Journal of Agricultural and Marine Sciences 2024, 29(2): 51- 63
DOI: 10.53541/jams.vol29iss2pp51- 63
Received 23 May 2023
Accepted 16 June 2024

RESEARCH PAPER

Screening of probiotics from indigenous Omani natural products for potential use in the aquaculture industry

Manal Al Sheriyani[1], Miyoung Cho[2], Hanan Al Balushi[1], Sharifa Al Julandani[1], Al Thuraiya Al Shidhani[1] and Gilha Yoon[1,*]

فحص البروبيوتيك من المنتجات الطبيعية العمانية الأصلية للاستخدام المحتمل في صناعة تربية الأحياء المائية

منال الشريانية[١]، ميونج تشو[٢]، حنان البلوشية[١]، شريفة الجلندانية[١]، الثريا الشيذانية[١]، جيلها يون[١]

ABSTRACT. Probiotics can enhance the growth performance and disease resistance of aquatic animals. Probiotics can influence digestive physiology through the secretion of exogenous digestive enzymes, facilitating the utilization of nutrients, and resulting in improvements in growth performance. The objectives of the present study were to reduce fish diseases, minimize the usage of antibiotics, and promote national policy on aquatic health management in Oman through enhancing immune stimulation mechanisms with probiotics from indigenous Omani products and finally to promote Oman's aquaculture industry. Thirty-five kinds of edible products were screened. After the primary screening process, only 3 products contained appropriate amounts of probiotics, therefore the present study focused on 3 probiotics from Omani products, i.e., dried date, date vinegar, and camel milk. Gram staining, VITIK2, and DNA tests were used for the identification and characterization of bacterial strains. DNA sequence revealed that the bacteria isolated from dried dates were identified as *Bacillus licheniformis*. Those from date vinegar were identified as *Bacillus vallismortis*. A viability test with pepsin, pH 3 showed that the bacteria isolated from camel's milk (*Bacillus stratospheric*) had a greater tolerance at low pH followed by bacteria isolated from dried date (*B. licheniformis*). Thus, these can be used as a probiotic for aquatic organisms.

KEYWORDS: Aquaculture, probiotics, Omani products, bacteria

الخلاصة: يمكن للبروبيوتيك أن يعزز أداء النمو ومقاومة الأمراض لدى الحيوانات المائية. يمكن أن تؤثر البروبيوتيك على فسيولوجيا الجهاز الهضمي من خلال إفراز الإنزيمات الهاضمة الخارجية، مما يسهل استخدام العناصر الغذائية، ويؤدي إلى تحسين أداء النمو. كانت أهداف هذه الدراسة هي الحد من أمراض الأسماك، وتقليل استخدام المضادات الحيوية، وتعزيز السياسة الوطنية بشأن إدارة الصحة المائية في عمان من خلال تعزيز آليات تحفيز المناعة باستخدام البروبيوتيك من المنتجات العمانية الأصلية، وأخيرا تعزيز صناعة تربية الأحياء المائية في عمان. وتم عرض خمسة وثلاثين نوعا من المنتجات الصالحة للأكل. بعد عملية الفحص الأولية، كانت ثلاث منتجات فقط تحتوي على كميات مناسبة من البروبيوتيك، وبالتالي ركزت الدراسة الحالية على ثلاثة بروبيوتيك من المنتجات العمانية، وهي التمر المجفف، وخل التمر، وحليب الإبل. تم استخدام تلوين جرام، فايتيك ، واختبارات الحمض النووي لتحديد وتوصيف السلالات البكتيرية. أظهر تسلسل الحمض النووي أن البكتيريا المعزولة من التمر المجفف تم تحديدها على أنها (عصية ليشنيفورمس). تم التعرف على تلك الموجودة في خل التمر على أنها (عصية فاليس مورتيس). أظهر اختبار الحيوية باستخدام البيبسين، الرقم الهيدروجيني ٣ أن البكتيريا المعزولة من حليب الإبل (عصية ستراتوسفيرك) كانت أكثر تحملاً عند الرقم الهيدروجيني المنخفض تليها البكتيريا المعزولة من التمرالمجفف (عصية ليشنيفورمس). وبالتالي، يمكن استخدامها كبروبيوتيك للكائنات الحية المائية.

الكلمات الرئيسية: الإستزراع السمكي، البروبيوتيك، منتجات عمانية، البكتيريا

[1]Department of Marine Science and Fisheries, Sultan Qaboos University, Oman
[2]Pathology research division, National Institute of Fisheries Sciences, R.O Korea
[*]Corresponding author: Dr. Gilha Yoon, (E-mail: ghyoon@squ.edu.om)

Introduction

Aquaculture has grown in importance as a source of income in several countries. Diseases and degradation of environmental conditions are common in large-scale production facilities where aquatic animals are subjected to stressful settings, resulting in significant economic losses. High mortality in aquaculture is one of the main problems facing this sector (Kjørsvik et al., 2011). The occurrence of these deaths varies, it may be due to the environment, diseases, and water quality, but the main cause of death is diseases, and especially what causes the highest rate of death in aquaculture is diseases caused by bacterial infection (Mohamad et al., 2019). The high density of the population in the culture causes the spread of bacteria among the fish, as well as the wrong practices during the transfer of fish between the juvenile and adult stages (Lim and Jang, 2004).

