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Streptococcus cristatus ArcA Interferes with *Porphyromonas gingivalis* Pathogenicity in Mice

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Abstract

Background and Objective—*Porphyromonas gingivalis* has been implicated as one of the major pathogens in chronic periodontitis, an infectious disease affecting the majority of the adult population. We have previously demonstrated that a surface protein, arginine deiminase (ArcA), of *Streptococcus cristatus* represses production of *P. gingivalis* long fimbriae and interrupts the formation of *P. gingivalis* biofilms *in vitro*. Our *in vivo* studies have also shown that the distribution of *P. gingivalis* and *S. cristatus* in human subgingival plaque is negatively correlated. The objective of this study is to determine if *S. cristatus* ArcA inhibits *P. gingivalis* colonization and attenuates its subsequent pathogenesis in alveolar bone loss in the murine oral cavity.

Material and Methods—A wild-type strain of *S. cristatus*, CC5A and its *arcA* knock-out mutant ArcAE were used as initial colonizers in the oral cavity of BALB/cByJ mice. Colonization of *P. gingivalis* on the existing *S. cristatus* biofilms was assessed by qPCR and *P. gingivalis*-induced alveolar bone loss was measured 6 weeks after *P. gingivalis* infection.

Results—The presence of *S. cristatus* CC5A, but not its *arcA* mutant, attenuated *P. gingivalis* colonization in the murine oral cavity. In addition, *P. gingivalis*-induced alveolar bone loss was significantly less in mice initially infected with *S. cristatus* CC5A than in those infected with the *arcA* mutant.

Conclusions—This study provides direct evidence that *S. cristatus* ArcA has an inhibitory effect on *P. gingivalis* colonization, which may in turn attenuate the pathogenecity of *P. gingivalis*.

Keywords

Porphyromonas gingivalis; *Streptococcus cristatus* ArcA; mouse; pathogenesis

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INTRODUCTION

Chronic periodontitis is among the most common infectious diseases in humans and affects not only the periodontium but also systemic conditions. Associations have been demonstrated between periodontitis and several systemic diseases such as cardiovascular diseases, preterm labor, and respiratory diseases (1-3). It has been generally accepted that the initiation of periodontitis depends on the existence of certain gram-negative species of bacteria and, *Porphyromonas gingivalis* is the predominant species implicated in periodontitis (4, 5).

P. gingivalis has been studied extensively *in vitro* and *in vivo* for its role in chronic periodontitis. The majority of *P. gingivalis* clinical isolates are fimbriated, especially when isolated from deep periodontal pockets (6, 7) and different FimA genotypes have been demonstrated (8-10). The major fimbriae (long fimbriae) of *P. gingivalis* composed of polymeric FimA subunit proteins are a well-studied virulence factor contributing to colonization, biofilm formation, cell invasion, bone resorption, and evasion of host defenses (11-19). Thus, reduction of FimA production in *P. gingivalis* has been considered as an attractive strategy for preventing the bacterial colonization and subsequent periodontal pathogenesis. We previously reported that arginine deiminase (ArcA) of *Streptococcus cristatus* is capable of selectively repressing FimA expression in *P. gingivalis* (20). As a surface protein of *S. cristatus*, ArcA was identified as the signaling molecule to which *P. gingivalis* responds by repressing expression of the *fimA* gene and production of the FimA protein. Our recent results also showed that the distribution of *S. cristatus* and *P. gingivalis* in dental plaque in periodontitis patients was negatively correlated (21). These studies suggested that an early colonizer of dental plaque, *S. cristatus*, may serve as a beneficial bacterium to block *P. gingivalis* accumulation in dental plaque in sufficient numbers as to reduce periodontal inflammation.

In the present study, we investigated the role of ArcA in *P. gingivalis* colonization and its pathogenesis in alveolar bone loss using a mouse model. Utilizing a wild-type *S. cristatus* strain CC5A and its *arcA* knockout mutant (22) as initial colonizers, we report here direct evidence that *S. cristatus* ArcA attenuates sequential *P. gingivalis* colonization and subsequent *P. gingivalis*-induced alveolar bone loss *in vivo*.

MATERIALS & METHODS

Bacterial Strains and Media

S. cristatus CC5A and its *arcA* knockout mutant, ArcAE, (erythromycin-resistant) (22) were grown in Trypticase peptone broth (TPB) supplemented with 0.5% glucose at 37°C under aerobic conditions. *P. gingivalis* 33277 was grown from frozen stocks in Trypticase Soy broth (TSB) or on TSB blood agar plates supplemented with yeast extract (1 mg/ml), hemin (5 μg/ml), and menadione (1 μg/ml) at 37°C in an anaerobic chamber (85% N_2, 10% H_2, 5% CO_2).

