MOL2NET, International Conference Series on Multidisciplinary Sciences
CHEMXEDIT-05, Chem. Exp., Edu., & Info. Tech., Bilbao, Spain-Paris, France-Texas, USA, 2021

In vitro Assessment of the probiotic characteristics of Bacillus strains from gut of gilt-head bream, *Sparus aurata*

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Abstract.
The rapid development of aquaculture, its intensification, and the occurrence of fish health problems on farms push to develop alternative methods to antibiotics and chemotherapy for controlling fish diseases. In this regard, the application of antibiotics as therapeutic and/or prophylactic strategies during fish production may lead to serious health and environment problems such as bacterial drug resistance development and dissemination, and food safety concerns. Probiotics may provide a potential alternative method to protect fish from opportunistic and pathologic bacteria and promote a balanced environment.
Evaluating the *in vitro* probiotic properties of nine bacteria of aquatic or gut of gilt-head bream for their application in aquaculture. Selection was was assayed by substractive screening with several criteria including, their antimicrobial activity (Bacteriocin) against fish pathogens, tolerant to low pH and bile salts, their secretion of enzymes and finally their in *vitro* safety assessment
Two of the nine bacteria isolated from several biotopes showed strong antibacterial activity against several pathogenic species such as *Lactococcus garvieae*, *Vibrio anguillarum*, *Vibrio harveyi*, *Aeromonas hydrophila* and *Aeromonas salmonicida*. These endospores-forming strains were withstand gastrointestinal conditions, produced a notable amylase, protease and lipase activities, showed high percentages of hydrophobicity, auto-aggregation and co-aggregation with fish pathogens and a strong adhesion to several fishes mucus and presented also a strong inhibition of pathogens adhesion. The tested strains showed a great heterogeneity respect to their safety and antibiotic susceptibility and were taxonomically identified by partial 16S rDNA gene sequencing.
Given their antimicrobial activity against fish pathogens and their safety, some of the tested strains may be considered as potential fish probiotics, and their effectiveness will be further tested in vivo.

Keywords: Aquaculture, pathogens, probiotics, screening, adhesion.

1. Introduction

World aquaculture is currently the fastest growing industry which makes a significant contribution to the rising demand for animal proteins in the last decades. Fish farming is increasing the economic activities in the developed and developing

countries. However, in order to provide nutritional and food security, the fish varieties are cultivated at high densities. This intensification damages not only the environment by changing in structures of aquatic habitats or in water quality but also the aquatic animals with a high stress condition. As a result the diseases are spread and the productivity is decreased leading to economic losses (Ashley 2007; Cabello 2006; Wang et al. 2008). The disease outbreaks are habitually caused by a wide range of pathogenic bacteria belonging to the genus: *Aeromonas*, *Vibrio*, *Edwardsiella*, *Pseudomonas*, *Streptococcus* and *Staphylococcus*... (Defoirdt et al. 2011; Toranzo et al. 2005). To sort out these problems, vaccines, antibiotics and chemotherapeutics are widely used as traditional methods (Cabello 2006; Gram et al. 2001). However, continuous and uncontrolled use of antibiotics may lead to the emergence and development of multidrug-resistant bacteria due to the contamination of aquaculture livestock and its surrounding environment. Therefore, an increasing number of researchers are going on the development of environmentally friendly treatments as a replacement of antibiotics usage. In this respect, the use of non-pathogenic bacteria (i.e. probiotics) has emerged as a viable prophylactic alternative to control diseases outbreaks without any side effects (Kaktcham et al. 2018; Midhun et al. 2018). Probiotics are defined as viable microorganisms conferring benefits to the health of the host when consumed in adequate concentrations (FAO/WHO/OIE 2006; Fuller 1989). Generally, probiotics are isolated from the indigenous and exogenous microbiota of aquatic animals(do Vale Pereira et al. 2017). Several recent studies have shown that the application of probiotics has many benefits including modifying the intestinal microbiota, improving digestion, metabolism and feed value by producing enzymes and enhancing growth promoting. They also stimulate the host's innate immunity and improve the water quality (Alonso et al. 2019; Balcázar et al. 2006; Kavitha et al. 2018; Nayak 2010a; Nayak 2010b).Besides, probiotics are capable of inhibiting pathogen's growth and adhesion through serval mechanisms such as secretion of inhibitory substances like bacteriocins, hydrogen peroxide, enzymes and organic acids as well as competitive exclusion. Earlier studies have demonstrated that a wide range of Gram-positive and Gram-negative probiotics strains have been used for aquaculture practices including lactic acid bacteria, (*Lactobacillus, Lactococcus, Leuconostoc,* and *Enterococcus*), *Pseudomonas sp., Bacillus sp., Vibrio sp.* and *Saccharomyces cerevisiae* species (Kaktcham et al. 2018; Zorriehzahra et al. 2016).

