Impact of acquired pellicle modification on adhesion of early colonizers

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Disclosure Statement

The authors have no conflicts of interest to declare.

Abstract

New preventive approaches against dental erosion caused by acidic drinks and beverages include fortification of beverages with natural polymers. We have shown that the mixture of casein and mucin significantly improved the erosion-inhibiting properties of the human pellicle layer. This study aimed to investigate the effect of pellicle modification by casein, mucin and a casein/mucin mixture on the adhesion of early bacterial colonizers. Test specimens of human tooth enamel were prepared, covered with saliva and coated with 0.5% aqueous casein, 0.27% aqueous mucin or with 0.5% aqueous casein/0.27% aq. mucin, after which the adhesion of *Streptococcus gordonii*, *Streptococcus oralis*, and *Actinomyces odontolyticus* was measured after incubation for 30 min and 2 h. Log10 colony forming units were compared by non-parametric tests. All three bacterial strains adhered in higher number to pellicle-coated enamel than to native enamel. The protein modifications of pellicle all decreased the counts of adhering bacteria up to 0.34 log10 /mm^2, the most efficient being casein/mucin mixture. In addition to the recently shown erosion-reducing effect by casein/mucin, modification of the pellicle may inhibit bacterial adherence compared to untreated human pellicle.

Caries is associated with bacterial processes leading to damage of the hard dental tissues (enamel, dentine and cementum) and remains one of the most common diseases worldwide [Kassebaum et al., 2015] Caries results from the interplay of three main factors: dietary carbohydrates, cariogenic bacteria on the dental surface and susceptibility of hard tooth tissues to demineralization. [Bradshaw and Lynch, 2013; Kutsch and Young, 2011].

The prevalence of dental erosion is steadily increasing in European countries and requires effective preventive solutions [Jaeggi and Lussi, 2014]. It is widely documented that acidic beverages as well as gastric juice may cause dental erosion [Lussi et al., 1995; Lussi et al., 2012; Sovik et al., 2015; Wilder-Smith et al., 2015]. New approaches to preventing dental erosion, which include fortification of beverages with natural polymers, or with dietary proteins such as ovalbumin from egg white, have been reported to reduce acid-induced hydroxyapatite dissolution [Barbour et al., 2008; White et al., 2011]. Furthermore, when added to commercial soft drinks or simple acidic solutions, casein subfractions (α-, β- and κ-) and ovalbumin reduced enamel erosion in the presence of the acquired pellicle [Hemingway et al., 2008]. The addition of dietary proteins to soft drinks is currently regarded as a promising preventive measure as it does not spoil the taste and carries no risk of dental staining. We proved in our previous study that modification of salivary pellicle by casein and casein/mucin mixture (mucin–salivary protein) increased the ability of the pellicle layer to protect against erosive enamel demineralization [Cheaib and Lussi, 2011]. Interestingly, application of casein–mucin mixture for pellicle modification showed greater anti-erosive efficacy than application of casein alone. In spite of the proven efficiency of these proteins in the inhibition of enamel demineralization and wear, it is not clear if incorporating them into the pellicle layer can also interfere with bacterial adhesion provoking or preventing caries. Thus, it is necessary to assess the preventive effect of proteins against dental erosion as well as against caries. Such complementary studies are much in demand in this field.

Bacterial colonization starts with the adhesion of early colonizers, known as pioneer bacteria, to the salivary pellicle of the teeth, as well as of the dental restorations, within minutes after tooth cleaning. These early colonizers, mostly streptococci and *Actinomyces* spp., contribute to plaque development and ultimately to oral diseases [Li et al., 2004]. The bacterial adhesion occurs via specific receptors located in the salivary pellicle layer and follows the typical stages of biofilm formation [Marsh, 2004].

The molecular composition and physicochemical properties of the pellicle are critical in determining the pattern of microbial colonization. As bacteria approach the pellicle layer weak physicochemical forces are generated. Within a short time these weak physicochemical interactions may become irreversible due to adhesins on the microbial cell surface, which are involved in specific interactions with complementary receptors [van der Mei et al., 2008]. Thus, a key event in biofilm formation is the initial adhesion of bacteria to matching host ligands.

The aim of this study was to investigate the effect of pellicle modification by casein, mucin and a casein/mucin mixture being a potential alternative in preventing dental erosion on the adhesion of early bacterial colonizers *Streptococcus gordonii*, *Streptococcus oralis* and *Actinomyces odontolyticus.*

Materials and Methods

The bacteria used for this study were: *Streptococcus gordonii* ATCC 10558, *Streptococcus oralis* ATCC 35037 and *Actinomyces odontolyticus* ATCC 17982. The strains were maintained on tryptic-soy-agar (Oxoid, Basingstoke, GB) with 5% sheep blood. They were always passaged 16 h before the experiment.

