s*ubtilis*; **T5** = *B. licheniformis*+ *B. subtilis*+ *B. subtilis* subsp. *subtilis*; 1: 1: 1 proportion. **C** = Control group, no bacteria or antibiotics added**.** Samples were randomly collected at days 7, 14, 21 and 28 to evaluate growth rate.

Growth rate

Growth was evaluated placing 30 samples per container on graph paper laminate, taking a photographic image to measure length and width using the Image-Pro Plus version 5.1 software (Media Cybernetics, Bethesda, MD, USA). At day 28, average final length was achieved in each treatment, as well as absolute growth (AG) according to Ziaei-Nejad *et al*. [29]:

$AG = L_f - L_i$

where L_f = final average length, and L_i = initial average length.

The coefficient of variation (CV) regarding length was calculated as:

$CV = S / X * 100$

where S = standard or variance deviation and X = average sample.

Statistical analysis

Group normality was analysed with the Kolmogorov-Smirnov test, and then with Bartlett test, for homogeneity of variance. Thereafter, one-way ANOVA was used to asses for significant differences in growth. When the data showed significant differences Tukey's *post hoc* honestly significant difference (HSD) analysis was used. Level of significance was set at $P \leq 0.05$ for all analyses. All statistical analyses were performed using Statistica 7.0 software (StatSoft, Tulsa, OK, USA).

Results

Growth of microalgae and bacteria

The interaction of *I. galbana* and *C. calcitrans* cultivated with *B. licheniformis* for seven days is shown in Figure 1. After co-culture with *B. licheniformis*, *C. calcitrans* showed a significant decrease ($P < 0.05$) in growth more than the control (*C. calcitrans* without bacterial strains) at days 5 and 7 (Fig. 1A) . However, the cell density of *C. calcitrans* co-cultured with *B. licheniformis* increased from 4.5×10^4 cell·mL^{-1} to 3.5×10^6 cell·mL^{-1} at day 7. The growth of *I. galbana* was not influenced by *B. licheniformis*. Figure 1B shows the growth of *B. licheniformis* cultured with *I. galbana* and *C. calcitrans*. The density of *B. licheniformis* significantly increased (P <0.05) with *I. galbana* and *C. calcitrans* cultures compared to the growth of *B. licheniformis* without microalgae (control) at days 5 and 7. The highest growth of *B. licheniformis* was reached with *C. calcitrans* at day 5 (Figure 1B).

Figure 2 shows growth of microalgae in co-culture with *B. subtilis* for seven days. The growth of *I. galbana* was not affected by *B. subtilis*, and cell density of *C. calcitrans* decreased significantly when compared to control at days 5 and 7 (Figure 2A). When *B. subtilis* was cultured with microalgae, growth significantly increased ($P < 0.05$) since day 3 (Figure 2B).

The growth of *I. galbana* was not negatively influenced by *B. subtilis subtilis* at seven days of culture (Figure 3A). However, when *C. calcitrans* was co-cultured with the bacteria, cell density decreased significantly ($P < 0.05$) more than control at days 5 and 7. Figure 3B shows a significant ($P < 0.05$) growth increase in *B. subtilis subtilis* when it had been exposed to both microalgal strains since day 3. The highest growth of *B. subtilis subtilis* was recorded in co-culture with *C. calcitrans*.

