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ORIGINAL RESEARCH

EFFECTIVENESS OF DIFFERENT SODIUM FLUORIDE CONCENTRATIONS AGAINST LACTOBACILLUS CASEI IN THE INHIBITION ZONE: A NOVEL INOCULATION MICROBIOLOGICAL STUDY

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ABSTRACT

Background: Lactobacillus Casei is a bacterium related to dental caries by producing acidification of the oral environment and leading to teeth demineralization. It appears that these bacteria correlate with another pathogenic microorganism, as evidenced by increased extracellular protein production. Sodium fluoride has been used as a preventive treatment to prevent acidification and reduce the risk of caries disease. At the same time, the optimal sodium fluoride concentration for prevention and treatment remains unclear, which also allows for safety in dental products. The present trial aims to find the best sodium fluoride concentration that is effective in eliminating Lactobacillus Casei.

Methods: To evaluate the inhibitory effect of sodium fluoride on Lactobacillus casei, we conducted a preliminary trial using distilled water (0% sodium fluoride) to refine experimental procedures. This trial revealed contamination from airborne dust, prompting adjustments such as working near a Bunsen burner flame to create a sterile environment and manually sterilizing all equipment with ethanol before and after use. Bacterial inoculation involved evenly spreading L. casei from yogurt onto MRS agar plates using sterile cotton swabs. Filter paper discs were prepared by dipping them into sodium fluoride solutions of varying concentrations (0.15%, 0.30%, 0.45%, and 0.60%) and placed on the agar. Plates were incubated at 32.5°C for 72 hours. The radius of the inhibition zone around each disc was measured using a digital caliper, and results were averaged for each concentration. Sodium fluoride solutions were prepared through serial dilutions, starting from 99% sodium fluoride powder mixed with distilled water.

Results: The results demonstrated a strong positive correlation ($r = 0.942$) between the sodium fluoride concentration and the radius of the inhibition zone, as illustrated by the non-linear trend in the processed data. At 0% sodium fluoride, a small inhibition zone (mean 0.58 mm) was observed due to contamination, but with increasing concentrations, the inhibition zone expanded significantly, peaking at 3.30 mm for 0.60% sodium fluoride. Standard deviations were minimal for most concentrations, indicating homogeneity in the data, except for 0% and 0.45%, which showed greater variability. The statistical analysis confirmed the significance of the correlation ($p = 0.016$), rejecting the null hypothesis and supporting the alternative hypothesis that higher sodium fluoride concentrations inhibit bacterial growth more effectively. Error bars highlighted the precision of the data, with the lowest error observed at 0.30% sodium fluoride (0.11 mm).

Conclusion: The most effective concentration of sodium fluoride was 0.60%. This concentration obtained the widest zone of inhibition against Lactobacillus Cassei, followed by 0.45% concentration.

Keywords*:* Sodium fluoride, Lactobacillus Casei, microbiology, inhibition zone, dental caries

