

# The Promising Efficacy of Probiotics, Casein Phosphopeptide and Casein Macropeptide as Dental Anticariogenic and Remineralizing Agents Part I; An *In vitro* Study

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## Authors' contributions

This work was carried out in collaboration between all authors. Authors HE and MAES designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors HS, HES and ES managed the analyses of the study. Authors HE and MAES managed the literature searches. All authors read and approved the final manuscript.

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## ABSTRACT

**Objectives:** To investigate the effect of different mixtures from nano casein phosphopeptides (CPP), amorphous calcium phosphate (ACP), probiotic *Lactobacillus rhamnosus* B-445 (*L. rhamnosus*) and casein glycomacropeptide (GMP) against *Streptococcus mutans* (*S. mutans*) growth and its adhesion.

**Methods:** CPP was prepared from the tryptic digest of bovine casein and GMP by the action of chymosin on casein solution. Four mixtures namely: Group 1) Nano CPP; Group 2) Nano CPP+

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ACP; Group 3) Nano CPP+ ACP + *L. rhamnosus*; and Group 4) Nano CPP+ ACP+ GMP were prepared and tested for its inhibitory activity against *S. mutans* growth and its adhesion to saliva-treated glass surfaces in comparison with a commercial product (GC MI paste plus) and chlorhexidine (0.2%) as a positive control. The particle size and zeta potential of nano CPP and its complex with ACP were evaluated. Furthermore, the viability of *L. rhamnosus* in its mixture was determined during two weeks of storage at pH 6.8 and 8 respectively.

**Results:** Revealed that Nano CPP had an average particle size (7.75 nm) and zeta potential (-8.43 mV) lower than that of CPP+ACP mixture. Probiotic containing mixture exhibited inhibitory activity slightly less than the positive control at pH 6.8. All tested mixtures reduced the adhesion of *S. mutans* to saliva-treated glass surfaces and the highest was that containing probiotic and GMP. *L. rhamnosus* showed acceptable stability in CPP+ACP mixture during storage period.

**Conclusions:** All these findings suggest the use of probiotic, CPP+ACP mixture as a dental anticariogenic and remineralizing agents.

**Keywords:** Probiotics; oral health; casein phosphopeptides; casein glycomacropeptide; adhesion; inhibitory activity; *S. mutans*.

## 1. INTRODUCTION

Dental caries is one of the most prevalent and expensive human disease of bacterial origin to treat all over the world [1]. It is well documented that the progress of a caries lesion is known as a disproportion in the demineralization and remineralization cycle [2]. Demineralization arises from the action of the organic acids that result from the fermentation of dietary sugar by *Streptococcus mutans* (*S. mutans*), the dental plaque odontopathogenic bacteria [3]. Dental plaque is a matrix-enclosed microbial community that develops on the tooth surface, whereas a large number of bacteria are involved and there is fierce competition for nutrients and attachment sites [4]. Several strategies have been proposed to prevent the incidence of dental caries based on the mediation of the growth and activity of odontopathogenic bacteria and/or increased tooth remineralization.

Recent reviews have reported the use of probiotic strains for the prevention of oral diseases, including caries [5,6,7]. Probiotics are administered to maintain or restore the natural saprophytic micro-flora against a pathogen invasion, which is central to the development of the major oral diseases (caries and periodontal disease). The beneficial action of probiotic is relied on their interaction with other bacteria in the plaque and hampers growth by pathogens through production of hydrogen peroxide, bacteriocins, and organic acids [8]. The effect of probiotics on dental caries and its related risk factors has been evaluated in several experimental studies, using different strains; *L. rhamnosus* GG, *L. casei*, *L. reuteri*, *L. plantarum*, *L. brevis* CD2, *Bifidobacterium* spp [5]. These

previous studies have demonstrated the capacity of probiotic to reduce *S. mutans* counts in saliva and/or plaque in short-term but randomized clinical trials on this topic were insufficient to provide scientific clinical evidence [5].

In the late 1950s Dairy products have been considered as effective food in preventing dental caries [9]. Phosphorus rich peptides (CPP) which is isolated from tryptic digest of bovine milk casein ( $\alpha$ s1-casein,  $\beta$ -casein and  $\alpha$ s2-casein), could stabilize calcium ions at wide range of pH (5.0-9.0) in a direct proportional relationship [1,10].

Amorphous calcium phosphate (ACP) is the initial solid phase that precipitates from a highly supersaturated calcium phosphate solution. In vivo, ACP is a good osteoconductivity, biodegradability and bioactivity but without cytotoxicity. However, unstabilized ACP undergoes rapid transformation to crystalline phases in the mouth where it may attribute to dental calculus [11,12]. Therefore, there is a need to stabilize ACP to retain its favorable properties. CPP was found bind to nanoclusters of ACP in supersaturated solutions, preventing the growth of ACP to the critical size required for phase transformation and precipitation. CPP-ACP nanoclusters work effectively as a remineralizing agent at wide range of pH [13]. Furthermore, CPP-ACP has the ability to inhibit cariogenic streptococci to the tooth surface inducing the formation of noncariogenic plaque [13]. Thus, the anticariogenic potency of CPP-ACP may act to promote its incorporation into food products and dental products as a novel tool in the fight against dental caries.

