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Probiotics Metabolism of Prebiotics: Selectivity and Biofunctional Metabolite Diversity Varies Under Variable Conditions.

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Abstract: Consumption of non-digestible dietary fibers, or prebiotics, is increasingly being prescribed, encouraged, and recommended by physicians, and dietary specialists to maintain health. Gut microorganisms selectively ferment prebiotics, producing specific metabolite profiles. We believe gut microbial populations and their metabolic activity can be modulated through targeted manipulation of prebiotic composition. Hence, we aimed to engineer gut metabolites using different fiber blends and oxygen supply as a versatile platform with clinically relevant bioactivities. In this study, the effect of commercially available prebiotics from various brands on the growth of probiotics is evaluated. The selective fermentation of prebiotics by different probiotics has been observed. Moreover, unique metabolic profiles produced by different probiotic strains in different prebiotics under aerobic and anaerobic conditions having different antimicrobial and antioxidant activities shed light on the gut modulation strategies to get the maximum benefit of gut microbial diversity. Levan (fructooligosaccharides), which was not an ingredient of any commercial prebiotic, but its effect on probiotics was evaluated in this study, exhibited high biological activities, demonstrating its excellent prebiotics promise for future uses. Our work provides a robust foundation for engineering gut metabolites to modulate host-microbiome interactions and supports the use of customized prebiotics as a worthwhile strategy for personalized medicine and clinical intervention.

Keywords: Probiotics; Commercial Prebiotics; Levan, Gut microbiota, Gut Metabolites, Antioxidant activity, Antimicrobial activity.

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1. Introduction

Probiotics are known as live microorganisms, which confer health benefits to their host when consumed in adequate amounts. They have gained an increased interest in many fields including clinical research, as they play a major role in modulating gut microbiota, maintaining and promoting metabolic homeostasis [1]. A prominent characteristic of probiotics is how they selectively ferment dietary fibers and prebiotics that results in boosting overall health. Prebiotics are non-digestible dietary fibers that selectively enhance growth of beneficial bacteria only and confer a beneficial effect on the host. Prebiotics predominantly include carbohydrate-based substrates such as inulin, fructooligosaccharides (FOS), galactooligosaccharides (GOS), and xylooligosaccharides (XOS) and some non-carbohydrate prebiotics like phenols and phytochemicals [2,3].

Soluble prebiotic fibers such as inulin and GOS are easily fermented, allowing probiotics to produce metabolites like SCFAs [4]. Contrary, insoluble fibers are less fermentable, but may facilitate cooperative microbial interactions, enhancing gut microbial diversity and stability [5].

Selective fermentation not only exhibits a beneficial impact on the gut microbiota but also leads to the production of unique secondary metabolites. The produced

metabolites are not only influenced by its metabolizing probiotics, but also by the composition and complexity of the prebiotics consumed. Additionally, prebiotics not only affect the gut microbiota, but also directly or indirectly affect the other distant organs and systems of the body. For instance, lactulose is a synthetic prebiotic disaccharide composed of galactose and fructose. It is fermented in the colon resulting in an acidic environment due to the production of short chain fatty acids (SCFAs). The resulting gut environment improves the solubility and bioavailability of minerals such as calcium and magnesium, facilitating their absorption, and subsequently ensuing in beneficial effect on bone health [6]. SCFAs, like acetate, propionate, and butyrate, are within the most produced and studied secondary metabolites, as they maintain gut epithelial integrity, modulate immune responses, and have anti-inflammatory effects [7]. In addition to SCFAs, probiotics can produce a wide variety of metabolites, like bacteriocins, exopolysaccharides, vitamins, phenols, and other bioactive compounds, which in return have biological activities such as antimicrobial, antiviral, antioxidant, immunomodulatory, and neuroprotective [8-10].

Since the significance of prebiotics in human health has been recognized recently and health practitioners recommend prebiotics to manipulate different diseases, several blends of prebiotics have been introduced into the market by different pharmaceutical industries. A combination of distinct dietary fibers/prebiotics may be necessary to enhance gut microbial diversity, as different fiber types possess unique properties that influence the growth of specific microbial species. This will facilitate selective fermentability, and by-products produced during microbial fermentations could be utilized by other microorganisms in the colon, consequently producing distinct metabolites through cross-feeding mechanisms [11]. However, currently, limited data is available about the effects of commercial prebiotic supplements on the growth of common gut microorganisms and if they have selective utilization of some of the components of a prebiotic blend. Moreover, it will be interesting to evaluate what type of metabolites could be produced after selective utilization of prebiotics and if these metabolites have some biological activities to have an impact on the host's health.

In this context, the aim of the present study was to access the selective utilization of commercial prebiotics, having a variety of prebiotic molecules, both carbohydrate and non-carbohydrate-based, by purified probiotic strains and a consortium of six abundant gut microorganisms under aerobic and anaerobic conditions in-vitro. We tested the effect of prebiotics on the growth of probiotic strains and also extracted their extracellular metabolites to evaluate their metabolic profile. We further accessed the biological activities of extracted metabolites to understand the impact of different prebiotic blends on human health through these metabolites. To our knowledge, this is the first study presenting the potential of modulating the metabolic profile of probiotics with diverse prebiotics to obtain unique biofunctional molecules in the laboratory for industrial applications.

2. Materials and Methods

2.1. Probiotic strains

The probiotic strains used in this study were carefully selected from different sources to ensure a diverse microbial composition. The strains included *Lactococcus lactis* (mentioned as Y strain), isolated from yogurt; *Bacillus sp., firmicutes* (mentioned as S strain) purified from a commercial probiotic sachet; and a bacterial consortium consisting of *Lactobacillus acidophilus, Lactobacillus casei, L. lactis, Bifidobacterium bifidum, Bifidobacterium infantis,* and *Bifidobacterium longum* obtained from ATCC.

2.2. Prebiotic samples

Nine prebiotic supplements with diverse compositions were collected from pharmacies and healthcare stores in the United Arab Emirates. Prebiotic supplements were selected based on their distinct formulations, as detailed in Table 1, to assess their impact on the growth of different probiotics and to evaluate their metabolic profile. *P*rebiotic

samples used in this study were available in either lyophilized or powdered form. Additionally, a potential prebiotic polysaccharide levan was also used in this study. Levan-producing bacterium *Bacillus paralicheniformis* LB1-1A was sourced from Nasir *et al.,* [12] and the molecule was synthesized and purified following the protocol described in their work.