In this present study, we have assessed the *in vitro* probiotic properties of isolates bacteria from marine origin, for their application in aquaculture. Selection was assayed by subtractive screening with several criteria including, their antimicrobial activity (Bacteriocin) against fish pathogens, sporulation and biofilm formation, tolerant to low pH and bile salts their hydrophobicity, auto-aggregation ,co-aggregation, adhesion and inhibition of pathogens adhesion capacities and finally their *in vitro* safety assessment which haemolytic, gelatinase mucinolytic activities, bile salt deconjugation ability, antibiotic susceptibility and detection of virulence genes (Onarheim et al. 1994).

Materials and Methods

2.1 Sampling procedure and Bacillus *sp.* Isolation

Bacteria used at this study were originally isolated from intestine gilt-head bream, *Sparus aurata* of local farm in Sfax (South Est of Tunisia). The intestinal content of gilt-head bream were aseptically removed by scrapping and opened. The intestines were washed with sterile saline solution to take away non-adherent microflora. One gram from all the samples was taken and homogenized in 9 ml phosphate buffered saline (PBS, pH=7.2). Series of dilution were then prepared from the homogenates. Purification was determined by the spread plate method using Tryptone Soya Agar (TSA) (Oxoid Ltd, Basingstoke, United kingdoom) and plates were incubated at (28±1)°C for 24 h (Dash et al. 2018).Then, 115 colonies were picked and purified by streak plating. Pure isolates was stored in tryptone soy broth (TSB) containing 1.5% NaCl (Panreac Quimica S.A.U, Barcelona, Spain) with 15% (v/v) glycerol (Sigma-Aldrich) at -20°C and -80°C until further routine use.

2.2 Pathogen collection and culture condition

All *vibrio.* sp used in this study were provided by Dr. MA Moriñigo (department of microbiology Faculty of Science, University of Malaga)**.** The remaining pathogens were obtained from Departmental of Nutrition and Food Science

(Nutrition, Bromatology, Hygiene and Food Safety, veterinary Faculty University of Complutense Madrid). The pathogenic strains were cultured in TSA (Oxoid) supplemented with NaCl 1.5% (w/v) at 28°C. Stock cultures in TSB were stored at -80°C and -20°C in 1.5% NaCl with 15% glycerol to provide stable inoculum throughout the study.

2.3 Screening for antimicrobial activity

The antimicrobial activity of putative probiotic bacteria was examined against indicator fish pathogens by colony overlay method described by (Seghouani et al. 2017). Individual colonies of pathogens strains were suspended in a sterile tube containing 5 ml saline solution (0.9% NaCl) to a turbidity of 0.5 in the McFarland Scale (*ca.* 10^8 cfu/mL). Liquid bacterial of each strain culture was dipped with a sterile cotton swab to streak the whole surface of fresh TSA media. Individual colonies of *Bacillus* were spotted at the plates. After 24 h at 28°C, antimicrobial activity was assessed by observing the presence of inhibition zones around colonies.

