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Time dependent growth pattern study of *Lactobacillus spp.* and *Bifidobacterium spp.* in the presence of Tartrazine; an azo dye

Abstract

Tartrazine is an azo dye that is widely used in the food and pharmaceutical industry. It is also known to have a potential impact on human health. Reports indicate possible carcinogenic and mutagenic effects of the dye. Joint expert WHO/ FAO committee on food additives, Codex Alimentarius Commission (CAC) have defined permissible limits for the use of tartrazine in fermented food products, including probiotic drinks, yet the dye finds wide usage as an additive. This study demonstrates the probable effect of tartrazine, at different concentrations on Lactic acid bacteria commonly used as starter cultures and probiotics. This study indicates the effect of tartrazine in particular on *Lactobacillus casei, Lactobacillus plantarum, Bifidobacterium bifidum*.

Keywords: Azo dye, *Bifidobacterium bifidum, Lactobacillus casei, Lactobacillus plantarum, Probiotic, Tartrazine*

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Introduction

Azo dyes are the large group of synthetic chemicals that are most commonly used colorant in food and pharmaceutical industries. Owing to their sensory attributes these dyes are being widely used as a food additive. The effect of these dyes on human health and the environment has been an emerging cause of concern.[1] Azo dyes, including Tartrazine, are chemically synthesized and substituted with N2 group. The intermediates of azo dyes are carcinogenic, mutagenic, and highly recalcitrant in natural water bodies impacting the ecosystem.[2] Aniline based intermediaries of azo dye metabolism have been reported to be toxic, carcinogenic and mutagenic.[3,4] Given the emerging concerns of toxicity of the azo dyes, in particular Tartrazine it is of relevance to study the possible effects of the dye on the beneficial microbiota that have been established to have a crucial role in the immune system, the digestion and nutrition, maintaining the integrity of the gut barrier and gastrointestinal tract, besides its detoxifying properties.[5-7]

The relevance of lactic acid bacteria as probiotics in fermented foods that adds to the gut flora has gained considerable importance in recent times.[8] Among the many effects of probiotics, its role in neuro-modulation and the underlying mechanism is the most intriguing.[9] Reports have stated that Dysbiosis (also called as dysbacteriosis or microbial imbalance or an impaired microbiota) of gut microbiome can be the cause to multiple ailments including mental illness.[10,11] *Lactobacillus Plantarum 299v, Bifidobacterium longum, Lactobacillus rhamnosusGG, L. paracasei, Bifidobacterium breve* have been reported to reduce symptoms of mental illnesses when supplemented with diet as probiotics.[12-16]

Tartrazine despite its wide usage as an approved synthetic food color is also known to have potential ill effects on human health.[17] Given the continued use of the dye in fermented foods and beverages on one hand and the emerging evidence of the toxicity of the dye on the other, it is imperative to study the effect of tartrazine on probiotic species. The aim of the current study is to understand the effect of tartrazine on probiotic species particularly, *Lactobacillus casei, Lactobacillus plantarum and Bifidobacterium bifidum.*

Material and methods

Cultures and chemicals

Lactobacillus casei (ATCC 12116), *Lactobacillus plantarum* (ATCC 8014) and *Bifidobacterium bifidum* (ATCC 29521) were procured from United States Department of Agriculture (USDA) Agricultural Research Service (ARS) Culture Collection (NRRL), USA and National collection of Industrial Microorganism(NCIM), Pune, India. The cultures were revived and identified using biochemical tests and refrigerated for further use. Culture Media including *Bifidobacterium* Selective agar (Himedia- M1734), Hichrome lactobacillus selective agar (Himedia - M2065) and Tryptone Soy Broth (Himedia- M011), and Tartrazine (Himedia- GRM431) were used.

Preparation of Tartrazine stock solution

Stock solutions in three different concentrations (1%, 2% and 3%) were reconstituted with demineralized water. Furthermore the concentrations T1 (100ppm), T2 (200ppm) and T3 (300ppm) were studied by diluting 1ml from stock solution of 1%, 2% and 3% respectively in broth.

