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Effect of Fructooligosaccharide Supplementation on Growth and Survival of *Bacillus coagulans* IS2 in Green Tea and Black Tea Infusion

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ABSTRACT: Fructooligosaccharide is a dietary fibre known to improve the survivability of probiotic microorganisms by digesting in the large intestine by the probiotics and species like Lactobacillus and Bifidobacterium are incapable of colonizing the large intestine as they are sensitive to acidity and bile salts. Being the most consumed beverage after water, tea can function as a carrier of probiotics for large amounts of the population. The objective of this study is to investigate the effect of Fructooligosaccharide on the growth and survival of *B. coagulans* in tea infusion. Green tea infusion (GTI) and black tea infusion (BTI) were divided into three combinations containing 1) 0% sucrose + 0% FOS; 2) 5% (w/v) sucrose; 3) 5% (w/v) sucrose + 5% (w/v) FOS. The Total polyphenol content (TPC) and Ferric-reducing antioxidant power (FRAP) of the combinations were determined and *B. coagulans* was inoculated into each group, and incubated at 45°C for 24 hours. Samples were collected every 6 hours to determine the log CFU/ml and average growth rate (log CFU/ml/hr). GTI exhibited higher TPC, and FRAP compared to BTI. Notably, the viable count of *B. coagulans* was 63.60% higher in BTI compared to GTI in the first combination, indicating a negative correlation (R^2 = 0.70) between TPC and *B. coagulans'* growth rate. Supplementation of FOS resulted in a 36.07% increase and a 4.32% increase in the viable count of *B. coagulans* in green tea and black tea formulations respectively which were positive correlations (R^2 = 0.97, R^2 = 0.98). Therefore, incorporating FOS into tea infusions, especially in green tea can enhance the growth and viable count of *Bacillus coagulans* suggesting potential applications in synbiotic beverages combining the antioxidant benefits of tea polyphenols.

Keywords: *Bacillus coagulans IS2*, Fructooligosaccharides (FOS); Probiotic, Prebiotic; Synbiotic, Tea, FRAP

INTRODUCTION

Prebiotics are non-viable food components that exert a benefit on the health of the host, associated with modulation of the intestinal flora (FAO/WHO 2002). On the other hand, probiotics are live microorganisms, administered in quantities adequate to confer health benefits (FAO/WHO 2002; Deshpande *et al.,* 2022). Synbiotics are a combination of probiotics (living bacteria) and prebiotics (food components they feed on) and are primarily used since pure probiotics do not survive well in the digestive system without their prebiotic food supply (Panesar *et al.,* 2009).

Currently, a plethora of thoroughly characterized strains of *Lactobacilli* and *Bifidobacteria* have been developed for human utilization, aiming to mitigate the potentiality of gastrointestinal (GI) infections or to provide remedial measures for such infections. (Nagpal *et al.,* 2012). However, these cultures are incapable of colonizing the human gut because they are sensitive to an acidic stomach and intestinal bile salts (Del Campo *et al.,* 2005). Among the various probiotic strains, *Bacillus coagulans*, a Gram-positive, catalase-positive, spore-forming, motile, facultative anaerobe rod, has gained prominence for its robustness and resilience, especially in the gastrointestinal environment (Garrison, 2019; Majeed *et al.,* 2021; Zhao *et al.,* 2023). *Bacillus coagulans* can aid in the creation of an anaerobic and acidic gut environment that is unfavorable to pathogens, allowing other beneficial probiotics to thrive (Cao *et al.,* 2019). *B. coagulans* strains can consume free oxygen in the colon and stomach and inhibit redox processes as facultative anaerobic bacteria (Abhari *et al.,* 2015). Particularly, *Bacillus coagulans* Unique IS2 is a commercial probiotic strain with well-established