The extracellular antimicrobial activity of cell-free supernatants was determined using an agar well diffusion test (ADT) Further, the neutralization was obtained using 1N Sodium hydroxide to pH=7. To determine the nature and thermosability of the antimicrobial compounds, the supernatants showing highest antimicrobial activity were treated with proteinase K at 10 mg/mL) (AppliChem GmbH, Germany) and then (2) heated at 100 ºC for 10 min. After treatments, samples were assayed for residual antimicrobial activity by an ADT using *Vibrio anguillarum CECT4344* and *V.harveyi* Lg16/00 as indicator microorganisms(Araújo et al. 2015).

2.4 Enzyme production

Proteolytic activity: strains were cultured in medium (pH=7.0) containing 0.5 % peptone, 0.3% yeast extract, 25% skimmed milk and 1.6% agar. Single colonies of each strain were spotted onto plates and then incubated at 28 °C for 24 h. The protease production was observed in terms of clear zone around colonies (Hmani et al. 2017)

Amylase activity: for the screening of amylase production, individual colonies of each strain were spotted in Luria-Bertani (LB) medium supplemented with 1% starch. After 24h at 28°C of incubation, plates were flooded with iodine to identify activity (Cai et al. 2019)

Lipase activity: strains were spotted onto LB plates containing 1% olive oil and 1% Rhodamine. After incubation for 24 h at 28 °C, plates were flooded with 1% Congo red. Lipase activity was observed by zone of clearance surrounding colonies(Dawood et al. 2017)

2.5 Preparation of vegetative cells and spores

For the preparation of vegetative cells, cultures of each isolate were taken from vegetative logarithmic phase cells. For the preparation of spores, broth cultures were centrifuged after incubation for 48 h at 28 °C and then supernatant was removed. To eliminate vegetative cells, spores were purified using lysozyme solution (4 mg/ml) and washed twice with Phosphate-buffered saline (PBS). Spores were then re-suspended in sterile deionized water and heated at 80 °C for 20 min. After centrifugation spores were re-suspended again in sterile deionized water.

Sporulation properties: Sporulation efficiency was determined through inducing sporulation and performing total viable counts of spore suspensions before and after heating (Zhou et al. 2019).

2.4 Acid and bile tolerances of the isolates strains

The tolerance of isolated strain to low pH and bile salt was assayed as described by Barbosa et al. (2005) and Hmani et al. (2017) with modifications. Briefly, an overnight culture was harvested and re-suspends cells in PBS .Essentially ~10^8 to 10^9 bacterial cells or spores ml^{-1} were resuspended in TSB broth containing bile salts (0–5% sodium cholate 50%, sodium deoxycholate 50%) (bio basic, Canada) or in TSB broth adjusted to pH 1, 2, 3 and 7.3 (control) with concentrated HCl. Aliquots were taken immediately and after 3 h for low pH and after 6 h for bile salt tolerance. Viable counts were determined by the spread plate method on TSB after incubation at 28 °C for 24 h.

2.5 Auto-aggregation and co-aggregation assays

Auto-aggregation assay was performed according to(Meidong et al. 2018). Overnight cultures grown were centrifuged, washed twice and resuspended in PBS followed by turbidity measurement to obtain OD600~1(A0). The bacterial suspensions were vortexed for 10 s and kept undisturbed at 28 °C for 6 h. Then each two hours OD600 of the upper suspensions (At) were measured. Auto-aggregation percentage (%Agg) was expressed as: $\%Agg = 1 - (At/A0) \times 100$, Where A_t represents the OD_{600} at time t = 2, 4 and 6 h and A_0 the OD_{600}at t = 0.