Table 1 Detailed composition of selected commercial prebiotic supplements

Samples	Formulations of commercial prebiotics supplements
Prebiotic 1	Inulin, fructooligosaccharides (FOS), burdock root, arabinoglactan bark. *L. acidophilus*, *Bifidobacterium lactis, B. longum, Lactobacillus plantarum,* slippery elm bark, hemicellulose, phytase, glucanase, xylanase, pectinase, gelatin and maltodextrin
Prebiotic 2	Acacia seyal
Prebiotic 3	Inulin, FOS, organic acacia fiber, organic Jerusalem artichoke fiber, organic green banana fiber and resistant starch
Prebiotic 4	Maltodextrin
Prebiotic 5	Psyllium seed husk, calcium carbonate, FOS, magnesium oxide, acacia gum, non-GMO soy fiber, gluten-free oat bran, apple fiber, Selenium amino acid chelate, USP kosher gelatin capsule
Prebiotic 6	Xylooligosaccharides (XOS), galactooligosaccharides, guar gum, tapioca starch, cellulose, coconut milk, xylitol, glyceryl behenate, natural flavor and silicon dioxide
Prebiotic 7	XOS, gum Arabic, cellulose, maltodextrin, mannitol, glucose, D-Xylose, stearic acid, croscarmellose sodium, arabinose, xylitol and silica
Prebiotic 8	Inulin, prebiotic fibers, polyphenolic compounds, glucose, fructose, sucrose, Jerusalem artichoke root extract, oat fiber, blueberry fruit extract, pomegranate fruit extract, steviol glycosides (stevia leaf extract), citric acid and natural flavors
Prebiotic 9	Soluble fibers, insoluble fibers, polyphenolic compounds, organic prebiotic fiber blend, vitamin C, limonene, pectic-oligosaccharides, flavonoids, pectin, acacia fiber (gum arabic), organic orange peel, organic apple peel, baobab (fruit), and organic cranberry (seeds)

2.3. Water solubility of prebiotics

Prebiotic fibers are categorized as either water-soluble or insoluble, each with distinct properties that influence human health in different ways. To assess the water solubility of each sample, 1 gram of each prebiotic supplement was dissolved in milli-Q water at room temperature (around 22 ℃) to prepare a 10% (w/v) solution in preweighted falocon tubes. The solution was thoroughly mixed and then centrifuged. The resulting fractions were separated into two tubes: one containing the soluble portion and another containing the insoluble pellet. The insoluble fraction was then dried in an incubator at 60 °C and weighed to determine the solubility percentage using the following formula.

$$\%\ insolubility\ = \frac{mass\ of\ insolube\ sample\ after\ drying\ (g)}{The\ intial\ mass\ of\ the\ sample\ \ (around\ 1g)} \times 100$$

2.4. Effect of prebiotic supplementation on the growth of L. lactis and Bacillus sp.

The effects of commercial prebiotic supplements and levan on the growth of *L. lactis* and *Bacillus sp.* were examined under aerobic conditions. For this, nutrient broth (NB) and de Man, Rogosa & Sharpe (MRS) media were prepared and sterilized by autoclaving for *Bacillus sp.* and *L. lactis*, respectively. One-fifth of the original concentration of the NB and MRS ingredients was used to prepare the media, allowing the prebiotics to serve as the carbon source. The prebiotic samples were sterilized using ultraviolet radiation and filter sterilization methods and transferred to the sterilized NB and MRS media (1% w/v). Pre-inocula were prepared and inoculated to respective media at a concentration of 1% v/v. The cultures were incubated at 37 °C and 170 rpm for 96 hours. NB and MRS media having the same cultures without prebiotic supplementions were used as controls. Aliquotes were taken out at regular time intervals (0, 4, 8, 12, 24, 48, 72 & 96 h), and the growth profile was monitored by measuring the optical density (OD_{600nm}) using a Shimadzu UV1900i spectrophotometer. The growth was then plotted as a function of time. At the end of incubation, pH was also measured for all the prebiotic-supplemented cultures. Gut microbial consortium of six strains was not cultured under aerobic conditions because of the presence of anaerobic strains in it.

2.5. Fermentation of prebiotics under anaerobic conditions

Since the gut environment is anaerobic, the fermentation capabilities of probiotics of selective prebiotics were evaluated under anaerobic conditions. Three commercial prebiotics, 2, 3, and 9, out of nine, were selected for this experiment on the basis of their water solubility and diversity of ingredients. Furthermore, lab-synthesized levan was also used to evaluate its prebiotic potential under anaerobic conditions. Both probiotic strains, *L. lactis, Bacillus sp.,* and gut microbial consortium consist of 6 strains of *Lactobacillus* and *Bifidobacterium* (*L. acidophilus*, *L. casei*, *L. lactis*, *B. bifidum*, *B. infantis*, and *B. longum*) were grown in the presence of three selective prebiotics. A one-fifth-strength dilution of NB medium was used for *Bacillus sp.*, and a one-fifth-strength dilution of MRS medium was prepared for *L. lactis* and the consortium. Both media were sterilized by autoclaving at 121 °C. The media were then supplemented with 5% of serilized prebiotics, followed by the addition of a 1% (v/v) inocula. Media containing probiotic strains (1% v/v) without adding prebiotics were used as a control. The inoculated cultures were immediately placed in an Anaerobic System Mark II (LE002-1NO) - Hi Media Jar equipped with an anaerobic gas pack (3.5 L, LE002A-5NO - Hi Media) to eliminate oxygen. The jar was securely sealed and incubated at 37 °C for 96 hours. After incubation, samples were collected for metabolite analysis, and the pH of all samples was measured to assess secondary metabolite production. The growth profile of the cultures could not be monitored at regular intervals due to the strict anaerobic environment in a sealed container.

2.6. Extraction of secondary metabolites

Secondary metabolites produced were extracted from both aerobic and anaerobic sample cultures using ethyl acetate (1:1 ratio). An equal volume of ethyl acetate was added to cultures and mixed vigorously for 5 minutes. The mixture was then left undisturbed on the bench to facilitate the separation of the organic and aqueous layers. The upper organic layer containing the secondary metabolites, was carefully collected, and the solvent was evaporated under a safety hood overnight to concentrate the metabolites.

2.7. Thin layer chromatography (TLC) analysis of metabolites

TLC analysis was conducted to evaluate the metabolic profile produced as a result of prebiotics fermentation, following the method described by Zahiruddin *et al.*, [13], with

slight modifications. Briefly, the concentrated secondary metabolites were redissolved in 1% w/v ethyl acetate, and 5 µL of each sample was separately applied onto the TLC plate (Silica gel 60 F254; Merck). TLC plate was positioned vertically in a TLC tank containing a mobile phase consisting of toluene, ethyl acetate, and formic acid in a 5:4:1 (v/v/v) ratio. Gallic acid and quercetin served as reference standards. After allowing the mobile phase to migrate on the TLC plate for approximately 30–40 minutes, the plate was air-dried and subsequently examined under UV light at wavelengths of 254 nm and 366 nm.

2.8. Antimicrobial activity assay

The antimicrobial activity of secondary metabolites was assessed against *Escherichia coli* using an agar well diffusion assay. Nutrient agar plates were prepared, and 50 µL of overnight-grown *E. coli* culture was spread evenly on the surface of the agar. Wells with a diameter of 8 mm were created. Extracted secondary metabolites (1 mg) from both aerobic and anaerobic fermentations were dissolved in 100 µL of sterilized deionized water and added to the wells. A 100 µL solution of 100 mg/mL ampicillin was used as the positive control, and sterilized water was used as the negative control. The plates were incubated at 37 °C overnight, and the clear zones of inhibition around the wells were measured in millimeters to determine the antimicrobial activity of the samples.