clinical efficacy without being harmful (Ahire *et al.*, 2020). It is also efficient in treating diarrhoea, IBD, IBS, constipation, hypercholesterolemia, depression, and dental caries (Adibpour *et al*., 2019; Majeed *et al.*, 2018; Satti *et al*., 2023; Shinde *et al*., 2020; Sudha *et al.*, 2012; Sudha *et al*., 2018; Sudha *et al.*, 2020). The therapeutic benefits of *Bacillus coagulans* may be attributed to its capacity for synthesizing antimicrobial compounds like bacteriocins (Abdhul *et al.*, 2015; Zhang *et al.*, 2022), which impede the growth of pathogenic bacteria (Rugji *et al*., 2022) and contribute to the equilibrium of microbiota populations. (Honda *et al.*, 2011).

Fructooligosaccharides (FOS), a subset of oligosaccharides, have arisen as prebiotic compounds with the ability to modulate the gut microbiota and augment the viability of advantageous microorganisms (Roberfroid, 2007). Prebiotics can bolster the subsequent: (1) creation of short-chain fatty acids (SCFA) and lactic acid as a result of fermentation; (2) proliferation of the probiotic bacteria along with an elevation in the concentration of minerals such as calcium, magnesium, etc. in the colon; and (3) the immune response of the host (production of IgA, modulation of cytokines, etc) (Dable-Tupas *et al.*, 2020). Different prebiotic compounds like FOS, galactooligosaccharides, inulin, and polydextrose were reported to be having synergistic growth-promoting effects on *B. coagulans* according to Majeed *et al.* (2019). Lesser explored prebiotics like fibers from cranberry, fenugreek, sugarcane, green banana, and chitooligosaccharides were also found to enhance the growth and survival of *B. coagulans* in the gastrointestinal tract. (Majeed *et al.*, 2018; Shinde *et al.*, 2019; Shinde *et al.*, 2020).

According to the International Scientific Association for Probiotics and Prebiotics (ISAPP) ISAPP, a synbiotic is now described as a combination of live microorganisms and specific substances that are utilized by the microorganisms present in the host, ultimately leading to a positive effect on the overall health of the host organism (Parhi *et al.*, 2022; Swanson *et al.*, 2020). The synergistic combination of probiotics and prebiotics is called synbiotics (Raksha *et al.*, 2022). Therefore, incorporating a suitable combination of both elements within a singular product should guarantee an enhanced outcome in comparison to the individual efficacy of probiotics or prebiotics alone (Bengmark, 2005). A combination of *Lactobacillus* or *Bifidobacterium* genus bacteria with FOS (fructooligosaccharides) is the most common example of a synbiotic (Markowiak *et al.*, 2017). The probiotics utilize the prebiotics as a source of energy, allowing them to live in the gastrointestinal tract for longer periods than they would otherwise be able to (Gibson and Roberfroid 1995; Dable-Tupas *et al*., 2020). Synergism also aids in the proper implantation of live microbial dietary supplements in the colon, as well as the growth of probiotics (Pandey *et al.*, 2015).

Green tea (*Camellia sinensis*) and black tea (*Camellia sinensis* var. assamica) are popular beverages globally, known for their antioxidant properties and health-promoting effects (Gramza *et al*., 2005; Sena *et al.*, 2020). Tea functions as an excellent carrier for *B. coagulans* Ganeden BC30 and the infusion treatments are well-tolerated by the spores of *B. coagulans* Ganeden BC30 (Poshadri *et al.*, 2022; Polo *et al.*, 2022). Tea polyphenols, especially catechins, are potent antimicrobial and antioxidant agents, with positive effects on human health (Almajano *et al.*, 2008). However, little is known about their impact on probiotic microorganisms, particularly *Bacillus coagulans*. Understanding how FOS and types affect the growth and survivability of *Bacillus coagulans* is of significant importance, as it can provide valuable insights into the development of functional beverages that combine the health benefits of probiotics and tea. *B. coagulans* was found to retain its viability when combined with various foods like jelly candies (Miranda *et al.*, 2020), date paste (Marcial-Coba *et al*., 2019), sweetener Nabat (Adibpour *et al*., 2019), orange juice, yogurt (Almada-Érix *et al.*, 2021), pasta (Fares *et al.*, 2015).

