Biofilm formation and genotypic characterisation of *Biofidobacteria* from yoghurt and food supplements

Acknowledgements

I would like to thank Almighty for making me capable of completing my research work. I would also like to thank Dr. Daniel Amund and the laboratory staff at Coventry University for guidance and support throughout my project.

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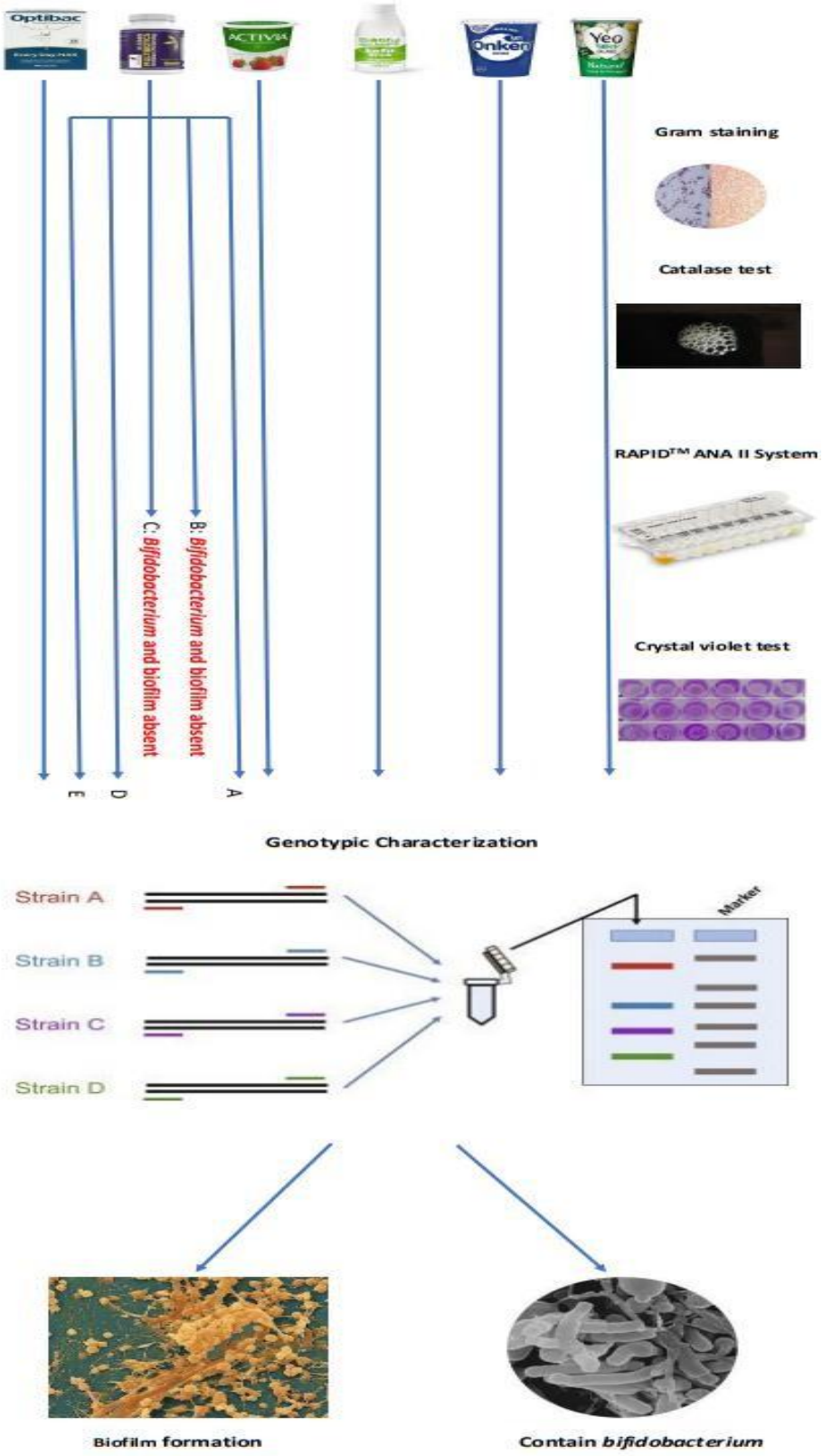
Graphical Abstract



Impact Statement

Biofilms are an important area of study for microbiologists due to their impact on human health. Biofilms are complex communities that are produced by potent probiotics as well as pathogenic bacteria. These biofilms can be advantageous or harmful to human health, depending on the microorganisms involved. The best probiotics would be comprised of biological strains that can compete with the pathogenic bacteria's capacity to form biofilm. Some bacteria, such as *bifidobacteria*, are considered to be beneficial to human wellness and can help to avoid the formation of harmful biofilms.

Bifidobacteria are a type of lactic acid bacteria that are prevalently present in the gastrointestinal tract of healthy humans. They are known for their ability to ferment lactose and produce lactic acid, which aids to maintain a healthy pH in the gut. Additionally, *bifidobacteria* have been found to promote digestion, boost immune system performance, and lower the risk of infection.

In this particular research, we aimed to examine the existence of *bifidobacteria* in yoghurt and dietary supplement samples and to evaluate their capacity to create biofilms in comparison to pathogenic microbes. Yoghurt is a great source of *bifidobacteria* and is an easy way to incorporate this beneficial probiotic into your diet. The study found that *bifidobacteria* were present in all the samples and had a propensity to create biofilms in almost all samples except two. This suggests that *bifidobacteria* could be used as a probiotic to stop the development of detrimental biofilms in the gut.

The study also emphasises the significance of recognising the role of biofilms in human health. Biofilms can be a major source of infection and can contribute to the emergence of long-term illnesses. By studying the formation and function of biofilms, researchers can develop new strategies for preventing and treating these conditions.

The current research concludes by providing useful information about the potential of *bifidobacteria* as a probiotic to prevent the growth of potentially hazardous biofilms. It also highlights the importance of incorporating fermented foods into the diet to promote gut health and overall wellbeing. Additional study is needed to completely comprehend the processes behind the probiotic effects of bifidobacterial and other beneficial microorganisms, but the findings of this study are a promising step in this direction.

1. Introduction

1.1. Probiotics

Advantageous, harmless bacteria known as probiotics have been used as food additives or supplements to cure or prevent a variety of illnesses (Guarner & Malagelada, 2003). Doctors are increasingly recommending probiotics as an effective therapeutic intervention for modulating digestive and immune function (Sanders, Merenstein, Merrifield, & Hutkins, 2018). Probiotics were first developed in the early 20th century, and research on them has advanced significantly during the past two decades in particular (Ritchie & Romanuk, 2012). Probiotics create complex communities called biofilms, which have a number of characteristics that help microbial populations expand in the face of a variety of abiotic and biotic pressures (Costerton, Stewart, & Greenberg, 1999). Take into account that bacterial colonization requires the bacteria to preferentially adhere to a certain epithelium, such as the intestinal mucosa, in order to prolong and stabilize their residence in the epithelium, assist in the exclusion of pathogenic bacteria by competitive inhibition or steric impediment, and possibly instigate the host cell's immune response (Saxelin, Tynkkynen, Mattila-Sandholm, & de Vos, 2005). A distinguishing characteristic of biofilms is the synthesis of an extracellular polysaccharide matrix; this matrix aids in resistance to antibiotics and enzymes and allows the creation of a niche for metabolic interaction among the population (Lewis & chemotherapy, 2001). Similarly, quorum sensing, which may regulate gene expression, is employed in bacterial communication (Schauder, Shokat, Surette, & Bassler, 2001). Mature biofilms have antibacterial activity and are more resistant to stomach pH than newly produced biofilms (Phillips et al., 2015). Researchers studying the potential of several bacterial species to form biofilms have shown that these organisms depend on a variety of abiotic surfaces for nutrition (mainly polystyrene or glass). Also, it has been demonstrated that they are successful in stopping the development of several harmful microorganisms (Villena, Hernández, Lara, & Martínez, 2009).

1.2. Health Benefits of Probiotics

Scientists have approved a consensual definition, and some of the mechanisms of action have been revealed, based on experimental data supporting the health advantages of probiotics and the quality of products (O'Toole, Marchesi, & Hill, 2017). The risk of acquiring oncogenic illnesses such as cancer may be deeply correlated to the growth of infectious microorganisms in the

gastrointestinal tract, activation of intestinal immunity, and synthesis of essential nutrients and/or biologically active food components (Dinparast-DjadidPhD, Frankland, & Saleh Azizian, 2015). Hence, microorganisms may have an impact on the various processes linked to a shift in cancer risk. Hence, a potential mechanism that could control disease severity is the replacement of the inflammatory bacteria by probiotics (Abreu & Peek Jr, 2014).

1.3. Probiotics for Disease Prevention

It appears that different probiotics can be employed in different ways to treat different diseases like cancer based on their capacity to alter gut flora in the short or long run. Studies showed that the probiotic strains of the useful bacteria *Lactobacillus* and *Bifidobacterium*, two well-known genera, can boost the immune system's defenses against intestinal infections, maintain the gut mucosal barrier, and prevent colon cancer (Kailasapathy, Chin, & biology, 2000; Ranji, Akbarzadeh, & Rahmati-Yamchi, 2015). In this regard, Perdigon et al. discovered that probiotics can improve the immune system's defense against colon cancer through the activation of interferon gamma (IFN-) and interleukin-10 (IL-10) in mice with colon cancer caused by 1, 2-dimethylhydrazine (DMH) (Perdigon, De Moreno de LeBlanc, Valdez, & Rachid, 2002). Also, animals with colon tumors have less tumor growth when routine oral administration of the microencapsulated *Lactobacillus acidophilus* (L. acidophilus) is used (Zhu, Luo, Jobin, & Young, 2011).

1.4. Probiotics in Food

The growth of both the food sector and human health is in danger from foodborne pathogens and associated biofilms. Between 40 and 80 percent of bacteria on Earth may create biofilms (Flemming & Wuertz, 2019). Because of the occurrence of numerous bacteria that may grow on food substrates and the food industry's infrastructure, there is a great potential for the formation of biofilms in the food industry. In the food manufacturing sector, microbial biofilms can grow on both food-contact and non-food-contact surfaces. Human diseases are among the biofilm-forming microorganisms prevalent in food plant environments, and they can build biofilm structures on numerous synthetic substrates (Abdallah, Benoliel, Drider, Dhulster, & Chihib, 2014; Colagiorgi et al., 2017), making this an especially salient issue in the food manufacturing sector. Biofilms, which can grow on the surfaces of food processing and medical equipment (Hall-Stoodley, Costerton, & Stoodley, 2004), have been shown to be a major cause of human immunodeficiency

virus and foodborne infections. These biofilms can host diseases and remain to be a cause of infection and cross-contamination. It is a big problem that threatens food safety that microbial biofilms form and grow in places where food is made (Galie, García-Gutiérrez, Miguélez, Villar, & Lombó, 2018; Han et al., 2017) because biofilms are responsible for roughly 60% of food-borne illnesses worldwide. Microbial biofilm growth and accumulation can cause food spoiling, posing major public health dangers as well as negative economic effects for customers (Coughlan, Cotter, Hill, & Alvarez-Ordóñez, 2016). Pollutants in food manufacturing plants often come from the atmosphere, machinery, or food surfaces, and if not properly cleaned, they can lead to food spoiling. Biofilms pose significant issues with innovation in the food sector because they can impede heat transfer through equipment surfaces, increase fluid frictional resistance on surfaces, cause physical crowding of fluid handling systems, and accelerate the rate of corrosion on metal surfaces, all of which reduce output (Meesilp, Mesil, & Biotechnology, 2019).

