

ADHESION OF PATHOGENIC BACTERIA TO POLYSTYRENE, SKIN AND GUT MUCUS OF GILTHEAD SEABREAM, INFECTIOUS CAPACITY AND ANTIBIOTICS SUSCEPTIBILITY

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Received: February 05, 2019

Approved: July 28, 2019

ABSTRACT

Linking properties of adhesion, infectious capacities and antibiotic resistance of pathogen bacteria could help to treat fish diseases. Adhesions of ten fish pathogenic bacteria were tested in microtiter plates vacant, coated with skin or gut mucus, fixed with methanol, stained with 2% crystal-violet and revealed by colorimetric method. Infectious capacity was performed by exposing gilthead seabream fibroblast cell line (SAF-1) to 10^7 - 10^8 CFU mL⁻¹ of pathogen bacteria. Cell viability was measured 3h, 9h and 20 hours post-infection. The sensitivity to antibiotics was executed by disk diffusion. Data showed that all the bacteria tested adhere to polystyrene. For skin mucus, *Vibrio harveyi*, *Vibrio alginolyticus*, *Halomonas venusta*, and *Aeromonas bivalvium* were moderately adherent. *Dietzia maris* was strongly adherent. For gut mucus, 60% of tested bacteria were weakly adherent and 40% were non adherent. For infection, *D. maris*, *V. harveyi* and *A. bivalvium* decreased the cells viability to 89% after only 3h. After 20h, the viability percentage ranged between 1% and 32%. All isolates presented resistance to 1000 mg ml⁻¹ of sulphonamide, 60% were resistant to sulfonamide and penicillin G. Present findings could be relevant in fish aquaculture and underscore the importance of the linkage between adhesion, infectious capacity, and antibiotic susceptibility of pathogen bacteria to avoid fish diseases.

Key words: pathogen adhesion; skin mucus; gut mucus; SAF-cell line; antibiotic susceptibility; gilthead seabream (*Sparus aurata* L.).

ADESÃO DE BACTÉRIAS PATOGÊNICAS A POLIESTIRENO, PELE E MUÇO INTESTINAL DE GILTHEAD SEABREAM, CAPACIDADE INFECCIOSA E SUSCEPTIBILIDADE A ANTIBIÓTICOS

RESUMO

Estudar o link entre propriedades de adesão, capacidades infecciosas e resistência a antibióticos de bactérias patogênicas pode ajudar a tratar doenças de peixes. A adesão de dez bactérias patogênicas foi testada em placas de microtitulação vazias, revestidas com muco da pele ou do intestino, fixadas com metanol, coradas com 2% de violeta cristal e reveladas pelo modo colorimétrico. A capacidade infecciosa foi realizada expondo a linha celular de fibroblasto de dourada (SAF-1) a 10^7 - 10^8 CFU mL⁻¹ de bactérias patogênicas. A viabilidade celular foi medida 3h, 9h e 20 horas após a infecção. A sensibilidade aos antibióticos foi executada por difusão em disco. Os dados mostram que todas as bactérias testadas aderem ao poliestireno. Para o muco cutâneo, *Vibrio harveyi*, *Vibrio alginolyticus*, *Halomonas venusta* e *Aeromonas bivalvium* foram moderadamente aderentes. *Dietzia maris* foi fortemente aderente. Para o muco intestinal, 60% das bactérias testadas eram fracamente aderentes e 40% não aderentes. Para infecção, *D. maris*, *V. harveyi* e *A. bivalvium* diminuíram a viabilidade celular para 89% após apenas 3h. Após 20h, o percentual de viabilidade variou entre 1% e 32%. Todos os isolados apresentaram resistência a 1000 mg mL⁻¹ de sulfonamida, 60% foram resistentes à sulfonamida e à penicilina G. Os achados atuais podem ser relevantes na aquicultura e ressaltam a importância da ligação entre adesão, capacidade infecciosa e suscetibilidade a antibióticos de bactérias patogênicas para evitar doenças em peixes.

Palavras-chave: adesão de patógenos; muco da pele; muco do intestino; linha de células SAF; sensibilidade a antibióticos; dourada (*Sparus aurata* L.).

INTRODUCTION

Gilthead seabream (*Sparus aurata* L.) aquaculture is one of the fastest-growing and most promising segments of fish production around the world including Brazil that have 9200 km of coastline. Gilthead seabream possesses many important nutritional qualities that allow this fish to be more focused in aquaculture such as their significant content in ω -3 fatty acids and micro constituents of fish lipids (Nasopoulou et al., 2011), their tolerance of husbandry procedure and resistance to diseases (Okte, 2002).

The intensive culture of food fish, such as gilthead seabream, has led to outbreaks of various bacterial diseases, resulting in annual economic losses to the aquaculture industry. Thus preventative and therapeutic approaches represents a significant challenge today.

