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FORMULATION OF PROBIOTIC CHOCOLATE USING - Arthrobacter globiformis AND FLAXSEED

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ABSTRACT

Concerns regarding food quality have arisen recently, and research on supplementation to increase food's nutritional worth is extensive. One such significant and often utilized functional food supplement is probiotics. *Arthrobacter globiformis* is a soil microorganism that can have the ability to act as a probiotic in the human body. A probiotic chocolate is made by combining chocolate, flaxseed powder, and *Arthrobacter globiformis*. Flaxseed rich in dietary fibre, can help us feel fuller for longer. Chocolate, on the other hand, may have anti-inflammatory, antioxidant, and heart-healthy properties as well as lower bad cholesterol, lower blood pressure, and improve brain function. Current research aims to isolate and characterize probiotic bacteria investigate the acid and bile tolerance capacity and utilize them as nutritional supplements by incorporating with chocolates. Probiotic bacteria were isolated from soil using ABM (Arthrobacter medium) medium. The isolated colonies were identified using standard techniques. The isolated microorganism was identified as *Arthrobacter globiformis*. The mass production of *Arthrobacter globiformis* was done and lyophilized for further study. The analytical techniques were performed to evaluate the produced probiotic chocolate and its effectiveness towards gut health.

INTRODUCTION

'Live microorganisms which, when provided in suitable numbers, confer health advantages to the host' is the definition given by FAO/WHO (2002) for probiotics. Probiotics, on the other hand, are described as live microbial feed supplements that enhance the intestinal microbial balance of the host animal beneficially. (Fuller and Appl *et al.*,1989)

Probiotics are typically added to food, drinks, and sauces at the right times throughout the fermentation process. It can be consumed orally as a probiotic meal or as a pill. Probiotic cells must be able to survive in the food carriers and adapt to the harsh environment of the gastrointestinal tract to provide a health effect. On the other hand, it needs to remain stable throughout the gastrointestinal tract and achieve a minimum cell count of 10^{-6} CFU g/l. (Ravinder, Ashwani *et al.*, 2012). Probiotic-based microbiological therapies have the potential to balance, enhance, or study the structure, composition, and function of microbial communities throughout colonization and development. Probiotics are more widely used in the food, feed, dairy and fermentation industries as non-pharmacological methods of managing health. (Anandharaj, Sivasankari *et al.*,)

Microorganisms must meet certain functional and technological requirements as well as adequate safety standards to be recognized as probiotic strains. Probiotics need to be safe for human use and recognized at the strain level. They must benefit the host, which is why they must be non-pathogenic to humans, resistant to stomach enzymes, have a low pH, and a high bile salt content. (Guarner *et al.*, 2005). One of the most significant probiotic species, *Lactobacillus acidophilus*, is phenotypically difficult to evaluate. *Bifidobacterium* sp. Probiotics are often severe anaerobes that are challenging to grow on milk or other dietary substrates.

A possible probiotic strain is expected to have specific characteristics for it to be able to carry out its advantageous effects. At present, those who are using in vitro testing to determine are

i. Tolerance to acid and bile, which appears to be essential for oral administration
ii. Adherence to epithelial and mucosal surfaces, a crucial characteristic for effective
immune regulation, pathogen competitive exclusion, and avoidance of pathogen adherence and colonization
iii. Antimicrobial efficacy against pathogenic bacteria
iv. Bile salt hydrolase enzyme activity.

Arthrobacter globiformis is a soil bacteria that is present in nitrogen-rich soil. *Arthrobacter globiformis* is a bacterium that belongs to the genus Arthrobacter and is a part of the Actinobacteria phylum. Its basonym is Bacterium *globiformis*, which was first identified by Conn in 1928. (Conn *et al.*, 1947). In 24-hour agar slant cultures, *Arthrobacter globiformis* appears as short rods, 0.6-0.5 x 1.0-1.5 µm in size. After 3-4 days, the cultures show Gram-positive cocci, 0.8-12 µm in diameter, which occur in pairs and tetrads. In liquid media, branching forms with similar cocci and also large spherical bodies (1 to 2 µm) appear. It lacks motility and spore forming ability. (Hans- Jurgan Busse, 2012). This culture is characterized by circular, smooth, raised, and glistening colonies on nutrient agar. The colonies are entire and not pigmented, with a cream color. In nutrient broth, the culture is turbid with sediment. This organism requires a mineral salt medium with ammonium and nitrate as nitrogen source and glucose as the carbon and energy source, optionally supplemented with biotin. It can grow in 0.5% NaCl and thrives at an optimum temperature of 28°C, but cannot grow at 37°C. It grows under aerobic conditions and can tolerate a pH range of

5.0 to 9.0. (Tero SAWAI *et al.*, 1976). *Arthrobacter globiformis* has the ability to strengthen our gut and support our immune system.

