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Ex vivo HNSCC clinical studies using saliva and antiviral or antibacterial chewing gums reveal reduction in carcinogenic microbes

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Higher abundance of oral human papilloma virus (HPV), *Porphyromonas gingivalis* (Pg) and *Fusobacterium nucleatum* (Fn) correlate with worse survival of head and neck squamous cell carcinoma (HNSCC). One-third of males globally are HPV-positive, one-fifth have high-risk HPV-16 and is transmitted through oral sex. In our ex vivo clinical study of HNSCC patients, HPV ELISA detected viruses in 100% of saliva and 75% of oral-rinse samples ($n = 44$). Cell culture studies showed 1000-fold higher Pg/Fn in their saliva and 100-fold higher in oral-rinse samples than non-cancer control participants. Bean-lectin (FRIL) gum (trapped) aggregated 93% of HPV in saliva and 80% in oral-rinse samples in ELISA. Single dose of bean gum + protegrin-1 (antimicrobial peptide) reduced Pg/Fn > 99% in saliva and oral-rinse samples ($n = 42$). Although protegrin was effective against anaerobic Pg/Fn, it did not kill capsule forming bacteria including Streptococci, providing selectivity and protection of oral commensal bacteria. These findings from ex vivo clinical studies support potential value of advancing these biologics to clinical trials as adjuncts to current therapeutic strategies or as prophylaxis to control pathogenic microbes associated with HNSCC.

Plants provide food, fiber, fuel, and oxygen to support life on earth but are also sources of medicines to treat human diseases[1–3]. Antiproliferation, induction of apoptosis, and reduction in tumor size by plant lectins in breast, colon, lung, ovarian, prostate, oral and nasopharyngeal carcinoma cancer treatments have been widely reported[4,5]. In addition to anticancer activity, Flt3 Receptor Interacting Lectin (FRIL) from Lablab bean has significant anti-viral properties[6,7]. FRIL has four carbohydrate-binding domains that bind efficiently to complex-type N-glycans and oligosaccharides present on virus surface, thereby entrapping virus into large aggregates of crosslinked FRIL and bound virion particles, blocking entry into host cells (Fig. 1A). In addition, FRIL blocks viruses at the endosome level in host cells upon entry[6,7]. Protegrin is an antimicrobial peptide that has both anticancer[8] and antimicrobial activities (Fig. 1B)[9,10]. The antimicrobial activity of protegrin is conferred by stability of b-hairpin conformation, stabilized by the two disulfide bonds (Fig. 1B) and is more potent against bacterial pathogens than human neutrophil defensins[9]. The cost of chemical synthesis of protegrin-1 is reduced by expression in plants[9,10]. Efficacy and safety of protegrin has been evaluated in several Phase III clinical trials in patients with chemotherapy-related oral mucositis[11–13]. Phase I clinical trials of lectins have been conducted in advanced cancer patients by injections (NCT03051477)[14] or to treat HIV/HPV infection by topical vaginal delivery (NCT02875119)[15].

A systematic review of 65 studies including 45,000 males from 35 countries identified one third of males in the global population are infected with Human Papilloma Virus (HPV), providing an important reservoir for continued virus infection and transmission[16]. One fifth are infected with high-risk HPV-16, the most common cancer-associated type of this virus[17,18]. Although an HPV vaccination program is available in > 100 countries[19], the incidence of oropharyngeal cancer (OPC) has significantly increased post vaccination[20]. HPV vaccines are systemic and not mucosal vaccines[19] delivered through intramuscular injection. Injected vaccines do not