INTRODUCTION

Nowadays, dental caries is still one of the most common diseases worldwide, affecting 60-90% of children and almost every adult. This disease seems to be dependent on socio-economic status, being more prevalent in low-income communities. Its risk factors are also dependent on the country and education, but it is common to observe a high intake of sugar with insufficient oral hygiene. Fluoridation is an effective preventive treatment to avoid enamel demineralization, reducing the prevalence of this disease in children who live in low and middle-income countries [1]. 5% sodium fluoride (NaF) varnish and 38% diamine fluoride are adequate against dental caries without statistical differences between the two products. Whereas, as behavior modifications significantly prevent this pathology and due to the public cost increase general fluoridation might lead to, health and oral education programs are essential for prevention [2]. Therefore, a recent meta-analysis has presented evidence that chemical disorders occur in saliva when caries are active, which could also aid in prevention. It was shown that during active caries, the levels of chloride, magnesium, and zinc are elevated in contrast to healthy patients, whose calcium, phosphorus, potassium, and sodium levels are higher. Fluoride level alterations are still controversial [3]. However, fluoride has been used in dentistry for years to deal with caries remineralization, dental erosion, and hypersensitivity [4]. Fluoride components have been included in mouthwashes, dentifrices, or varnishes [2,4]. Also, in orthodontic patients, fluoride has been used to prevent white spot lesions, which are prevalent after debonding and are considered the initial stage of caries development [5]. Previously, NaF concentrations were studied in order to optimize their effects. It was shown that 2.800 ppm fluor ion as NaF in dentifrices had statistically higher effectiveness than 1.1000 ppm concentrations after one, two, and three years of use [6]. This treatment aims to prevent the demineralization of the enamel caused by cariogenic bacteria. The most common and studied cariogenic bacteria is Streptococcus Mutans. Cariogenic bacteria have high tolerance to acidification and also acidify the oral environment leading with demineralization and cavitation of the tooth. In addition, they have the capability of modifying cell-to-cell communication by affecting extracellular deoxyribonucleic acid (DNA). Other cariogenic microorganisms are Lactobacillus Casei (LC) and Candida Albicans [7]. Lactobacillus are the second most cariogenic bacteria, and they have high adhesion capability to zirconia and metallic porcelain crowns [8]. LC has also been linked to oral carcinoma and to DNA replication increases of other pathogens [9]. In addition, patients suffering from carcinoma and treated with head and neck radiotherapy often suffer

Radiation caries. It has been studied as a topic of fluoridation for those patients, but its use is controversial, and its optimum concentration is still unclear in the literature [10]. Fluorides, even being necessary for bone structures and teeth development, are able to cause high body damage if human exposure is excessive [11].

The purpose of this trial is to compare the effectiveness of five different concentrations (0%, 0.15%, 0.30%, 0.45%, and 0.60%) of Sodium Fluoride against *Lactobacillus Casei* bacteria during 72 hours of bacteria inoculation.

2. METHODOLOGY

2.1 Trial design

A preliminary trial was conducted with distilled water to simulate 0% sodium fluoride to assess the trial methodology during the experiment, trying to avoid errors and risks. In the control trial, it was shown that the agar plate contained an abnormal bacterial growth after 72 hours, caused by dust air contamination. This side effect could have been avoided by using a laminar flow hood. After that, the technique was modified, working closer to the Bunsen burner flame, which prevents external contamination by heat application. Moreover, before and after using any device, a manual sterilization process was performed to avoid another kind of contamination, which might affect the results of the study.

Controlled variables

Table 1: Showing a list of controlled variables along with brief reasoning.

Variables

Table 2: Showing independent and dependent variables with justification.

Table 3: Showing material required and quantity in (-)

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Controlled variables

Table 1. Showing a list of controlled variables along with brief reasoning.

Variable	Why control ?	How to control	Possible effect
Type of Lactobacillus (casei)	Because different types of lactobacillus respond differently to the inhibitory effect of sodium fluoride due to their physiological characteristics, hence the result will be affected.	The same type of lactobacillus was used during the entire experiment by taking samples from the same yogurt pot.	If the variable is left uncontrolled, it will cause inaccurate comparisons, when comparing the different concentrations of sodium fluoride.
Time	Because prolonged exposure typically correlates to a larger zone of inhibition. (10)	Each concentration of sodium fluoride was exposed to a lactobacillus for a 72-hours period.	If the variable is left uncontrolled, it will cause an inaccurate comparison, therefore giving unreliable data.
Petri medium (MRS Agar plates)	Because different types of Petri dishes may contain a different Petri medium that can be less or more suited environment for bacterial growth.	The same types of Petri medium were used for each trial, during the entire investigation.	If different types of Petri dishes with different mediums are used, it may affect bacterial growth causing comparison to be inaccurate.
Temperature when growing	Because most bacteria grow best at a temperature between 30-40°C.	The inoculated agar plates were kept all together in a stable environment at 32.5°C.	If the variable is not kept under control it will heavily affect the bacterial growth.
Volume of distilled water	Because it will affect the dilution of sodium fluoride.	100 ml measuring cylinder is a good choice with an uncertainty of 0.5±1.0	If left uncontrolled it will highly affect the zone of inhibition.