Probiotic chocolate is made by combining chocolate, flaxseed powder, and *Arthrobacter globiformis*. Flaxseed rich in dietary fibre, can help us feel fuller for longer. Flaxseed contains a high concentration of lignin, soluble fiber, phenolic compounds, alpha-linolenic acid, and high-quality protein, it has the potential to be used as a functional food. Chocolate, on the other hand, may have anti-inflammatory, antioxidant, and heart-healthy properties as well as lower bad cholesterol, lower blood pressure, and improve brain function. Probiotics are currently in style as dietary supplements on the global market. The desire to consume it is associated with its classification as a natural or functional food. Due to the probiotics established therapeutic benefits, consumer awareness is growing quickly. Probiotics have been linked to several health advantages, including improved immunity, nutrient contribution, growth promotion, and antibacterial activities against harmful microorganisms. (Verschuere *et al.*, 2000)

OBJECTIVE

The objective of this project is to develop and evaluate a functional probiotic chocolate enriched with *Arthrobacter globiformis* and flaxseed powder. The study aims to characterize *Arthrobacter globiformis* by analyzing its morphological, physiological, and biochemical properties to confirm its probiotic potential, including acid and bile tolerance, epithelial adherence, antimicrobial activity, and bile salt hydrolase enzyme activity. Additionally, the project seeks to formulate a nutritionally enhanced chocolate by combining *Arthrobacter globiformis* with flaxseed powder, ensuring the survival of probiotic cells during processing and storage. The nutritional composition of the chocolate will be evaluated, focusing on dietary fiber, protein, lignin, and phenolic content, while assessing its potential antioxidant, anti-inflammatory, and gut-health-enhancing properties. Furthermore, sensory analysis will be conducted to determine consumer acceptability, along with an assessment of consumer awareness and perception of probiotic-based functional foods. Finally, the therapeutic and health benefits of the probiotic chocolate, including its impact on gut health, immune support, and antibacterial activity, will be investigated to position it as a novel and appealing product in the functional food market.

MATERIALS AND METHODS

3.1. Collection of samples (Harney *et Al.*, 2015)

The soil sample was collected from nitrogen-rich fields for the isolation of bacteria in suitable media

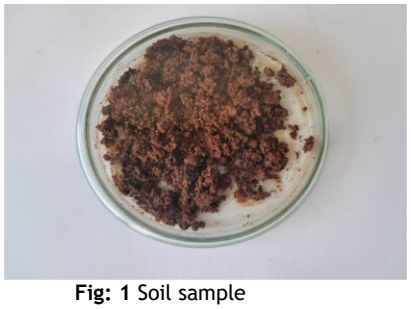

Fig: 1 Soil sample

3.2. Isolation of bacteria (Harney *et Al.*, 2015)

The samples were serially diluted (10^{-1} dilution to 10^{-8}) and was plated on ABM agar plates. The plates were kept at 37℃ for 24-48 hours.

Composition of ABM Medium:

Yeast extract	0.2g
Peptone	0.5g
Soil extract	5ml
Agar	1.5g
Distilled water	95ml
Final pH at 25℃	7

3.3 Characterization of isolates (Tero SAWAI *et al.*, 1976)

Arthrobacter globiformis isolates were characterized using microscopical and cultural characteristics on ABM medium

3.3.1 Microscopical Characterization of isolates (sunil kumar jha, 2022)

The cultures were examined under a microscope with magnification of 45X and 100X to study the morphological characteristics. The characterization was facilitated by preparing slides using Gram staining. Gram staining helps in the differentiation of bacteria based on the presence of the cell wall.

3.3.2 Gram staining procedure

The standard Gram staining protocol was followed for all isolated colonies. A bacterial smear was gently heat fixed onto a clean glass slide. Crystal violet solution was applied to the heat fixed smear for 1minute. The smear was rinsed with water and then stained with Gram's Iodine. Decolorization was achieved using 95% ethyl alcohol and the smear was then rinsed with water. The smear was counterstained with Safranin for 60-80 seconds, then rinsed with water. Cells were then examined under a microscope.

