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**Research Article**

# Biofilm Formation and Genotypic Characterization of *Bifidobacteria* from Yoghurt Samples and Food Supplements

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## Abstract

This study evaluates the ability of yoghurt and supplement isolated *Bifidobacterium* speciesto generate biofilms through characterization of ITS region genes associated with biofilm formation. The objective of this project is to gain a better understanding of the ability of probioticBifidobacteria to build biofilms, which is thought to be beneficial for maintaining intestinal microbial balance and reducing the amount of pathogenic and food-spoilage microorganisms. Thiswork is distinctive because it aims to close a knowledge gap on the ability of *Bifidobacterium* species isolated from yoghurts and supplements to form biofilms. Giving important details on prospective applications for these probiotic *Bifidobacterium* species, particularly in the biomedical and food industries. Samples including yoghurt samples and food supplements will be cultured using RCA (Reinforced Clostridial Agar) media. The *Bifidobacteria* will be isolated and then identified using morphological and biochemical analysis. 9 controls of bacteria with established biofilm forming potential will be run. The crystal violet assay will be used to evaluate the ability of various microbes to produce biofilms, and the DNA extraction for the samples and controls willbe performed following ITS-PCR for the genotypic characterization. The DNA of the isolated *Bifidobacterium* species will be run through a gel electrophoresis procedure to determine its size. Comparison of the biofilm forming potential of *Bifidobacterium* species isolated from yoghurt andfood supplements with that of pathogenic bacteria known to build biofilms may provide light on the utilization of probiotics to avoid infections from several pathogens.

***Keywords:*** *Bifidobacteria; Yoghurt Samples; Biofilm*

## Introduction

Probiotics are defined as live microorganisms that, when supplied in suitable proportions,have been proved to promote the health of the host (Sánchez et al., 2017). Probiotic bacteria like *Lactobacillus acidophilus*, *Bifidobacterium spp*., and *Lactobacillus casei* have been linked to a multitude of health advantages (Rubin et al., 2022). Since lactic acid bacteria (LAB) have been shown to have positive health effects when consumed, they are routinely added to foods, especiallydairy products, and sold commercially in large quantities as probiotics (Ljungh & Wadstrom, 2006). Novel strains of *Lactobacillus* and *Bifidobacterium* have been assessed for probiotic potential and in vitro effects. The effects of these strains on the human intestine HT-29 cell line were also analysed, and it was found that *Lactobacillus plantarum* PBS068, *Lactobacillusrhamnosus* PBS070, and *Bifidobacterium animalis subsp. lactis* PBSO75 had the most potent probiotic qualities (Chen, Hsieh, Huang, & Tsai, 2017).

These organisms are being added to dairy products more frequently because of the possiblehealth benefits. Yoghurt consumption has been demonstrated to result in quantifiable health advantages associated to the presence of live bacteria (Mckinley, 2005).

Human studies show timeand time again that consuming yoghurt with live bacteria (*Streptococcus thermophilus* and *Lactobacillus delbrueckii sp. bulgaricus*) improves lactose digestion and eliminates lactose intolerance symptoms (Marco et al., 2017). Some of these results include maintaining intestinal structure and restoring gut flora, enhancing immunological barrier functions, and decreasing intestinal inflammation. Therefore, it is obvious that these cultures meet the current definition of probiotics (Guarner et al., 2005).

Numerous bacterial species, including members of the *Enterococcus, Enterobacter, Escherichia, Bifidobacterium*, and *Lactobacillus* families, colonize the human gastrointestinal tract (Koleva, Kim, Scott, & Kozyrskyj, 2015). The majority of the indigenous bacterial species found in the human gut, *Bifidobacteria*, are perhaps the most important for understanding the potential health benefits of this microbiota (Conlon & Bird, 2014). In addition to its amazing capacity to cling to epithelial cells and to utilize the host's metabolic pathways to metabolize glycans, this bacterium also exhibits remarkable physiological and genetic features. A number of beneficial health effects are associated with the microbiota, including regulation of intestinal microbial homeostasis, inhibition of pathogens and harmful bacteria that colonize and/or infect thegut mucosa, modulation of local and systemic immune responses, inhibition of pro-carcinogenic enzymatic activities within the microbiota, vitamin production, and bioconversion of a number ofdietary compounds into bioactive molecules (Rossi & Amaretti, 2010). The most common speciesof gut-colonizing bacteria passed from mothers to their offspring are *Bifidobactreium bifidum* and *Bifidobacterium breve* (Cukrowska, Bierła, Zakrzewska, Klukowski, & Maciorkowska, 2020).