In aquatic environment, several pathogenic or commensal bacterial species can form a complex multicellular structure on surfaces known as biofilm (Hall-Stoodley and Stoodley, 2005; Doi et al., 2015; Ballesteros et al., 2016). The adherence of bacteria to surfaces, including mucosal surfaces, is considered a prerequisite for microbial colonization, infection or both (Otto, 2014). Successful establishment of infection by bacterial pathogens requires previously adhesion of such bacteria cells to host, colonization of tissues, and in certain cases, bacteria can perform cellular invasion. Usually, this invasion is followed by bacterial intracellular multiplication, dissemination to other tissues, or persistence (Ben Hamed et al., 2018; Pizarro-Cerda and Cossart, 2006).

Bacterial biofilm population could be formed with rod shaped or cocci-shaped bacteria, likewise it could be formed with a mixed consortium bacteria. Biofilm formation conveys a selective advantage to certain pathogens by increasing their ability to persist under diverse environmental conditions (Hall-Stoodley and Stoodley, 2005). Some studies considered that adhesion can be a prerequisite for successful infection, and once adhesion occurs, the bacteria can induce the expression of other virulence genes and cause the activation of host cell signaling pathways (Pizarro-Cerdá and Cossart, 2006; Ovando Fraiha et al., 2019). Bacterial adhesion and colonization depend on several factors, among them: the bacterial species, the bacterial surface, chemical and physical interactions between the potential substratum and the polymeric adhesive (Tang et al., 2009). In addition, the surface topographical feature has also a strong influence of the bacterial adhesion. Bacteria are more able to attach to crevices and pits where they are protected from unfavorable environmental disturbances (Hsu et al., 2013). In fact, it has been hypothesized that bacteria preferentially stick to rougher surfaces because of a higher surface area available for attachment and besides that a chemical change on this surface type causes preferential physicochemical interactions (Scheuerman et al., 1998).

As well, the bacterial resistance to antibiotic depends on many factors. Some of them are related to the physic-chemical and environmental conditions while others are related to the bacterial strain properties. Ceri et al. (1999) demonstrated that the antibiotic susceptibility of certain bacteria in biofilm is 1,000 times the minimum inhibitory concentration (MIC) compared to susceptibility measured for free-floating bacteria.

It is known that the indiscriminate use of antibiotics in aquaculture may cause toxic effects, bacterial resistance and accumulation of residues in tissues of individuals, as well as other potentially negative effects on human health and environmental quality which have raised public concern, resulting in strict regulations of their use (Buschmann et al., 2012).

In aquaculture industry, when fish diseases are diagnosed, the treatments start with a global identification of the pathogen and subsequently with the antibiotic administration in water or mixed with food pellet to eradicate the pathogen. Unfortunately, these administration methods of antibiotics usually neglected the adhesive capacity of the bacteria and their ability to arrange in a biofilm (Stewart and Costerton, 2001). In bacteriological diagnosis studies, the majority of authors have focused on identification of the pathogen but information is lacking about the adhesion of these pathogens to the environment of fish, to the skin and gut mucus.

In our opinion, we hypothesizes that combination of some bacterial proprieties essentially adhesion, cell infection and resistance to antibiotic could be useful for fish disease treatment. Thus, the aim of the present study was to check the adhesion capacity of several common pathogens bacteria (*Aeromonas bivalvium*, *Dietzia maris*, *Halomonas venusta*, *Pseudoalteromonas* sp, *Pseudomonas* sp, *Serratia marcescens*, *Tenacibaculum mesophyllum*, *Vibrio alginolyticus*, *Vibrio harveyi* and *Vibrio kanaloae*) which infect fish and cause damages in fish farms. For this, the adhesion capacity was evaluated to polystyrene, to skin mucus and to gut mucus of gilthead seabream. Additionally, the infectious capacities of these bacterial strains were tested on gilthead seabream fibroblast cell line (SAF-1 cells) and the sensitivity of them was verified against thirteen antibiotics used frequently in fish aquaculture.

MATERIAL AND METHODS

Fish care and maintenance and mucus collection

Specimens of the hermaphroditic protandrous seawater teleost gilthead seabream (*Sparus aurata* L.) obtained from the local farm (Murcia, Spain) were randomly placed in seawater aquaria (250 L). A quarantined period was induced for one month, with daily veterinary control, before the start of the study without showing any disease. Furthermore, fish were allowed to acclimatise, in new seawater aquaria (250 L), for 15 days before the start of the experimental trial. Fish (mean body weights 25-30 g) were reared in seawater aquaria (250 L) in the Marine Fish Facility at the University of Murcia in recirculation systems. The water was maintained at 20 ± 2 °C with a flow rate of 900 L h⁻¹ and 28‰ salinity. The photoperiod was of 12 h light: 12 h dark and fish were fed with a commercial pellet diet (Skretting) at a rate of 2% body weight day⁻¹.