3.4. Biochemical test (sunil kumar jha, 2022)

3.4.1. Indole Test (Collins *et. al.*, 1898)

Isolated colonies were inoculated to the suspension tubes containing sterile tryptophan broth and incubated at 37℃ for 24 hrs. 0.5ml of Kovac's reagent was added after the incubation along the walls of the test tube. A bright red color ring was observed.

3.4.2. Methyl Red Test (Collins *et. al.*, 1898)

Test organisms were inoculated in MR broth and the tubes were incubated for 24 hrs at 37℃, after 24 hrs methyl red indicator was added to the tubes and a color change of the broth was observed.

3.4.3 Vogus Proskauer Test (Collins *et. al.*, 1898)

The isolated colonies were inoculated to the tubes containing sterile MR-VP broth and incubated for 24 hrs at 37℃. 0.5 ml of Barret's reagent was added to the tubes. The color change was observed.

3.4.4. Citrate Utilization Test (Collins *et. al.*, 1898)

Simmon citrate agar medium was prepared the test organism was inoculated and the tubes were incubated for 24 hrs at 37℃. After 24 hrs the colour change was observed.

3.4.5. Catalase test (Collins *et. al.*, 1898)

A loop full of isolated bacterial colonies was transferred from the agar plate to a clean glass slide. Immediately a drop of 3% hydrogen peroxide was added to the culture and rapid evolution of effervescence was observed indicating the production of catalase produced by the organism.

3.5. Screening for probiotic organism

3.5.1. Assay for acid tolerance (Gardini *et al.*, 2001).

The acid tolerance capability of the isolated microorganisms was investigated utilizing a modified protocol. The isolates were cultivated in MRS broth at 37℃ and then centrifuge at 1800 rpm for 5 mins. Cell pellets will be harvested and washed twice in sterile phosphate buffer saline (PBS) and the pellet will be diluted in a ratio of 1:100 in PBS in various pH ranges (3-6). Samples were incubated at 37℃ and viable bacterial cells were determined in various time intervals (1, 2, 3 hours) by inoculating in MRS broth. Simultaneously the bacterial growth was monitored by measuring absorbance with a spectrophotometer at 600nm.

3.5.2. Assay for bile salt tolerance (Gardini *et al.*, 2001).
All the isolates were grown in MRS agar plate with various ranges of bile salt as 0.5%, 1%, 3%, 4%, and 5% were incubated at 37℃ for 24 hrs. Viable counts of *Arthrobacter globiformis* were determined by the spread plate technique.
3.5.3. NaCl Tolerance Test (Gardini *et al.*, 2001).
NaCl tolerance test of isolated *Arthrobacter globiformis* was determined using different concentrations of NaCl and bromocresol purple as indicators. *Arthrobacter globiformis* was inoculated into MRS broth with 0.5%, 2%, 4%, and 6.5% NaCl and incubated for 7 days. The change in color from violet to yellow, representing acidification of the media, confirmed that the strains were able to survive at the designated salt concentration
3.5.4. Antimicrobial activity (Gardini *et al.*, 2001).
The inhibitory effect of *Arthrobacter globiformis* was determined by the well-diffusion method. In the agar well diffusion assay, an overnight culture of indicator strains (*Staphylococcus aureus, Escherichia coli, Pseudomonas* sp.) was swabbed into Muller Hinton Agar (MHA) and the extracellular and intracellular were added into the wells and incubated for 24 hrs.
Wells of 4mm diameter were cut into agar plates and 20ul of *Arthrobacter globiformis* culture supernatant fluid that probably contained antibacterial activity was added to each well and incubated at 37℃for 24 hrs the zone of inhibition was noted.
Composition of MHA:

Beef extract	2g
Acid Hydrolysate of casein	17.5g
Starch	1.5g
Agar	17g
Distilled water	1000ml

3.6. Flaxseed powder
Flaxseed was dry roasted by tossing them constantly over medium-high heat until they started to crackle and until they reached puffy. After cooling, grind them into a fine powder using a dry grinder.
3.7. Probiotic Chocolate (Nagamani Kathiresan *et al.*, 2021)
The *Arthrobacter globiformis* was dried. Both flaxseed powder and dried powder of *Arthrobacter globiformis* were added to the chocolate they were mixed thoroughly and cooled in the refrigerator, and after a few hours, the probiotic chocolate was ready.
3.8. Nutritional study (Kharat and Deshpande, 2017.)
The nutritional value of the prepared chocolate was studied after the preparation of Probiotic Chocolate. The major components that have to be present are Carbohydrates, Phenolic compounds, Fiber, Fat have to be present in the prepared chocolate.
RESULT
4.1. Mass dilution of the soil sample
The soil sample was collected from nitrogen-rich soil and serially diluted in ABM medium for 24 hours at 37℃ were represented in Fig 2.