Variables

Table 2. Showing independent and dependent variables with justification

Independent variable	Significance/Justification
Concentration of sodium fluoride (0%, 0.15%, 0.30%, 0.45%, and 0.60%)	Increasing the concentration of sodium fluoride will significantly affect bacterial growth as stated in the alternative hypothesis. Concentration will be changed by diluting sodium fluoride (99%) using distilled water. Calculation for finding out after concentration is stated in the methodology section.
Dependent variable	**Significance/Justification**
Zone of inhibition	The zone of inhibition will be measured after a 72 hours period, by measuring the radius using a digital caliper. Zone of inhibition will be affected by the concentration of sodium fluoride.

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Table 3. Showing material required and quantity in (-)

Material Required		
Digital caliper (1)	Hole puncher (1)	Measuring cylinder (1)
Bunsen burner (1)	Forceps (1)	Sterilized cotton swap (1 pack)
MRS agar plates (30)	Spatula (1)	Autoclave (1)
Filtered paper (1 pack)	Mass Balance (1)	Beaker (5)
Yogurt with *L.casei* (1)	99% Sodium fluoride concentration	Distilled water
Plastic gloves (1 pack)	95% Ethanol	Disinfectant spray

Trial justification and risk assessment

The range of sodium fluoride concentration was chosen from 0% to 0.60%. The 0% concentration was chosen as a sample control, allowing for comparison with higher concentrations. The range from 0.15% to 0.60% was chosen because it is the most common concentration range found in toothpastes. The concentration was not increased more than 0.60% because it might be harmful to people's intake, as it is not recommended to exceed 6000 ppm. De Man-Rogosa-Sharpe (MRS) agar plates were selected as the concrete solid surface used for bacteria inoculation. In the agar plates, it is also possible to provide bacteria with the adequate nutrients they need for their multiplication [12].

2.3 Interventions

The methodology aims to assess the effectiveness of five different sodium fluoride concentrations against LC bacteria using the Kirby-Bauer method and measuring the zone of inhibition (13). The protocol performed in this trial has been explained below step by step, starting with SC bacteria inoculation.

I. First, use a black marker to divide the Petri dish into four equal segments.

II. Inoculate the dish with the bacteria LC following the aseptic technique. First, take a sterile cotton swab and gently dip it into the yogurt pot for exactly 2 seconds.

Take the cotton swab with the bacteria and cover the entire surface area of the plate, moving the cotton swab back and forth. Do this back-and-forth process 4 times to ensure the LC is well distributed correctly over the dish.

III. Then, place the chemical disc according to the following instructions.

a. Carefully set up a Bunsen burner by ensuring the flame is set to a safe level by regulating the oxygen flow.

b. Take a pair of forceps and sterilize them by gently dipping them into a 95 % concentration of ethanol.

c. Modify the flame intensity of the Bunsen burner and carefully pass the forceps through the flame so the ethanol on the forceps volatilizes. In that way, the forceps will be totally sterile.

IV. Use the sterile forceps to pick up one of the sterile filter paper discs.

V. Dip half of the filter paper disc into the diluted 0.15% sodium fluoride concentration (Table 5 and Table 6).

VI. Place the disc into the middle of one of the four segments of the plate.

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VII. Repeat III to V instructions, but put the disc in a different segment of the plate. The entire process must be repeated five times for each sodium concentration (0.15%, 0.30%, 0.45% and 0.60%) with the same yogurt pot.

VIII. After 72 hours, an inhibition zone will be able to be noticed around each paper disc. Measure the radius of each zone of inhibition using a digital caliper (± 0.4mm) for each segment.