Fish were sacrificed by using an overdose of MS-222 (Sandoz, 100 mg mL⁻¹ water). Skin mucus samples were collected from naïve specimens using the method of Guardiola et al. (2015) with some modifications. Briefly, skin mucus were collected by gentle scraping the dorso-lateral surface of naïve seabream specimens using a cell scraper into a small amount of phosphate buffer saline (PBS), with enough care to avoid contamination with

blood and urino-genital and intestinal excretions. For intestinal mucus, the intestine was separated during autopsy and mucus was collected and homogenized in PBS. All mucus preparations were centrifuged (12,000 x g, 10 min, 4 °C) to remove particulate and cellular material (Balcázar et al., 2007), being the supernatants aliquoted and stored at -20 °C until use.

Bacterial growth conditions

Bacterial isolates were kindly donated by Dr. M.A. Morinigo from the Department of Microbiology (University of Málaga, Spain). These bacteria were isolated from specimens of gilthead seabream manifesting symptoms of several diseases. All isolates are Gram negative, rod shaped, motile except *D. maris* which is Gram positive. The diseases symptoms of these strains are essentially hyperpigmentation, skin and/or intestinal ulcers, anemia and hemorrhages in the muscle, gills, fins, nares (Minana-Galbis et al., 2007). All the bacterial isolates used in this study were grown in Tryptic Soy Broth (TSB) (Difco Laboratories, Detroit, MI) with 2% NaCl (TSBS) and incubated at 22 °C for 24 h with agitation 150 rpm (Balebona et al., 1995). Bacteria were harvested by centrifugation (2,000 x g for 10 min), washed twice with phosphate-buffered saline (PBS; 10 mM phosphate, pH 7.2), and resuspended in PBS. Bacterial suspensions were adjusted to an absorbance (600 nm) of 0.25 ± 0.05 that corresponded to 10^7 - 10^8 CFU mL⁻¹ (Basson et al., 2008).

Bacterial adhesion tests

Wells of sterile 96-well U-bottomed microtiter plates (Deltalabs S.L, Barcelona, Spain) were each filled with 90 µL TSBS and 10 µL of each bacterial suspension (prepared above), in triplicate. Negative control wells contained only broth. Microtiter plates were incubated aerobically at room temperature in a shaker (150 rpm) for 24h. Contents of each well were aspirated, washed three times with 250 µL sterile PBS and the remaining cells were fixed with 200 µL of methanol for 15 min. After air-drying, wells were stained with 150 µL of 2% crystal violet for 5 min. Dye bound to adherent cells was resolubilized with 150 µL of 33% (v/v) glacial acetic acid, and the optical density (OD) of each well was obtained at 595 nm using an automated microtiter-plate reader (Microplate Reader model 680, BioRad Laboratories Inc., Hercules, California). All the tests were done in triplicate on three separate occasions and the results averaged (Basson et al., 2008).

Bacterial adhesion to the skin and intestinal mucus was determined as described by Balcázar et al. (2007) with some modifications. Briefly, 100 µL of skin or intestinal mucus was immobilized on polystyrene microtiter plate wells by overnight incubation at 4 °C. The wells were washed twice with 250 µL of PBS to remove unbound mucus. Aliquots of 90 µL PBS and 10 µL of each bacterial suspension (prepared as it was described above), were added to each well. After incubation for 1 h at 22 °C, the wells were also washed twice with 250 µL PBS to remove unbound bacteria. The cutoff OD (OD_c) for the microtiter plate test was defined as three standard deviations above the mean OD of the negative control. Isolates were classified as follows: OD ≤ ODC = non-adherent, ODC < OD ≤ (2 × ODC) = weakly

adherent; (2 × ODC) < OD ≤ (4 × ODC) = moderately adherent and (4 × ODC) < OD = strongly adherent. Adhesion of the bacteria was performed in triplicate for polystyrene and for each type of mucus (Basson et al., 2008). The polystyrene microtiter plate is an abiotic surface used as a positive control for the bacterial adhesion (Nicolau Korres et al., 2013).

SAF-1 cell culture

The pathogenicity of isolated bacteria was tested on the non-phagocytic epithelial SAF-1 cell line provided by the European Collection of Cell Cultures (ECCC). The culture and maintaining of SAF-1 cell was realized following the protocol detailed by Béjar et al. (1997). SAF-1 cells were cultured in Leibowitz L-15 medium (Gibco) supplemented with 10% of fetal bovine serum (FBS; Gibco), 2 mM L-glutamine (Gibco), 100 IU mL⁻¹ penicillin (Gibco) and 100 µg mL⁻¹ streptomycin (Gibco). Cells were seeded in culture flasks (T-25 unventilated Nunc) of plastic with approximately 7 mL of culture medium and maintained in an incubator at 25 °C. When the crop was at confluence, about every 2-3 days, the cells were subcultured using standard trypsinization method (2.25% trypsin/0.53 mM EDTA; Invitrogen).

