



Characterization of two potent probiotic strains: *Bacillus* sp. R2 and *Planococcus* sp. R11 isolated from the gastrointestinal tract of sea bream (*Sparus aurata*)

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ABSTRACT

Probiotics are beneficial microorganisms administered in aquacultures to control pathogens and enhance feed utilization, survival, and growth rate of farmed fish. This study aimed to get the probiotic bacteria from the gastrointestinal tract which had protease, amylase and lipase activities to improve growth performance of sea bream (*Sparus aurata*). The most potent selected isolates were identified- using biochemical and molecular sequencing of 16s rRNA gene method- as *Bacillus* sp. R2 and *Planococcus* sp. R11 and were characterized to ensure their safety and efficiency. The two bacterial strains were characterized as potential probiotic for hemolytic activity, antibiotic susceptibility, growth pattern, antagonistic activity against fish pathogens, cell surface hydrophobicity and tolerance to acidity and alkalinity. The two strains showed antagonism against the fish pathogen *Vibrio alginolyticus* isolated from a fish rearing tank. This is the first time to report the probiotic property of genus *Planococcus*.

INTRODUCTION

Probiotics are identified as live microorganisms that confer a health benefit on the host when administered in adequate amounts (Jahangiri & Esteban, 2018). In aquatic culture environments, administration of probiotics can be applied to inhibit pathogens. It enhances survival, feed utilization and growth rate of farmed species with no side effects on treated organisms (Huynh *et al.*, 2017).

The selection criteria of potential probionts for applications with fish species are listed, some of which are essential (E) and some are considered as merely favorable (F). The more of these characteristics that are fulfilled by a candidate probiotic species, the more appropriate that species shall be considered and thus more likely to be an effective fish probiont (Ibrahim, 2015). A probiotic candidate should meet a number of requirements, including safety, must not be pathogenic, not only with regards to the host species but also with regards to aquatic animals in general and human consumers (E). It must be also

with regards to aquatic animals in general and human consumers (E), must be resistant to bile salts and low pH (E), should be able to adhere to and/or grow well within intestinal mucus (F), must be free of plasmid-encoded antibiotic resistance genes (E), should display advantageous growth characteristics (e.g. short lag period, a short doubling time and/or growth at host rearing temperatures) (F), should exhibit antagonistic properties towards one or more key pathogens (F), should produce relevant extracellular digestive enzymes or produce vitamins (F), should be indigenous to the host or the rearing environment (F) and should remain viable under normal storage conditions and robust enough to survive industrial processes (F) (Merrifield *et al.*, 2010; García-Hernández *et al.*, 2016)

Exogenous enzymes such as amylase, protease, lipase and cellulase produced by probiotic bacteria can increase the activity of the digestive tract enzymes and feed digestibility (Widanarni *et al.*, 2015). The existence of these exogenous enzymes will help the endogenous enzymes in hydrolyzing feed nutrients and to break down long chains of macromolecules into simpler molecules, so they will be easier to be absorbed by the intestine (Sánchez-Ortiz *et al.*, 2015).

In aquaculture, Gram-negative and Gram-positive bacteria, bacteriophages, yeasts, and unicellular algae were examined for application as probiotics (Lara-Flores, 2011). Members of genus *Bacillus*- belong to endospore-forming- have wide applications (Hong *et al.*, 2005), especially *Bacillus subtilis* used in aquaculture (Hai, 2015). Up to our knowledge, no available publication are found on the probiotic property of *Planococcus*.

Potential probiotics may be obtained from various sources such as the gastrointestinal tracts of aquatic animals (Ramesh *et al.*, 2015) and aquatic environment such as water or sediment (Del'duca *et al.*, 2013).

In this work, it is aimed to isolate bacterial strains from the GI tracts of fish and evaluate their potential probiotic properties for possible use in aquaculture.

MATERIALS AND METHODS

Bacillus sp. R2 and *Planococcus* sp. R11 used in this study were isolated from the gastrointestinal (GI) tract of sea bream collected from Damietta Governate in Egypt (El-Ratoma fish farm, salinity 34 ppt). They were identified using 16S rDNA phylogenetic analysis.