Antibiotic-resistant microorganisms, according to the World Health Organization, have emerged as a result of the widespread and careless use of antibiotics, e.g., vancomycin resistanceexhibited by *Pediococcus* and *Leuconostoc* species (Zhang, Xu, Yang, Chou, & He, 2022). Clinicalinfections caused by MRSA were successfully treated with vancomycin (Micek, 2007). Therefore,the medical breakthroughs of the last century may be lost as a result of the rapid and widespread development of antibiotic resistance in bacteria. Assuming this tendency continues, antibiotic therapy may become ineffective in the coming years, leading to more severe illnesses among the general population. Probiotics can serve as a source of avoiding the infections and limiting the useof antibiotics which will serve as a way of controlling antibiotic resistance issue (Cars, Hedin, & Heddini, 2011).

Bacterial cells can increase their chances of surviving in difficult settings by forming biofilms, which operate as a protective mechanism (Chmielewski, Frank, & safety, 2003). Therefore, it is reasonable to develop methods for producing *bifidobacteria*l biofilms that are inspired by the properties of such biofilms in nature. To prevent the spread of pathogenic and spoilage microorganisms, *bifidobacteria*l biofilms may one day be used in both industrial and medical settings (Speranza, Liso, Russo, & Corbo, 2020). One hundred eighty isolates from sevendifferent *Bifidobacterium* species were examined in vitro using a subtractive technique to identifygood and undesirable features (Delgado, O'sullivan, Fitzgerald, & Mayo, 2008). About 20% of these isolates could grow at pH 3–5, and about 45% of them could grow in 2% bovine bile. Unwanted enzymatic activity, such as those of N-acetyl-glucosaminidase, glucuronidase, and chymotrypsin, was not found (Delgado et al., 2008).

The present study aims to isolate and genotypically characterize the *Bifidobacteria* for biofilm formation potential, found in yoghurt and food supplements. Yoghurt is well established source of a variety probiotic bacteria including *lactobacillus* species, the present study aims the genotypic characterization of probiotic *Bifidobacterium* strains isolated from yoghurts and dietarysupplements for its potential to generate biofilms as compared to pathogenic organisms known toform biofilms. It will also find the presence of biofilm forming genes in *Bifidobacterium* species isolated from yoghurts and dietary supplements. The study will provide an insight towards use of*Bifidobacteria* in yoghurts and other fermented food supplements as the bacteria has beenassociated with excellent probiotic properties.

## Methods

### Samples

4 yoghurts samples and 2 food supplements, as mentioned in table 1. will be selected for the isolation of Bifidobacterium and the yoghurts and food supplements will be purchased from theTesco UK and Amazon.

**Table 1.** 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 | Activin Strawberry Yoghurt |
| 5. | Food Supplement | NutriZing (16 Strain Multibiotics: 30 CFU/serving) |
| 6. | Food Supplement | Optibac Probiotics Everyday Max |

### Isolation and Identification of *Bifidobacteria*

One of the most used diluents for counting *Bifidobacteria* in dairy products water with saline (Roy, 2001). The yoghurts and food supplements will be diluted in normal saline. To get reach a certain type of bacterium, researchers use selective culture mediums. This type of media is used to prevent the growth of unwanted microorganisms by including a variety of inhibitors in the growth conditions (Harrigan & McCance, 1976). For the culturing of the Bifidobacterium RCA(Reinforced Clostridial Agar) medium with the addition of lithium mupirocin will be used(Modesto, 2018).

For the identification of *Bifidobacterium* morphological analysis and biochemical testing will be employed. Gram staining technique will be used to confirm the presence of *Bifidobacterium*in the isolated bacterial colonies. Catalase test will be done for the biochemical characterization of*Bifidobacterium* (Behrad, Yusof, Goh, Baba, & Technology, 2009). In addition to this, RapID™ ANA II System will be utilized to positively identify the isolated *Bifidobacterium* species.

### Experimental Controls and Culturing of the Controls

The pathogenic controls will also be employed in the study. The 9 control groups with theconfirmed biofilm forming potential will be used, according to table 2.

