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Remineralization of Incipient Enamel Lesions with Probiotics Versus Casein Phosphopeptide Amorphous Calcium Phosphate with Fluoride for Treatment of Demineralization of Maxillary Bovine Anterior Teeth: An *in Vitro* Study

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Abstract

Objectives:*To evaluate and compare the remineralization potential of two types living of bacteria probiotic and casein phosphopeptide amorphous calcium phosphate with fluoride (CPP-ACPF) on in vitro incipient enamel lesion and the stablility of remineralization produced by these agents when subjected to demineralizing solution immediately and after three months.*

Methods: *Six specimens sounds, freshly extracted bovine central incisor teeth were used in the study. Each tooth of the six specimens incorporated four windows (5×5 mm) one of which will be a control (sound enamel), the remaining three windows were of demineralized enamel, CPP-ACPF (GC MI Paste plus), and probiotic. The teeth were tested at four different time periods; at baseline, after demineralization, after remineralization immediately, and after three months of remineralization. The specimens were soaked in the demineralizing solution hydrochloric acid (HCl) pH 2.49 for 10 minutes at room temperature. Each block was individually placed in 25 ml of the solution in a numbered plastic container, then the specimens were rinsed with distilled water, and then the two agents were applied according to manufactures' instructions.*

(The Probiotic paste was applied to the predetermined marked zone and left undisturbed for 20 minutes. Then CPP-ACPF was applied to the predetermined marked zone and left undisturbed for 3 minutes. All the tested specimens were incubated in artificial saliva for one hour at 37°C.)

All specimens were tested for mineral content (calcium and phosphorus content in wt. %) using Scanning Electron Microscopy with Energy Dispersive X-ray Analysis (SEM-EDX) attachment before any treatment (control), then after each treatment.

One-Way ANOVA used to compare between tested groups and interaction between variables for mean mineral content. Kruskal Wallis test used to compare between tested materials for % of change in mineral content. Statistical analysis was performed with IBM® SPSS®[*]

Results: *At different periods immediately, and after 3 months the Ca/P ratios in both probiotic and CPP-ACPF groups, were not significantly different. However at immediate period both agents showed higher Ca/P ratios than the demineralizing, as also after 3 months, probiotic group showed higher ratio than CPP-ACPF, yet not significant.*

Conclusions: *Within the limitations of this study it can be concluded that the use of probiotic as an adjunctive, might provide appropriate oral environment for the remineralization process to occur.*

Clinical significance: *The probiotic, being a safe and relatively easy procedure, can be used as a good home-based adjuvant to supplement the action of remineralizing agents. However more clinical trials are needed to determine the optimal probiotic strains, daily doses, concentrations, and vehicles for safely improving oral health. Further studies with a mix of probiotic strains in various dosages on the long periods are needed to explore the potentiality of effecting on incipient enamel lesions.*

Key words: *Probiotic, remineralization, incipient enamel lesions, CCP-ACPF.*

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I. Introduction

Dental caries is a major problem in most countries, affecting the majority of adults. The most common type of bacteria in oral cavity is *Streptococcus mutan* that produce a bacteriocin ***(Elgamily et al., 2015)***. These micro-organisms have an ability to create the acid that lead to drop in pH in dental plaque causing demineralization on enamel surface that appear in early stage as white spots. This is known as an early caries lesion.

Remineralization is the natural repair process for non-cavited lesions. It relies on calcium and phosphate ions, assisted by fluoride, to rebuild a new surface on the existing crystal remnants in the subsurface.

Dental health organizations support prophylactic and preventive measures such as dietary alterations as sugar free chewing gums such as xylitol, and regular oral hygiene as using antibacterial mouth rinses, that neutralize the acidity in the mouth to preserve on the oral environment from growth the bacteria that cause destruction of tooth structures ***(Koli et al., 2013)***. The use of fluoride alone to treat the demineralization lesions is not enough due to the restricted effect of fluoride that need sufficient quantity of saliva or calcium and phosphate ions to produce fluorapatite from fluoride ions. With the advancement in the reparative dental materials; invented materials can overcome the limited effect of the fluoride such as casein (extracted from milk of bovine) this material conjugate the fluoride to become (CPP-ACPF) that establish high concentration of calcium and phosphate with fluoride ions to join with pellicle on the surface of the demineralized tooth.

Scientists were then looking for another approach that can inhibit oral pathogens without affecting on other beneficial oral flora. It was known that milk fermented with lactic acid bacteria inhibits the growth of proteolytic bacteria because of its low pH due to the fermentation of lactose. Based on these facts, it was suggested that consumption of fermented milk in the intestine with harmless lactic acid bacteria, modifies the intestinal pH thereby suppressing the growth of proteolytic bacteria.

Different probiotics have been used in the treatment of gastro-intestinal diseases. Recently, probiotics have been used as a treatment to promote oral health ***(Mehanna et al., 2009)***.There has also been a change in the management of the oral disease process because of better understanding of the microbiology of the oral cavity ***(Sudhakar et al., 2011)***.