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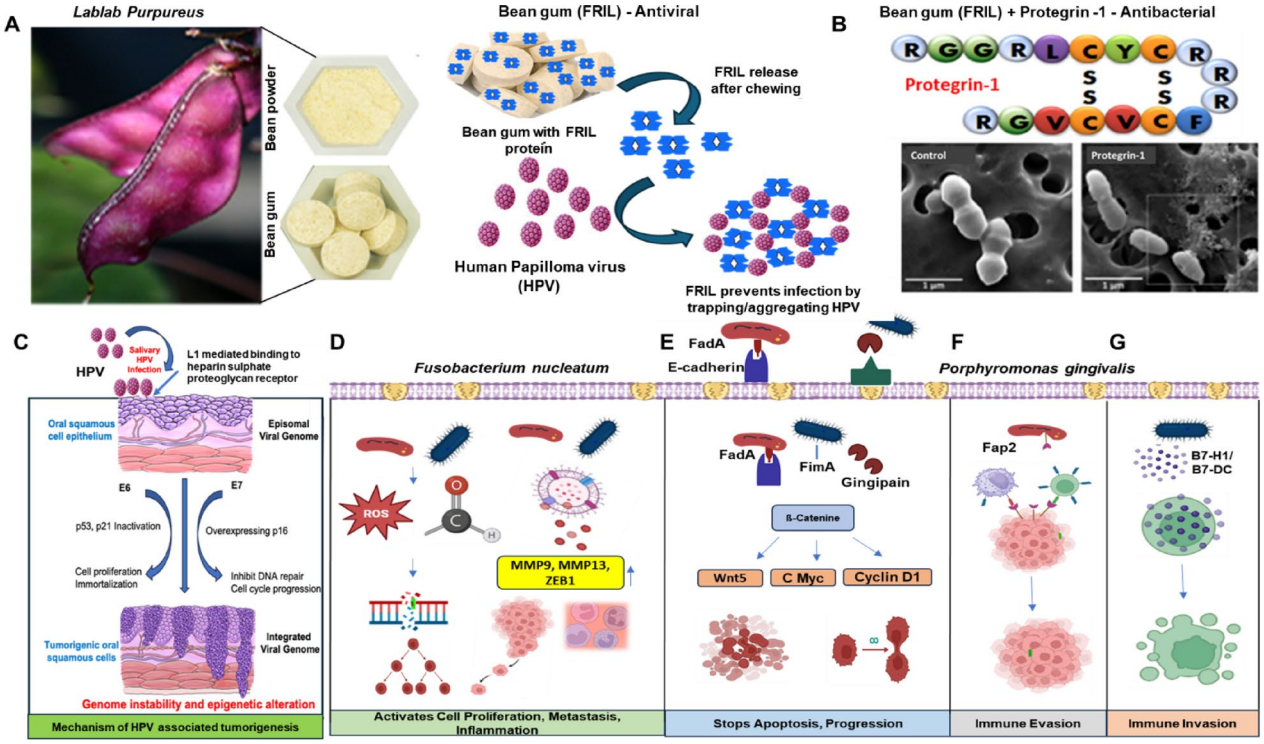

Fig. 1. Mechanisms of antiviral bean-gum/antibacterial protegrin-1 and HPV, Pg and Fn in Oral Squamous Cells Carcinoma. (**A**) Antiviral action of FRIL - Flt3 Receptor Interacting Lectin (FRIL) present in lablab bean is facilitated by the four carbohydrate-binding domains that bind efficiently to complex-type N-glycans and oligosaccharides present on virus surface, thereby entrapping virus into large aggregates of crosslinked FRIL and bound virion particles, blocking entry into host cells. In addition, FRIL blocks viruses at the endosome level in host cells upon entry (Daniell et al. 2022). (**B**) Antibacterial action of protegrin-1 (PG1) is conferred by its b-hairpin conformation, stabilized by two disulfide bonds and is more potent against bacterial pathogens than human neutrophil defensins (previously evaluated in several human clinical trials). (**C**) HPV L1 protein mediates internalization by binding with heparin sulphate proteoglycan receptor on epithelial cells. The viral genome initially exists in the episomal form. Expression of oncogenic proteins E6 and E7 inactivates p53 and pRb pathways, respectively which induce neoplastic transformation and tumor progression. Integration of the viral genome with the host genome causes genome instability and genic alteration facilitating mutation and hyperproliferation of the tumorigenic cell. (**D**) *F. nucleatum* (Fn) and *P. gingivalis* (Pg) produce oncometabolites, reactive oxygen species and outer membrane vesicles inducing cell proliferation, metastasis and inflammation. (**E**) Fn and its attachment protein FadA binds to E-cadherine and activate ß-Catenine pathway. FimA and gingipain of Pg activates ß-Catenine pathway inducing progression of HNSCC. (**F**) Fap2 protein of Fn attaches via TIGIT on T cells and natural killer cells evading immune surveillance. (**G**) B7-H1/ B7-DC receptors of Pg partially evade immune system by inducing T cell apoptosis.

produce secretory IgA on mucosal surfaces and therefore do not prevent reinfection or transmission[21]. Fully vaccinated individuals with breakthrough infections have peak viral loads similar to unvaccinated individuals and efficiently transmit virus in household settings. This has been repeatedly reported during the coronavirus pandemic[22]. Because HPV spreads primarily through mucosal contact, prevention of HPV transmission from infected individuals to healthy individuals remains challenging. Global increase in OPC is linked with HPV-16 infection and the popularity of oral sex[23]. Therefore, new approaches are needed to reduce oral transmission of HPV.