Strains used for antimicrobial activity were *Vibrio fluvialis*, *Vibrio cholerae* and *Vibrio parahaemolyticus* kindly obtained from the Microbiology lab, Marine Environment Division, National Institute of Oceanography and Fisheries, Alexandria, Egypt. *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 9027, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212 and *Candida albicans* ATCC 10231 were kindly provided from the Department of Pharmaceutical Microbiology, Faculty of Pharmacy, Alexandria University. The fish pathogen *Vibrio alginolyticus* was isolated from infected rearing tank and identified using Bruker Daltonik MALDI Biotyper at Proteomic research lab, medical research centre, Faculty of Medicine, Alexandria University .

Isolation of bacterial strains

Bacteria were isolated from the gastrointestinal tract (GI) tract of sea bream by diluting samples in sterile saline solution (0.85% NaCl) and 100 µl were spread on seawater nutrient agar (SWNA) containing (g/L): Beef extract 1.0; yeast extract 3.0; peptone, 5.0; sodium chloride, 5.0; Agar 15. pH was adjusted to 7.0 then incubated at temperature range 30, 35 and 37°C for 48 h. Selected isolates were purified and maintained as subcultures on the same media.

Screening of bacterial isolates for enzymatic activity

Production of extracellular enzymes was assessed as described by **Widanarni *et al.*, (2015)**. Bacteria were cultivated on SWNA agar plates amended with 2% skim milk for proteolytic property, 2% olive oil for lipolytic property and 2% starch for amylolytic property. Plates were incubated at 35°C for 24-48 h. The clear zone formed around the colony in skimmed milk plates indicates proteolytic activity. Lipolytic property was detected by the formation of bright green color around the colony of olive oil plates after adding saturated copper sulphate solution. Amylolytic activity was identified by clear zone formation around the colony in starch plates after adding 1-2 drops of iodine solution.

Phenotypic characterization of the two bacterial isolates

The two bacterial isolates that had high enzymatic activity were selected and their phenotypic characteristics were determined for identification which included culture morphology, Gram stain and some biochemical tests.

Citrate utilization: Simmon's citrate medium was inoculated with isolates then incubated at 37°C for 2 days after which the medium was examined for colour change.

Urease production: Urease broth was inoculated with isolates then incubated at 37°C. The broth was examined after 2 days. A pink colour reflects the alkaline conditions developed as a result of ammonia production and hence indicated a positive result.

Triple sugar iron: Triple sugar iron medium was inoculated with isolates then incubated at 37°C for 2 days after which the medium was examined for colour change

Indole production: Tryptone broth (1%) was inoculated with isolates and then incubated at 37°C. The broth was examined after 2 days by addition of 10-12 drops of Kovac's reagent. A red layer at the top of culture reflects tryptophane hydrolysis to indole and indicate positive result.

Lysine iron agar: Lysine iron agar medium was inoculated with isolates then incubated at 37°C for 2 days after which the medium was examined for colour change.

Genotypic characterization

DNA isolation, PCR amplification of 16S rDNA region and sequencing were carried out by Sigma Scientific Services Co., 6 of October City, Egypt. Sequences of 16S rRNA genes were compared to other sequences in the NCBI database. BLAST program was used to assess the DNA similarities and multiple sequence alignment. The molecular

phylogeny was performed and phylogenetic tree was reconstructed by BioEdit software (Hall, 1999).

Probiotic properties of *Bacillus* sp. R2 and *Planococcus* sp. R11.

The safety profile of the two isolates was determined by their hemolytic activity and antibiotic susceptibility.

Hemolytic test

To assess the safety of isolated bacterial strains, blood hemolysis test was performed. The hemolytic reaction was determined on nutrient agar medium containing 5% blood by streaking on blood agar plates then incubated at 35°C for 48 h and observed for lysis or complete digestion of red blood cell contents surrounding bacterial colonies.

Antibiotic susceptibility:

Antibiotic susceptibility was determined by disk diffusion according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2012). The following antibiotics were tested: Amoxicillin 10µg, colistin sulphate 10µg, Nalidixic acid 30µg, Bacitracin 10µg, cephalixin 30 µg, Piperacillin 100µg, Rifampin 5µg, Metronidazole 5µg and Fusidic acid 10µg. Presence or absence of inhibition zones was defined as sensitivity or resistance, respectively.