Casein glycomacropeptide (GMP) is a blend of glycosylated and non-glycosylated peptides of the same peptide chain which originates from the influence of chymosin on  $\kappa$ -casein [14]. Because of the great antimicrobial effects of GMP including inhibitory adhesion of oral Actinomyces and Streptococci to surfaces, it was suggesting to be as inhibitor against dental caries [15]. Moreover, GMP exhibited a superior tooth protection against erosion than CPP at pH 3 while CPP exhibited better performance at pH 4.5 [16].

Previous studies reported that one of aforementioned agent could prevent dental caries; however, there is no reference has been cited on the use of combined agents. Hence, the aim of this study was to assess the effect of different combinations of probiotics, CPP-ACP, and GMP as anticariogenic or/and remineralizing agents against *S. mutans* growth and its adhesion on saliva-coated glass surfaces simulating the pellicle-covered enamel surfaces.

## 2. MATERIALS AND METHODS

### 2.1 Separation of Casein Phosphopeptide (CPP)

Bovine casein solution (1%) in phosphate buffer (pH 8 0.1M) was hydrolyzed with trypsin (1:100, enzyme: protein ratio) at 45°C/5 hrs. Then, pH was adjusted to 4.6 and finally centrifuged at 1500 g/15 min. Calcium chloride (0.2%) was added to the clear supernatant followed by ethanol to precipitate CPP and then lyophilized [17].

### 2.2 Determination of the Purity of CPP by HPLC

HPLC analyses of CPP were done using high purity water (Millipore, Bedford, MA, USA), methanol and acetonitrile (Redel and Fluka, Germany), also utilizing Trifluoroacetic acid (TFA; Merck, Darmstadt, Germany). Aliquot of a filtrate containing (0.1 g) of the lyophilized CPP with 10 mL of 0.1% (V/V) TFA was injected into the HPLC system. Chromatographic analysis was carried out using HPLC Agilent 1100 integrated system equipped with a G1313A automated injector, a G1311A pump and G1315B multi wavelength diode-array detector (DAD) operated at 220 nm. The separation was carried out at 20°C and flow rate of 0.5 ml/min. using linear gradient from 0.1% (V/V) TFA acid to

a solution containing 0.1% and 80% acetonitrile (V/V). The chromatographic data was analyzed using Agilent Chemstation.

### 2.3 Measurement of the Phosphorus Content of CPP

This was conducted by following the standard method [AOAC, 1990] [18] and using phosphomolybdic acid.

## 3. PREPARATION OF CASEIN GLYCOMICROPEPTIDE (GMP)

Bovine casein was dissolved at pH 8 to make 1% solution with the aid of 0.1% NaOH, then adjusted to pH 6.6 and finally, 0.2% CaCl<sub>2</sub> was added. Chymosin (Ch.Hansen Lab, Denmark) was added at the ratio of 0.3g/l casein solution and incubated at 40°C until the formation of a coagulum. The reaction mixture was heated to 70°C to inactivate the enzyme, coagulum was broken and the curd was separated by filtration, then finally freeze dried [14].

## 4. *Lactobacillus rhamnosus* (L. rhamnosus) CULTURE AND ENUMERATION

Probiotic *L. rhamnosus* B-445 was provided in lyophilized form by the Northern Regional Research Laboratory, Illinois, USA. Bacterial activation was carried out through inoculation in MRS medium broth and anaerobic incubation (Gas Generating Kit Anaerobic System, Oxoid, UK) at 37°C for 48 hrs. Then cultured bacteria were centrifuged at 5000 rpm/20 min to obtain pure cell pellet. The number of the live bacteria cells in one gram of the previously obtained pellet was determined by colony counting method and the total live cell numbers per one gram of pellet were calculated using the following formula; Live cells (CFUs/g) = number of colonies x dilution factor [19].

## 5. PREPARATION OF DIFFERENT EXPERIMENTAL MIXTURES

Different combinations from Nano (CPP), amorphous calcium phosphate (ACP), *L. rhamnosus* and (GMP) were prepared to form four groups as follows; Group 1: Nano CPP (5.0 g) was dissolved and made up to 100 ml with phosphate buffer at pH 6.8 and 8, Group 2: Nano CPP (2.0 g) and (0.375 g) nano ACP (<150 nm particle size, Sigma-Aldrich, USA) were

dissolved and made up to 100 ml with phosphate buffer at pH 6.8 and 8, Group 3: Nano CPP (2.0 g) and nano ACP (0.375 g) were dissolved followed by inoculation of  $10^9$  CFU/g of *L. rhamnosus* B-445, and then were made up to 100 ml phosphate buffer at pH 6.8 and 8, Group 4: nano CPP (2.0 g), nano ACP(0.375 g) and GMP (2.0 g) were dissolved and made up to 100 ml phosphate buffer at pH 6.8 and 8, Group 5: Commercial group (GC MI paste plus; Contains 0.2% (900 ppm) Fluoride and Recaldent (cpp-acp)), Group 6 (Positive control): % 0.2 Chlorhexidine diacetate (Sigma-Aldrich, USA, Product Number C 6143).