The term "Probiotic" meaning "for life", and defined by the World Health Organization are live micro-organisms, which, when administered in adequate amounts, confer a health benefit on the host. The mechanism of probiotic action might be disruption of plaque biofilm formation through competition for bacteria binding sites and their nutrients, a production of antimicrobial compounds such as bacteriocins that inhibit the harmful oral lactic acid producing bacteria, ***(Meurman 2005)***. So, probiotic can be used in dentistry not only to enhance oral health but also to prevent oral disease like: caries, gingivitis, periodontitis, and halitosis. The potential application of probiotics for oral health has recently attracted the attention of several teams of researchers but area of research is still in its infancy and need more research in oral remineralization.

Therefore, the aim of this study was to search about the effectiveness of two probiotic species compared to commercially casein phosphopeptide amorphous calcium phosphate with fluoride paste regarding remineralization of the early demineralized lesions immediately and after three months in vitro study.

II. Materials and Methods

Materials used in this study were as follows:

For remineralization, two test materials were used:

1. Probiotic:

Lactobacillus and Bifidobacterium species

2. Casein phosphopeptide amorphous calcium phosphate fluoride (CPP-ACPF) remineralizing agent:

GC MI Paste plus *(GC Corporation, Japan)*

Six sounds, freshly extracted bovine central incisor teeth were selected. The teeth that showed no evidence of white spot lesion, enamel cracks, or caries on visual inspection were taken for evaluation then examined using magnifying loupes at 3X and head-light to ensure the absence of deformity, teeth were thoroughly cleaned to remove blood, debris and any attached periodontal tissue using a hand scaler, then stored in distilled water until use.

The selected teeth were marked by a pencil at the cemento-enamel junction then the roots of the teeth were molded in acrylic blocks.The molded specimens were sectioned with water-cooled diamond saw at the demarcation line to separate coronal part from root portion**,** using accuracy cutting machine

The coronal specimens were embedded in self-cure acrylic blocks and numbered horizontally on the back of each block with a permanent marker from one to six, with facial surface upward. Four equal zones were marked by a ruler on the facial surface where zone (1): was used as control, zone (2): demineralizing solution, zone (3): demineralized area followed by probiotic application, zone (4): demineralized area followed by CPP-ACPF application.

All specimens were tested for mineral content (calcium and phosphorus content in wt. %) using Scanning Electron Microscopy with Energy Dispersive X-ray Analysis (SEM-EDX) attachment before any treatment (control), then after each treatment.

Six specimens were used in the study. Each tooth of the six specimens incorporated four windows (5×5 mm) one of which will be a control (sound enamel); the remaining three windows were areas of demineralized enamel, probiotic, and CPP-ACPF. The teeth were tested at four different time periods; at the baseline, after demineralization, after immediate remineralization and after three months of remineralization.

The specimens were soaked in the demineralizing solution hydrochloric acid (HCl) for 10 minutes at room temperature ***(Ivanoff et al., 2012)***; the pH of the solution was adjusted at 2.49 with sodium hydroxide to completion of the reaction. The pH of the solution was checked using a digital pH meter to ensure stability. The volume required for each specimen was calculated as follows: each $1mm^2$ required 1ml of demineralizing solution, thus each block was soaked in 25 ml of solution to avoid the production of under or over demineralization of enamel lesion in a numbered plastic container ***(Ivanoff et al., 2012)***.

The specimens were rinsed with distilled water, and then the two agents were applied according to manufactures.

The Probiotic paste was applied to the predetermined marked zone, using stainless steel sterilized dental cement spatula, approximately 1gram, measured with a dental discoid excavator, rubbed for 5-10 seconds and left undisturbed for 20 minutes.

Then CPP-ACPF was applied to the predetermined marked zone according to manufacturer's instructions. Where, the CPP-ACPF cream was applied with a regular size disposable micro-applicator, standardized amount approximately 1gram ***(Vashisht et al., 2013)***, measured as mentioned previously rubbed for 5-10 seconds and left undisturbed for 3 minutes ***(Mehta et al., 2014).*** All the tested specimens were incubated in artificial saliva for one hour at 37°C. ***(Ivanoff et al., 2012)***

Mineral content was evaluated using scanning electron microscope (SEM). To identify the structural analysis that is used in conjunction with (EDX) Energy Dispersive X-ray analysis. To determine element analysis of calcium and phosphorus content in weight % of control, demineralized and remineralized enamel in each group. Each zone was measured separately to calculate surface of the tested materials.

The specimens were incubated in artificial saliva at 37°C for three months, during this period the tested materials were applied twice daily on the specimens, and the artificial saliva was replaced daily to avoid being oversaturated by the investing materials ***(Majstorović et al., 2013), (Jo et al., 2014)***, and pH of the solution was regularly checked using a digital pH meter to ensure stability during daily refreshment of the solution. Finally, at the end of the three months period (SEM) and (EDX) analysis were used to evaluate the remineralization mineral contents of specimens.