1.5. Fermented Foods as source of Probiotics

For thousands of years, people all around the world have enjoyed the health benefits of consuming fermented foods and drinks like yoghurt. In some circumstances, when probiotic bacteria are added, these foods improve in nutritional value and health benefits to the point where they are advised for regular intake. Despite its importance for basic and gastric health and disease prevention, dietary guidelines have not included nutritional guidance on the use of fermented foods and milk - based products (Bell, Ferrão, & Fernandes, 2017). Even though yoghurt has a long history of consumption in various regions and cultures, the frequency with which it is consumed differs substantially from one country to the next. In 2012, consumers in Finland, France, Spain, and Germany consumed the most yoghurt. Whereas just about 6% of the population in the United States consumes yoghurt every day, the vast majority of people in France consumes at least one serving daily. So adding the probiotics to the yoghurt is a good way of probiotic introduction in the diet for daily consumption.

1.6. Yoghurt as a rich Probiotic

Yoghurt and other fermented dairy products have been linked to improved health in a number of studies. It's no surprise that these foods make up a large part of the Mediterranean diet, either (Aznar et al., 2013; H. Wang, Livingston, Fox, Meigs, & Jacques, 2013). Yoghurt and other

fermented milk products serve a purpose in this diet that goes over merely being a nutritious substitute for typical dairy products (Vasilopoulou, Dilis, Trichopoulou, & Metabolism, 2013). There is a lot of data suggesting that the strains of bacteria employed in the fermentation of dairy products have healing capabilities that can prevent infections and reduce inflammation (Adolfsson, Meydani, & Russell, 2004). Researchers discovered that yoghurt and other fermented milk products can help manage blood cholesterol, particularly LDL cholesterol, and have a positive influence on the human digestive system. (Morelli, 2014; Sah, Vasiljevic, McKechnie, Donkor, & nutrition, 2018).Yoghurt and fermented milk have been demonstrated to aid in the digestive process and reduce the severity and frequency of gastrointestinal infectious diseases and antibiotic-associated diarrhea (Magro et al.; Merenstein et al., 2010). Regardless of the reality that substantial evidence already exists favouring the advantageous impact of yoghurt usage on gut health, the reported results may be inconsistent with variations in the strains of LAB used, differences in the methodology, or the absence of an explicit description of "gut health." (Adolfsson et al., 2004). Industry and researchers have begun looking at this product, which is well-known to include live bacteria, as a carrier for potentially advantageous substances due to its widespread public acceptability (Tapsell, 2015). Hence, Yoghurt and fermented milk are among the most prevalent methods through which probiotics are delivered to the human population (Haleh Sadrzadeh-Yeganeh et al., 2010).

1.7. *Bifidobacteria* as Probiotic

There are multiple reports of success with using *bifidobacteria* as probiotics for treating newborns (Costeloe, Hardy, Juszczak, Wilks, & Millar, 2016). For instance, it has been shown that *Bifidobacterium animalis* (BB-12 strain) and *longum* species strains are effective against mild rotavirus diarrhea in hospitalised infants, particularly by boosting the immune response and shortening the length of illness (Picard et al., 2005). Furthermore, *Bifidobacterium bifidum* and *B. animalis* strains have shown clinical favourable results for therapy of necrotising enterocolitis (NEC) in premature and low birth weight newborns (Picard et al., 2005). Probiotics like *Lactobacillus* and *Bifidobacterium* have shown promise in preventing influenza infection in mouse models by stimulating natural killer (NK) cell activity, according to a few studies (Iwabuchi, Xiao, Yaeshima, Iwatsuki, & Bulletin, 2011).

1.8. Biofilm Formation by Disease causing Microbes

Staphylococcus aureus is an extremely widespread pathogen that is regularly isolated from non-food contact surfaces in food manufacturing facilities, such as stainless steel (Pastoriza et al., 2002). The most prevalent symptom of *S. aureus* related food poisoning is intoxication from eating enterotoxins produced in food by enterotoxigenic *S. aureus* strains (Normanno et al., 2007). In the field of food safety, technological advancement and the finding of new anti-staphylococcal biofilm agents are viewed as potentially successful research fields (Schillaci, Napoli, Cusimano, Vitale, & Ruberto, 2013).

The ineffectiveness of antibiotics and disinfectants due to the production of biofilms by pathogens of clinical significance is a severe concern for animal and human health (Clutterbuck et al., 2007; Jacques, Aragon, & Tremblay, 2010; Parsek & Singh, 2003). Biofilms have universal qualities that call for unconventional methods of biofilm-related illness prevention, diagnosis, and therapy. Discovering and creating chemicals that can inhibit biofilm formation or eliminate established biofilms could increase the efficacy of biocontrol agents that are currently mainly useless against biofilm-forming bacteria (Rendueles, Kaplan, & Ghigo, 2013). There is also an increasing interest in finding non-biocidal anti-biofilm compounds, as doing so should greatly lessen the selective pressure bacteria face in adapting to withstand non-biocidal chemicals (Worthington, Richards, Melander, & chemistry, 2012).

Since their introduction in 1944, aminoglycosides such as streptomycin, neomycin, gentamicin, and tobramycin have proved crucial in battling a wide range of Gram-positive and Gram-negative bacteria that cause disease in humans (e.g., Pseudomonas aeruginosa, Staphylococcus aureus, Escherichia coli, etc.) (Henry-Stanley, Hess, & Wells, 2014; Krause, Serio, Kane, & Connolly, 2016; Labby & Garneau-Tsodikova, 2013; Mulani, Kamble, Kumkar, Tawre, & Pardesi, 2019). The long-term usage and overuse of aminoglycosides has led, like the pointless and excessive use of other antibiotics, the formation of various antibiotic-resistant bacterial species (Mulani et al., 2019; Pérez-Rodríguez & Mercanoglu Taban, 2019).

1.9. Biofilm Formation by *Bifidobacteria*

Many studies have shown that the introduction of the recently identified process of learned immunity through the use of natural components such as probiotics is a safe alternative

preventative and curative strategy for regulating immune responses (Hori, Kiyoshima, Shida, Yasui, & Immunology, 2002). Bacteroides such as *Bifidobacterium bifidum* are abundant in the colon and have been found to change the make-up of gut microbiota and the host's immunological responses for the better (Servin, 2004). Early childhood probiotic supplementation is superior for disease prevention and treatment because it promotes healthy microbial colonisation of the gut microbiota (Rodríguez et al., 2015). One of the most popular infant probiotics, *Lactobacillus reuteri,* has been demonstrated in multiple studies to be effective in preventing and treating gastrointestinal issues such colic, regurgitation, vomiting, and constipation (Weizman, Asli, & Alsheikh, 2005). Infants with colic have shown betterment in symptoms and a decline in the numbers of anaerobic Gram-negative bacteria, *Enterobacteriaceae*, and *enterococci* when exposed to this species (Savino et al., 2017). In addition, *L. reuteri ATCC 55730* was helpful in reducing swelling of the mucosa and modifying mucosal expression levels of certain cytokines associated in the intestinal inflammation in children with distal active ulcerative colitis (UC) (Oliva et al., 2012). Children with infectious diarrhea benefited from a supplement of *Lactobacillus* and *Saccharomyces* strains (*L. casei CG*, *L. reuteri ATCC 55730*, and a strain of *S. boulardii*) in their rehydration therapy (Cruchet et al., 2015).

1.10. Genotypic Characterization of *Bifidobacteria* for Biofilm Formation

In *Bifidobacteria*, the genetic control of extracellular polysaccharide production and the transition from a planktonic to an aggregative and biofilm-forming state have both been connected to c-di-GMP. (Jenal, Reinders, & Lori, 2017). Nevertheless, overexpression of EAL domain genes leads to the opposite symptoms, including a high cellular c-di-GMP concentration that substantially encourages the creation of biofilm matrix components and interferes with cell movement (Hengge, 2009).

Although biofilm formation has been extensively investigated (Flemming et al., 2016), its mechanism in *bifidobacterium* species is poorly understood (Barzegari et al., 2020). Genes involved in bifidobacterial biofilm production can now be studied using gene-trait matching analysis because to advances in next-generation sequencing technologies and bioinformatics (Barriuso & Martínez, 2018). Combining experimental phenotype data with comparative genome

analysis, gene-trait matching analysis is a crucial tool for pin-pointing candidate genes responsible for a given phenotype (Ostrov, Sela, Belausov, Steinberg, & Shemesh, 2019).

Zongium et al. (2021) undertook a time-series transcriptome sequencing analysis of *Bifidobacterium longum* FGSZY16M3 biofilm and planktonic cells to find potential genes involved in biofilm development. Genes involved in the SOS response (dnaK, groS, guaB, ruvA, recA, radA, recN, recF, pstA, and sufD) were shown to be related with the initial stage of biofilm development when a protein-protein interaction network was analyzed using 1296 differentially expressed genes during biofilm formation. At this point of biofilm development, the transcription of genes that produce extracellular polymeric substances (epsH, epsK, efp, frr, pheT, rfbA, rfbJ, rfbP, rpmF, secY, and yidC) increased. The genes involved in biofilm formation were subsequently studied using weighted gene co-expression network analysis (WGCNA), which revealed nine WGCNA modules and 133 genes involved in response to stress, regulation of gene expression, quorum sensing, and a two-component system. This research shows that biofilm production in *B. longum* is a complex process including structural development, regulatory mechanisms, and the organism's reaction to stress (Liu, Li, Wang, et al., 2021).

1.11. Aims & Objectives of this Study

In the current research, recognition of the *bifidobacterium* was done using PCR for ITS region. Inside the DNA of eubacterial cells, the rRNA genetic loci consist of 16S, 23S, and 5S rRNA genes. Internally transcribed spacer (ITS) sections isolate these genes from one another. It is common knowledge that the DNA sequences found in the 16S-23S intergenic spacer region display a significant amount of sequence and length diversity (Barry, Colleran, Glennon, Dunican, & Gannon, 1991; Navarro, Simonet, Normand, & Bardin, 1992). It has been demonstrated that changes in this region are helpful in distinguishing between distinct kinds of prokaryotes. PCR-restriction fragment length polymorphism analysis of the ribosomal intergenic spacer has recently been found to be an effective tool for distinguishing between various strains of prokaryotes (Regnery, Spruill, & Plikaytis, 1991).

While yoghurt is a widely recognized source of a variety of probiotic bacteria, including *lactobacillus* species, there are other sources of probiotic bacteria, the present investigation seeks the identification of *bifidobacteria* in yoghurt. The purpose of this research was to isolate and

describe the *bifidobacteria* found in yoghurt samples. Due to the fact that *bifidobacteria* has been linked to having exceptional probiotic characteristics, the research provided light on the possibility of using the bacteria in yoghurt and other fermented foods. There are multiple yoghurt brands and food supplements were taken in this research and the bacteria used as probiotics in those food products were assessed for the biofilm formation capacity and the capacity of those probiotics was compared with the biofilm forming capacity of pathogenic bacteria. The pathogenic controls were used in this study which included *Pseudomonas aerugonisa*, *Klebsiella Pneumoniae*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Coronobacter sakazakii*, *Bacillus subtilis* and *Escherichia coli.* These pathogens are linked to various diseases. This study will provide an insight of the better yoghurt with effective probiotics that can be utilized by the people to attain protection from several pathogenic bacteria.