2.89. Antioxidant activity using DPPH assay

The DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging capability of the metabolites was assessed using a method previously described [14]. Briefly, 0.1 mM DPPH solution was prepared in methanol, while the samples and ascorbic acid (used as the standard) were dissolved in an appropriate solvent (water/methanol) at a concentration of 25 µg/mL. The reaction mixture (200 µL) was set up in a sterile 96-well plate by combining the DPPH solution with the sample in a 1:1 ratio. A separate well containing 200 µL of 0.1 mM DPPH alone served as the control. The plate was incubated at room temperature in the dark for 30 minutes. After incubation, the absorbance was recorded at 517 nm using a microplate reader (Hidex Sense). The scavenging activity of the samples was calculated using the following equation:

$$Antioxidation = \frac{A_o - A_1}{A_o}\ x\ 100$$

Where Ao represents the absorbance of control and A1 denotes the absorbance of the sample. A standard curve was plotted for ascorbic acid using 5, 10, 15, 20, and 25 µg/ml concentrations (Figure 1).

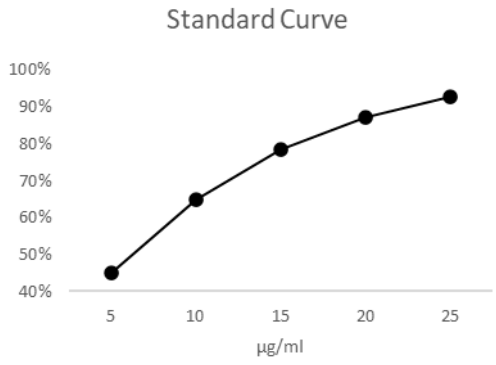

Figure 1. Standard curve for DPPH assay using ascorbic acid with different concentrations

3. Results

3.1. Water solubility analysis of the prebiotics

The solubility of each prebiotic sample was determined by measuring the amount of insoluble part in milli-Q water. As shown in Table 2, prebiotic 4 was completely soluble in water, whereas prebiotics 1 & 6 exhibited the least solubility of approximately 70%. The enhanced solubility of prebiotic 4, composed of maltodextrin, may be attributed to the physicochemical properties of the hydroxyl groups present in its glucose molecules. The insolubility of certain prebiotics may be attributed to their composition, which includes diverse plant extracts, hemicellulose, gelatin, and similar components that are insoluble at room temperature.

Table 2. Water solubility analysis of the prebiotic products in water. *Absorbed all water, provided a gel, thus excluded from the study.

Prebiotic No.	1	2	3	4	5	6	7	8	9
Sample mass (g)	1.0004	1.0045	1.0013	1.0003	1.0039	1.0097	1.0012	1.0067	1.0048
Mass of insoluble portion after drying (g)	0.3086	0.0104	0.1189	0	*	0.3054	0.0449	0.1441	0.0975
% Insolubility	30.8 %	1.0%	11.9%	0	*	30.2%	4.5%	14.31%	9.7%

3.2. Fermentation of prebiotics by L. lactis and *Bacillus sp. in aerobic environment*

3.2.1. *Growth profile*

To assess the capability of *Bacillus sp.* and *L. lactis* to utilize different prebiotics as a carbon source, the growth profile was observed at different time intervals. Figure 2 shows the growth profile of *Bacillus sp.* when supplemented with commercial prebiotics or levan. In general, bacterial growth was increased over time, and maximum bacterial growth, determined as OD_{600nm}, was observed at 96 h. Interestingly, for most prebiotics tested, the growth profiles were comparable with the control containing a complete set of ingredients in the growth medium. The observations suggest that the prebiotics effectively replaced the growth medium's carbon source. However, few exceptions were observed with prebiotics 6 and 8, which supported comparatively lower bacterial growth. This data strongly suggests the selective utilization of different prebiotics when fermented by a single bacterium.

A more pronounced trend was observed in the growth pattern of *L. lactis* (Figure 3), with most prebiotics promoting enhanced growth compared to the control group. Interestingly, while *Bacillus sp.* and *L. lactis* exhibited comparable optical densities (approximately 1.5–1.8) after 96 h of incubation, their growth profiles differed significantly over time. For instance, *Bacillus sp.* efficiently fermented prebiotic 9 within the first 24 h of incubation, as evident from the exponential trendline (Figure 2). In contrast, for the same prebiotic 9, *L. lactis* followed a linear trendline between 8 and 48 h of incubation; and a significant increase in OD was observed only after 48 h, suggesting a slower rate of digestion. *Bacillus sp*. didn't show high growth in levan however, levan supported the growth of *L. lactis*. These data suggest that the growth profile of different bacteria is determined not only by the structure and composition of the prebiotic substrates but also by the probiotic strains used.

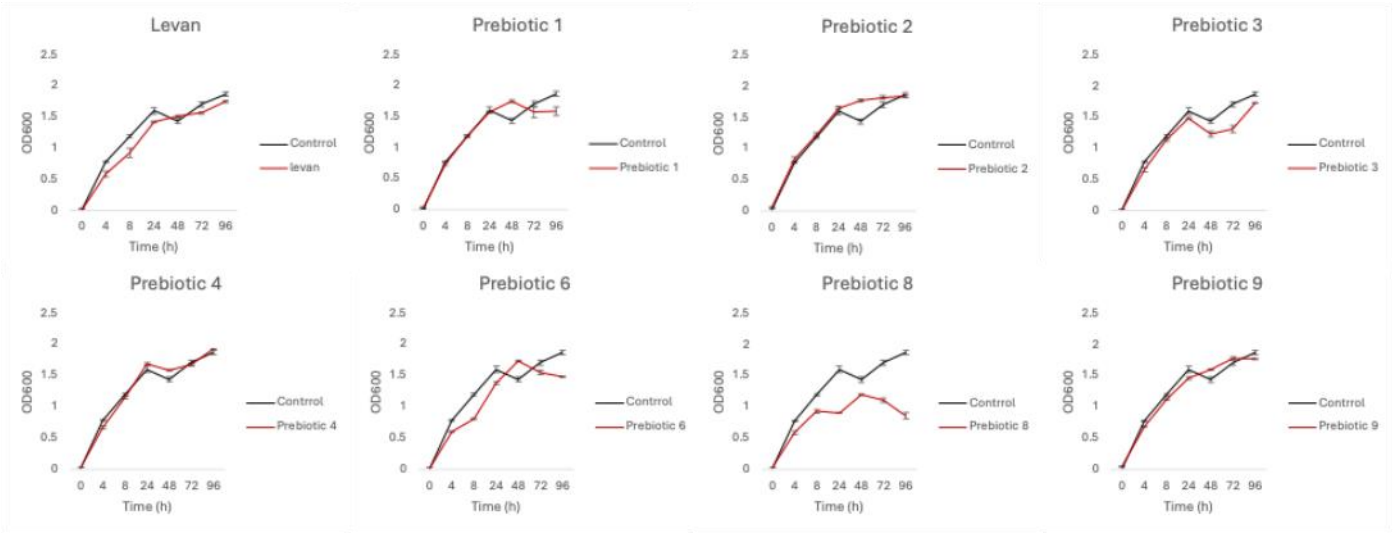

Figure 2. Growth profile of *Bacillus sp.* supplemented with different prebiotics