Experimental study

Effect of T1, T2, and T3 were studied for their effect on the test organisms *Lactobacillus casei* [LC], *Lactobacillus plantarum* [LP] & *B. bifidum* [BB] at specific time intervals as shown in Table 1. Viable cell counts were determined using standard plate count method[18] in broths at fixed intervals. *Lactobacillus casei* (1%) was inoculated in four 100ml TSB, each made-up to Tartrazine concentration T1, The cell density of LC at a concentration of 1ml/100ml of the broth was estimated at 0hrs. Thereafter, cell density of LC at intervals of 6h, 24h & 30h were estimated from both controls and the tests [LC+T1]. The same was followed with other concentration [LC+T2, LC+T3]. A similar setup was designed and performed using *Lactobacillus plantarum* and *Bifidiobacterium bifidum*.

Table 1 Experimental design with bacterial cultures and tatrazine concentrations

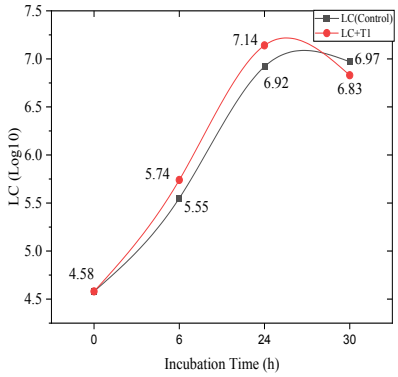
	0h 6h 24h 30h	0h 6h 24h 30h	0h 6h 24h 30h
SET 1	LC(Control)	LP(Control)	BB(Control)
	LC+T1 (Test)	LP+T1 (Test)	BB+T1 (Test)
SET 2	LC(Control)	LP(Control)	BB(Control)
	LC+T2 (Test)	LP+T2(Test)	BB+T2 (Test)
SET 3	LC(Control)	LP(Control)	BB(Control)
	LC+T3 (Test)	LP+T3(Test)	BB+T3(Test)

(LC, *Lactobacillus casei;* LP, *Lactobacillus plantarum;* BB, *Bifidobacterium bifidum;* T1: 100ppm tartrazine T2: 200ppm tartrazine T3: 300ppm tartrazine)

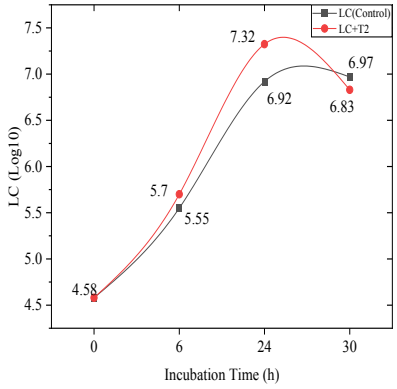
Results

Growth pattern of *Lactobacillus casei* with tartrazine

The growth pattern of *Lactobacillus casei* as control (LC) and with the concentrations of 100, 200, 300ppm of dye as test (denoted as LC+T1, LC+T2 and LC+T3 respectively) is as shown in Figure 1: A-C. The log10 concentration of *L. casei* when exposed to tartrazine at concentrations T1, T2, T3, exhibited marginal though, increased growth compared to control at 6h, which then decreased with time. Log10 concentrations of control (LC), test 1(LC+T1), test 2 (LC+T2) and test was 5.55, 5.74.5.7 and 5.62 at 6h respectively (Figure 1A-C). There was no significant difference observed in the growth pattern of *L.casei* in test with different concentration of tartrazine (T1, T2, T3) at 24h and 30h as compared to control (LC).



(A)



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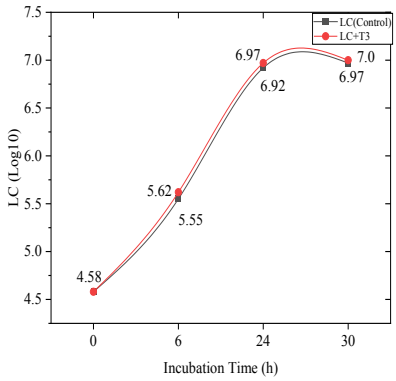


(C)

Figure 1 Growth pattern of LC (A) With T1 (B) With T2 (C) With T3.

