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FLUORIDE EFFECT ON MINIMUM INHIBITORY CONCENTRATION AND GROWTH DYNAMICS OF *LACTOBACILLUS ACIDOPHILUS* AND *LACTOBACILLUS SALIVARIUS*

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ABSTRACT

Fluoride is the simplest fluorine anion. Fluoride is required for the human beings to prevent early dental disorders and to meet body's minimum Fluoride levels. It is supplied to human beings through several sources such as water, food, green tea, tooth paste, mouth varnishes and gels etc. Of all the mentioned sources, drinking water is a major source of Sodium fluoride. Fluoride is added to the drinking water supplies by a process known as "Water fluoridation". Sodium fluoride is a common agent used in water fluoridation. When drinking water, Fluoride concentration exceeds required levels in the body, Fluoride commence bacteriostatic activity against beneficial flora in the gastrointestinal tract. Two such commonly effected organisms are *L.acidophilus* and *L.salivarius*. These are probiotic organisms that help to maintain immunogenic gut against several pathogenic organisms. Hence, Minimum inhibitory concentration (MIC) and growth dynamics were assessed on *L.acidophilus* and *L.salivarius* in the presence of different Sodium fluoride concentrations. During the course of research, *L.acidophilus* and *L.salivarius* were observed to be inhibited at 20 mM and 40 mM Sodium fluoride concentrations respectively. At the same concentrations, growth densities of both the probiotic organisms were significantly reduced. The performed research evaluated growth dynamics of organisms in the media supplemented with Sodium fluoride. Addition of Sodium fluoride to the media exerted significance not only on the MIC and growth dynamics but also on the other metabolic activities of the organism uncertainly.

KEYWORDS: Probiotics, Sodium fluoride, *Lactobacillus acidophilus*, *Lactobacillus salivarius*.

INTRODUCTION

Sodium fluoride is an inorganic form of Fluoride used for both systemic and topical applications. Fluoride is an electronegative element of halogen family occurring naturally in the earth's crust. Fluoride sources include air, soil, water, food, green tea, tooth paste, mouth rinses and fluoride drugs respectively. Controlled addition of fluoride to public water supplies to minimize dental diseases is called "water fluoridation". Fluoridation can occur naturally or artificially by the addition of Sodium fluoride, Sodium fluorosilicate and Hydrofluorosilicic acid.[1] Fluoride is considered as an effective agent in reducing dental caries.[2] Fluoride action is part of cariostatic activity and its presence in saliva is critical in preventing dental caries.[3]

It is significant to realize that fluoride is a cumulative poison. Once ingested, fluoride is absorbed into gastrointestinal tract and further enters into cellular tissues. Fluoride combines with hydrogen ions to form hydrogen fluoride (HF), which easily crosses the gastric epithelium, and is the major form in which fluoride is absorbed from the stomach. GI tract is the first and commonly affected body system and then urogenital tract, since fluoride is excreted mainly through kidneys.[4] Remaining percentage accumulates in teeth, bones, pineal gland, and other tissues, including blood vessels.

Ingestion of excess amounts of fluoride by human and animal species affects micro flora. It shows impact on enzymes and regulatory proteins which plays important physiological role of the organism like Enolase, ATPase, catalase, antioxidant enzymes etc. Fluoride causes acidification of cytoplasm in bacterial cells making the environment acidic for the crucial enzymes.[2] Sodium fluoride inhibits *L.acidophilus* by inhibition of enolase enzyme.[5] Enolase plays a crucial role in Glycolysis. The effect of fluoride on enolase is mainly due to acidification of cytoplasm than the binding of fluoride to enolase.[2]

Probiotics

A probiotic is a live microorganism which beneficially affects the host animal by improving its intestinal microbial balance.