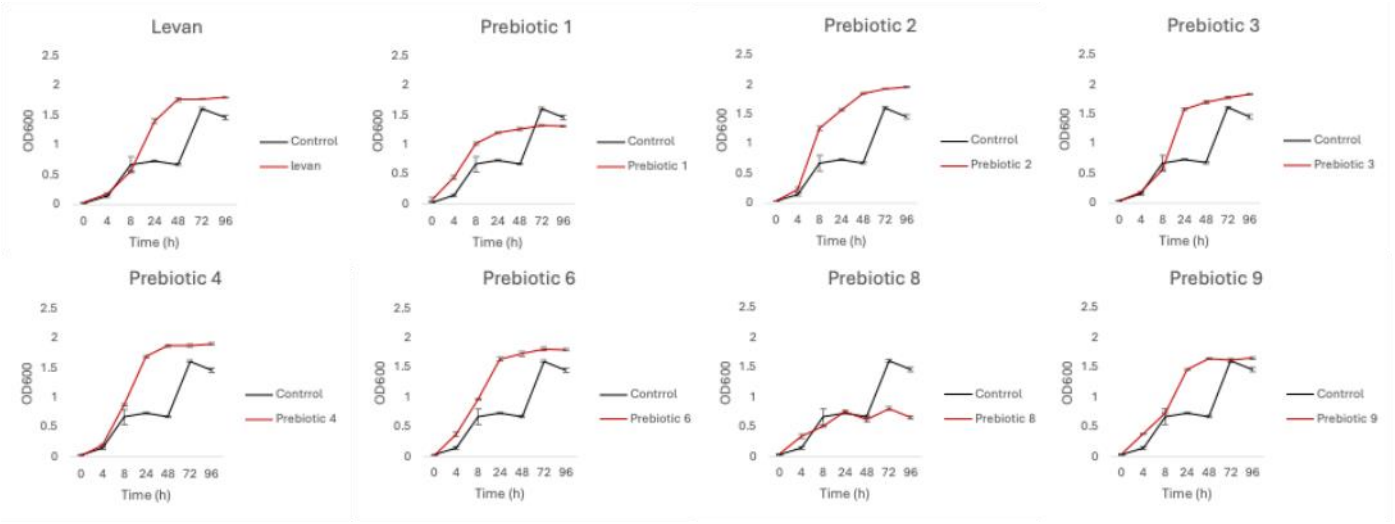

Figure 3. Growth profile *L. lactis* supplemented with different prebiotics

3.2.2. *pH change*

A change in pH of the culture media was observed after 96 h of fermentation under aerobic conditions. Initially, the pH of NB and MRS media was neutral at the time of inoculation. Interestingly, after 96 h of incubation, an alkaline change was observed for most of prebiotics supplements when fermented by *Bacillus sp.* and *L. lactis* (Table 3). However, a reduced pH of 6.0 and 5.5 was detected when prebiotic 8 was used as a nutrient source for *Bacillus sp.* and *L. lactis*, respectively (Table 3). These results further highlight the selective utilization of prebiotics, influencing the metabolic end products formed during fermentation.

Table 3. pH changes in culture media after 96 h of incubation under aerobic conditions, inoculated with different bacterial strains and a combination of various prebiotics.

pH	Pre. 1	Pre. 2	Pre. 3	Pre. 4	Pre. 6	Pre. 7	Pre. 8	Pre. 9
Before incubation	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0
Bacillus sp. at 96 h	8.5	8.5	8.5	8.5	8.5	8.5	6.0	8.5
L. lactis at 96 h	8.5	8.5	8.5	8.5	8.5	8.5	5.5	8.5

Note: Pre. stands for prebiotic

3.2.3. *TLC analysis*

TLC analysis was performed to separate the phenolic metabolites of various extracts of different prebiotic fermentations. Figure 4 shows TLC profiling of various extracts visualized under UV 254 and 366nm. *Bacillus sp.* didn't produce many metabolites in the control sample when visualized under both ranges of UV light. However, multiple metabolites were observed at 254 nm and 366 nm in the presence of levan and commercial prebiotic products. The TLC metabolites fingerprinting comparison of *Bacillus sp. a*nd *L. lactis* strain at 254nm (Figure 4 top and bottom TLCs at left-hand side) showed distinct spots. The phenolic profile of prebiotics 3 and 4 was similar for both stains and showed 4 bands at equidistant spaces. In the case of levan fermentation, four distinct bands were observed for *Bacillus sp.*, while seven thick bands were detected at different positions for *L. lactis*. Prebiotic 1 was extensively fermented by *Bacillus sp.* but not by *L. lactis*, as evident from Figure 4. For prebiotic 9, *Bacillus sp.* produced four distinct metabolite bands, while *L. lactis* produced seven bands, indicating a broader range of metabolites generated by the latter.

Additionally, different metabolites were observed when the TLC plates were analyzed under UV light at 366 nm compared to 254 nm. This indicates the presence of different metabolic products detectable at different wavelengths. The TLC plate analyzed under UV light at 366 nm further showed that prebiotic 1 was only utilized by *Bacillus sp*. but not by *L. lactis*. This characteristic between the two strains was evident as the *Bacillus sp.* produced nine highly fluorescent bands, while the *L. lactis* produced 5 bands with slightly different positions and reduced fluorescence intensity. In general, most of the prebiotics fermented by *L. lactis* produced a higher number of phenolic metabolites when compared to *Bacillus sp.*, as observed under UV light at 366 nm.

