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ARAŞTIRMA MAKALESİ **RESEARCH ARTICLE**

A Comparative Study of *Bacillus* Spp. Isolated from Various Sources and Commercial Food Supplements and Evaluation of Some Probiotic Properties

Çeşitli Kaynaklardan ve Ticari Gıda Takviyelerinden İzole Edilen *Bacillus* Türlerinin Bazı Probiyotik Özelliklerinin Karşılaştırmalı Değerlendirilmesi

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Abstract

Bacillus species are gram-positive, aerobic, peritrically flagellated and endospore-forming bacteria. They can be found everywhere in the environment, especially in soil (its common habitat), water, dust or in the air. Probiotics, which have beneficial health effects, constitute an important group of *Bacillus* species. This study aimed to isolate *Bacillus* from various sources, identify it molecularly and determine its probiotic properties.For this purpose, eight *Bacillus subtilis*, *Bacillus coagulans* and *Bacillus clausii* strains among 58 isolates from fish intestine, soil, ripened cheese and commercial probiotic supplements were identified and their probiotic properties were characterized. Firstly, *Bacillus* strains were molecularly identified by 16S rRNA PCR analysis. The growth of *Bacillus* isolates at various temperatures, salt concentrations, and pH levels, as well as tests for esculin hydrolysis, starch hydrolysis, nitrate reduction, and gas generation from glucose, were all investigated to assess the isolates' physiological and biochemical characteristics. In terms of probiotic potential of *Bacillus* isolates; tolerance of bile salt, cell surface hydrophobicity, auto aggregation, antibiotic susceptibility tests were conducted. In all analyses, strains obtained from food supplements showed high levels of hydrophobicity and auto-aggregation properties, and the highest values following these strains were observed in *Bacillus* subtilis strains (F1 and S2) isolated from fish intestines and soil, respectively. All strains showed strong growth features in bile salt conditions. It has been determined that antibiotic sensitivity varies depending on the strain. Overall, high sensitivity to tetracycline has been observed. In summary, this study revealed the potential probiotic properties of *Bacillus* isolates obtained from different sources. The study also compared these probiotic properties with probiotic *Bacillus* strains isolated from food supplements.

Keywords: *Bacillus*, Biochemical characterization, Molecular identification, Probiotics

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Öz

Bacillus türleri gram pozitif, aerobik, peritrik olarak kamçılı ve endospor oluşturan bakterilerdir. Çevrede her yerde; özellikle toprak (yaygın habitatı), su, toz veya havada bulunabilirler. *Bacillus* türleri içerisinde önemli bir grubu ise sağlığa faydalı etki gösteren probiyotikler oluşturmaktadır. Probiyotik *Bacillus*'ları içeren gıdalar ve yemler, genellikle; insanlar için besin takviyesi, hayvanlar için büyümeyi teşvik edici, su ürünleri için ise büyüme düzenleyici veya hastalıklara karşı direnç sağlayıcı olarak kullanılmaktadır. Bu çalışmada, çeşitli kaynaklardan probiyotik *Bacillus* izolasyonu, moleküler tanımlanması ve probiyotik özelliklerinin belirlenmesi amaçlanmıştır. Bu amaçla, balık bağırsağından, topraktan, olgunlaştırılmış peynirden ve ticari probiyotik gıda katkılarından elde edilen 58 *Bacillus* izolatından 8 adet *Bacillus subtilis*, *Bacillus coagulans* ve *Bacillus clausii* suşu tanımlanmış ve probiyotik özellikleri karakterize edilmiştir. İlk olarak, *Bacillus* suşları 16S rRNA PCR analizi ile moleküler olarak tanımlanmıştır. *Bacillus* cinsine ait izolatların fizyolojik ve biyokimyasal özelliklerini belirlemek için farklı sıcaklıklarda, tuz konsantrasyonlarında ve pH derecelerinde büyümeleri incelenmiş, ardından eskülin hidrolizi, nişasta hidrolizi, nitrat redüksiyonu, glikozdan gaz oluşumu testleri yapılmıştır. *Bacillus* izolatlarının probiyotik potansiyelinin değerlendirilmesi açısından; safra tuzu toleransı, hücre yüzeyi hidrofobikliği, oto-agregasyon, antibiyotik duyarlılık testleri yapılmıştır. Tüm analizlerde, gıda takviyelerinden elde edilen suşlar yüksek düzeyde hidrofobiklik ve oto-agregasyon özellikleri göstermiştir ve bu suşları takip eden en yüksek değerler sırasıyla balık bağırsağından ve topraktan izole edilen *Bacillus subtilis* suşlarında (F1 ve S2) gözlemlenmiştir. Tüm suşlar, safra tuzu koşullarında güçlü gelişme özellikleri göstermiştir. *Bacillus* suşlarının antibiyotik duyarlılığını suşa özgü özellikler belirlemiştir. tetrasikline karşı yüksek düzeyde duyarlılık gözlenmiştir. Özetle, bu çalışma çeşitli kaynaklardan izole edilen *Bacillus* suşlarının potansiyel probiyotik özelliklerini ortaya koymuş ve bu probiyotik özellikler gıda takviyelerinden izole edilen *Bacillus* suşları ile karşılaştırılmıştır.