Saliva collection

Whole saliva was collected from healthy volunteers by paraffin stimulation and pooled. Saliva collection was approved by the ethics committee of the University of Bern. To remove debris the saliva was centrifuged for 20 minutes at 4 °C and 4,000 *g*. The supernatant was sterilized using UV radiation for 30 minutes. The processed, sterile saliva sample (tested by cultivation) was then stored at −80 °C; aliquots of saliva were defrosted immediately before the experiment.

Preparation of human tooth specimens

After patients had given informed consent for the use of their extracted teeth for research purposes (in accordance with the regulations of the ethics committee of the University of Bern), cavity-free human premolars were selected and viewed under a stereomicroscope (Leica, Zoom 2000, USA; 25× magnification) to choose specimens with a sound undamaged surface. Tooth crowns were separated from the roots using an IsoMet® Low Speed Saw (Buehler, Düsseldorf, Germany). The buccal sites were then divided into small cubical slabs, and polished on a Kuntch-Rotor polishing machine with carbide paper (30 µm, 18 µm and 6 µm grain size) under water cooling. The cubes had a size of about 2 × 2 × 0.75 mm and consist of enamel only (checked by stereomicroscopy).

Samples were stored in a mineral solution (1.5 mmol/l $CaCl_2$, 1.0 mmol/l KH_2PO_4, 50 mmol/l NaCl, pH 7.0). Prior to the experimental procedures, the samples were disinfected in ethanol (70%) for 1 h, and washed in sterile deionized water for a further 1 h.

Calculation of specimen surface area

The determination of surface area was performed using a light microscope (Leica M 420). Every side of each tooth specimen was manually outlined using Software IM500, and the surface was measured taking into account the corresponding magnification factor of the microscope. The total surface area was calculated as the sum of the areas of all the faces.

In vitro bacterial adhesion assay

For each bacterial strain, the enamel specimens were randomly selected and divided into eight groups: 1) native enamel (E, n=20); 2) pellicle layer (P, n=20); 3) enamel incubated in 0.5% aq. casein ((casein from bovine milk, Merck for analysis) C, n=20); 4) enamel incubated in 0.27% aq. mucin ((gastric mucin from pig, Merck for analysis) M, n=20); 5) enamel incubated in 0.5% aq. casein/0.27% aq. mucin (CM, n=20); 6) pellicle incubated in casein 0.5% aq. (PC, n=20); 7) pellicle incubated in 0.27% aq. mucin (PM, n=20); 8) pellicle incubated in 0.5% aq. casein/0.27% aq. mucin (PCM, n=20). The chosen concentrations are based on the results of the study made before and provide buffering potential similar to human

salivary proteins [Cheaib and Lussi, 2011]. To prepare protein and protein-modified pellicle coatings, enamel was incubated in the corresponding aqueous solution of the proteins casein, mucin and casein/mucin mixture for 2 h, or in sterile human saliva for 2 h followed by protein aqueous solution for 2 h for pellicle coated by protein groups (or left in saliva for 2 h), at room temperature under sterile conditions immediately before starting the experiments. Samples were then contaminated with bacterial suspension (OD600=1.05; equivalent to 10^8 bacteria/ml) for 30 min or 2 h in Dulbecco's modified Eagle's medium **(**DMEM; Gibco**,** Invitrogen**)** under slow motion irrigation before being carefully dipped in DMEM to eliminate non-adhering bacteria. Samples were soaked in NaCl 0.9%, and bound bacteria were dispersed by sonication for 30 s. The samples were vortexed for a further 30 s, serially diluted and spread over a tryptic-soy-agar plate. The media were incubated at 37 °C with 5% carbon dioxide for 48 h and bacterial colonies were then counted using an Acolyte Super Count Colony Counter (Synbiosis, UK). The number of bacteria was measured as colony-forming units (CFU). Results are expressed as $\log_{10}$ CFU per mm^2. Each two specimens per coating and time were independently included in one run. Meaning, at least 10 runs per bacterial strain were conducted.

Statistical analysis

All statistical analysis was performed with R, Version 2.15.1, using the extension package exact RankTests. A global significance level of alpha = 0.05 had to be achieved.

Since the data did not fulfil the assumptions of a parametric ANOVA (heteroskedastic groups, i.e. unequal variances), a nonparametric approach known as the aligned rank transformation (ART) method (Higgins 2004) was applied [Higgins, 2004]. Comparisons between different groups were made by using Kruskal-Wallis test.

Results

Eight groups at two different sampling times per bacterial strain (n=20/group) were compared in the experiment. Slabs of native enamel, enamel slabs coated by a pellicle layer, by casein, mucin and casein/mucin mixture, and enamel slabs coated first by pellicle layer followed by

casein, mucin and casein/mucin mixture were prepared. All test groups were incubated for 30 min or 2 h with *S. gordonii* ATCC 10558, *S. oralis* ATCC 35037 or *A. odontolyticus* ATCC 17982.