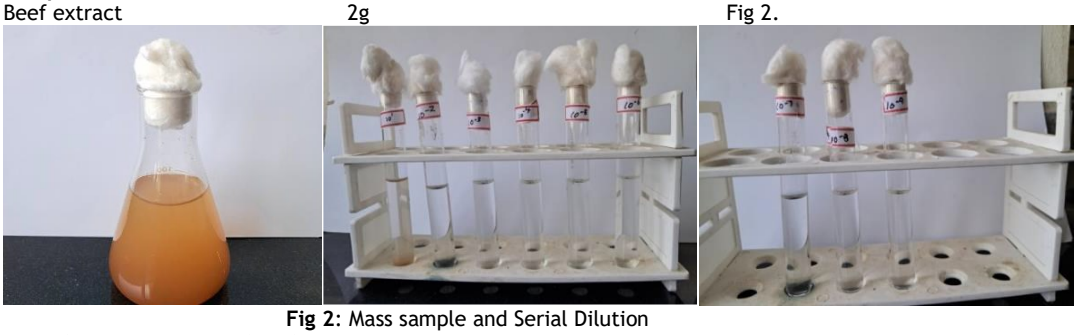

Fig 2: Mass sample and Serial Dilution
4.2. Identification of isolated organism
Colony morphology:
The colony morphology of isolated bacteria was Circular and smooth, and white cream color colonies were observed in ABM media.
4.3. Preliminary test
4.3.1 Gram staining
Gram staining was performed to study the morphological characteristics and the result was Gram-positive cocci as shown in Fig: 3

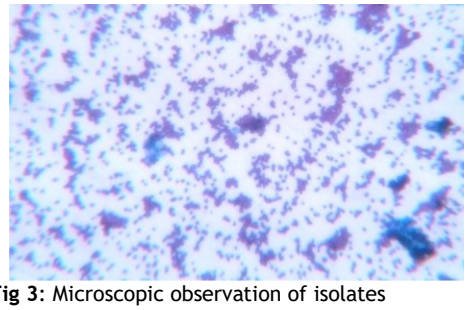

Fig 3: Microscopic observation of isolates
4.3.2 IMViC test and Catalase test results
The results of the IMViC test for isolated *Arthrobacter globiformis* bacteria as shown in Table: 1

Table: 1 IMViC test result

Organism	Indole test	Methyl red test	Voges Proskauer test	Citrate test	Catalase test
Arthrobacter globiformis	-	-	-	-	+

According to Bergey's manual, Determinative Bacteriology, and morphological & biochemical assays showed that the isolated organism is confirmed as *Arthrobacter globiformis.*
4.4. Preparation of Pure culture
The pure culture of *Arthrobacter globiformis* was obtained from a serially diluted plate as shown in Fig: 4. The plates showed white creamy colonies which proved that the isolates were *Arthrobacter globiformis.*

Fig 4: Pure culture

4.5 Screening of probiotic organisms

4.5.1. Assay for acid tolerance

An examination was carried out on the isolated organism *Arthrobacter globiformis* to determine its cell viability and optical density pH range after treatment for 1, 2, and 3 hours at pH levels of 3, 4, and 5. The results, as shown in **Fig:** 5.

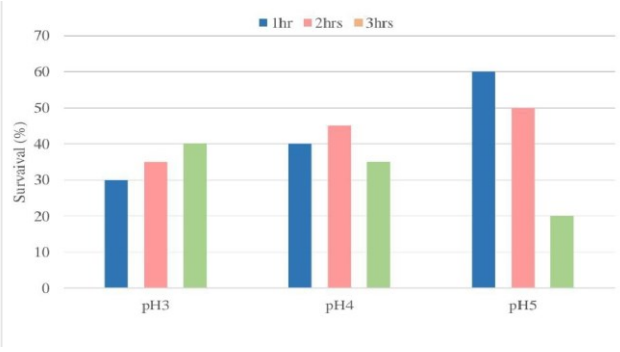

Fig: 5 *Arthrobacter globiformis* pH tolerance levels and its survival %

4.5.2. Assay for bile tolerance

The study showed that the isolated organism was able to withstand lower concentrations of bile salts, but as the concentration of bile salts increased, it was unable to survive. *Arthrobacter globiformis* could tolerate concentrations of 0.5%, 1%, and 3% of bile salts as shown in Fig: 6.