Antibiotics are produced in the United States in large quantities for medical and agricultural applications, with 12,600 tons used for non-therapeutic treatments of animals to stimulate growth. Throughout 1997, 1600 tons of antibiotics were used for growth promotion purposes in the European Union and Switzerland, accounting for nearly 30% of overall antibiotic use in farm animals. Antibiotics in such large quantities have put a severe selection pressure on resistance of bacteria, which have adapted to the circumstance mostly through a horizontal and promiscuous transfer of resistance genes (Manage, 2018). Probiotics, or good bacteria, are considered as an alternative to antibiotic treatment since they suppress pathogens through a variety of processes. Probiotics have long been used in human and animal nutrition, and they have only lately begun to be used in aquaculture. Probiotics is a term that refers to bacteria that aid in the health of other species. Probiotics are live bacteria that impart a health benefit to the host when taken in sufficient proportions (FAO, 2001). In general, probiotic strains have been isolated from the microbiota of aquatic animals, both exogenous and indigenous. Gram-negative facultative anaerobic bacteria like *Pseudomonas* and *Vibrio* make up most of the indigenous microbiota of a wide range of marine fish species (Lara-Flores, 2011). In contrast to saltwater fish, members of the genera *Aeromonas* and *Plesiomonas*, members of the family Enterobacteriaceae, and obligate anaerobic bacteria of the genera *Bacteroides*, *Fusobacterium*, and *Eubacterium* dominate the indigenous microbiota of freshwater fish species (Mohamad et al., 2019).

Intensification is used to boost production by adding commercial diets, growth stimulants, antibiotics, and a variety of other additives. The employment of these techniques results in high output without a doubt, but the most concerning element is that regular use of these items creates severe difficulties, to the point where their long-term viability is in jeopardy. Probiotics have been employed in aquaculture for a long time, but in the recent years, they have become a key part of culture methods for boosting growth and disease resistance. This technique has numerous advantages, including the ability to overcome the restrictions and adverse effects of antibiotics and other medications, as well as increased production due to improved growth and disease prevention. Aside from the health and nutritional benefits, some probiotics used as water additives can help with organic matter decomposition, phosphorus, and nitrogen reduction, also ammonia, nitrite, and hydrogen sulfide control.

Probiotics support feed conversion efficiency and live weight gain in fish, as well as confer pathogen protection through competitive exclusion for adhesion sites, the production of organic acids, hydrogen peroxide, and a variety of other compounds such as antibiotics, siderophores, bacteriocins, and lysozyme, and modulation of physiological and immunological responses. Most previous studies in fish focused on probiotics' capacity to promote growth and protect against illness. However, in recent years, there has been a lot of focus on the immune-modulating effects of probiotics in the aquatic system. Many immunological investigations have

been conducted in a variety of fish with various probiotics, and their ability to enhance immunity both *in vitro* and *in vivo* is noteworthy. According to a review of the literature, numerous probiotics, either separately or in combination, can improve both systemic and local immunity in fish (Manage, 2018).

The objectives of this present study were to reduce fish diseases, minimize the usage of antibiotics, and promote national policy on aquatic health management in Oman through enhancing immune stimulation mechanisms with probiotics from indigenous Omani products. Finally, to promote environmentally friendly Oman's aquaculture industry.

Materials and Methods

Identification Bacteria

Primary Test: Using gram staining the type of bacteria was identified, as the pink denotes gram-negative bacteria, and the violet hue means the gram-positive bacteria. By using a sterilized loop, one colony was taken from a TSA plate, 3 drops of saline water was mixed with the colony in the glass slide, and the smear was fixed via flame, the smear was dried in the air for 10 minutes. Then, the prepared smear was covered by crystal violet for 1 minute, distilled water was used to wash the smear. After that, Iodine was used to continue the process of stain smear and washed after 1 minute. To decolorize the smear, alcohol was added for 1 minute. The smear was covered with safranin and washed with saline water after 1 minute; the slide was dried in the air for 20 minutes. Then, we used a light microscope to observe the smear in ×100 magnification.

Secondary Test: First, a Gram stain film from isolated, fresh (12-24 h), and pure colonies from a plate was prepared. Second, according to the result of the Gram stain the type of card was selected. Then, the homogenous organism suspension in 3 ml saline (0.45-0.5%) in a plastic tube was prepared, and the DensiCHECK plus was used to check the turbidity of the suspension.

After that, the suspension was inoculated into cards within 30 minutes after preparation, the suspension tube and ID card were placed in the cassette, and the managed cassette view was entered. The virtual cassette icon was clicked, the cassette number was selected, the card barcode was scanned, and automatically the card type column was filled. By filling in the cassette number, the isolate was identified, and the cassette in the filler station was inserted and the door was closed. After that, the fill on button was pressed (filling is 70 second cycle), the cassette was entered into the load station and the door was closed, then, the card was automatically ejected after analysis.