In this context, the present study seeks to advance the current understanding of the interaction between *Bacillus coagulans* and FOS by incorporating Unique IS2 strain into green tea and black tea. Additionally, it seeks to compare the effects of these teas on the growth and viability of *B. coagulans*, shedding light on their suitability as probiotic carriers.

MATERIALS AND METHODS

A. Bacterial strain and culture conditions

The spores-containing product of *Bacillus coagulans* Unique IS2 (1.8×10^9 CFU/gram) was obtained from Unique Biotech Limited, Hyderabad, India. Glucose Yeast Extract Agar (GYEA), de Man, Rogosa, and Sharpe (MRS) broth were purchased from HiMedia, Mumbai, India. The bacterial strain was cultured in MRS broth at 37°C for 24 hours. After incubation, bacterial cells were harvested by centrifugation and resuspended in sterile saline. From this solution of sterile saline, 5% (w/v) of culture would be added to flasks containing sterile Green tea and Black tea infusions.

B. Preparation of tea infusion

Green tea and Black tea in powdered form were purchased from the local market. 90% Fructooligosaccharide powder was purchased from TATA Chemicals Ltd, India and sucrose was purchased from HiMedia, Mumbai, India. Green tea infusion (GTI) and black tea infusion (BTI) were prepared by brewing 1.5 grams of tea powder in 150 ml of sterile distilled water at 90°C for 5 minutes which were filtered using Whatmann filter paper.

C. Experimental design

The experiment consisted of following formulations.

Table 1: Composition of green tea and black tea formulations.

Formulation		Composition				
		Green tea infusion (ml)	Black tea infusion (ml)	Sucrose (%w/v)	FOS (%w/v)	*Bacillus coagulans* (log CFU/ml)
With GTI	N_1	50	-	-	-	3.20-3.25
	C_1	50	-	5	-	3.20-3.25
	T_1	50	-	5	5	3.20-3.25
With BTI	N_2	-	50	-	-	3.20-3.25
	C_2	-	50	5	-	3.20-3.25
	T_2	-	50	5	5	3.20-3.25

All six combinations were sterilized, followed by inoculation with *Bacillus coagulans* culture (5% w/v) at 90°C and incubated in an orbital shaker incubator at 130 r.p.m for 24 hours at 40°C. The formulations N_1 and N_2 were regarded as negative controls for GTI and BTI, whilst the sucrose-containing formulations C_1 and C_2 were considered positive controls for the GTI and BTI. For the subsequent analyses, samples from all the formulations were taken at 0, 6, 12, 18, and 24 hours.

D. Determination of total polyphenols and antioxidant activity

The total polyphenol content (TPC) expressed as mg GAE (Gallic acid equivalent)/gram was analyzed for the tea formulations using the Folin-ciocalteu (Khanum *et al*., 2017) method. The antioxidant activity of the formulations expressed as mg ascorbic acid equivalent (AAE)/gram was determined using FRAP (Ferric ion reducing antioxidant power) assay (Kiran *et al.*, 2018) using ascorbic acid as standard.

E. Viable count of B. coagulans

The viable count of *B. coagulans* was determined by serial dilution and plating on GYEA agar. Colony-forming units were counted after 24 hours of incubation period at 40°C (Majeed *et al.*, 2016; Majeed *et al.*, 2019).

F. Average growth rate of B. coagulans per hour

The average growth rate of *B. coagulans* per hour in 24 hours was calculated according to the following equation assuming linearity.

$$\text{Average growth rate(log cfu/ml)} = \frac{\text{Final count - Initial count}}{\text{Total time period in hours}}$$

G. Statistical analysis

All the assays were conducted in independent triplicates. The results were statistically analyzed in one-way analysis of variance (ANOVA) followed by Tukey's test using Jamovi desktop statistical software (version 2.3.28, https://www.jamovi.org/), and statistical significance was determined at $p < 0.05$.