Fig: 6 Assay of Bile tolerance

4.5.3. NaCl tolerance test

The studied organism indicated tolerance to salts at lower concentrations but could not survive as the salt concentration increased. *Arthrobacter globiformis* could tolerate and grow at salt concentrations of 0.5% and 2% but was unable to grow at 4% and 6.5% salt concentrations as shown in Fig: 7.

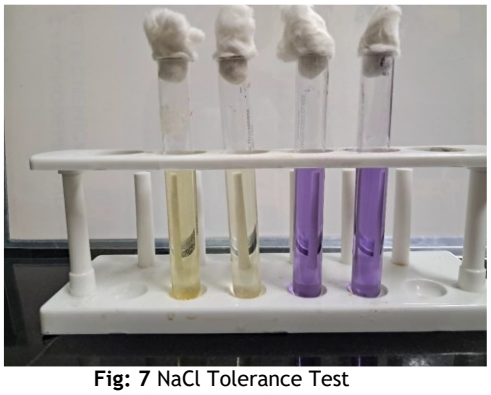

Fig: 7 NaCl Tolerance Test

4.5.4. Antimicrobial Activity (Well diffusion method)

The result of the antimicrobial test for *Arthrobacter globiformis* can inhibit *Klebsiella* and *E. coli* so the zone of incubation was formed about 4mm and 3mm. But it cannot inhibit the growth of *S. aureus* & *Pseudomonas* sp so no zone is formed as shown in table: 2

Table: 2 Antimicrobial Activity of *Arthrobacter globiformis*

Strain	*S.aureus*	*Pseudomonas* sp	*Klebsiella* sp	*E.coli*
Isolated organism	-	-	4mm	3mm

4.6. Mass production of *Arthrobacter globiformis*

Mass Production of isolated organisms was prepared using a starter culture as shown in Fig: 8 and Fig: 9.

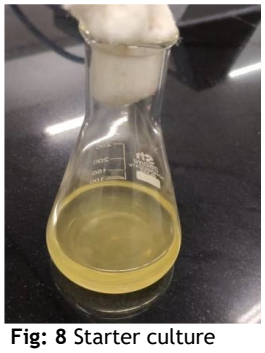

Fig: 8 Starter culture

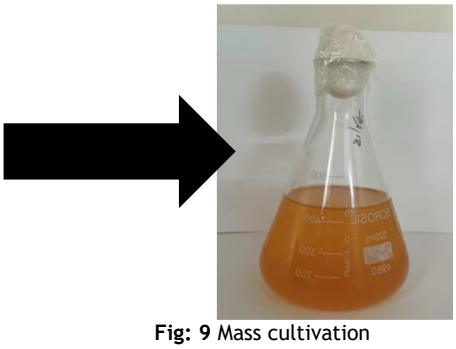

Fig: 9 Mass cultivation

4.7. Lyophilization of bacterial culture

Lyophilization of mass culture was done to obtain *Arthrobacter globiformis* in powder form to be incorporated in dark chocolate as shown in Fig: 10.

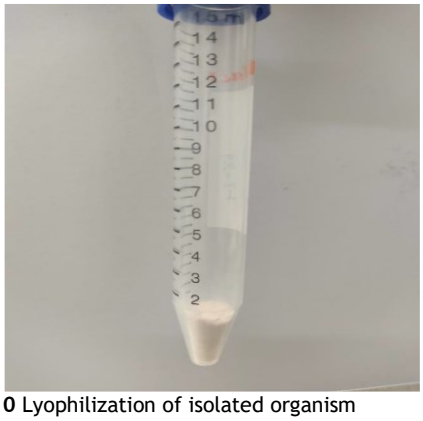

Fig: 10 Lyophilization of isolated organism

4.8. Preparation of chocolate

Chocolate was prepared with cocoa powder and cocoa butter, then mixed with roasted Flaxseed powder and lyophilized *Arthrobacter globiformis* powder as shown in Fig: 10, Fig: 11, and Fig: 12.