DNA Test: From plates to sample tubes, a part of a pure colony of bacteria (500 mg) was transferred. 3 ml CTAB extraction buffer and 5 ul protease k were added, and the mixture was kept at 55 C for 12 hours. After cooling the samples to room temperature, 5 ul RNase (10 mg/ml) was added. After that, it was incubated for 30 minutes at 37 degrees Celsius. PCI (25:24:1) was added in an equal volume and centrifuged for 10 minutes at 12000 rpm. The supernatant was then transferred to a new Eppendorf tube. An equal volume of cold isopropanol was added, and the mixture was centrifuged for 10 minutes at 12000 rpm. The supernatant was discarded, and the pellet was cleaned in 70% ethanol. The pellets were dried and re-suspended in 70 microliters of autoclaved distilled water. Finally, for a short time, it was kept at 4 degrees Celsius. All bacterial DNA was extracted. For all samples, the polymerase chain reaction (PCR) was used to make multiple copies of DNA segments. The DNA sequence of the target gene (16S rRNA) was then determined by amplifying it using a sequencer.

Viability Test: These steps were done for all bacteria, in the first period the process was repeated every hour for 8 hours (from 12 pm - 7 pm), in the second period, these steps were also repeated for each type of bacteria every 24 hours for a week.

Pepsin Preparation: 90 ml of distilled water and 3 g of pepsin powder were mixed in a beaker

and the lactic acid was added to adjust the pH3.

Bacteria Preparation: A pure colony of bacteria was taken and mixed with 5 ml of distilled water in a beaker, the reading of bacteria concentration was taken from the spectrophotometer, and then the bacterial mixture was added to the pepsin solution.

Dilution: Using a micropipette 1 ml of the final mixture was transferred into the empty watchmen glass, another four watchmen glasses were filled with 0.9 ml of saline water by micropipette. For dilution, 0.1 ml was transferred from the original bacteria into (10^{-1}) dilution and mixed, then 0.1 ml was transferred from (10^{-1}) dilution into (10^{-2}) dilution, these steps were repeated till (10^{-3}) dilution.

Bacterial Culture: From each dilution, 0.1 ml was transferred into the TSA plate that was labeled by bacteria name and dilution factor, three replicated TSA plates were set for each dilution.

Statistical Analysis

Two-way ANOVA test, one-way ANOVA test, and Tukey's-test were performed in order to test the potency and viability of bacteria. The results of the potency test were analyzed through the mean values and the standard error of the mean. The differences were analyzed using the one-way ANOVA. The viability test of bacteria strains and daily changes were compared with the Two-way ANOVA test. All tests were applied to determine a significant difference level at $P \leq 0.05$ with Excel 2021.

Results

Identification and Characterization of Bacteria Strain

After the primary and secondary tests, the results of the VITEK2 did not clearly identify the isolated bacteria. The present study, therefore, used Gram staining and DNA sequencing results.

Bacteria Isolated from Date Vinegar

The Gram staining result showed that the isolated bacteria were gram-positive and rod-shaped. In addition, they were observed to grow very fast compared with other bacteria and were difficult to dilute with distilled water. The shape of the colony was irregular. DNA sequence revealed that the bacteria isolated from date vinegar were identified as *Bacillus vallismortis*, and the nucleic acid sequence showed in Table 1.

Bacteria Isolated from Commercial Probiotic

DNA test was not conducted for these bacteria, since this bacterium is known by the produced company as *Lactobacillus* spp. Bacteria isolated from commercial probiotic is also gram-positive and rod-shaped.

Bacteria Isolated from Dried Date

The rod-shaped and purple color bacteria were found from the dried dates. By DNA sequence this bacteria was identified as *Bacillus licheniformis* (Table 2).

Bacteria Isolated from Camel Milk

Based on gram staining, these isolates were found to be gram-positive and rod-shaped bacteria, one of the characteristics of these bacteria is that they are fast-growing and they grow in regular shapes in TSA. After DNA of these isolates was sequenced, this bacterium was identified as *Bacillus stratosphericus* (Table 3).

Viability Test

Figure 1 shows the viability test of *B. vallismortis* in pepsin per hour. The X-axis displays the time elapsed (hours), y-axis displays the concentration of viable bacteria (10^6) per 1 ml. Generally, there is a gradual decrease in the concentration of bacteria in the pepsin-free medium; on the other hand, we notice a significant decrease in the concentration of bacteria in the pepsin medium per day. The initial concentration of bacteria is 5×10^7/ml. After 24 hours, the concentration of bacteria in pepsin is 1.8×10^7/ml, while 3.2×10^7/ml without pepsin. The concentration of viable bacteria after 48 hours is 1.5×10^7/ml without pepsin, 2×10^6 in pepsin. On the last day, the abundance of viable bacteria in pepsin is

Table 1. DNA sequence of bacteria isolated from date vinegar.

Bactria source	Date vinegar
Bacteria name	*Bacillus vallismortis*
DNA sequence	AAGCAGGGCGCTAACTGCAGTCGAGCGGACAGATGGGAGCTTGC- TCCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGC- CTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATG- CTTGTTTGAACCGCATGGTTCAGACATAAAAGGTGGCTTCGGCTAC- CACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACG- GCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGC- CACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAG- TAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCG- CGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAA- CAAGTGCCGTTCAAATAGGGCGGCACCTTGACGGTACCTAACCAGAAAGC- CACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAG- CGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTC- TGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGG- GGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGT- GAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTG- GTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGAT- TAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGG- GGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGG- GAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCA- CAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTAC- CAGGTCTTGACATCCTCTGACAATCCTAGAGATAGGACGTCCCCTTCGGG- GGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGAT- GTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCAT- TCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTG- GGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGC- TACAATGGACAGAACAAAGGGCAGCGAAACCGCGAGGTTAAGC- CAATCCCACAAATCTGTTCTCAGTTCGGATCGCAGTCTGCAACTCGACTG- CGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAA- TACGTTCCCGGGCCTTGTACACACCGCCCATCACACCACGAGAGTTTGTAA- CACCCGAAGTCGGTGAGGTAACCTTTAGAGCCGCCGCGAAGTGCAGGTT

Table 2. DNA sequence of bacteria isolated from commercial probiotic.