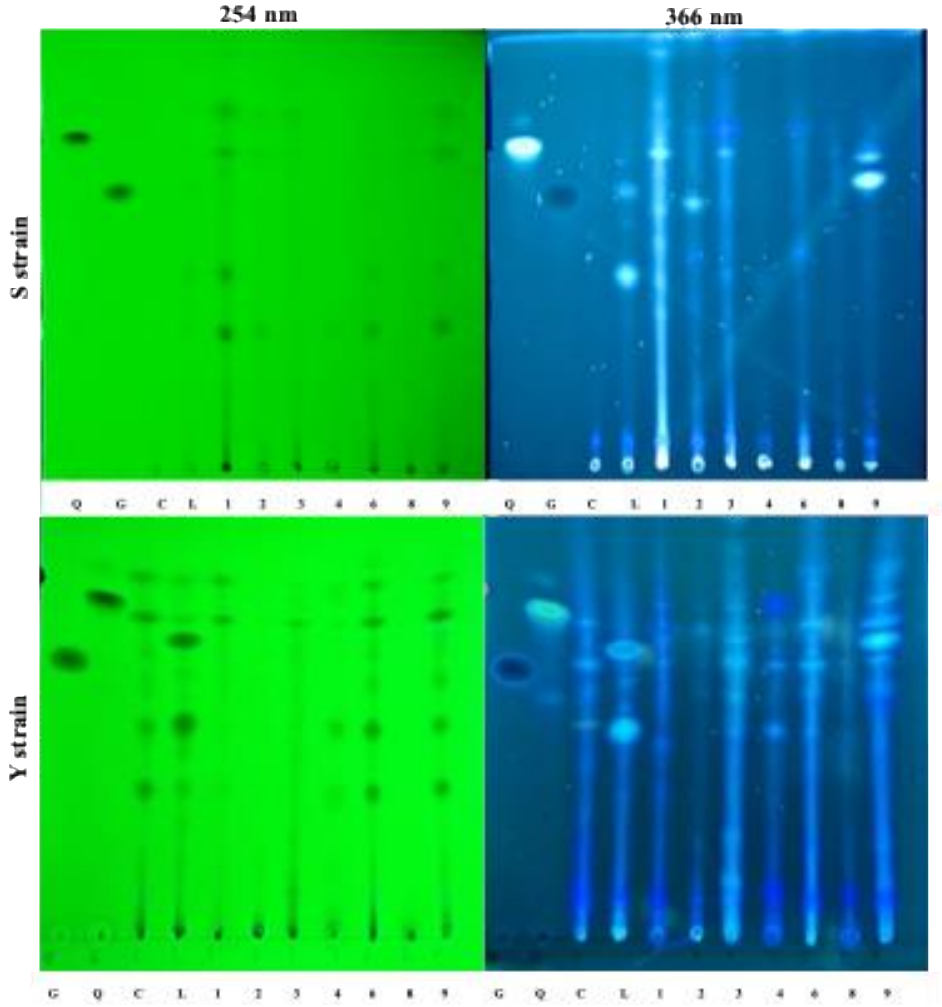

Figure 4. TLC profile of metabolite at 254 nm and 366 nm from both *Bacillus sp.* (S strain) and *L. lactis* (Y strain) in levan and different prebiotic supplements under aerobic conditions. Q: quercetin, G: gallic acid, C: control with probiotic strain containing no prebiotic, L: levan, 1: prebiotic 1, 2: Prebiotic 2, 3: Prebiotic 3, 4: prebiotic 4, 6: prebiotic 6, 8: prebiotic 8, 9: prebiotic 9.

3.2.4. Evaluation of antimicrobial activity

The antimicrobial activity of the metabolites extracted from all samples was evaluated using the well diffusion method against *E. coli*. As shown in Figure 5, none of the extracted metabolites from *Bacillus sp*. and *L. lactis* exhibited a clear zone of inhibition against *E. coli*. These results suggest that no antibacterial compounds were produced during the fermentation of prebiotics under aerobic conditions by both strains.

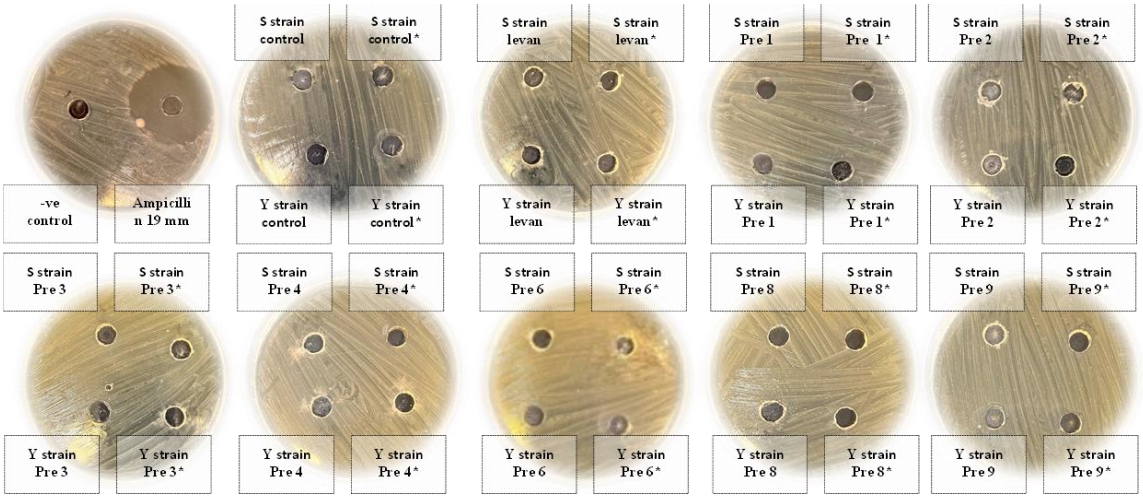

Figure 5. Antimicrobial activity extracted metabolites of *s Bacillus sp.* (S strain) and *L. lacti* (Y strain) against *E. coli*

3.2.5. Evaluation of antioxidant activity

The DPPH scavenging activity of 1% metabolites extracted from cultures of *Bacillus sp.* and *L. lactis* is presented in Figure 6. All the metabolic products exhibited antioxidant activity to varying extents. The %inhibition of DPPH radicals by metabolites produced from the *L. lactis* was higher than that of metabolites produced by *Bacillus sp.* Also, the antioxidant activity of prebiotics 1 and 8 fermented by *L. lactis*, was found to be comparable to that of the standard ascorbic acid (vitamin C). Notably, prebiotics 1 and 8 contain inulin, which may explain their enhanced activity. The observed free radical scavenging activity is likely attributed to the polyphenolic compounds present in the metabolites, which are known for their antioxidant properties.

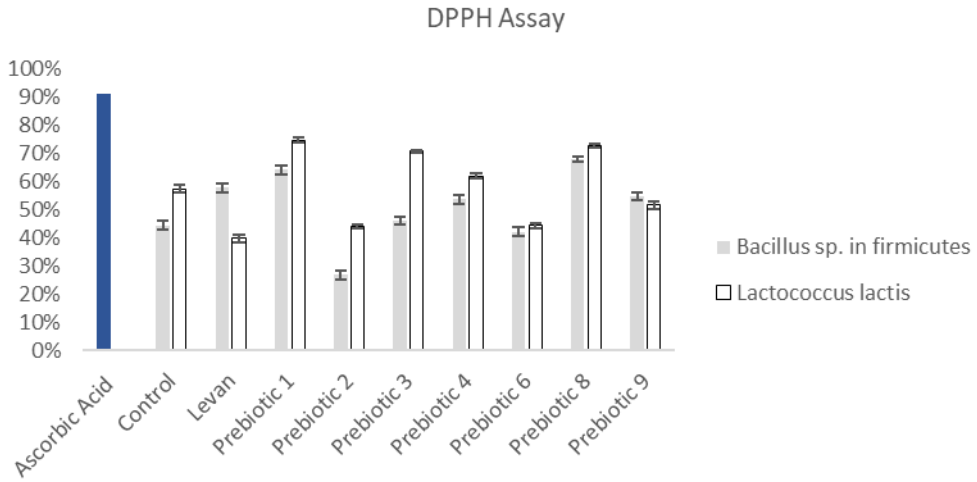

Figure 6. Antioxidant activity for *L. lactis* and *Bacillus sp.* metabolites produced under aerobic conditions. Standard = ascorbic acid 25 µg/ml.

3.3. Fermentation of prebiotics by L. lactis and *Bacillus sp. under anaerobic conditions*

Only three prebiotic samples instead of eight were utilized for anaerobic fermentation to test the hypothesis of selective fermentability of fiber by probiotics and the consequent production of unique metabolites having diverse biological activities.

3.3.1. *pH change*

The change in pH of the fermentation media for different prebiotics fermented by *Bacillus sp.* and *L. lactis* after 96 h is shown in Table 4. Generally, a decrease in pH was observed for all substrates when fermented by both strains under anaerobic conditions. Except for prebiotic 2, fermentation of the other prebiotics resulted in a significant pH drop of approximately 4.0 compared to the control. This decrease in pH indicates the production of short chain fatty acids (SCFAs) during fermentation. Notably, the results under anaerobic conditions contrast with those observed under aerobic conditions, where an increase in pH towards basic was recorded. This suggests that SCFAs, which contribute to the acidification of the medium, are produced by both strains primarily under anaerobic conditions during the fermentation of prebiotics.