## 6. DETERMINATION OF PARTICLE SIZE DISTRIBUTION AND ZETA POTENTIAL

The size and zeta potential of the droplets in the prepared CPP containing particles were evaluated by Zetasizer var. 704 instrument (Malvern Instruments, Malvern, UK). Sample was diluted with MQ water before measurement its light scattering for a laser beam (633 nm) at an angle of 173 at 25°C over time intervals. The changes in laser beam scattering versus time was used to determine the particle size distribution and polydispersity index (PDI) were calculated from the particle size distribution. Zeta potential was measured in the same sample by electrophoresis and results were expressed as mV. and (PDI) [20].

## 7. MICROBIOLOGICAL ASSESSMENT

*S. mutans* was isolated from the dental plaque of high caries index patients by swabbing method according to Sanchez et al. [21]. Selective media Mitis salivarius-bacitracin agar (MSB; BD Difco, Paris) was used to isolate and grow *S. mutans* (Dairy microbiological Lab., National Research Center, Giza, Egypt). A single colony of *S. mutans* was isolated and placed in a test tube containing 10 ml of Tryptone Soya broth (TSB; Difco laboratories, Detroit, MI, USA) and incubated at a temperature 37°C for 24 hrs. The cultures were then diluted with fresh media until a concentration  $10^6$  CFU/ml was obtained and used as working microbial solution [22].

### 7.1 Screening of Antimicrobial Activity Using Well Diffusion Method

The antimicrobial activity of eighty-four specimens of the previously prepared four experimental groups were divided into twenty-eight specimens at pH6.8 and other twenty-eight

at pH 8 (n=7) as well the fifth commercial group (n=14) and the sixth positive group (n=14) was evaluated on the profile growth of *S. mutans*. Nutrient agar medium was poured into sterile petri dishes (15 ml each) and 20 µl of the formerly made microbial solution was spread over each agar plate and allowed to dry for 5 min. A sterile 6 mm cork-borer was used to cut six wells at equidistance in the plate and the first four wells, each was filled with 50 µl of each four experimental groups, while the fifth well with 50µl of commercial product and the sixth well with 50µl of positive group. The agar plates were incubated at 37°C for 24 hrs and the inhibition zones around the wells were measured [23].

### 7.2 Survival Profile of Probiotic Strain in the Experimental Group 3

The viability of *L. rhamnosus* B-445 in its mixture at pH6.8 and pH8 of Group 3 was determined at different storage period (after 24 hrs, 72 hrs, and 2 weeks). Live bacterial cells in one ml of mixture were enumerated using colony counting method as aforementioned [19].

### 7.3 Adherence Inhibition Assay

The previously prepared microbial solution was poured onto anaerobic agar plates and incubated at 37°C for 18-20 hrs. The colonies were then harvested and dispersed into 30 ml Brain Heart Infusion (BHI, Oxoid) broth containing 5% (w/v) sucrose. The turbidity of the suspension was adjusted spectrophotometrically at 550 nm to an absorbance of about 0.144. At this absorbance, the concentration of cell is standardized to about CFU/ml for use in the anti-adherence study. Also, whole saliva (WS) was collected from a single donor by expectoration after chewing a piece of rubber band, and then clarified by centrifugation (17,000 ×g, 30 min) [24].

#### 7.3.1 Determination of the adherence affinities of *S. mutans* on the saliva-coated glass surfaces

Saliva-coated glass culture tubes were prepared by treating its inner surface with two ml of clarified saliva for two min. Then the surface was rinsed with sterile distilled water and the rinse was used as control. After that, two ml of bacterial suspension was added to saliva-coated glass culture tubes incubated for 18-20 hrs at 37°C. The tube contents were removed and its turbidity (A) was measured spectrophotometrically at 550 nm. The emptied

tube was then washed with two ml of distilled water, suspension was collected and its turbidity (B) was measured at 550 nm. The sum of A and B turbidity (Aub) corresponds to the amount of the total unbound cells. The concentration of the adherent *S. mutans* was determined by adding two ml of distilled water into the emptied tube, sonicated for ten sec and the turbidity (Ab) of the suspension was measured at 550 nm [25].

### **7.3.2 Determination of the anti-adherence effect of different groups on *S. mutans* attached to saliva-treated glass surfaces**

Two ml of each of the four experimental groups and the fifth commercial group was added into previously treated saliva-coated culture tubes and left for two min. The inner surfaces were then rinsed with sterile distilled water and the procedure was repeated as above described. The percentage of adherence was calculated as follows:

$$\text{Adherence \%} = \text{Ab} / \text{Ab} + \text{Aub} \times 100$$

Where, Ab and Aub are the absorbance of bound and unbound cells at 550 nm respectively.