Bactria source	Dried date
Bacteria name	*Bacillus licheniformis*
DNA sequence	AAGCAGCGCGCTAACTGCAGTCGAGCGGACAGATGGGAGCTTGCTCC-CTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTG-TAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGCTTGAT-TGAACCGCATGGTTCAATTATAAAAGGTGGCTTCTGCTTCCCCTTTC-CAAAGGACCCCCCGCCCCATAACCAATTTGTGAAGTAACCGCTCCCC-CAGGGCACCAATCCTAACCCAACTTAAAAGGTGATCCGCCCCCCTGG-GACTGAAAACCCGCCCAAAACCCCACCGGAAGGAACCATAAGGAATC-CTCCCCCATGGAACAAAATCCGAACGAACCACCCCCCCTGAATTAA-TAAAGGTTTCCGAACCTAAAACTCCTTTGTTAAGGAAAAACCAATAC-CGTTCCAATAAGGGGGTACCTTTACCGGACCTAACCCAAAAGCCCC-CGCTAAATACCTTCCAACCACCCCCGTAATACCTAAGTTGCAAACC-TTTTCCCGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTTCTTAAGTC-TGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGG-GGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGT-GAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTG-GTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCGAACAGGAT-TAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAG-GGTTTCCGCCCTTTAGTGCTGCAGCAAACGCATTAAGCACTCCGCCTGGG-GAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCA-CAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTAC-CAGGTCTTGACATCCTCTGACAACCCTAGAGATAGGGCTTCCCCTTCGGG-GGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGAT-GTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAG-CATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAG-GAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTA-CACACGTGCTACAATGGGCAGAACAAAGGGCAGCGAAGCCGCGAGGC-TAAGCCAATCCCACAAATCTGTTCTCAGTTCGGATCGCAGTCTGCAAC-TCGACTGCGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGC-CGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACAC-CACGAGAGTTTGTAACACCCGAAGTCGGTGAGGAACCTTTGAGCCGCCG-CGAAGGCAGGG

Table 3. DNA sequence of bacteria isolated from camel milk

Bactria source	Camel milk
Bacteria name	*Bacillus licheniformis*
DNA sequence	GGCAGGGCGCTATACTGCAGTGAGCGGACAGAAGGGAGCTTGCTCC-CGGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTG-TAAGACTGGGATAACTCCGGGAAACCGGAGCTAATACCGGATAGTTC-CTTGAACCGCATGGTTCAAGGATGAAAGACGGTTTCGGCTGTCAC-TTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGG-CTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGC-CACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAG-CAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGC-CGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGG-GAAGAACAAGTGCAAGAGTAACTGCTTGCACCTTGACGGTACCTAAC-CAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTG-GCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTC-TTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTG-GAAACTGGGAAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTG-TAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGG-CGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAG-CGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGC-TAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAG-CACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAAT-TGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAG-CAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAACCCTAGAGA-TAGGGCTTTCCCTTCGGGGACAGAGTGACAGGTGGTGCATGGTTGTCGT-CAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACC-CTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCG-GTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCC-CTTATGACCTGGGCTACACACGTGCTACAATGGACAGAACAAAGGGC-TGCGAGACCGCAAGGTTTAGCCAATCCCACAAATCTGTTCTCAGTTCG-GATCGCAGTCTGCAACTCGACTGCGTGAAGCTGGAATCGCTAGTAATCG-CGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCC-CGTCACACCACGAGAGTTTGCAACACCCGAAGTCGGTGAGGAACCTTAG-GAGCCGCCGCCGAGTGCAGTAAA

Figure 1. Viability test result of *B. vallismortis* with pepsin and without pepsin for 72 hours