In co-aggregation assay (Meidong et al. 2018), the suspension of isolated strains and fish pathogens were prepared the same as auto-aggregation analysis above. Equal volume of *Bacillus* spp. and pathogen strains were mixed and incubated at 28 °C for 4 h. The OD_{600} of the mixtures and controls (unmixed cultures of *Bacillus* and fish pathogens) were measured after incubation. Co-aggregation (Co-agg) was calculated as:

$$\% Co - agg = [(Apat + Aisolate) - 2(Amix)/(Apat + Aisolate)] \times 100$$

Where A_{pat} and $A_{isolate}$ represent the OD_{600} of each bacterial suspension alone and A_{mix} represents the OD_{600} of mixed suspension pathogen and isolated strains.

2.6 Taxonomic identification of the strains

Bacterial DNA was extracted using the InstaGene Matrix resin (Bio-Red Laboratories Inc: Hercules CA USA).Strains were taxonomically identified by DNA sequencing of the PCR amplified genes encoding the 16S rRNA subunit(16S rDNA)(Araújo et al. 2015).The corresponding species identity was obtained by comparative sequence analysis (BLASTN) against available sequence data in the National Center for Biotechnology Information (NCBI) database.

2.7 In vitro safety assay

Antibiotic susceptibility assay

The susceptibility of isolated strains was determined by a broth microdilution test(Araújo et al. 2015).Tested antibiotics were ampicillin (0.12–8 mg/L), vancomycin (0.5–32 mg/L), gentamicin (2–128 mg/L), kanamycin (4–256 mg/L), streptomycin (4–256 mg/L), erythromycin (0.12–8 mg/L), clindamycin (0.25–16 mg/L), tetracycline (0.5–32 mg/L) and chloramphenicol (1–64 mg/L). Individual colonies were suspended in 5 mL of saline solution (0.9% NaCl) to reach an optical density of 1 in the McFarland Scale (*ca.* 3×10^8 cfu/mL) and diluted in LSM broth (Iso-Sensitest (IST) (Oxoid) and MRS broth; IST/MRS, 9:1; pH 6.7). Fifty microliters of suspension strains were inoculated in each microplate well containing decreasing concentrations of the corresponding antibiotic diluted in LSM. Then, the inoculated plates were incubated at 28°C for 18 h. The minimum inhibitory concentrations (MICs) were read as the lowest antibiotic concentration inhibiting the growth of bacteria, and were compared with the cut-off values established by EFSA for *Bacillus* spp. (Additives and Feed 2012). Resistant strain was defined when the observed MIC value for one antibiotic was higher than the respective established breakpoint. *Staphylococcus aureus* CECT 794 and *Enterococcus faecalis* CECT 795 were used for quality control.

Hemolytic Activity

The haemolytic activity was screened as follows: Bacillus sp. strains previously cultured in TSB with 1.5%NaCl were streaked on horse blood agar plates (BioMérieux, Marcy l'Etoile, France) .After incubation at 28°C for 1-2 days. The presence of clear zones of hydrolysis around the colonies revealed β-hemolysin(Hmani et al. 2017)

2. Results and Discussion

3.1 Screening for antimicrobial activity

A total of 30 bacteria were isolated and purified from gut of gilt-head bream, *Sparus aurata*. All selected strains were found to be Gram-positive, catalase positive, oxidase negative, rod-shaped, motile and endospore-forming. These spore-forming strains possessed typical characteristics of *Bacillus* species.

All strains except D16, D17, D19, D22, D24 and D29 exerted direct antimicrobial activity with cross streak test represented as score (Table 1). Isolated strains have shown heterogeneity activity at gram+ and gram– pathogenic bacteria. Nine strains were found that inhibit the growth of at least one of the 9 pathogens tested. A wide range of inhibitory spectrum was found. The strongest inhibition was found in strains D6 and D9, which showed antimicrobial effect against majority of indicators studied. The activity 9 selected bacteria CFS by ADT is presented as score (fig. 1). Cumulative maximum and minimum scores were 10 and 2, respectively. Based on total score, D6 and D9 showed a strong antagonistic activity against all tested pathogens with score 10 (data not shown).