Major properties and functions of probiotics

Table 1: Functions of Probiotics.[2]

Adherence to host epithelial tissue	Safety, non-pathogenic and non-carcinogenic
Acid resistance and bile tolerance	Improvement of intestinal micro flora
Antagonism against pathogens in the host	Acts as Biosurfactants
Elimination of pathogens by competition with enteric pathogens for adhesion sites on gastrointestinal tract and also for nutritional sources	Inhibition of bacterial toxins production and their action
Production of inhibitory compounds like acids, hydrogen peroxide and bacteriocins that are antagonistic to pathogen growth	Ability to co-aggregate with pathogens and stimulation of IgA

Probiotic organisms selected for research

Lactobacillus acidophilus* and *Lactobacillus salivarius

Both organisms are gram-positive, non-spore forming, rod shaped, obligatory homo fermentative organisms that occurs naturally in the human intestines, oral cavities and vagina. It is said to be non-pathogenic and used as a probiotic in preventing infections. They used to produce lactic acid in fermented foods.[6,7] These species helps to enhance immunity and fight against infection. *L. acidophilus* lacks cytochromes, porphyrins and respiratory enzymes and is acidogenic, aciduric and produces lactic acid as the main product of metabolism. Lactic acid helps in the inhibition of unwanted intestinal microbes.[7] Probiotics are now emerged as vital category of supplements found in conventional, medicinal and dietary products.[8]

In current study, our effort is to identify Fluoride impact on probiotic organisms by studying the MIC and growth curves of the Fluoride treated and untreated organisms.

MATERIALS AND METHODS

Culture collection

Lactobacillus acidophilus

The starter culture of lyophilized probiotic bacterium '*Lactobacillus acidophilus*' (MTCC 10307) was procured from IMTECH, Chandigarh, India.

Lactobacillus salivarius

The starter culture of *L.salivarius* culture was prepared by using dietary supplement capsules of make R Garden.

Cultivation of bacterial strains

The lyophilized *L. acidophilus* culture was activated by dissolving in 0.85% saline whereas *L. salivarius* capsules were used directly for culture propagation. Both strains were cultivated with de Man Rogosa Sharpe (MRS) medium which is specific for the growth of *Lactobacillus* species.

Composition of de Man Rogosa Sharpe (MRS) medium

The medium was prepared by adding the following chemicals.

Table 2: Composition for one litre preparation of MRS medium.

S. No.	Chemical description	Required quantity
1	Peptone	10 g/L
2	Beef extract	10 g/L
3	Yeast extract	5 g/L
4	Na_2HPO_4	2 g/L
5	$NaCH_3CO_2.3H_2O$	5 g/L
6	Tri ammonium citrate	2 g/L
7	$MgSO_4.7H_2O$	0.2 g/L
8	$MnSO_4.4H_2O$	0.2 g/L
9	Glucose	20 g/L
10	Tween 80	1 mL/L
11	Glycerol	12%
12	Agar	15 g/L

pH was adjusted to 6.2 - 6.6 (DeMan *et al*., 1960). Prepared media was sterilized by autoclaving at 121˚C for 15minutes at 15 lbs pressure. *L. acidophilus* and *L.salivarius* were first cultured in MRS broth and then streaked onto MRS agar plates and incubated for 24 hrs at 37˚C under aerobic conditions. After 24 hours, the plates were stored at 4˚C for further use and preservation.

Determination of Minimum inhibitory concentration of *L. acidophilus* and *L. salivarius*

Preparation of Sodium fluoride concentrations

The minimum inhibitory concentration of *L.acidophilus* and *L.salivarius* was determined by diluting different concentrations of sodium fluoride.

Preparation of inoculums

For laboratory research, MRS medium is the commonly used medium to grow *Lactobacilli*. Higher growth rate of *L.acidophilus* and *L. salivarius* in MRS medium indicates media fulfill the growth demands of organisms. Colonies (*L. acidophilus* and *L. salivarius*) were taken

from prepared agar plates for MIC analysis. A 0.5 McFarland standard was used for visual comparison of culture suspensions to a density equivalent of 1.5 X108 CFU/ml.

McFarland Standard preparations

McFarland Standards are turbidity standards used to determine approximate density of bacteria present in a liquid suspension. These are determined by visual comparision of bacterial suspensions with appropriate McFarland standard.

0.5 McFarland standard = 0.05 mL of 1.175% of barium chloride +9.95 mL of 1% sulphuric acid

Mixing the two compounds forms a barium sulphate precipitate, causing turbidity in the solution. The volumes of the two reagents are adjusted to prepare standards of different turbidity that represent different concentrations of bacteria.

Table 3: Composition of McFarland standards.