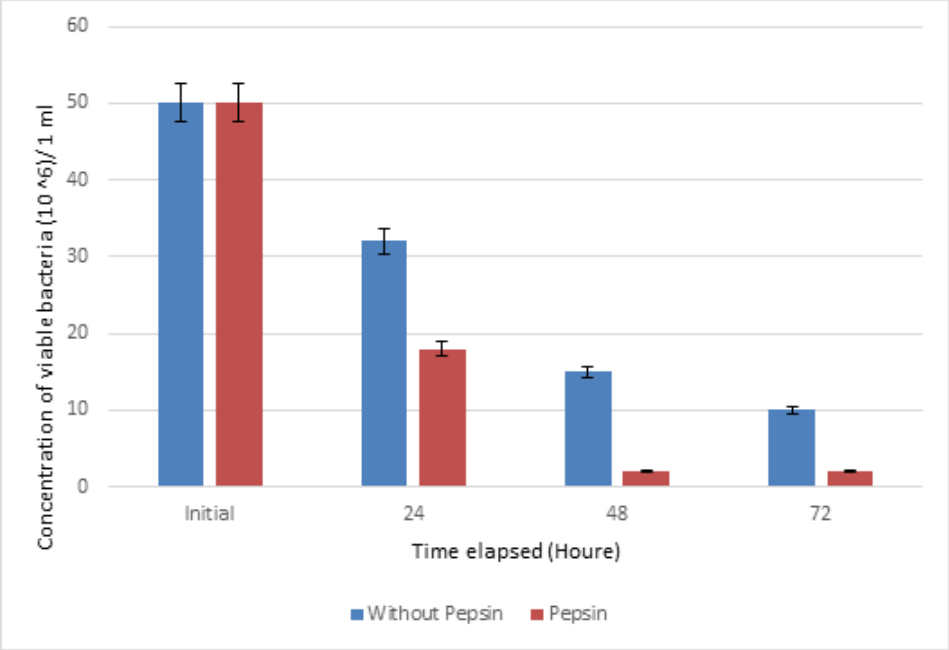
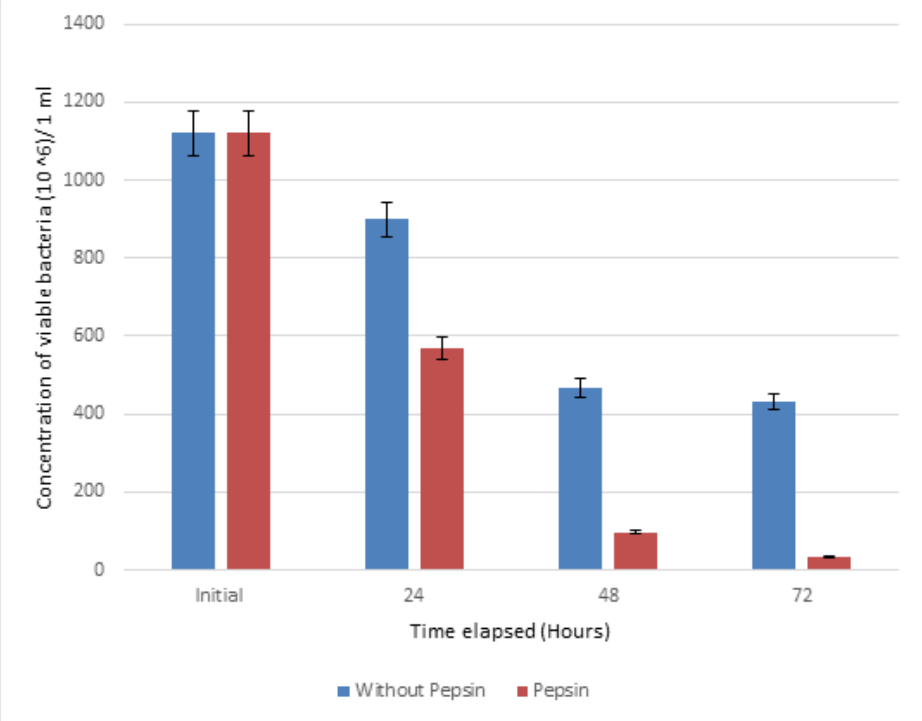


Figure 2. Viability of *Lactobacillus* sp. with pepsin and without pepsin for 72 hours



2×10^6/ml. Figure 2 illustrates the viability test of *Lactobacillus* sp. in pepsin per hour. The x-axis shows the time elapsed (h), y-axis displays the concentration of viable bacteria per 1 ml. The initial concentration of bacteria is 1.1×10^9/ml. After 24 h the growth rate of bacteria decreased to 5.7×10^8 per ml in the pepsin medium, 8.1×10^8/ml without pepsin. After 48 hours, bacteria concentration halved in the medium without pepsin, and 9.7×10^7 in pepsin medium. Figure 3 represents the viability test of *B. licheniformis*. in pepsin per hour. The x-axis displays the time elapsed (h), the y-axis displays the concentration of viable bacteria per 1 ml. The initial concentration of bacteria is 1.6×10^8/ml. Generally, there is a gradual decrease in the concentration of bacteria in the pepsin-free medium, on the other hand, we notice a significant decrease in the concentration of bacteria in the pepsin medium per day. After 72 h, the concentration of viable bacteria is 2.2×10^8/ml in pepsin medium, while 5.4×10^8 without pepsin. Figure 4 shows the viability test of *B. stratospherious* in pepsin per hour. The x-axis represents the time elapsed (hours), y-axis displays the concentration of viable bacteria per 1 ml. The initial concentration of bacteria is 1.96×10^{10}/ml. After 24 h, the concentration of viable bacteria decreased to 1.59×10^{10}/ml in the pepsin medium, 1.8×10^{10}/ml without the pepsin medium. The concentration of viable bacteria after 48 hours and 72 h in pepsin is (9.8×10^9/ml and 3.3×10^9/ml, respectively).