Bacterial infectivity of SAF-1 cells

Preliminary tests were performed beforehand to ensure that all bacteria could grow on the medium used for the culture of SAF-1 cells without penicillin G and streptomycin. The adherence of bacteria to SAF-1 cells was skilled following the protocol of Acosta et al. (2009) with some modification. Briefly, SAF-1 cells were plated at a density of 10⁵ cells per well into 24-well tissue culture plates at 20 °C. Afterwards, Leibowitz L-15 medium (Gibco) supplemented with 10% of foetal bovine serum (FBS; Gibco), 2 mM L-glutamine (Gibco) was added. Cells were incubated for 10 h before infection test to form a monolayer. Bacteria were grown for 48 h at 25 °C in TSBS; then 5 µL of bacterial cultures (10⁷-10⁸ CFU mL⁻¹) were added to each well containing SAF-1 cells. A time course of bacterial infectivity of SAF-1 cells was performed (3, 9 and 20 h) to determine the effect of time of incubation on the viability of SAF-1 cells.

The integrity of the monolayers was examined by light microscopy and cell viability was assessed using trypan blue exclusion and cell counter (TC20 Bio-Rad). Control wells without inoculum were also included to ensure cell viability in experiments conditions.

Bacterial sensitivity to antibiotics

The bacterial sensitivity was measured according to the Clinical Laboratory Standards Institute (CLSI, 2015a, 2015b) and EUCAST methods (Matuschek et al., 2014) which are recommended for the determination of antimicrobial bacterial sensitivity. Bacterial suspensions (equivalent to 0.5 McFarland turbidity standards of the isolates) were prepared and applied on Mueller Hinton agar supplemented with NaCl (2%), in triplicate. After 15 min, commercial round disks impregnated with specific antibiotic were placed aseptically on a dry round Petri plate. Each plate contained three impregnated disks and one blank disk which are separated

from each other and from the edge of the plate. Antibiotics were prepared by serial dilutions from a stock solution of 10 mg mL⁻¹. They were initially diluted in ethanol or sterile milli-Q water as it was appropriate. Whatman paper disks of 420 g m⁻² and 6 mm in diameter were used to be impregnated with antibiotic. The antibiotics tested were kanamycin, gentamicin, sulfonamide, streptomycin, oxolinic acid, erythromycin A, flumequine, oxytetracycline, sulfadimethoxine, sarafloxacin, polymyxin b, penicillin G and chloramphenicol (Sigma). The assays were performed with 10 µL of different concentrations of antibiotics (10, 50, 100 µg mL⁻¹) according to EUCAST and CLSI disk diffusion methods (Matuschek et al., 2014); however in the cases where was obtained a resistance to antibiotics were tested higher concentrations (1,000 and 10,000 µg mL⁻¹).

Cultures were covered and incubated with antibiotics at 22 °C for 24 h. The incubation was prolonged to 48 h in cases where it was not possible to determine sensitivity or resistance during the first 24 h. Zone diameters was measured and interpreted as Susceptible (S), Intermediate (I) or Resistant (R) based on criteria from CLSI/EUCAST (Matuschek et al., 2014).

Statistical study

All the bioassays were done in triplicate, and the mean ± standard error (SE) for each group was calculated. Data were statistically analysed by one-way analysis of variance (ANOVA) to determine differences between groups. Normality of the data was previously

assessed using a Shapiro-Wilk test and homogeneity of variance was also verified using the Levene test. Non-normally distributed data were log-transformed prior to analysis and a non-parametric Kruskal-Wallis test, followed by a multiple comparison test, was used when data did not meet parametric assumptions. Statistical analyses were conducted using SPSS 19.0 and differences were considered statistically significant when $p \leq 0.05$.

RESULTS

Bacterial adhesion to polystyrene, skin mucus and gut mucus

The degree of adhesion of the different bacteria cells was studied to polystyrene, to skin mucus and to gut mucus (Table 1). All the tested bacteria adhered to polystyrene. However, the degree of adhesion was variable while *Pseudoalteromonas* was weakly adherent other bacteria such as *V. harveyi* and *H. venusta* were strongly adherents. The rest of the bacteria were moderately adherents. Regarding adherence to skin mucus, five of the studied bacteria were weakly adherents, four were moderately and only one bacterium was strongly adherent (*D. maris*). Interestingly, any studied bacteria were neither moderately nor strongly adherent to gut mucus. Thereby, four bacteria were non adherents while six bacteria were weakly adherents.

Table 1. Bacterial adhesion to polystyrene, skin mucus and gut mucus.