McFarland Standard No.	0.5	1	2	3	4
1.0% Barium chloride (ml)	0.05	0.1	0.2	0.3	0.4
1.0% Sulfuric acid (ml)	9.95	9.9	9.8	9.7	9.6
Approx. cell density ($1X10^8$ CFU/mL)	1.5	3.0	6.0	9.0	12.0
% Transmittance*	74.3	55.6	35.6	26.4	21.5
Absorbance*	0.08 to 0.1	0.257	0.451	0.582	0.669

*at wavelength of 600 nm

Effect of Sodium fluoride on Minimum inhibitory concentration of *L. acidophilus* and *L. salivarius*

For determination of Sodium fluoride minimum inhibitory concentration, 2 sets of 6 sterile 50 ml conical flasks, total 12 were taken for MIC analysis. For each flask in 12 flasks, 10 ml of MRS broth was added. For both *L.acidophilus* and *L.salivarius*, different sodium fluoride concentrations were added. For the two sets of flasks, increasing concentrations of sodium fluoride at 0 mM, 10.0 mM, 20.0 mM, 30.0 mM, 40.0 mM, and 50.0 mM were added. After addition of media and sodium fluoride to the flasks, flasks were kept for autoclaving at 121˚C/15 minutes/15 lb's pressure.

Approximately 10 µL of inoculum from each organism (which is equal to O.D 0.5 McFarland standard) was aseptically added to each set of different concentrations of Sodium fluoride. Cultures of *L.acidophilus* and *L.salivarius* were taken from prepared agar plates. Then, the flasks were incubated at 37˚C for 24 hrs under aerobic conditions. After incubation, the optical densities for each concentration were recorded at 600 nm. The lowest concentration of

sodium fluoride that is completely preventing the growth of *L.acidophilus* and *L.salivarius* was analyzed.

Evaluation of growth curves

Bacterial population growth studies were performed by the inoculation of *L.acidophilus* and *L.salivarius* into sterile broth media. Inoculated cultures are incubated under optimal growth conditions and required time. Under these conditions, cells divided rapidly and the microbial growth was analysed by means of growth curve. Analysis was done by plotting cell densities versus incubation time to outline growth of organisms.

Growth curves of *L.acidophilus* and *L.salivarius* were determined by the amount of biomass produced in relation to time. Strains of *L.acidophilus* and *L.salivarius* were grown in MRS broth at 37˚C for 24 hours and approximately 0.1 ml of each strain were inoculated into conical flasks containing 49.9 ml of MRS broth with sodium fluoride (test) and without Sodium fluoride (control) and incubated at 37˚C. The bacterial growth (OD values) of control and test cultures was measured at 600nm absorbance in the spectrophotometer for every one hour interval upto 30 hours where stationary phase and decline phases were observed.

RESULTS AND DISCUSSION

Determination of Sodium fluoride Minimum Inhibitory Concentration (MIC) on *Lactobacillus acidophilus* and *Lactobacillus salivarius*

The effect of sodium fluoride on the growth of probiotic strains was determined by MIC. Concentrations of sodium fluoride in the range of 10 mM, 20 mM, 30 mM, 40 mM and 50 mM were tested on *L.acidophilus* and *L.salivarius*. The growth of *L.acidophilus* and *L.salivarius* were correlated with that of cells grown in the absence of Sodium fluoride in MRS broth, which acts as control.

The MIC of Sodium fluoride was observed to be probable at 20 mM for *L.acidophilus* and 40 mM for *L.salivarius*. This indicates there is growth inhibition of *L.acidophilus* and *L.salivarius* at low sodium fluoride concentrations. At milli molar fluoride concentrations, the growth inhibition effect on the organisms was increased with increase in the incubation time.

It is interesting to know the inability of *L.acidophilus* and *L.salivarius* organisms, to grow at milli molar concentrations of Sodium fluoride.