2. Methods

2.1. Ethical Approval

Ethical approval certificate by Coventry University ethics board was obtained on 8th December 2022 (Project reference number P146451) and the investigation was conducted according to the experimental method outlined in the ethics application.

2.2. Experimental Samples

The number of samples employed for the experiment which served as a source of *bifidobacteria* was 6 (n=6) which included four yoghurt samples and two samples from the food supplements.

Table 1. Experiment Samples

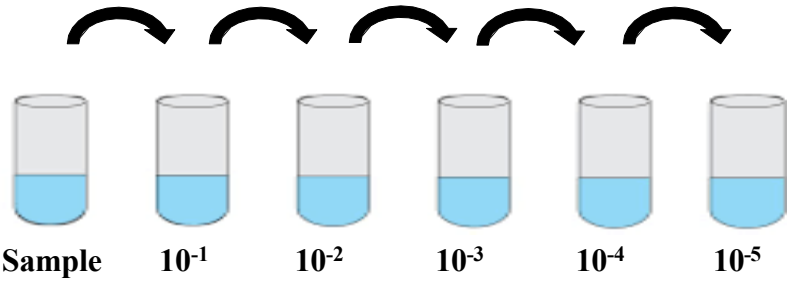
Sr. No.	Sample	Sample Name
1.	Yoghurt	Yeo Valley Natural Yoghurt
2.	Yoghurt	Onken Natural Set Yoghurt
3.	Yoghurt	Bio-Tiful Dairy Kefir Drink Original
4.	Yoghurt	Activia Strawberry Yoghurt
5.	Food Supplement	Nutrizing (16 Strain Multibiotics: 30 CFU/serving)
6.	Food Supplement	Optibac Probiotics Every Day Max

The samples (Table 1.) were selected on the basis of the nutritional values and the presence of said probiotics. All the samples were purchased from the Tesco UK and Amazon.

2.3. Isolation of *Bifidobacteria*

2.3.1. Dilution of samples

In order to isolate *bifidobacteria* from samples. The samples were made in dilutions**.** To make dilutions of yoghurt samples 2mL of sample was mixed with 18mL normal saline (0.9%). For the food supplement dilutions, 1 pill was diluted with 9 mL of regular saline (0.9%). 10 fold dilutions were prepared by mixing 1 mL of the solution with 9 mL of regular saline to a concentration of 10-5.



The dilutions 10^{-3}, 10^{-4} and 10^{-5} were used for the spreading of the samples on media plates.

2.3.2. Spreading

For the isolation of *bifidobacteria* the selective media was used. Reinforced Clostridial Agar (RCA) with lithium mupirocin was prepared and petri plates were prepared for the culturing of bacteria. The 100µL samples were spread on the agar plates with the help of the spreader. The plates were incubated in anaerobic chamber for 48hrs at 37°C.

2.3.3. Sub culturing

The colonies were picked from the cultured RCA plates and streaking was done further for the isolated colonies of *bifidobacteria*. With the aid of the loop, the colonies were chosen. Streaking was done on RCA media plates and the plates were again placed in hypoxic box for 48hrs. Colony characteristics of all samples were studied and compared with control.

2.4. Identification of *Bifidiobacterium*

Biochemical and morphological assays were used to distinguish the isolated colony that had grown on RCA plates. Gram staining, catalase test, and the RapID™ ANA II System Cat# 3502282; Thermo Scientific, were used for the identification of the isolated bacteria.

2.4.1. Gram Staining

From each RCA media plate, a loopful of colonies was taken and distributed in the shape of a smear in a circular pattern on the glass slides. The heat-fixed, air-dried smear of cells was treated with reagents for Grams' staining. The slides were observed under the microscope for the confirmation of *bifidobacterium.*

2.4.2. Catalase Test

For the biochemical identification of the *bifidobacterium*, Catalase test was used. Using a dropper, one drop of 3% H_2O_2 was applied to each individual organism on microscope slides.

2.4.3. RapID™ ANA II System; Thermo Scientific™

A sufficient amount of growth from the six agar plate cultures was suspended in six RapID Inoculation Fluid (1 ml) tubes using an inoculating loop with the purpose of achieving apparent turbidity hat was equal to a 3.0 McFarland turbidity standard or equivalent. Separate RapID ANA II Panels were inoculated with 6 samples and 1 BB12 control. The panels were then incubated at 35-37°C in a non CO_2 incubator for a maximum of 4 hours. The label covers over the reaction cavities was removed by drawing the lower right hand tab up and to the left, while the RapID ANA II panels were still firmly planted on the benchtop. Cavities 1 (URE) through 10 (PO4) were read and scored using the interpretation guide without the use of reagents. Test scores for bifunctional exams were written in the appropriate boxes on the report form using the test code located above the bar. 10's cavity of each panel was treated with two drops of RapID Spot Indole Reagent (IND). Adding 2 drops of RapID ANA II Reagent to cavities 3 (LGY) through 9 (PYR) of each panel. It was incubated for 30 seconds to allow color development. Then, cavities 3 through 10 were read through Labtech biofilm reader (LT-4500) and scored, and the scores were written in the appropriate report form boxes via the test codes below the bar for bifunctional tests. The obtained microcode's were compared to a chart for the identification of bacteria (Blairon et al., 2010).

2.5. Culturing of Pathogenic Controls

The University of Coventry offered nine potentially biofilm-forming pathogenic microorganisms. *Escherichia coli K12* (8797), *Streptococcus mutans, Staphylococcus aureus* 10442 (MRSA), *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* 10442 (MRSA) were among these microorganisms. *Bacillus subtilis* 8054, *Listeria monocytogenes*, *Cronobacter sakazakii, Klebsiella pneumonia* NTCT 13368, *Bifidobacterium animalis* subsp. *lactis* (BB-12), and *Klebsiella pneumonia*.

Tryptone Soya Agar (Lot no. 3128386) was used to grow *Cronobacter sakazakii*. A variety of bacteria were grown on Nutrient Agar, including *Escherichia coli K12* (8797), *Klebsiella pneumonia* NTCT 13368*, Listeria monocytogenes*, *Staphylococcus aureus* 10442 (MRSA),

Pseudomonas aeruginosa ATCC 27853 and *Bacillus subtilis* 8054. *Streptococcus mutans* was grown on Blood Agar, whereas *Bifidobacterium animalis* subsp. *lactis* (BB-12) was grown on Reinforced Clostridium Agar.

2.6. Determination of Biofilm Formation Potential

On cultured biofilms in microtiter plates, the production of biofilms was quantified using the traditional microbiological crystal violet test (Amund, Ouoba, Sutherland, & Ghoddusi, 2014). 180 µL of Reinforced Clostridial Media (RCM) were added in 96-well flat bottom microtiter plate. 20µL of bacterial suspensions were added into 180 µL of Reinforced Clostridial Medai (RCM), 200 µL of RCM served as negative control. The plate was incubated at 37°C for 48hrs under anaerobic conditions.

After removing the media from the plates, they were washed three times with PBS (Phosphate Buffer Saline). The bacteria were heat-fixed by exposing the microtiter plate wells at near to the Bunsen burner for one hour. Microtiter plate bacteria were stained with 1% Crystal Violet in 150 µL. A 15-minute incubation at room temperature was performed. The stain was removed, and the wells were rinsed with water thrice. The plate was air-dried at room temperature, then 200 L of 5% acetic acid was added, and the plate was placed at room temperature for 1.5 hours to solubilize the crystal violet.

100µl of solution was picked from the microtiter plate and added into a fresh 96-well flat bottom microtiter plate to calculate the OD value. The optical density (OD) was determined for each well using a Labtech International LT-4500 Absorbance Microplate Reader set at 595 nm. The OD value coincided with the crystal violet generated by the cells and provided a rough estimate of the biofilm forming capability of the bacterial cultures. 2 replicates of the experiment were done and the mean OD value was calculated for each sample.

2.7. DNA Extraction

The following protocol by InstaGene Matrix Cat# 7326030 BioRad Laboratories, for DNA extraction of isolated *bifidobacterium* species and control was followed. 1 mL of sterile milli-Q H2O was added to the Eppendorf tube and the bacterial colony was suspended in it. The supernatant was collected after the tube was centrifuged for 1 minute at 12000 rpm. 200 L of InstaGene matrix was added to the pellet, which was then incubated for 30 minutes at 56°C in a

heating block, followed by a 10-second vortex and a further 8 minutes at 100°C. The tube was centrifuged for 3 minutes at 12000 revolutions per minute. The DNA-containing supernatant was submitted to the nanodrop method for measurement.

2.8. DNA Quantification

For the DNA quantification the supernatant was used. The nanodrop was calibrated with 1 µL of sterile water which served as a blank. After that, 1 µL of the extracted DNA sample was added on the sampling arm of the nanodrop. The absorbance was calculated at 230nm.

2.9. Identification of Bifidobacteria through ITS-PCR

With the use of primers (Table 2.) against the 16S-23S rDNA ITS gene, the recovered DNA was determined to be *bifidobacterium* by ITS-PCR using thermocycler (Product Code: 5PRMEG/05).

Table 2. ITS Primers

Forward Primer	GTCGTAACAAGGTAGCCGTA	55°C Annealing Temperature
Reverse Primer	CAAGGCATCCACCGT	55°C Annealing Temperature

The PCR master mix was prepared as mentioned in Table 3.

Table 3. PCR Reaction Mixture

Sr. No.	Reagent	Volume	Volume for 15 Reactions
1.	10X PCR Buffer including 20 mM $MgCl_2$	2.5µL	37.5 µL
2.	1.25 mM dNTPs mix	4.0µL	60 µL
3.	5U Taq DNA Polymerase	0.1µL	1.5 µL
4.	10 pmol/ µL Forward Primer	1.0µL	15 µL
5.	10 pmol/ µL Reverse Primer	1.0µL	15 µL
6.	Sterile Milli-Q Water	15.4µL	231 µL
7.	DNA Template	0.1µL	1.5 µL

After making the reaction mixture the PCR was run under the following conditions (Table 4.).

Table 4. PCR Conditions

Steps	Time	Cycles	Temperature
Initial Denaturation	5 min		95°C
Denaturation	45 sec	25 Cycles	94°C
Annealing	1 min		55°C
Elongation	1 min		72°C
Final Extension	10 min		72°C

2.10. Determination of PCR Product Size via Gel Electrophoresis

Gel electrophoresis was used to measure the size of the extracted DNA from the various *bifidobacterium* species that had been isolated. For ITS-PCR 1.2% agarose gel by using agarose (Lot no. 43403-43532) was prepared and 2 µL of GelRed stain was added in the gel. After the solidification of the gel, the wells were loaded with 10 µL sample and 2 µL of loading dye. The gel was run for one hour at 120 volts in 1x Tris-Borate-EDTA (TBE) buffer. DNA molecular marker was run along with the samples for the determination of the size of PCR product. After the completion of gel running, the gel was observed under UV trans-illuminator to see the PCR bands and images were taken in GelDoc.

3. Results

3.1. Isolated Colonies of Bifidobacteria

In the figure 1. **A** is the Nutrizing A, **B** is the Nutrizing B, **C** is the Nutrizing C, **D** is the Nutrizing D, **E** is the Nutrizing E, **F** is the Kefir, **G** is Activia A, **H** is the Optibac B, **I** is the Yeovalley, **J** is the Onken and **K** is the BB-12 which is the *bifidobacterium* control.