HPV presence in basal cells of tonsil or gingival tissues triggers the malignant transformation of oropharyngeal SCC[24]. HPV L1 protein mediates internalization through binding heparin sulphate proteoglycan receptor on epithelial cells. The viral genome initially exists in episomal form. Expression of oncogenic proteins E6 and E7 inactivates p53 mediated growth arrest and retinoblastoma protein, resulting in neoplastic transformation and tumor progression (Fig. 1C). In tumorigenic cells the viral genome integrates into the host genome causing genome instability and genic alteration which facilitate mutation and hyperproliferation[25,26].

In addition to HPV, two anaerobic bacterial species play a major role in initiation or metastatic oral cancer. *Porphyromonas gingivalis* (Pg) induces dysbiosis shift, promoting progression of HNSCC and generating a tumor friendly microenvironment (Fig. 1D–G). Pg is invasive and replicates within tumor cells and influences oncogenic signaling of HNSCC[27,28]. *Fusobacterium nucleatum* (Fn) is also associated with oral cancer[29–32]. Fn and Pg produce oncometabolites, reactive oxygen species and outer membrane vesicles inducing cell proliferation, metastasis and inflammation[28–32]. Mechanistically, Fn triggers cancer progression by internalizing

in the host tissue through FadA and upregulating oncogenes (Cyclin D1, Myc, MMP1, MMP9) responsible for inflammation, cell proliferation, invasion, metastasis (Fig. 1D–G). The Fap2 protein helps evade immune surveillance by interacting with TIGIT on T cells and natural killer cells facilitates tumor survival and growth (Fig. 1F)[32]. Fn induces resistance to Oxaliplatin, 5-fluorouracil due to increased autophagy and apoptosis blockade[33].

Survival rates of recurrent or metastatic head and neck squamous cell carcinoma (HNSCC) after surgery and adjuvant therapies continue to be low[34–38]. The 2022 GLOBOCAN estimates cumulative oral cancer cases to be the second highest behind lung cancer among men living in 185 countries and 50% of cases died after treatments[39], with an estimated 890,000 new cases per year[40]. Higher abundance of oral HPV, Pg and Fn correlate with worse survival of HNSCC patients[34–37]. Recent studies show that nearly all deaths averted from cervical cancer were by screening and removal of precancerous growth[41,42] and most cancer drugs granted accelerated approval did not significantly improve overall survival[43].

DNA/RNA PCR amplification doesn't distinguish living from dead microbes or episomal and integrated HPV[44–49] and cancer associated bacteria are present in healthy individuals but at lower density. Therefore, in this ex vivo study we quantify pathogenic live bacteria by culturing or virions by ELISA and develop therapeutic approaches to reduce their load to attenuate HNSCC initiation or recurrence. Particular attention has been paid to develop affordable drugs to meet global needs and increase access to healthcare. Biologics made in plant cells approved by FDA cost < 3% of newly launched biologics produced in other systems[2,50–53]. Clinical grade biologics developed in this project should facilitate further clinical advancement as adjuvants with current treatments or as prophylaxis to prevent infection and transmission.

Results

Collection of saliva and oral rinse samples from oral cancer patients

Salivary samples (5 to 6 ml) were collected from non-cancer and oral cancer patients after obtaining IRB approval (study #1615727) from Veterans Administration of Greater Los Angeles Healthcare System (VAGLAHS), Los Angeles, California. Informed consent approval was obtained from all research cancer patients. Samples were spun at 1300 × g for 20 min at 4 °C to collect cells and supernatants. Aliquots were frozen at − 80 °C. Deidentified salivary samples were divided into two parts and analyzed for HPV viral load changes or in vitro bean gum treatment and microbial load before and after bean gum (166.6 mg/mL) + protegrin-1 (100 µg/mL).

The Institutional Review Board at the University of Kansas Medical Center approved the acquisition of oral rinse samples after reviewing compliance with ethical standards (IRB# STUDY00001732). Samples collected from HNSCC patients and cancer-free participants, were deidentified after obtaining written informed consent. Oral cancer patients or cancer-free participants were asked to rinse their oral cavity with 10 ml sterile isotonic saline for 30 s. The oral rinse samples were collected, aliquoted in 1 ml and stored at − 80 °C.

