



## Preliminary Protein Profiling of Sodium Fluoride treated *Lactobacillus acidophilus* and *Lactobacillus salivarius*

Sandhyapriya Parthasaradhi, Chenchu Lakshmi Kandati and Jasti Pramoda Kumari\*

Department of Microbiology, S.V. University, Tirupati, Andhra Pradesh, India.

**Abstract:** Sodium fluoride is a trace element required for human beings to prevent early dental disorders and to meet body's minimum Fluoride levels. It is signified as a nutritional supplement for the prevention of dental caries in children of areas with inadequate Fluoride concentration in the drinking water. When Fluoride concentration exceeds required levels in the body, it commences bacteriostatic activity against beneficial flora in the gastrointestinal tract. Two such commonly affected organisms are *L. acidophilus* and *L. salivarius*. These are probiotic organisms that help to maintain immunogenic gut against several pathogenic organisms. In our previous study, Minimum inhibitory concentration (MIC) and growth dynamics were assessed on *L. acidophilus* and *L. salivarius*, in the presence of different Sodium fluoride concentrations. *L. acidophilus* and *L. salivarius* were observed to be inhibited at 20 mM and 40 mM Sodium fluoride concentrations respectively. These inhibitory concentrations were selected for further analysis. The proteins were isolated from such Sodium fluoride treated and untreated cells, the protein concentration was estimated by Bradford assay and protein profiling was done by 1D Gel Electrophoresis. The protein concentration is found to be higher in Sodium fluoride untreated organisms and below 3 kDa proteins of Sodium fluoride treated samples. Whereas low protein concentration was observed in the above 3 kDa protein samples (*L. acidophilus* treated protein sample above 3 kDa and *L. salivarius* treated protein sample above 3 kDa) of fluoride treated organisms. *L. acidophilus* and *L. salivarius* showed difference in protein expression under fluoride stress. Protein expression is high in *L. salivarius* than *L. acidophilus*. This is an indication that these strains have different capabilities for adapting to varying environmental conditions. we conclude that there is no impact on below 3 kDa protein samples in Sodium fluoride treated organisms and impact was there on the above 3 Kda proteins which are inhibited.

**Keywords:** Probiotics, Sodium fluoride, *Lactobacillus acidophilus*, *Lactobacillus salivarius*, 1D Gel Electrophoresis.

### \*Corresponding Author

Jasti Pramoda Kumari\*, Department of Microbiology, S.V. University, Tirupati, Andhra Pradesh, India.



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## I. INTRODUCTION

Sodium fluoride is often referred to as a “Double edged sword”, because in small doses it acts as an essential trace element with notable protective effect in preventing *dental caries* and *osteoporosis*. On the other hand, excess exposure to fluoride exerts harmful effects on the organism.<sup>1</sup> Fluoride is signified as a nutritional supplement for prevention of dental caries in children<sup>2</sup> and increases bone strength in all the ages of human beings. In teeth, this agent may also inhibit acid production by commensal oral bacteria.<sup>3</sup> On the exterior tooth enamel, Fluoride binds to calcium ions in the hydroxyapatite and helps in preventing deterioration of tooth enamel by acids.<sup>4</sup> Water fluoridation is a process of adding fluoride to the drinking water systems as a public health measure.<sup>5</sup> Sodium fluoride is used as a supplement in areas where the level of naturally occurring fluoride is inadequate.<sup>6</sup> But ingestion of excess amounts of fluoride affects micro flora in human and animal species. It shows impact on enzymes and regulatory proteins which plays an 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.<sup>7</sup> Sodium fluoride inhibits *L. acidophilus* by inhibition of enolase enzyme.<sup>8</sup> 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. Probiotics are considered to be “live microorganisms that give health benefits to the host when administered in adequate amount.”<sup>9</sup> These are beneficial and are naturally found in the human gastrointestinal tract. They are often called “good” or “helpful” bacteria because they help to maintain healthy gut. The term probiotic is derived from the Latin preposition “pro,” which means “for” and the Greek word “biotic” meaning “bios” or “life”.<sup>10</sup> Probiotics are now emerged as a vital category of supplements found in conventional, medicinal and dietary products.<sup>11</sup> The risk of several chronic diseases like inflammatory bowel disease, obesity, type 2 diabetes, cardiovascular disease, and cancer are reduced by the role of intestinal microbiome.<sup>12</sup> The different Adhesion mechanisms of probiotics to the intestinal mucosa, antagonism against pathogens, simulation and modulation of the immune system are well explained.<sup>13</sup>