Table 1: Direct antimicrobial activity against fish pathogens

strains	Lg16/00	lg26/01	Va	Vv	Ah	As	Lg5807	Sa	Se	Scores
D1	0	0	0	0	1	1	0	1	0	3
D2	0	0	2	0	0	0	0	2	0	4
D3	1	1	1	0	0	0	0	0	0	3
D4	0	2	0	0	0	0	0	4	0	6
D5	0	2	0	0	0	0	0	2	0	4
D6	1	1	2	0	1	1	1	2	1	10
D7	0	3	0	0	0	0	1	4	1	9
D8	0	0	0	0	1	0	1	1	0	3
D9	0	3	2	0	0	1	0	4	0	10
D10	1	0	0	0	0	0	0	0	0	1
D11	1	0	0	0	0	0	0	0	0	1
D12	0	2	0	0	0	0	0	0	0	2
D13	0	0	1	0	0	0	0	0	0	1
D14	0	0	1	0	0	0	0	0	0	1
D15	0	0	2	0	0	0	0	0	0	2
D16	0	0	0	0	0	0	0	0	0	0
D17	0	0	0	0	0	0	0	0	0	0
D18	0	0	1	0	0	0	1	0	0	2
D19	0	0	0	0	0	0	0	0	0	0
D20	0	0	0	0	1	0	0	0	0	1
D21	0	0	0	0	0	0	1	0	0	1
D22	0	0	0	0	0	0	0	0	0	0
D23	0	0	0	0	0	0	1	1	0	2
D24	0	0	0	0	0	0	0	0	0	0
D25	0	0	0	0	0	0	2	0	0	2
D26	0	0	1	0	0	0	0	0	0	1
D27	0	0	0	0	1	1	0	0	0	2
D28	0	0	2	0	0	0	0	0	0	2
D29	0	0	0	0	0	0	0	0	0	0
D30	0	1	1	0	0	0	0	0	0	2

Zones of inhibition (halo diameter) were presented as scores; 0 (0–5 mm), 1 (low, 6–10 mm), 2 (moderate, 11–20 mm), 3 (high, 21–24 mm) and 4 (very high, ≥25 mm. 0-no antagonistic activity. All indicator strains were assayed at least twice. Ah, *Aeromonas hydrophila*; As, *A. salmonicida*; Lg 5807, *Lactococcus garvieae CECT* 5807; Va, *V. anguillarum.* CECT4344; Lg16/00 and Lg26/011, *V.harveyi* ; Vv, *V. vulnificus;* Sa,*Staphylococcus aureus*;Se,*Staphylococcus*.

The antimicrobial activity of cell free supernatant exerted by all the strains persisted after adjustment of the pH and heat treatment, but disappeared, partially or completely, after proteinase K treatment, thus confirming the proteinaceous nature of the antimicrobial compounds(Table 2).

Fish diseases caused by Aeromonas and Vibrio species have been reported in aquaculture (Kuebutornye et al. 2020) Earlier reports indicated that several species of Bacillus have antimicrobial properties against several Gram-positive and Gram-negative pathogenic bacteria(Kavitha et al. 2018; Kuebutornye et al. 2019; Ramesh and Souissi 2018).The occurrence of bacteria- producing inhibitory compounds in the intestine of the host, on its surface, or in its culture medium creates a barrier against the proliferation of opportunistic pathogens(Verschuere et al. 2000).The following aspects, either individually or in combination caused the antagonistic effect of bacteria: bacteriocins, production of antibiotics, lysozymes, proteases, siderophores, and hydrogen peroxide and the change of pH values by the production of organic acids). (Kuebutornye et al. 2019; Verschuere et al. 2000).The selected isolates produce inhibitory substances against certain pathogenic microorganisms. Therefore, they can improve the immune response and disease resistance of their host.