Anahtar Kelimeler: *Bacillus*, Biyokimyasal karakterizasyon, Moleküler tanımlama, Probiyotikler

1. Introduction

The genus *Bacillus* consists of aerobic, spore-forming, gram positive bacteria that shows heterotrophic or autotrophic growth using a variety of carbon sources. *Bacillus* genus includes around 200 species, some of them have been classified based on new biological data. (Logan and De Vos, 2009). *Bacillus* species are generally considered soil organisms as they have spores that can be isolated from soil. However, it has been shown that *Bacillus* species are not only soil-based and can sustain their viability in many environments through their spores. It has been suggested that *Bacillus* may be an undiscovered gastrointestinal system (GIS) commensal since these bacteria can maintain their viability in the GIS of animals that ingest *Bacillus* spores through digestion (Hong et al., 2009). Because of their spores' outstanding resistance and dormancy, which allows them to survive in any ecosystem longer than vegetative organisms, *Bacillus* species are widely distributed in a variety of habitats. *Bacillus* Probiotics, a significant category of *Bacillus* species that have beneficial effects on health (Sui et al., 2020). Foods and feeds containing probiotic *Bacillus* are generally used as nutritional supplements for humans, growth promoters for animals, and growth regulators or disease resistance providers for aquaculture (Cutting, 2011). Thanks to the antagonistic effects of *Bacillus* species, its use as a biological seed has also been proposed (Güldoğan et al., 2022) It has also been determined that due to the potent antagonistic activities of some *Bacillus* spp. isolates, citrus fruits have the potential to be used as biofungicides in the fight against post-harvest disease agents (Soylu et al., 2022) It has been stated that *Bacillus* probiotics are suitable for human consumption (Urdaci et al., 2004; Nithya and Halami, 2013). Most commercially available probiotic products consist of various microorganisms, especially *Lactobacillus* sp., *Bifidobacterium* sp., *Streptococcus* sp. However, the biggest weakness that makes it difficult to use these species as probiotics in the food industry is their susceptibility to harsh environments. Probiotic bacteria such as *Lactobacillus* and *Bifidobacteria* are highly sensitive to normal physiological conditions such as the highly acidic environment of the stomach and bile salts, and their survival rate under such conditions is 20-40% (Bezkorovainy, 2001). In addition, the viability of these bacteria can be affected by the production method, transportation, and storage conditions (Ljungh and Wadström, 2006; Endres et al., 2009;). In this context, spores formed by *Bacillus* species have an important effect on their evaluation as probiotics, thanks to their durability in difficult conditions. Their survivability in the digestive system and their thermal stability makes *Bacillus* probiotics attractive and their use is increasing (Cutting, 2011).

Although there is a lot of literature on the identification and examination of the properties of commonly used probiotics such as *Lactobacillus* and *Bifidobacteria*, there is a lack of isolation and investigation of the properties of *Bacillus* probiotics. In addition, even though studies on *Bacillus* probiotics have increased in the last 25 years, they have not gained high popularity compared to *Lactobacillus* species.

This study aims to identify *Bacillus* species obtained from different sources by molecular identification test (PCR) and to determine their biochemical, probiotic and technological properties. For the probiotic potential of *Bacillus* isolates, it is aimed to conduct in-vitro tests like tolerance of bile salt, cell-surface hydrophobicity test, antibiotic sensitivity test, hemolytic activity and lecitinase activity determination. In addition, the fact that the preparations currently sold in the trade and the *Bacillus* bacteria isolated from probiotic foods were also examined in this study makes the study different and interesting.

2. Materials and Methods

2.1. Isolation of Bacillus strains and molecular identification

2.1.1. Sample collection

Soil samples were collected from Istanbul and Edirne, Turkey in 2020. Following the cleaning of top surface of the soil, eleven soil samples from different locations were taken from approximately 4-5 cm depth with a sterile spatula and placed in sterile plastic bags. For the fish samples, nine fresh fish samples, including sea bream, salmon and sea bass, were purchased from local market, placed in sterile plastic bags and kept at + 4°C until the deriving of the intestinal samples. In addition, two ripened Mihaliç cheese samples were also obtained for *Bacillus* isolation.