Growth characteristics

An inoculum (1% v/v) of bacterial suspension ($OD_{600} = 0.8-1$) was introduced into 25 mL of SWNB in 100 mL Erlenmeyer flask and incubated at 35°C at 150 rpm. Growth was monitored regularly at time intervals and specific growth rate (μ) was determined as the slope of the best-fit equation corresponding to the exponential phase of growth and doubling time (t_d) was calculated according to the following equation $t_d = \ln 2 / \mu$ (Widdel, 2010).

Antimicrobial activity

Screening of antimicrobial activity was performed by disc diffusion method according to Chantharasophon *et al.*, (2011). This was achieved by placing paper discs saturated with bacterial suspensions ($OD_{650} = 0.8-1$) on SWNA plates previously inoculated with 24 h old indicator strains. All plates were incubated at 35°C for 24 h. Antagonistic activity was observed by measuring the inhibition zone diameter around the paper disc.

Cell surface hydrophobicity

According to Tyfa *et al.*, (2015), bacterial cultures in stationary phase were pelleted at 5000 g for 10 min, washed twice with phosphate buffer saline (PBS) (pH 7.4) consisted of (g/L) NaCl, 8; KCl, 0.2; $Na_2HPO_4 \cdot 2H_2O$, 1.44; KH_2PO_4 , 0.24) and resuspended in 3 mL of the same buffer solution. The bacterial concentration was adjusted with PBS (pH 7.4) to $OD_{560nm} = 1.0$ (A_{b0}). One ml of this suspension was added to 0.2 mL of toluene, mixed for 2 min and incubated for 1h at 35°C. The aqueous phase was removed and the

OD_{560nm} was determined again (A_{b1}). Percentage of Microbial Adhesion to Solvents (MATS) was calculated using the following equation:

$$\% \text{ MATS} = (A_{b0} - A_{b1}) / A_{b0} \times 100$$

Where A_{b0} represents absorbance of bacteria at stationary phase, A_{b1} represents absorbance of adhering bacteria to organic solvent.

Resistance to acidic and alkaline condition

Growth of *Bacillus* sp. R2 and *Planococcus* sp. R11 was monitored in SWNB of different pHs (2.5, 7 and 8) and estimated as colony forming unit (CFU) by plate count method (Ngatirah *et al.*, 2000).

RESULTS

Isolation of probiotic bacteria: Eleven autochthonous isolates were obtained from the GI tract of sea bream (*Sparus aurata*). They were subjected to screening of enzymatic production to select the most potent isolates to be characterized as probiotics.

The enzymatic profile of the eleven bacteria was tested. Data in Fig.1 show that only one isolate (11S) produced amyolytic activity, whereas, 2S produced higher protease activity with clear zone 2cm and none of them showed lipase activity.

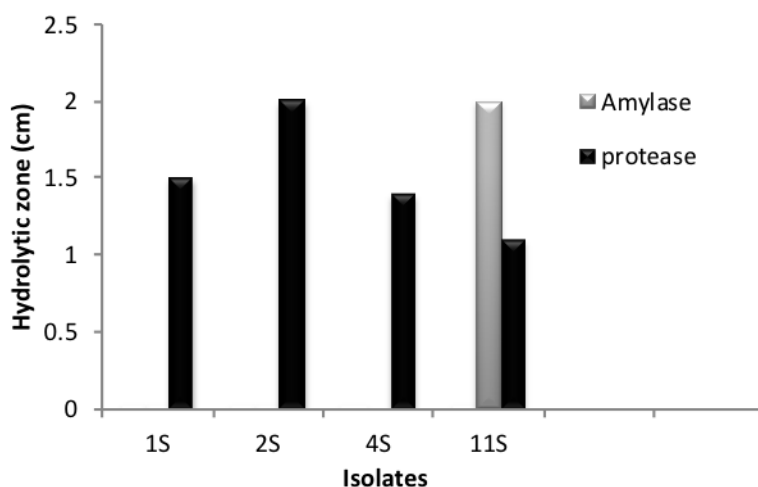


Fig.1 Enzymatic activity (hydrolytic zone diameter, cm) of bacterial isolates

Phenotypic characterization of the most potent isolates

On the basis of the enzymatic profile, strains 2S and 11S were selected and phenotypically characterized. Strain 2S formed large circular mucoid and entire shaped colonies with pale yellow color on SWNA plates. Cells were Gram +ve spore-forming bacilli. While, strain 11S formed small circular smooth raised and entire shaped colonies with yellow orange color on SWNA plates. Cells were Gram +ve non-sporing coccobacilli (**Plate 1**). Some characteristics of the two strains are given in **Table 1**