Table 2: Nature of antibacterial activity against *Vibrio anguillarum* CECT4344 and *V.harveyi* Lg16/00

Strains	Va				Lg16/00			
	I	II	III	VI	I	II	III	VI
D1	1	1	0	0	3	3	1	1
D2	2	2	1	0	2	2	1	1
D3	1	1	1	0	3	3	1	1
D4	2	2	0	2	3	3	2	2
D5	1	1	0	0	3	3	1	1
D6	3	3	0	2	3	3	2	1
D7	2	2	0	1	3	3	1	1
D8	2	2	0	0	1	1	1	1
D9	3	3	0	0	4	4	0	1

I: supernatant without any treatment, **II**: pH neutralization treatment, **III**: heat treatment at 100 ° C, **VI**: proteolytic enzyme treatment. Zones of inhibition (halo diameter) were presented as scores; 0 (0–5 mm), 1 (low, 6–10 mm), 2 (moderate, 11–20 mm), 3 (high, 21–24 mm) and 4 (very high, ≥25 mm. 0-no antagonistic activity.

3.2 Enzyme production and sporulation

Extracellular enzyme production varied among the 9 selected strains. Amylase activity was noticed only with D3 and D6 (Table 3). The strain D6 exhibited highest protease and amylase activity.The extreme resistance of spores allow them to pass drastic stress on the gastrointestinal tract (GIT). Lately, *Bacillus* spores are being used as animal probiotic. So it is important to study the sporulation. All of isolated strains showed good sporulation efficiencies (Table 3) and showed spore titres in order of 10^7-10^8 spores/ mL (data not shown). Unlike lactic acid bacteria, *Bacillus* sp. is considered to be very stable due to their sporulation ability. Their endospores are resistant to harsh conditions, including high temperature, UV and acidity, drought, freezing, radiation and rising oxygen levels. Relatively, Bacillus spores are capable to survive throughout the simulation of GIT. (Adibpour et al. 2019; Mingmongkolchai and Panbangred 2018). Besides, bacterial strains that can only inhibit pathogens are not considered as effective probiotic strains. The bacteria that is capable to produce enzymes such as amylase, protease, lipase, and cellulase can be beneficial since the extracellular enzymes may help in the digestion of major food contents in the diet. The present study effectively screened the production of extracellular enzymes by the bacterium. The probiotic strain capable of producing extracellular enzymes not only helps in the survival of the bacterium but also facilitate feed digestion (Midhun et al. 2018)

Table3: Extracellular enzymes production and sporulation

	Protease	Lipase	Amylase	Sporulation	Hemolytic activity
D1	+++	+	-	-	α
D2	-	-	-	-	β
D3	++	+	-	+++	α
D4	++	+	+	++++	β
D5	++	+	-	++	β
D6	+++	+	++	++	-
D7	++	+		++	β
D8	-	-	-	-	β
D9	++	+	-	+	-

3.3 Acid and bile tolerances of the isolates strains

As probiotics are intended to colonize the (GIT) of fish and to restore the balance of intestinal microflora, the candidate strains should tolerate GIT conditions. In fact, ingested probiotic strains need to survive acidity of stomach and bile in small intestine.In the present study, the vegetative cells and spores of selected strains were tested in GIT conditions. No vegetative cells could tolerate pH1.0 while, the majority of tested cells withstand low pH above2.0 and wide range of bile concentration up to 5%. D6 and D9 showed the highest viability of selected strains in GIT conditions. Unlike other probiotics, Bacillus species produce spores that are more heat tolerant resistant to low pH and a high percentage of bile concentration and have the abilities to germinate and survive in the gut of fish Gastric (low pH) and intestinal (high bile

concentration) tolerance are the prerequisites for probiotics to survive and colonize the gut to produce beneficial traits (Barbosa et al. 2005; Kuebutornye et al. 2020)