RESULTS AND DISCUSSION

A. Total polyphenol content (TPC) and Ferric reducing antioxidant power (FRAP)

The TPC and FRAP of all the formulations were analyzed at 0 hours and 24 hours after inoculation with *B. coagulans*.

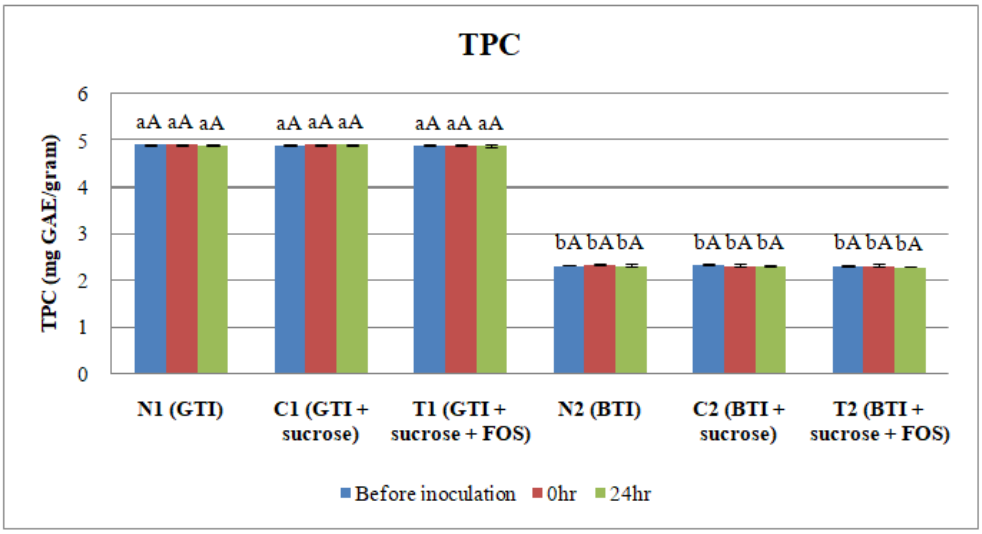

Fig. 1. Total polyphenol content (TPC) (mg GAE/gram) of the formulations before inoculation with *B. coagulans* and at 0 hours and 24 hours after inoculation with *B. coagulans*. Values with different capital letters indicate significant differences within each formulation before inoculation, and at 0 hours and 24 hours after inoculation by Tukey's test ($p < 0.05$). Values with different lowercase letters indicate significant differences between formulations by Tukey's test ($p < 0.05$).

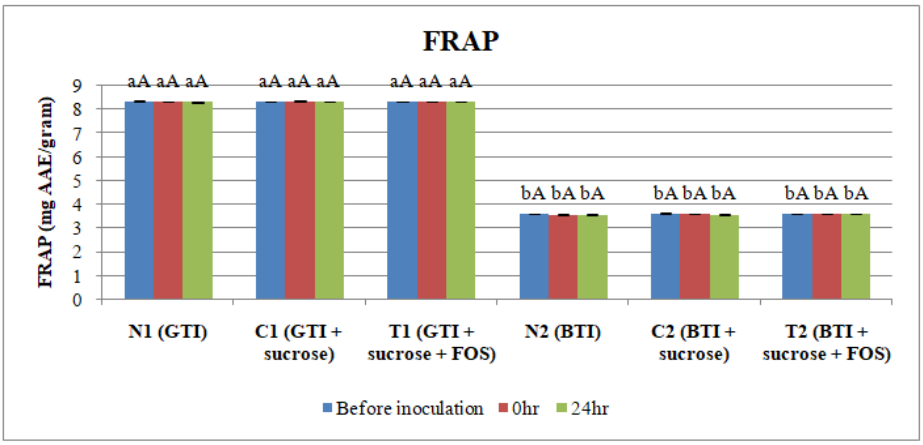

Fig. 2. Ferric reducing antioxidant power (FRAP) (mg AAE/gram) of the formulations before inoculation with *B. coagulans*, and at 0 hours and 24 hours after inoculation with *B. coagulans*. Values with different capital letters indicate significant differences within each formulation before inoculation, and at 0 hours and 24 hours after inoculation by Tukey's test ($p < 0.05$). Values with different lowercase letters indicate significant differences across formulations by Tukey's test ($p < 0.05$).