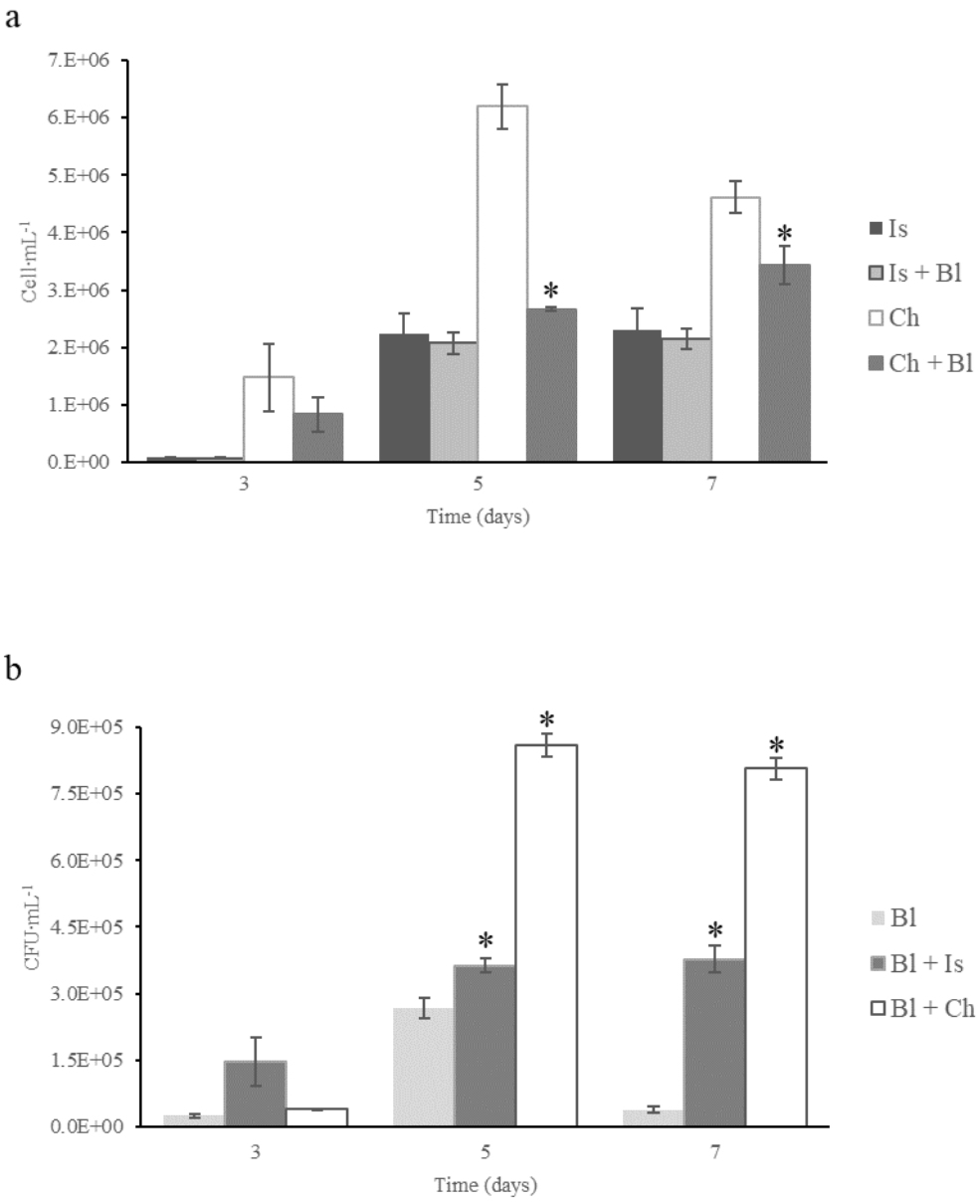

Figure 1 (**a**) Microalgal growth in co-culture with bacteria. **Is** = *Isochrysis galbana* (control); **Is + Bl** = *I. galbana* + *Bacillus licheniformis*; **Ch** = *Chaetoceros calcitrans* (control); **Ch + Bl** = *C. calcitrans* + *B. licheniformis*. (**b**) Bacterial growth in co-culture with microalgae. **Bl** = *B. licheniformis* (control); **Bl + Is** = *B. licheniformis* + *I. galbana*; **Bl + Ch** = *B. licheniformis* + *C. calcitrans*. Bars correspond to standard error (SE). (*) significantly ($P < 0.05$) different than control.