IX. Finally, calculate the average radius of each NaF concentration inhibition zone.

After SC bacteria inoculation, a serial dilution was performed in order to observe the fluoride effect.

- Label four beakers for each sodium fluoride concentration (0.15%, 0.30%, 0.45% and 0.60%).
- Take a measuring cylinder and pour 100ml of distilled water.
- Measure carefully 150 mg of sodium fluoride (99%) on a mass balance (± 0.01gr).
- Transfer the 100 ml of distilled water and 150 mg of sodium fluoride into the 0.15% labeled beaker and mix gently until the powder is dissolved.
- Repeat this entire process, but this time change the concentration of sodium fluoride according to the diluting formulae (Table 4 and Table 5).

Table 4. Showing the calculations for diluting the sodium fluoride (99%)

Formulae:	**Calculations:**
Formula = $\frac{Concentration you want \times 10}{Initial volume needed}$	Formula = $\frac{0.15 \times 10}{100}$ = 0.015g → 150 mg

Table 5. Showing the concentration and the amount of sodium fluoride (99%) needed.

Concentrations	0.15%	0.30%	0.45%	0.60%
Amount of sodium fluoride (99%) in mg	150mg	300mg	450mg	600mg

Some precautions must be taken in order to perform this methodology safely (Table 6).

Table 6. Showing safety issues and precautionary measures.

Safety issues:	**Precautionary measures:**
Chemicals such as sodium fluoride were harmful to skin and eyes and toxic if ingested in great amounts.	Rubber gloves and a lab coat were used for skin protection while eye goggles were used for eye protection.
Liquids like ethanol are highly flammable and they were used next to a Bunsen burner in the sterilization process when handling the forceps.	Ethanol was handled carefully and the sterilization process was strictly followed. (see methodology part 3)
Glass equipment was used during the investigation, which could have caused minor injuries if broken.	Glassware was handled extremely carefully and kept safe from any risk.
Agar plates are biohazard, as they contain bacterial growth.	All agar plates were autoclaved before disposal.

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RESULTS

3.1 Processed data

The mean of all inhibition zones was calculated, and also the percentage error using the standard deviation (STDEV(σ)/(Mean) ×100) as calculated in the table above. The percentage error for some means, such as 0% turned out to be extremely high as the standard deviation was greater than the mean. Furthermore, the standard deviations for 0.15%, 0.30%, and 0.60% are closer to zero, indicating that the data points are very close to the mean. This shows homogeneity in the results obtained in the trial. In contrast, the standard deviation for 0% and 0.45% is closer to one, which implies that the data points are spread further away from the mean, so they are more heterogeneous. The non-linear graph was drawn (using Google Sheets) to show possible trends in the zone of inhibition with different sodium fluoride concentrations.