The data shows that the mean TPC value of green tea formulations is 2.1 times higher (Fig. 1) and the mean FRAP value is 2.3 times higher (Fig. 2) than that of black tea formulations which were in agreement with the results demonstrated by Kaur *et al.* (2015); Kiran *et al.* (2018). There was no significant change ($p > 0.05$) in TPC and FRAP of the tea formulations containing *B. coagulans* after the fermentation period of 24 hours compared with the samples without *B. coagulans*. The data of this experiment suggested that the addition of probiotic *B. coagulans* IS2 did not affect the functional properties of both green and black tea even after 24 hours of fermentation which was essential to preserve the antioxidant properties of tea, similar to observations reported by Majeed *et al.* (2019).

B. Viable count of Bacillus coagulans in tea

The viable count of *Bacillus coagulans* was assessed over 24 hours in all the formulations. The growth curve was obtained by plotting the viable count of bacteria against time (hours).

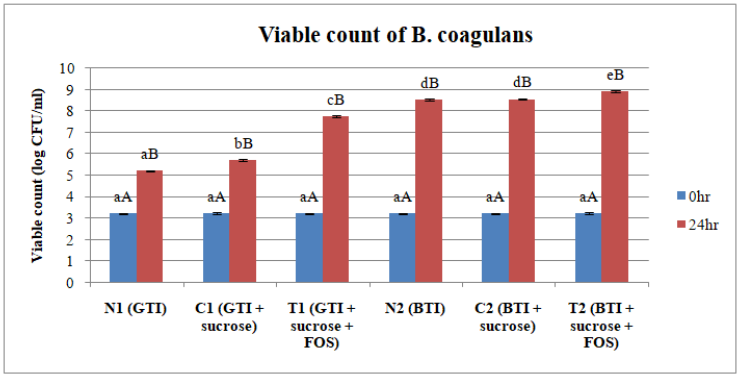

Fig. 3. Effect of FOS on viable count (log CFU/ml) of *B. coagulans* in different formulations at 0 hours and 24 hours after inoculation. Values with different capital letters indicate significant differences within each formulation at 0 hours and 24 hours by Tukey's test ($p < 0.05$). Values with different lowercase letters indicate significant differences across formulations by Tukey's test ($p < 0.05$).

At 0 hours there was no significant difference ($p > 0.05$) in the viable count of *B. coagulans* IS2 across all the formulations which ranged from 3.21 ± 0.04 to 3.23 ± 0.03 log CFU/ml (Fig. 3). With green tea and black tea as sole nutritional sources, the viable count of *B. coagulans* after 24 hours of fermentation was 63.60% higher ($p < 0.05$) in black tea infusion (N_2) compared to green tea infusion (N_1). With the addition of sucrose, the difference has reduced to 49.91% with C_2 (BTI + sucrose) exhibiting a higher viable count ($p < 0.05$) than C_1 (GTI + sucrose). It can be noted that after supplementation with FOS, the difference in viable count between black tea and green tea further reduced to 14.92% with T_2 (BTI + sucrose + FOS) exhibiting a higher value ($p < 0.05$) than T_1 (GTI + sucrose + FOS). Comparing the viable count of *B. coagulans* after 24 hours of fermentation between green tea formulations C_1 (GTI + sucrose) and T_1 (GTI + sucrose + FOS) showed that the addition of FOS along with sucrose resulted in a 36.07% significant increase ($p < 0.05$) in the viable count. However comparing the same parameter between black tea formulations C_2 (BTI + sucrose) and T_2 (BTI + sucrose + FOS) after 24 hours of fermentation resulted in a mere 4.32% increase in the viable count of *B. coagulans*, which is significant ($p < 0.05$) but lower than what was observed in the case of