Three different probiotic supplements which claiming to contain *B. coagulans*, *B. clausii* and *B. subtilis*, sold commercially in Turkey and the USA were purchased. Two out of the three products were gummy samples, and one was a suspension. Commercial samples were also subjected to isolation and identification process similar to the soil and food samples.

2.1.2. Isolation of Bacillus strains from distinct samples

Each sample was initially diluted with peptone-water in 1:10 ratio before thoroughly mixing by the stomacher (VWR Star blender LB 400, England). Diluted suspensions in peptone water were heated at 80 °C for 20 minutes to kill vegetative cells. Isolation was carried out by streaking heat treated cultures on TSA (tryptic soy agar) for aerobic spore-formers (Ghosh et al., 2002, Gatson et al., 2006). Then the samples were subjected to serial dilution (up to 10^{-7}), and the 0.1 ml aliquots were aseptically plated on the TSA. The Petrie dishes were incubated at 37°C for 24 and 48 hours. Randomly selected colonies of various morphologies were purified and kept at -80 °C in TSB (tryptic soy broth), which contains 40% glycerol. Pure cultures were subjected to Gram staining to select Gram-positive rod-shaped bacteria. Subsequently, catalase test and spore staining tests were performed in these isolates to represent possible *Bacillus* isolates. Determination of catalase activity was conducted by resuspending the culture in a 3% hydrogen peroxide solution.

2.1.3. Bacterial identification

The isolates were grown in TSB under gentle agitation at 37 °C for 24 hours. The EcoSpin Bacterial Genomic DNA Kit (EcoTech, Turkey) was used to extract DNA. First, with the help of primers specific to *Bacillus* species, Bsub 5F (5'-AAGTCGAGCGGACAGATGG-3') and Bsub3R (5' -CCAGTTTCCAATGACCCTCCCC-3') (Mohd Isa et al., 2020), it was determined whether the isolates were *Bacillus* species or not.

To select distinct strains, randomly amplified polymorphic DNA (RAPD) test was implemented with the GTG 5 primer (5'-GTGGTGGTGGTGGTG-3') (Freitas et al., 2008). The following PCR parameters were used: 30 cycles of 94 °C for 1 minute, 40 °C for 1 minute, and 72 °C for 2 minutes, followed by one cycle of 72 °C for 8 minutes. Fingerprints of the isolates were recorded under UV light after running in agarose gel and similar strains were detected. One representative sample from each similar group formed was used for sequence analysis.

Potential distinct colonies were then subjected to identification process. For this, AMP_F (5'-GAGAGTTTGATYCTGGCTCAG-3') and AMP_R (5'-AAGGAGGTGATCCARCCGCA-3') primers were used the amplify to 16S ribosomal RNA (rRNA) section (Baker et al., 2003). PCR mixtures prepared with 1 µL of DNA template, 5 µL of 5× PCR buffer, 4 µL of dNTPs, 1 µL of 20 mmol/L primers, 0.125 µL of Taq polymerase and up to 50 µL of sterile H_2O. For the amplification of DNA, PCR (Bio-Rad T100 Thermal Cycler, USA) was used with the conditions of: Denaturation at 95 °C for 2 minutes is followed by 20 cycles of 95 °C for 30 seconds, 53 °C for 1 minute, and 72 °C for 30 seconds, with a final extension step at 72 °C for 5 minutes. The PCR products obtained were run on a 1% agarose gel and visualized with a gel imaging system.

Sequention of the samples were conducted at the Medsantek genomics sequencing laboratory. (Medsantek, Turkey). Using the Basic Local Alignment Search Tool (BLAST), the acquired nucleotide sequences were compared to the sequences of *Bacillus* species that were included in the National Center for Biotechnology Information (NCBI) database (Altschul et al., 1990).

2.1.4. Determination of physiological and biochemical properties

The degree of growth of *Bacillus* isolates at various pH (5, 6, 7, 8, and 10) temperature, (5, 30, 40, 65 °C) and salt concentrations (2, 5, 7, 10%) was investigated. In addition, various biochemical tests such as starch hydrolysis, nitrate reduction, esculin hydrolysis, formation of gas from glucose, acid formation from various carbohydrates were performed (Smith, 1981).

2.2. Probiotic properties of Bacillus strains

2.2.1. Growth at different concentrations of bile salt

Growth at changing bile salt concentrations of *Bacillus* isolates analyzed according to the method by Nithya and Halami (2013) with some modifications. The growth of *Bacillus* isolates was tested at different bile salt (0.0, 0.3, 0.5, 1 1.0, and 2.0) concentrations. The samples incubated for 24 hours at 37°C and then the growth of the test cultures was examined by determining the optical density (OD) with using a spectrophotometer (Optizen Pop Bio Uv/Vis Spectrophotometer, Korea).