One-Way ANOVA used to compare between tested groups and interaction between variables for mean mineral content. Dependent t-test used to compare between follow-up periods for mean mineral content. Kruskal Wallis test used to compare between tested materials for % of change in mineral content. Statistical analysis was performed with IBM® SPSS®*

III. Results

I. Calcium (Ca)

I.1. Difference between tested groups on mean Ca wt%:

Mean and standard deviation (SD) for Ca wt% for different tested groups are presented in Table (1) and Figure (1). On examine the test groups of the study, it was shown that at the immediate testing period appear non significance different as follows, but were significantly higher than demineralization (36.99±4.47) at p-value 0.007.

Table (1): Mean and standard deviation (SD) for Ca wt% for different tested groups

		Groups								p-value
		Control		Demineralization		Probiotic		CPP-ACPF		
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	
Ca	Immediate	40.68	2.83	36.99	4.47	41.09	1.87	41.26	2.05	0.07 NS
	3 Months	40.68^{b}	2.83	36.99^{a}	4.47	43.37^{b}	1.62	42.48^{b}	2.17	0.007*

Means with the letter indicating insignificant difference within each row at $p \geq 0.05$*=significant, NS= Non-Significant

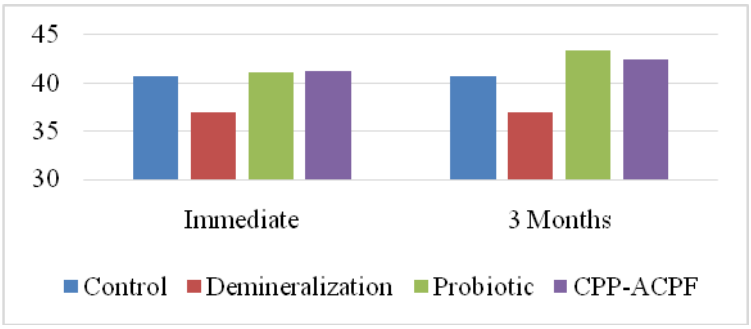

Figure (1): Bar chart showing the mean Ca wt% for different tested groups

I.2. Difference between tested groups on mean Ca wt% regardless effect of storage time intervals:
Mean and standard deviation (SD) for Ca wt% for different tested groups were presented in Table (2) and Figure (2). The mean of Ca wt% for control, probiotic and CPP-ACPF were not significantly different (40.68±2.83), (42.23±2.05), and (41.87±2.11), but were significantly higher than demineralization (36.99±4.47) at a P-value 0.003.

Table (2): Mean and standard deviation (SD) for Ca wt% for different tested groups regardless effect of storage time intervals

	Groups								p-value
	Control		Demineralization		Probiotic		CPP-ACPF		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
Ca	40.68[a]	2.83	36.99[b]	4.47	42.23[a]	2.05	41.87[a]	2.11	0.003*

Means with the letter indicating insignificant difference within each row at p ≥ 0.05*=significant

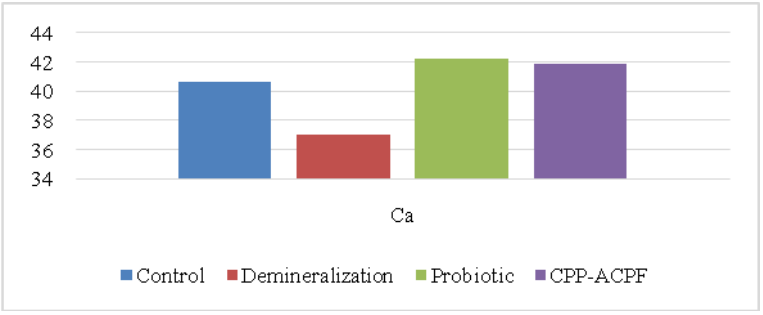

Figure (2): Bar chart showing the mean Ca wt% for different tested groups regardless effect of storage time intervals

I.3. Difference between storage time intervals on means Ca wt% regardless the remineralizing agents:
Mean and standard deviation (SD) for Ca wt% for follow up periods were presented in Table (3) and Figure (3). The mean for Ca wt% was non-significant but the mean at 3 months (42.93±1.88) higher than mean of immediate (41.18±1.88) at p-value 0.075.

Table (3): Mean and standard deviation (SD) for Ca wt% for different storage time intervals regardless the remineralizing agents:

	Time				p-value
	Immediate		3 Months		
	Mean	SD	Mean	SD	
Ca	41.18	1.88	42.93	1.88	0.075 NS

NS= Non-Significant

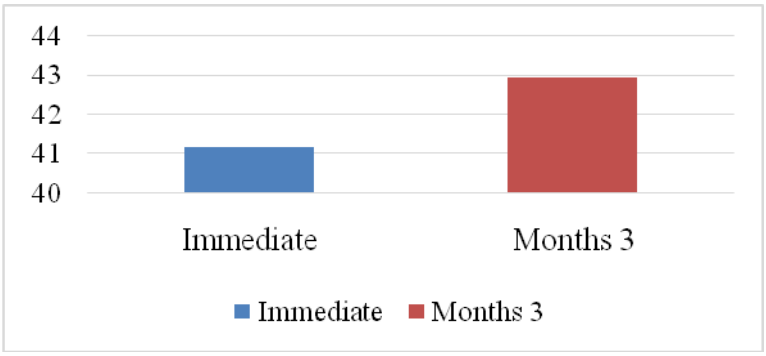

Figure (3): Bar chart showing the mean Ca wt% for different storage time intervals regardless the remineralizing agents

I.4. Difference between storage time intervals on mean Ca wt%:

Mean and standard deviation (SD) for Ca wt% for different follow-up periods were presented in Table (4) and Figure (4). The Ca wt% was in probiotic group significantly higher at 3 months than immediate period, whereas in CPP-ACPF was revealed insignificant difference between two periods at p-value 0.282.