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Infection of Mice

Specific-pathogen-free BALB/cByJ male mice (Jackson Laboratory, Bar Harbor, ME, USA) were maintained in the Laboratory Animal Facility of the University at Buffalo, State University of New York. The experimental protocols were approved by the Institutional Animal Care and Use Committee of the University at Buffalo. Infection timeline and the experimental groups are listed in Table 1. Briefly, 6 week old mice (8 mice per group) were treated with kanamycin (Sigma-Aldrich, Saint Louis, MO) at 1 mg/ml in water ad libitum for 7 days, followed by a three-day antibiotic-free period (23). Mice were infected orally with micropipettes using 2×10^9 cfu of live *S. cristatus* CC5A or its *arcA* knock out mutant (ArcAE) twice a day for 5 days in 50 μl of PBS with 2% carboxymethyl cellulose (PBS-CMC). *P. gingivalis* 33277 cells in late log phase were pelleted and re-suspended in PBS-CMC. Inoculation of *P. gingivalis* (5×10^9 cfu in 50 μl PBS-CMC intraorally by micropipettes, three inoculations at two day intervals) was carried out 7 days after the last inoculation of *S. cristatus* and was repeated two weeks after the last *P. gingivalis* inoculation. Control (sham-infected) mice received the antibiotic treatment and the intraoral PBS-CMC inoculation without the bacteria. Mice were kept away from food and water for 1 h after each inoculation of bacteria.

Bacterial Quantitation

The colonization of *S. cristatus* or *P. gingivalis* in the murine oral cavities was examined at 7 or 14 days after the final inoculation of bacteria, respectively (see Table 1). The plaque samples were taken from tooth surfaces and surrounding gingival mucosa using cotton applicators and immersed in 1 ml of Tris-EDTA (TE) buffer (pH 7.5). The suspensions were dispersed by vortex at full speed for 30 seconds and bacteria were harvested by centrifugation. The samples obtained were boiled for 20 minutes to release chromosomal DNA. To minimize experimental error, plaque samples from the oral cavity were collected by a single investigator (JH). The final volume per sample before DNA extraction by boiling was fixed at 50 μl in TE buffer. A fixed volume of 5 μl DNA per sample was utilized in qPCR. *P. gingivalis* and *S. cristatus* cells were enumerated by using a QuantiTect SYBR Green PCR Kit (Qiagen, CA) with *P. gingivalis* species-specific 16S rDNA gene primers, (TGTAGATGACTGATGGTGAAA and ACTGTTAGCAACTACCGATGT) (24) or *S. cristatus* 23S rDNA gene primers (ACTGCAATGTGGACTCAGAATTTAT and TACAGAATCTATTTAAAATACGAGGCTCT). Standards used to determine numbers of *P. gingivalis* or *S. cristatus* cells were prepared using genomic DNAs from the wild type strain 33277 or CC5A (21). A fresh culture of bacteria was serially diluted in PBS and plated to enumerate colony forming units at each dilution. Chromosomal DNA was isolated from the dilutions and a qPCR assay was run to determine cell numbers. *S. cristatus* colonization in the oral cavity was confirmed by colony morphology. The colonization of *S. cristatus* in the murine oral cavities was examined 7 days after the final inoculation of the bacteria. The plaque samples were taken from tooth surfaces and surrounding gingival mucosa using cotton applicators (from 3 mice per group that have not be sampled for qPCR) and smeared on Mitis Salivarius (MS) agar plates. Blue colonies representing *S. cristatus* were counted after 36-48 h incubation at 37°C in candle jars.

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Alveolar Bone Loss Measurement

To examine alveolar bone loss, the mice were sacrificed on day 69 (see Table 1). The maxillae from the sacrificed mice were removed, autoclaved, and mechanically defleshed to remove all the soft tissue. The maxillae was then immersed overnight in 3% hydrogen peroxide and stained with 1% methylene blue. The distance between the cementoenamel junction (CEJ) and alveolar bone crest (ABC) were measured for a total of 14 buccal sites on the right and left maxillary molars [3 sites on first molar, 2 sites on second molar and another 2 sites on third molar, as described by Sharma et al (25)]. Measurement was made under a dissecting microscope with an Aquinto imaging measurement system. Alveolar bone loss per experimental group was calculated as the average of 14-site total CEL-ABC distances for each group.

