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Effect of ultraviolet (UV) radiation on the abundance and respiration rates of probiotic bacteria

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Abstract

Effects of ultraviolet radiation (UV) on probiotic bacteria (*Bacillus subtilis* and *B. licheniformis*) were tested in two experiments, with the following treatments: (i) UV treatment – using fluorescent and UV-lamps and (ii) Control – CTRL, using fluorescent lamps. Bacterial abundance and respiration were evaluated every 24 h for 3 days for Experiment 1, and at 0, 6 and 24 h for Experiment 2. In the Experiment 1, total UV dose was 4 336.41 mW cm^{-2}. UV treatment presented small respiration rates only on day 3, while in the CTRL oxygen consumption was always high. On all days, the abundance of the *Bacilli* exposed to UV was significantly smaller than that of the CTRL. The second experiment, with total UV dose of 1 445.47 mW cm^{-2}, presented oxygen consumption in the UV treatment only during the first 6 h. In the CTRL, oxygen consumption increased from the beginning due to the bigger abundance *Bacilli* cells. Small coccus-shaped bacteria ocurred in the UV treatment of both experiments. It may be concluded that exposure to UV, normally used for water disinfection, can inactivate probiotic bacteria.

Keywords: UV, probiotic, *Bacillus*, abundance, respiration

Introduction

Microorganisms are now used to improve the water quality of aquaculture systems (bioremediation), as antagonists to pathogenic bacteria (biocontrol), and to enhance the health of cultivated organism (probiotic) (Gatesoupe 1999; Balcázar, Blas, Ruiz-Zarzuela, Cunningham, Vendrell & Múzquiz 2006; Gutierrez-Wing & Malone 2006; Rijn, Tal & Schreier 2006; Wang, Li & Lin 2008). In addition, microorganisms, especially bacteria, play an important role in biofloc technology, which is used to allow the growth of fish and crustaceans at high density with reduced, or no water exchange (Wasielesky, Atwood, Stokes & Browdy 2006; Ballester, Abreu, Cavalli, Emerenciano, Abreu & Wasielesky 2010).

Probiotics are defined as viable microorganisms that positively affect the health of raised organisms by improving the indigenous microbial flora present in the gastrointestinal tract (Ouwehand, Tölkkö, Kulmala, Salminen & Salminen 2000). These microorganisms have the potential to enhance food digestibility and increase the immunity of the host species (Chen & Chen 2001; Decamp & Moriarty 2006; Picchietti, Fausto, Randelli, Carnevali, Taddei, Buonocore, Scapgloati & Abelli 2009; Velmurugan & Rajagopal 2009).

The probiotics currently used in aquaculture include a range of microorganisms, such as bacteria and yeasts (Decamp & Moriarty 2007). Bacterial species used as probiotics generally form spores that allow these microorganisms to be added to feed and still remain viable for long periods (Kesarcodi-Watson, Kaspar, Lategan & Gibson 2008). The use of *Bacillus* species as probiotics in aquaculture is common (Rengpipat, Rukpratanporn, Piyatiratitivorakul & Menasaveta 2000;

Rengpipat, Tunyanun, Fast, Piyatiratitivorakul & Menasveta 2003; Ziaei-Nejad, Rezaei, Takami, Lovett, Mirvaghefi & Shakouri 2006). For example, *Bacillus subtilis* can improve the immune conditions, growth and/or survival of fish (Kumar, Mukherjee, Prasad & Pal 2006) and shrimp (Vaseeharan & Ramasamy 2003; Tseng, Ho, Huang, Cheng, Shiu, Chiu & Liu 2009). The potential of the *B. licheniformis* as a probiotic organism was tested with good results for shrimp (Li, Zheng, Tian, Xi, Yuan, Zhang & Hong 2007). In addition, Bagheri, Hedayati, Yavari, Alizade and Farzanfar (2008) reported the efficiency of commercial probiotics composed by *B. subtilis* and *B. licheniformis* for use in culture of rainbow trout (*Oncorhynchus mykiss*).