Determination of Sodium fluoride MIC on *L.acidophilus*

Table 4: Minimum Inhibitory Concentration of Sodium fluoride on *L.acidophilus*

S. No	Concentration of NaF (mM)	OD Values at 600 nm
1	0	0.54
2	10	0.22
3	20	0.17(MIC)
4	30	0.04
5	40	0.02
6	50	0.02

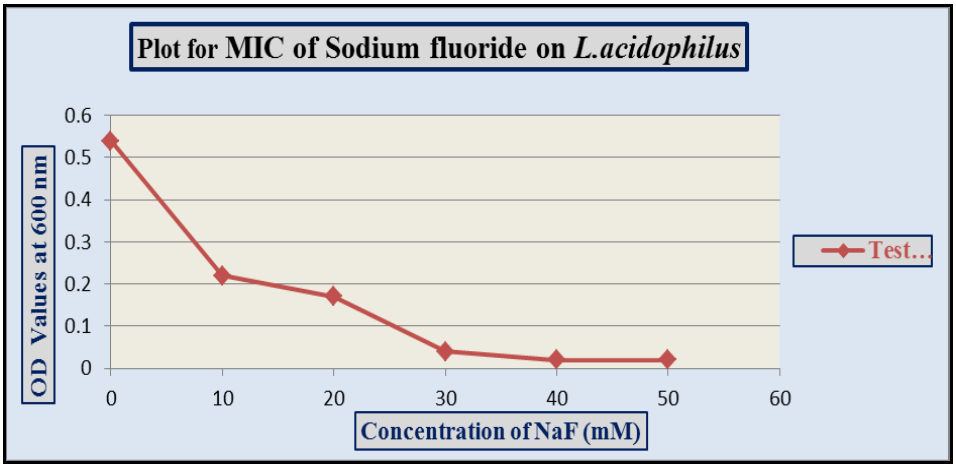

Figure- 1: Plot for inhibition of *L.acidophilus* by Sodium fluoride.

The above graph represents at 20 mM Sodium fluoride concentration, the growth of *L.acidophilus* was observed to be inhibited. Hence, the MIC of sodium fluoride on *L.acidophilus* is 20 mM.

Determination of Sodium fluoride MIC on *L.salivarius*

Table 5: Minimum Inhibitory Concentration of Sodium fluoride on *L.salivarius*

S. No	Concentration of NaF (mM)	OD Values at 600 nm
1	0	0.54
2	10	0.38
3	20	0.23
4	30	0.21
5	40	0.16 (MIC)
6	50	0.02

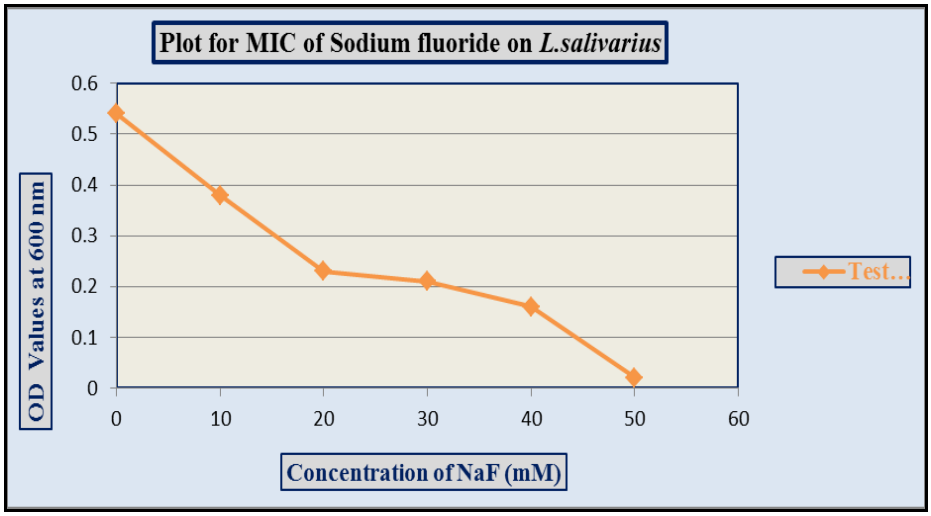

Figure 2: Plot for inhibition of *L.salivarius* by Sodium fluoride

The above graph represents at 40 mM Sodium fluoride concentration, the growth of *L. salivarius* was observed to be inhibited. Hence, the MIC of sodium fluoride on *L.salivarius* is 40 mM.

Evaluation of *L.acidophilus* and *L.salivarius* growth curves against Sodium fluoride stress

The lag, log, stationary and decline phases of *L.acidophilus* and *L.salivarius* were analysed in presence (Test) and absence (control) of Sodium fluoride stress conditions. During the experiment, growth results of both organisms were collected for every one hour time interval. Calorimetric measurements were used for measuring turbidity, as an index of increasing cellular mass.The decrease in microbial cell density caused by sodium fluoride action was observed in both *L.acidophilus* and *L.salivarius*.