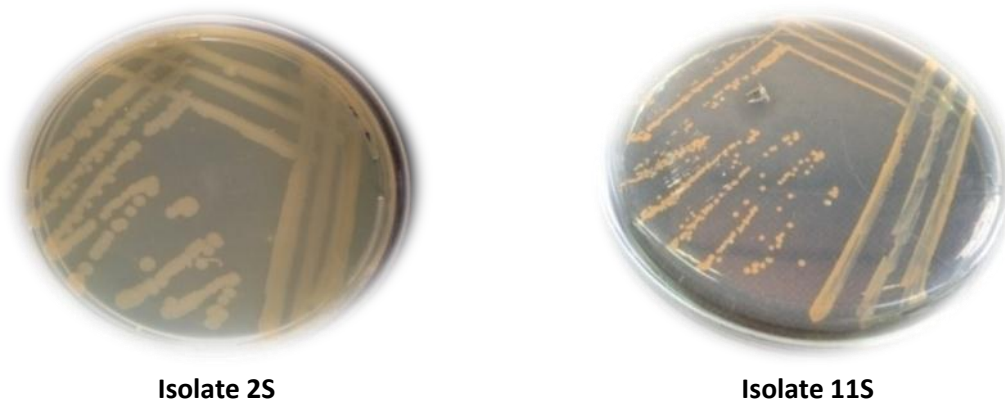


Plate 1 Culture morphology of 2S and 11S on SWNA plates after 2 days incubation at 35°C

Table 1 Some characteristic properties of the selected isolates

Isolate	2S	11S
Triple sugar iron	red with black H ₂ S	Red/yellow
Simmons citrate	Blue and green	Green
Lysine iron agar	violet with black H ₂ S	Violet/yellow
Urea hydrolysis	Pink	Yellow
Indole production	Yellow	Yellow

Genotypic characterization

16S rDNA sequence analysis revealed the affiliation of strain 2S to members of genus *Bacillus* showing the highest sequence homology (99%) to *Bacillus firmus* strain SQUN, while that for strain 11S showed 98% similarity to *Planococcus massiliensis* strain ES2. Accession numbers and similarity percent to nearest sequences are depicted in **Table 2**. Phylogenetic trees for the two strains are illustrated in **Figs 3** and **4**. Strains 2S and 11S were thus designated as *Bacillus* sp. R2 and *Planococcus* sp. R11, respectively.

Hemolytic activity

The two strains *Bacillus* sp. R2 and *Planococcus* sp. R11 did not show clear zone on blood agar and proved to be non-hemolytic.

Antibiotic susceptibility

Bacillus sp. R2 showed sensitivity to Amoxicillin (AML10), Nalidixic acid (NA30), cephalexin (CL30), Piperacillin (PRL100), Rifampin (RD5) and Fusidic acid (FA10) and resistance to colistin sulfate (CT10), Bacitracin (B10) and Metronidazole (MTZ5) while *Planococcus* sp. R11

showed sensitivity to Amoxicillin (AML10), Bacitracin (B10), cephalixin (C130), Piperacillin (PRL100), Rifampin (RD5) and Fusidic acid (FA10) and resistance to colistin sulfate (CT 10) , Nalidixic acid (NA 30) and Metronidazole (MTZ 5).

Growth patterns

As shown in **Fig.5** *Bacillus* sp. R2 began to grow exponentially immediately after inoculation and this phase was extended till 26 h where maximum growth ($OD_{600}=1.737$) was achieved, pH gradually increased by time reaching a value of 9.9 after 30 h due to release of metabolites produced into the liquid broth. On the contrary, *Planococcus* sp. R11 exhibited a lag phase of 5 h, followed by exponential phase which extended till 26 h with maximum growth of $OD_{600}=2.76$. pH gradually increased by time reaching a value of 9.76 after 30h.