Adhesion of *Streptococcus gordonii* ATCC 10558 to native enamel and pellicle-covered enamel with different coatings

The adhesion rate of *S. gordonii* ATCC 10558 was significantly higher to pellicle coated enamel than to native enamel after 30 min and 2 h incubation ($p=0.017$; $p<0.001$). After 30 min, coating with casein and with casein/mucin decreased the adhesion of *S. gordonii* ($p=0.006$; $p=0.005$) compared to native enamel and after 2 h incubation there was still less adhesion after casein/mucin coating ($p=0.040$) (all: $p=0.010$, $p=0.155$). When comparing pellicle-covered enamel, all modifications significantly decreased the adhesion of *S. gordonii* after 30 min (casein $p=0.002$, mucin and casein/mucin both $p<0.001$) and after 2 h the lowest rate was found after casein/mucin with a reduction in median by up to 0.43 log10 CFU (or 63%, $p=0.001$) (all: $p<0.001$, $p=0.003$). However, counts were higher when comparing modified pellicle with native enamel coated with the respective proteins (casein after 30 min and 2 h ($p<0.001$; $p=0.006$), mucin and casein/mucin after 2 h ($p=0.011$; $p=0.009$) (figure 1).

Adhesion of *Streptococcus oralis* ATCC 35037 to native enamel and pellicle-covered enamel with different coatings

The adhesion rate of *S. oralis* ATCC 35037 was significantly higher in pellicle-coated enamel than in native enamel after 30 min and 2 h incubation ($p<0.001$; $p=0.001$). After 30 min and 2 h incubation, coating with casein ($p=0.002$; $p=0.013$), mucin ($p=0.034$; $p=0.002$) and with casein/mucin ($p<0.001$; $p=0.006$) decreased the adhesion of *S. oralis* compared to native enamel (all: $p=0.001$, $p=0.009$). With regard to pellicle-covered enamel, mucin and casein/mucin modifications significantly decreased the adhesion of *S. oralis* after 30 min ($p=0.038$; $p<0.001$), the difference was still significant for casein/mucin after 2 h ($p=0.020$) (all: $p=0.001$, $p=0.057$). All bacterial counts were higher when comparing modified pellicle

with native enamel coated with the respective proteins (each $p<0.001$) after 30 min and 2 h of incubation (figure 2).

Adhesion of *Actinomyces odontolyticus* ATCC 17982 to native enamel and pellicle-covered enamel with different coatings

The adhesion rate of *A. odontolyticus* ATCC 17982 was significantly higher in pellicle-coated enamel than in native enamel after 30 min and 2 h incubation (both $p<0.001$). Coating with casein, mucin or with casein/mucin mixture did not change the adhesion of *A. odontolyticus* to native enamel (all: $p=0.348$, $p=0.247$). In the case of pellicle-covered enamel, modifications with casein significantly decreased the counts of *A. odontolyticus* after 30 min ($p=0.009$) and modifications with mucin after 2 h ($p=0.004$). Casein/mucin mixture inhibited bacterial adhesion after 30 min ($p=0.003$) and 2 h ($p<0.001$) (all: $p=0.011$, $p=0.001$). Counts were higher when comparing modified pellicle with native enamel coated with the proteins (casein after 2 h ($p=0.020$), mucin after 30 min and 2 h ($p<0.001$; $p=0.006$) and casein/mucin after 30 min ($p=0.015$) (figure 3).

Comparison between adhesion of bacterial strains after 30 min and 2 h incubation

After 2 h, the counts of attached bacteria were always higher than after 30 min; the difference ranged between 0.14 log10 cfu/mm^2 (*S. oralis* ATCC 35037 coating with casein) and 0.59 log10 cfu/mm^2 (*S. oralis* ATCC 35037 coating with casein/mucin).

The rate of bacterial adhesion to native and pellicle-coated enamel with and without modifications was always the highest for *A. odontolyticus* 17982. The rate was always significantly different ($p<0.001$) from those of *S. oralis* ATCC 35037 and *S. gordonii* ATCC 10558, both after 30 min and after 2 h. Furthermore, *S. oralis* adhered in greater numbers than *S. gordonii* to native enamel at 2 h ($p=0.007$), and to pellicle-covered enamel with and without modifications after 30 min (without modifications $p=0.001$; casein and mucin both $p<0.001$, casein/mucin 0.003) and after 2 h (without modifications $p=0.026$; casein $p<0.001$; mucin $p=0.001$, casein/mucin 0.006). For native enamel coated with casein/mucin, after 30

min, *S. gordonii* adhered in greater numbers than *S. oralis* (p=0.007). There was no difference between the adherence of the two streptococcal strains on native enamel after 30 min, native enamel coated with casein and mucin at either time and native enamel coated with casein/mucin mixture after 2 h.

Discussion

We studied the effect of pellicle modification by casein, mucin or a mixture of the two on formation of biofilm on human enamel. Our previous study showed that the mixture of casein and mucin increased the original ability of pellicle to protect against dental erosion [Cheaib and Lussi, 2011].