Evaluation of growth curves of *L.acidophilus* under normal and Sodium fluoride stress

The relation between OD at 600nm and time in hours (Two hours interval for plots) helps us to understand the growth characteristics of *L.acidophilus* and *L.salivarius*. Increase in OD represents increase in cell number over a period of time. This relation was used to estimate the concentration of *L.acidophilus* and *L.salivarius* live cells in MRS broth under normal and stress conditions.

Table 6: *L.acidophilus* growth curve results under 20 mM Sodium fluoride stress for two hours interval

Time Interval (hrs)	OD values	
	Control	Test (20 mMNaF)
0	0	0
1	0.03	0
2	0.06	0.02
4	0.08	0.04
6	0.13	0.08
8	0.18	0.15
10	0.24	0.19
12	0.35	0.24
14	0.42	0.36
16	0.52	0.37
18	0.63	0.4
20	0.67	0.53
22	0.76	0.5
24	0.76	0.5
26	0.76	0.5
28	0.68	0.42
30	0.62	0.36

In the above table, control represents culture without Sodium fluoride, test represents culture with fluoride (with 20 mMNaF).

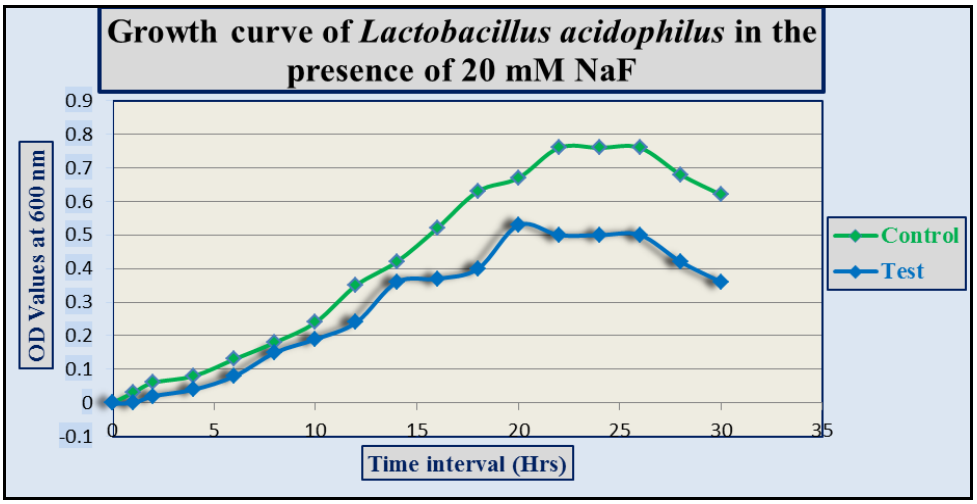

Figure 3: *L. acidophilus* growth curve under 20 mM Sodium fluoride stress.

The above growth curve indicates profile of *L.acidophilus* up to 30 hrs period of time under normal and 20 mM sodium fluoride stress conditions. All phases i.e. lag, log, stationary and decline phases were clearly understood from the graph, which is a sigmoidal curve.

Growth curve phases for *L.acidophilus* control (Without fluoride)

In *L.acidophilus*, lag phase was observed for four hours, log phase for eighteen hours, stationary phase for four hours and followed by death phase for four hours.

Growth curve for *L.acidophilus* test (With fluoride)

In *L.acidophilus*, lag phase was observed for four hours, log phase for sixteen hours, stationary phase for six hours and followed by death phase for four hours.

Evaluation of growth curves of *L.salivarius* under normal and Sodium fluoride stress

Table 7: *L.salivarius* growth curve results under 40 mM Sodium fluoride stress for two hours interval.

Time Interval (hrs)	OD values	
	Control	Test (40 mMNaF)
0	0	0
1	0.04	0.02
2	0.11	0.05
4	0.14	0.08
6	0.17	0.14
8	0.25	0.18
10	0.34	0.26
12	0.48	0.38
14	0.54	0.46
16	0.82	0.52
18	0.93	0.68
20	0.9	0.74
22	0.9	0.74
24	0.9	0.74
26	0.72	0.62
28	0.6	0.58
30	0.58	0.52

In the above table, control represents culture without fluoride, test represents culture with fluoride (with 40 mMNaF).