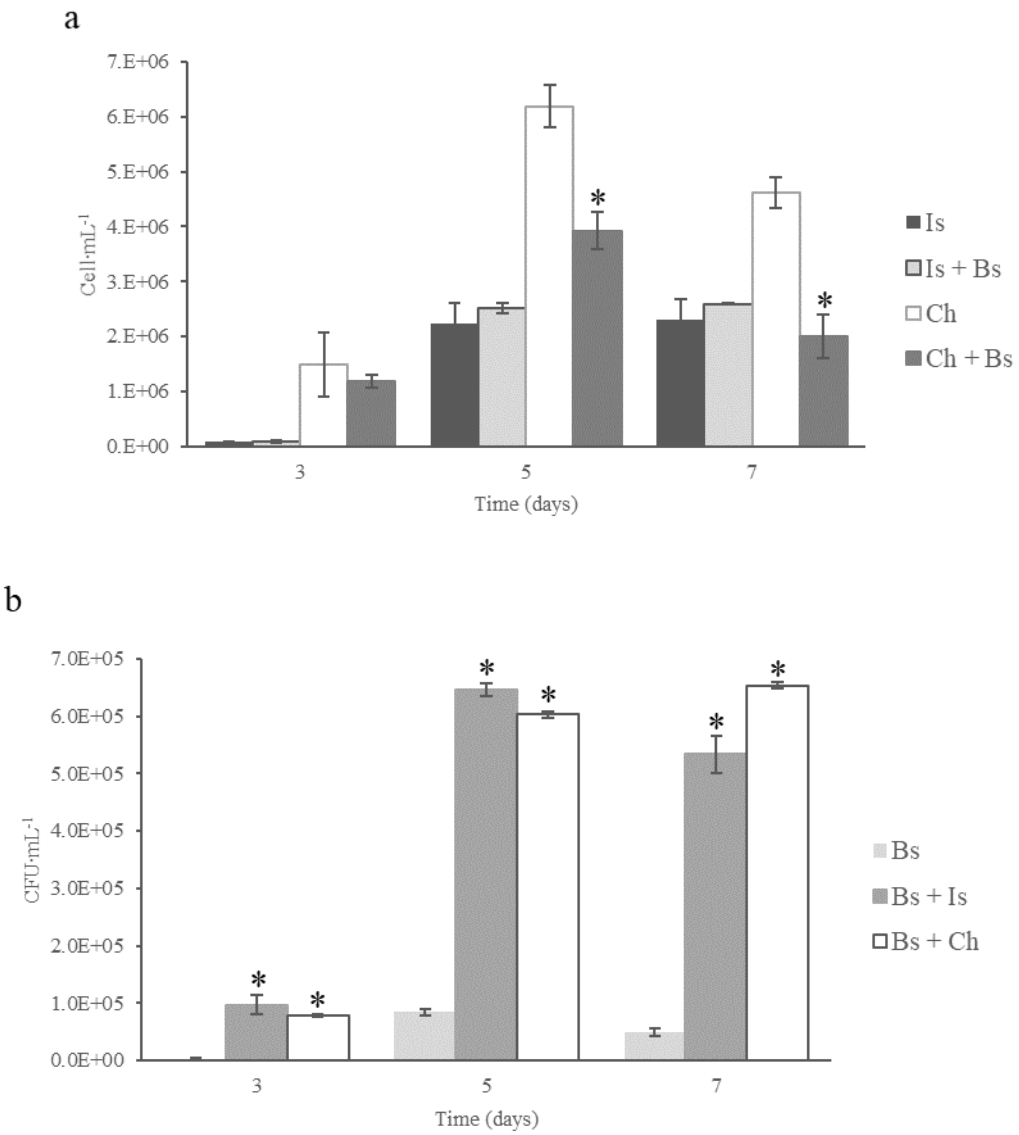

Figure 2 (**a**) Microalgal growth in co-culture with bacteria. **Is** = *Isochrysis galbana* (control); **Is + Bs** = *I. galbana* + *Bacillus subtilis*; **Ch** = *Chaetoceros calcitrans* (control); **Ch + Bs** = *C. calcitrans* + *B. subtilis*. (**b**) Bacterial growth in co-culture with microalgae. **Bs** = *B. subtilis* (control); **Bs + Is** = *B. subtilis* + *I. galbana*; **Bs + Ch** = *B. subtilis* + *Ch. Calcitrans*. Bars correspond to standard error (SE). (*) significantly ($P < 0.05$) different than control.