	Non adherent OD≤0.032	Weakly adherent 0.032≤OD≤0.064	Moderately adherent 0.064≤OD≤0.0128	Strongly adherent 0.128≤OD
Polystyrene	No bacteria	<i>Pseudoalteromonas</i> sp rHS str 402	<i>D. maris</i> <i>S. marcescens</i> <i>V. alginolyticus</i> <i>V. kanaloae</i> <i>Pseudomonas</i> sp <i>A. bivalvium</i> <i>T. mesophyllum</i>	<i>V. harveyi</i> H28 <i>H. venusta</i>
Skin Mucus	No bacteria	<i>S. marcescens</i> <i>V. kanaloae</i> <i>Pseudomonas</i> sp <i>T. mesophyllum</i> <i>Pseudoalteromonas</i> sp rHS str 402	<i>V. harveyi</i> H28 <i>H. venusta</i> <i>V. alginolyticus</i> <i>A. bivalvium</i>	<i>D. maris</i>
Gut Mucus	<i>V. harveyi</i> H28 <i>S. marcescens</i> <i>V. kanaloae</i> <i>A. bivalvium</i>	<i>Pseudoalteromonas</i> sp rHS str 402 <i>Pseudomonas</i> sp <i>T. mesophyllum</i> <i>D. maris</i> <i>H. venusta</i> <i>V. alginolyticus</i>	No bacteria	No bacteria

Viability of SAF-1 cells after contamination with pathogen bacteria

After infection of SAF-1 cells with the different pathogen bacteria used in this study, *D. maris*, *V. harveyi* and *A. bivalvium* seems to be the more pathogens bacteria to this fish cell line (Table 2). For the whole test, the percentage of viability of SAF-1 cells incubated with those bacteria decreased in a way significant compared to negative control. However was observed a significant decrease of viability of SAF-1 cells after 9 hours of exposure to all the pathogen bacteria except *V. alginolyticus*. The cell viability percentage was less than 79.66% while for the negative control it was 97%. In the case of *Pseudomonas* sp, *T. mesophyllum* and *S. marcescens*, these bacteria registered the lowest percentages of SAF-1 cells viability: 7%, 24% and 39%, respectively. Interestingly, the viability of SAF-1 cells incubated with all bacteria tested after 20 hours was significantly decreased comparing to negative control. For instance, the viability percentage of SAF-1 cells after exposure to *D. maris*, *V. harveyi*, *H. venusta* and *Pseudoalteromonas* sp was around of 1%. Similarly, exposure to *Pseudomonas* sp and *V. kanaloae* evidenced a viability SAF-1 cells percentage between 2 to 4%, respectively. Differently, when SAF-1 cells were incubated during 20 h with *S. marcescens*, *T. mesophyllum* and *A. bivalvium*, the percentages of viability of SAF-1 cells were between 17 and 32%.

Bacterial sensitivity to antibiotics

The bacterial sensitivity to antibiotics was determined using 13 antimicrobial agents that were selected as relevant in human medicine and veterinary: chloramphenicol (C), erythromycin A (E), gentamicin (GM), kanamycin (K), oxolinic acid (OA), oxytetracycline (OTC), polymyxin B (PB), penicillin G (PG), streptomycin (S), sarafloxacin (SAR), sulfonamide (SSS), sulfadimethoxine (SUL), flumequine (UB) (Table 3). All the bacteria studied in the present work presented resistance to sulfonamide and 60% of them were resistant to both sulfonamide and penicillin G. Except *D. maris*, all the bacteria studied were

sensitive to a concentration of oxalinic acid (OA). In the case of *V. harveyi* and *Pseudoalteromonas* sp, these bacteria were resistant to sulfonamide, penicillin G and sulfadimethoxine. *A. bivalvium*, *Pseudoalteromonas* sp, *V. harveyi*, *V. kanaloae* were sensitive (S) or intermediate (I) to 1000 µg mL⁻¹ of the following antibiotics: Kanamycin, streptomycin, erythromycin A, sulfonamide and sulfadimethoxine. Notably, *A. bivalvium* was inhibited only with a high concentration (10,000 µg mL⁻¹) of chloramphenicol. A concentration of 100 µg mL⁻¹ of polymyxin B was inhibitory of all the isolates tested in this study except for *D. maris*. Oxolinic acid, flumequine and sarafloxacin seems to be the more efficient antibiotics since they were able to inhibit 80 to 90% of all the bacteria studied with the lower concentration tested (10 µg mL⁻¹).

DISCUSSION

In aquaculture, whatever the pathogen bacterial specie present, important losses of fish may occur if the relevant treatment delayed. Generally if any infectious warning sign emerged in aquaculture, one or mixture of antibiotics with broad spectrum are used. Unfortunately inadequate and prolonged antimicrobial prophylaxis increases resistance to antimicrobial drugs (Harbarth et al., 2000). Subsequently, the challenge occurring in the aquaculture domain is how to maintain or increase the production of this food source and, concomitantly, avoid the uses of antibiotics which very often are also used due to their effects as growth promoters (Angulo et al., 2005). In aquaculture, the uses of antibiotics often fail to eradicate the pathogen bacteria because of the aggregation strategy that they have. Indeed, after adhesion to host fish, pathogen bacteria aggregate, produce an extracellular exopolysaccharides (EPS) that form a matrix and serves for biofilm formation and invading (Limoli et al., 2015). Bacteria within biofilms exhibit differences in the expression of surface molecules, nutrient utilization and virulence factors. Likewise, the fish immune responses are only directed toward those antigens on the outer surface of the biofilm because antibodies and other proteins or factors present in fish serum may fail to penetrate the biofilm.