Microbial adhesion on tooth surfaces is always preceded by the adsorption of a salivary pellicle [Hannig and Joiner, 2006; Lendenmann et al., 2000]. After adhering, the early colonizers such as *Actinomyces* spp., *S. oralis*, *Streptococcus mitis*, or *S. gordonii* influence the formation and the composition of the mature [Kolenbrander, 2000]. Thus, they provide optimal conditions and attachment substrates for the secondary colonizers, i.e., *Streptococcus mutans*, *Fusobacterium nucleatum*, or *Veillonella* spp. In this study, *S. gordonii* ATCC 10558, *S. oralis* ATCC 35037 and *A. odontolyticus* ATCC 17982 were used for evaluating early biofilm formation on the protein-modified enamel and protein-modified salivary pellicle. The number of adhered bacteria was higher in the pellicle-coated enamel group than in the native enamel group after 30 min and 120 min incubation. This could be explained by nonspecific and specific interactions which operate together on the pellicle layer leading to a stronger adhesion of *S. gordonii* ATCC 10558, *S. oralis* ATCC 35037 and *A. odontolyticus* ATCC 17982 to the pellicle surface. This is in agreement with our previous study [Mai et al., 2014] showing that pellicle promotes more bacterial adhesion than human enamel.

The primary interactions of bacteria with the tooth surface can result from diffusion, sedimentation, liquid flow, or from active movement of bacteria. Interactions between the surface and the bacteria include electrostatic, hydrophobic, and van der Waals forces, and lead to firm nonspecific but reversible adhesion of the bacteria to the pellicle [Kolenbrander, 2000] The irreversible attachment of bacterial cells to the tooth involves specific, stereochemical interactions between bacterial adhesins and complementary receptors in the acquired pellicle.

Furthermore, specific interactions strengthen more over more extended periods of time than nonspecific ones [van der Mei et al., 2008]

The number of bacteria that had adhered to pellicle and protein-coated pellicle was higher for *A. odontolyticus* ATCC 17982 than *S. gordonii* ATCC 10558 and *S. oralis* ATCC 35037 after both 30 min and 2 h incubation. This could be explained by stronger nonspecific and specific interactions with *A. odontolyticus* ATCC 17982 than for the two other strains. *Actinomyces* spp. have two distinct types of fimbriae: type 1 fimbriae mediate adherence to proline-rich proteins and to statherin, whereas type 2 fimbriae are involved in the adherence of bacterial cells to already attached bacteria [Hallberg et al., 1998]. *A. odontolyticus* expresses a sialic acid binding specificity potentially related to type-2 fimbriae [Drobni et al., 2006]. This specific interaction with the protein layer via fimbriae may result in stronger binding sites. Adhesins on *S. gordonii* can bind to α-amylase [Rogers et al., 2001]. S. *gordonii* and *S. oralis* express LPXTG-linked adhesins which binds to saliva-coated surfaces [Davies et al., 2009; Dorkhan et al., 2012]. The 30 min and 2 h incubation times in this study were chosen to acquire first-hand knowledge about nonspecific and specific interactions of bacteria with protein-modified enamel and pellicle.

For *S. gordonii* ATCC 10558, smaller numbers of bacteria adhered to pellicle modified by casein, mucin or a mixture of the two, or to enamel modified by proteins, than to non-modified pellicle. The difference was statistically significant at the two incubation times. Also, for *A. odontolyticus* ATCC 17982, the number of adhered bacteria was lower in casein-coated pellicle after 30 min incubation and in mucin-coated pellicle after 2 h incubation compared to pellicle layer. For *S. oralis* ATCC 35037, incubation of enamel in saliva followed by protein treatment resulted in lower counts in the casein/mucin-coated pellicle group than in the pellicle group.

Nonspecific interactions are mainly altered upon addition of casein to pellicle layer, as the effect had already occurred at 30 min. However, in the case of *A. odontolyticus* ATCC 17982, specific interactions may be affected upon addition of mucin. The modification of pellicle by casein/mucin mixture resulted in lower numbers of all the bacteria tested than counted for pellicle at the two incubation times. Casein forms micelle-like structures on the pellicle [Cheaib and Lussi, 2011]. The addition of casein promoted adsorption of mucin and it is known that mucin interacts weakly with the pellicle layer on salivary pellicle in vitro [Cheaib

and Lussi, 2011]. Therefore, the formation of a protein complex between casein and mucin and its interaction with pellicle could explain a better shielding barrier between the pellicle layer and oral bacterial cells.