2.2.2. Cell surface hydrophobicity

The cell surface hydrophobicity of *Bacillus* strains was determined according to the method described previously by İspirli et al. (2015) with some modifications. The cultures grown overnight were obtained by centrifugation (at 3000 and 4 °C for 10 minutes) and suspended again in PBS tampon to observe an OD600 of 1.0. The bacterial cell suspension (3 mL) and 0.6 mL of chloroform were then mixed. This mixture was vortexed for 1 minute and afterwards it is kept undisturbed for 30 minutes to allow separation of phases completely. Then the aqueous phase was separated and OD at 590nm was carefully measured. The percentage of hydrophobicity was calculated according to the following equation:

$$Hydrophobicity\ (\%) = (1 - A_1/A_0) \times 100 \qquad \text{(Eq.1).}$$

In this equation A_0 refers to the initial absorbance of the bacterial suspension while A_1 is the absorbance which is measured after 30 minutes of incubation.

2.2.3. Autoaggregation assay

The autoaggregation of *Bacillus* isolates was calculated using to method of Patel et al. (2009). *Bacillus* cultures which were grown at 37 °C for 24 hours in nutrient broth was centrifugated, washed, and resuspended in PBS to get absorbance 0.5 at 595 nm. The 4 ml of cell suspension was mixed by vortex and incubated at 37 °C for 1 h. After the incubation, the upper layer was measured at 595 nm. Then finally autoagregation was calculated as:

$$Autoaggregation\ (\%) = (1 - A_t/A_0) \times 100 \qquad \text{(Eq.2).}$$

In this equation A_t represents the absorbance after incubation and A_o the initial absorbance.

2.2.4. Antibiotic susceptibility assay

Antibiotic susceptibility of *Bacillus* isolates determined with disc diffusion method according to the the National Committee for Clinical Laboratory Standards (NCCLS 1997). Resistance of *Bacillus* strains against tetracycline (30 µg), vancomycin (30 µg), rifampicin (30 µg), amoxicillin (10 µg), penicillin G (10 µg), was determined using antibiotic discs. When inhibition zones present around the disks, the length of the disks were measured in centimeters (Chaiyawan et al., 2010).

2.2.5. Lecithinase and hemolytic activity test

For lecithinase test, bacteria are streaked on a medium prepared with egg yolk and incubated at 37 °C for 24-48 hours. The results were evaluated for the formation of a white opaque zone around the colonies (McClung and Toabe, 1947). Hemolysis was determined on Blood agar base (Liofilchem, Italy) supplemented with 5% sheep blood after incubation at 37ºC for 24 hrs α-haemolysis, β-haemolysis or non-haemolytic properties were determined with the examination of the plates (Chaiyawan et al., 2010).

2.2.6. Statistical analysis

Statistical analysis was implemented by one-way analysis of variance (ANOVA). Tukey's multiple comparison test is conducted using JMP 6.0. The values were given with means ± standard deviations. The level of significance was selected to be 0.05.

3. Results and Discussion

3.1. Isolation and identification of distinct strains

3.1.1. Isolation of spore-forming bacteria

In the study, 58 different isolates were selected from colonies belonging to fish, soil and cheese samples. Among these isolates, 35 catalase-positive, gram-positive, rod-shaped and spore-forming strains were evaluated as possible *Bacillus* and selected for further studies.

3.1.2. Identification of Bacillus by 16S rRNA sequence analysis

Following the RAPD PCR analysis, 16S rRNA gene analysis was used to identify different strains. The isolates have 99% sequence similarity with *Bacillus* species according to the 16S rRNA sequencing analysis. Using the

Basic Local Alignment Search Tool (BLAST) Program, the acquired sequences (about 1,500 bp) were deposited in the National Center for Biotechnology Information (NCBI) gene bank and accession numbers are given *Table 1*. The commercial probiotics isolated in this study were *Bacillus subtilis* (PB1), *Bacillus coagulans* (PB2) and *Bacillus clausii* (PB3).

Table 1. Bacillus strains isolated and identified in this study and their sources

***Bacillus* isolates**	**Sources**	**Gene bank accession number**
F1- *Bacillus subtilis*	Fish intestine	OM807211
F2- *Bacillus subtilis*	Fish intestine	OM807212
S1- *Bacillus subtilis*	Soil	OM807213
S2- *Bacillus subtilis*	Soil	OM807214
C1- *Bacillus coagulans*	Kashar cheese	OM867479

3.1.3. Physiological and biochemical properties

Biochemical and physiological properties of isolated *Bacillus* species were given in *Table 2*. Physiological tests were performed to observe temperature, NaCl and pHs effect on *Bacillus* isolates. The findings showed that the *Bacillus* isolates could grow easily in alkaline, salt-containing environments. The best growth was observed between pH 6 and 8. *Bacillus* strains have increasing growth rates from pH 1 to 7 and have promising tolerance with their survival at different acidic-basic degrees (Kavitha et al., 2018). In addition, growth at 10% NaCl concentration exhibited that these species could resist high salt concentrations (Satapute et al., 2012). The best