Discussion

The probiotic market is constantly growing, and new products are being created all the time. *Lactobacilli* and yeast make up the majority of formulations. Spore-containing probiotics based on *Bacillus* spp. are making a significant contribution to the worldwide nutraceutical and pharmaceutical sector, and have a long history of usage in several countries. Different genetic techniques and biological assays have been developed for discriminating more than 300 species belonging to the genus *Bacillus*, which has experienced si-

Figure 3. Viability test of *B. licheniformis* with pepsin and without pepsin for 72 hours

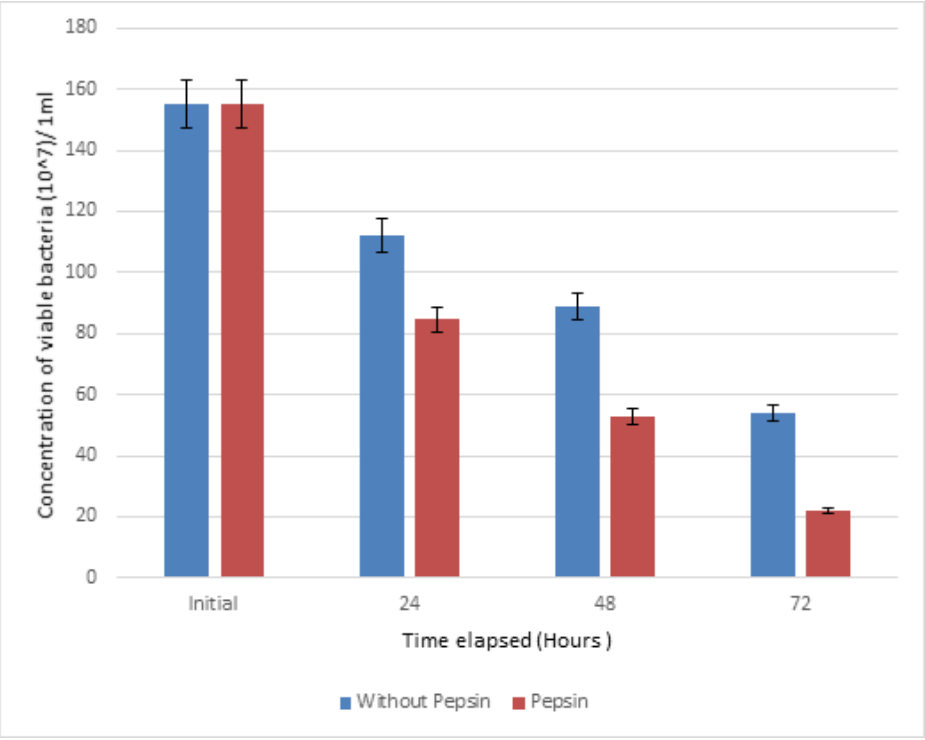
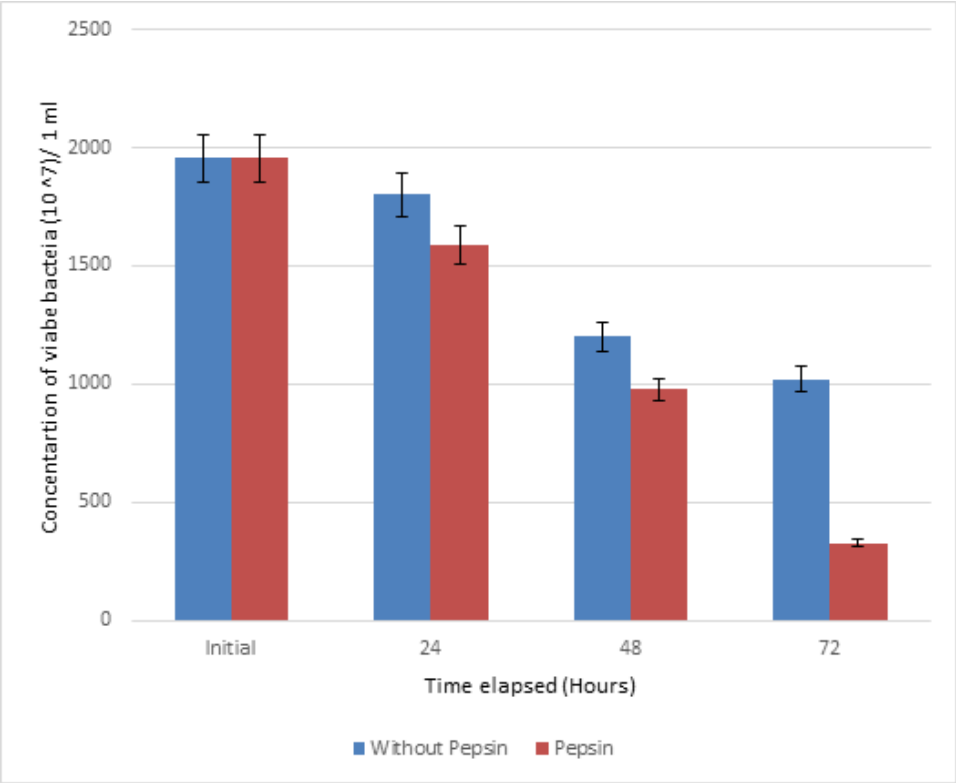


Figure 4. Viability test of *B. stratospherious* with pepsin and without pepsin for 72 hours



gnificant taxonomic alterations.