**Table 2.** Pathogenic Controls with Growth Media.

| Sr. No | Control Name | Media for Culturing |
|---|---|---|
| 1. | *Bacillus subtilis* 8054 | Nutrient Agar |
| 2. | *Escherichia coli* K12 (8797) | Nutrient Agar |
| 3. | *Staphylococcus aureus* 10442 (MRSA) | Nutrient Agar |
| 4. | *Klebsiella pneumonia* NTCT 13368 | MacConkey agar |
| 5. | *Streptococcus mutans* | Nutrient Agar |
| 6. | *Cronobacter sakazakii* | Tryptone Soy Agar |
| 7. | *Pseudomonas aeruginosa* ATCC 27853 | Nutrient Agar |
| 8. | *Listeria monocytogenes* | Blood Agar |
| 9. | *Bifidobacterium animalis* subsp lactis (BB-12) | TOS-MUP agar |

### Determination of Biofilm Formation Potential

Biofilm assay procedure will be utilized to determine the potential of *Bifidobacterium* to form biofilm. Crystal violet technique is a potential method to test the biofilm formation potentialof *Bifidobacterium* (Riedel et al., 2009). The samples and the controls will be subjected to biofilmassay.

### DNA Extraction

The bacterial colonies from the samples and controls will be taken and treated to extract the DNA. The DNA extraction protocol will be followed after the literature review and the appropriate method mentioned by (Matsuki, Watanabe, & Tanaka, 2003) will be applied.

### Genotypic Characterization of *Bifidobacteria*

For confirmation at the genetic level PCR will be performed. The ITS-PCR followed for the genotypic characterization of *Bifidobacteria*. The primers for the ITS region will be made andthe PCR will be run. The primers for the ITS-PCR will be used for 16S-23S rDNA ITS gene, table3.

After the PCR, the PCR product will be subjected to gel electrophoresis along with a marker to identify the size of the PCR product which will confirm the presence of *Bifidobacteria* in the yoghurts and food supplements.

**Table 3.** ITS Primers.

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

### Statistical Analysis

One-way ANOVA and Post Hoc Test will be performed for the statistical analysis. This will compare the biofilm formation potential of *Bifidobacteria* from yoghurts & food supplementsand controls. The analysis will be performed using SPSS (Statistical Program for the Social Sciences). The p values ($p < 0.05$) will be considered as significant.