### 1.1 Probiotic organisms selected for research - *Lactobacillus acidophilus* and *Lactobacillus salivarius*

Both bacteria are gram-positive, non-spore forming, rod shaped obligate homo fermentative bacteria 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 are used to produce lactic acid in fermented foods.<sup>14</sup> 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.<sup>15</sup> In the current study, our effort is to identify Fluoride impact on probiotic organisms by isolation, quantification of protein by Bradford assay method and protein profiling by ID of the fluoride treated and untreated organisms.

## 2. MATERIALS AND METHODS

### 2.1 Culture collection of *Lactobacillus acidophilus* and *Lactobacillus salivarius*

The starter culture of lyophilized probiotic bacterium ‘*Lactobacillus acidophilus*’ (MTCC 10307) was procured from IMTECH, Chandigarh, India and the starter culture of *L. salivarius* culture was prepared by using dietary supplement capsules of make R Garden.

### 2.2 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.

### 2.3 Analysis of protein extraction

Eight samples from *L. acidophilus* and *L. salivarius* were extracted for further analysis.

**Table 1 Samples for protein extraction**

S. No	Organism	Sample description
1	<i>L. acidophilus</i>	Control above 3 Kda (Without Sodium fluoride)
2	<i>L. acidophilus</i>	Control below 3 Kda (Without Sodium fluoride)
3	<i>L. acidophilus</i>	Test above 3 Kda (With Sodium fluoride)
4	<i>L. acidophilus</i>	Test below 3 Kda (With Sodium fluoride)
5	<i>L. salivarius</i>	Control above 3 Kda (Without Sodium fluoride)
6	<i>L. salivarius</i>	Control below 3 Kda (Without Sodium fluoride)
7	<i>L. salivarius</i>	Test above 3 Kda (With Sodium fluoride)
8	<i>L. salivarius</i>	Test below 3 Kda (With Sodium fluoride)

In the above table (Table 1), Test sample refers to cultures grown in the presence of Sodium fluoride whereas Control refers to culture without Sodium fluoride. The inoculated cultures of *L. acidophilus* and *L. salivarius* were removed from 20mM and 40mM fluoride treated culture by centrifugation (5,500 rpm, 10 min and 4°C). The pellet was suspended in 3ml lysis buffer<sup>16</sup> and sonicated for 5 min at 45 Hz with an interval of 30s. The cell lysate was subjected to centrifugation at 10,000 rpm for 10 minutes. The

supernatant was treated as protein sample. The sample was further subjected to filtration with 3kDa cut off by using Amicon centrifugal filters. Cut-off filter along with supernatant sample was placed in the centrifuge tube and centrifuged (5,000 rpm for 5 minutes). Protein samples below 3 kDa migrate through the filter membrane and collected in centrifuge tube whereas protein samples above 3 kDa left over in the cut off filter which was collected in an eppendorf tube. After extraction the concentration of

obtained protein was determined by Bradford protein assay.<sup>17</sup>

## 2.4 Protein quantification by Bradford protein assay method<sup>17</sup>

In Bradford assay, for protein quantification 8 samples mentioned in the Table: I were analysed and Blank (Distilled water and Bradford reagent)

## 2.5 Preparation of protein samples for SDS-PAGE (1D)

One dimensional gel electrophoresis (SDS PAGE) 12% was performed in 1.0 mm thick discontinuous gel by Laemmli's procedure (1970) at 40mA constant current (Bio Rad). 10ml of 12% resolving gel was prepared and allowed to polymerize for 20-30 min. Added 1ml of 5% stacking gel onto it. 15 well comb was placed and allowed to polymerize. 15µl of protein sample was mixed gently with the protein loading dye and loaded into wells. Protein marker with known molecular weight was added in a well for reference.