Planococcus sp. R11 displayed a faster growth rate compared to *Bacillus* sp. R2 ($0.265h^{-1}$ and $0.127 h^{-1}$, respectively, and also a higher doubling time (5.46 h and 2.61 h for R11 and R2, respectively).

Antagonism against fish pathogens

Indeed, a good probiotic candidate is selected based on its inhibitory activity against target pathogens *in vitro* (Cao *et al.*, 2011). Data illustrated in **Fig.6** indicate that *Planococcus* sp. R2 exhibited antimicrobial activity against *Vibrio parahaemolyticus* and *Vibrio alginolyticus*. On the other hand, *Bacillus* sp. R2 showed a broader spectrum, being antagonistic against same pathogens in addition to *Vibrio fluvialis* and *Staphylococcus aureus*.

Cell surface hydrophobicity

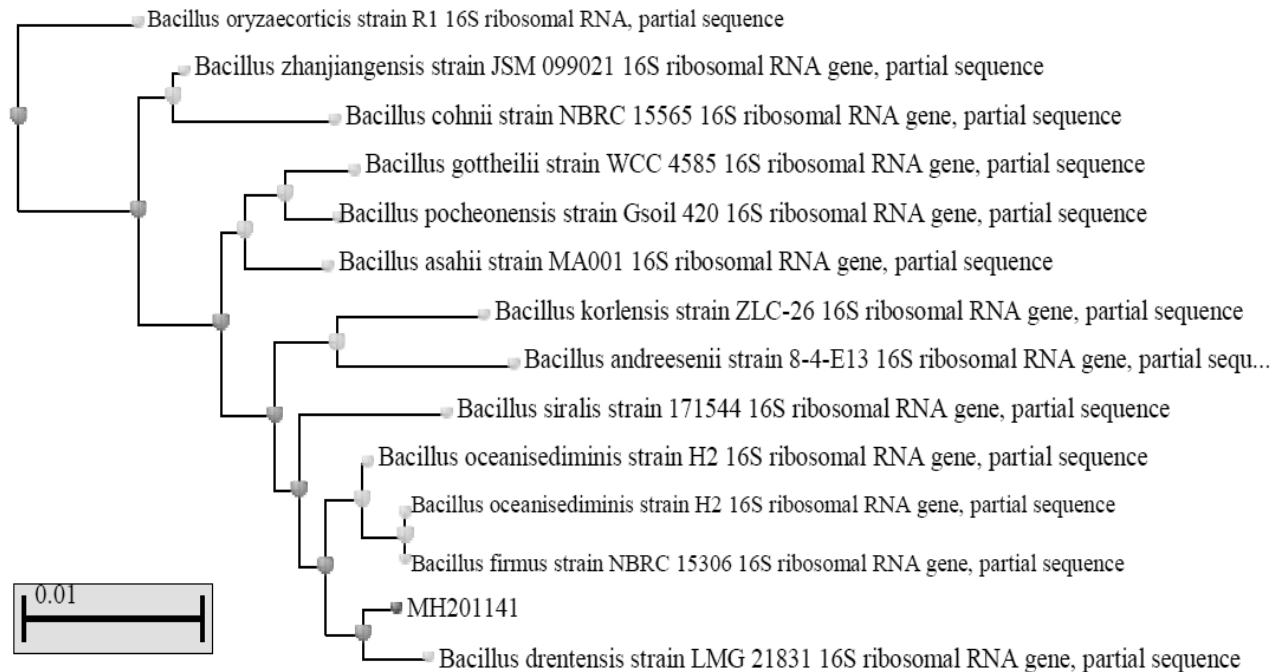
Data presented in **Table 3** show the values of hydrophobicity (MATS %) of the tested strains. Accordingly, they are both classified as moderately hydrophobic.

Tolerance to acidic and alkaline condition

As depicted in **Table 4**, *Bacillus* sp. R2, survived after 24 h of exposure at pH 2.5, and 8 with slight loss in viability compared to pH 7. *Planococcus* sp. R11 showed better growth at low and high pHs compared to R2 after 24 h of incubation.