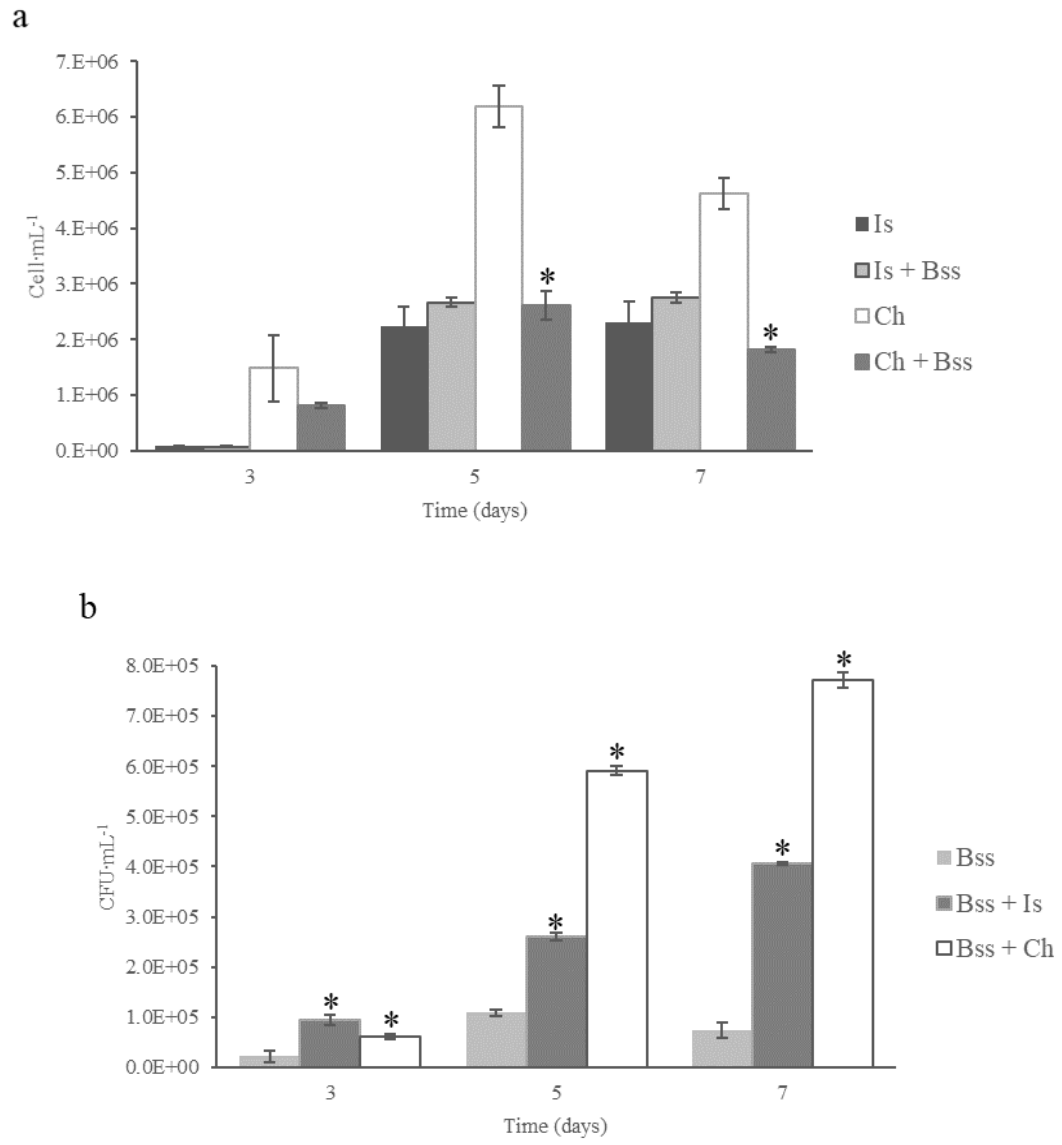

Figure 3 (**a**) Microalgal growth in co-culture with bacteria. **Is** = *Isochrysis galbana* (control); **Is + Bss** = *I. galbana* + *Bacillus subtilis subtilis*; **Ch** = *Chaetoceros calcitrans* (control); **Ch + Bss** = *C. calcitrans* + *B. subtilis subtilis*. **(b**) Bacterial growth in co-culture with microalgae. **Bss** = *B. subtilis subtilis* (control); **Bss + Is** = *B. subtilis subtilis* + *I. galbana*; **Bss + Ch** = *B. subtilis subtilis* + *C. calcitrans*. Bars correspond to standard error (SE). (*) significantly ($P < 0.05$) different than control.