3.2 Data analysis and trends

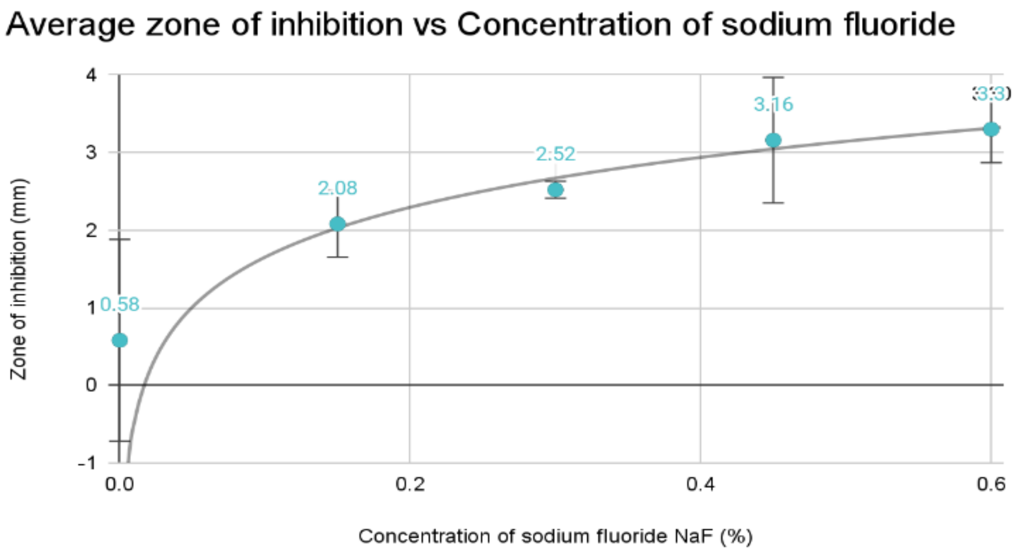

Figure 1. Shows the mean zone of inhibition for each concentration of sodium fluoride.

The data processed through the inquiry reveals a strong positive correlation, which can be highlighted in the graph above (Figure 1). Firstly, a mean inhibition zone of 0.58 mm was obtained with a 0% sodium fluoride concentration, where there should be no inhibition because there was no fluoride in the agar plate. Then, the inhibition zone mean increased to 2.08 mm when the NaF concentration was increased to 0.15%. This increase in the inhibition zone is the highest found in the graph. Another increase of the inhibition zone was observed with 0.45% NaF concentration, but it was less significant (3.16 mm). Finally, the graph reaches its peak at 0.60% NaF concentration, showing a mean of 3.3 mm in the inhibition zone radius. Moreover, the graph allows for the visualization of the error resulting from each mean. That provides a graphical representation of the data precision and homogeneity. It can be seen in the graph that the error bar for 0% NaF was relatively higher (1.30 mm) compared to the other concentration errors, indicating the heterogeneity of its results. The error bar in 0.30% NaF was the lowest, with a value of 0.11 mm. Finally, the results shown in Table 7 reveal a strong positive correlation between NaF concentration and the inhibition zone radius. To further substantiate this correlation, the Pearson correlation coefficient was calculated, yielding a value of $r = 0.942$. That mathematically asses there was a strong positive correlation between both variables. Additionally, in order to finally reject the null hypothesis, the P-values were also calculated ($p<0.05$). The p-value was 0.016, which is smaller than the critical value, indicating statistical significance in the correlation between the inhibition zone and NaF concentration. Because of all that, the null hypothesis, which said there were no differences between inhibition zone radius and NaF concentration, was totally rejected.

3.3 Trial limitations and accuracy

In addition, the limitations of this trial are shown in Table 8 as well as the improvements needed for further similar trials corresponding with the random errors which affected the precision and accuracy.

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Table 7. Showing the average mean, the standard deviation, and the percentage error of the zone of inhibition of each concentration of sodium fluoride, rounded to 2 d.p.

Concentration of Sodium Fluoride (NaF) (± 0.02%)	Radius of the zone of inhibition (±0.4mm)					Mean (mm)	Standard deviation	Percentage error (%)
	T1	T2	T3	T4	T5			
0.00	2.90	0	0	0	0	0.58	1.30	224.14
0.15	2.10	1.80	1.70	2.80	2.00	2.08	0.43	20.67
0.30	2.70	2.50	2.50	2.40	2.50	2.52	0.11	4.29
0.45	2.90	4.60	2.80	2.80	2.70	3.16	0.81	25.63
0.60	4.00	2.90	3.40	3.10	3.10	3.30	0.43	13.03