Table 2 Strain code, accession number and similarity % of 2S and 11S to the nearest neighbors

Isolate	GenBank accession number	Similarity %	Nearest neighbor (s)	The proposed name
2S	MH201141	99%	<i>Bacillus firmus</i> strain SQUN	<i>Bacillus</i> sp. R2
11S	MH201142	98%	<i>Planococcus massiliensis</i> strain ES2	<i>Planococcus</i> sp. R11

**Fig.3 Phylogenetic tree of isolate 2S based on 16S rDNA sequence analysis.**

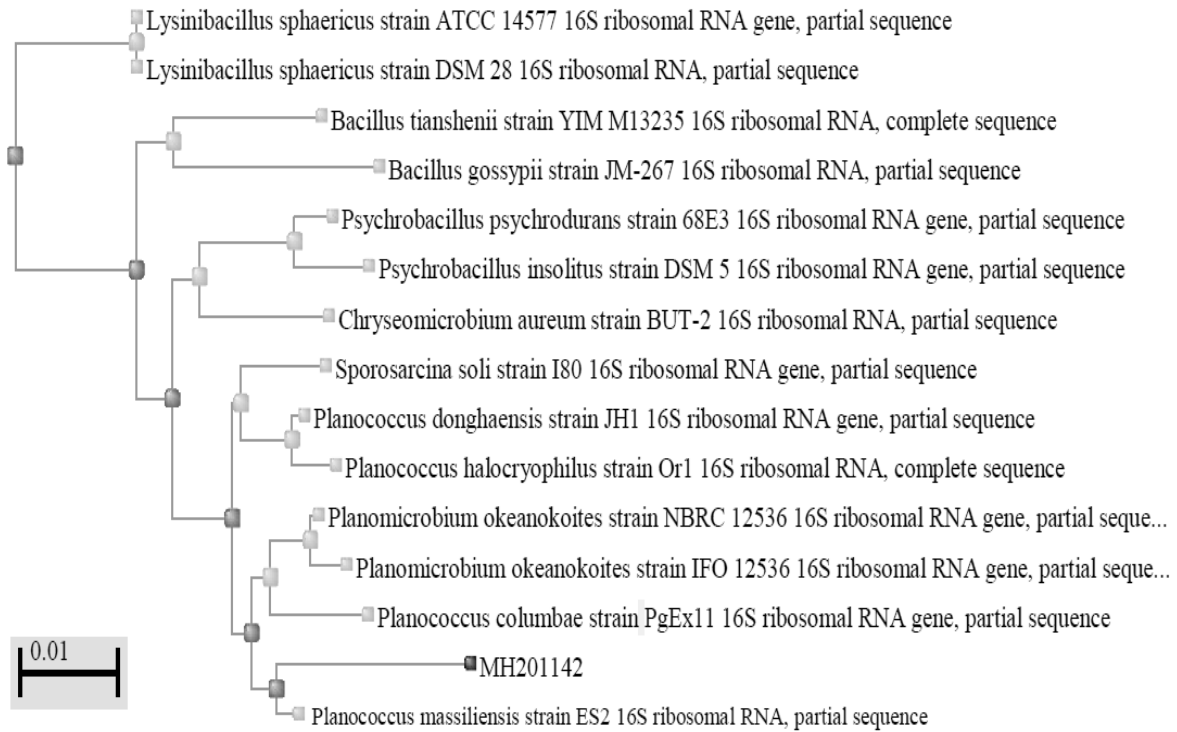


Fig.4 Phylogenetic tree of isolate 11S based on 16S rDNA sequence analysis.