Table 2. Viability of SAF-1 cells after infection with bacteria at 3, 9 and 20 hours.

Bacteria	SAF-1 cell viability (percentage %)		
	3 h	9 h	20 h
Negative Control	97.66 ± 0.33 ^a	97.0 ± 0.57 ^a	97.0 ± 0.57 ^a
<i>A. bivalvium</i>	89.66 ± 0.33 ^b	55.0 ± 5.03 ^b	32.0 ± 6.65 ^b
<i>D. maris</i>	89.0 ± 1.51 ^b	50.33 ± 4.48 ^b	1.0 ± 0.15 ^b
<i>H. venusta</i>	95.0 ± 0.26 ^a	73.33 ± 1.21 ^b	1.33 ± 0.88 ^b
<i>Pseudoalteromonas</i> sp rHS str 402	91.33 ± 1.21 ^a	79.66 ± 3.17 ^b	1.66 ± 0.33 ^b
<i>Pseudomonas</i> sp	94.66 ± 1.33 ^a	7.0 ± 0.57 ^b	2.66 ± 1.21 ^b
<i>S. marcescens</i>	96.66 ± 0.88 ^a	39.66 ± 2.02 ^b	29.66 ± 4.9 ^b
<i>T. mesophyllum</i>	96.33 ± 0.66 ^a	24.66 ± 1.21 ^b	17.66 ± 0.88 ^b
<i>V. alginolyticus</i>	95.33 ± 0.66 ^a	85.33 ± 0.88 ^a	6.0 ± 3.05 ^b
<i>V. harveyi</i> H28	89.0 ± 3.78 ^b	55.0 ± 3.78 ^b	1.0 ± 0.17 ^b
<i>V. kanaloae</i>	96.66 ± 0.88 ^a	53.66 ± 3.17 ^b	4.33 ± 2.33 ^b

Data represent the mean ± S.E. (n=6). Different letters denote significant differences between experimental groups and negative control (P≤0.05).

Table 3. Bacterial sensitivity to antibiotics.

ATB	<i>A. bivalvium</i>			<i>D. maris</i>			<i>H. venusta</i>			<i>Pseudoalteromonas</i>		
	Cc(μgml^{-1})	IZ	S									
C	10000	30	R	1000	36	S	1000	12	S	50	9	S
E	1000	19	S	50	15	S	1000	16	S	1000	9	I
GM	100	8	S	10	13	S	10	9	S	1000	15	S
K	1000	14	S	10	8	S	50	13	S	1000	11	S
OA	10	12	S	1000	20	I	10	13	S	10	9	S
OT	1000	12	S	10	10	S	50	8	S	50	11	S
PB	100	12	S	1000	10	S	100	12	S	100	6	S
PG	1000	14	R	10	12	S	1000	14	R	R	R	R
S	1000	14	I	50	6	S	50	5	S	1000	11	R
SAR	1000	22	N	50	14	N	10	32	N	10	3	N
SSS	R	R	R	R	R	R	R	R	R	R	R	R
SUL	1000	10	S	1000	10		1000	10	S	R	R	S
UB	10	9	S	1000	12	S	10	14	S	10	8	S
ATB	<i>Pseudomonas sp.</i>			<i>S. marcescens</i>			<i>T. mesophyllum</i>			<i>V. alginolyticus</i>		
	Cc(μgml^{-1})	IZ	S									
C	50	20	S	1000	36	S	10	18	S	1000	28	S
E	10	8	S	50	15	S	50	6	S	50	13	S
GM	10	8	S	10	13	S	50	6	S	10	8	S
K	50	12	S	10	8	S	100	8	S	50	11	S
OA	10	14	S	1000	20	I	10	11	S	10	17	S
OT	100	12	S	10	10	S	100	12	S	100	19	S
PB	100	12	S	1000	10	S	100	8	S	100	12	S
PG	10	8	S	10	12	S	R	R	R	R	R	R
S	50	11	S	50	6	S	10	6	S	50	10	S
SAR	10	20	N	50	14	N	10	28	N	10	34	N
SSS	R	R	R	R	R	R	R	R	R	R	R	R
SUL	1000	10	S	1000	10		1000	10	S	1000	10	S
UB	10	11	S	1000	12	S	50	14	S	10	12	S
ATB	<i>V. harveyi</i>			<i>V. kanaloae</i>								
	Cc(μgml^{-1})	IZ	S	Cc(μgml^{-1})	IZ	S						
C	50	12	S	100	8	S						
E	1000	11	I	1000	19	S						
GM	1000	14	I	100	9	S						
K	1000	10	S	1000	15	S						
OA	10	9	S	10	13	S						
OT	50	11	S	100	14	S						
PB	100	3	S	100	12	S						
PG	R	R	R	R	R	R						
S	1000	12	I	1000	11	R						
SAR	10	8	N	10	14	N						
SSS	R	R	R	R	R	R						
SUL	R	R	S	1000	12	S						
UB	10	9	S	10	10	S						