Table 8. Limitations and sources of errors affecting accuracy

	Potential effect on data	Improvements
Random errors affecting precision:		
The amount of sodium fluoride that went into the paper disc was improperly maintained during the investigation. **HIGH SIGNIFICANCE**	It might have affected the inhibitory effect of the enzyme enolase.	The volumetric pipet could have been used instead of dipping half of the paper disc.
The amount of *L.casei* in the agar plate. **HIGH SIGNIFICANCE**	It might have affected the inhibitory effect of the enzyme enolase.	A volumetric pipet could have been used instead of dipping the sterile cotton swab in the yogurt pot.
Systematic errors affecting accuracy:		
The mass balance was very sensible to any air movement or vibrations. **LOW SIGNIFICANT**	It could have caused small imprecision during the dilution process.	Anti-vibrator equipment could have been used or an air shield, to prevent any external factor affecting the weighting.
When measuring the zone of inhibition there could have been a parallax error. **MID SIGNIFICANT**	It could have caused the zone of inhibition to be either smaller or bigger.	The transilluminator could have been used in order to make the zone of inhibition more clear and reduce any type of parallax error.
Limitation of data:		
This investigation was limited to only one type of lactobacillus (Casei).	This research is limited to only one type of lactobacillus, however different types of lactobacillus would have responded differently but followed the same trend because they all contained enolase.	More research could have been done using different types of lactobacillus. This could have produced more data to support the investigation.
Concentration was limited between 0% and 0.60%.	Investigation is limited to a certain range of concentration so the data collection is limited to a range. However, this is the most common concentration that is found in toothpaste.	Having a bigger range of concentration bearing in mind the safety issues could have provided more context and data to answer the research question.

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4. DISCUSSION

The trial shows that the concentration of sodium fluoride affects the zone of inhibition when cultivating the bacteria Lactobacillus Casei. The highest zone of inhibition was 4.0 mm, adding 0.60% sodium fluoride. Whereas the lowest zone of inhibition was 0mm, while using 0% concentration of sodium fluoride. Although it should have performed some improvements in data quality, the p-value from Pearson shows a statistically significant correlation between the zone of inhibition and the concentration of sodium fluoride, allowing us to reject the null hypothesis and accept the alternative hypothesis. Although the standard deviation values in Table 7 suggest there is a low level of random errors in the experiment, evidencing the accuracy of the data collected, the standard deviation value in 0% concentration of sodium fluoride is the highest (1.30). That probably shows an iatrogenic contamination of the agar plate affecting the inhibition zone. Whereas, if a laminar flow had been used, this fact could have been avoided. Additionally, it has been shown a high r-value (0.942), indicating a strong positive correlation between the zone of inhibition and the concentration of sodium fluoride. It might be due to the fluoride ions present in sodium fluoride. These ions act as an enolase competitive inhibitor and prevent the substrate from binding to the receptors. Therefore, LC metabolism is increased, and its spread is higher as a result. This is shown in Table 7, where the widest inhibition zone was 4.0 mm using the highest concentration of sodium fluoride (0.60%).

The presence of fluoride acts to reduce the capability of LC growth, reducing its environmental pH. LC normally grows in a 7.0-3.2 pH range without fluoride, but this range is reduced to 7.0-5.4 with only 20mg NaF. Whereas, it has been observed that LC requires a higher NaF concentration in order to inhibit LC metabolism, in contrast with other cariogenic bacteria. It might have happened because LC is able to acquire tolerance to NaF at a lower pH by phenotypic changes. This tolerance seems to be totally turned with seven oral pH. Fluoride has the capability to inhibit glycolysis at the enolase step and reduce the sugar transport system. Therefore, it was said that a significant concentration of NaF would be necessary in acid environments [14]. However, high amounts of NaF lead to several injuries in the intestinal epithelium by ferroptosis promotion, affecting gut microbiota alterations [15]. In addition, researchers have studied fluorosis because it has been shown that high concentrations of fluoride in water at an early age could lead to thyroid and mental alterations. The thyroid gland seems to change its capability to store fluorides and iodines when fluorosis occurs, leading to hormonal changes and a higher risk of thyroid cysts [16].