## 3. RESULTS

### 3.1 Growth inhibition of *L. Acidophilus* and *L. salivarius* by sodium fluoride

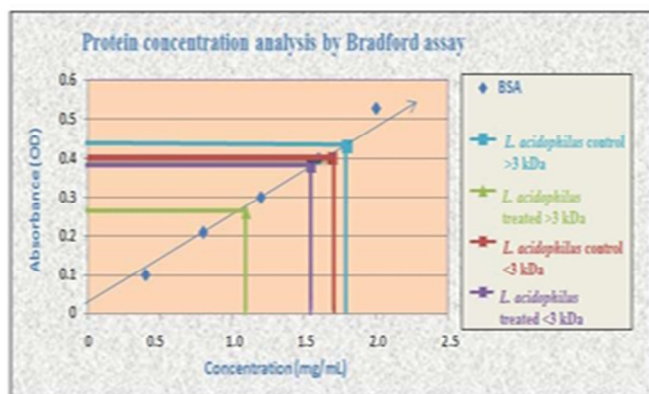
In our previous study, effect of Sodium fluoride on the growth inhibition of *L. acidophilus* and *L. salivarius* was investigated<sup>18</sup>. These ions exerted approximately 50% inhibition at the concentrations of 20mM and 40 mM respectively. These inhibitory concentrations were selected for further analysis.

### 3.2 Quantitative analysis of protein content of *L. acidophilus* and *L. Salivarius* under sodium fluoride stress by Bradford assay

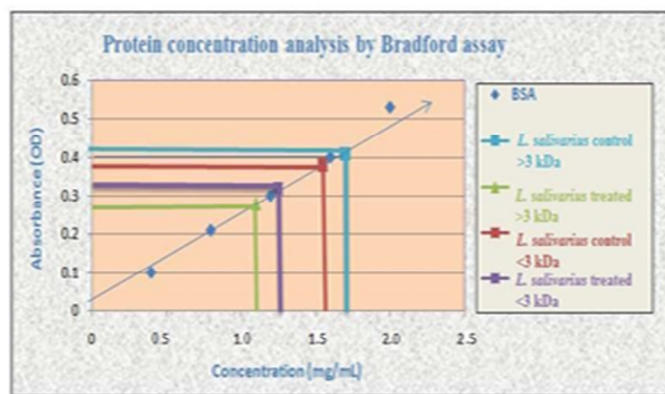
For the analysis of the protein concentration, proteins were isolated from respective samples (control *L. acidophilus* and Fluoride treated, control *L. salivarius* and Fluoride treated) (Table 2).

**Table 2 Quantitative analysis of protein concentration of *L. acidophilus* and *L. salivarius* under Sodium fluoride stress**

<i>L. acidophilus</i>			<i>L. salivarius</i>		
Concentrations (µg/ml)		ABS (OD values)	Concentrations (µg/ml)		ABS (OD values)
BSA	4	0.1	BSA	4	0.1
	8	0.21		8	0.21
	12	0.3		12	0.3
	16	0.4		16	0.4
	20	0.53		20	0.53
<i>L. acidophilus</i> control >3 kDa (10 µl)		0.43	<i>L. salivarius</i> control >3 kDa (10 µl)		0.41
<i>L. acidophilus</i> control <3 kDa (10 µl)		0.40	<i>L. salivarius</i> control <3 kDa (10 µl)		0.38
<i>L. acidophilus</i> treated >3 kDa (10 µl)		0.26	<i>L. salivarius</i> treated >3 kDa (10 µl)		0.28
<i>L. acidophilus</i> treated <3 kDa (10 µl)		0.38	<i>L. salivarius</i> treated <3 kDa (10 µl)		0.32