Table 2. Biochemical and physiological characterization of isolated Bacillus species

Biochemical characteristics	***Bacillus* isolates**							
	P1	**P2**	**P3**	**F1**	**F2**	**S1**	**S2**	**C1**
Gram staining	+	+	+	+	+	+	+	+
Motility	+	+	+	+	+	+	+	+
Ellipsoidal	+	+	+	+	+	+	+	+
Spore formation	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+
Nitrate reduction	+	-	+	+	+	+	+	+
Egg yolk reaction	-	-	-	-	+	-	+	-
Hydrolysis of starch	+	+	+	+	+	+	+	+
Esculin hydrolysis	+	+	+	+	+	+	+	+
Gas production from glucose	-	-	-	-	-	-	-	-
Growth at pH 5	+	+	+	+	+	+	+	+
6	+	+	+	+	+	+	+	+
7	+	+	+	+	+	+	+	+
8	+	+	+	+	+	+	+	+
10	+	+	+	+	+	+	+	+
Growth in NaCl: 2%	+	+	+	+	+	+	+	+
5%	+	+	+	+	+	+	+	+
7%	+	+	+	+	+	+	+	+
10%	+	-	+	+	+	+	+	-
Growth at 5 °C	-	-	-	-	-	-	-	-
30 °C	+	+	+	+	+	+	+	+
40 °C	+	+	+	+	+	+	+	+
65 °C	-	-	-	-	-	-	-	-

temperature values of *Bacillus* isolate to grow have been determined as 30 °C – 40 °C. Studies have shown that the growth ranges of *Bacillus* species vary from mesophilic to moderately thermophilic (54 °C). (Łubkowska et al., 2023) It was also understood that the isolates did not produce gas from glucose. Total number of bacterial strains could hydrolyze esculin and starch and utilize catalase. Moreover *B. subtilis* and *B.clausii* isolates were able to reduce nitrate, *B. coagulans* isolates were varied according to the subspecies for the reduction of nitrate to nitrite.

3.2. Characterization of probiotic properties of Bacillus strains

3.2.1. Growth at different bile concentrations

The survivability and growth of the strains in the high concentration of bile salts is an important parameter for probiotic selection. Resistance to bile compounds is one of the most widely used assays in survival and growth studies of probiotic organisms reported by FAO/WHO (FAO/WHO, 2002). Bile tolerance tests are usually performed at a concentration of 0.3%, as it is like human bile juice (Conway et al., 1987; Nithya and Halami, 2013). Probiotic strains must be resistant to bile to function in the intestines (Sharma et al., 2023). In this study, growth of *Bacillus* strains was observed in bile salt concentrations of 0.3%, 0.5%, 1% and 2%. According to the results, all the tested *Bacillus* strains were grown with changing concentrations of bile salts (*Figure 1*). It was observed that strains performed well in especially 0.3% and 0.5% bile concentrations. Similar studies also reported that *Bacillus* isolates showed high survival rates even in the presence of 6% and 10% bile (Giri et al., 2012, Thankappan et al., 2015). The PB1 strain showed good resistance to increased bile salt, while the C1 strain was found to be more sensitive. It is noteworthy that *Bacillus subtilis* species were more resistant to bile salt than *Bacillus coagulans* and *Bacillus clausii* species.