We studied adhesion of initial colonizers to modified pellicle. Independly of the used strain, application of pellicle increased bacterial counts by 0.13 - 0.34 log10 cfu/mm^2 and modification by 0.27% mucin/0.5% casein reduced bacterial adhesion up to 0.33 log10 cfu/mm^2. This suggests a non-selective effect of 0.27% mucin/0.5% casein resulting in a slight inhibition of bacterial adhesion. Following a disturbance of bacterial homoeostasis might be excluded. Bacterial homoeostasis in the oral cavity include the integrity of host defence and the consumption of sugars [Marsh, 2006]. Pulsing of a microbial community with glucose in uncontrolled pH conditions led to a decrease of acid-sensitive microorganisms among them *S. gordonii* and *S. oralis* by favoring acidogenic bacteria e.g. *S. mutans* [Bradshaw and Marsh, 1998]. Sequence analysis of severe caries confirmed a decrease of *S. gordonii*, while *S. mutans* increased and *Actinomyces* sp. remained unchanged [Gross et al., 2010]. Casein inhibits the adherence of cariogenic bacteria to saliva-coated surfaces. Sodium caseinate, casein phosphopeptide, and glycol-macropeptide inhibited adherence of oral streptococci to saliva-coated hydroxyapatite beads. Anticariogenicity of these proteins and peptides was accomplished by selectively inhibiting adhesion of streptococci to the teeth; microbial composition of dental plaque was modulated to favour establishment of less cariogenic species, such as oral *Actinomyces* spp. [Neeser et al., 1994]. Milk and individual caseins (α, β, and κ caseins) were studied to determine adherence of *S. mutans* to saliva-coated hydroxyapatite discs. Milk inhibited in vitro adherence of *S. mutans* GS-5 [Vacca-Smith et al., 1994]. Individual caseins were also examined. No effect on streptococcal adherence was observed when α- or β-casein was incubated with hydroxyapatite beads. However, κ-casein inhibited adherence of *S. mutans* GS-5 Inhibitory properties were attributed to a 40 kDa glycoprotein [Vacca-Smith et al., 1994]. It has also been reported that α-, β- and κ- caseins of bovine origin inhibit adhesion of *S. mutans* to saliva-coated hydroxyapatite [Malkoski et al., 2001]. Another study, however, found clear inhibition of adhesion of *S. mutans* strains to saliva-coated hydroxyapatite by the C-terminal fraction of β-casein [Danielsson Niemi et al., 2009].

In a study in rats, micellar casein was shown to prevent oral colonization by *Streptococcus sobrinus* and to promote colonization by *Actinomyces viscosus*. Sodium caseinate was not as effective as micellar casein at inhibiting streptococcal colonization [Guggenheim et al., 1999].

Our adhesion model used sterilized human saliva to provide a close representation of the natural growth conditions. It has been suggested that bacterial attachment in the mouth is sensitive to the ionic strength of saliva. The ionic strength used in this study was 15 mM for mucin and 11 mM for casein, which is in line with the strengths used in previous studies [Mai et al., 2014]. The present in vitro study has some limitations because it investigated only the simple adhesion of bacterial strains without considering the influence of other strains. To have standardized conditions, pooled saliva from healthy subjects was used. Saliva from healthy subjects and patients with dental erosion do not differ in the total protein amount [Bardow et al., 2014; Carpenter et al., 2014], however the content of calcium and proteins is reduced in newly formed acquired pellicle on enamel in dental erosion [Carpenter et al., 2014].

Recently we have shown that pellicle formed with saliva containing a mixture of 0.27% mucin and 0.5% casein clearly inhibits loss of microhardness of enamel already after two cycles of 2 h incubation when being exposed to a 0.65% citric acid solution [Cheaib and Lussi, 2011]. This modification of the pellicle did not only reduce erosion of enamel [Cheaib and Lussi, 2011] but also slightly reduced bacterial adherence compared to untreated human pellicle. This may be of practical significance in prevention, as on the one hand bacterial homoeostasis might be not seriously affected and on the other hand as both erosion and caries are widespread around the world. More extensive investigations are required to understand the interaction of a complex oral bacterial community incl. cariogenic bacteriawith the 0.27% mucin/0.5% casein mixture as a potential additive to soft drinks in preventing dental erosion.

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Author Contributions

Z. Cheaib, E. Rhakmatulina, A. Lussi and S. Eick conceived and designed the study. Z. Cheaib performed the experiments. Z. Cheaib, E. Rhakmatulina, A. Lussi and S. Eick analysed the data. Z. Cheaib, E. Rhakmatulina, A. Lussi and S. Eick wrote the manuscript.

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Legends

Figure 1

Number (minimum, maximum, quartiles) of *Streptococcus gordonii* ATCC 10558 adhering to different surfaces: native enamel (E), pellicle-covered enamel (P-E), enamel coated with casein (C), enamel coated with mucin (M), enamel coated with casein/mucin (CM), enamel covered with pellicle modified by casein (PC), enamel covered with pellicle modified by mucin (PM) and enamel covered with pellicle modified by casein and mucin (P-CM) after 30 min (A) and 2 h (B) incubation. * $p<0.05$; ** $p<0.01$; *** $p<0.001$ pellicle-covered enamel each compared to the same coating without pellicle

¶ $p<0.05$; ¶¶ $p<0.01$ coating each compared to enamel

† $p<0.05$; †† $p<0.01$; ††† $p<0.001$ coating each compared to pellicle-covered enamel

Figure 2

Number (minimum, maximum, quartiles) of *Streptococcus oralis* ATCC 35037 adhering to different surfaces: native enamel (E), pellicle-covered enamel (P-E), enamel coated with casein (C), enamel coated with mucin (M), enamel coated with casein/mucin (CM), enamel covered with pellicle modified by casein (PC), enamel covered with pellicle modified by mucin (PM) and enamel covered with pellicle modified by casein and mucin (P-CM) after 30 min (A) and 2 h (B) incubation.