Three native *Bacillus* spp. previously isolated from different food sources and identified as *B. subtilis, B. licheniformis and B. vallismortis* were used, these bacterial cultures were identified by biochemical and 16S rRNA gene sequencing (Zulkhairi et al., 2020). In our study, *B. vallismortis* isolated from dates vinegar and *B. licheniformis* isolated from dried dates, bacteria were identified by using DNA gene sequencing.

Previous studies isolated lactic acid bace teria (LAB) from raw camel's milk included *Leuconostoc mesenteroides*, *Lactobacillus plantarum*, *Weissella paramesenteroides* and *Weissella confuse*. Antagonistic activity of isolated LAB against two pathogenic bacteria showed that they had more inhibitory activity against *S. aureus* subsp. *aureus* PTCC 1431 than *E. coli* ATCC 25922 (Edalati et al., 2019). In the present study, we found that raw camel's milk contains another potential bacteria (*Bacillus stratosphericus*), which is gram-positive bacteria, According to (Karthik et al., 2015), the *B. stratosphericus* strain was isolated from marine sediment samples. This strain has the strongest antagonistic activity against different seafood bacterial pathogens.

Bacillus has been widely investigated in vertebrates, especially aquatic animals, as a safe and high-quality feed addition and potential antibiotic alternative. Improvements in growth performance, immunology, antioxidant ability, and disease resistance are all connected with the use of *Bacillus* as probiotics in aquaculture (Rahman et al., 2021). *Bacillus* sp. has been shown in numerous studies to minimize harmful microorganisms in aquaculture. When challenged with *Vibrio harveyi* at 10^3-10^4 CFU/ml for 1 h, *Penaeus monodon* immersed in *Bacillus subtilis* BT23 at a density of 10^6-10^8 CFU/ml for 6 days demonstrated a 90% reduction in accumulated mortality (Faragi, 2012). This study focused on finding other sources of *Bacillus* species in Oman to develop it as a probiotic and use it as an alternative

to antibiotics, which contributes to enhancing the sustainability of fish farming.

Probiotics must remain alive during intake and transit before reaching the large intestines in order to exercise their therapeutic effects on the host. Tolerance to unfavorable conditions, such as low pH, is therefore critical for lactobacillus and *Bacillus* species survival (Karthik, 2015). In the present study, the pH of simulated pepsin fluid was maintained at pH 3 for 72 h, as the low pH mimics the conditions in the stomach during fasting and during digestion. According to our results, the viability of bacteria in the medium without pepsin was gradually decreased, this is due to a lack of nutrients that bacteria need for growth. It is clear from the results that *B.stratospheric* have greater tolerance at pH 3 followed by *B. licheniformis*, *Lactobacillus* spp., and *B. vallismortis*. The significant decrease in the concentration of bacteria in the pepsin medium can be explained by pH 3, as this value is considered high for the growth of probiotics according to previous studies. In a previous study, they found that a low pH level of 2 had consistent effects on the viability of the probiotic cells, with higher viability log reduction compared to higher pH levels of 3 and 4 (Tham et al., 2021).

The survivability of *Bacillus* spp. in gastric juice depends on their ability to tolerate low pH, which is an important probiotic characteristic. *B. stratospherious* and *B. licheniformis* tested cultures including control showed survivability at pH 3. Ran et al. (2021) studied the survival and probiotic effects of 21 *Bacillus* strains in the intestine of catfish, their results showed that strains with excellent intestinal persistence conferred significant benefit in reducing catfish mortality when challenged by *Edwardsiella tarda. Bacillus subtilis, B. amyloliquefaciens, Bacillus licheniformis, and B. vallismortis* are phylogenetically and phenetically closely related species, which have been referred to as *B. subtilis* species made complex. When growing fish in high-tech breeding systems, it is exposed to many stress factors, in a previous study, to reduce stress, and they used spore forms of microorganism *B. licheniformis*, possessing probiotic properties. Antioxidant defense enzyme activity when using *B. licheniformis* increased, indicating activation of the defense system against oxidative stress, the results demonstrated the high efficiency of the use of spore forms *B. licheniformis* when growing catfish in high-tech fish farming systems (Romanova et al., 2020). Accordingly, these bacteria extracted from dried dates in the present study have the potential to be developed as a probiotic and applied to fish farms in Oman as an alternative to antibiotic. To our information, there are no previous studies yet about isolating this bacterium from dried dates. Therefore, this project can be completed in the future and new probiotics can be developed for aquatic organisms. A good alternative to chemical antimicrobials for disease prevention in shrimp aquaculture is the use of effective probiotics. A previous study proved the effect *of B. vallismortis*, a native probiotic isolated from the digestive tract of *Penaeus vannamei*, on pathogenic *Vibrio harveyi* in vitro and in vivo. Co-cultivation of *V. harveyi* and *B. vallismortis* significantly decreased the growth of *V. harveyi* in the treatment groups compared to the control (Mahjoub et al., 2019). For future studies, these bacteria isolated from date vinegar can be tested in *in-vivo* and *in-vitro* systems to measure the extent of their activities to enhance the non-specific immunity of shrimp.