All the samples were streaked on the RCA media and the streaking was done. After the 48h the plates were observed and separate colonies were observed. The colonies were smooth, round, milky white and raised.

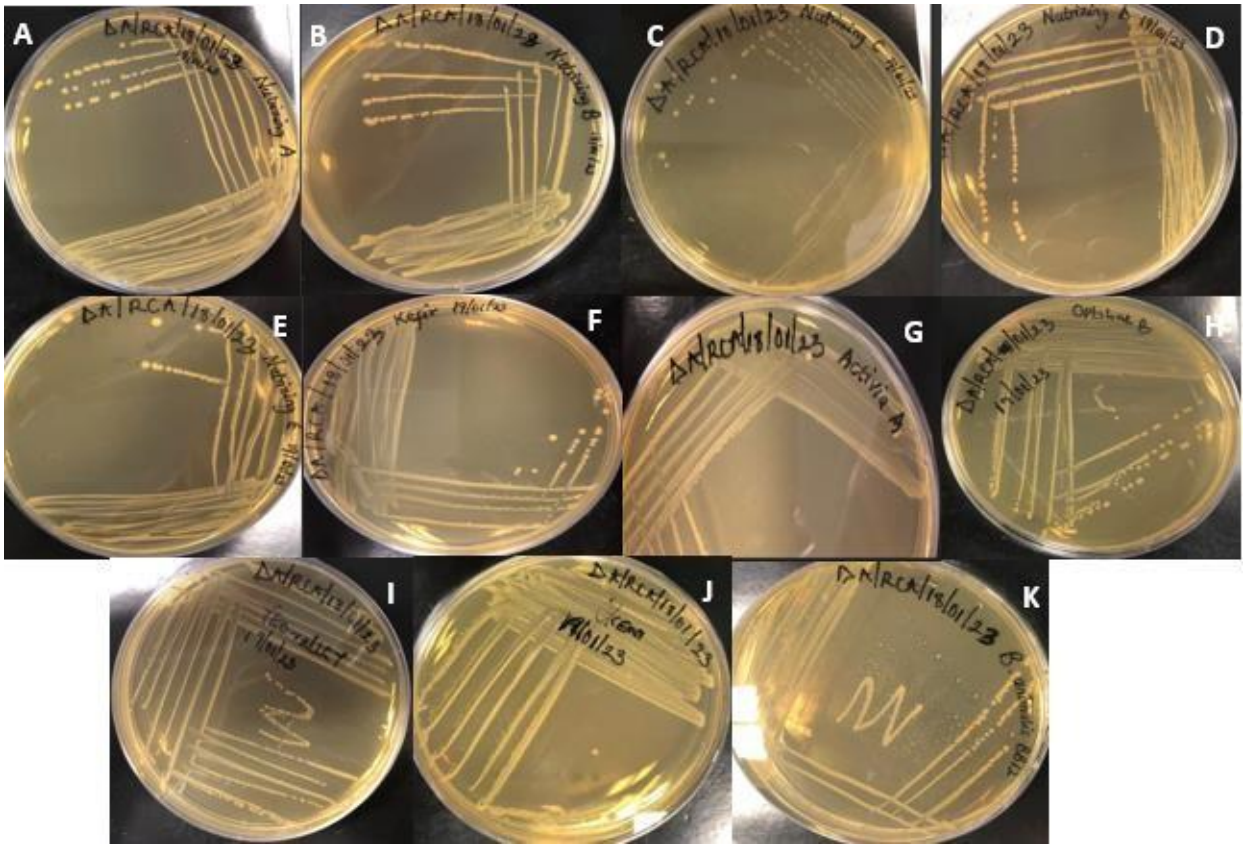


Figure. 1. Isolated Colonies of Bifidobacteria grown on RCA Media and incubated for 48 hrs. at 37°C under anaerobic conditions

3.2. Gram Staining

In the figure 2. **A** is Yeovalley A, **B** is Yeo Valley B, **C** is Kefir, **D** is Onken, **E** is Activia A, **F** is Activia B, **G** is Optibac A, **H** is Optibac B, **I** is Nutrizing A, **J** is Nutrizing B, **K** is Nutrizing C, **L** is Nutrizing D, **M** is Nutrizing E and **N** is BB-12. All the samples showed gram positive, purple, short branched roads, confirming the presence of *bifidobacteria* in the isolated bacterial cultures from the yoghurt and food supplement samples.

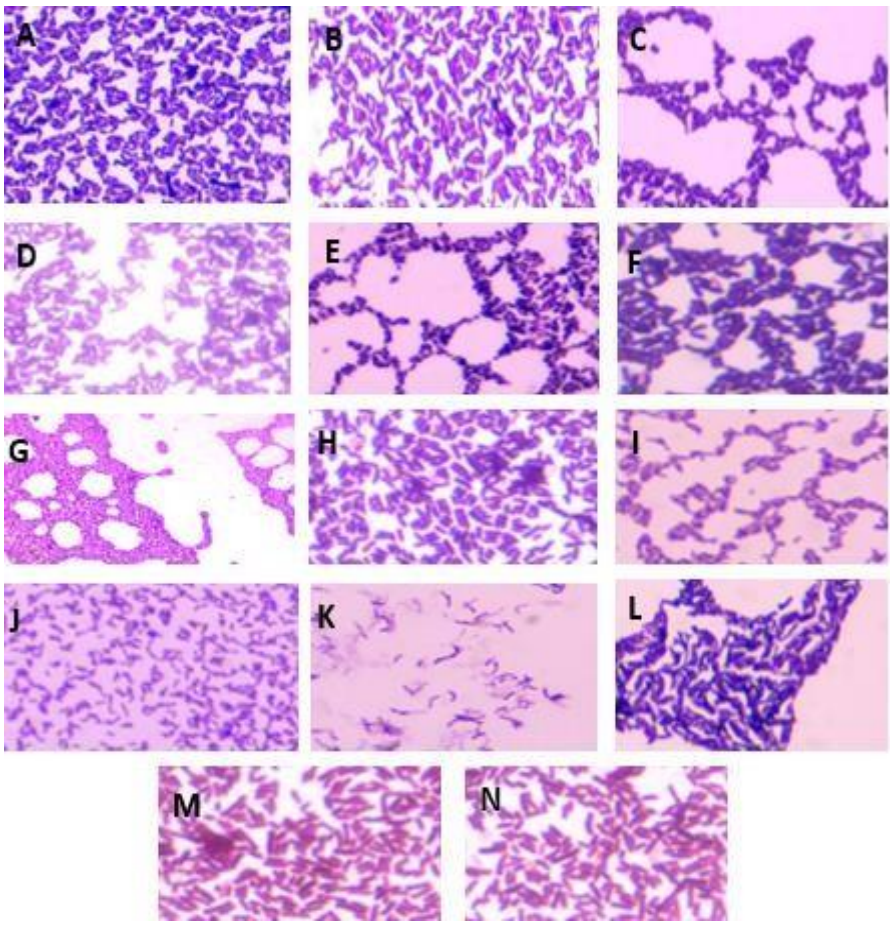


Figure. 2. Grams' staining of the Bifidobacteria colonies obtained on RCA plates

3.3. Catalase Test

The catalase test for the *bfidobacterium* samples was negative. For all the samples and control the results of the catalase test was negative. That showed that the isolated bacteria were *bifidobacteria*.

3.4. RapID™ ANA II System

RapID ANA II System confirmed the presence of bacterium found in different samples (Table 5). The samples from Activia B and Yeovalley A showed maximum similarity of 98.84% with *bifidobacterium* sp. Nutrizing C was also 97.93% similar to *bifidobacterium* sp. These results showed the samples from yoghurt and food supplements contain *bifidobacerium* as probiotic.

Table 5. RapID™ ANA II System

Sample	Code	Specie Name	Similarity Percentage
Onken	071671	*Actinomyces israelii*	99.63%
Kefir	471371	*Actinomyces israelii*	96.66%
Activia B	471071	*Bifidobacterium sp.*	98.84%
Optibac B	471471	*Bifidobacterium sp.*	83.95%
Yeovalley A	471071	*Bifidobacterium sp.*	98.84%
Nutrizing C	461071	*Bifidobacterium sp.*	97.93%
Nutrizing E	071471	*Actinomyces israelii*	76.07%
Bifidobacterium animalis	471671	*Actinomyces israelii*	99.79%

3.5. Biofilm Assay

The biofilm assay of the samples was done and the mean OD values were recorded (Figure 3). The cut off value is 0.1. Nutrizing B, Nutrizing C, and RCM showed values less than 0.1 while the rest of the samples showed a value greater than 0.1. The greatest value was found in the Yeovalley B sample, which was nearly identical to that obtained by the BB-12 *bifidobacterium* sample used as a positive control.

Nutrizing A, Nutrizing D and Onken A showed lower values but slightly greater than the cut off value. The values obtained for Activia B, Kefir and Octibac A were intermediate. The OD values confirmed by the Biofilm Assay shows the bacteria isolated from the yoghurt samples exhibit biofilm forming capacity except the Nutrizing B and Nutrizing C samples.

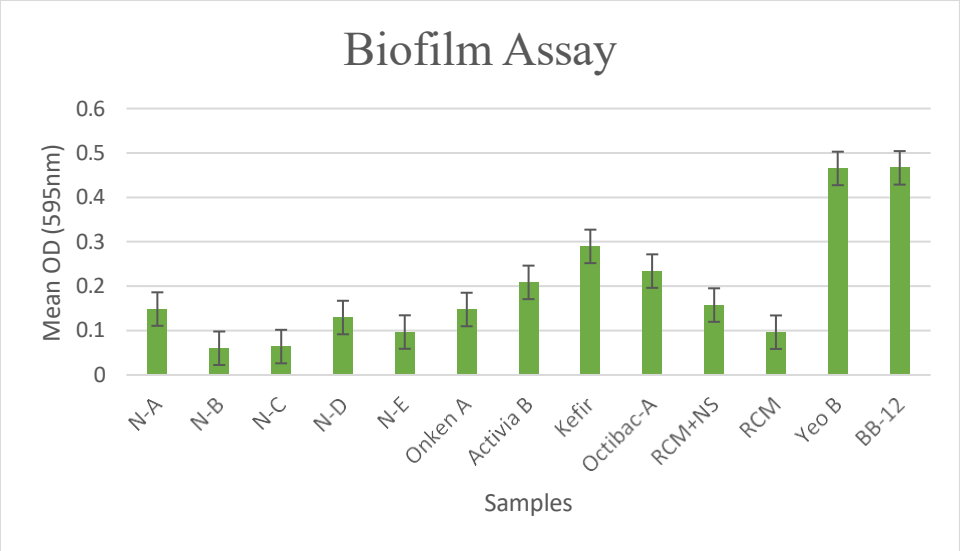


Figure 3. Biofilm Assay of Yoghurt samples and Food Supplements with RCM serving as negative control and BB-12 serving as positive control

The pathogenic controls were grown in BHI and the OD values were recorded from the cultures from samples from both media. The OD values represent that the highest biofilm forming potential was observed in the *Pseudomonas aerugonisa*. *Klebsiella Pneumoniae, Staphylococcus aureus* and *Listeria monocytogenes* showed almost same OD values that were lower but close to the cut off value. The lowest biofilm forming potential was observed in the pathogenic bacteria *Cronobacter sakazakii. Bacillus subtilis* and *Escherichia coli* also had lower values than the cut off value showing a lower potential to form biofilms in these pathogenic bacteria (Figure 4).