Statistical Analysis

ANOVA analysis and Student's *t*-test were performed to determine statistically significant difference in colonization and alveolar bone loss among different experimental groups. A *p* value < 0.05 was considered significant.

RESULTS

Colonization of *S. cristatus* in the Murine Oral Cavity

To establish the feasibility of *S. cristatus* colonization in the murine oral cavity, wild-type *S. cristatus* CC5A and its *arcA* knockout mutant ArcAE were introduced into the murine oral cavities. Quantification of colonized *S. cristatus* was carried out using qPCR, with *in vitro* cultured *S. cristatus* CC5A as a standard control. Both the wild-type CC5A and the knockout mutant consistently colonized the murine oral cavity (positive qPCR was obtained from every mouse infected, data not shown). There was no significant difference in colonization efficiencies between the two groups [$(13.0 \pm 3.4) \times 10^3$ vs. $(14.2 \pm 2.9) \times 10^3$); $p > 0.05$] (Fig. 1).

Colonization of *S. cristatus* strains in the murine oral cavity was also determined by culturing the bacteria on MS agar plates; streptococcal colonies were observed in the samples retrieved from CC5A or its *arcA* mutant infected mice, but not from sham-infected mice. This result further confirmed the colonization of *S. cristatus* in the murine oral cavity (Fig. 2). We also confirmed that the streptococcal colonies on MS agar plates were indeed the *S. cristatus* strains, using qPCR with the *S. cristatus* 23S rDNA primers (data not shown).

S. cristatus ArcA Attenuated the Colonization of *P. gingivalis* in the Murine Oral Cavity

Formation of dental plaque on human enamel chips has been shown to be a sequential process with streptococci as the dominating initial colonizers (26, 27). Based on our earlier observations that ArcA of *S. cristatus* inhibits formation of *P. gingivalis* biofilms *in vitro*, we examined the role of *S. cristatus* biofilms on *P. gingivalis* colonization in the murine oral cavities. Colonization of *P. gingivalis* was determined using qPCR, with 16S rDNA primers as probes. As shown in Fig. 3A, at 2 weeks post *P. gingivalis* inoculation, the colonization of *P. gingivalis* in the wild-type *S. cristatus* CC5A colonized mice was significantly less

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efficient than that in the *arcA* mutant ArcAE colonized mice [(4.48 ± 2.54) x 10^3 vs. (24.93 ± 2.08) x 10^3; $p = 0.0062$], indicating that ArcA of *S. cristatus* interferes with *P. gingivalis* colonization in the murine oral cavity.

We also examined the persistence of initial *S. cristatus* colonization in the mixed *S. cristatus*-*P. gingivalis* biofilms. As shown in Fig. 3B, *S. cristatus* colonization persisted for 4 weeks after the last *S. cristatus* inoculation and 2 weeks after the sequential *P. gingivalis* inoculation. There was no statistically significant difference in quantity between the wild-type *S. cristatus* CC5A and its *arcA* knockout counterpart [(8.94 ± 2.65) x 10^3 vs. (10.93 ± 3.04) x 10^3; $p = 0.2245$].

Our results demonstrated that *S. cristatus* and *P. gingivalis* are able to co-exist in the murine oral cavity and the expression of ArcA by *S. cristatus* inhibits *P. gingivalis* colonization.

S. cristatus ArcA Prevented *P. gingivalis*-induced Alveolar Bone Loss in Mice

We next examined *P. gingivalis*-induced alveolar bone loss in the presence of *S. cristatus* CC5A or *S. cristatus* ArcAE mutant on day 69 (6 weeks after *P. gingivalis* infection). As shown in Fig. 4, no significant bone loss was observed in the mice infected only with *S. cristatus* CC5A or ArcAE, relative to the sham-infected mice (0.076 ± 0.019 mm, 0.077 ± 0.028 mm, 0.073 ± 0.018 mm; $p > 0.05$). *P. gingivalis*-induced alveolar bone loss was significantly more prominent in the *S. cristatus* ArcAE group than in the comparable CC5A group (0.120 ± 0.037 mm, 0.092 ± 0.033 mm; $p = 0.011$). These results indicated that ArcA serves as a potent inhibiter for *P. gingivalis* pathogenesis.