Fig: 11 Flaxseed powder

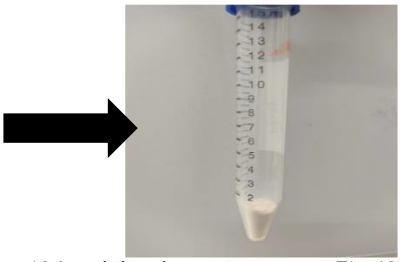

Fig: 12 Lyophilized organism

Fig:13 Probiotic chocolate

4.9. Fourier transform infrared spectroscopy

The FT-IR spectrum of the Probiotic chocolate was analyzed. The frequency ranges observed were 1050.89 cm^{-1}, 1140.15 cm^{-1}, 1459.86 cm^{-1}, 1638.19 cm^{-1}, 1743.48 cm^{-1}, 2852.87 cm^{-1}, 2922.14 cm^{-1}, 3321.02 cm^{-1}. The FTIR result for prepared probiotic chocolate is represented in Fig. 14

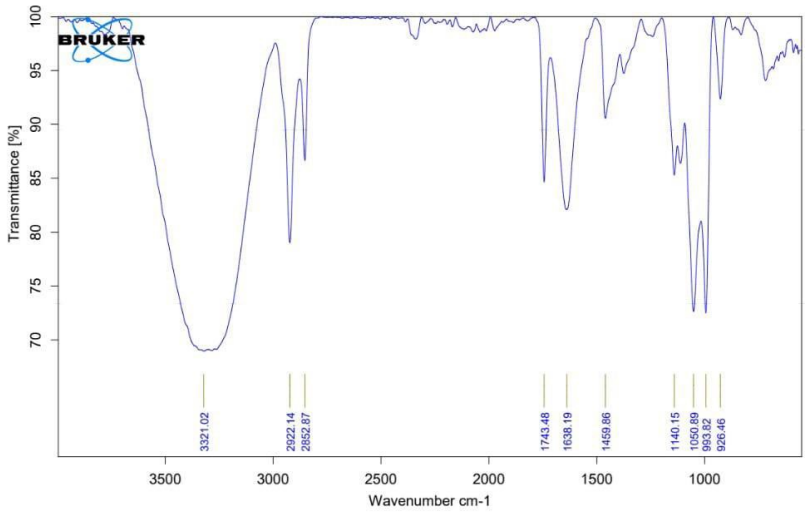

Fig: 14 FTIR analysis of the prepared probiotic chocolate

Table: 3 FTIR analysis for the prepared probiotic chocolate

S.NO	Frequency	Bond	Functional Group
1	1050.89 cm^{-1}	C-N stretch	Aliphatic amines
2	1140.15 cm^{-1}	C-N stretch	Aliphatic amines
3	1459.86 cm^{-1}	C-C stretch	aromatics
4	1638.19 cm^{-1}	N_H bend	1^0 amines
5	1743.48 cm^{-1}	C=O stretch	esters,saturated aliphatic
6	2852.87 cm^{-1}	C-H stretch	alkanes
7	2922.14 cm^{-1}	C-H stretch	alkanes
8	3321.02 cm^{-1}	O-H stretch, H-boned	alcohols, phenols

The spectral features of chocolate were obtained by FTIR analysis and are shown in **Table**:3. Bands referring to the phenol group were observed in the regions 3321.02 cm^{-1} (attributed to the stretch of O-H) and 2852.87 cm^{-1} to 2922.14 cm^{-1} (attributed to C-H stretch). The bands related to the amine were observed in the regions 1638.19 cm^{-1} (attributed to the stretch of the N_H), bands related to the Aliphatic amines were observed in the regions 1050.89 cm^{-1} to 1140.15 cm^{-1} (attributed to C-N stretch), bands related to the esters, saturated aliphatic were observed in the regions 1743.48 cm^{-1} (attributed to the C=O stretch) and the band 1459.86 cm^{-1} (attributed to the C-C stretch of the aromatic ring).

4.10. Characterization of Probiotic chocolate by Nutritional analysis

Nutritional analysis of prepared probiotic chocolate from *Arthrobacter globiformis* and flaxseed is shown in table:4.