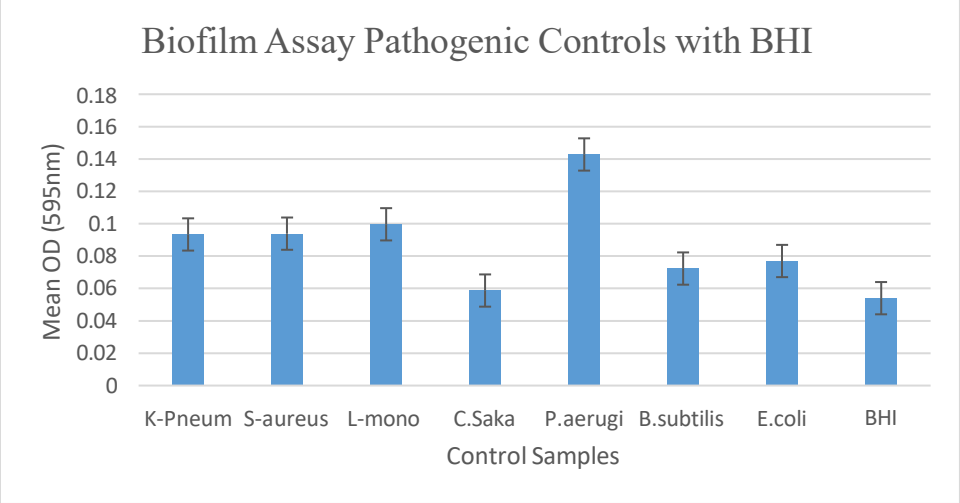


Figure 4. Biofilm Assay of Pathogenic Controls with BHI serving as negative Control

The pathogenic controls were grown in TSB and the OD values were recorded from the culture samples from both media. The OD values represent that the highest biofilm forming potential was observed in the *Pseudomonas aerugonisa*. *Klebsiella Pneumoniae, Staphylococcus aureus* and *Listeria monocytogenes* showed almost same OD values that were lower but close to the cut off value. The lowest biofilm forming potential was observed in the pathogenic bacteria *Cronobacter sakazakii. Bacillus subtilis* and *Escherichia coli* also had lower values than the cut off value showing a lower potential to form biofilms in these pathogenic bacteria (Figure 5).

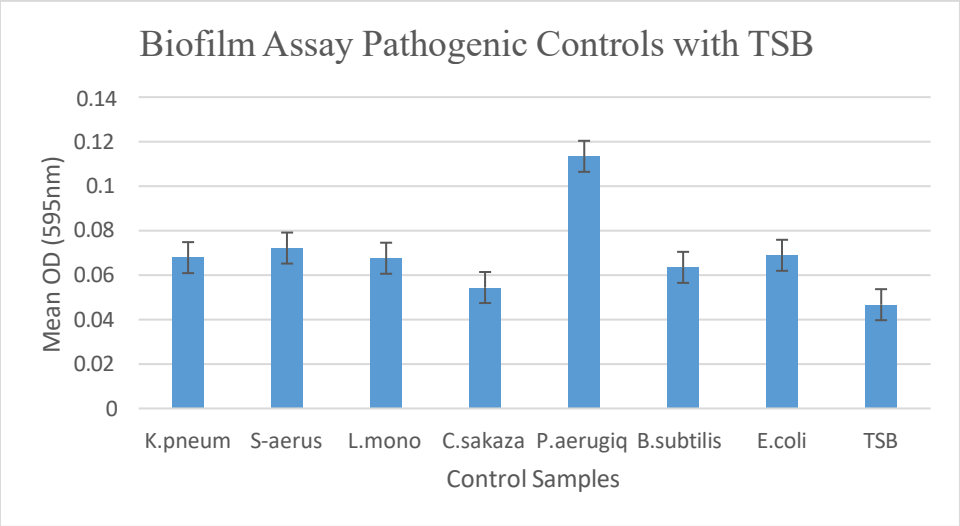


Figure 5. Biofilm Assay of Pathogenic Controls with TSB serving as negative control

3.6. Nanodrop

In the Figure 6. the sample is Nutrizing A and the concentration was 23.3 ng/ul. The A260/A280 value obtained was 1.64 that shows purity of DNA sample and A260/A230 value recorded was 0.96, which shows the presence of organic contaminants. The nanodrop results depict good quality and quantity of DNA in the extracted samples and the samples of DNA were further processed for PCR analysis.

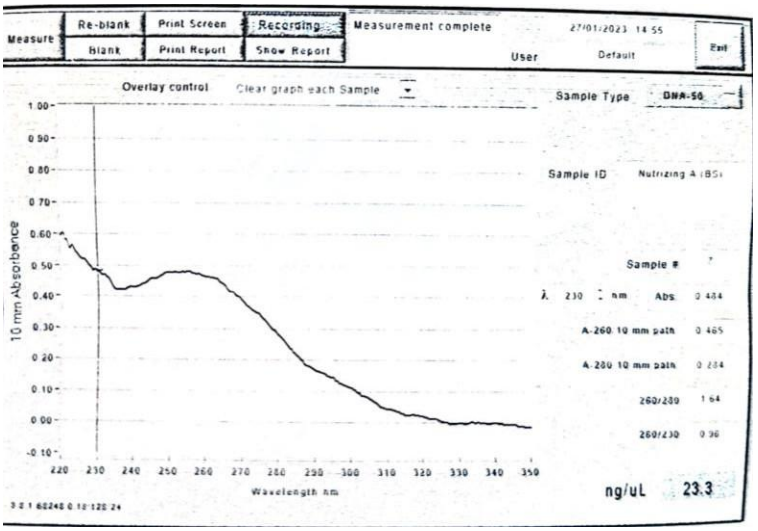


Figure 6. Nanodrop of Nutrizing A

The sample, Nutrizing B, in Figure 7. had a concentration of 54.8 ng/ul. The obtained A260/A280 value of 2.21 indicates the DNA sample's purity, and the A260/A230 value of 1.08 indicates the presence of organic contaminants. The DNA samples were then processed for PCR analysis, and the nanodrop results show good quality and amount of DNA in the extracted samples.

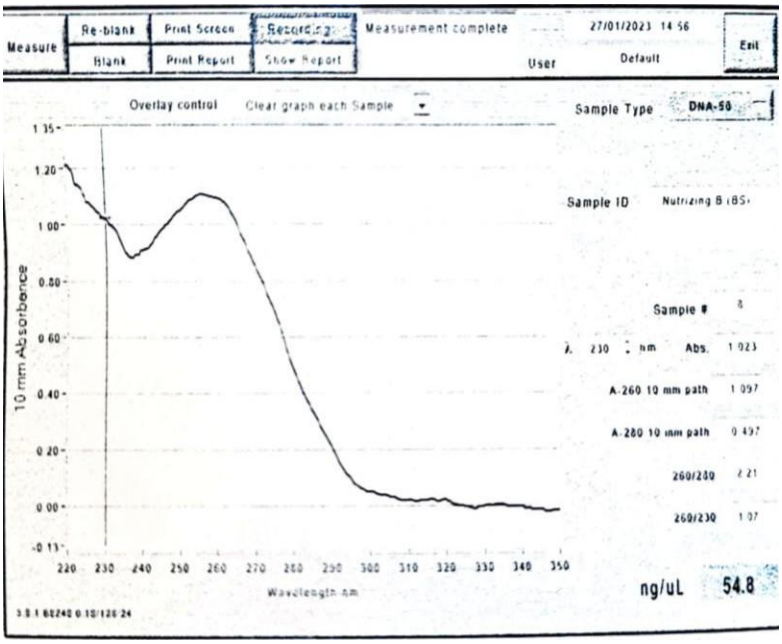


Figure 7. Nanodrop of Nutrizing B

Nutrazing C, with a concentration of 23.7 ng/ul, is the sample shown in Figure 8. The DNA sample's purity was demonstrated by an A260/A280 ratio of 1.74, whereas an A260/A230 ratio of 0.30 indicated the presence of organic impurities. The extracted DNA samples were processed further for PCR analysis, and the findings showed high quality and quantity of DNA.

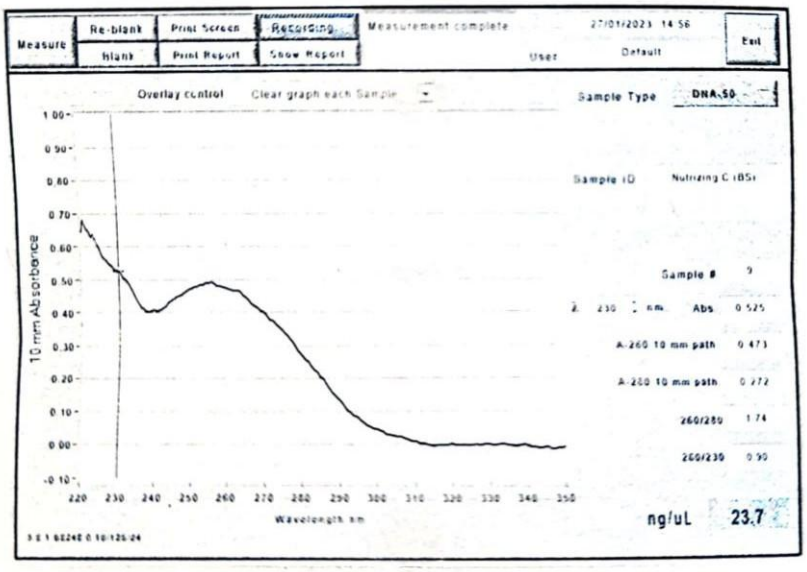


Figure 8. Nanodrop Nutrizing C.

The Nutrizing D sample shown in Figure 9 had a concentration of 30.4 ng/ul. The DNA sample obtained an A260/A280 ratio of 2.11, indicating its high purity, whereas the A260/A230 ratio recorded 1.49, indicating the absence of organic impurities. After processing the DNA samples for PCR analysis, the nanodrop results showed that both the quality and quantity of the extracted DNA were high.

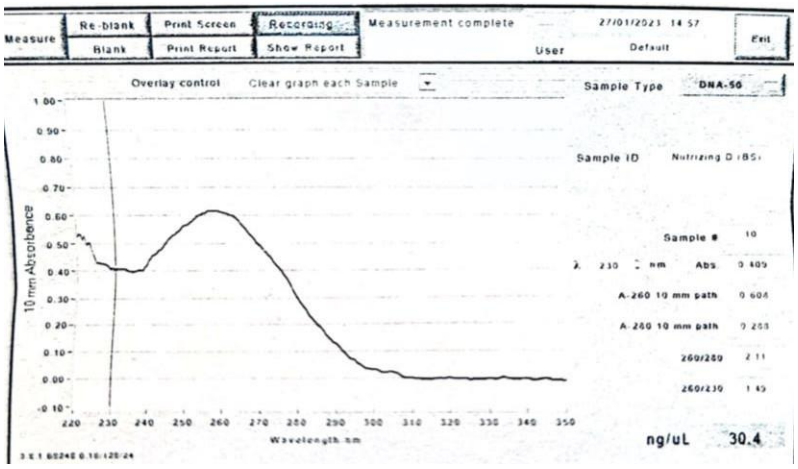


Figure 9. Nanodrop of Nutrizing D

In the Figure 10. the sample is Nutrizing E and the concentration was 98.6 ng/ul. The A260/A280 value obtained was 2.12 that shows purity of DNA sample and A260/A230 value recorded was 1.43, which shows the absence of organic contaminants. The nanodrop results depict good quality and quantity of DNA in the extracted samples and the samples of DNA were further processed for PCR analysis.