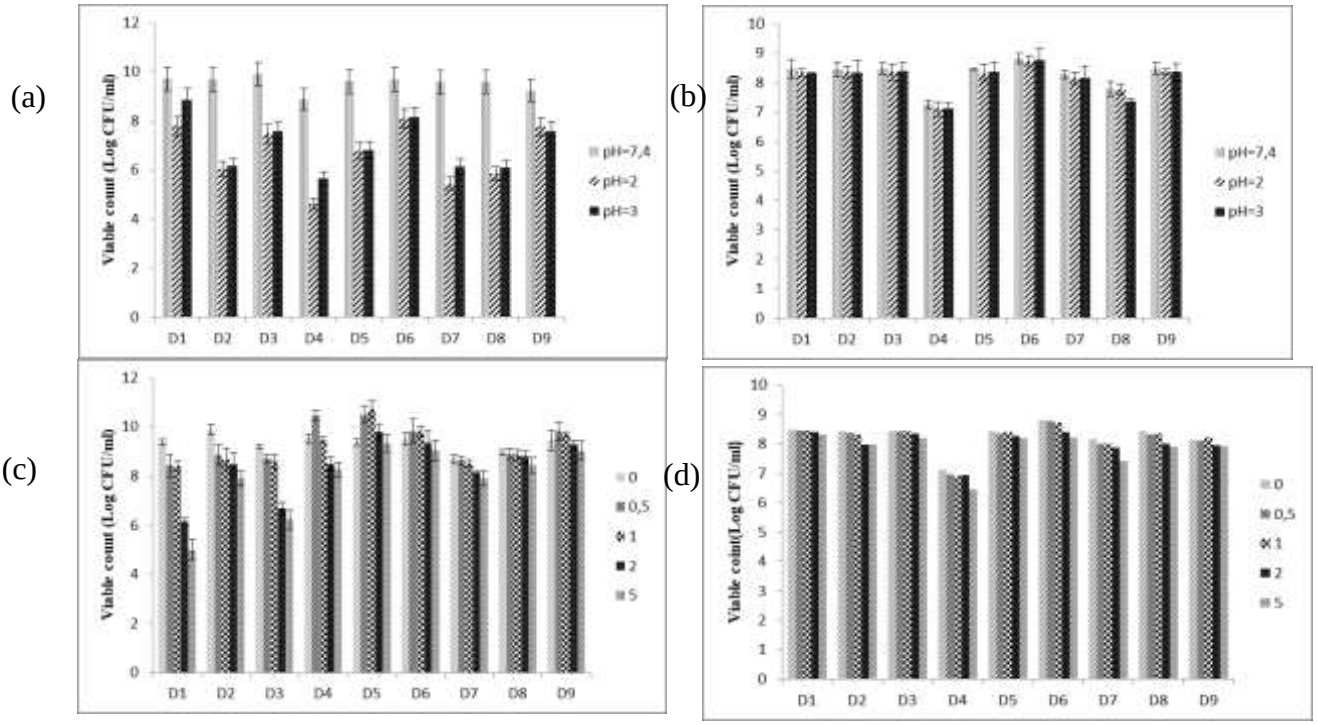

Fig1: Tolerance of vegetative cells (a), (b) and spores (c), (d) at different pH conditions and at different bile concentrations for 3h at 28°C (log CFU/ml)