* $p<0.05$; ** $p<0.01$; *** $p<0.001$ pellicle-covered enamel each compared to the same coating without pellicle

¶ $p<0.05$; ¶¶ $p<0.01$; ¶¶¶ $p<0.001$ coating each compared to enamel

† $p<0.05$; ††† $p<0.001$ coating each compared to pellicle-covered enamel

Figure 3

Number (minimum, maximum, quartiles) of adhered *Actinomyces odontolyticus* ATCC 17982 to different surfaces surfaces: native enamel (E), pellicle-covered enamel (P-E), enamel coated with casein (C), enamel coated with mucin (M), enamel coated with casein/mucin (CM), enamel covered with pellicle modified by casein (PC), enamel covered with pellicle modified by mucin (PM) and enamel covered with pellicle modified by casein and mucin (P-CM) after 30 min (A) and 2 h (B) incubation.

* $p<0.05$; ** $p<0.01$; *** $p<0.001$ pellicle-covered enamel each compared to the same coating without pellicle

†† $p<0.01$; ††† $p<0.001$ coating each compared to pellicle-covered enamel

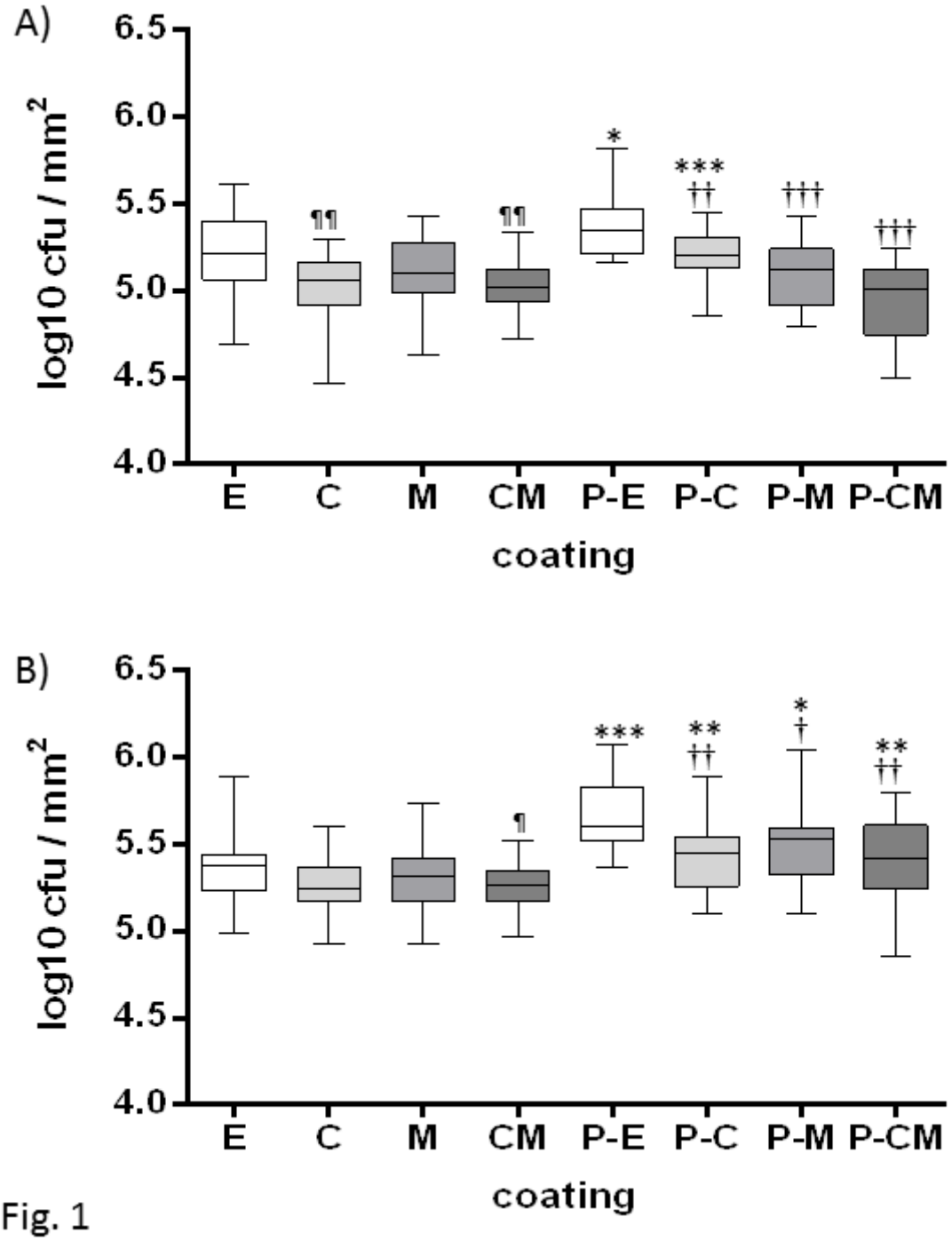

A)
log10 cfu / mm2
6.5
6.0
5.5
5.0
4.5
4.0
¶¶
¶¶
*
***
††
†††
†††
E
C
M
CM
P-E
P-C
P-M
P-CM
coating
B)
log10 cfu / mm2
6.5
6.0
5.5
5.0
4.5
4.0
¶
***
**
††
*
†
**
††
E
C
M
CM
P-E
P-C
P-M
P-CM
coating