Table (4): Mean and standard deviation (SD) for Ca wt% for different storage time intervals

		Time				Difference		p-value
		Immediate		3 Months				
		Mean	SD	Mean	SD	Mean	SD	
Ca	Probiotic	41.09	1.87	43.37	1.62	-2.28667	1.90675	0.032*
	CPP-ACPF	41.26	2.05	42.48	2.17	-1.21333	2.46652	0.282 NS

*=significant, NS= Non-Significant

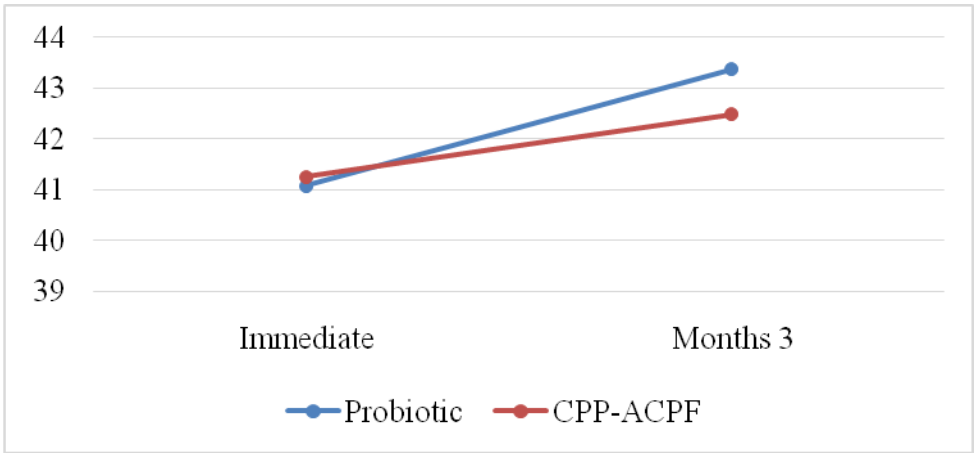

Figure (4): Line chart showing the mean Ca wt% for different storage time intervals

I.5. Interaction between variables on mean Ca wt%:

Mean and standard deviation (SD) for Ca wt% for interaction between all tested groups were presented in Table (5) and Figure (5). The highest mean for Ca wt% in all tested groups was in probiotic after 3 months (43.37±1.62), followed by CPP-ACPF after 3 months (42.48±2.17), CPP-ACPF at immediate (41.26±2.05), probiotic at immediate (41.09±1.87), control (40.68±2.83), and the lowest was in demineralization (36.99±4.47) at p-value 0.007.

Table (5): Mean and standard deviation (SD) for Ca wt% for interaction between variables in descending order

		Ca		Rank	p-value
		Mean	SD		
Interaction	Demineralization	36.99	4.47	b	0.007*
	Control	40.68	2.83	a	
	Probiotic+ Immediate	41.09	1.87	a	
	CPP-ACPF+ Immediate	41.26	2.05	a	

	CPP-ACPF+ 3 Months	42.48	2.17	a	
	Probiotic+ 3 Months	43.37	1.62	a	

*=significant

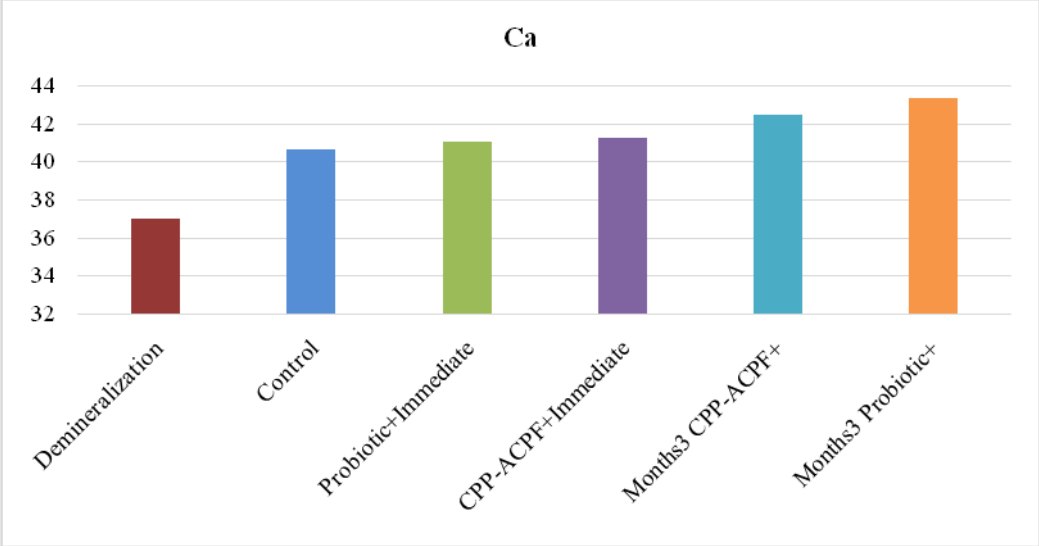

Figure (5): Bar chart showing the mean Ca wt% for different storage time intervals between variables in descending order