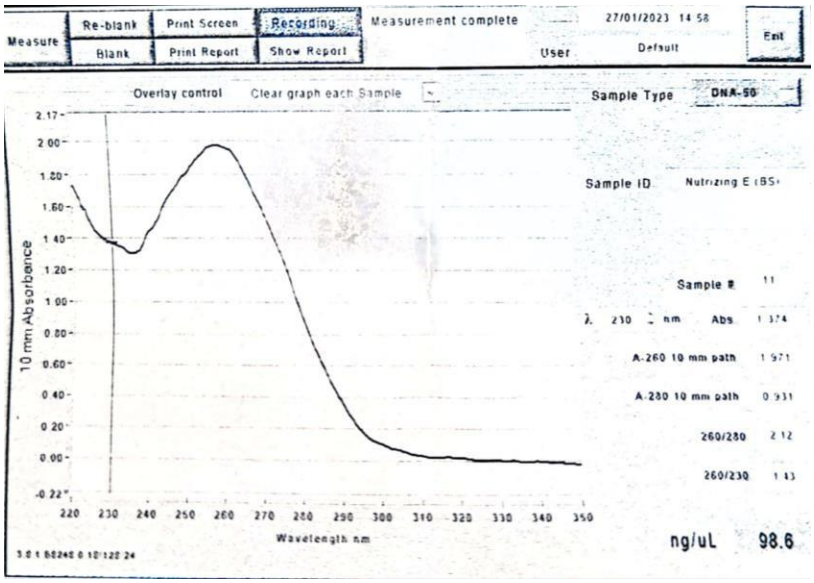


Figure 10. Nanodrop of Nutrizing E

The Optibac sample shown in Figure 11 had a concentration of 132.3 ng/ul. The DNA sample's purity was demonstrated by an A260/A280 ratio of 2.09, while an A260/A230 ratio of

1.55 indicated the absence of organic impurities. The extracted DNA samples were processed further for PCR analysis, and the findings showed high quality and quantity of DNA.

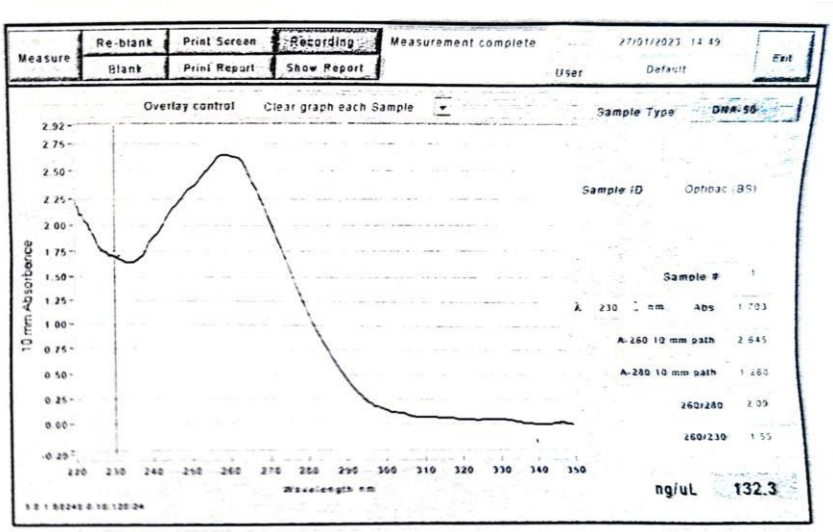


Figure 11. Nanodrop of Optibac

In the Figure 12. the sample is Yeovalley and the concentration was 126.3 ng/ul. The A260/A280 value obtained was 2.96 that shows purity of DNA sample and A260/A230 value recorded was 1.59, which shows the sample was free of organic contaminants. The nanodrop results depict good quality and quantity of DNA in the extracted samples and the samples of DNA were further processed for PCR analysis.

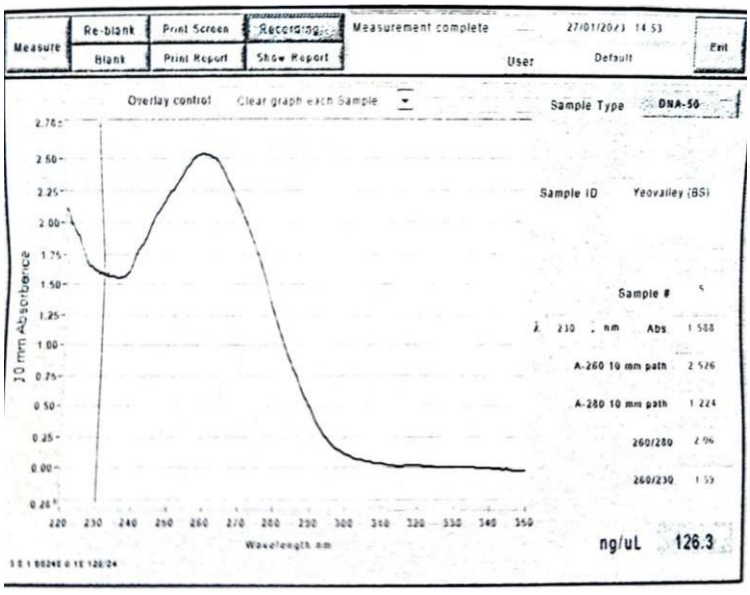


Figure 12. Nanodrop of Yeovalley

In the Figure 13. the sample is Onken and the concentration was 35.8 ng/ul. The A260/A280 value obtained was 1.99 that shows purity of DNA sample and A260/A230 value recorded was 1.24, which shows the presence of organic contaminants to some extent in the sample. The nanodrop results depict good quality and quantity of DNA in the extracted samples and the samples of DNA were further processed for PCR analysis.

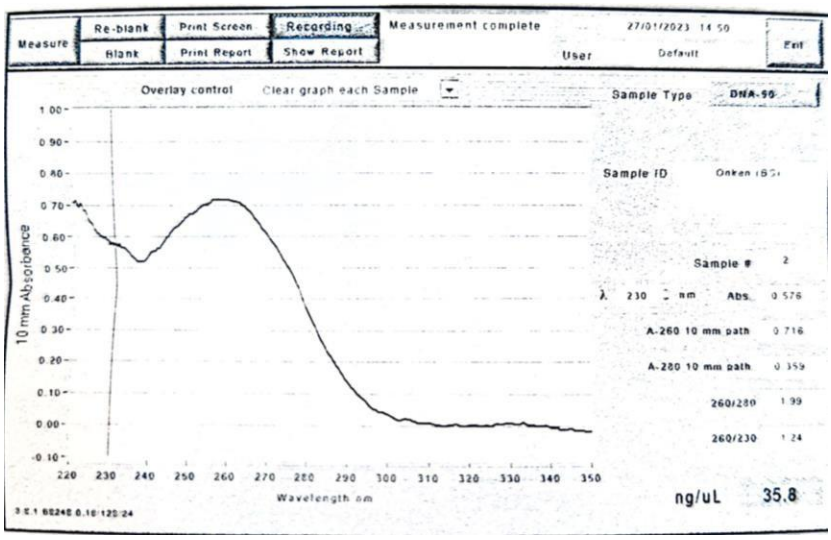


Figure 13. Nanodrop of Onken

In the Figure 14. the first sample is Kefir and the concentration was 84.1 ng/ul. The A260/A280 value obtained was 2.16 that shows purity of DNA sample and A260/A230 value recorded was 1.59, which shows the presence of organic contaminants. The nanodrop results depict good quality and quantity of DNA in the extracted samples and the samples of DNA were further processed for PCR analysis.

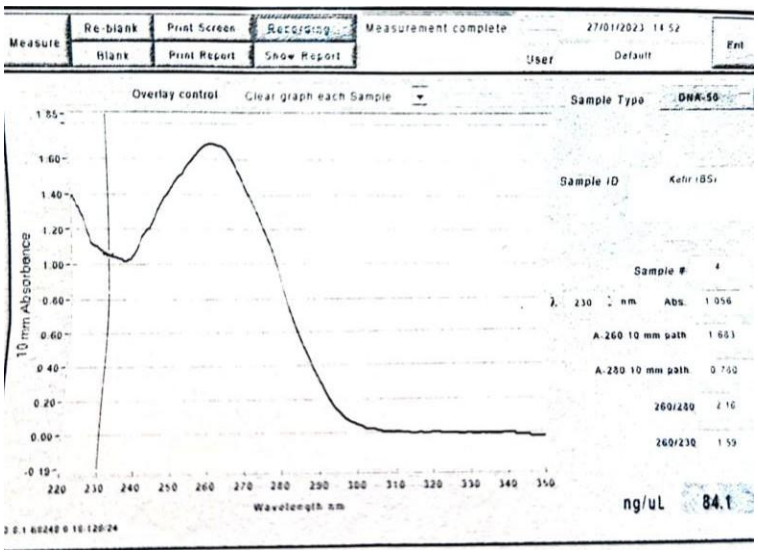


Figure 14. Nanodrop of Kefir

In the Figure 15. the sample is B. animalis and the concentration was 108.8 ng/ul. The A260/A280 value obtained was 2.02 that shows purity of DNA sample and A260/A230 value recorded was 1.56, which shows the presence of organic contaminants. The nanodrop results depict good quality and quantity of DNA in the extracted samples and the samples of DNA were further processed for PCR analysis.

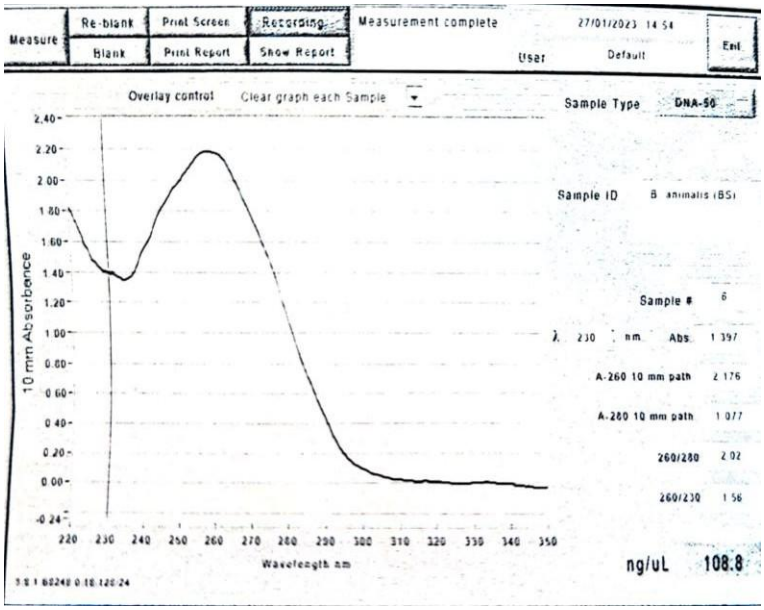


Figure 15. Nanodrop of B. animalis

In the Figure 16. the sample is Activia and the concentration was 56.8 ng/ul. The A260/A280 value obtained was 1.94 that shows purity of DNA sample and A260/A230 value recorded was 1.38, which shows the presence of organic contaminants. The nanodrop results depict good quality and quantity of DNA in the extracted samples and the samples of DNA were further processed for PCR analysis.