Conclusion

The Gram-positive probiotic *Bacillus* is thought to be an important method for promoting sustainable aquaculture because they have significant advantageous effects on the aquaculture industry. *Bacillus* has a number of beneficial impacts, including improving aquaculture species' growth performance, suppressing irritation and disease infestation by inducing immunity, enhancing nutrition, and improving aquatic environments for fish and shrimp development and reproduction. The study of different sources for extracting beneficial bacteria to be developed as an alternative to antibiotics is the basis for the probiotic manufacturing process. Based on the results of

our study, *Lactobacillus* sp., *B. stratosphericus*, and *B. vallismortis* have potential value as probiotics. All of these showed good colonization and tolerance to acid and low pH medium, as well as the production of potential antimicrobial substances towards certain enteric pathogens. These bacteria could be further assessed for possible benefits *in vivo* and used as probiotics for the development of aquaculture. Aquaculture is becoming more and more integrated with other agricultural activities, and a larger use of probiotic *Bacillus* instead of hazardous synthetic chemicals would promote environmentally friendly low-input sustainable aquaculture for the food and nutritional security of the world's growing population.

Acknowledgments

The authors would like to thank the fund from FURAP (2020-2021), Research Council that helped in conducting this study.

References

Edalati E, Saneei B, Alizadeh M, Hosseini SS, Bialvaei AZ, Taheri K. 2019. Isolation of probiotic bacteria from raw camel's milk and their antagonistic effects on two bacteria causing food poisoning. New Microbes and New Infections 27: 64-68.

Faragi JK. 2012. Isolation and identification of *Bacillus subtilus* as probiotic from intestinal microflora of common carp *Cyprinus carpio* L. Iraqi Journal of Veterinary Medicine 36(0E): 355-361.

Hill C, Guarner F, Reid G, Gibson GR, Merenstein DJ, Pot B, Sanders ME. 2014. Expert consensus document: The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nature reviews Gastroenterology & Hepatology 11(8): 506-514.

Ina-Salwany MY, Al-saari N, Mohamad A, Mursidi FA, Mohd-Aris A, Amal MNA, Zamri-Saad M. 2019. Vibriosis in fish: a review on disease development and prevention. Journal of Aquatic Animal Health 31(1): 3-22.

Karthik R, Pushpam AC, Chelvan Y, Vanitha MC. 2015. Efficacy of Cheatoceros Calcitrans, Enriched *Artemia salina*, *Bacillus stratosphericus* (amet1601) nov., and Nitrifier Bacterial Consortium as Probiotics on *Litopenaeus vannamei* Culture. Journal of Fisheries Sciences 9(4): 41-48.

Lafferty KD, Harvell CD, Conrad JM, Friedman CS, Kent ML, Kuris AM, Saksida SM. 2015. Infectious diseases affect marine fisheries and aquaculture economics. Annual Review of Marine Science 7: 471-496.

Lara-Flores M. 2011. The use of probiotic in aquaculture: an overview. Journal of Microbiology 2(12): 471-478.

Lim HJ, Park JH, Jang IK. 2004. Effect of probiotics on water quality in the shrimp (*Fenneropenaeus chinensis*) ponds. Korean Journal of Fisheries and Aquatic Sciences 37(2): 91-97.

Manage PM. 2018. Heavy use of antibiotics in aquaculture; emerging human and animal health problems–a review. Sri Lanka Journal of Aquatic Sciences 23(1): 13-27.

Mahjoub M, Mirbakhsh M, Afsharnasab M, Kakoolaki S, Hosseinzadeh S. 2019. Inhibitory activity of native probiotic *Bacillus vallismortis* IS03 against pathogenic *Vibrio harveyi* under in vitro and in vivo conditions in *Litopenaeus vannamei*. Iranian Journal of Aquatic Animal Health 5(2): 54-66.

Nguyen HQ, Le TL, Tran MT, Sorgeloos P, Dierckens K, Reinertsen H, Svennevig N. 2011. Cobia *Rachycentron canadum* aquaculture in Vietnam: recent developments and prospects. Aquaculture 315(1-2): 20-25.

Rahman A, Shefat SHT, Chowdhury MA. 2021. Effects of Probiotic *Bacillus* on Growth Performance, Immune Response and Disease Resistance in Aquaculture. Journal of Aquaculture Research & Development 12(4): 634-643.

Ran C, Carrias A, Williams M, Capps N, Dan BCT, Newton JC, Kloepper JW, Ooi L, Browdy CL, Terhune JS, Liles MR. 2012. Identification of *Bacillus* strains for biological control of catfish pathogens. PLOS One 7(9): 1-9 (Article e45793).

Romanova E, Spirina E, Romanov V, Lyubomirova V, Shadyeva L. 2020. Effects of *Bacillus subtilis* and *Bacillus licheniformis* on catfish in industrial aquaculture. In E3S Web of Conferences, Vol.

175, p. 02013.

Tham CSC, Peh KK, Bhat R, Liong MT. 2012. Probiotic properties of Bifidobacteria and lactobacilli isolated from local dairy products. Annals of Microbiology 62(3): 1079-1087.

Zulkhairi AFA, Sabri S, Ismail M, Chan KW, Ismail N, Mohd Esa N, Zawawi N. 2020. Probiotic properties of Bacillus strains isolated from stingless bee (*Heterotrigona itama*) honey collected across Malaysia. International Journal of Environmental Research and Public Health 17(1): 278-280.