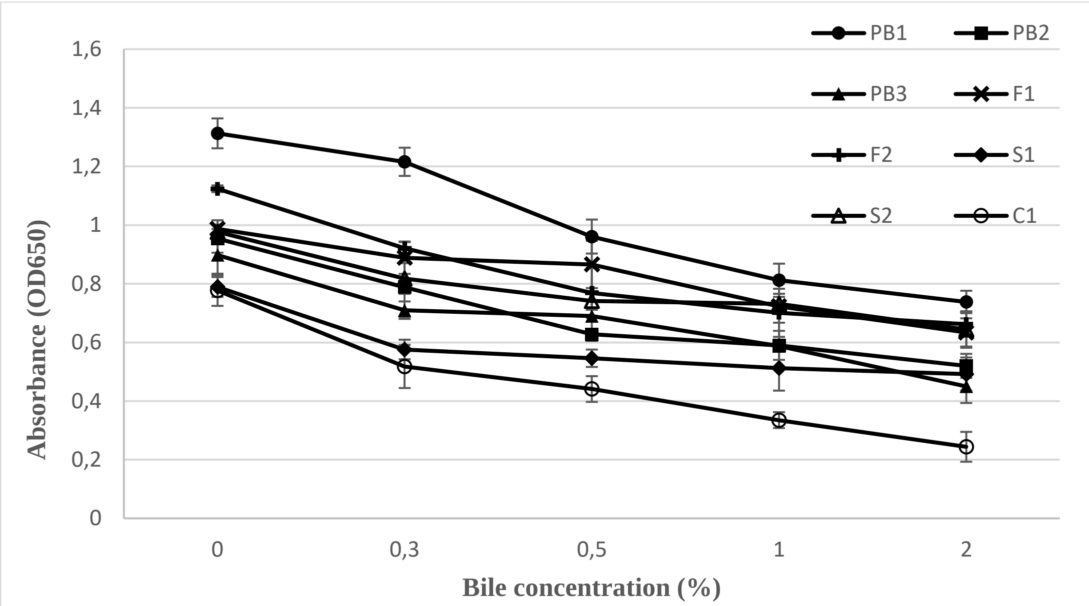

Figure 1. Growth of Bacillus isolates at different concentration of bile salt

3.2.2. Cell surface

It is very important that probiotic strains adhere to epithelial cells of the intestine to colonize in the gastrointestinal system, which prevent elimination by peristalsis, and provide an advantage for competition in microflora (Kos et al., 2003). The chloroform adhesion capacity was used to determine the hydrophobicity of *Bacillus* strains. (*Figure 2a)*. The adhesion percentage of *Bacillus* strains showed differences between 51.18% and 25.7% among the strains tested. The strains PB1 and PB3 from commercial probiotic products showed the highest hydrophobicity capacity. *Bacillus subtilis* F1 strain, which is the fish isolate, gave the closest value to these with 44.6%. The lowest hydrophobicity capacity was observed for fish isolate F2 which was nearly half that of the highest degrees of hydrophobicity tested. In general, significant statistical differences were observed between the degree of hydrophobicity of the isolates. Previous studies evaluating the hydrophobicity of *B. coagulans*, *B. licheniformis*, *B. flexus*, *B. subtilis* isolates used xylene as the hydrocarbon and reported adhesion values ranging from 30% to 90%. (Nithya and Halami, 2013). In another analysis performed using toluene, the cell surface hydrophobicity value varied between 73.62 and 95.3% among the *Bacillus* strains tested (Dabire et al., 2022). Solvents such as xylene and ethyl acetate have also been used in other studies, resulting in different degrees of hydrophobicity of *Bacillus* strains (Patel et al., 2009; Nithya and Halami, 2013). In this study all isolates exhibited remarkable affinity to chloroform, a monopolar acidic solvent and electron acceptor. Since fish and soil and cheese isolates showed different adhesion degrees among themselves, no generalized results were obtained that would allow a comprehensive comparison in terms of isolates source.

3.2.3. Auto-aggregation assay

Auto-aggregation is a crucial functional trait of probiotic strains, along with hydrophobicity. It was stated that the isolates' surface characteristics, such as auto-aggregation and hydrophobicity, contributed to the adhesion property. The surface properties such as auto-aggregation and hydrophobicity exhibited by the isolates contribute to the adhesion property (Kos et al., 2003). In general auto-aggregation ability is related to cell adhesion properties and also provide their ability to survive and endurance in the digestive system. (Vlková et al., 2008). The auto-aggregation activity of the isolates varied from 28.4 to 35.4% (*Figure 2b*). *Bacillus subtilis* PB2 which is the commercial probiotic isolate showed the highest auto-aggregation activity. Similar to cell surface hydrophobicity,