DISCUSSION

Dental plaque formation is generally programmed beginning with the initial colonization by gram-positive aerobic bacteria, followed by a succession of gram-negative anaerobic bacteria. The transition from commensal bacterial accumulation to periodontopathic plaque involves the colonization of certain species of gram-negative anaerobic bacteria such as *P. gingivalis*. Evidence is accumulating that some early colonizers of dental plaque provide a favorable environment for *P. gingivalis*, at least with regard to attachment sites and lower oxygen tension. This in turn facilitates retention and multiplication of *P. gingivalis*, ultimately leading to the progression of adult periodontitis (14). Although less well understood, antagonistic relationships have been reported between/among oral bacteria. *P. gingivalis*, for example, does not grow with *Streptococcus oralis* in two-species biofilms (28) and does not co-aggregate with *Streptococcus mutans* (29). We have reported earlier that S. *cristatus* represses expression of *fimA* in *P. gingivalis* and attenuates bacterial colonization *in vitro* (22, 30). The present study was designed to test our hypothesis that *S. cristatus* ArcA interferes with *P. gingivalis* colonization in the murine oral cavity and attenuates the subsequent *P. gingivalis*-induced alveolar bone loss, an indicator of *P. gingivalis* pathogenesis in periodontitis.

P. gingivalis-induced alveolar bone loss in rodent models has been widely accepted as a valid model for *P. gingivalis* pathogenicity critical for periodontitis. Furthermore, studies utilizing these rodent models have indicated that *P. gingivalis* – induced alveolar bone loss

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depends on the expression of FimA protein in the bacterium (17, 18, 31). These studies have shown that isogenic mutants lacking FimA or its associated minor proteins are less virulent and induce significantly less alveolar bone loss relative to the wild-type bacterium. Since we have previously demonstrated that ArcA of *S. cristatus* represses *fimA* gene expression in *P. gingivalis in vitro* (20, 22, 30), in this study we intended to determine if biofilms of *S. cristatus* CC5A, relative to those of a isogenic mutant lacking ArcA would interfere with the pathogenesis of *P. gingivalis* in the murine oral cavity.

S. cristatus is a component of earlier colonizers in human dental plaque and has been detected in the 8-h biofilms on freshly cleaned enamel chips (26). In this study, we demonstrated that *S. cristatus*, like some other oral streptococci (32-34) could colonize the rodent oral cavity. The presence of *S. cristatus* in the oral cavity could be detected as monobiofilms (Fig. 1) and also as heterotypic biofilms with *P. gingivalis* (Fig. 3B).

The objective of this study is to determine if *S. cristatus* ArcA attenuates *P. gingivalis* colonization and subsequent *P. gingivalis*-induced alveolar bone loss *in vivo*. Therefore, *P. gingivalis* infection was carried out in the oral cavity with existing biofilms of wild-type *S. cristatus* CC5A or its *arcA* knockout mutant. This experimental design allows us to test our hypothesis, since the only difference between the two groups is the presence or absence of *S. cristatus* ArcA. As demonstrated in Fig. 4, the wild-type CC5A strain expressing ArcA is more potent than the ArcA deficient mutant in blocking *P. gingivalis*-induced alveolar bone loss. Since *P. gingivalis* was not recovered from *P. gingivalis* alone infected group two-weeks post infection, in contrast to the *S. cristatus* colonized groups where *P. gingivalis* was recovered persistently, the *P. gingivalis* alone infected group was considered an inappropriate control for the study and was excluded. However, in light of our findings, it would be interesting to compare the *P. gingivalis* alone infected group with the *S. cristatus* precolonized groups in the bone loss experiments in the future.

Inter-species interactions play important roles in the initiation and development of oral infectious diseases, since it can affect the pathogenecity of a particular pathogen. Our results provide direct evidence that *S. cristatus* ArcA attenuates *P. gingivalis* biofilm formation (Fig. 3A) and subsequent pathogenesis (as indicated by *P. gingivalis*-induced alveolar bone loss) *in vivo* (Fig. 4). The presence of *S. cristatus* CC5A did not completely eliminate *P. gingivalis* colonization and *P. gingivalis*-induced alveolar bone loss in mice. However, both the quantity of colonized *P. gingivalis* and *P. gingivalis*-induced bone loss were significantly less in the *S. cristatus* CC5A-infected group than in the corresponding *S. cristatus* ArcAE-infected animals. Further *in vivo* studies are warranted to determine if purified *S. cristatus* ArcA or a synthetic ArcA functional domain attenuates *P. gingivalis* pathogenesis, as they did in *P. gingivalis* biofilm formation *in vitro* (20, 30