3.4 Auto-aggregation and co-aggregation assays

Colonization and adhesion to epithelial cells and mucosal surfaces are important characteristics of good probiotics since it guarantees the ability to resist the fluctuation of the intestinal content and it also inhibits the pathogenic bacteria adhesion by occupying all the space of the intestine as well as inhibits inflammatory reactions. An indirect method of determining the adhesion ability of probiotic bacteria is the determination of the auto-aggregation. Besides, there is a strong correlation between auto-aggregation and cell adhesion to the digestive tract, which is one of the prerequisites for a good probiotic bacteria. (Butt and Volkoff 2019; Kuebutornye et al. 2020; Mingmongkolchai and Panbangred 2018; Shinde et al. 2019; Soltani et al. 2019).In the present study, Auto-aggregation was investigated for selected strains (Fig 2a). Auto-aggregation for selected strains increased with incubation period and varied from 60% to 90% .D6 and D9 exhibited the strongest auto-aggregation ability (90%); 85%, respectively) after 6h of incubation. In addition, all tested strains were able to co-aggregate with pathogens significantly, showing co-aggregation percentages above 40% (Fig 2b). Among the isolates, D6 showed the highest percentage of co-aggregation against the majority of indicator pathogens after 4h of incubation followed by D9 with percentage >50%.

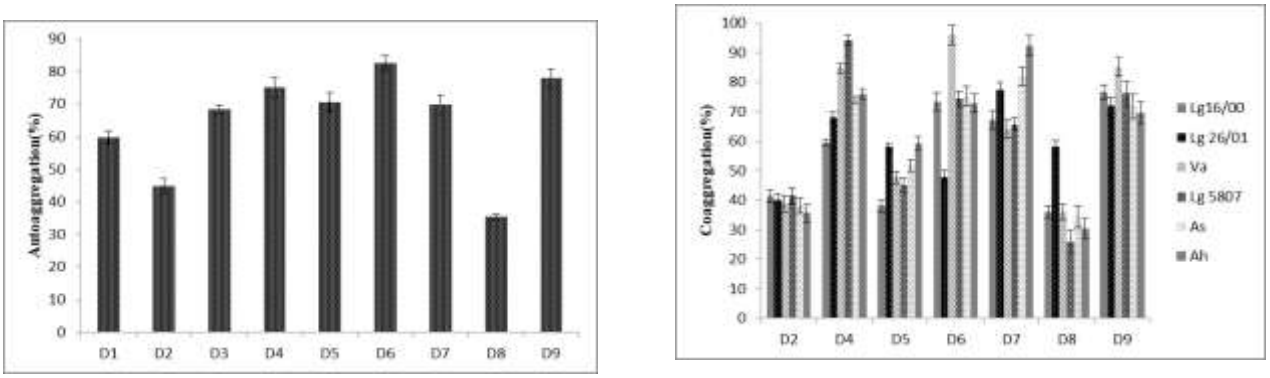

Fig2: Auto-aggregation of selected strains after 6h and their co-aggregation percentages with bacterial pathogens after 4h of incubation

2.5 *In vitro* safety assay

The antibiotic resistance profile of selected strains indicated that all strains are sensitive to all selected antibiotics as suggested by EFSA (data not shown). Regarding hemolytic activities, only D6 and D9 showed γ- haemolysis.

The safety prerequisites for the selection of a probiotic strain are the absence of haemolytic activity, and antibiotic resistance as haemolysin is considered a virulent factor due to its ability to initiate infection by entering small lesions in the mucous membranes and skin of any host. No or γ-haemolysis and α-haemolysis are considered to be safe, and β-haemolysis is considered harmful. In this study, isolates D6 and D9 showed γ-haemolysis. A similar observation was made by (Kuebutornye et al. 2020; Shinde et al. 2019).Followed by BLAST analysis, the partial 16S rRNA gene sequence of selected strains showed 99.9% similarity to D6 , *Bacillus subtilis* and D9, *B pumilus* for strain**.**

Conclusions

The present study confirms probiotic properties and *in vitro* safety of *B. pumilus* D9 and *B.subtilis* D6 bacteria isolated from intestine of gilt-head bream. Selected strains exhibited antimicrobial activity against various gut pathogens tolerated gastric low pH and bile salts and showed a strong sporulation. D6 and D9 also showed a high auto-aggregation and co-aggregation abilities. Selected strains are sensitives to antibiotics and showed the safety assessment (absence hemolysis). Therefore, *in vivo* evaluation studies are required to determine its real-life applications.

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