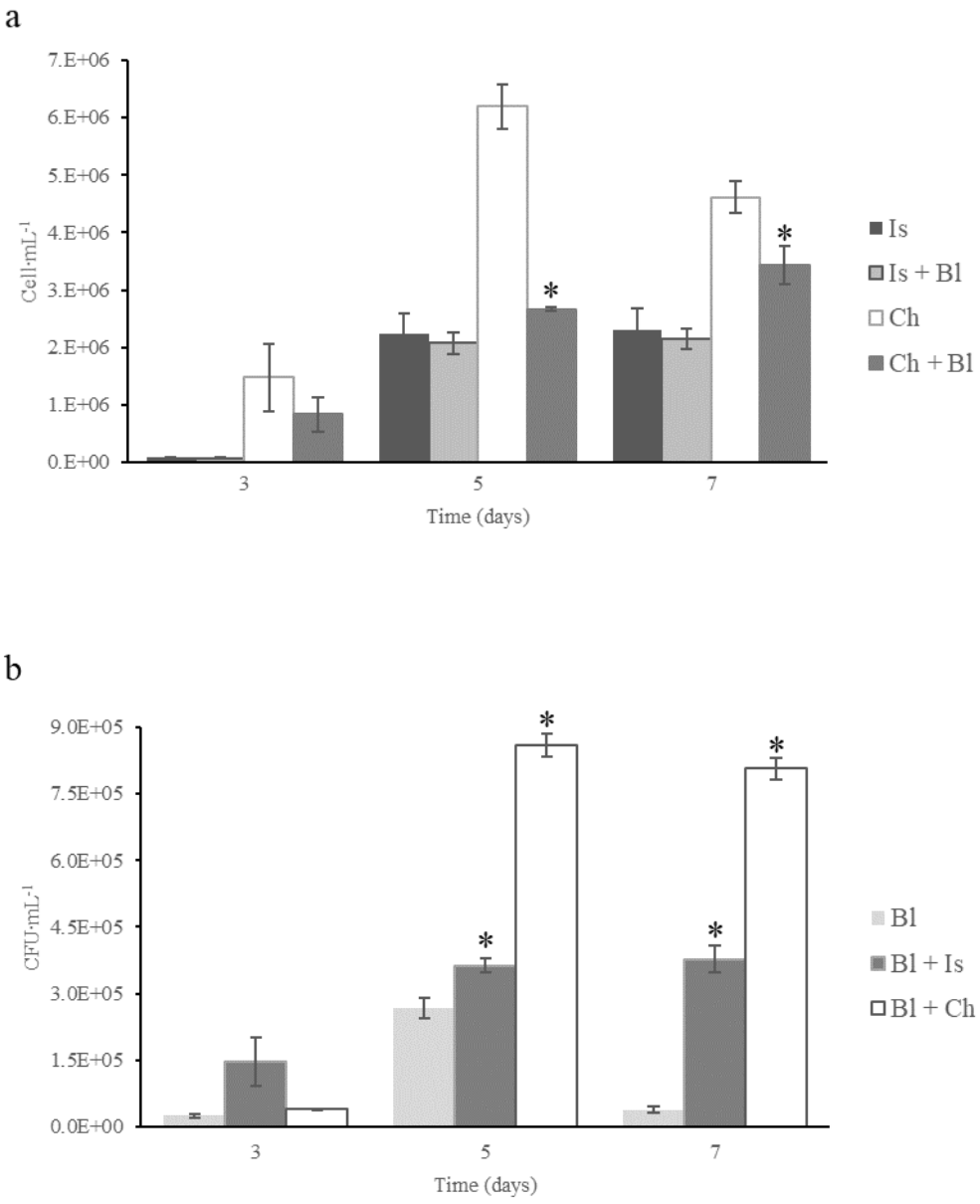

Figure 4 (**a**) Microalgal growth in co-culture with bacteria. **Is** = *Isochrysis galbana* (control); **Is + Bl** = *I. galbana* + *Bacillus licheniformis*; **Ch** = *Chaetoceros calcitrans* (control); **Ch + Bl** = *C. calcitrans* + *B. licheniformis*. (**b**) Bacterial growth in co-culture with microalgae. **Bl** = *B. licheniformis* (control); **Bl + Is** = *B. licheniformis* + *I. galbana*; **Bl + Ch** = *B. licheniformis* + *C. calcitrans*. Bars correspond to standard error (SE). (*) significantly ($P < 0.05$) different than control.