Table: 4 Nutritional compounds present in prepared chocolate are shown below

Raw Material	Moisture %	Crude Protein %	Fat %	Ash %	Fibre %	Carbohydrate%
Chocolate	4.2	3	9.7	2.4	3.8	23.7

DISCUSSION

Probiotics are currently in style as dietary supplements on the global market. The desire to consume it is associated with its classification as a natural or functional food. Probiotics have been linked to several health advantages, including improved immunity, nutrient contribution, growth promotion, and antibacterial activities against harmful microorganisms. (Verschuere *et al.*, 2000). *Arthrobacter globiformis* were isolated from Nitrogen-rich soil samples and screened for probiotic properties including Gram's staining, catalase test, and biochemical tests, The isolated organisms were Gram-positive cocci, the IMViC result for isolated bacteria was negative, and it was catalase positive. According to Bergey's manual, the isolated organism was confirmed as *Arthrobacter globiformis*. (Nagamani Kathiresan *et. al.*, 2021). Bile, acid, and NaCl tolerance, and antimicrobial activity were performed. Antimicrobial effect of *A. globiformis* against selected pathogens bacteria *S. aureus Pseudomonas* sp, *Klebsiella* sp., and *E.coli* because they can occasionally be found as the food-borne microorganism that can cause gastroenteritis. *A. globiformis* showed antimicrobial potential against *E. coli* and *Klebsiella* sp. (Villanova, 2017)

Then mass production of *A. globiformis* was done. The organism was Lyophilized into powder and incorporated with Flaxseed and chocolate. Flaxseed rich in dietary fibre, can help us feel fuller for longer. Flaxseed contains a high concentration of lignin, soluble fiber, phenolic compounds, alpha-linolenic acid, and high-quality protein, it has the potential to be used as a functional food. Chocolate, on the other hand, may have anti-inflammatory, antioxidant, and heart-healthy properties as well as lower bad cholesterol, lower blood pressure, and improve brain function.

Probiotic organisms found in soil can regulate the overgrowth of harmful bacteria, which can reduce symptoms of constipation and Irritable Bowel Syndrome. After the preparation of chocolate, it was observed for the presence of potential compounds present in it by Fourier Transform Infrared Spectroscopy (FTIR). The total antioxidant capacity and the total phenolic compounds in probiotic chocolate were successfully predicted using FTIR. Nutritional analysis study was further conducted. The results of the Nutritional analysis of the prepared probiotic powder moisture (4.2%), protein (3%), crude fat (9.7%), ash (2.4%), fiber (3.8%) and Carbohydrate (23.7%).

CONCLUSION

The *Arthrobacter globiformis* was isolated from nitrogen-rich soil, serially diluted, and plated on Arthrobacter media for further study. Gram-positive was performed, and Gram-positive cocci-shaped bacilli were found in 3 days of culture media, and biochemical tests were done. *Arthrobacter globiformis* was IMViC negative and Catalase positive. According to Bergey's manual, the isolated organism was confirmed as *Arthrobacter globiformis*. pH tolerance of *Arthrobacter globiformis* was studied with pH levels of 3, 4, 5, and 6 for 1, 2, and 3 hours. *Arthrobacter globiformis* shows growth in all pH levels in the given time interval. Tolerance towards bile salt showed that *Arthrobacter globiformis* survived at 0.5%, 1%, and 3% of bile salts, but its growth was reduced when the concentration of bile increased. Tolerance against NaCl showed *Arthrobacter globiformis* could tolerate and grow at salt concentrations of 0.5% and 2% but was unable to grow at 4% and 6.5% salt concentrations. The antimicrobial activity of *A. globiformis* against common pathogens (*Escherichia coli, Staphylococcus aureus, Pseudomonas* sp, and *Klebsiella*) was studied and it showed antimicrobial activity against *Escherichia coli* and *Klebsiella* sp. *Arthrobacter globiformis* was further lyophilized and incorporated with chocolate using the standard method. FTIR was performed to observe the presence of potential compounds present in prepared probiotic chocolate.The band related to the aromatic ring was observed which represent the potential compound present in the prepared probiotic chocolate. The nutritional analysis result showed that the percentage of moisture, protein, crude fat, ash, and Carbohydrate of Prepared probiotic chocolate was 4.2, 3, 9.7, 2.4, 3.8 and 23.7 respectively.

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CONFLICT OF INTEREST

There is no conflict of interest among the authors for publishing this manuscript.

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