Optimization of HPV quantitation and viral aggregation by ELISA

ELISA detects HPV L1 surface protein in six different HPV types by utilizing multivalent antibody against HPV types 1, 6, 11, 16, 18 and 31[54]. FRIL present in the bean gum cross-links and forms higher-order structures and entrap viruses in large aggregates of crosslinked FRIL and virion particles[6,7] (Fig. 1A). We observed similar viral loads either before or after sonication used to disrupt epithelial cells, confirming that viruses were released already from cells, due to freeze thaw cycle (Fig. 2A). In the bean gum virus trap/aggregation assay, we distinguished the role of centrifugation from the aggregation of HPV caused by FRIL by spinning samples at 21,000 × g for 2 h without the bean gum. HPV in absence of bean gum extract was not precipitated even after centrifugation at 21,000 × g for 2 h, while in presence of bean gum extract HPV aggregated and precipitated after brief centrifugation (Fig. 2B).

ELISA evaluation of HPV types 1, 6, 11, 16, 18, 31 in cancer patient samples

By following the optimized protocol of HPV detection by ELISA we investigated presence of HPV in salivary samples of 14 HNSCC patients and 12 non-cancer patients visited/treated at UCLA Medical Center, Los Angeles, California. HPV was detected in 100% HNSCC patients and viral load was much higher in HNSCC patient samples than controls ($^*p \leq 0.050$) (Fig. 3A). Because samples were collected at the VA hospital from veterans, all non-cancer patients were male except one and age group was 33–70 years old. All cancer patients were also male in the age group of 42–89 years old. Among 12 patients recruited at VA Hospital, six patients each were OPC and OC. Among Kansas University Medical Center participants, fifteen male patients were in the age group 60–79 and fifteen females were in the age group 51–71). When the cut off ELISA reading of salivary cell samples absorbance at > 0.5 at 450 nm/mL was used as HPV positive readout, 8 samples out of 12 were HPV positive, 4 each of OPC and OC. One out of 12 non-cancer samples were also HPV positive. High percentage of HPV positive in OPC cancer patients in this study is not surprising. Data reported here is from Phase I clinical trial. In Phase IIA clinical studies conducted at UCLA/VA, in a cohort of 24 patients, there were 11 oropharyngeal cancers and 10 were HPV16 positive (91%). Of the 13 oral cavity cancers, 4 were HPV16 positive[55]. High prevalence of HPV in OPC (> 90%) has been reported in several previous studies[56]. As mentioned above, ELISA detected HPV L1 surface protein in six different HPV types. Therefore, HPV positive patients include both high risk and lower risk HPV types. However, HPV prevalence is > 90% in OPC patients, irrespective of the diagnostic method used (PCR or ELISA).

The bean gum extracts aggregated HPV in ~ 93% HNSCC patient samples (13 out of 14 evaluated patient saliva samples) (Fig. 3B). Among the bean gum treated samples > 85% HPV aggregation was observed in 80% patients (in 5 patients) 100% aggregation observed while in other 5 patients aggregation observed in the range of 86–99%. The HPV aggregation of 61% was observed in one patient while < 50% aggregation was observed in two patients. Viral aggregation efficacy is based on viral count and density. Overall, consistent HPV reduction