green tea formulations(C_1 and T_1). In black tea formulations N_2 (BTI) and C_2 (BTI + sucrose), the addition of sucrose did not cause any significant change ($p > 0.05$) in the viable count of *B. coagulans* whereas in the case of green tea formulations N_1 (GTI) and C_1 (GTI + sucrose) addition of sucrose caused a slight increase of 9.38% ($p < 0.05$) in the viable count of *B. coagulans*.

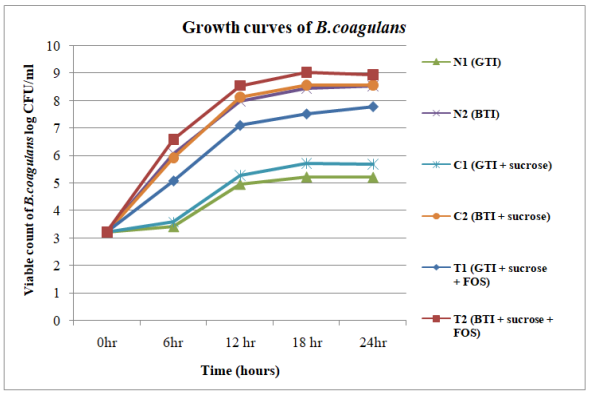

Fig. 4. Growth curves of *B. coagulans* with viable count (log CFU/ml) plotted against time (hours).

Fig. 4 demonstrates the growth curve of *B. coagulans* in different green and black tea formulations, where the viable count (log CFU/ml) of *B. coagulans* is plotted against the fermentation time in hours. The results suggested that *B. coagulans* utilized FOS more effectively in green tea which has higher TPC (total polyphenol content) to reach the viable count comparable to that of black tea, whereas in the case of black tea which has lower TPC, the viable count had already reached the peak level and the addition of FOS resulted in comparably lower difference.

C. Effect of FOS on growth rate of Bacillus coagulans

The average growth rate of *B. coagulans* was calculated as Log CFU/ml/hour in all the formulations.

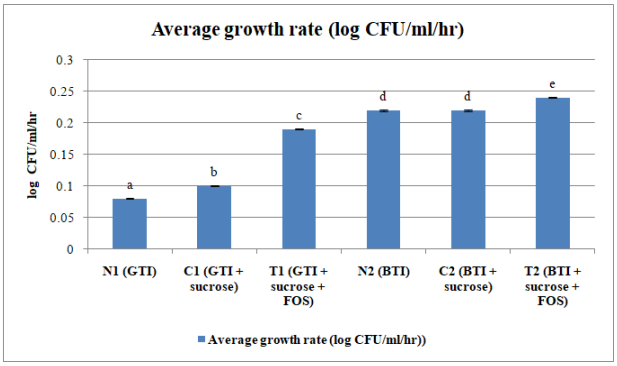

Fig. 5. Effect of FOS on the average growth rate of *B. coagulans* expressed as log CFU/ml/hour. Values with different lowercase letters indicate a significant difference ($p < 0.05$).

In the case of green tea formulations N_1 (GTI) and C_1 (GTI + sucrose), the addition of sucrose resulted in a 25% increase ($p < 0.05$) in the average growth rate of *B. coagulans* (figure 5). Also in T_1 (GTI + sucrose + FOS), the average growth rate of *B. coagulans* was 90% higher ($p < 0.05$) compared to C_1 (GTI + sucrose) which might be due to the addition of FOS. In the case of black tea, the addition of sucrose did not affect the growth rate of *B. coagulans* significantly ($p > 0.05$). However, the addition of FOS affected the growth rate, with the average growth rate 9.1% higher ($p < 0.05$) in T_2 (BTI + sucrose + FOS) compared to C_2 (BTI + sucrose). Even though the percentage increase in growth rate upon the addition of FOS was significant ($p < 0.05$) in the case of black tea formulation, it was 9.9 times lower ($p < 0.05$) than that of green tea formulation. When compared between green tea and black tea, the black tea formulation N_2 (BTI) supported a 175% higher growth rate of *B. coagulans* compared to the green tea formulation N1 (GTI). However, in formulations containing FOS, this difference had diminished to 26.31%, with black tea formulation T_2 (BTI + sucrose + FOS) still supporting a higher growth rate than green tea formulation T_1 (GTI + sucrose + FOS).