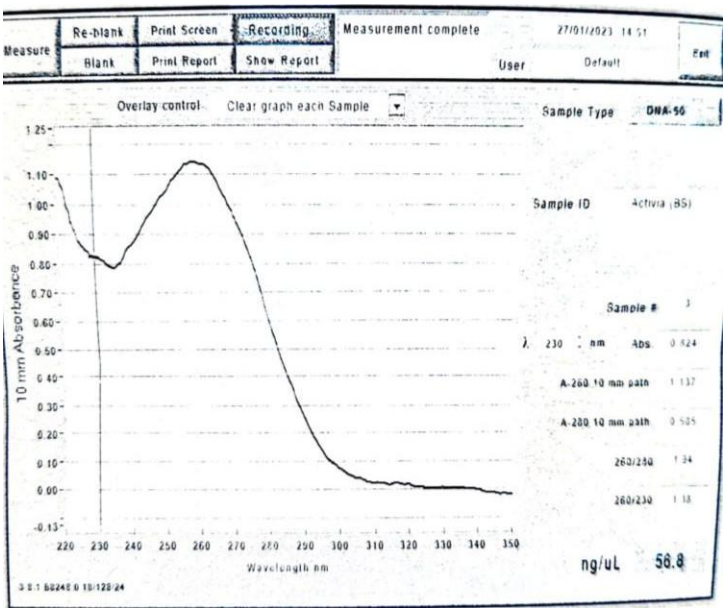


Figure 16. Nanodrop of Activia

3.7. ITS-PCR

In Figure 17 and 18, the PCR results showed the band size of 766 bps according to the primers designed for the 16S-23S r DNA ITS. The BB-12 serving as positive control for *bifidobacterium* showed a band size of 766 bps and the samples were also found positive for this gene that is responsible for the biofilm development in the *bifidobacerium.* No band was found in Nutrizing C showing the absence of this gene in the sample which represents the absence of *bifidobacteria* in Nutrizing C samples.

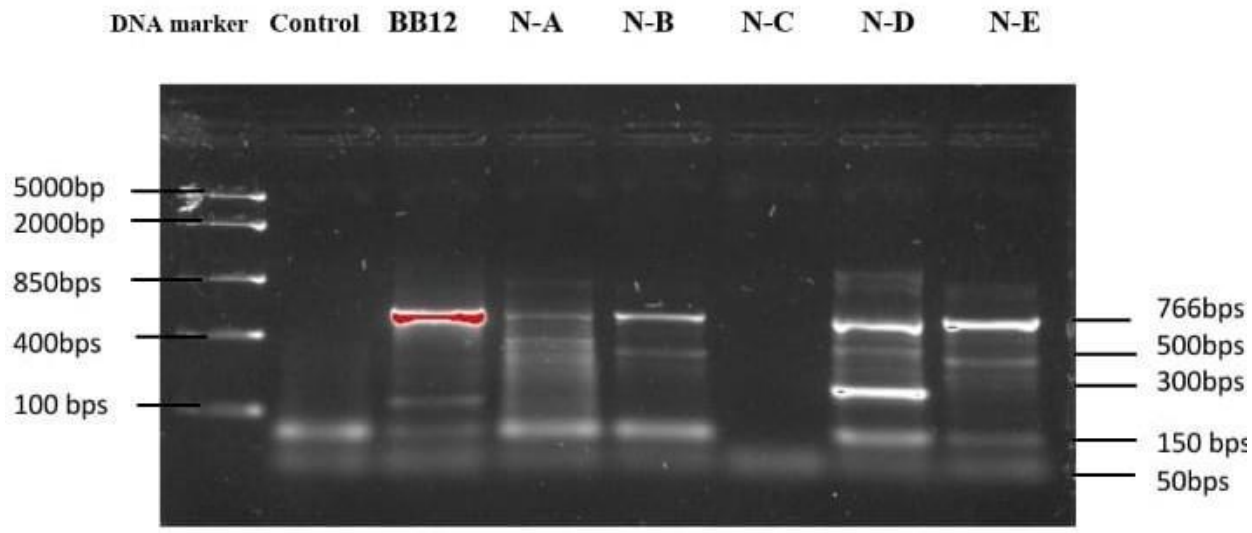


Figure 17. PCR for Sample 1-5

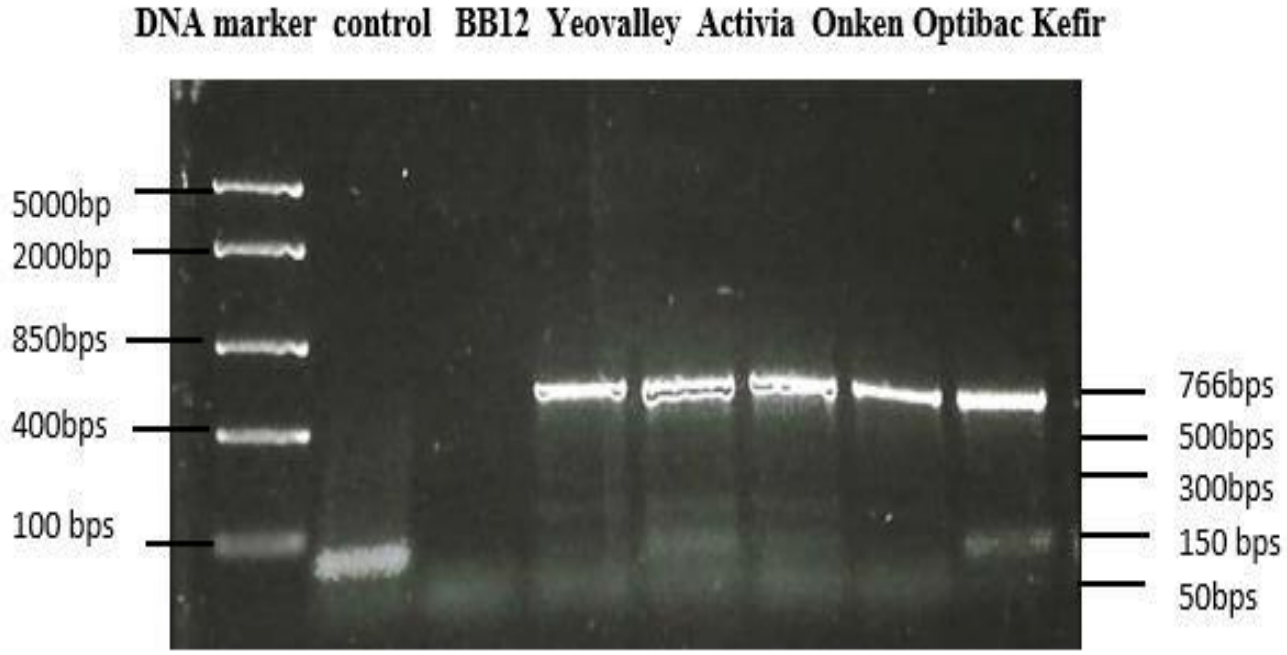


Figure 18. PCR for Sample 6-10

4. Discussion

4.1. Microbial diversity in Yoghurt and food supplements

Yoghurt contains a complex microbial ecosystem that includes LAB, yeast, and other bacteria. LAB are the dominant microorganisms in yoghurt and are responsible for its acidic taste and texture. In addition to *L. bulgaricus* and *S. thermophilus*, other LAB species such as *Lactobacillus acidophilus*, *Lactobacillus casei*, and *Bifidobacterium bifidum* can also be present in yoghurt. Yeasts such as *Saccharomyces* and *Kluyveromyces* can also be found in some types of yoghurt (Lourens-Hattingh & Viljoen, 2001). Other bacteria such as *Acetobacter* and *Streptococcus lactis* may also be present.

The microbial diversity of yoghurt can be influenced by several factors, including the milk source and processing, the starter culture used, and the fermentation conditions. For example, yoghurt made from raw milk may contain a more diverse microbial population than yoghurt made from pasteurized milk. The starter culture used can also affect the microbial diversity of yoghurt, as some cultures contain a wider range of microorganisms than others. The diversity of yoghurt's microbial population can also be impacted by fermentation factors like temperature, pH, and time.

Assessment of the microbial diversity was the goal of the research that was presented of a variety of food supplements and yoghurts, with a particular emphasis on determining whether or not these samples contained bifidobacterial characteristics. The popularity of *Bifidobacterium* in a variety of commercial products can be attributed to the fact that these bacteria have a probiotic effect. *Bifidobacterium* species such as *B. breve, B. longum, B. bifidum, B. infantis,* and *B. lactis* are frequently utilized in the production of probiotic products (Matsuki, Watanabe, & Tanaka, 2003).

In order to isolate and identify *bifidobacteria* in the yoghurt and food supplement samples, a number of different tests were carried out. The bifidobacteria colonies that were isolated and grown on RCA medium displayed smooth, round, milky white, and raised colonies (Figure 1), which are the typical characteristics of *bifidobacteria* (HENDRATI, KUSHARYATI, RYANDINI, & OEDJIJONO, 2017). Additionally, Gram staining of the isolated colonies revealed gram-positive, purple, short-branched rods, which verified the presence of *bifidobacteria* in the cultured samples (Figure 2). which accords with results of prior studies that have characterised

bifidobacteria as Gram-positive rods (Butta, Sardana, Vaishya, Singh, & Mendiratta, 2017). The presence of *bifidobacteria* in the isolated bacterial cultures was further supported by the results of the catalase test, which came back negative for all of the samples. In the scientific literature, *bifidobacteria* are referred widely as catalase-negative bacteria (HENDRATI et al., 2017; Tham, Peh, Bhat, & Liong, 2012).

The RapID^{TM} ANA II System was then utilized to validate the findings that *bifidobacteria* were present in the samples. With the assistance of the Remel RapID ANA II system, it is possible to recognize anaerobes that are of clinical significance. This system employs a chromogenic, enzyme technology-based method that uses a single substrate for its qualitative analysis (Chow, 2019). The results indicated that the samples from Activia B and Yeovalley A showed a maximum similarity of 98.84% with Bifidobacterium sp., while Nutrizing C was 97.93% similar to *Bifidobacterium sp.* (Table 5). However, the presence of other species of bacteria i-e., *Actinomyces israelii* in samples (Onken, Kefir, and Nutrizing E, *Bifidobacterium animalis* with 99.63%, 96.66%, 76.07%, 99.79% similarity respectively) instead of *bifidobacteria* raises the question about the quality of the source sample.

Actinomyces israelii is a type of filamentous anaerobic Gram-positive bacteria found in the mouth and upper gastrointestinal tract and is part of normal microflora in these areas (Eslick, 2019). Since a long time ago, *A. israelii* has been identified as the infectious agent responsible for actinomycosis (Tsuzukibashi et al., 2022). It is believed that the capacity of *Actinomyces israelii* to produce biofilms contributes to its virulence. This is because biofilms may give protection to *Actinomyces israelii* against antimicrobial agents as well as the immune system of the host. In a research, it was shown that *A. israelii* is capable of producing EPSs and of forming biofilm in vitro on the copper surfaces of intrauterine implanted contraceptive devices when the devices were submerged in a fluid that was meant to resemble uterine fluid and the conditions were anaerobic (Carrillo et al., 2010).