Probiotics have been used in organisms raised in closed or recirculating water systems (McIntosh, Samocha, Jones, Lawrence, Mckee, Horowitz & Horowitz 2000; Taoka, Maeda, Jo, Jeon, Bai, Lee, Yuge & Koshio 2006). These systems utilize the water more efficiently with several cycles of production per year (Ridha & Cruz 2001). In recirculation systems, the water may be colonized by pathogens due to the high load of organic matter with relatively little dilution (Sharrer, Summerfelt, Bullock, Gleason & Taeuber 2005). In these cases, water disinfection is needed (Liltved, Hektoen & Efraimsen 1995). Some methods used for disinfection include treatment by heat, ozone and ultraviolet radiation (Douillet & Pickering 1999; Ouwehand *et al.* 2000).

Moran and Zepp (2000) and Summerfelt (2003) described the different effects of UV radiation on microorganisms. For example, UV radiation can damage DNA, causing lethality or defects in growth rates. In addition, it can damage other molecules such as proteins associated with cell membranes, which can also have a significant impact on growth and reproduction. Ultraviolet radiation is very effective in inactivating a variety of microorganisms (Liltved *et al.* 1995). However, the effect of UV radiation on specific probiotic organisms remains unclear because factors such as germicidal wavelength, exposure period and type of microorganism can give different results (Chen, Craik & Bolton 2009).

The main objective of this study was to evaluate the possible effects of UV radiation on the abundance and respiration rates of the probiotic bacterial species *B. subtilis* and *B. licheniformis* used in closed aquaculture systems.

Material and methods

Two experiments were carried out at the Aquaculture Marine Station of the Federal University of Rio Grande, southern Brazil. The first experiment lasted for 3 days, and the second experiment took 24 h. Both experiments were composed of two treatments: (i) UV treatment – with fluorescent and UV lamps (T8 – Germicidal NARD Light Express), and (ii) Control (CTRL) with only fluorescent lamps.

The commercial probiotic (PRO-Sanolife W® INVE, Salt Lake City, UT, USA) composed of *Bacillus subtilis* and *B. licheniformis* spores ($< 5 \times 10^{10}$ cfu g^{-1}) were added (0.1 g L^{-1}) to 12 L of autoclaved and filtered (glass fibre filter, GF 1-A, Schleicher & Schuell) seawater (30 g L^{-1}). According to the manufacturer, bacterial germination occurs from 2 to 8 h after addition in the water. No nutrients were added to the water, except that of the commercial probiotic.

The solution of probiotic and seawater was distributed in 12 sterilized 1 L beakers. Six beakers were stored in an incubator (Tecnal TE - 401, Brazil) equipped with two T8 fluorescent lamps (15 W) and two UV lamps (GL/Germicidal NARD Light Express – 15 W) (UV treatment). UV irradiance (μW cm^{-2} s^{-1}) was measured in the subsurface of water with an International Light IL-1400A (UV-A and UV-B) and a MRUR-203 (UV-C). Total UV dose was obtained by multiplying the UV irradiance by exposure time (mW cm^{-2}).

The other six beakers were stored in another similar incubator equipped only with four fluorescent lamps (CTRL). Both incubators were kept at a constant temperature of 26°C (±0.1°C). All beakers received gentle aeration to ensure water circulation. Water from each treatment was sampled every 24 h in Experiment 1 and at 0, 6 and 24 h in Experiment 2 for further analyses of bacterial abundance and respiration rates.

In each sampling period, water samples were taken and fixed using 4% (v/v) formalin (final concentration) and stored in 100 mL glass bottles. Afterwards, 1 mL aliquots were filtered through polycarbonate membranes filters (Nuclepore, Kent, UK, 0.2 μm pore size) previously darkened with 12% Irgalan Black. Cells were dyed with acridine orange (1 μg mL^{-1}) (Hobbie, Daley & Jasper 1977) and counted in thirty randomly chosen fields using a Zeiss Axioplan epifluorescence microscope (Oberkochen, Germany) equipped with a blue filter

Table 1 UV dosages used in the Experiments 1 and 2

	Experiment 1			Experiment 2	
UV dose (mW cm^{-2})	Day 1	Day 2	Day 3	6 h	24 h
UV-A	1,416.96	2,833.92	4,250.88	354,24	1,416.96
UV-B	1.72	3.45	5.18	0.43	1.72
UV-C	26.78	53.56	80.35	6.69	26.78
Total	1,445.47	2,890.94	4,336.41	361.36	1,445.47

set (487709 – BP 450-490, FT 510, LT 520) and a CCD Watec (Watec Co., Yagamata, Japan) (0.0003 Lux).