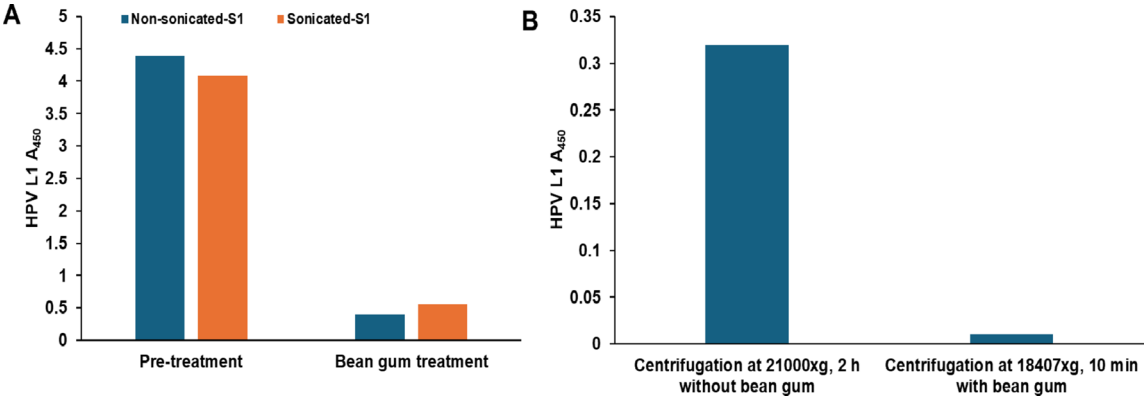

Fig. 2. Optimization of HPV quantitation and viral aggregation by ELISA. (**A**) Effect of sonication on the release of HPV in saliva sample of cancer patient S1 analyzed by ELISA. Sonication was done by using probe sonicator (2 cycles, 10 s on, 15 s off at 80% amplitude). (**B**) Effect of centrifugation to precipitate HPV of cancer patient oral rinse sample (O1) analyzed in the presence and absence of bean gum extract. Cancer sample HPV incubated with bean gum was centrifuged at 18,407xg for 10 min. While cancer sample HPV in absence of bean gum, centrifuged at 21,000xg for 2 h. Presence of HPV in the supernatant was evaluated by ELISA using multivalent antibody that binds to surface protein L1 of HPV. Bean gum extracted at 666 mg chewing gum / mL concentration in buffer (100 mM NaCl, 200 mM Tris-Cl (pH-8.0), 400 mM Sucrose, 2 mM PMSF and 0.5x Protease Inhibitor Cocktail). For evaluation of sonication and centrifugation effects, bean gum stored at ambient temperature for 583 and 575 days, respectively was used.

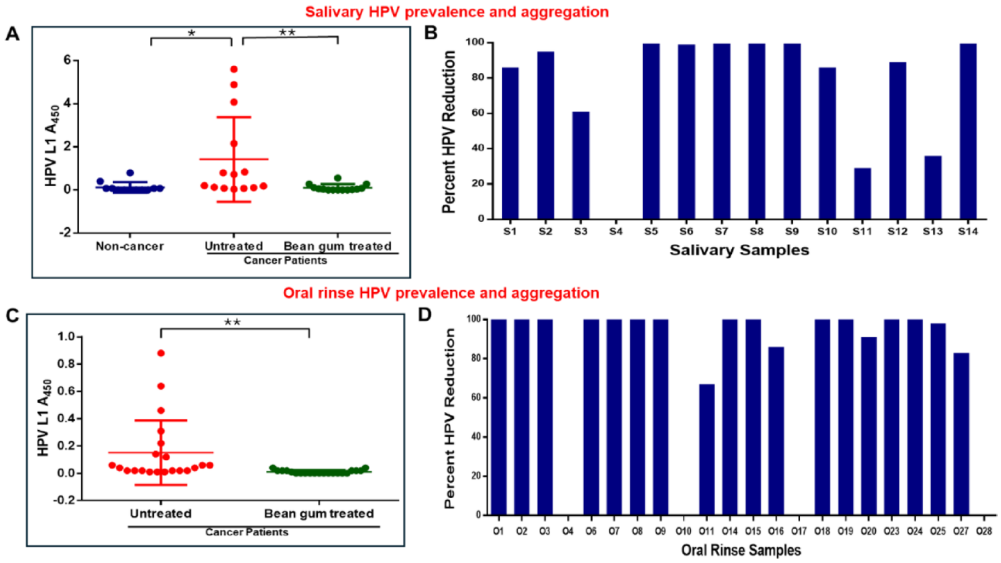

Fig. 3. Effect of bean gum on HPV in saliva and oral rinse samples of cancer patients. (**A**) Comparison of HPV prevalence in saliva samples of non-cancer ($n = 12$) and cancer patients ($n = 14$), and HPV aggregation after bean gum treatment. (**B**) Percent HPV reduction after bean gum treatment in saliva samples of cancer patients. (**C**) Comparison of HPV prevalence in untreated and bean gum treated group of oral rinse HPV positive cancer samples ($n = 21$). (**D**) Percent HPV reduction in oral rinse samples of HPV positive cancer patients after bean gum treatment ($n = 22$). In HPV aggregation assay, 665 mg bean gum/mL was extracted in buffer (100 mM NaCl, 200 mM Tris-Cl (pH-8.0), 400 mM Sucrose, 2 mM PMSF and 0.5x Protease Inhibitor Cocktail) and cancer patient oral rinse or saliva samples were incubated for 1 h. After centrifugation at 18,407xg for 10 min viruses in the supernatant were detected by ELISA. ELISA was performed with multivalent antibody against major surface protein L1 of HPV type 1, 6, 11, 16, 18 and 31. Significance was determined by t-test ($^{**}p \leq 0.010$; $^{*}p \leq 0.050$).