The percentage of adherence reduction of cells in the presence of experimental group or commercial group was calculated as follows:

$$\text{Adherence reduction \%} = \text{Ab} - \text{Abt} / \text{Ab} \times 100$$

Where, Ab and Abt are the absorbance of bound cells without and with treated groups respectively at 550 nm.

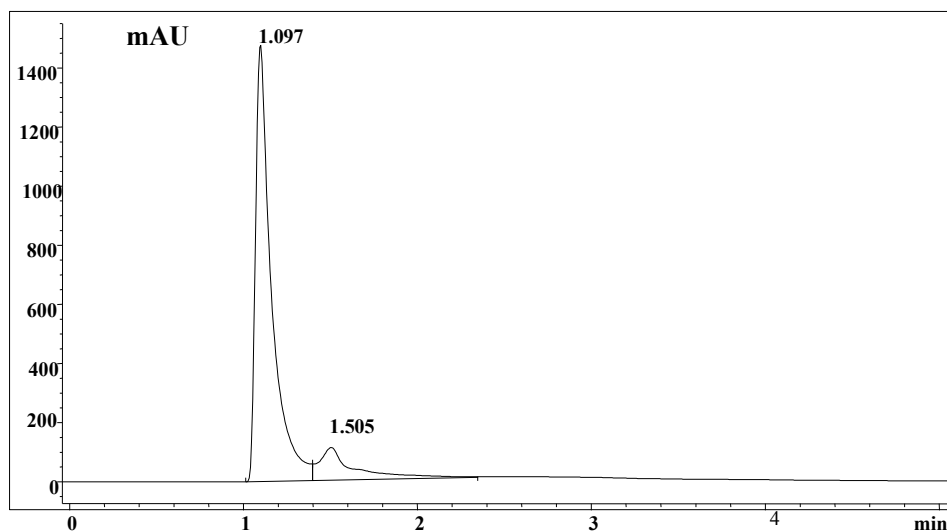
## **7.4 Statistical Analysis**

Data were explored for normality using Kolmogorov-Smirnov and Shapiro-Wilk tests and showed parametric distribution. For parametric data; One-way ANOVA followed by tukey post hoc test was used for comparison among the groups in non-related samples. Repeated measure ANOVA and paired sample t-test was used for comparison among the related samples. The significance level was set at  $P \leq 0.05$ . Statistical analysis was performed with IBM® SPSS® Statistics Version 20 for Windows.