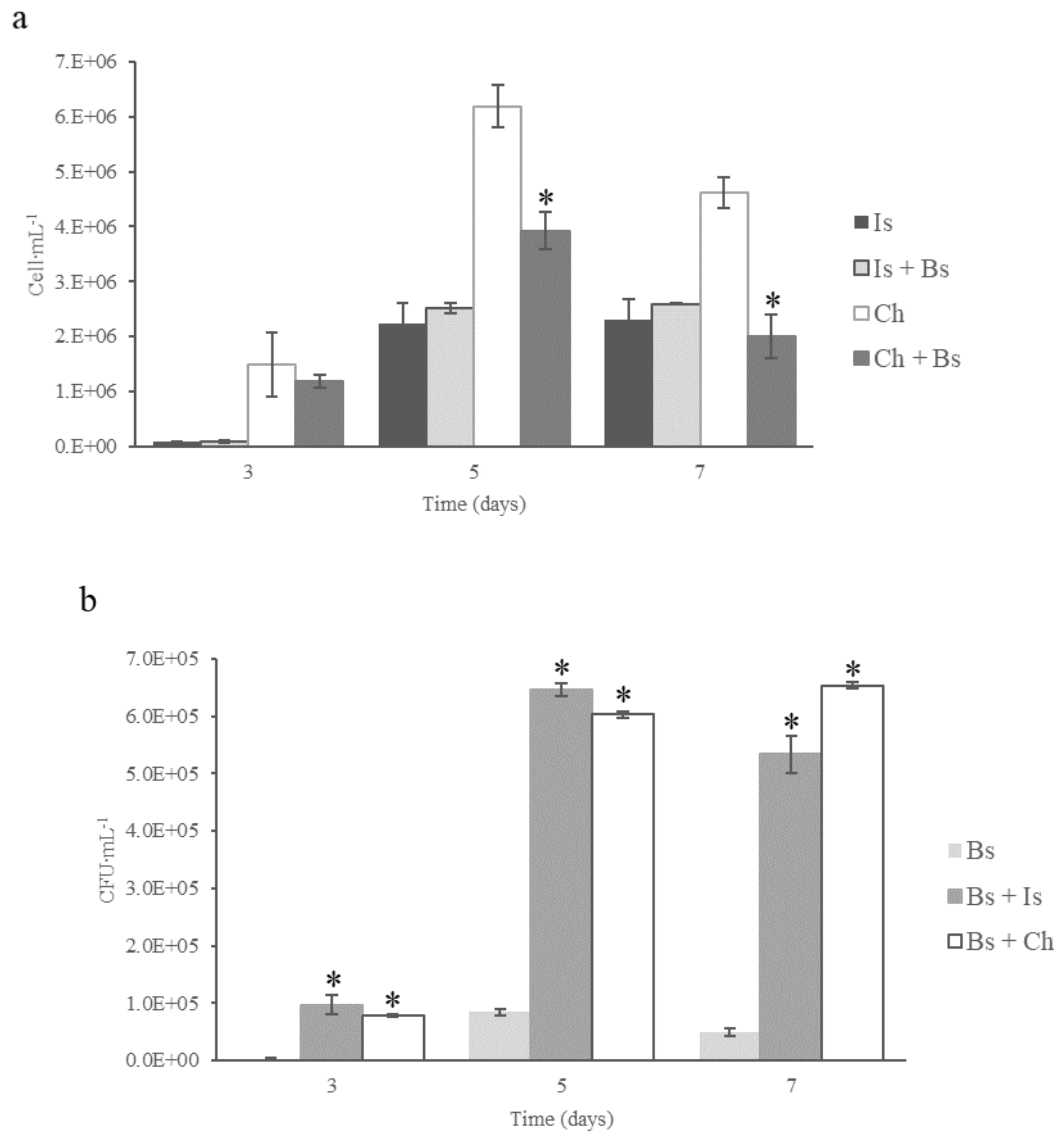

Figure 5 (**a**) Microalgal growth in co-culture with bacteria. **Is** = *Isochrysis galbana* (control); **Is + Bs** = *I. galbana* + *Bacillus subtilis*; **Ch** = *Chaetoceros calcitrans* (control); **Ch + Bs** = *C. calcitrans* + *B. subtilis*. (**b**) Bacterial growth in co-culture with microalgae. **Bs** = *B. subtilis* (control); **Bs + Is** = *B. subtilis* + *I. galbana*; **Bs + Ch** = *B. subtilis* + *Ch. Calcitrans*. Bars correspond to standard error (SE). (*) significantly ($P < 0.05$) different than control.