These side effects of fluoride pose a challenge in the dentistry field because they invite finding the best match concentration of NaF, which is capable of avoiding caries, but without side effects. Dental materials include NaF in order to avoid caries because of its stability in oral conditions. Whereas its highest effect appears in the beginning, over time, its effect starts to decrease, showing bacterial tolerance. Nevertheless, NaF still shows a significant effect against cariogenic bacteria in the current evidence [17]. A similar article studied inhibition zones against LC and SM using glass ionomer with antibiotic and fluoride release. They obtained a 0 mm of LC inhibition zone in the group control, which is opposite to the present trial. A 14.04 ±1.65mm was obtained with 1% concentration and 21.96 ±2.16mm with 2%, both with statistical significance ($p<.001$). Their results seem to be higher due to the antibiotic paste used in addition to fluoride (18). Due to that, introducing antibacterial agents into glass ionomer cements has been recommended in order to increase the zone of LC inhibition [19]. Silver diamine fluoride (SDF) has also been used to avoid enamel demineralization. SDF 38% has been used before glass ionomer cements to increase the anticariogenic effect [20]. In addition, hydroxyapatite toothpastes were presented as a good substitute for fluoride ones, but their effectiveness is still controversial. It did not show better results than NaF toothpastes [21]. Moreover, the inhibition zones against SM and LC were studied in vitro with different fluoride (NaF) toothpastes concentrations (500 ppm, 1000ppm, 1100ppm, 1450 ppm, 1480 ppm, 1490 ppm, and 1500 ppm). The widest inhibition zone against LC was obtained with a 1000 ppm NaF (Parodontax Protect Neocosmed (Thailand)). This product obtained 25.92 ±0.91mm of inhibition zone against LC. Also, 0.12% chlorhexidine alone showed interesting results inhibiting 20.6 ±0.89 wide in the LC plate [22,] and NaF mixed with xyitol and erythritol showed an effective reduction of the inhibition zones [23]. But, evidence is still controversial about the optimum concentration of NaF in toothpastes because it seems that the active component interacts with other compounds, resulting in controversial results in the literature [24]. In the present study, only NaF was inoculated to avoid those interactions. On the other hand, the possibility of prescribing oral probiotics and prebiotics for high-risk caries children, added to NaF toothpaste usage, in order to reduce this condition has been presented [25, 26]. In addition, nano-silver diamine products are actually studied due to their effectiveness in caries reduction, especially in pediatric children [27]. Silver diamine fluoride has been studied with microcomputed tomography evaluation and microbiology, showing significant reduction of SM amounts and radicular caries [28]. Also, the silver nanoclusters from silver diamine showed high long-term stability and antioxidant properties [29]. The effect of silver diamine was confirmed in both gram-negative and gram-positive bacteria. [30-32].

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Within the limitations of the present in vitro study, fluoride (NaF) has shown effectiveness against Lactobacillus Casei. The width of the inhibition zone appears to increase with the increase in NaF concentration.

5. CONCLUSION

The widest zone of inhibition was obtained with 0.60% sodium fluoride concentration, followed by 0.45%. The novel technique of inoculation appears to be an accurate and reproducible method for studying material effectiveness against Lactobacillus Casei.

Compliance with Ethical Standards:
The authors declare that they have no conflict of interest. This research received no external funding.

Impact Statement

This study investigates the effectiveness of varying concentrations of sodium fluoride against *Lactobacillus casei*, contributing to the understanding of fluoride's antimicrobial potential in dental health. By utilizing a novel microbiological inoculation method, the research provides insights into optimizing fluoride usage for caries prevention, potentially guiding clinical and preventive strategies.

Authors' Contributions Statement
J.F.F. and A.B.M. designed and coordinated the study. F.S. conducted microbiological experiments. L.F., G.F., and G.C. contributed to data analysis and interpretation. C.G. provided critical revisions of the manuscript. All authors participated in writing and approved the final manuscript.

Data Availability Statement
The data supporting the findings of this study are available from the corresponding author, A.B.M., upon reasonable request.

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