II. Phosphorus (P)

II.1. Difference between tested groups on mean P wt%:

Mean and standard deviation (SD) for P wt% for different tested groups are presented in Table (6) and Figure (6). On examine the test groups of the study, it was shown that at the immediate testing period appear non significance different as follows; control (19.33±.63), demineralization (2.13±19.98), probiotic (19.98±.84) and CPP-ACPF (19.88±.73) at p-value 0.191. While after 3 months testing period, it was revealed that all groups were significant difference but between groups were non-significant difference while, both probiotic and CPP-ACPF (20.38±1.01), (20.55±.47) did not differ significantly, but were significantly higher than control and demineralization although was highly significance than demineralization (19.33±.63), (18.52±2.13) respectively at p-value 0.034.

Table (6): Mean and standard deviation (SD) for P wt% for different tested groups

		Groups								**p-value**
		Control		**Demineralization**		**Probiotic**		**CPP-ACPF**		
		Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	
P	**Immediate**	19.33	.63	18.52	2.13	19.98	.84	19.88	.73	0.191 NS
	3 Months	19.33^{ab}	.63	18.52^{a}	2.13	20.38^{b}	1.01	20.55^{b}	.47	0.034*

Means with the letter indicating insignificant difference within each row at p≥0.05
*=significant, NS= Non-Significant

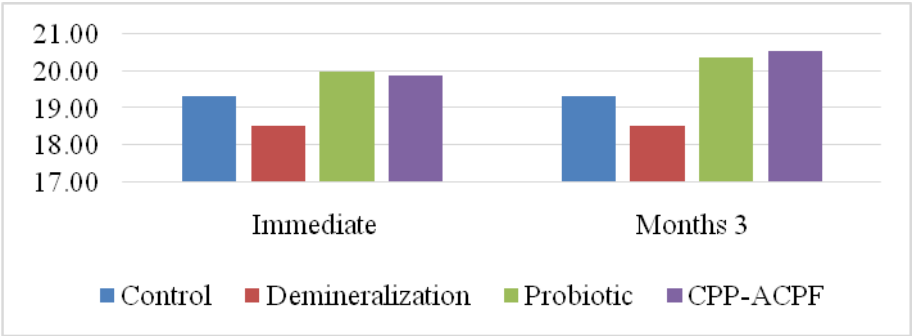

Figure (6): Bar chart showing the mean P wt% for different tested groups

II.2. Difference between storage time intervals on mean P wt%:

Mean and standard deviation (SD) for P wt% for different follow-up periods were presented in Table (7) and Figure (7). The P wt% was non-significant in both group while, at 3 months probiotic and CPP-ACP were highly significant than immediate period.

Table (7): Mean and standard deviation (SD) for P wt% for different storage time intervals

		Time				Difference		p-value
		Immediate		3 Months				
		Mean	SD	Mean	SD	Mean	SD	
P	Probiotic	19.98	.84	20.38	1.01	-.39500	1.28853	0.487 NS
	CPP-ACPF	19.88	.73	20.55	.47	-.67500	.83116	0.103 NS

NS= Non-Significant

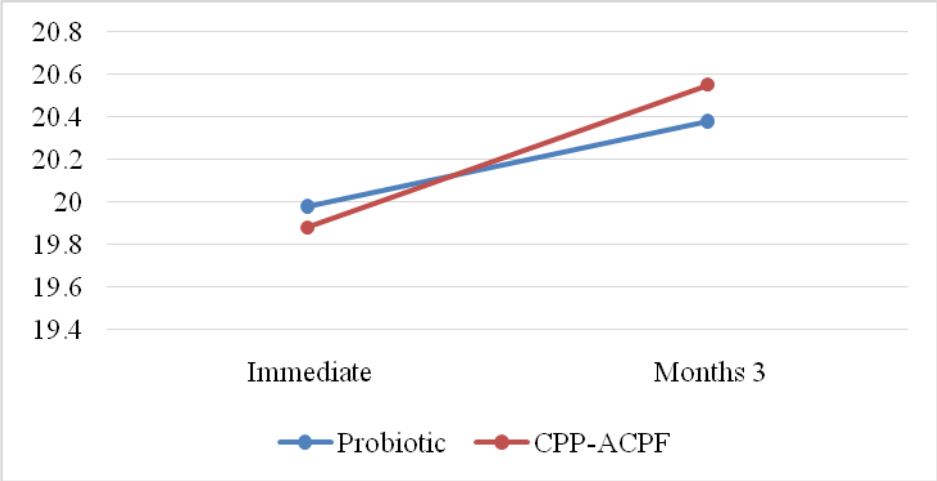

Figure (7): Line chart showing the mean P wt% for different storage time intervals