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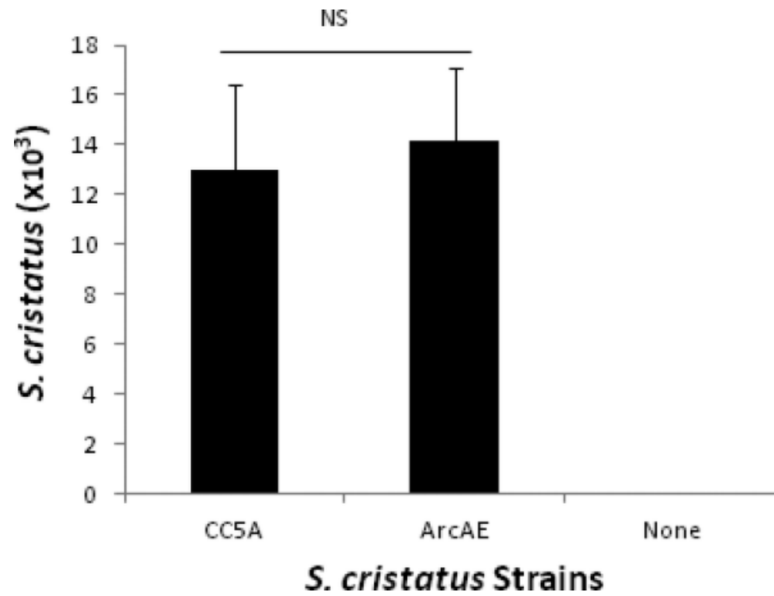

Figure 1.

Colonization of *S. cristatus* in murine oral cavities. The plaque samples were taken from tooth surfaces and surrounding gingival mucosa 7 days after the last inoculation of *S. cristatus* in the oral cavities. DNA extracted from these plaque samples (50 μl final volume/ sample in TE buffer) was examined for quantitation of *S. cristatus* using qPCR with primers corresponding to 23S rDNA as probes. ANOVA analysis revealed no significant difference in colonization efficiencies between the wild-type CC5A and the knockout mutant ArcAE ($p > 0.05$). Data represent the means ± S.D. from 5 mice per group in Experimental Groups 3, 4, and 5.

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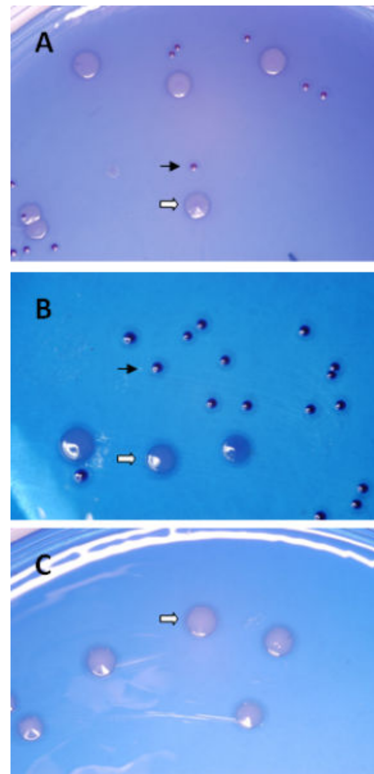

Figure 2.

S. cristatus morphology on MS agar plates. The plaque samples were taken from tooth surfaces and surrounding gingival mucosa 7 days after the last intraoral inoculation of *S. cristatus* in the oral cavities. The bacteria on the cotton applicator were streaked on the MS plates and incubated for 36-48 hours anaerobically. Photographs are the representatives of each experimental group (3 mice/group sampled from Experimental Groups 3, 4, and 5). A, CC5A-infected; B, ArcAE-infected; and C, sham-infected. The small blue colonies represent colonized *S. cristatus* (solid arrows) and large white colonies were residual endogenous murine bacteria (open arrows).

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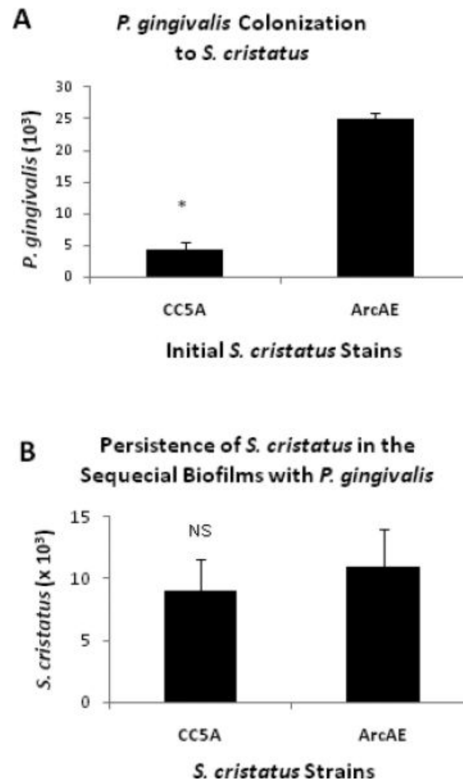

Figure 3.