For respiration measurements, three samples of each treatment were poured into 300 mL dark BOD bottles. Dissolved oxygen concentrations were measured in each bottle before (DO_0) and after (DO_1) incubation in the dark at 26 ± 0.1°C for 24 h. The measurements of the dissolved oxygen concentrations were carried out using a calibrated oxymeter (Digimed DM4P, Brazil). The respiration rates were calculated as difference between DO_1 and DO_0 and expressed as mg O_2 L^{-1} h^{-1}.

The Kruskal–Wallis nonparametric analysis was employed to identify significant differences between treatments in both experiments ($P < 0.05$) (Sokal & Rohlf 1995).

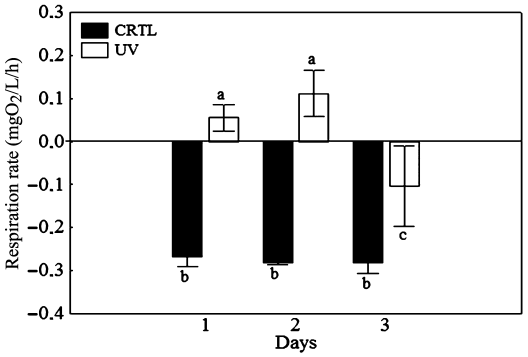

Figure 1 Respiration rates of the ultraviolet and control treatments on days 1, 2 and 3. Vertical lines represent one standard deviation and different letters show significantly statistic difference ($P < 0.05$).

Results

Experiment 1

UV dose used in Experiment 1 is shown in the Table 1. On days 1 and 2, no respiration was observed in the UV treatment, but there was a small amount of oxygen production (0.06 ± 0.03 and 0.11 ± 0.06 mg O_2 L^{-1} h^{-1}), while in the CTRL treatment, oxygen levels decreased in all bottles from the beginning of the experiment (−0.27 ± 0.02 and −0.28 ± 0.0 mg O_2 L^{-1} h^{-1}). On day 3, both treatments presented bacterial respiration, although respiration rates in the UV treatment (−0.10 ± 0.10 mg O_2 L^{-1} h^{-1}) were smaller than in the CTRL treatment (−0.28 ± 0.03 mg O_2 L^{-1} h^{-1}) (Fig. 1). Mean respiration values were significantly different between treatments during the study period ($P < 0.05$).

The abundance of *Bacilli* was not significantly different between days for each treatment. Nevertheless, the abundance of the *Bacilli* was significantly lower in the UV treatment (0.66 ± 0.49–1.16 ± 1.20 × 10^5 cells mL^{-1}) than in the CTRL treatment (13.36 ± 38.41–34.46 ± 19.66 × 10^5 cells mL^{-1}) during all experiments. The abundance of other bacteria (small coccus) significantly increased in the CTRL treatment on day 2 (1.15 ± 1.30–13.36 ± 3.84 × 10^5 cells mL^{-1}). In contrast, in the UV treatment, the abundance of the small coccus significantly increased only on day 3 (1.21 ± 1.24–13.36 ± 3.84 × 10^5 cells mL^{-1}) (Fig. 2).

Experiment 2

The UV dose applied in this experiment is also shown in the Table 1. At 0 h, the respiration rates were not significantly different between the UV (−0.28 ± 0.01 mg O_2 L^{-1} h^{-1}) and CTRL (−0.25 ± 0.02 mg O_2 L^{-1} h^{-1}) treatments. The respiration rates at 12 h in the UV treatment (−0.31 ± 0.02 mg O_2 L^{-1} h^{-1}) were significantly higher than that in the CTRL treatment (−0.24 ± 0.01 mg O_2 L^{-1} h^{-1}). At 24 h, the oxygen consumption in the CTRL treatment was not significantly different compared with the other sampling periods (−0.27 ± 0.02 mg O_2 L^{-1} h^{-1}). However,

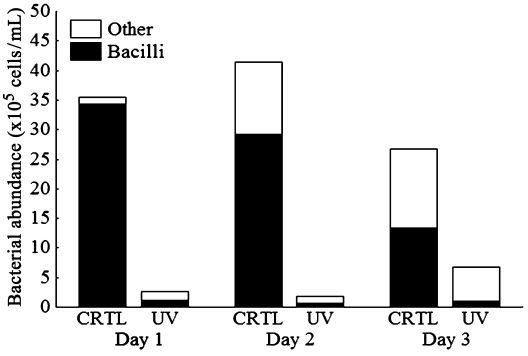

Figure 2 Mean abundance (10^5 cells mL^{-1}) of the *Bacilli* and other bacteria in the control and ultraviolet treatments on the days 1, 2 and 3.