**Figure 1a**



**Figure 1b**

**Fig 1. Protein concentration analysis of 1a) *L. acidophilus* and 1b) *L. salivarius* by Bradford Assay**

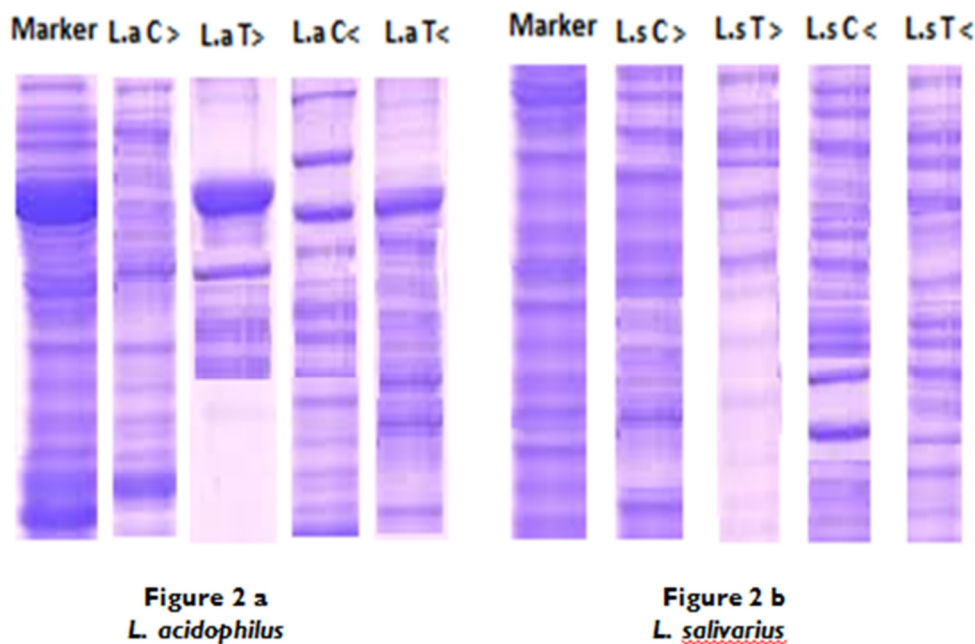
Based on figure 1a), protein concentration of *L. acidophilus* control above 3 kDa protein sample (10 µl) is 1.80 µg/µl and *L. acidophilus* test above 3 kDa protein sample (10 µl) is 1.07 µg/µl. Whereas protein concentration of *L. acidophilus* control below 3 kDa protein sample (10 µl) is 1.70 µg/µl and *L. acidophilus* test below 3 kDa protein sample (10 µl) is 1.53 µg/µl. Based on graph 1b), protein concentration of *L. salivarius* control above 3 kDa protein sample (10 µl) is 1.7 µg/µl and *L. salivarius* test above 3 kDa protein sample (10 µl) is 1.1 µg/µl. Whereas protein concentration of *L. salivarius* control below 3 kDa protein sample (10 µl) is 1.52 µg/µl and *L. salivarius* test below 3 kDa protein sample (10 µl) is 1.20 µg/µl.

### 3.3 Protein Profiling Of *L. Acidophilus* and *L. Salivarius* By 1 D Gel Electrophoresis (SDS-PAGE)

Protein expressions of the extracted proteins from Sodium fluoride treated and untreated *L. acidophilus* and *L. salivarius* were studied by SDS-PAGE. The SDS-PAGE bands showed differences in the expression of proteins, both in the presence and absence of Fluoride. Bands in SDS-PAGE showed less expression of proteins in the presence of Fluoride, whereas protein expression was high in control samples without Fluoride. With SDS-PAGE results, it can be concluded that there might be an involvement of Sodium fluoride stress during the expression of proteins. As per studies done by other researchers, there might be a stress on Enolase

enzyme of glycolysis, which shows impact on metabolism and finally

on the protein expression.