Sequential *P. gingivalis* colonization on preexisting *S. cristatus* biofilms in the murine oral cavity. The plaque samples were taken from tooth surfaces and surrounding gingival mucosa 2 weeks post *P. gingivalis* inoculation. DNA extracted from these plaque samples (50 μl final volume/sample in TE buffer) was examined for quantitation of *P. gingivalis* and *S. cristatus*, using qPCR with primers corresponding to 16S or 23S rDNA as probes. Data represent the means ± S.D. from 8 mice per group. The variances between the groups were analyzed by ANOVA. A, *P. gingivalis* colonization on *S. cristatus* biofilms; B, Persistence of *S. cristatus* in the sequential biofilms with *P. gingivalis*.

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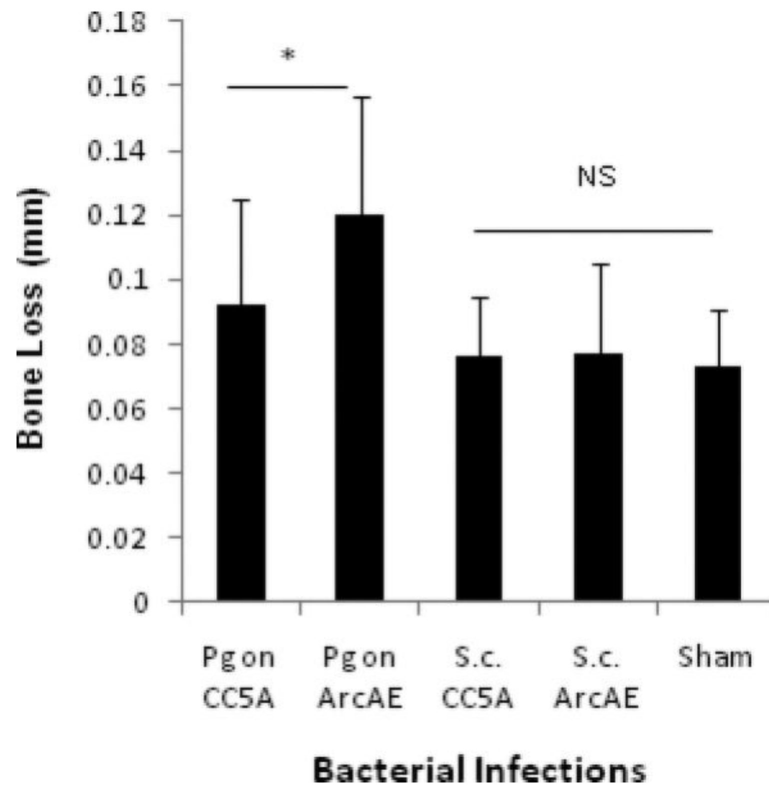

Figure 4.

Effect of *S. cristatus* ArcA on *P. gingivalis*-induced alveolar bone loss in BALB/cByJ mice. Data represent the means ± S.D. from 8 mice per group. Alveolar bone loss was calculated as the average of 14-site (3 sites on first molar, 2 sites on second molar, and 2 sites on third molar, at both right and left sides of the maxilla) total CEJ-ABC distances for each group. ^{NS}, no significant difference in alveolar bone loss when compared with the sham-infected group *, the difference in alveolar bone loss was significant ($p < 0.05$) between the two *P. gingivalis* infected groups

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Table 1

Timeline and experimental groups.

Days	**1-7**	**8-10**	**11-16**	**23**	**23, 25, 27 41, 43, 45**	**41**	**69**
	Antibiotics	**Antibiotics**	***S. cristatus* infection**	**qPCR & Colony morphology**	***P. gingivalis* infection**	**qPCR**	**Assessment of bone loss**
Group 1	Yes	No	CC5A	No	Yes	Yes	Yes
Group 2	Yes	No	ArcAE	No	Yes	Yes	Yes
Group 3	Yes	No	CC5A	Yes	No	No	Yes
Group 4	Yes	No	ArcAE	Yes	No	No	Yes
Group 5	Yes	No	PBS-CMC	Yes	No	Yes	Yes