Table 4. pH changes in culture media after 96 h of incubation under anaerobic conditions, inoculated with different bacterial strains and a combination of various prebiotics

pH	Control	Prebiotic 2	Prebiotic 3	Prebiotic 9
Before incubation	7.0	7.0	7.0	7.0
After incubation				
Bacillus sp.	6.5	5.0	4.0	4.0
L. lactis	6.5	5.0	4.0	4.0

3.3.2. TLC analysis

TLC analysis of the phenolic metabolites produced by *L. lactis* and *Bacillus sp.* in the presence of three different prebiotics under anaerobic conditions is shown in Figure 7. Under anaerobic conditions, almost no phenolic compounds were detected. However, when prebiotic 2 was fermented with *Bacillus sp*. (S2 in Figure 7), multiple bands were observed upon visualization under 254 and 366 nm UV light. Interestingly, although the decrease in pH during the fermentation of prebiotic 2 under anaerobic conditions was relatively small, a higher number of phenolic metabolites were produced (Section 3.2.3). This suggests that when phenolic metabolites are generated, there is a lower production of SCFAs. The observed alkaline pH shift under aerobic conditions, coupled with significant phenolic metabolite production, further supports this hypothesis.

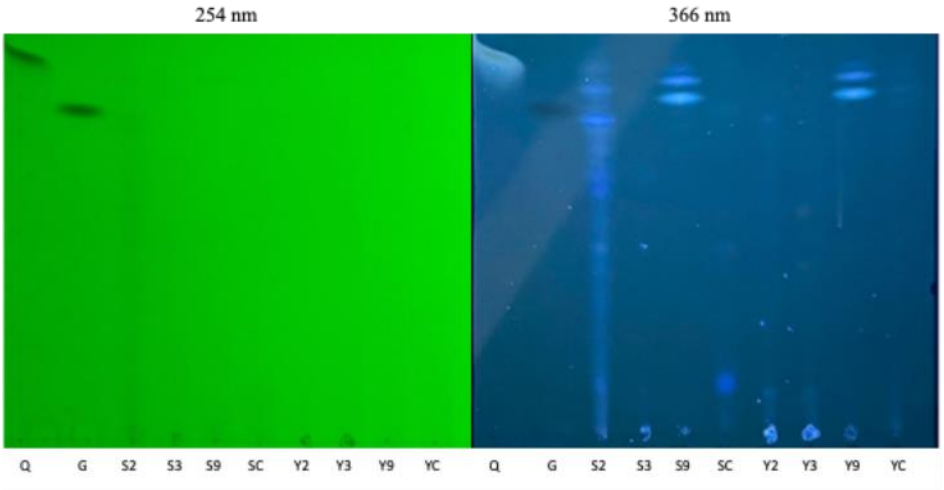

Figure 7. TLC of metabolites profile at 254 nm and 366 nm from both *Bacillus sp.* and *L. lactis* strains in different prebiotic supplements under anaerobic conditions. Q: quercetin; G: gallic acid; *Bacillus*

sp. (SC: control having no prebiotic, S2: prebiotic 2, S3: prebiotic 3, S9: prebiotic 9); *L. lactis* (YC: control having no prebiotic, Y2: prebiotic 2, Y3: prebiotic 3, Y9: prebiotic 9).

3.3.3. *Evaluation of antimicrobial activity*

The metabolic extracts produced under anaerobic conditions were found to be almost insoluble in water. The water-soluble fraction of the metabolites was dissolved in sterile water and tested for antimicrobial activity against *E. coli* using the well diffusion method. However, no antimicrobial activity was observed as it can be observed in Figure 8a. Subsequently, the extracts were dissolved in methanol and applied to sterile 8 mm filter paper discs, followed by assessment for antibacterial activity against *E. coli* using the disc-diffusion assay. As shown in Figure 8b, a potent antimicrobial effect (zone of clearence) was observed under the discs for all the metabolites. The clear zone under the disk could be confidently attributed to the antimicrobial activity of the metabolites attached to the disk since methanol was completely evaporated by drying the disk before putting it on the plate. This notable difference in antimicrobial activity observed when metabolites are dissolved in organic solvent compared to the water can be attributed to the presence of lipophilic metabolites produced under anaerobic conditions, which are better solubilized in organic solvents.

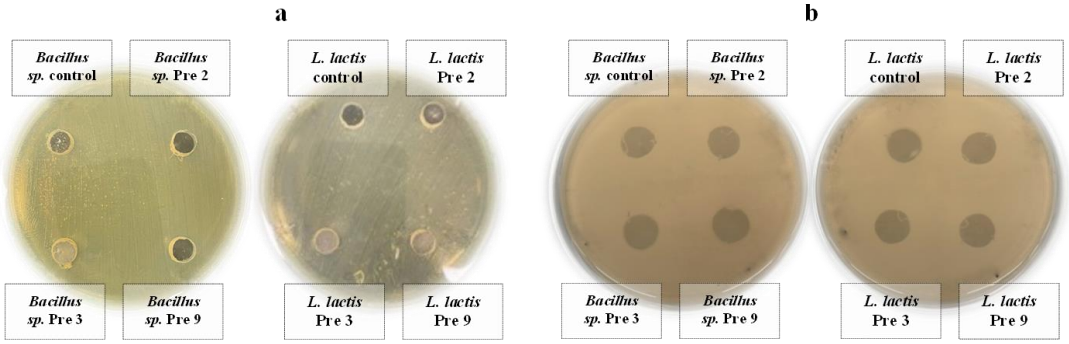

Figure 8. Antimicrobial activity using a) well diffusion technique and metabolites dissolved in water, and b) disc diffusion technique and metabolites dissolved in organic solvant.

3.3.4. *Evaluation of antioxidant activity*

Metabolites produced under anaerobic conditions showed significantly higher DPPH radical scavenging activities (Figure 9) as compared to the metabolites produced under aerobic conditions (Figure 6) by *Bacillus sp.* and *L. lactis* in the presence of the same prebiotic samples. Notably, metabolites derived from prebiotic 9 by *Bacillus sp.* demonstrated a significantly higher antioxidant property compared to those produced by *L. lactis.* Additionally, metabolites produced by *Bacillus sp.* in the presence of prebiotic 2 under anaerobic conditions exhibited an impressive 83% radical scavenging activity. This amount was substantially higher than the 27% antioxidant activity observed for metabolites produced by the same strain and prebiotic under aerobic conditions. These findings highlight the strain-specific and fermentation condition-dependent selective fermentation and digestibility of prebiotics, which can influence the bioactive properties of the resulting metabolites.