Fig. 1

473

474

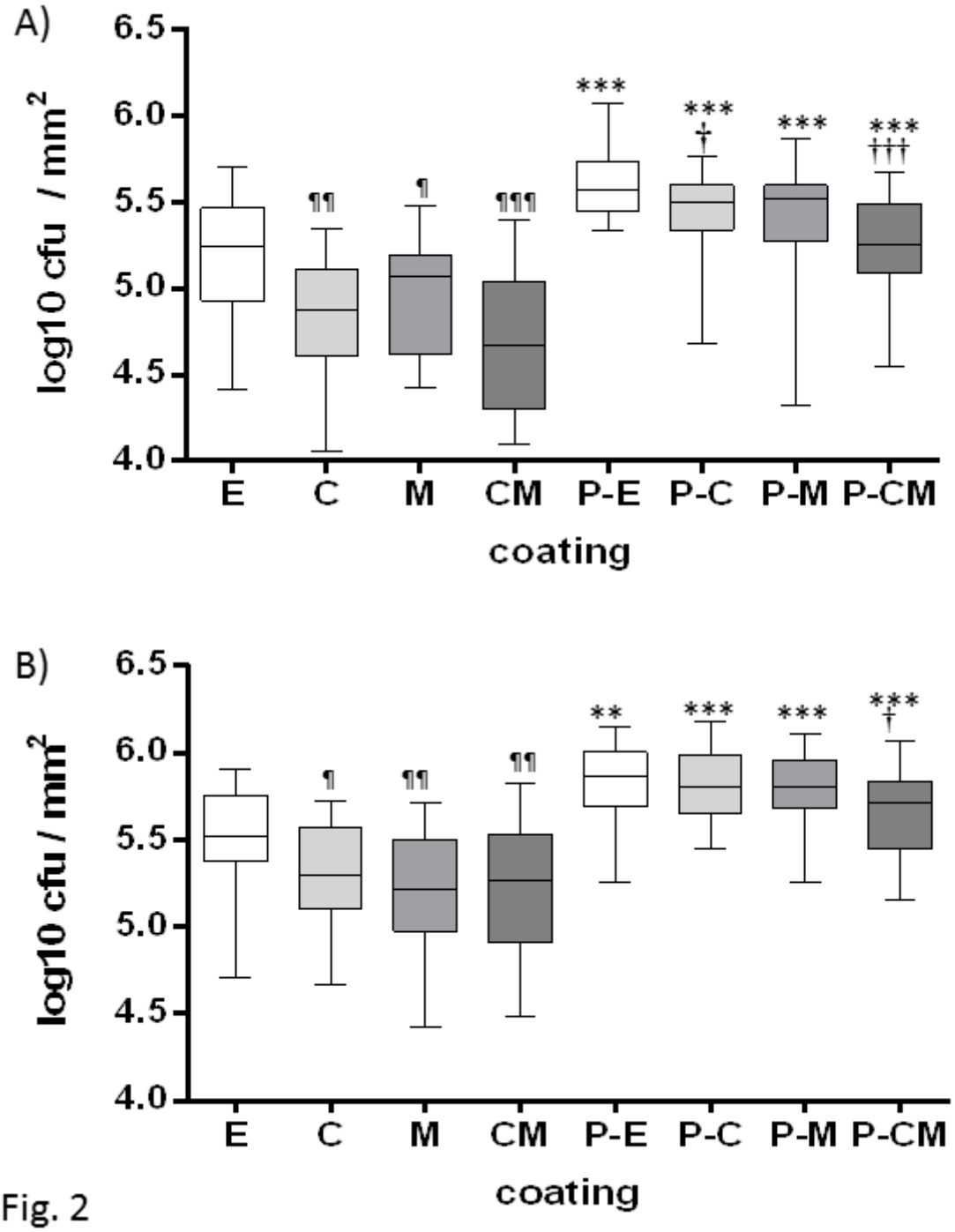
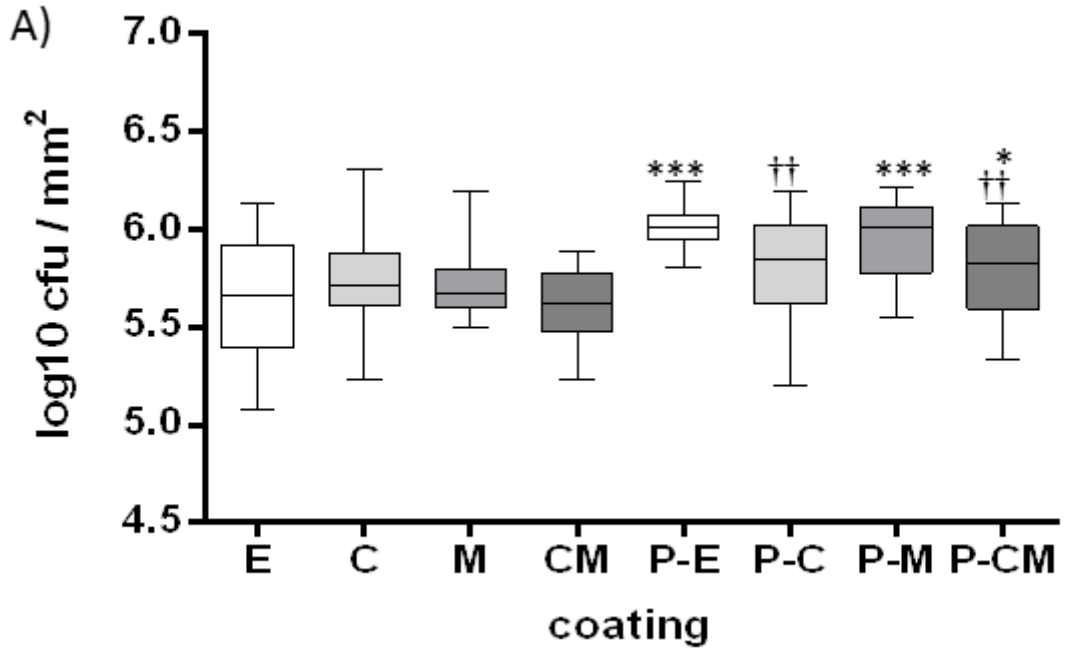