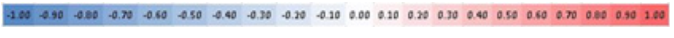
Growth pattern of *Lactobacillus plantarum* with tartrazine

The growth pattern of *Lactobacillus plantarum* with T1, T2 and T3 is shown in Figure 2A-C. The cell concentration (Log10) of *L. plantarum* was 4.13 at 0h with T1. The concentrations of control (LP) were 4.97, 5.97 and 5.4 at 6, 24 and 30 hours respectively. Similarly the concentrations of the test-1 (LP+T1) were 4.57, 6.2 and 5.5 at 6, 24 and 30 hours respectively (Fig 2-A). With test-2 (LP+T2) the initial cell concentration was 6.70 (0 hours). The cell concentrations of the test-2 (LP+T2) were 7.2, 7.53 and 7.3 as against the cell concentrations 7.23, 7.9 and 7.3 of the control (LP) at 6, 24 and 30 hours respectively (Fig 2 B). Cell concentration of test-3 (LP+T3) was estimated to be 5.4(0 hours), 5.7, 6.33 and 6.33 as against 5.73, 6.43 and 6.4 of the control (LP) at 6, 24 and 30 hours respectively (Figure 2C). Interestingly, it was observed that growth of *L. plantarum* when exposed to T1, increased with time. Hence, as the concentrations of tartrazine increased (T2 and T3), the cell density, which was unaffected initially (6 hours), showed a decline with time. The difference in log 10 concentration of tests and control at 24h were 0.23, -0.03 and -0.1) for test-1, 2 and 3 respectively (Table 2). Decline in growth was observed at 24 and 30hrs respectively as compared to the control.

Table 2 Variation in Log10 concentration of bacterial species with tatrazine and control

	LC1	LC2	LC3	LP1	LP2	LP3	BB1	BB2	BB3
6h	0.19	0.15	0.07	-0.4	-0.03	0.03	0.04	-0.04	-0.12
24h	0.22	0.4	0.05	0.23	-0.37	-0.1	0	0.04	0.06
30h	-0.14	-0.14	0.03	0.1	-0.03	-0.07	0.03	-0.06	-0.22

Colour Scale (log10 values from -1.0 to 1.0)



LC1: (LC+T1)-(LC Control); LC2: (LC+ T2)-(LC Control); LC3: (LC+T3)-(LC Control); LP1: (LP+T1)-(LP Control); LP1: (LP+T1)-(LP Control); LP2: (LP+T1)-(LP Control); LP3: (LP+T1)-(LP Control); BB1: (BB+T1)-(BB Control); BB2: (BB+T2)-(BB Control); BB3: (BB+T3)-(BB Control)

Growth pattern of *Bifidobcaterium bifidum* with tartrazine

The growth pattern of *Bifidobacterium bifidum* with T1, T2 and T3 is shown in Figure 3A-C. The cell concentration (Log10) of *B. bifidum* was 6.1 at 0h with T1. The log10 concentrations of control (BB) and test-1 (BB+T1) were the same, i.e.,7.4, 8.07, and 8.17 at 6, 24 and

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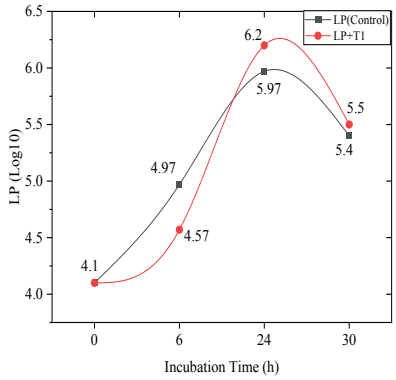
30h respectively (Figure 2A). With test-2 (BB & BB+T2) the initial cell concentration was 6.0 (0 hours). The cell concentrations of the test-2 (BB+T2) were 7.17, 7.26, 7.3 and 7.08 at 6, 24 and 30 hours as against the cell concentrations 7.18, 7.22 and 7.36 of the control (BB) at 6, 24 and 30 hours respectively (Figure 2B). Cell concentration of test-3 (BB+T3) was estimated to be 5.55 (0h), 7.1, 7.31 and 7.2 as against 5.56, 7.24 and 7.31, 7.42 of the control (BB) at 6, 24 and 30 hours respectively (Figure 2C). *B. bifidum* when exposed to 100ppm showed no change in growth pattern. However, there was marginal decline in growth with 200ppm and 300ppm TZ.