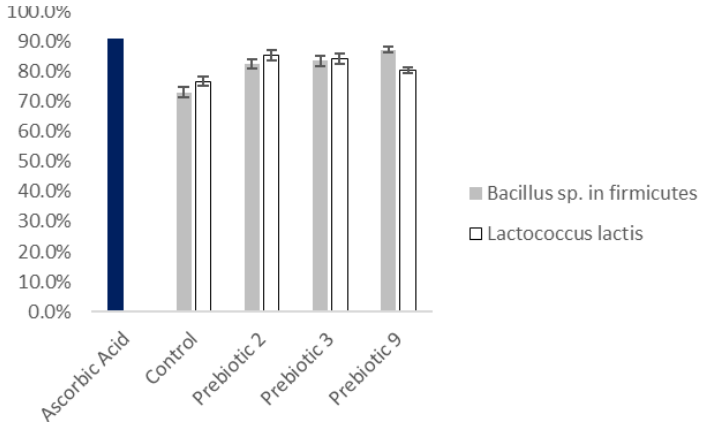

Figure 9. Antioxidant activity for *L. lactis* and *Bacillus sp.* metabolites produced under anaerobic conditions. Ascorbic acid (25 μg/ml) was used as standard.

3.4. Fermentation of levan and commercial prebiotics by a consortium of microorganisms

3.4.1. pH change

Species of *Lactobacillus* and *Bifidobacterium* were used as a consortium to evaluate the metabolic changes under anaerobic conditions. The pH changes during fermentation with levan, prebiotic 2, prebiotic 3, and prebiotic 9 are presented in Table 5. The pH of all samples decreased to a range of 4.0 to 4.5. Notably, the pH drop observed in all the samples (levan, commercial prebiotics, and control), represented the most substantial decrease recorded across all previous experiments. This suggests that the consortium's cross-feeding mechanism led to the production of huge amounts of SCFAs.

Table 5. pH drop observed for bacterial consortium under anaerobic conditions using different prebiotics

pH	Control	Levan	Pre. 2	Pre. 3	Pre. 9
Before incubation	7.0	7.0	7.0	7.0	7.0
After incubation	4.0	4.5	4.0	4.0	4.0

3.4.2. TLC analysis

Figure 10 shows the production of phenolic compounds visualized under 254 and 366 nm, respectively. Under UV light at 254 nm, only a few lighter bands were observed for the metabolites. However, a prominent spot at the starting point of the TLC analysis was observed in all samples, indicating the presence of polyphenols. Noteably, when the same TLC plate was visualized under UV light at 366 nm, multiple distinct bands were observed across all samples. This suggests the presence of phenolic compounds with absorption maxima at longer wavelengths, further highlighting the diversity of phenolic metabolites produced during fermentation. The production of high amounts of both phenolic compounds and SCFAs after the fermentation by consortium further supports the existence of a cross-feeding mechanism. This mechanism facilitates metabolic interactions between species, leading to the generation of distinct metabolic end products compared to those observed when fermentation is carried out by a single bacterial strain.

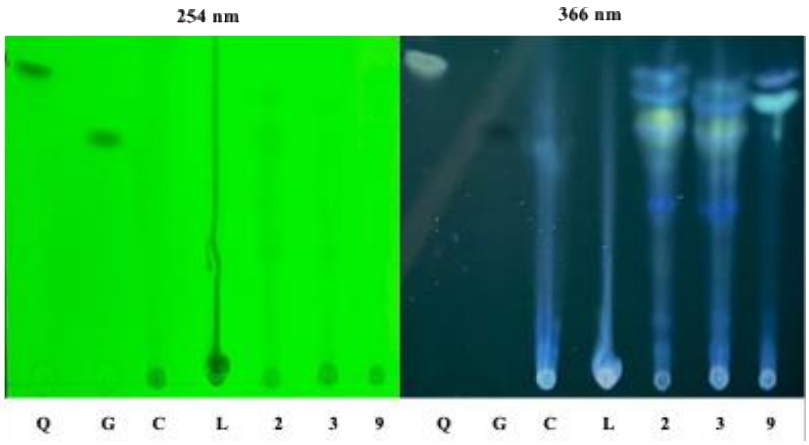

Figure 11. TLC of metabolites profile at 254 nm and 366 nm from bacterial consortium in different prebiotic under anaerobic conditions. Q: quercetin, G: gallic acid, C: Control meabolites, L: levan metabolites, 2: prebiotic 2 metabolites, 3: prebiotic 3 metabolites, 9: prebiotic 9 metabolites.

3.4.3. Evaluation of antimicrobial activity

At the end of the fermentation process of prebiotics by the microbial consortium, both the supernatant from fermentation media and the metabolic extracts were evaluated for antibacterial activity against the common pathogen *E. coli*. The antimicrobial activity of the supernatants was tested using the agar-well diffusion assay, and the results are presented in Figure 12a. Interestingly, all the samples including control, displayed antimicrobial activity. Zones of inhibition ranging from 4.0 to 6.0 mm were porduced in all the samples.

Similarly, when the metabolic extracts were tested, all the sample demonstrated antibacterial activity by producing zones of clearance (Figure 12b). However, the size of the zones differed between the supernatants and the metabolic extracts for the same prebiotics fermented by the same microbial consortium. These differences in zone size suggest that the antimicrobial compounds present in the supernatants and the extracts may differ in concentration or composition. The different composition may include the production of both polar and lipophilic antimicrobial compounds as a result of the fermentation process.

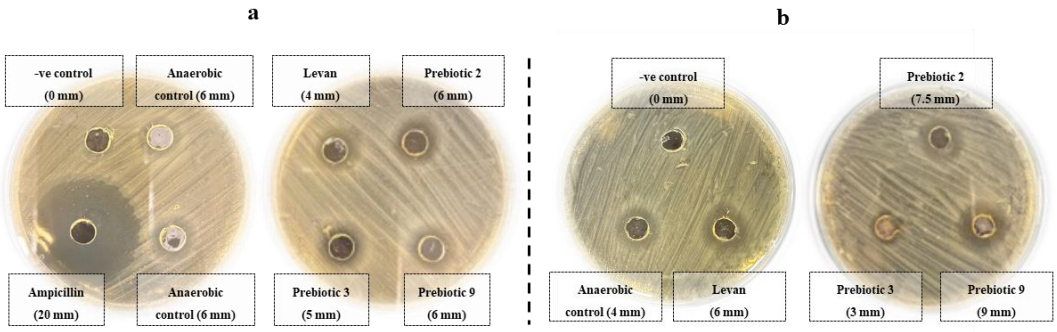

Figure 12. Antimicrobial activity of metabolites produced by gut microbial consortium. (a) Antimicrobial activity exhibited by media supernatant (b) Antimicrobial activity of extracted metabolites.

3.4.4. Evaluation of antioxidant activity

The antioxidant activity of metabolic extracts exhibited that levan fermented product contained the highest antioxidant activity of approximately 94%, while metabolites produced from prebiotic 3 showed the lowest antioxidant activity 67%. Interestingly, metabolites from the control, prebiotic 2, and 9 demonstrated approximately 75% antioxidant activity, as shown in Figure 13.

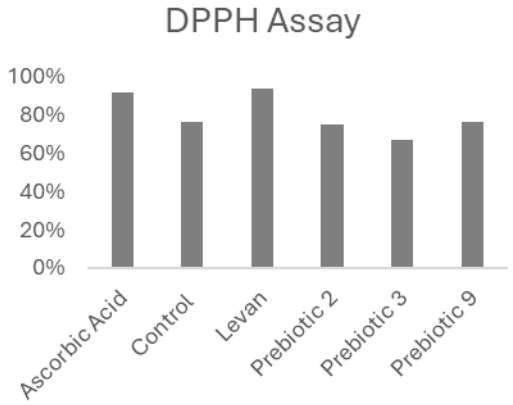

Figure 13. Antioxidant activity of microbial consortium metabolites produced under anaerobic conditions. Ascorbic acid (25 μg/ml) was used as standard.