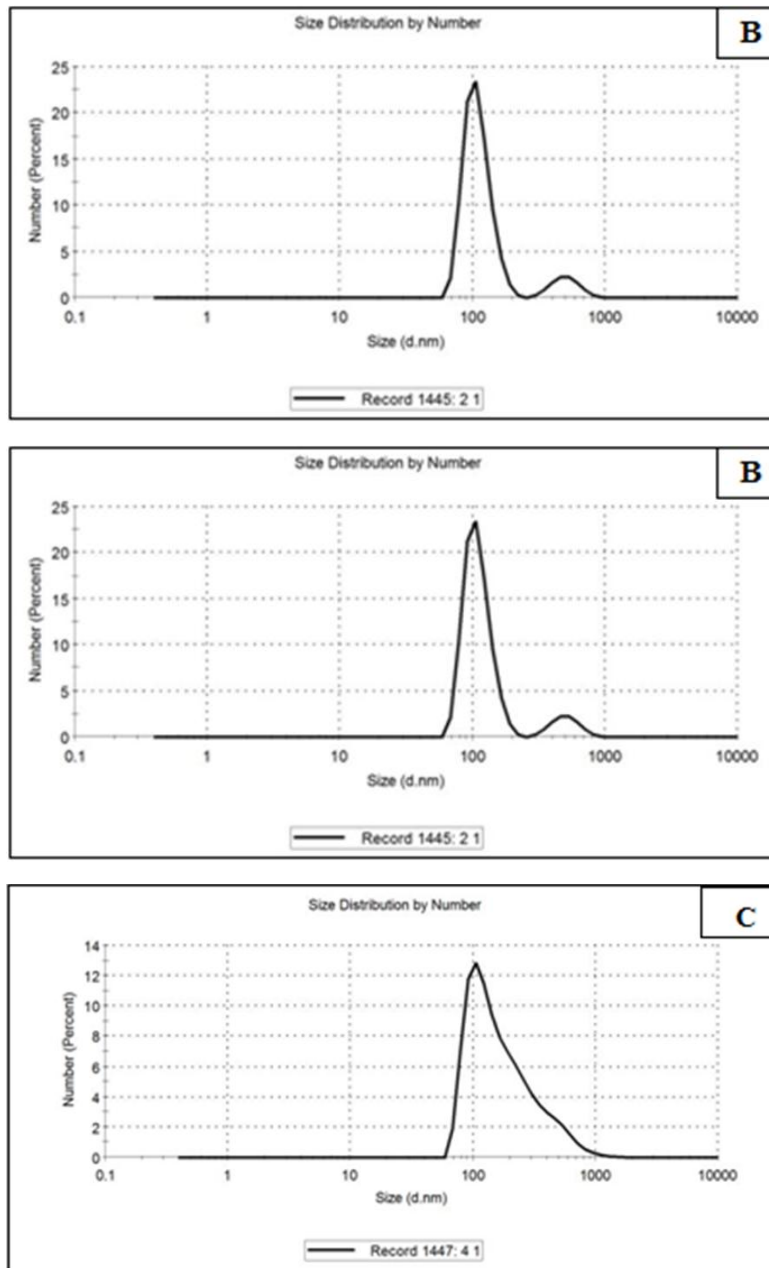
## **8. RESULTS**

Regarding preparation and purity of Casein phosphopeptide (CPP); the phosphorus content of the prepared CPP was found to be 2.32%, and HPLC separation of the prepared CPP demonstrated the presence of major and minor peaks respectively (Fig. 1).

The Particle size of CPP nanoparticles in Group 1 was  $7.75 \text{ nm} \pm 1.506 \text{ nm}$  and 0.561 for PDI (Fig. 2A). While 90% of particles in Group 2 had an average size of  $109.7 \text{ nm} \pm 25.66 \text{ nm}$  and 10% of the total particles had an average size of  $509.5 \pm 122.4 \text{ nm}$  (Fig. 2B) In Group 4, 100% of particles number had an average size



**Fig. 1. HPLC chromatogram of prepared CPP**

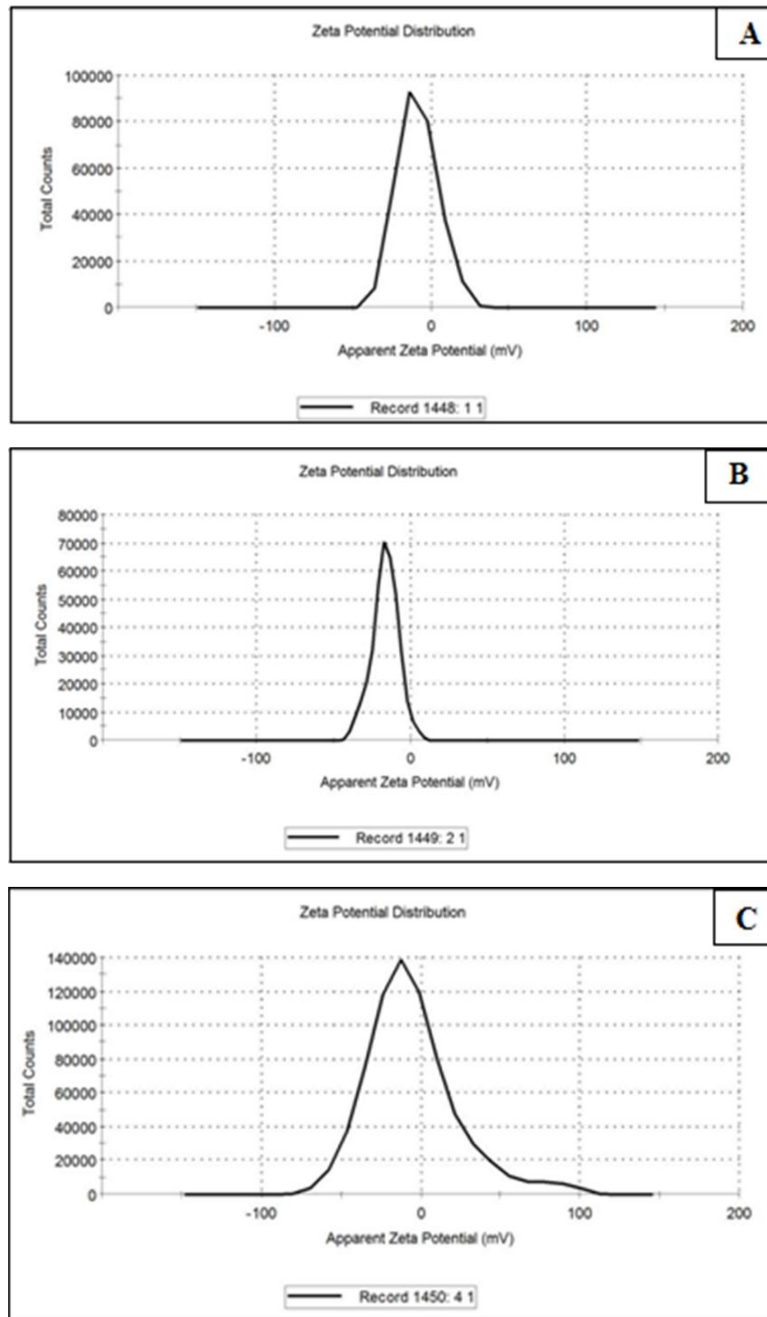


**Fig. 2. Particle size distribution of (A) CPP solution (Group 1) (B) CPP+ACP solution (Group 2) and (C) CPP+ACP+GMP (Group 4) solution by number**

199.6±156.9 nm and 0.471 for PDI (Fig. 2C). For the Zeta potential values of CPP, Group 1 followed normal distribution curve with -8.43 ±13.0 mV and Group 2 was revealed an average Zeta potential of -6.5 ± 8.73 mV. While low average values -5.40 ±29.3 mV was observed in Group 4 (Fig. 3).