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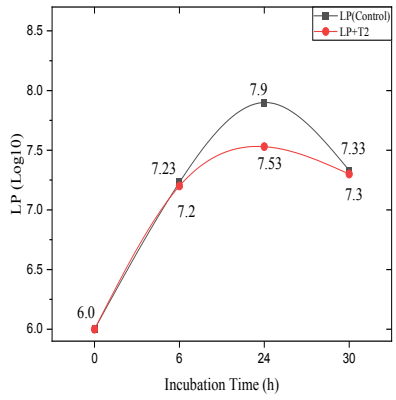

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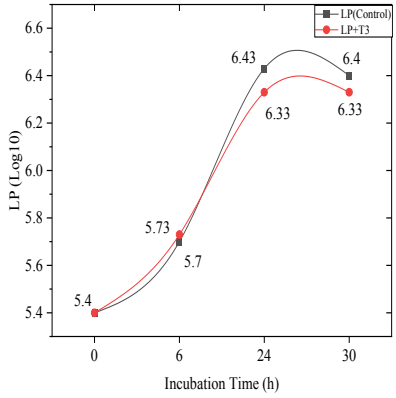
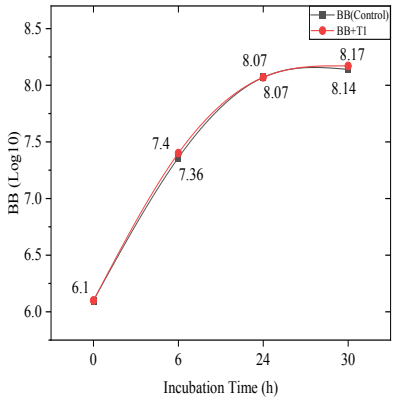
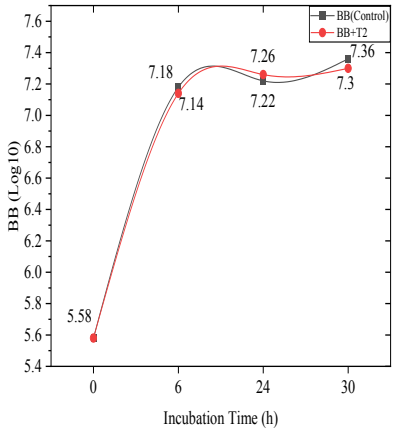


(C)

Figure 2 Growth pattern of LP (A) With T1 (B) With T2 (C) With T3.



(A)



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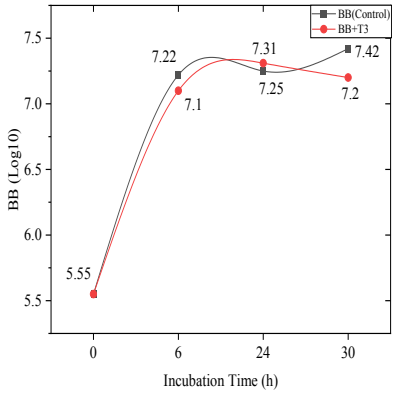

(C)

Figure 3 Growth pattern variation of BB (A) With T1 (B) With T2 (C) With T3.

Discussion

The implications of excessive usage of food additives and their health impact has been well established.[19-21] Literature indicates

permissible limits defined by the Codex Alimentarius Commission,[22] United States Food and Drug Administration[24] and other regulatory agencies (Table 3) on the use of tartrazine. While the effect of the dye has been a subject of concern with reference to human health, their effect on beneficial which includes the gut microbiota and probiotic species used in food and food supplements, is an aspect that has not garnered the due attention. The demand for Probiotic foods owing to its health benefits is emerging, needless to mention the growth of commercial market with probiotic foods. Probiotic foods and drinks also include additives in their compositional chart. Among others tartrazine is a food colour that is being used in probiotic drinks.

Table 3 Permissible limit of tartrazine for fermented foods

Regulatory agencies	Permissible limits
Codex Alimentarius International food Index(2018)	300mg/kg
United states food & Drug administration(2022)	300mg/kg
Food and Drug Regulations, Canada (2021)	300mg/kg
Food Safety Standard Authority of India(2020)	100mg/kg
Food Safety Standards for Food Additive, China(2015)	50mg/kg

The issue of relevance in this context is to understand the effect of additives such as tartrazine on the microbiota in use in the probiotic foods (drinks). The effect of additives may have bearing to the beneficial attributes of probiotics on their growth, proliferation and molecular expression.