III.Calcium Phosphorus (Ca/P)

III.1. Difference between tested groups on mean Ca/P:

Mean and standard deviation (SD) for Ca/P for different tested groups are presented in Table (8) and Figure (8). On examine the test groups of the study, it was shown that at the both testing period immediate and at 3 months appear non significance different as follows; in immediate control and demineralization (2.10±.14), (2.00±.08) respectively, while probiotic and CPP-ACPF (2.06±.08), (2.08±.10) respectively at p-value 0.337. After 3 months testing period, it was revealed; probiotic (2.13±.15) was higher than immediate testing period, while CPP-ACPF asymptotically to immediate (2.07±.09) at p value 0.250.

Table (8): Mean and standard deviation (SD) for Ca/P for different tested groups

		Groups								p-value
		Control		Demineralization		Probiotic		CPP-ACPF		
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	
Ca/P	Immediate	2.11	.14	2.00	.08	2.06	.08	2.08	.10	0.337 NS
	3 Months	2.11	.14	2.00	.08	2.13	.15	2.07	.09	0.250 NS

Means with the letter indicating insignificant difference within each row at p≥0.05

NS= Non-Significant

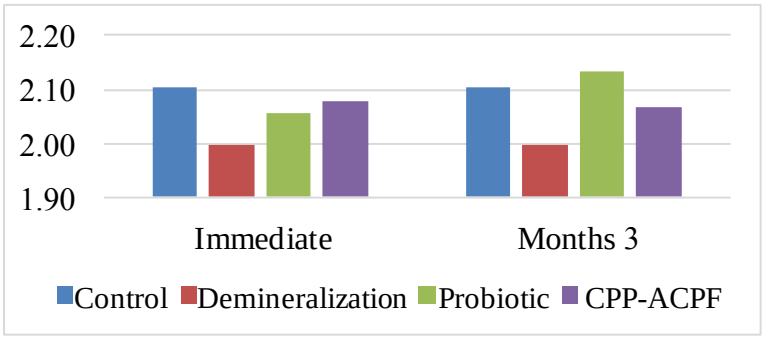

Figure (8): Bar chart showing the mean Ca/P for different tested groups

III.2. Difference between storage time intervals on mean Ca/P:

Mean and standard deviation (SD) for Ca/P for different follow-up periods were presented in Table (9) and Figure (9). The Ca/P wt% was non-significant in both group probiotic and CPP-ACP but highly at 3 months than immediate period in the probiotic, at 3 months revealed (2.13±.15) compared to (2.06±.08) at immediate at p-value 0.227. While in CPP-ACPF the values were asymptotically (2.07±.09), and (2.08±.10) at 3 months and immediate respectively at p-value 0.874.

Table (9): Mean and standard deviation (SD) for Ca/P for different storage time intervals

		Time				Difference		p-value
		Immediate		3 Months				
		Mean	SD	Mean	SD	Mean	SD	
Ca/P	Probiotic	2.06	.08	2.13	.15	-.07647	.13618	0.227 NS
	CPP-ACPF	2.08	.10	2.07	.09	.01029	.15115	0.874 NS

NS= Non-Significant

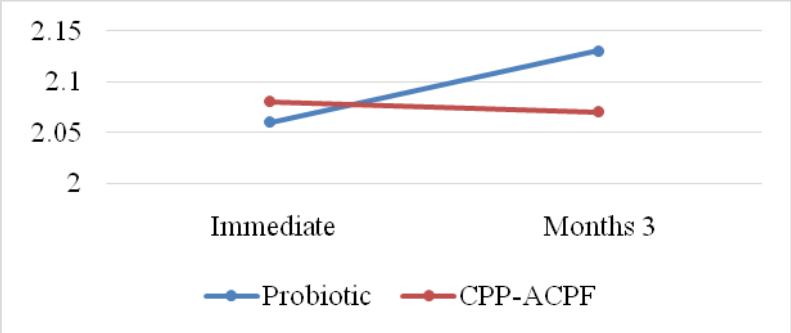

Figure (9): Line chart showing the mean Ca/P for different storage time intervals

III.3. Interaction between variables on mean Ca/P wt%:

Mean and standard deviation (SD) for Ca/P wt% for interaction between all tested groups were presented in Table (10) and Figure (10). The highest mean for Ca/P wt% in all tested groups was in probiotic after 3 months (2.13±.15), followed by control (2.11±.14), CPP-ACPF at immediate (2.08±.10), CPP-ACPF at 3 months (2.07±.09), probiotic at immediate (2.06±.08), and the lowest was in demineralization (2.00±.08) at p-value 0.416.