D. Correlation tests

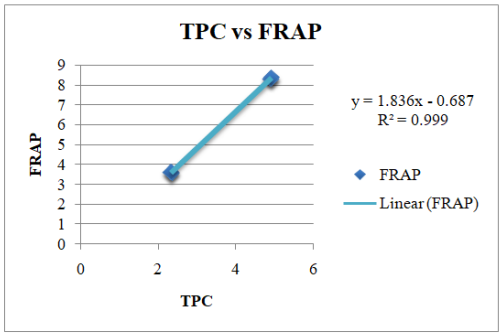

Fig. 6. Correlation between Total polyphenol content (TPC) and ferric-reducing antioxidant power (FRAP) in different tea formulations.

Fig. 6 shows that TPC and FRAP of teas had a very high positive correlation (R^2 = 0.99). Similar results were reported by Kiran *et al.* (2018) where a highly positive correlation was reported between polyphenol content and antioxidant activity of green tea and black tea. According to Almajano *et al.* (2008), the higher antioxidant activity of green tea could be due to the presence of catechins like Epi gallo catechin gallate (EPCG) and Epi gallo catechin (EPC). In the case of black tea, the antioxidant property was attributed to thearubigins, phenolic acids, catechins, and theaflavins. The polyphenol content of plant extracts has been attributed as the cause for their antioxidant properties (Benavente-garicia *et al*., 1997). Consequently, plants that possess a high concentration of polyphenols hold significant value as natural antioxidants (Zahra *et al.*, 2017). As reported by Tolambiya *et al.* (2023), the phenolic and flavonoid groups are highly responsible for the antioxidant activity. So, there was a linear correlation between Total Flavonoids, Total phenolic content, and antioxidant activity.

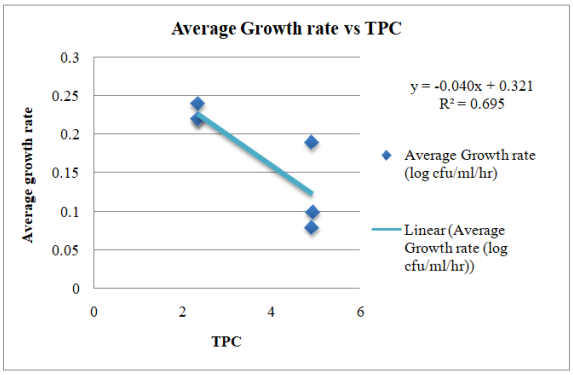

Fig. 7. Correlation between Average growth rate of *B. coagulans* and total polyphenol content (TPC) in tea.

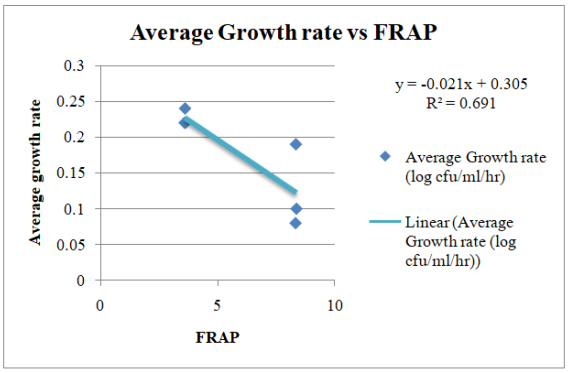

Fig. 8. Correlation between Average growth rate of *B. coagulans* and Ferric reducing antioxidant power (FRAP) in tea.

