Effect of bacterial epiflora on egg hatching of the Atlantic sardine (*Sardina pilchardus*)

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Abstract

The aim of this work was to study the influence of bacterial epiflora on egg hatching of the sardine (*Sardina pilchardus*) obtained from a natural environment (Ría de Vigo, Spain) during the spawning season of the sardine (from January to June). Total bacteria, viable bacteria or the presence of specific potential pathogens for eggs, such as *Pseudoalteromonas piscicida* and *Tenacibaculum (Flexibacter) ovolyticus*, did not affect the viability of sardine eggs. Additionally, no relationship was observed between the presence of *Vibrio* spp., pathogenic for fish larvae, and the egg hatching. This was probably because the amount of bacteria associated with the eggs were between 10^2 and 10^4 orders lower than those found so far on the eggs of different fish species in rearing systems. Therefore, epiphytic bacteria did not affect the wild sardine eggs and, hence, in the area studied, it is probably not an important factor affecting annual recruitment success of this pelagic fish species.

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1. Introduction

Although catches have been decreasing in recent decades [1] the sardine (*Sardina pilchardus*) is one of the most important pelagic fish species of the northwestern coast of the Iberian Peninsula. It has been observed that year-to-year sardine recruitment variability in this area is mainly determined by oceanographic and climatic factors, such as water column stability in February; larval transport offshore in March–April; the input of nutrients during May to August of the preceding year; and the North Atlantic Oscillation winter index [1]. However, when favourable environmental conditions have promoted a high year-class, other factors such as predation [2] and parasites [3], might likely come into play during the egg and larval stages. The surface of marine fish eggs is an excellent substrate for colonization by bacteria [4]. Furthermore, the existence of opportunistic pathogens associated with the surface of fish eggs can affect their viability and their later egg development [4–9].

In studies of fish eggs in rearing systems, it has been shown that some bacteria associated with the egg surface could damage egg membrane through exoenzymatic activities, excessive consumption of oxygen or the production of toxic metabolites [4,10].

To date, the only reported study concerning microbial community associated with sardine eggs in natural environments [11] showed that members of the genera *Vibrio*, *Alteromonas/Pseudoalteromonas*, *Pseudomonas...
and *Moraxella* dominated the culturable adherent microflora, whereas the genera *Aeromonas*, *Tenacibaculum* (*Flexibacter*), *Flavobacterium* and *Cytophaga* were detected as minor components of associated microbial community. Our previous study [11] showed the presence of *Vibrio anguillarum* and *V. fischeri*, pathogens of fish larvae [12], and also *Tenacibaculum ovoleticum* and *Pseudoalteromonas piscicida*, pathogens of fish eggs [7,13], however the influence of these bacteria on the egg hatching was not discussed.

The purpose of this study was to determine the possible relationship between the load and composition of bacteria on surface of eggs on their viability for the sardine (*S. pilchardus*).

### 2. Materials and methods

#### 2.1. Sampling

During the sampling period, that is from January to June 2000, samples of sardine (*S. pilchardus*) eggs were collected monthly. Samples were collected from the Ría de Vigo in Galicia coast (NW, Spain) (Fig. 1). The sampling was performed as described by Miguez and Combarro [11].

#### 2.2. Enumeration, isolation and characterization of bacteria

The method of Hansen and Olafsen [4] was used to isolate the bacteria from the eggs. This entailed three successive washings of the eggs in sterile seawater before being homogenized. To facilitate the removal of surface bacteria 10 µg/ml of Tween 80 (Panreac) were added. Two types of counts were completed using decimal dilutions of filtered and sterile seawater obtained from the homogenate: total direct count (TDC) and heterotrophic bacterial count (HBC). Direct counts of bacteria were determined by epifluorescence microscopy with acridine orange (Sigma) using the procedures of Kepner and Pratt [14]. Bacteria were counted at 1250× magnification with an Olympus BH2-RFC fluorescence microscope using blue light excitation. An ocular 10×10 grid was used and a total of 60 fields were counted. The results were expressed as cells per milliliter. For heterotrophic bacterial counts, 0.1 ml aliquot of each dilution was plated in triplicate on Marine Agar (MA: Cultimed). After five days incubation at 15 °C, the numbers of colonies on both media were counted and the average number of colony forming units (CFUs) per egg was calculated. Total direct count and heterotrophic bacterial count were considered in the viability study.

The identification of the isolates from marine agar was done as described by Miguez and Combarro [11]. Of all the genera associated with sardine eggs, only the percentages obtained for the genera *Vibrio*, *Pseudoalteromonas* and *Tenacibaculum* (*Flexibacter*) were considered in the viability study.