### Research flowsheet

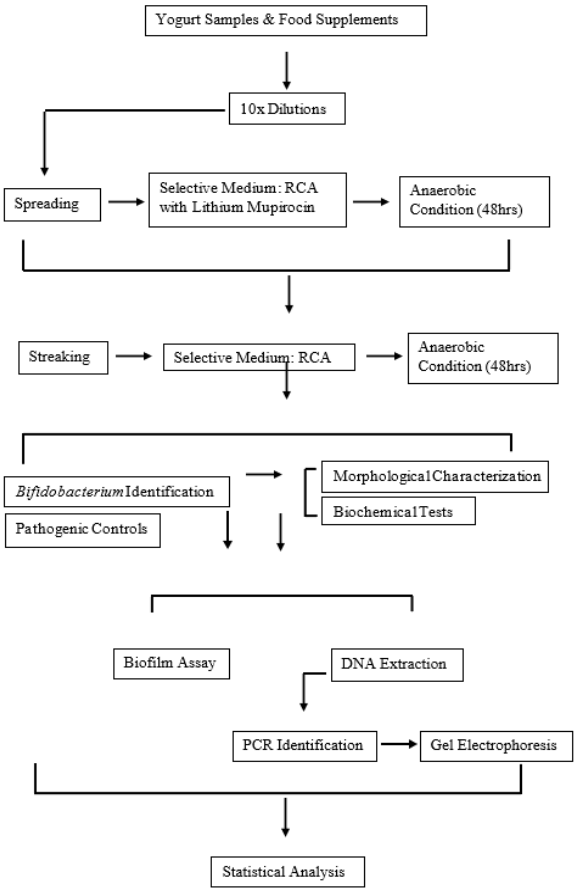


**Figure 1. Research Flowsheet**

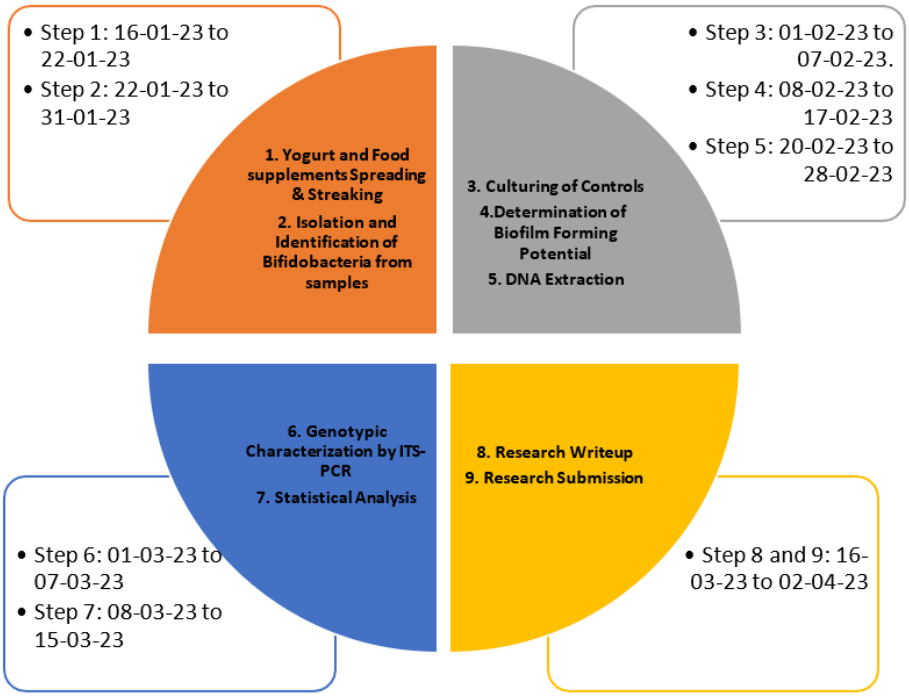


**Figure 2:** Research Parts divided according to the time frame.

## List of Equipment

| Sr. No | Name |
|---|---|
| **1.** | Petri Plates |
| **2.** | Flasks |
| **3.** | Test Tubes |
| **4.** | Inoculating Loops |
| **5.** | Bunsen Burner |
| **6.** | Reagent Bottles |
| **7.** | Spreader |
| **8.** | Slides |
| **9.** | Microscope |
| **10.** | Eppendorf |
| **11.** | ELISA Flat Bottom 96-wells plate |
| **12.** | PCR Machine |
| **13.** | Gel Tanks |
| **14.** | Water Bath |
| **15.** | Heating Blocks |
| **16.** | Gel Doc |
| **17.** | Centrifuge Machine |
| **18.** | Anaerobic Chamber |
| **19.** | Incubator |
| **20.** | Fridge -4°C |
| **21.** | Weighing Balance |

**List of Chemicals**

| Sr. No | Name |
|---|---|
| 1. | 100% Ethanol |
| 2. | Acetic Acid |
| 3. | TBE Buffer |
| 4. | Agarose |
| 5. | Crystal Violet |
| 6. | Phosphate Buffer Saline |
| 7. | GelRed Nucliec Acid Stain |
| 8. | 10X PCR Buffer |
| 9. | Gram Iodine |
| 10. | Safranin |
| 11. | Alcohol |
| 12. | DNA Molecular Markers |
| 13. | Reinforced Clostridial Agar (RCA) |
| 14. | Lithium Mupirocin Supplement |
| 15. | Brain Heart Infusion Broth |
| 16. | Trypton Soy Broth |
| 17. | Hydrogen Peroxide |
| 18. | Sodium Chloride |
| 19. | Methanol |
| 20. | McFarland Standards |
| 21. | MRS Medium |
| 22. | Methanol |
| 23. | Yoghurts Samples |
| 24. | Food Supplements |
| 25. | dNTPs |
| 26. | Taq Polymerase |
| 27. | Nutrient Agar |
| 28. | Instagene Matrix |
| 29. | RapID ANA II System |
| 30. | RapID Spot Indole Reagent |
| 31. | RapID 1mL Inoculation Fluid |
| 32. | *Listeria monocytogenes* |
| 33. | *Klebseilla pneumonia NTCT13368* |
| 34. | *E.coli K12 (8797)* |
| 35. | *Cronobacter sakazakii* |
| 36. | *Bacillus subtilis 8054* |
| 37. | *Bifidobacterium animalis subsp. Lactis (BB-12)* |
| 38. | *Streptococccus mutans* |
| 39. | *Staphylococcus aureus 10442 (MRSA)* |
| 40. | *Pseudomonas aeruginosa ATCC27853* |

## Research Hypothesis

*Bifidobacterium* strains isolated from yoghurts and dietary supplements contains genes forbiofilm formation and have more potential to generate biofilms as compared to pathogenicorganisms known to form biofilms.

## Adaptation of Research to Non-Laboratory Format

Due to the possibility for COVID-19 to cause a delay in the previously specified goals, this research will be conducted as a systematic review combined with bioinformatics analysis. Primarypapers for the systematic review will be gathered from reliable sources like PubMed, Google Scholar, Elsevier, ResearchGate, etc. These publications' data and information will be carefully crafted to shed light on the study's objectives. The identification and determination of potential genes connected to biofilm formation in *Bifidobacterium* species will be done using bioinformaticsanalysis.

## Ethical Consideration and COSH

All significant legal, moral, and social obligations connected to this research endeavour have beenthoroughly assessed, and any potential health and safety concerns have been appropriately taken into account.

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