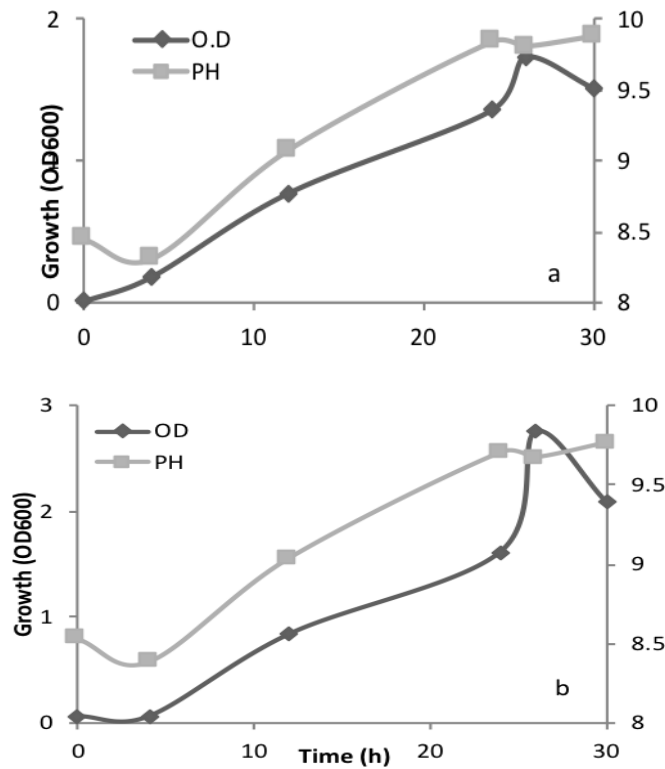


Fig.5 Growth pattern of *Bacillus* sp. R2 (a) and *Planococcus* sp. R11 (b) grown on SWNB for 30 h at 35°C under shaken condition (150 rpm)

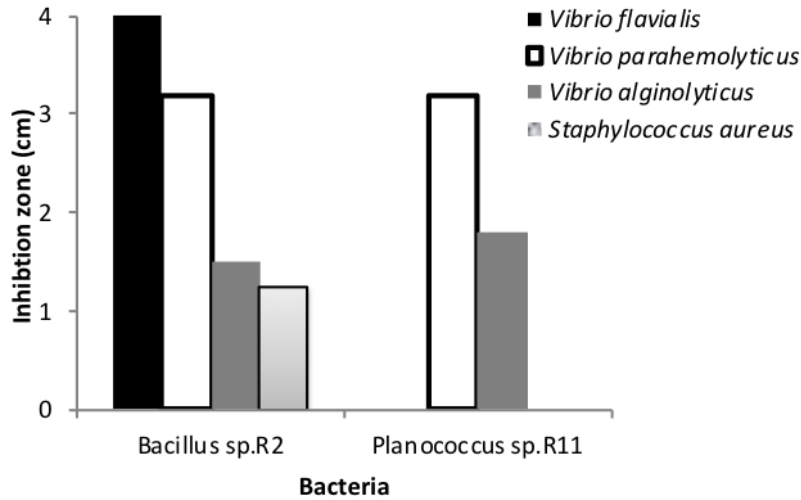


Fig.6 Antimicrobial activity of the bacterial strains against some pathogens

Table 3 Cell surface hydrophobicity assessed by MATS

Bacterial strain	MATS (%)	Hydrophobicity
<i>Bacillus</i> sp. R2	26.75%	Moderately hydrophobic
<i>Planococcus</i> sp. R11	38.00%	Moderately hydrophobic

Table 4 Bacterial count (CFU/mL) of *Bacillus* sp. R2 and *Planococcus* sp. R11 in acidic, alkaline and normal pHs

Bacterial strain	<i>Bacillus</i> sp. R2	<i>Planococcus</i> sp. R11
Acidic pH (2.5)	2×10^3	6×10^5
Normal pH (7.2)	1×10^7	1.3×10^7
Alkaline pH (8)	4×10^4	3.6×10^6

DISCUSSION

Administration of Commercial probiotic to aquatic organisms is less effective because it is isolated from non-fish source and during the growing phase of fish, they cannot be able to survive in a high density in the intestines . Therefore, Isolation of probiotic bacteria from the fish host perform better in their natural habitat than those derived from terrestrial hosts (Wanka *et al.*, 2018). Eleven autochthonous isolates were isolated from the gastrointestinal tract of Sea bream (*Sparus aurata*). They were subjected to enzymatic activity to select the most potent to be used as probiotics. The most potent bacterial isolates were selected and identified by 16S rDNA as *Bacillus* sp.R2 and *Planococcus* sp.R11 and characterized as potential probiotic. Although plenty of reports are available

for the use of *Bacillus* species as probiotics (Anyanwu & Ariole, 2019), no publication is available on the *Planococcus* as a probiotic candidate.