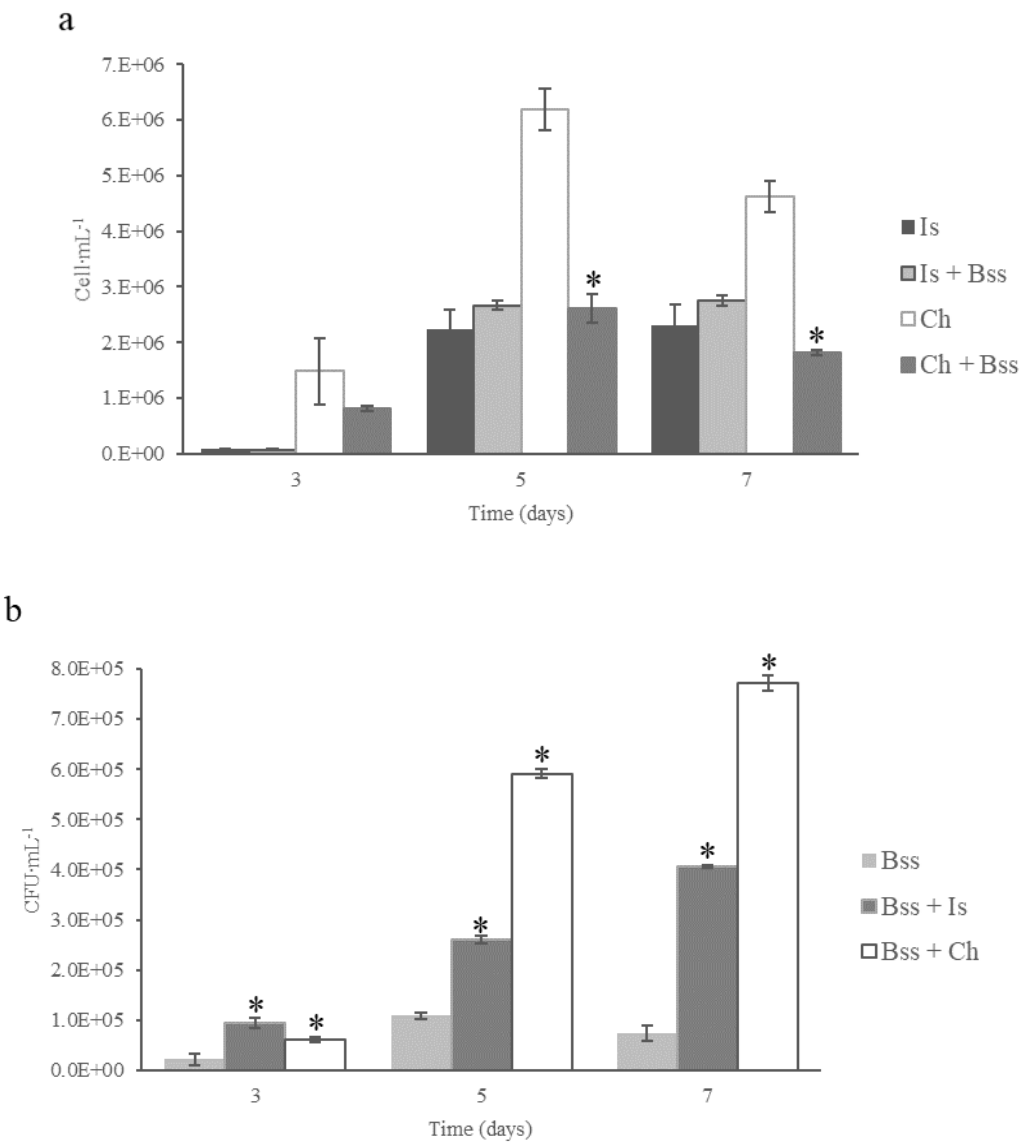

Figure 6 (**a**) Microalgal growth in co-culture with bacteria. **Is** = *Isochrysis galbana* (control); **Is + Bss** = *I. galbana* + *Bacillus subtilis subtilis*; **Ch** = *Chaetoceros calcitrans* (control); **Ch + Bss** = *C. calcitrans* + *B. subtilis subtilis*. **(b**) Bacterial growth in co-culture with microalgae. **Bss** = *B. subtilis subtilis* (control); **Bss + Is** = *B. subtilis subtilis* + *I. galbana*; **Bss + Ch** = *B. subtilis subtilis* + *C. calcitrans*. Bars correspond to standard error (SE). (*) significantly ($P < 0.05$) different than control.

Growth of Crassostrea sikamea

The growth and survival of *C. sikamea* spat treated with beneficial bacteria for 28 days is shown in Table I. Juvenile oysters treated with *B. subtilis* (T3) significantly ($P < 0.05$) increased in length more than the control group. However, absolute growth ($F_{0.05(5,320)} = 2.559$, p= 0.0273) and survival ($F_{0.05(5,12)} = 13.962$, p= 0.00012) of oysters treated with bacteria were significantly higher than those in the control and antibiotic groups. The highest absolute growth was recorded in *C. sikamea* treated with *B. subtilis* and highest survival in spat treated with bacterial mix (T5).

Table I Growth of juvenile *Crassostrea sikamea* reared for 28 days with: **(**Control**)** untreated oysters; (T1**)** Antibiotic; **(**T2*) Bacillus licheniformis***; (**T3*) Bacillus subtilis***; (**T4*) Bacillus subtilis subtilis***; (**T5*)* Mixed bacilli. Different letters indicate significant differences ($P < 0.05$).

Treatment	Length (mm)	Absolute Growth	Survival (%)
C	$6.38^{ab} \pm 0.16$	1.38^{a}	$33^{a} \pm 1.15$
T1	$6.26^{a} \pm 0.13$	1.26^{a}	$32^{a} \pm 1.89$
T2	$6.74^{ab} \pm 0.11$	1.74^{b}	$76^{b} \pm 0.89$
T3	$6.83^{b} \pm 0.12$	1.82^{b}	$63^{b} \pm 2.09$
T4	$6.74^{ab} \pm 0.09$	1.73^{b}	$77^{b} \pm 1.01$
T5	$6.66^{ab} \pm 0.09$	1.65^{b}	$81^{b} \pm 0.21$

Discussion

Feeding bivalve mollusks with live microalgae plays an essential role in larval and juvenile development, providing important nutrients for growth [30]. However, to improve these safe features, the use of antibiotics is a regular practice [31], which may destroy microalgal cells, avoid adequate feeding of farmed organisms and decline the quality of microalgae [32]. In this sense, Campa-Córdova *et al*. [33] reported that higher doses than 6 $mg.mL^{-1}$ of chloramphenicol or erythromycin significantly affected growth of *Isochrysis galbana* and *Chaetoceros gracilis*. Thus, the use of beneficial bacteria may be an alternative prophylactic method to that of antibiotics for mollusk farming. However, the interaction between bacterial populations and microalgae may also affect the development of some microalgal species. In this study, the growth of *I. galbana* with