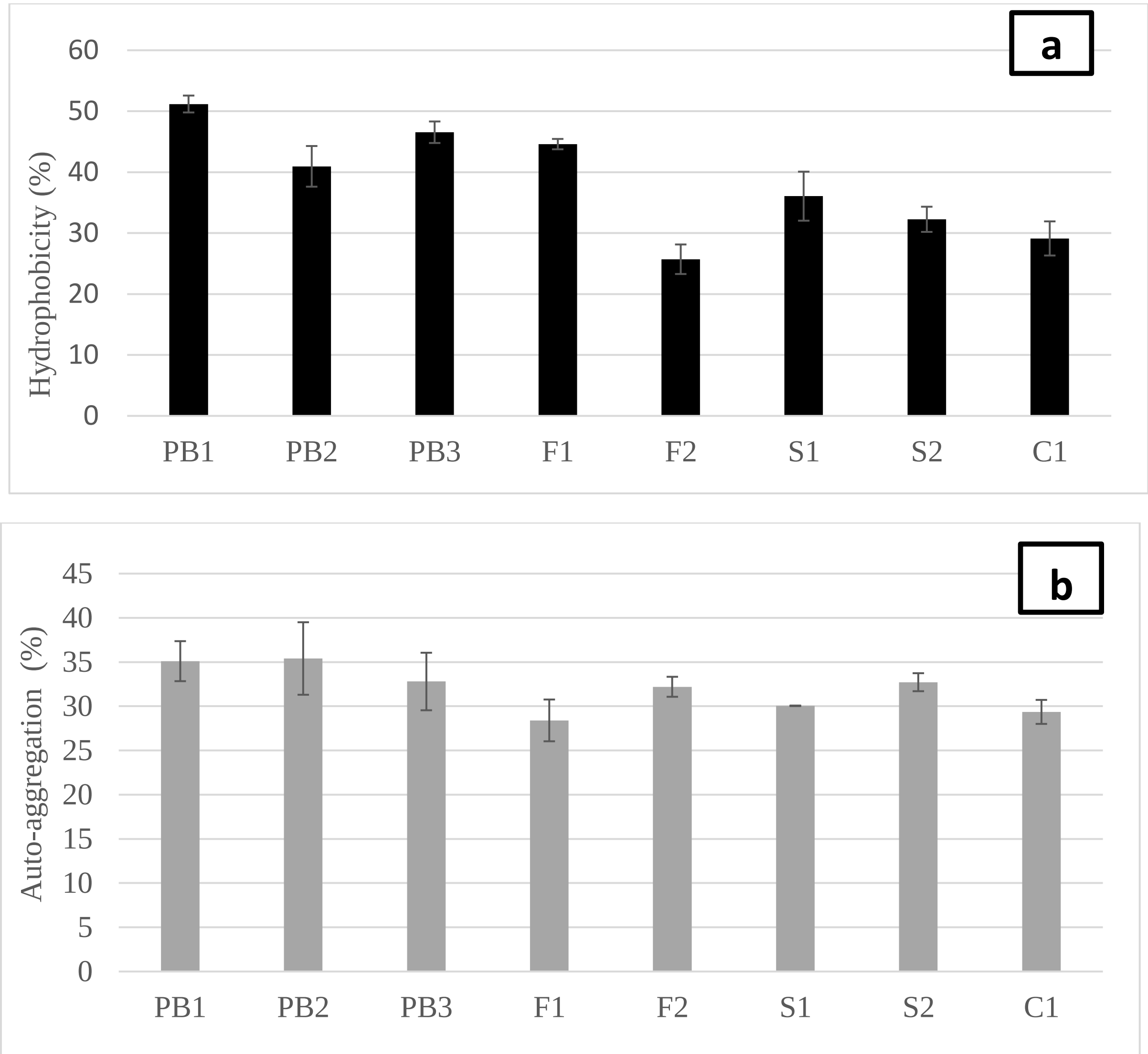

Figure 2. Cell surface hydrophobicity (a) and auto-aggregation (b) properties of the Bacillus isolates

commercial probiotic strains showed higher auto-aggregation activity. However, no statistically significant difference was observed between the auto-aggregation values of other isolated *Bacillus* strains. In this study, we report that *Bacillus* strains isolated from fish (F2) intestine and soil (S2) showed good auto-aggregation percentage with 32.2% and 32.7% levels, respectively. Previous studies have reported a wide range of auto-aggregation values for *Bacillus* species ranged between 20% to 98%. (Nithya and Halami, 2013; Nwagu, et al., 2020; Dabire et al., 2022). As stated in other studies, these properties can provide colonization of *Bacillus* in the gastrointestinal tract and competition against pathogens (Thankappan et al., 2015).

3.2.4. Antibiotic susceptibility assay

The safety of probiotics is of primary importance, as their resistance to antibiotics can be one of the possible threats. The presence of transferable antibiotic resistance genes generates a safety hazard (Sharma et al., 2014). *Table 3* displays the outcomes of the bacterial strains' tests for antibiotic sensitivity. All the eight isolates were susceptible (>1cm of zone of inhibition) to all tested antibiotics which are tetracycline, vancomycin, rifampicin, amoxicillin, penicillin G in various degrees. Patel et al. (2009) stated in their study that vulnerability against antibiotics is an important probiotic feature. The *Bacillus* isolates examined in the study do not show antibiotic resistance, which is an important finding in terms of inability to transfer the plasmid gene that triggers pathogenicity and enterotoxin formation.

Almost all isolates were more sensitive (S+++) to tetracycline and sensitive (S++) to Vancomycin. In addition, the isolates exhibited different susceptibility degree to rifampicin, penicillin G and amoxicillin antibiotics according to the bacterial type. *Bacillus* strains were sensitive to antibiotics which indicates that these isolates evaluated as probiotics are safe. These findings are consistent with previous research (Zeng et al., 2022; Lei et al., 2023). Previous studies examined different probiotic products that were commercially available and found that some of them contained a different strain of *Bacillus* than indicated, and that the bacteria showed high levels of resistance to antibiotics such as penicillin G, tetracycline, rifampin and ampicillin (Green et al., 1999; Hoa et al., 2000; Senesi et al., 2001).