Interestingly, the correlation between the TPC and the average growth rate of *B. coagulans* was moderately negative (R^2 = 0.6958) (Fig. 7). Almost similar was the correlation between the growth rate of *B. coagulans* and FRAP of tea (R^2 = 0.6917) (Fig. 8). These results suggest that TPC and FRAP might have a hindering effect on the growth rate of *B. coagulans*, which might have acted along with any other possibly negative factors that affected the growth rate of *B. coagulans*.

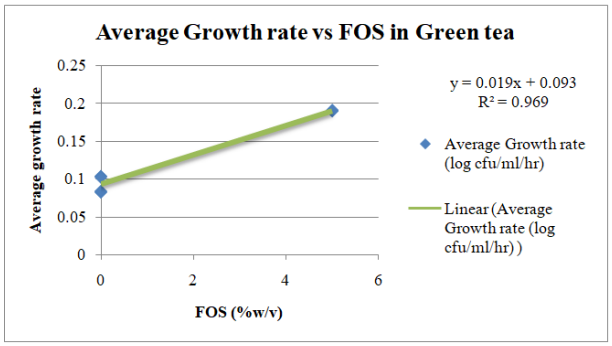

Fig. 9. Correlation between Average growth rate of *B. coagulans* and FOS in Green tea.

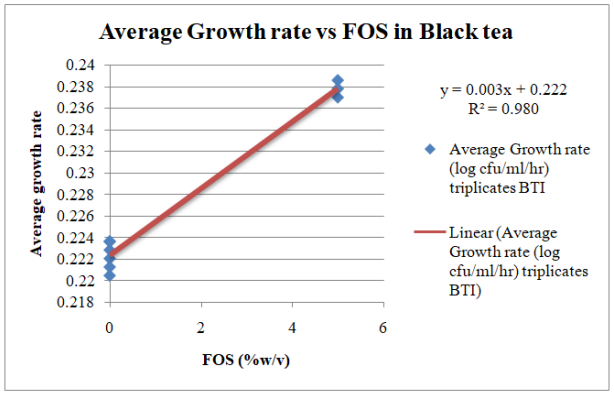

Fig. 10. Correlation between Average growth rate of *B. coagulans* and FOS in Black tea.

A highly positive correlation between the supplementation of FOS and the average growth rate of *B. coagulans* in both green tea (R^2 = 0.9691) and black tea (R^2 = 0.9804) was observed (Fig. 9 and 10) highlighting the growth-promoting effect of the prebiotic FOS on *B. coagulans*. Majeed *et al.* (2019) reported similar findings stating that the addition of water-soluble prebiotic fibers like fructooligosaccharide, galactooligosaccharide, etc, increased the growth of *B. coagulans* MTCC 5856 suggesting that soluble fibres had a synergistic effect on the growth of *B. coagulans* MTCC 5856 spores after brewing.

CONCLUSIONS

In conclusion, a highly positive correlation was observed between the supplementation of FOS and the average growth rate of *Bacillus coagulans* IS2 in both green and black tea formulations. Interestingly, a moderately negative correlation was observed between polyphenol content and the growth rate of *B. coagulans* IS2 in tea formulations. The growth-enhancing effect of FOS on *B. coagulans* was more pronounced in green tea where the probiotic *Bacillus coagulans* might have utilized the prebiotic FOS as a substrate to compensate for the growth hindering effect of polyphenols. Therefore, incorporating FOS into tea infusions, especially in green tea can compensate for the inhibitory effect of polyphenols and enhance the growth and viability of *Bacillus coagulans* suggesting important implications for the development of functional beverages, as tea-based probiotic and synbiotic products could offer health benefits associated

with both synbiotics and antioxidants of tea. However, further detailed studies may be required to determine an underlying mechanism to establish the effect of polyphenols on the growth of *B. coagulans* IS2.

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Conflicts of Interest. None.

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