This work was carried out to study the effect of tartrazine as an additives on the growth and proliferation of *L casei* (LC), *L. plantarum* (LP), and *B. bifidum* (BB). Based on the permissible limits (Table 1), the concentration range of tartrazine was decided to be between 100 to 300ppm.

Lactobacillus casei when exposed to tartrazine at concentrations 100ppm (T1), 200ppm (T2) and 300ppm (T3) exhibited; marginal though, increased growth at T1, which then decreased with time. These observations are in concurrence to an earlier report by Seesuriyachan *et al*.,[25] in their report suggested that *L. casei* strain exhibited the role in degrading sulphonated azo dye by converting it into two end products N, N-dimethyl-p-phenylenediamine and 4-aminobenzenesulfonic acid. It could be inferred from our findings that *L.casei* probably could be degrading tartrazine. Further, it is also of relevance to mention that Azo dye undergo degradation into potential carcinogenic amines,[2] which in turn may prove a major health risk and therefore warrants more study.

Interestingly, it was observed that growth of *L. plantarum* when exposed to T1, increased with time. But when tartrazine concentration increased (T2 and T3), the cell density, which was unaffected initially (6 hours), showed a marginal decline with time. From the reports by Seesuriyachan *et al*.,[25] where it was reported that the derivatives of azo dye degradation have antimicrobial properties, it could be hypothesized that the derivatives of the azo dye degradation could be responsible for decline in the population density of *L. plantarum.* Decline in growth was observed at 24 and 30hrs respectively as compared to the control. The reduction of azo dyes into its derivatives by *L. plantarum* is in turn affecting *L. plantarum* itself. It is evident that the probability of degradation of tartrazine by the *bacterium* exists and that the concurring effect of the derivative of the degradation on the bacterium itself both need further investigation. This would support the application of probiotics in degradation of azo dyes in the environment as has been hypothesized by Elbanna *et al*.,[26] Similar observations were made with *B. bifidum*, when exposed to 100ppm, which showed no change in growth pattern. However, there was marginal decline in growth with 200ppm and 300ppm Tartrazine. The cell concentration of *L. casei* and *B. bifidum* were observed to have no effect when exposed to tartrazine. It is of relevance to discuss the recent findings on the expression of specific, beneficial molecules by the test organisms used in the study. There have been reports of expression of metabolites beneficial for mood regulation in humans by the test organisms.[27,28] It would be pertinent to study the molecular expression of the test organisms in the presence of tartrazine.

The role of gut microbiota and probiotic supplements for maintaining the health of individuals has been a subject matter of deliberation in the recent past. Reports have established the relationship between human health and probiotics. It is therefore of relevance to understand the possible effects of food ingredients, including additives on this beneficial microbiota. This study reports the effect of tartrazine on the growth of *L. casei, L. plantarum* and *B. bifidum*. It is inferred from the above stated results, that the derivatives of tartrazine could be inhibitory to beneficial bacterial species, specifically to probiotics. Interestingly, Gut flora has been observed to metabolize tartrazine into sulfanilic acid and 4-sulfophenylhydrazine in animal and excreted as aminopyrazolone and sulfanilic acid[29] and it is noteworthy to mention that Sulfanilic acid has shown to have antimicrobial activity.[30] This gives us a reason to belive that the derivatives of tartrazine degradation, which are likely to accumulate in cultures after hours of incubation, as has been reported[29] may have inhibitory effect on the cultures. This needs further study.

Conclusion

The result of the study is indicative of possible inhibitory effect of tartrazine or the degraded form of the dye on the growth of probiotic species *L casei, L. plantarum*, and *B. bifidum*. Also, the findings prompt to explore the possible role of the probiotic species in the degradation of tartrazine. The study is novel owing its objective of understanding the impact of widely used food additive, tartrazine in probiotic foods. In light of the current research findings and in concurrence to the earlier reports it would be of relevance to investigate the intermediates formed during degradation of tartrazine, accumulation of its derivatives and its effect on probiotic species and gut flora.

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Conflicts of interest

The author declares that there is no conflict of interest.

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