In Table 1, the antibacterial efficacy of CPP containing solutions at pH 8 against *S. mutans*

growth showed a significant difference between Group 6; (positive control) and all other groups ( $P \leq .001$ ), while there were no inhibition zones in Groups 1, 2, 3, 4 and 5 at lower pH value of 6.8, inhibition zones value was found in Group 6 (16.00 ± 0.00) followed by Group 3 (10.29 ± 3.55) ( $P \leq .001$ ), where no inhibition zones were found in Groups 1, 2, 4 and 5 (0.00 ± 0.00) (Table 1).



**Fig. 3. Zeta potential of (A) CPP solution (Group 1) (B) CPP+ACP solution (Group 2) and (C) CPP+ACP+GMP (Group 4) solution**

For the survival profile of *L. rhamnosus* in the solution containing CPP + ACP in Group 3 at pH 8, results revealed that the highest survival mean value was after 72 hours storage period ( $231.25 \times 10^{-7} \pm 13.15 \times 10^{-7}$ ) followed by after 2 weeks ( $216.75 \times 10^{-7} \pm 11.35 \times 10^{-7}$ ), then after 24 hours ( $179.00 \times 10^{-7} \pm 8.60 \times 10^{-7}$ ), while the least mean value was in Base line ( $156.50 \times 10^{-7} \pm$

$22.34 \times 10^{-7}$ ) (Table 2). On contrary, the probiotic survival in mixture at pH 6.8 showed highest mean values in Base line ( $129.50 \times 10^{-7} \pm 12.37 \times 10^{-7}$ ) followed by after 24 hours ( $82.25 \times 10^{-7} \pm 9.03 \times 10^{-7}$ ) and 72 hours ( $73.25 \times 10^{-7} \pm 10.90 \times 10^{-7}$ ), while the least mean value was found after 2 weeks ( $73.00 \times 10^{-7} \pm 7.02 \times 10^{-7}$ ) (Table 2).

**Table 1. Mean and standard deviation (means  $\pm$  SD) values of the inhibition zones diameter (mm) were exhibited by different groups against *streptococcus mutans* growth at pH 8.0 and pH 6.8.**

Constituent in solution	pH 8.0	pH 6.8
CPP (Group 1)	0.00	0.00
CPP+ACP (Group 2)	0.00	0.00
CPP+ACP+Probiotic (Group 3)	0.00	10.3 $\pm$ 3.6 <sup>b</sup>
CPP+ACP+GMP (Group 4)	0.00	0.00
Commercial group (Group 5)	0.00	0.00
Chlorhexidine diacetate (Group 6) positive control	14.4	16.0 $\pm$ 2.3 <sup>a</sup>
<b>P value</b>		<b>&lt; .001</b>

\*Different letters in the same column indicate statistically significant difference at  $P < .05$

**Table 2. Mean Survival of *L. rhamnosus* (CFU/ml) in the solution containing CPP + ACP (Group 3) at different storage periods at pH 8.0 and pH 6.8**

Storage time	pH 8.0	pH 6.8
Base line	156.50 $\pm$ 22.34 <sup>a</sup>	129.50 $\pm$ 12.37 <sup>a</sup>
24 h	179.00 $\pm$ 8.60 <sup>a</sup>	82.25 $\pm$ 9.03 <sup>b</sup>
72 h	231.25 $\pm$ 13.15 <sup>b</sup>	73.25 $\pm$ 10.90 <sup>b</sup>
2 wk	216.75 $\pm$ 11.35 <sup>b</sup>	73.00 $\pm$ 7.02 <sup>b</sup>
<b>P value</b>	<b>&lt; .001*</b>	<b>&lt; .001*</b>

• Values are given as (mean  $\pm$  SD)  $\times 10^7$

• Different letters in the same column indicate statistically significant difference

• \*: significant ( $P < .05$ )

When comparing the mean percent of cell adherence values for different experimental solutions (Groups 1, 2, 3 and 4) and the commercial one (Group 5), the highest mean value of percent of cell adherence was found in (Control) (33.35%  $\pm$  0.82) followed by Group 2 (23.00%  $\pm$  0.82), Group 1 (18.60%  $\pm$  1.63), Group 5 (17.50%  $\pm$  0.82) and Group 4 (15.00%  $\pm$  0.83) while least percent of cell adherence was found in Group 3 (14.80%  $\pm$  0.79) (Fig. 4). Accordingly, the highest mean value of % of reduced adherence was found in Group 3 (65.00%  $\pm$  2.06) followed by Group 4 (64.00%  $\pm$  3.27), Group 5 (57.00%  $\pm$  0.82), Group 1 (55.70%  $\pm$  0.82) and Group 2 (42.00%  $\pm$  1.63) while least percent of reduced adherence was found in (Control) (Fig. 5).