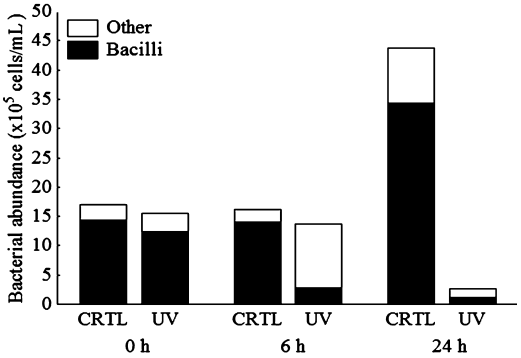

Figure 4 Mean abundance (10^5 cells mL^{-1}) of the *Bacilli* and other bacteria in the control and ultraviolet treatments at 0, 6 and 24 h.

in the UV treatment, there was oxygen production (0.06 ± 0.03 mg O_2 L^{-1} h^{-1}) in this sampling period, differing significantly from the CTRL treatment (Fig. 3).

The abundance of the *Bacilli* species in the control treatment did not vary significantly between 0 h (14.34 ± 7.29 × 10^5 cells mL^{-1}) and 6 h (13.98 ± 8.80 × 10^5 cells mL^{-1}), but increased significantly at 24 h (34.36 ± 19.46 × 10^5 cells mL^{-1}). In the UV treatment, however, the abundance of the *Bacilli* was significantly reduced from 0 h (12.45 ± 7.17 × 10^5 cells mL^{-1}) to 6 h (2.86 ± 1.33 × 10^5 cells mL^{-1}) and to 24 h (1.15 ± 1.22 × 10^5 cells mL^{-1}). The bacterial abundance was not significantly different between treatments at 0 h, while at 6 h and 24 h, the abundance in the UV treatment was significantly lower than that in the CTRL treatment (Fig. 4). The abundance of other bacteria (also small coccus) significantly increased in the UV treatment at 6 h (1.45 ± 0.9–10.89 ± 3.99 × 10^5 cells mL^{-1}) and in the CTRL treatment at 24 h (2.19 ± 3.21–9.30 ± 4.44 × 10^5 cells mL^{-1}) (Fig. 4).

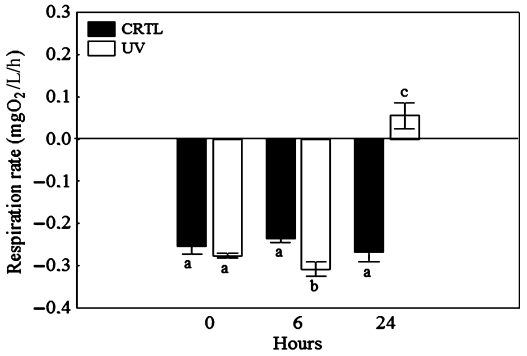

Figure 3 Respiration rates of the ultraviolet and control treatments at 0, 6 and 24 h. Vertical lines represent one standard deviation and different letters show significantly statistic difference ($P < 0.05$).

Discussion

Bacterial activity can be affected by ultra violet radiation in different ways. For example, lethal effects occur when the DNA and other molecules, such as proteins associated with cell membranes, are damaged. This may affect metabolic functions or generate mutations in essential genes, resulting in the death of the microorganism. Sub-lethal effects, on the other hand, do not cause cell death, but can negatively influence bacterial growth and metabolism. For instance, when cell membranes are injured, the permeability and hence the transport of molecules may be affected, and this can lead cell inactivation. Similarly, some components of the electron transport chain can absorb UV radiation and disrupt the gradient of electrons, stopping the mechanism of energy production, the so called proton-motive force (Moran & Zepp 2000; Summerfelt 2003).