Cc: Antibiotic concentration ($\mu\text{g/ml}$); IZ: Inhibition zone (mm); S: Sensitivity; R: Resistant; I: Intermediate; N: not found in CLSI . ATB: Antibiotic; The CLSI antibiotic abbreviation was adopted in our study; C: Chloramphenicol, E: Erythromycin A, GM: Gentamycin, K: Kanamycin, OA: Oxolinic acid, OT: Oxytetracycline, PB: Polymyxin B, PG: Penicillin G, S: Streptomycin, SAR: Sarafloxacin, SSS: Sulfonamide, SUL: sulfadimethoxine, UB: Flumequine.

Fish phagocytes are unable to effectively engulf a bacterium growing within a complex polysaccharide matrix (Cortes et al., 2011). In our belief, one of the main steps to achieve the objective of fish protection from bacterial diseases is the knowledge of the adherence behaviour of the pathogenic bacteria, necessary as an initial step of colonization and/or infection of fish. Afterward, the infecting capacity of the bacteria. Finally, their sensitivity to various antibiotics with different mode of action.

In the present study we opted to measure the degree of adhesion of ten pathogen bacteria isolated from infected gilthead seabream specimens, to polystyrene, to skin mucus and to gut mucus of gilthead seabream. Polystyrene of microtiter plate is an abiotic surface and is used usually as a positive control in adhesion tests (Nicolau Korres et al., 2013). Skin and gut mucus were select as principal routes of attachment, adhesion and interaction of pathogen bacteria with host fish (Benhamed et al., 2014).

This study showed that all the tested bacteria adhered to polystyrene, 90% were moderately and strongly adherent. Many studies indicate that surface roughness influences bacteria adhesion (Lorite et al., 2011; Dussud et al., 2018). Baker and Greenham (1988) found that roughening the surface of either glass or polystyrene with a grindstone greatly increased the rate of bacterial colonization. Quirynen et al. (1993) showed that rough surfaces harbored 25 times more bacteria. In aquaculture, we suggest that it should be important to monitor the biofilm formation in the tank wall or any other abiotic material.

For skin mucus adhesion test, five bacteria were weakly adherent, four bacteria were moderately adherent and only one bacteria was strongly adherent (*D. maris*). Since skin mucus constitute the first frontline barriers protecting the fish body, the weak adhesion rate of the pathogenic bacteria could be explicated by the presence of antimicrobial proteins (AMPs), proteases and mucosal lysozyme secreted by epidermal mucus cells that displays bacteriolytic action against a variety of pathogens (Dash et al., 2018).

V. harveyi and *V. alginolyticus* were moderately adherent but *V. kanaloae* was weakly adherent this confirm that the adhesion is a specific interaction that depend on the bacterial specie and other environmental factors (Bordas et al., 1998).

For gut mucus adhesion test, interestingly, any bacteria were moderately or strongly adherent. Four bacteria were non adherent and six bacteria were weakly adherent. This weak adherence could be explicated by the environment present in the gut. Indeed it appears that populations of bacteria in the intestinal tract are very controlled by substrate competition (Lee et al., 2013), competition for nutrients or adhesion receptors (Balcazar et al., 2007), inhibition of virulence gene expression, and enhancement of the immune response (Gomez and Balcazar, 2008).

Our results seem to underline a potential difference between skin and gut mucus of gilthead seabream. Further studies are needed to determine the possible differences and coincidences between these important routes for entry pathogens in fish and their function as infection limiting barrier. Nevertheless, weak, moderate or strong adherence to skin or gut mucus is considered a prerequisite for microbial colonization or infection or both (Otto, 2014). Adhesive capacity has been shown to be necessary

although not sufficient for some bacteria to induce diseases. Adhesion degree recorded for bacteria tested in this study could be in relation with the probiotics strains present in the gut. In fact, Tuomola et al. (1999) demonstrated that two strongly adhesive strains of lactobacilli significantly increased *in vitro* adhesion of *S. typhimurium* to human intestinal mucus. In other studies, it has been demonstrated that the two fish pathogen isolates *V. anguillarum* and *A. hydrophila* were able to adhere to skin and intestinal mucus (Garcia et al., 1997). This clearly demonstrates that each pathogen isolate should be judged on its own merits and that extrapolation from related strains is not acceptable, as suggested by previous studies (Balcazar et al., 2007).