## 9. DISCUSSION

Casein phosphopeptide (CPP) is a mixture of peptides containing clusters of phosphate groups and emerged from the action of proteolytic enzymes on the different casein fraction. In the present study, Trypsin was chosen to hydrolyze bovine casein because of its high specificity and its capability to release phosphopeptides from casein [17]. As it was reported in the literature that several phosphopeptides can be released from the different casein fraction and precipitated together by the applied procedure [17]. Therefore, in this study, the phosphorus content of the prepared CPP was found to be 2.32% and indicated that almost the phosphorus content was concentrated in the prepared CPP. Regarding HPLC separation of the prepared CPP (Fig. 1), the results revealed the presence of major and minor peaks respectively indicating the high purity of the prepared CPP.

Solutions containing CPP nanoparticles were examined for their particle sizes and the results are presented in CPP (Fig. 2). Previous study by Fitz [17] was demonstrated that CPP are small molecular weight peptides composed of 20-25 amino acid residues, and this latter observation explain the low molecular size of CPP particles in Group 1 (7.75 nm  $\pm$  1.506 nm) and high homogeneity of the tested CPP molecules (Fig. 2A). For Group 2 the recorded particle size of the major number (109.7 nm  $\pm$  25.66 nm) was less than in the pure ACP (150 nm) which was incorporated into the CPP-ACP solution Group 2. The previous results revealed that the interaction of CPP with ACP could be attributed in a complex of nano size particles with small amount of larger aggregates that apparent in 10% of the total particle number in Group (2) (509.5  $\pm$  122.4 nm) (Fig. 2B). Concerning Group 4 where GMP incorporated into CPP+ACP solution, a significant change in the particle size distribution pattern was observed in (Fig. 2C) where a broad shoulder in this distribution curve indicating the presence of large aggregates. Comparing results between Group 2 and Group 4 indicated that the addition of GMP resulted in significant increase in major number of particle size due to possible aggregation of the three components in the solution.

For Zeta potential measurement of CPP in Group 1 (-8.43  $\pm$  13.0 mV) and Group 2 (-6.5  $\pm$  8.73 mV) (Fig. 3A and 3B), the results could be explained according to a previous research conducted by Reynolds [10] who demonstrated that CPP to



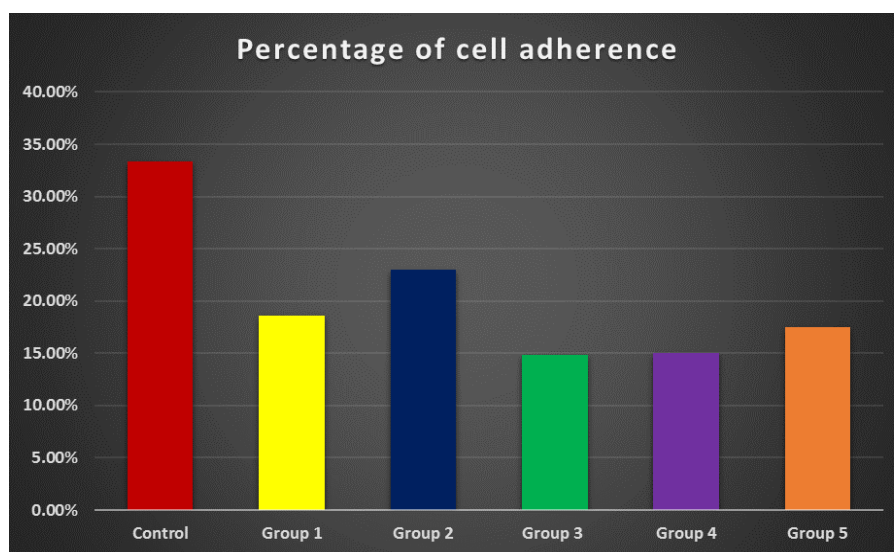


Fig. 4. Column chart of the mean values of the cell adherence percentage between different groups