**Fig 2. Protein profiling by one dimensional gel electrophoresis for 2a) *L. acidophilus* and 2b) *L. salivarius***

In the above figure, L.a C > represents *L. acidophilus* control above 3 kDa protein sample  
 L.a T > represents *L. acidophilus* test above 3 kDa protein sample  
 L.a C < represents *L. acidophilus* control below 3 kDa protein sample  
 L.a T < represents *L. acidophilus* test below 3 kDa protein sample  
 L.s C > represents *L. salivarius* control above 3 kDa protein sample  
 L.s T > represents *L. salivarius* test above 3 kDa protein sample  
 L.s C < represents *L. salivarius* control below 3 kDa protein sample  
 L.s T < represents *L. salivarius* test below 3 kDa protein sample

With the help of one-dimensional gel electrophoresis with protein markers (figure 2), we can analyse Sodium fluoride impact where expression of proteins in *L. salivarius* is higher than the expression of proteins in *L. acidophilus*. Thus with the comparison of 1-D protein profiles, we can evaluate fluoride treated and untreated samples showed differences in the expression of protein bands.

### 3.4 Comparison of protein expression profiles in response to sodium fluoride in *L. Acidophilus* and *L. Salivarius*

When the proteins bands in 1D were observed, proteins above 3 kDa expressed the least in *L. acidophilus* compared to *L. salivarius*. This may be due to the involvement of sodium fluoride stress on the protein expression of *L. acidophilus*.<sup>19</sup> It is clearly evident from the MIC and growth curve, because 20 mM sodium fluoride concentration inhibited the growth of *L. acidophilus*. But in the case of *L. salivarius*, proteins present in *L. salivarius* were suppressed and stress response proteins were expressed due to fluoride stress. So it explains the response of *L. acidophilus* and *L. salivarius* protein profiles to fluoride stress. As like physiological studies (MIC and Growth curve), *L. acidophilus* and *L. salivarius* showed difference in protein expression under fluoride stress. Protein expression is high in *L. salivarius* than *L. acidophilus*. This is an indication that these strains have different capabilities for adapting to varying environmental conditions.

## 4. DISCUSSION

The current research started with MIC and growth curve studies of *L. acidophilus* and *L. salivarius*. Minimum inhibitory concentration of *L. acidophilus* and *L. salivarius* was observed at minimal Sodium fluoride concentrations i.e., at 20 mM and 40 mM respectively<sup>18</sup>. Based on the results of MIC and growth curve studies, research was further proceeded to protein extraction, protein quantification by Bradford's assay and SDS-PAGE analysis. In protein isolation, for the isolated protein samples molecular weight cut-off separation was performed to know the impact of sodium fluoride on protein expression of two different sizes (Above and below 3 Kda proteins). In Bradford assay, protein concentration is found to be higher in Sodium fluoride untreated organisms<sup>19</sup> and below 3 kDa proteins of Sodium fluoride treated samples. Whereas low protein concentration was observed in the above 3 kDa protein samples (*L. acidophilus* treated protein sample above 3 kDa and *L. salivarius* treated protein sample above 3 kDa) of fluoride treated organisms. In SDS-PAGE analysis, fewer bands were observed in sodium fluoride treated protein samples, especially in the above 3 kDa protein samples of both organisms. Whereas in sodium fluoride untreated protein samples and treated protein samples of below 3 kDa, more bands were observed. Hence based on the results of Bradford assay and SDS-PAGE, it was concluded that there is no impact on below 3 kDa protein samples in Sodium fluoride treated organisms. Addition to the earlier studies, current research is supporting inhibition of Enolase enzyme<sup>20</sup> (Molecular weight: 80000-120000

Daltons) by Sodium fluoride, as above 3 Kda proteins are inhibited in the current study.

## 5. CONCLUSION

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. *Lacidophilus* 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. *Lacidophilus* and *L.salivarius* play essential functions as human microflora; it was interested to know Sodium fluoride impact

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on the growth. From the present study, we conclude that there is no impact on below 3 kDa protein samples in Sodium fluoride treated organisms and impact was there on the above 3 Kda proteins which are inhibited.

## 6. ACKNOWLEDGEMENT

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## 7. AUTHORS CONTRIBUTION STATEMENT

Mrs. Sandhya Priya gathered data with regard to this work. Dr. Pramoda kumari analysed the data and necessary inputs were given towards the designing of the manuscript. All the 3 authors discussed the methodology, results and contributed to the final manuscript.

## 8. CONFLICT OF INTEREST

Conflict of interest declared none.

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