probiotic bacteria was not significantly affected, but co-culture of *C. calcitrans* with probiotic bacteria resulted in a significantly reduced microalgal growth when compared to the control (growth of *C. calcitrans* without bacteria) group. Nevertheless, the *in vitro* growth of both microalgal strains did not stop during the 7-day co-culture with the tested *Bacillus* sp. In this sense, Grossart *et al*. [34] reported higher growth of *Thalassiosira rotula* when exposed to marine bacteria; Grossart and Simmon [35] concluded that increased growth of microalgae depended on the bacterial species and nutrient and vitamin concentrations in the environment. Other studies have reported advantages of using co-culture of microalgae and beneficial bacteria. Toi *et al*. [36] reported an improved biomass production of *Artemia* sp. exposed to heterotrophic bacteria with a low microalgal feeding regime, concluding that bacterial role could be an additional nutrient source. De Paiva-Maia *et al*. [37] enhanced phytoplankton concentration in an intensive recirculation system of *Litopenaeus vannamei* farming treated with a commercial probiotic. Pacheco-Vega *et al*. [38] showed that the use of both, microalgae *Schizochitrium* sp. and *Lactobacillus plantarum* in rearing *L. vannamei* avoided the periodical probiotic supplementation and reduced the use of molasses (sugar cane) during farming. In this study, the growth of probiotic bacteria significantly improved in co-culture of *I. galbana* or *C. calcitrans* when compared to the control (growth of bacteria without microalgae). This result could be used to increase probiotic bacteria density in culture systems, and in consequence reduce potential pathogenic bacteria. Microalgal-bacterial interactions have been previously described as a mutualistic relationship in which both microorganisms benefit each other [21,22,39]. The positive effect observed on bacterial growth in co-culture with microalgae may represent a proposal for biocontrol because the increased bacterial growth with probiotic potential and the antibacterial effect have been evident in some microalgae against pathogens for aquaculture [40].

In aquaculture, the genus *Bacillus* spp. have been successfully used to improve water quality, reduce the load of harmful bacteria, and maximise the host immune response [41]. Moreover, using mixes of different *Bacillus* strains instead of monospecific ones offers advantages for enhancing the overall health of the host, likely in response to the specific synergy of the mix [42]. In this study, *C. sikamea* spat treated with *Bacillus* strains (individually or mixed), significantly improved absolute growth and survival, compared to the control and antibiotic groups. The studies with the use of *B. subtilis* and *B. licheniformis* as probiotic dietary supplements in aquaculture systems are rapidly increasing [43]. For example, a mixture of *B. subtilis* and *B. licheniformis* improves

resistance against *V. alginolyticus* activating the unspecific immune system of *Penaeus japonicus* shrimp [15]. The synergy effect has been shown in other studies in which only mixtures of *Bacillus* spp. (*B. endophyticus*, and *B. tequilensis*) improved growth of juvenile white shrimp *L. vannamei* [44] and the mixture of *B. subtilis*, *B. firmus*, and *B. flexus* also improved the growth and condition of white shrimp [45].

Separately, *B. subtilis* was reported as a probiotic in aquaculture [46], for example, to improve growth of Nile tilapia and immune modulation with respect to neutrophil adherence and lysozyme activity [47] or improve growth, digestive enzyme activity, resistance to *Vibrio harveyi* and upregulation of immune gene expression when fed in equal proportions to juvenile white shrimp for eight weeks at 10^8 cells g^{-1} of feed [48]. The species that have been most extensively examined on their responses to probiotics are *B. subtilis*, *Bacillus clausii, Bacillus cereus, Bacillus coagulans* and *B. licheniformis*. For more information, a review of the use and benefit of *Bacillus* sp. in aquaculture is available in Nemutanzhela *et al*. [41]. The increased growth in *C. sikamea* spat could be given through improved nutritional status and feed digestibility in the host associated to the addition of beneficial bacteria [49] and the additional proteins and lipids provided by *Bacillus* [17].

Organisms that improve their immune status by adding beneficial bacteria find themselves less stressed, so they reduce their energy consumption, having more energy available for growth; these organisms are also less susceptible, maintaining a higher metabolic homeostasis, so mortality decreases even before pathogen invasion [50]. In this study, the increased survival of juvenile *C. sikamea* treated with beneficial bacteria may be due to an induced better nutritional and immune status than untreated juveniles (control group) and those treated with the antibiotic.

Conclusion

The addition of selected *Bacillus* strains in culture of *Isochrysis galbana* did not affect growth significantly, and *C. calcitrans* resulted more sensitive to the presence of bacteria. Probiotic bacteria increased cell density in co-culture with microalgae and increased growth and survival of *C. sikamea* spat.

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