UV radiation has been successfully used for the elimination of pathogenic bacteria and viruses in seawater in closed, recirculating, water systems (Liltved *et al.* 1995; Sharrer *et al.* 2005; Sharrer & Summerfelt 2007). However, the time necessary for this radiation to affect probiotics remains unknown (McIntosh *et al.* 2000; Taoka *et al.* 2006). Currently, UV lamps must be turned off before probiotic bacteria are added to the system due to their lethal and sub-lethal effects on bacteria. Thus, the frequency of probiotic addition in closed-culture systems equipped with UV must be determined and

will be different from that recommended by the manufacturers for open systems.

UV dose used in aquaculture systems has great variation. For example, Wedemeyer (1996) reported that UV dosages may vary from two to more than 230 mW s cm^{-2}. On the other hand, Sharrer *et al.* (2005) had mentioned that more 1 800 mW s cm^{-2} can reduce about 98% of heterotrophic bacteria abundance in a recirculating water system. For one of the probiotic species tested in this study, Hijnen, Beerendonk and Mederna (2006) observed that a UV dose varying between 5 and 78 mW s cm^{-2} is enough to inactivate *B. subtilis.* Total UV (A, B and C) dose used in this study were 4 336.41 mW cm^{-2}, at the end of the first experiment and 1 445.47 mW cm^{-2} after 24 h of the second test. Both doses were sufficient to guarantee the elimination of most bacteria in the water.

The bacterial abundance and respiration rates of both experiments indicate that the exposure to UV radiation killed most of the probiotic bacteria added to the seawater in the first day of Experiment 1, generating a decrease in oxygen consumption. In Experiment 2, it was observed that the deleterious effects of UV on both tested *Bacillus* species were most pronounced after 6 h of exposure to the UV radiation, as indicated by the decrease in *Bacilli* abundance (Figs. 2 and 4).

Considering the fact that *Bacillus subtilis* and *B. licheniformis* spores take 2 to 8 h to germinate, one could assume that UV would act only on active cells. *Bacillus subtilis* spores are 10- to 50-fold more resistant to UV radiation than active growing cell (Setlow 2006). However, it is noteworthy that respiration rates after 6 h in the UV treatment were higher than in the beginning of the experiment. It may be explained by the fact that some genes that regulate physiological processes, such as respiration, remain active for some time after UV exposure (Hamkalo & Swenson 1969). In addition, high respiration rates could also be stimulated by this radiation because many bacterial species show high metabolic activity after UV exposure (Blatchley & Peel 2001), although they lose their capacity for reproduction (Lopez, Li, Kataria, Russel & Neu 2008).

It is also likely that the high respiration rates measured after ultra violet treatment in both experiments were due to the presence of non-*Bacilli* cells in the water. Contamination of the experimental system with non-*Bacilli* bacteria likely originated from the air used to homogenize the water and/or due to contaminated sampling tools. It is unclear why UV did not affect these contaminating cells, as they showed an increase in abundance from 0 h to 6 h in Experiment 2 (Fig. 4). The non-*Bacilli* bacteria consisted mainly of small coccus-shaped cells. It is likely that the reduced bacterial surface probably led to smaller UV doses per unit volume than that received by the probiotic cells (Häder, Kumar, Smith & Worrest 1998; Madigan, Martinko & Parker 2003). We currently have no clear hypothesis as to why some samples from the UV treatment presented an increase in oxygen concentration after incubation. However, oxygen production in dark BOD bottles was previously reported as the action of catalase enzyme on hydrogen peroxide (Pamatmat 1997). More recently, Cory, McNeill, Cotner, Amado, Purcell and Marshall (2010) reported that when samples with dissolved organic matter are exposed to UV and visible light, an increase in DOM constituents with higher oxygen contents occur and the release of H_2O_2 are observed.

It must be considered that this experiment did not completely mimic real conditions of closed or recirculating aquaculture systems, especially regarding the possible UV protective effects of high turbidity and water colour found in aquaculture conditions. However, the results clearly indicate that the use of UV treatment must be carefully considered if the use of probiotics is necessary. The primary conclusion of this study is that the addition of *B. subtilis* and *B. licheniformis* to closed culture systems with UV lamps must be carried out at least every 6 h because after this period, these bacteria suffer lethal effects of the UV radiation. It is now necessary to evaluate the costs and benefits generated by the use of probiotics and UV treatment because they show antagonistic effects. There is no doubt that the use of UV radiation in close recirculating system is an effective way to guarantee disinfection of the water. However, the use of this equipment can hamper the positive effects of additional probiotic bacteria on the raised organisms.

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