The morphology of bacteria belonging to the genera *Actinomyces* and *Bifidobacterium* is very similar (Fortner, 1928). Both of these bacterial groups are gram-positive, anaerobic, and have a propensity to form branching rods and filaments. This is the primary reason *A. israelii* wasn't identified in the initial biochemical tests. The use of the RapID ANA II system for the purpose of microbial identification in the current investigation may not have been the best choice because of

its lower efficacy in the previous research that has been published. In a comprehensive performance study that had been done previously by Marler et al., it was determined whether or not the RapID ANA II test was capable of accurately identifying 566 anaerobes. There were a total of 566 anaerobic isolates, and using this method, 68% of them were correctly identified to the genus and species level (Marler et al., 1991). In a different study, the RapID ANA II panel incorrectly identified a total of 9 clinical isolates out of a total of 300 clinical isolates. This represents a 3% error rate (Celig & Schreckenberger, 1991). Therefore, the identification of *Actinomyces israelii* in samples of Onken, Kefir, and Nutrizing E, as well as *Bifidobacterium animalis*, could also be attributed to an error in the efficacy of the kit. However, a clear explanation cannot be provided for this observation, and further research may be needed to elucidate this.

In conclusion the results of the research proposes that *bifidobacteria* are present in various food supplements and yoghurts, making them a potential source of probiotics. However, the possible presence of other species of bacteria in samples, as indicated by the Nutrizing C sample's results, shows the necessity for further research to identify and evaluate the microbial diversity of such products. Overall, the study offers useful information about microbial diversity of food supplements and yoghurts and emphasizes the importance of identifying and evaluating potential probiotics' sources.

4.2. *Bifidobacterium's* ability to produce biofilms in contrast to pathogenic bacteria

The capacity of microorganisms to create biofilms has been the subject of a great deal of study, however most of these researches have emphasized on biofilm development by pathogens and/or spoilage microbes only (Micrococcus, Streptococcus, Bacillus, Enterobacter, Listeria, and Pseudomonas)(Corbo, Bevilacqua, & Sinigaglia, 2017; Salo et al., 2006; Sharma & Anand, 2002; Waak, Tham, & Danielsson-Tham, 2002). Antibiotic resistance is on the rise, necessitating the development of novel intervention tactics to address microbial pathogenesis. Recently, probiotic biofilms have gained attention as a potential method of preventing the spread of pathogens.

The biofilm assay is a method that is frequently utilized in order to determine the potential of bacterial isolates to form biofilms. Crystal violet staining of the biofilm, followed by measurement of its optical density, is used in this method for determining in vitro studies which

strains are capable of producing biofilm (OD). By determining the optical density (OD) of the biofilm matrix and cells, this allows for an indirect determination of the amount of biofilm present (Ebert et al., 2021). There is a correlation between biofilm mass (measured as total carbon and cell mass) and biofilm optical density, which is measured as the intensity reduction of a light beam that is transmitted through the biofilm. Measuring optical density for biofilm quantification is more sensitive method than other methods that are commonly used to determine the extent of biofilm mass accumulation, and it requires less labor than those other methods (Bakke, Kommedal, & Kalvenes, 2001).

In this study, a biofilm assay was used to investigate the possibility of biofilm development by a variety of bacterial strains that had been isolated from yoghurt and food supplements, in addition to pathogenic controls. The results of the test provide useful information about the potential for these bacterial strains to produce biofilms and the implications these findings have for the health of human beings.

The majority of the isolated *bifidobacteria* from the yoghurt samples, with the exception of the Nutrizing B and Nutrizing C samples, were shown to be able to form biofilms with variable potential according to the present research. The sample from Yeovalley B had the greatest potential to form biofilms and was nearly identical to the positive control BB-12. It indicates that the *bifidobacterium* species found in this sample is extremely significant and should be further analysed in order to determine the precise strain used. It is important to mention here that probiotic yoghurt often contains *Bifidobacterium lactis* strain BB-12, a probiotic bacteria (Ejtahed et al., 2011). So, the *bifidobacterium* strain present in the Yeovalley B sample could be *Bifidobacterium lactis.* This microorganism is believed to enhance glucose tolerance in pregnancy (Laitinen, Poussa, & Isolauri, 2009; Luoto, Laitinen, Nermes, & Isolauri, 2010), reduce serum LDL cholesterol in people with type 2 diabetes (H. Sadrzadeh-Yeganeh et al., 2010) raise HDL (good) cholesterol in adult women(Sur et al., 2011), and improve HDL cholesterol in probiotic yoghurt. Moreover, it has been shown that giving BB-12 to premature newborns causes them to excrete more faecal secretory IgA (Mohan et al., 2008).

Previous research that evaluated the ability of different bifidobacterium strains to generate biofilms. In one study, six species of *bifidobacteria* (*Bifidobacterium adolescents, Bifidobacterium breve, Bifidobacterium breve longum, and Bifidobacterium bifidum*) were tested for their capacity

to produce biofilms on grape seed flour (GSF). All strains were able to form biofilms with variable strengths (Liu, Li, Fang, et al., 2021). From these findings it seems that various species of *bifidobacterium* have varying degrees of potential for the development of biofilm.

The biofilm-forming potential of pathogenic controls, including *Pseudomonas aeruginosa, Klebsiella pneumoniae, Staphylococcus aureus, Listeria monocytogenes, Cronobacter sakazakii, Bacillus subtilis,* and *Escherichia coli*, was also assessed. In the current study *Pseudomonas aeruginosa* was shown to have the greatest biofilm producing capacity among the pathogenic strain in both BHI and TSB media. This agrees with prior research that found it to be the most prevalent in clinical biofilm formation (Mathur et al., 2018). *Cronobacter sakazakii* was identified as the bacterium which showed the lowest potential for biofilm formation. *Klebsiella pneumoniae, Staphylococcus aureus,* and *Listeria monocytogenes* showed similar OD values that were lower but close to the cut off value. *Bacillus subtilis and Escherichia* coli had lower values than the cut off value, indicating a lower potential to form biofilms.

In comparison to these pathogenic strains, the isolates from all the experimental samples (Onken, Kefir, Activia B, Optibac B, Yeovalley A, and Nutrizing E) apart from Nutrizing B and Nutrizing C, have shown promising biofilm potential and hence probiotic properties.

4.3. The Genetics of *Bifidobacteria's* Probiotic Effect: Insights from PCR and ITS Fingerprinting in Yoghurt Samples

One important part of the research was the use of PCR for the identification of ITS region to detect *bifidobacteria* in yoghurt samples. The primers used for the PCR were specific for 16S-23S rDNA intergenic spacer (ITS) region of *bifidobacteria*. It is crucial for the manufacturers of probiotic products to accurately identify the microorganisms responsible for probiotic effect. Phenotypic tests are widely used in most laboratories as the primary means of bacterial identification. Fermentation of carbohydrates, analysis of cell walls, and detection of particular enzymes are commonly employed methods. However, this type of identification takes a long time and does not always produce definitive results. Comparing *bifidobacterium* species like *B. animalis* and *B. lactis,* or *B. infantis and B. longum*, both of which are used in commercial products, can be challenging using only phenotypic tests (Masco et al., 2004; Roy & Sirois, 2000; Roy, Ward, & Champagne, 1996). The correct identification and characterisation of bacteria, including *bifidobacterium*, is essential for microbiologists, researchers, and producers. Molecular methods

have been developed to aid in this process. Species of *bifidobacterium* important to humans (*B. bifidum, B. infantis, B. breve, B. longum, B. angulatum,B. adolescentis, B. catenulatum group, B. dentium, B. gallicum* and *B. dentium*) (Matsuki et al., 1999; Matsuki, Watanabe, Tanaka, & Oyaizu, 1998; Roy et al., 1996; Silvi, Verdenelli, Orpianesi, & Cresci, 2003; R.-F. Wang, Cao, Cerniglia, & microbiology, 1996), as well as strains used in commercial products (*B. lactis and B. animalis*) (Prasad, Gill, Smart, & Gopal, 1998; Marco Ventura, Reniero, Zink, & microbiology, 2001), can be detected using specific primer pairs based on 16S rDNA sequences or on 16S to 23S internal transcribed spacer sequence. In contrast to the traditional culture approach, the polymerase chain reaction (PCR) does not call for any anaerobic conditions, which is the primary benefit of using this method to identify *bifidobacterium* (Bielecka, Markiewicz, Wasilewska, & sciences, 2003).

Using the PCR amplification and gel electrophoresis, the extent of the diversity in the 16S–23S rDNA intergenic spacer (ITS) sequences of the individual isolates of 6 samples and the BB-12 control was examined in this part of research. Formerly, the most commonly used housekeeping marker for the identification purpose was the 16S rRNA gene. However, because of the high similarity that exists in the 16S rRNA gene sequence, several researchers have encountered into resolution issues at the genus and species level (Gonçalves & Rosato, 2002; Janda & Abbott, 2007). This resulted in the search for a new phylogenetic marker, which led to the discovery of the 16S-23S rDNA intergenic spacer (ITS). The genes that are responsible for the production of ribosomal RNAs in prokaryotic cells are organized in an operon following the sequence 5'-16S-23S-5S-3'. These genes are separated from one another by two spacer sections that are referred to as the ITS (Condon, Squires, & Squires, 1995). Due to its greater diversity within a genus compared to the nearby 16S and 23S rRNA genes, ITS may be a more effective target for species-level identification (García-Martínez, Martínez-Murcia, Antón, & Rodríguez-Valera, 1996; Khan, Selvaraju, & Yadav, 2005).

The 16S-23S rDNA ITS gene is present in *bifidobacterium* and plays an important role in the development of biofilm by *bifidobacterium*. These genes have been utilised in the past for the purpose of bacterial identification and typing due to the fact that they are present in all organisms, are highly conserved within the bacterial kingdom, have numerous copies in each genome, and are subject to spacer variation between different strains, species, and genera (Tsai, Lai, Yu, & Tsen,

2008). ITS fingerprints (i.e. Relative intensities and number of bands) generated from bacterial DNA isolated from various samples may vary, indicating that bacterial populations across samples vary significantly. It is thought that the probiotic effect of *bifidobacteria* may have a genetic basis, and that gaining an understanding of its genome sequence through the use of molecular techniques such as ITS-PCR could reveal the molecular adaptations that enable this gut commensal to thrive in its extremely competitive environment (M. Ventura, Turroni, Lugli, & van Sinderen, 2014).

Electrophoresis of the ITS amplified products revealed that all of the samples along with BB-12 control, with the exception of one, displayed the expected band at 766 bp. Nutrizing C, indicated a lack of the gene when amplified and run on gel. Previously no biofilm potential was seen for this sample and the lack of biofilm development in the Nutrizing C sample was verified by both the biofilm test and the ITS-PCR, which is an intriguing finding of the current investigation. Understanding which strain of *bifidobacterium* is represented in this sample will be a fascinating exercise in further characterisation studies. It will help in comprehending genetic variations in the context of biofilm production capacity. Moreover, the existence of multiple bands in the Nutrizing D and other samples may indicate the presence of two or more distinct strains of *bifidobacterium.*

In the future, a method of comparative genomics known as pan-probiosis (Barh, Soares, Tiwari, & Azevedo, 2020) may be utilized to locate probiotic-related genes that are shared by the strains that are present in positive samples or that are unique to a particular bacterial strain. This will allow for the identification of probiotics that are not only beneficial but also preventative.

It is vital to point out that although while the PCR results are suggestive of the presence of *bifidobacteria*, more study, such as sequencing could be used in order to find the presence of exact strain of *bifidobacterium*. In addition, the study only looked at a limited number of samples; hence, more research with larger sample sizes is required in order to arrive at findings that are more certain.

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