Table (10): Mean and standard deviation (SD) for Ca/P wt% for interaction between variables in descending order

		Ca/P		p-value
		Mean	SD	
Interaction	Demineralization	2.00	.08	0.416 NS
	Probiotic+ Immediate	2.06	.08	
	CPP-ACPF+ 3 Months	2.07	.09	
	CPP-ACPF+ Immediate	2.08	.10	
	Control	2.11	.14	
	Probiotic+ 3 Months	2.13	.15	

NS= Non-Significant

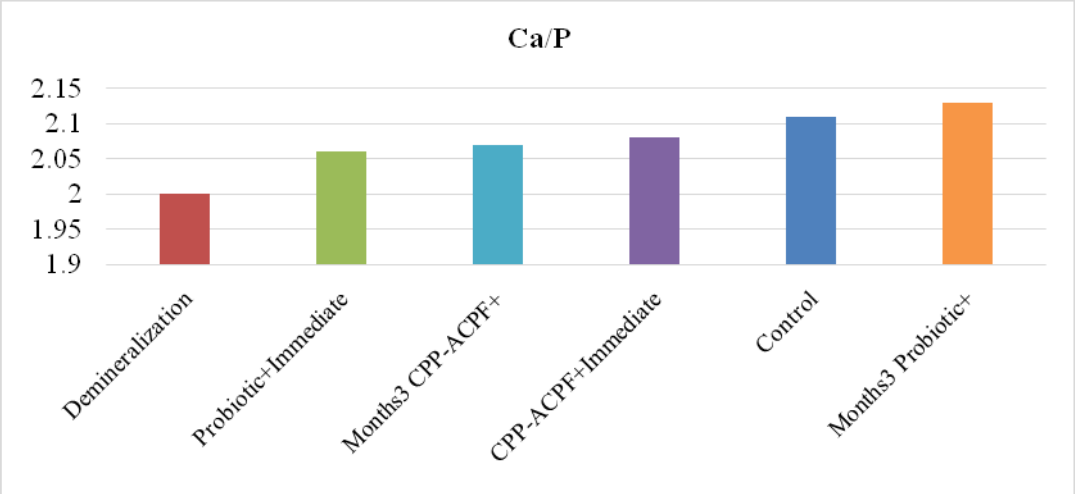

Figure (10): Bar chart showing the mean Ca/P wt% for different storage time intervals interaction between variables in descending order

IV. Discussion

This in vitro study was performed to evaluate and compare the remineralization potential of living bacteria (probiotic) and casein phosphopeptide amorphous calcium phosphate fluoride (CPP-ACPF) on demineralized enamel lesion immediately and after three months.

In vitro model has its advantages along with disadvantages. For example, in vitro experiments could be performed and completed in a short period of time, require fewer staff than in situ studies, avoid participant compliance issues, and are relatively inexpensive. ***(Patil et al., 2013)***

Bovine teeth were used in the current study as they are easy to obtain in large quantities and in good conditions, and showed less variability in composition than in human teeth. Moreover, The mineral distribution in caries lesion in bovine teeth reportedly similar to that found in human teeth, and structural changes in human and bovine teeth are compatible ***(Wang et al., 2012)***.

The demineralizing procedure was intended to produce artificial incipient caries lesion ***(Hegde and Moany, 2012)***. The utilized acid as (HCl), is a strong acid, requires a shorter time (10 min) and a less quantity of the solution ***(Amoras et al., 2012)***.

pH of artificial saliva acts as a natural buffer which contains calcium ions, phosphate ions, buffering agents, and other substances ***(Patil et al., 2013)***

Probiotic administration is considered a potential strategy for improving or maintaining oral health. According to ***(WHO/ FAO, 2001)***, the World Health Organization/ Food Agriculture Organization) probiotics are "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host". ***(Wattanarat et al., 2015)***

Probiotic paste; a (*Lactobacillus and Bifidobacterium*) were the most commonly used probiotic bacterial strains. The safe use of these bacteria is supported by their long history of consumption in fermented milk products and growing knowledge of their taxonomy and physiology ***(Jäsberg et al., 2016)***. It relies on the concept of using harmless or good bacteria to suppress or eradicate pathogenic bacteria causing harmful diseases mainly *Stereptococcus mutans* ***(Kour et al., 2015)***, which have been considered major pathogens associated with early caries development ***(Wattanarat et al., 2015***), as *S. mutans* has three virulence factors; water-insoluble glycans, acid tolerance, and production of lactic acid. These bacteria can rapidly metabolize dietary sugars to acid, creating locally a low pH ***(Ashwin et al., 2015).*** So probiotic maintain a healthy equilibrium in the oral microbial ecosystem. ***(Jäsberg et al., 2016)***

GC MI Paste plus; a casein phosphopeptide amorphous calcium phosphate fluoride cream (CPP-ACPF), was used because CPP make localization of (ACP) at the tooth surface which buffers the free calcium and phosphate ion activities by preventing of pH reduction in oral micro-enviroment ***(Grosiman et al., 2015)***, thereby helping to maintain a state of supersaturating with respect to tooth enamel, enhancing remineralization and preventing demineralization ***(Sathe et al., 2014)***.