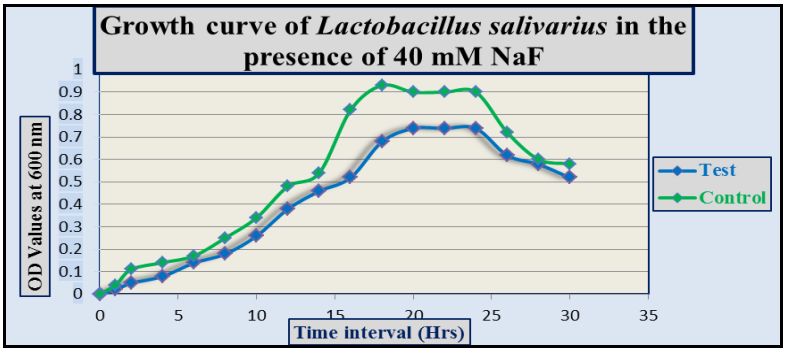

Figure 4: *L. salivarius* growth curve under 40 mM Sodium fluoride stress.

The above growth curve indicates the growth profile of *L.salivarius* upto 30 hrs period of time under normal and 40 mM Sodium fluoride stress conditions. All phases i.e. lag, log, stationary and decline phases were clearly understood from the graph which is a sigmoidal curve.

Growth curve phases for *L.salivarius* control (Without fluoride)

In *L.salivarius*, Lag phase was observed for four hours, log phase for fourteen hours, stationary phase for six hours and followed by death phase for six hours.

Growth curve for *L.salivarius* test (With fluoride)

In *L.salivarius*, Lag phase was observed for four hours, log phase for fourteen hours, stationary phase for six hours and followed by death phase for six hours.

In *L.salivarius* strain, timings of different growth curve phases in the presence and absence of Sodium fluoride are similar, but notable difference was observed between the OD's which represents cell densities.

The objective of the study was to observe the effect of Sodium fluoride on growth characteristics of test organisms using MIC and growth curve. Although a large number of minerals are essential for growth, some can be harmful for living cells in excess quantities like Fluoride. This is mainly due to the fact "Fluoride inhibits the enzymes of metabolism" thus making cells inactive. Sodium fluoride is detrimental to *L.acidophilus* and *L.salivarius* even at millimolar concentrations present in the growth medium. Primarily, the preliminary characteristics of strains like cell growth and minimum inhibitory concentration were analysed.

Increased concentrations of Sodium fluoride have inhibitory effects on growth of the organism. In normal growth medium, both *L.acidophilus* and *L.salivarius* were grown well and when incubated with media containing Sodium fluoride have impact on the microbial growth.

In the current study, Minimum Inhibitory Concentration (MIC) is the lowest concentration of Sodium fluoride that inhibits the growth of *L.acidophilus* and *L.salivarius*. That specific MIC was selected as concentration for the subsequent proteomic analysis of both *L.acidophilus* and *L.salivarius.*

CONCLUSION

Drinking water is the largest source of Fluoride. Several countries in the world lack appropriate Fluoride content in drinking water required for living creatures. Lack of appropriate Fluoride content in the water causes dental caries and other fluoridation diseases. To avoid such circumstances, world countries are using Sodium fluoride in the drinking water utilities, food products and dental products etc.

When the concentration of Sodium fluoride exceeds in intake, Fluoride starts impacting human system and probiotic flora in the body. *L.acidophilus* and *L.salivarius* are sensitive to Sodium fluoride at excess concentration. Fluoride is known to impact cellular respiration of flora by inhibiting metabolic enzymes like *Enolase*, *ATPase* which are key enzymes in glycolytic catabolism and energy generation**.** Inhibition of glycolysis and ATP synthesis results lack of ATP for further subsequent metabolic and molecular processes. This would impact the survival of the probiotic organism. *L.acidophilus* and *L.salivarius* play essential functions as human microflora; it was interested to know Sodium fluoride impact on the growth.

The current study started with MIC and growth curve of probiotic organisms (*L.acidophilus* and *L.salivarius*). Minimum inhibitory concentration of *L.acidophilus* and *L.salivarius* was observed at minimal concentrations of Fluoride i.e., 20 mM and 40 mM respectively. The same concentrations of Sodium fluoride were used for growth curve analysis. Upon growth curve analysis, it was found there was a greater impact on growth phases of Sodium fluoride treated *L.acidophilus* and *L.salivarius* strains than the untreated strains.

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