4. Discussion

The use of prebiotics/non-digestible fiber for modulation of the gut microbial equilibrium is an emerging therapy for the manipulation of diseases. The improvement in growth and diversity of gut microorganisms with prebiotics have been mentioned in previous studies[15]. This non-digestible fiber (mainly carbohydrates) can be converted into carbon sources in the intestine for probiotics, which cannot grow and reproduce without carbon sources, thus regulating the composition of probiotics. The current study observed the selective utilization of prebiotic blends by specific microbial strains. The controversy of selective stimulation of only one or a limited number of probiotics by a specific prebiotic agent in the past has been addressed in recent years. For instance, fructose-based carbohydrates stimulated the growth of *B. thetaiotaomicron* that could utilize leven efficiently among various *Bacteroides spp*. (*B. caccae, B. vulgatus, B. uniformis, B. fragilis, B. ovatus*) tested by Sonnenburg et al.[16]. However, *B. vulgatus* was not able to grow on levan or inulin, indicating the fiber selectivity of different gut microbial species. Vázquez-Rodríguez et al.[17] reported that polysaccharide-based prebiotics isolated from brown seaweed Silvetia compressa could proliferate *Bifidobacterium* and *Lactobacillus* like inulin and increase the synthesis of total SCFAs. The selective growth stimulation of probiotics, *Lactobacillus* bacteria and *Bifidobacterium* and *Akkermansia muciniphila*, by polysaccharide-based prebiotics in male and female mice, respectively, is reported by Shang et al.[18]. Our study shows that most of the commercial prebiotics, including lab-synthesized levan, are effective for *Lactococcus* compared to the *Bacillus* strain. Although the impact of prebiotics on the growth of probiotics has been known for decades, the specificity and mechanisms underlying the selective fermentation of prebiotics by probiotics have not been elucidated fully. However, the presence and absence of different types of hydrolases to breakdown polysaccharides in gut microbial species, especially *lactobacillus* and *Bifidobacterium*, and the existence of polysaccharide utilization loci (PULs) in *Bacteroides spp.*, which correspond to different capacities for glycan utilization could be attributed to the selective fermentation of prebiotics by gut microbes[19]. The selective utilization of prebiotics leads to the production of unique metabolites by each species, which contributes to the balance of metabolites in the gut. Gut microorganisms can produce organic acids such as, lactate, pyruvate and

succinate, gases e.g., H_2, H_2S, CO_2, and CH_4) and most importantly short chain fatty acids (SCFAs) like acetate, propionate, and butyrate etc. The production of acidic metabolites reduces the pH of the colon and nitrogenous end products. The production of diverse metabolites by different strains under aerobic and anaerobic conditions could be the result of the selective fermentation of prebiotics. These metabolites can stimulate intestinal bacterial growth directly or can stimulate the growth of other members of the gut microbiota. *Bifidobacterium* and *Lactobacillus* spp., produce lactate and acetate as the major fermentation products. The drop in pH in the current study could be due to the production of these molecules when probiotics were cultured under anaerobic conditions and metabolites produced were not visualized on TLC plates stained for phenolic compounds (Figure 7). Different metabolic profiles of probiotics have been observed under aerobic and anaerobic conditions. The metabolic shift is due to the shift to pathways utilized under aerobic conditions in the microbial species that partially utilize oxygen, like *Lactobacillus* and *Bifidobacterium*. These microbes prefer anaerobic conditions and utilize fermentative pathways to produce bioactive SCFs and lactic acid with enhanced biological effects however, production of these molecules would be reduced under aerobic conditions, and increased production of oxidative byproducts like acetoin and diacetyl could be observed[20]. The metabolic profile is not only changed by oxygen availability but also through a type of fiber blend supplied to the probiotic strains, as can be observed in the TLC images above. The metabolites produced under aerobic conditions are less bioactive compared to metabolites produced under anaerobic conditions because SCFs, which are highly biologically active, are produced under anaerobic conditions. However, strong biocidal properties of some metabolites produced under aerobic conditions were observed by the disc diffusion method against *E.coli* (Figure 8b). Very interestingly, the *E.coli* was not grown in the disc zone even after 30 days of leaving the plates at room temperature on the bench, indicating the presence of some strong antimicrobial metabolites other than SCFs produced under this condition utilizing specific prebiotics. *Lactobacillus* species are known to exhibit antimicrobial properties through the production of organic acids and bacteriocins[21]. They contribute to the production of a diverse range of bioactive compounds, such as enzymes, vitamins, conjugated linoleic acid, exopolysaccharides, and gamma-aminobutyric acid (GABA)[22]. Antioxidants are molecules with a high electron density that neutralize free radicals by donating an electron, effectively stabilizing the reactive species and preventing oxidative damage in the cell[23]. Gut microbiota contributes to the antioxidant capacity of the host through their metabolites. Uchiyama et al. found that gut microorganisms belonging to the *Lachnospiraceae* and *Ruminococcaceae* families enhance the antioxidant capacity of the host by generating reactive sulfur species (RSS)[24]. Metabolites isolated during the current study under all variable conditions showed free radical scavenging activity by DPPH assay. Very interestingly, the metabolites produced by levan alone under anaerobic conditions by gut microbial consortium showed highest DPPH activity. This recognizes the potential of modulation of the microbiome therapeutically through the use of prebiotics. However, the possibly diverse mechanisms by which specific gut microbial strain and their products elicit their distinct beneficial metabolites remain unknown. Therefore, identifying molecular pathways exploited by prebiotics in a specific gut microorganism using in vitro large-scale culture techniques would allow for the more targeted use of prebiotics or exploitation of microbial derived small biomolecules.

Further investigation and identification of metabolites produced under different conditions in different prebiotics is highly recommended. In our opinion, this understanding of prebiotic-mediated stimulation of specific probiotic strains and preliminary investigations of the corresponding metabolites and their biological activity will guide the production of unique bioactive metabolites and promote the rational application of prebiotics and purified metabolites in the health, pharmaceutical, and food sectors.

5. Conclusions, limitations, and future directions

The selective fermentation of dietary fibers by probiotics results in the production of diverse metabolites with potent antimicrobial and antioxidant activities. These metabolites not only inhibit pathogen growth but also protect against oxidative stress, making fiber-probiotic systems a cornerstone of functional nutrition and therapeutic strategies for gut health and beyond. Although very clear evidence of fiber selectivity and production of diverse metabolites having different levels of biological activity has been demonstrated in the current study, it is based on a limited number of strains and growth conditions, which are too narrow to decipher reliable ecological predictions and would require further, more extensive in-vitro investigations, including a larger set of strains. Identification of biologically active metabolites by mass spectrometer will expand the scope of metabolites' biological activities to assess their impact on different disorders. In-vivo studies should be done to gain a further understanding of the complexity of the gut microbiome and its interactions with diverse commercial blends of prebiotics. However, the data suggests that novel biomolecules can be produced in vivo by probiotics using customized prebiotics as natural therapeutics. Overall, continued research and innovation in this area will pave the way for personalized and sustainable health interventions.

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