CPP contain a cluster of phosphoseryl residues that bind and stabilize nanoclusters of ACP in metastable solution and prevents their growth to the critical size required for nucleation and precipitation, so remineralization process occurs by diffusion of calcium and phosphate ions through the protein/water-filled pores of the carious surface enamel into the body of the enamel lesion ***(Somasundaram et al., 2013)***.The acid diffused out of the lesion and down a concentration gradient. So, CPP-ACP would consume this acid and would

aid in remineralization by generating more calcium and phosphate ions, thus maintaining their high concentration gradients into the enamel subsurface lesion. ***(Hegde and Moany, 2012)***

(CPP-ACPF) stabilizes calcium, fluoride, and phosphate at the tooth surface in a slow-release amorphous form, thus enhancing deeper remineralization of demineralized lesion, in addition to increase remineralization potential when combined with fluoride due to a synergistic effect ***(Huang et al., 2013)***. The ability to deliver calcium phosphate and fluoride ions in the correct ratio into the subsurface lesion may be attributed to the ability of the CPP to localize and stabilize the ions at the tooth surface in the correct ratio (Ca: PO_4: F = 10:6:2) are required to form one unit cell of fluorapatite ($Ca_{10}(PO_4)_6F_2$). ***(Vanichvatana 2013)***

Using Scanning Electron Microscopy with Energy Dispersive X-ray Analysis (SEM-EDX) was done to estimate the mineral content in the samples. All (SEM) microphotographs taken before demineralization (control) showed normal enamel surface (regularly smooth) in all the specimens. (SEM) took after 1 h of demineralization displayed irregular surface with loss of some surface enamel in all the study specimens, revealed as an etching pattern. Remineralization of demineralized enamel surface after treatment with the two agents (probiotic and CPP-ACPF) in immediate period appear as large remineralization globules and after 3 months the remineralized enamel showed regular surface that showed improvement. ***(Patil et al., 2013)***

(EDX) is a micro-analytical technique that is used in conjunction with (SEM) to estimate structural, morphological variation and quantitatively estimate the amounts of mineral calcium and phosphorus content in % weight of sound, demineralized, and remineralized enamel in each group ***(Sathe et al., 2014)***.

The results of the current study with EDX analysis showed that, the Ca/P ratio for all tested groups were not significantly different. Although that in the probiotic and CPP-ACPF group the effect of remineralization of demineralization enamel surface obscured the etched pattern created by HCL and this was confirmed by SEM.

Several studies concluded that there are positive effects of various strains of *Lactobacilli* probiotics in the oral cavity such as dental caries prevention and maintenance of periodontal health ***(Deo and Deshmuk, 2015),***Other studies suggested that probiotics could control plaque by decreasing periodontopathogens or *S. mutans* ***(Toiviainen et al., 2015)***. As well as role of probiotic in the future as a therapeutic and prophylactic agent ***(Kour et al., 2015)***.

The exact mechanism of action of probiotic is not very clear but some theories are presented that probiotics can prevent diseases through several mechanisms including direct interaction competitive, as inhibition of specific pathogens through: production of various anti-microbial substances which are: (organic acids, hydrogen peroxide, carbon peroxide, and bacteriocin). Also, it can inhibit adhesion of pathogen that is involved in the metabolism of substrates, and prevent colonization. As well as formation of a protective lining for oral tissues against oral diseases via keeping the bacterial pathogens off the oral tissues by filling the spaces where the pathogens would invade ***(Chaturvedi and Jain, 2015)***. Moreover, probiotics play a role in modulating pH by enhancement of remineralization. They promoted the health bacteria to address the imbalance in the oral environment by competitively inhibiting the pathogens and shifting the oral milieu to a higher pH, thereby reversing the demineralization. ***(Kour et al., 2015)***

The main probiotic preparations currently on the market are the lactic acid-producing bacteria. The *LB. rhamnosus* is safe and useful in its purposed uses as an ingredient for storage stability in refrigerated milk-based foods ***(Ashwin et al., 2015)***.

To be accepted the probiotic should have a concentration of 10^8 to 10^9 colony forming unit (CFU/g) probiotic microorganisms. ***(Deo and Deshmukh, 2015)***

To date, there is no sufficient evidence that probiotic can remineralize enamel, and it is almost a little previous studies concerning to the subject were found in the literature. Although, it seems to has ability as remineralizing agent used in the present study to increase Ca/P percentage even at a low rate of treated enamel immediately and after 3 months of daily use.

Therefore, due to some limitation in in vitro study it should be predictive of further clinical studies of the characteristics of probiotic strains and the host responses are required to determine their appropriate applications, doses and treatment durations to accurately determine the effect of probiotic on the enamel remineralization process.

V. **Conclusions**

Within the limitations of this study, the following conclusions can be determined:

1. Use the probiotic as an adjunctive therapy, might provide appropriate oral environment for the remineralization process.
2. The ability to inhibit harm bacterial growth depends on specific-strains which are derived from probiotic.
3. Application of CPP-ACPF paste containing fluoride causes saturation of the most outer layer of enamel which leads to inhibition of remineralization process in the inner layer.

4. Probiotic and CPP-ACPF are used as preventive methods not as treatment.
5. Increasing time generally has a direct effect on remineralization.

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