Hemolysis remain the major virulence factors among pathogenic bacteria and hence, as the safety aspects that have been evaluated for the two bacterial strains in the current study, the *in vitro* test for hemolytic activity has been considered first, the next one in the probiotic safety issues being the antibiotic susceptibility ; the probiotic bacteria should be sensitive to antibiotics so as to be inept disseminating the resistance property to other pathogenic bacteria in the same niche, or the antibiotic resistance among them should be innate and non-transferable (Halder *et al.*, 2017). *Bacillus* sp. R2 and *Planococcus* sp. R11 had no hemolytic activity, a property similar to *Lactobacillus* strains selected for *in vivo* application as probiotic in aquaculture (Kaktcham *et al.*, 2017). . The two strains were sensitive to Amoxycillin (AML10), Cephalexin (CL30), Piperacillin (PRL100) , Rifampin (RD5) and Fusidic acid (FA10) while *Planococcus* sp. R11 was sensitive to Bacitracin (B10) and *Bacillus* sp. R2 was sensitive to Nalidixic acid (NA30) and the two strain were resistant to Colistinsulphate (CT10) and Metronidazole (MTZ5). Georgieva *et al.*, 2015 reported the sensitivity of natural isolates of *lactobacilli* (*L. acidophilus*, *L. brevis*, *L. fermentum* and *L. plantarum*) to Am, Gm, erythromycin (Em) and Tc, and intrinsic resistance to Vm (non-transferable), and suggested their use as probiotics appropriate in clinical practice.

One of the very important parameters for selection of probiotics is the high growth rate within the gastrointestinal ecosystem to compete for nutrients and adhesion sites, to exert their beneficial effects in the host and to reduce their elimination from the digestive tract (Ibrahim, 2015). *Planococcus* sp. R11 displayed a faster growth rate compared to *Bacillus* sp. R2 (0.265h^{-1} and 0.127h^{-1} , respectively, and also a higher doubling time (5.46 h and 2.61h for R11 and R2, respectively). In comparison, the two probiotics *Pediococcus pentosaceus* (LB-12 and LB-25) and *Lactobacillus pentosus* (LB-31) strains (García-Hernández *et al.*, 2016) had higher growth rates (0.5h^{-1}) and lower doubling times (1.3 h) than our strains.

Probiotics should possess antagonistic activities against fish pathogens. Both strains showed antimicrobial activity against *Vibrio parahaemolyticus*. Moreover, *Bacillus* sp.R2 had antimicrobial activity against *Vibrio fluvialis* and *Staphylococcus aureus*. There are a good agreement with our data and those recorded with *Bacillus* species (Silva *et al.*, 2015)

The two strains possessed antagonistic action against *Vibrio alginolyticus* isolated from fish tank . Our results coincide with Widanarni *et al.*, (2015) who introduced four selected isolates from gastrointestinal tract of Pacific white shrimp *Litopenaeus vannamei* had antagonistic activity against pathogenic bacteria *V. harveyi*, .

They also showed moderate surface hydrophobicity (26.75% and 38% for *Bacillus* sp. R2 and *Planococcus* sp. R11, respectively) .The value of MATS recorded for *Planococcus* sp. R11 is similar to other *Lactobacilli* (Xu *et al.*, (2009). Sánchez-Ortiz *et al.*, (2015) who introduce two bacterial isolates from gastrointestinal tract from adult mangrove cockle *Anadara tuberculosa* and identified as *Citrobacter koseri* and *Bacillus*

subtilis subtilis with high hydrophobicity (60%) and with medium hydrophobicity (35%) respectively, reached their stationary phase after 48 h of culture.

Bacillus sp. R2 and *Planococcus* sp. R11 had small difference of the log bacterial number to the bacterial number in normal condition that mean that these two bacterial strain had the ability to survive in acidic and alkaline pH. The data obtained coincide with **Widanarni *et al.*, (2015)** who reported that the initial number of probiotic candidates in normal, acidic and alkaline pH was in range of 1.1×10^7 - 2.9×10^8 CFU/ mL and **Vieira *et al.*, (2013)** who reported growth loss in *Lactobacillus plantarum* at pH2, whereas no growth loss at pH 8 and 9.

CONCLUSION

The present work provides an evidence that the gastrointestinal tract of sea bream (*Sparus aurata*) could be a good source for probiotic bacteria. All studied characteristics of the isolated bacteria confirm their possible use as probiotic. This is the first report on evaluation of *Planococcus* as potential probiotic. *In vivo* application of the two strains in fish rearing tanks is considered in future work.

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