A)
log10 cfu / mm2
6.5
6.0
5.5
5.0
4.5
4.0
¶¶
¶
¶¶¶
***
***
†
***
***
†††
E
C
M
CM
P-E
P-C
P-M
P-CM
coating
B)
log10 cfu / mm2
6.5
6.0
5.5
5.0
4.5
4.0
¶
¶¶
¶¶
**
***
***
***
†
E
C
M
CM
P-E
P-C
P-M
P-CM
coating

Fig. 2

475

476


A)
log10 cfu / mm2
7.0
6.5
6.0
5.5
5.0
4.5
***
††
***
*
††
E
C
M
CM
P-E
P-C
P-M
P-CM
coating

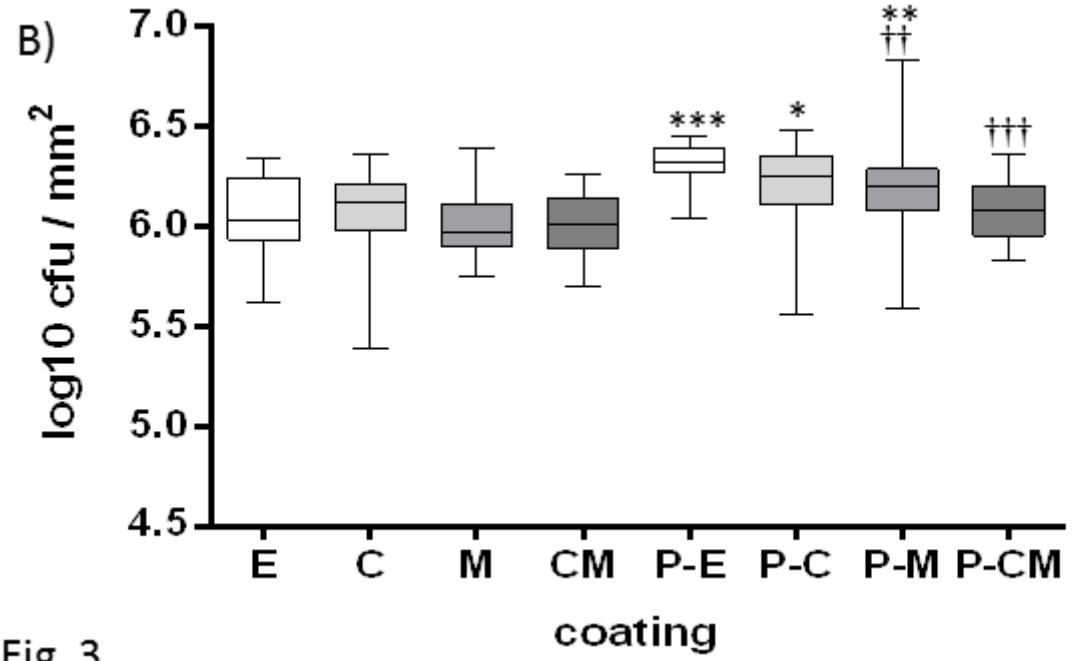

B)
log10 cfu / mm2
7.0
6.5
6.0
5.5
5.0
4.5
***
*
**
††
†††
E
C
M
CM
P-E
P-C
P-M
P-CM
coating

Fig. 3

477