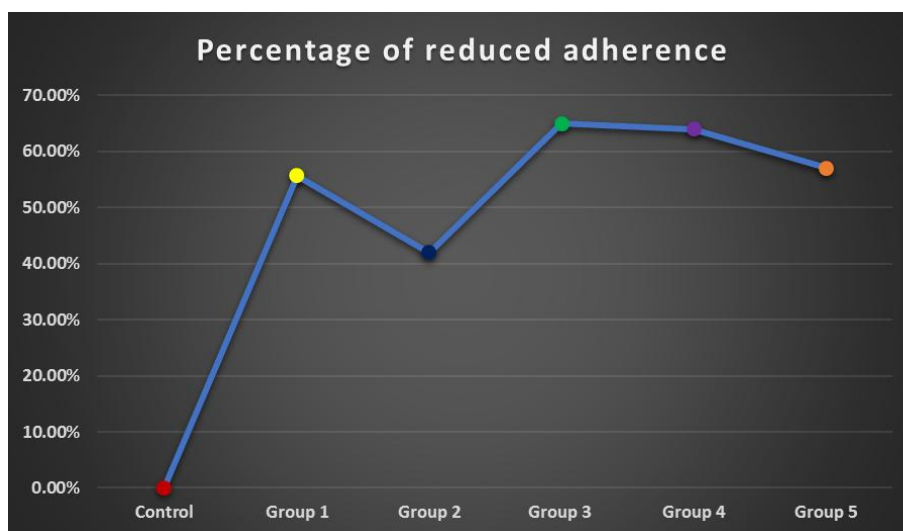


Fig. 5. Line chart of the mean values of the reduced adherence percentage between different groups

bind ACP forming clusters in metastable solution preventing their growth to the critical size required for their flocculation and precipitation. Furthermore, Uskoković et al. [26] reported that the increase of  $\text{HPO}_4$  groups to calcium ions could increase the negative zeta potential of the solution. While for Group 4, the addition of GMP to the CPP+ACP solution resulted in wide variation of the zeta potential of the solution with low average of  $-5.40 \pm 29.3$  mV (Fig. 3C). This later result could be related to the previously observation with variation in its particle size (Fig. 2. C).

For the antimicrobial activity of the four experimental groups (Groups 1, 2, 3 and 4) at pH 8.0, the results exhibited no inhibitory activity against *S. mutans* except for the positive control which had an inhibitory zone of  $(14.43 \pm 3.41$  mm). While at lower pH value of 6.8., the results revealed that both Group 3 and Group 6 exhibited clear inhibitory zones against *S. mutans*. The effect of pH on the growth and activity of probiotic bacteria has been documented by Rault et al. [27] and Lambert [28] whereas most bacteria exhibit maximum activity at pH 6-7. Accordingly, the present results

suggest that pH plays an important role in the inhibitory activity of the probiotic containing solution in Group 3. This observation is in agreement with Deepika et al. [29] who demonstrated that increasing the pH during fermentation from 5 to 6 changes the surface composition of *L. rhamnosus* whereas it increased its hydrophobicity and decreased its adhesion to caco-2-cells. These results support the assumption that pH 6.8 is the best pH for the inclusion of probiotic *L. rhamnosus* in oral health preparations as a natural therapeutic mean to control oral pathogen *S. mutans* while pH 8.0 is inappropriate for this purpose.

Concerning, the survival profile of *L. rhamnosus* in the solution containing CPP + ACP (Group 3), the count of *L. rhamnosus* stored at pH 6.8 decreased rapidly after 24 hours losing about 37% of its original count and then slightly decreased to reach  $(73.00 \times 10^7 \pm 7.02 \times 10^7)$  after 2 weeks and this indicates reasonable stability under the tested conditions. While *L. rhamnosus* storage at pH 8.0 showed an increase in its viable count up to 72 hours and then decreased slightly. This observed increase may be attributed to the possible disaggregation of cell aggregates due to the presence of hydroxyl ion in the medium.

The anti-adherence activities of CPP containing solutions against *S. mutans* attached to saliva-treated glass surface at pH 6.8 showed that, all tested experimental solutions (Groups 1, 2, 3 and 4) and the commercial product (Group 5) exhibited anti-adherence activities against attached *S. mutans* with variable degrees. For Group 1 the adherence of the pathogen was reduced by  $(55.70\% \pm 0.82)$  which is close to the reduction percentage exhibited by the commercial product (Group 5)  $(57.00\% \pm 0.82)$ . Both solutions containing the probiotic (Group 3) and GMP (Group 4) exhibited high adherence reduction percentages of  $(65.00\% \pm 2.06)$  and  $(64.00\% \pm 3.27)$  respectively. This is in agreement with a previous study conducted by Marttinen et al [30] who demonstrated that Lactobacilli could reduce streptococcal adhesion in particular to saliva-coated hydroxyapatite. Also, it has been reported in the literature by Neeser [15] that GMP reduces the adhesion of toxins, this may explain the anti-adherence potential of Group 4.

## 10. CONCLUSION

The present in vitro study confirmed the anti-adhesive and antibacterial activities of the

experimental solution containing mixture of probiotic *L. rhamnosus* in CPP-ACP against *S. mutans*. Moreover, *L. rhamnosus* showed high stability in this solution for a practical period of time study. Therefore, this mixture can be considered a promising ingredient in the oral health preparations.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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