Table 3. Diameters of inhibition zone (cm) exhibited against test bacteria of standard antibiotics

Isolates	Vancomycin (30 µg)	Rifampicin (30µg)	Penicillin G (10 µg)	Amoxicillin (10 µg)	Tetracycline (30 µg)
PB1	1.85±0.07^{bc}	1.40±0.00^{f}	1.50±0.00^{e}	1.55±0.07^{c}	2.55±0.07^{bc}
PB2	1.85±0.21^{bc}	1.55±0.07^{e}	1.55±0.07^{de}	1.55±0.07^{c}	2.65±0.07^{b}
PB3	1.75±0.07^{c}	2.50±0.00^{cd}	1.75±0.35^{cde}	2.25±0.35^{b}	2.90±0.14^{a}
F1	2.10±0.00^{a}	2.60±0.14^{c}	2.25±0.07^{ab}	2.75±0.07^{a}	2.45±0.07^{c}
F2	2.05+0.07^{ab}	2.85±0.07^{b}	2.45±0.21^{a}	2.35±0.21^{b}	2.60±0.14^{bc}
S1	2.00±0.14^{ab}	2.45±0.07^{d}	2.05±0.07^{bc}	2.30±0.00^{b}	2.50±0.00^{bc}
S2	2.05±0.07^{ab}	3.05±0.07^{a}	1.85±0.07^{cd}	2.85±0.07^{a}	3.05±0.07^{a}
C1	1.90±0.14^{abc}	1.60±0.00^{e}	1.65±0.07^{de}	1.65±0.07^{c}	2.55±0.07^{bc}

*Where, inhibition zone diameter <0.5 cm, resistant (R); inhibition zone diameter between 0.6-1.5 cm Susceptibility (S+), inhibition zone diameter between 1.6-2.5 cm Susceptibility (S++), and inhibition zone diameter > 2.6 cm Susceptibility (S+++). **Different letters show significant ($p < 0.05$) differences between samples.

3.2.5. Lecithinase and hemolytic test

Strong hemolytic and/or lecithinase activity could be a sign that cytotoxic phopholipases are present, which affect the virality of bacteria. (Sorokulova et al., 2008). Therefore, the absence of hemolytic and lecithinase activities in these isolated bacteria is important in terms of being evaluated as probiotics. Our findings show that all *Bacillus* isolates from commercial supplements together with *Bacillus subtilis* F1 and *Bacillus coagulans* C1 were lecithinase negative which is important parameter for consideration as probiotic but *Bacillus subtilis* S1, S2, and F2 showed lecithinase activity. However, it has been stated that not all lecithinase positive strains are necessarily toxigenic (Obi, 1980). Hemolytic activities of 8 tested isolated were assessed on blood agar plates. Analysis of hemolytic capacity of tested samples demonstrated that no strains showed α hemolytic or ß hemolytic activity on sheep blood agar. Hemolytic activity is critical parameter for evaluating the biosafety of probiotics. Hemolysis and erythrocyte abnormalities can be caused by certain pathogenic bacteria that lyse red blood cells (Mondal et al., 2023). In the present study, *Bacillus* isolates not show any lysis of the blood cells. The strains showed γ hemolytic, or negative depending on strain specific conditions. Likewise, several investigators have shown that distinct *Bacillus* strains exhibit no hemolytic activity (Banerjee et al., 2017, Pahumunto et al., 2021, Zeng et al., 2021).

4. Conclusions

In this investigation, five potential probiotics from fish intestine, soil, and cheese and three probiotics from commercial food supplements were successfully isolated. Commercial food supplement isolates generally exhibited the best probiotic properties among all the isolates, as they are expected to deliver on their promises. All isolates displayed antibiotic susceptibility and exhibited good survival at 0.3% bile salt concentration. Analysis of hemolytic activity of tested strains demonstrated that isolates were non-hemolytic. Due to its strong bile salt resistance, highest hydrophobicity, and good auto-aggregation activity among the five isolates, *Bacillus* subtilis F1 demonstrated the most promise for practical application. This strain will be further tested in different food formulation especially for the development of probiotic confectionery products.

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Ethical Statement

There is no need to obtain permission from the ethics committee for this study.

Conflicts of Interest

We declare that there is no conflict of interest between us as the article authors.

Authorship Contribution Statement

Concept: Arıcı M.; Design: Kahraman B., Dertli E.; Data Collection or Processing: Kahraman B., Şenol B. M.; Statistical Analyses: Kahraman B.; Literature Search: Kahraman B., Şenol B. M.; Writing, Review and Editing: Kahraman B., Arıcı M., Dertli E.

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