#### 2.3. Egg hatching estimation

Of all eggs collected each month, approximately 90 in the stage II were randomly selected and individually incubated in 10 ml of filter-sterilized (0.2 µm) seawater. The eggs were incubated at three different temperatures (12, 14 and 16 °C), 30 eggs for each temperature. Egg hatching was estimated as the percentage of eggs hatched onto healthy larvae. The mean hatching time, the period between the beginning of the incubation until the egg hatched onto the larva, was 77.04 h for 12 °C, 65.51 h for 14 °C and 48.28 h for 16 °C.

#### 2.4. Statistical analysis

A correlation analysis was carried out for each of the two bacteria counts and the egg hatching estimation, and for each of the three genera and the egg hatching estimation.

### 3. Results

The percentages of egg hatching ranged between 90.4% and 94.3%, these values remained relatively constant throughout the sampling period. TDC and HBC were considered in the viability study. No relationship was found between TDC and the hatch of the eggs ($r = 0.13, p = 0.84$) (Fig. 2(a)) and neither
was a relationship observed between HBC and egg hatching \((r = 0.23, p = 0.71)\) (Fig. 2(b)).

The dominant genera in the adherent microbial community of sardine eggs collected from the Ría de Vigo were *Vibrio*, *Alteromonas/Pseudoalteromonas*, *Pseudomonas* and *Moraxella*. The genera *Flavobacterium*, *Tenacibaculum (Flexibacter)*, *Aeromonas* and *Cytophaga* were detected as minor components [11]. No relationship was detected between the percentage of the genera that included species potentially pathogenic for fish eggs (*Flexibacter*, \(r = 0.40, p = 0.51\); *Pseudoalteromonas*, \(r = 0.07, p = 0.91\)) and the viability of the eggs (Fig. 3(b) and (c)). Neither was a relationship observed between the genus that included species potentially pathogenic for larvae (*Vibrio*, \(r = 0.34, p = 0.58\)) and the egg hatching (Fig. 3(a)).

### 4. Discussion

All studies that have intended to link the existence of possible pathogens with the mortality of fish eggs have been undertaken in rearing systems, where it is relatively easier to obtain and control them. The excessive bacterial growth associated with the surface of fish eggs can produce hypoxia of the embryo [4,15,16], degradation of the shell due to the
exoenzymatic activity of the associated bacteria [10, 17] or even affect their ability to float [5]. The counts of viable bacteria associated with sardine eggs obtained from natural environment [11] were lower than counts obtained in previous studies carried out in rearing systems with others fish species [8, 18]. In this context, Keskin et al. [8], Hameed [19] and Hansen and Olafsen [18] observed in their respective studies that bacterial colonization associated with the surface of the fish eggs was considerably higher after the incubation. From this fact it is deduced that the existence of special conditions in intensive rearing systems facilitate high levels of bacterial colonization, explaining the presence of a greater bacterial load in these rearing systems in respect to that in natural ecosystems. The low count of bacteria obtained for the sardine eggs collected from a natural environment is probably one of the main factors that explain the absence of a relationship between the counts of associated bacteria and the viability of these eggs, as it has been shown in our results.

This study demonstrates that, in this case, cultivable viable bacteria do not influence the egg hatching. Furthermore, the fact that there is no relationship between total bacteria and the viability of the eggs, could suggest that the range of viable but non-cultivable bacteria that could affect the quality of the eggs does not have a relevant role.

From the genera identified associated with sardine eggs [11], the genera *Vibrio*, *Pseudoalteromonas* and *Tenacibaculum* (*Flexibacter*) were considered in the viability study. Among the species identified as vibrios, two must be pointed out. These are *V. anguillarum*, an important fish pathogen [20] and *V. fischeri*, which was also previously detected associated to cod and halibut eggs in rearing systems [4]. No relationship was found between the presence of these genera and the viability of the eggs. These results agree with those observed in their respective studies that bacterial colonization, explaining the presence of a greater bacterial load in these rearing systems in respect to that in natural ecosystems. The low count of bacteria obtained for the sardine eggs collected from a natural environment is probably one of the main factors that explain the absence of a relationship between the counts of associated bacteria and the viability of these eggs, as it has been shown in our results.

We conclude that the bacterial load present on the sardine eggs is not a determining factor in their viability when compared with the various biotic and abiotic factors present in their natural ecosystem [1]. However, the microbial community of sardine eggs is diverse and contains several pathogens. In our previous study an effect of abiotic factors on bacterial counts associated with the surface of sardine eggs was not observed [11], however, there were no important changes of these abiotic factors during the period studied. Therefore, it is not possible to decline the idea that abundance of bacterial epiflora may change in an environment with higher seasonal variability in abiotic conditions and/or in bacterial community profiles in the water column, thus affecting sardine egg viability.

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References