In this study, we choose SAF-1 cells to measure the infectious capacity of each bacteria. SAF-1 was used because it's good adaptation for growth in a standard medium without special requirements. Besides, the adaptation of cell lines to *in vitro* conditions is often associated with dynamic chromosomal changes affecting chromosome number and/or morphology, giving rise in some cases to marker chromosomes which are line-specific (Ghosh and Chaudhuri, 1984). In the SAF-1 line neither chromosome changes nor differences between passages have been detected, suggesting that besides having a normal karyotype, this line is rather stable. In our results, the low viability of SAF-1 cells recorded after 20 h of exposure to the different isolates, confirms their pathogenicity. Nevertheless, after exposure of SAF-1 cells to *S. marcescens*, *T. mesophyllum* and *A. bivalvium*, for 20 h, SAF-1 cells viability were 29%, 17%, and 32% respectively. These percentages could be explained by their low capacity of adhesion. In fact, *S. marcescens* is moderately adherent to polystyrene, weakly adherent to skin mucus and non-adherent to gut mucus. *T. mesophyllum* is weakly adherent to skin and gut mucus. *A. bivalvium* was moderately adherent to skin mucus and non-adherent to gut mucus. The viability of SAF-1 along time infection with *D. maris* was 89%, 50% and 1% after 3, 9 and 20 h, respectively. This strain was weakly adherent to gut mucus and strongly adherent to skin mucus. The viability of SAF-1 infected with *Pseudoaeromonas* sp was 9%, 79%, and 1% respectively after 3 h, 9 h and 20 h. Bejar et al. (1997) suggest that cytotoxic effects could be observed within 3-6 h after inoculation of the pathogen bacteria but results were recorded after 24 h to avoid discarding some late positive effects. The differences of cell viability after infection and the adherence results showed that these two proprieties (adhesion and infection capacity) depend on bacteria specie and substratum composition.

Considering that significant infectious degree results could be obtained within 3 h to 9 h, it is important to highlight the use of cell lines in aquaculture diagnosis to test *in vitro* pathogenicity as alternative way to avoid animal trials.

Besides the adhesion and infectious capacities of these pathogen bacteria, we proceeded to analyze their sensitivity to different concentrations of thirteen antibiotics selected as representative of different classes of antimicrobial agents that could be eventually used in aquaculture in case of infection.

Taking into account the results proved by many studies, we have enough evidence that the advisable antibiotic is that having the lower inhibiting concentration. Our study showed that oxolinic

acid, flumequine and sarafloxacin seems to be the most efficient since they were able to inhibit 80 to 90% of the bacteria studied with the low concentration used (10 µg mL⁻¹).

The two isolates *Pseudomonas* sp and *Pseudoalteromonas* sp necessitated high concentrations (1,000 µg mL⁻¹ for each antibiotic), or they were resistant to respectively 46% and 53% of the antibiotics used. Interestingly these two bacteria have weakly capacity of adhesion to skin and gut mucus but high infectious capacity to SAF-1 cells after 20 hours. We suggest that the infection mode of these gram negative bacteria could be via secretion of extracellular substances. Likewise, *V. harveyi* and *V. kanaloae* resisted or necessitated high concentration of the 53% of the antibiotics used. These two bacteria were non-adherent to gut mucus and have high infectious capacity to SAF-1 cells after 20 hours (cell viability of 1% and 4% respectively). Present results demonstrated that *Pseudomonas* and *Vibrio* are multidrug-resistant pathogenic bacteria. In fact, Matyar (2007) demonstrated that these bacteria isolated from sea bass (*Dicentrarchus labrax*) showed a multidrug resistance to trimethoprim-sulfamethoxazole, cephalothin, tetracyclin and streptomycin. Blasco et al. (2008) observed that some isolates of *Aeromonas* sp. have a total of 41 different multiresistant patterns. The same authors demonstrated that isolates of *P. aeruginosa* presented resistance to more than two antibiotics with resistance to amoxicillin/clavulanate, cefoxitin, clindamycin, erythromycin and nitrofurantoin (Blasco et al., 2008).

According to these results obtained for the ten pathogen bacteria, we could not confirm the hypothesis suggesting that the adhesion is a key for infection for SAF-1 cells *in vitro* or determine the infection degree. As well, the sensitivity of these bacteria to antibiotics is not related to adhesion or infectious capacity but may be to the surface properties of the bacteria. More investigation will be needed to confirm these results *in vivo*.

CONCLUSIONS

This study demonstrated that, *in vitro*, there is not strict link between adhesion propriety, the infection capacity, the time needed for infection and resistance to antibiotics. With this study we could highlight that all these bacterial proprieties depend on the bacterial specie. For aquaculture, this study could be important for pathogen bacterial investigation, fish disease treatment and suitable uses of antibiotics. In order to put out this relation between adhesion, infection, biofilm and resistance to antibiotic, raised recently by many studies, additional studies *in vivo*, should be undertaken.

ACKNOWLEDGEMENTS

F.A. Guardiola wants to thank the Fundação para a Ciência e Tecnologia (Portugal) for grant (SFRH/BPD/104497/2014). This work was supported by the Spanish Ministry of Economy and Competitiveness (Grant no. AGL2014-51839-C5-1-R); S. B. Hamed gratefully acknowledge the Postdoctoral scholarship provided by the UE-MARE NOSTRUM (204195-EM-1-2011-1-ES-ERA MUNDUS-EMA21).

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