was observed in the saliva samples treated with bean gum. patients at the University of Kansas Medical Center (KUMC). ELISA assay detected major surface L1 protein of HPV by using multivalent antibody. Among 30 HNSCC patient oral rinse samples, ~ 75% were HPV positive (Fig. 3C). The bean gum extract aggregated ~ 80% of HPV positive cohort that was in the range of 67 to 100% (Fig. 3D). Interestingly, bean gum was able to

aggregate 100% of HPV in 13 oral rinse cancer samples among 22 HPV positive cohort. Again, viral aggregation efficacy is based on viral count and density. Overall, compared to untreated samples HPV was significantly decreased (**$p \leq 0.010$) by the treatment of bean gum extract (Fig. 3C).

Optimization of bean gum extract in Fn and Pg cultures

We evaluated the effects of different concentrations of bean gum extract (83.3, 166.6, and 333.3 mg/mL) on Pg and Fn using ATCC strains. Additionally, the impact of initial cell density (10^5 and 10^6 CFU/mL) on Fn was assessed with different bean gum concentrations. The maximum reduction observed for both strains was 1 log at 166.6 mg/mL. No bactericidal effect was observed at any concentration of bean gum extract (Fig. 4A). Initial cell density had a marginal inhibitory effect on Fn. Interestingly, the 333.3 mg/mL concentration of bean gum extract induced growth in both bacteria (Fig. 4A). Similar results were observed for Pg. Bean gum extract alone reduced Pg counts from 10^4 to 10^3 CFU/mL on blood agar plates, indicating one log reduction (Fig. 4B). The slight increase in total viable counts for Fn and Pg at higher bean gum concentrations could be due to free sugars or unknown components in lablab bean powder promoting growth.

FRIL, a novel lectin in the bean gum extract, has known antiviral efficacy against various viruses, including flu, coronavirus and HSVs[6,7,57] but antibacterial effect has not yet been investigated. Due to the complex cell wall or capsular exopolysaccharide matrix of Pg and Fn, a combination treatment approach using bean gum extract with protegrin-1 was evaluated in clinical samples from oral cancer patients or non-cancer volunteers.

Optimization of potegrin-1 with bean gum extract in non-cancer clinical samples

Human clinical samples positive for Pg and Fn, as determined by PCR, were obtained from OralDNA labs. High-density samples were selected to optimize an antimicrobial peptide cocktail containing bean gum and protegrin-1. The total viable counts of Pg and Fn were analyzed for samples treated with two different concentrations of protegrin-1 (50 and 100 µg/mL) along with 166.6 mg/mL bean gum extract for 1 h at 37 °C. Both untreated (negative control) and treated samples were plated using the selective agar plate method, and viable counts were measured. The percentage kill was determined for the two different concentrations of protegrin-1 combined with bean gum extract. The combination treatment of 50 µg/mL protegrin-1 and 166.6 mg/mL bean gum extract reduced Pg and Fn counts by 2–3 logs, whereas 100 µg/mL protegrin-1 resulted in complete elimination of Pg and Fn (4–5 log reduction) (Fig. 4C). Consequently, this dose was selected to assess antibacterial efficacy for oral cancer samples.

Antibacterial effect of bean gum + protegrin – 1 in cancer samples

The antibacterial effect of bean gum+ protegrin – 1 was evaluated by incubating the oral cancer samples with antimicrobial peptide cocktail for 1 h at 37 °C and counting colony forming units for both saliva and oral rinse samples on different screening media before and after treatment. P. GING agar (Pg), Crystal Violet Erythromycin Agar (Fn), StrepA Agar (*Streptococcus* spp.), Bacteroides Bile Esculin Agar (BBE) for *Bacteroides* spp., CHROMagar™ for Candida spp, CHROMagar™ Campylobacter and Brucella agar for *Leptotrichia buccalis* were used for microbiological evaluation of clinical samples[58,59].

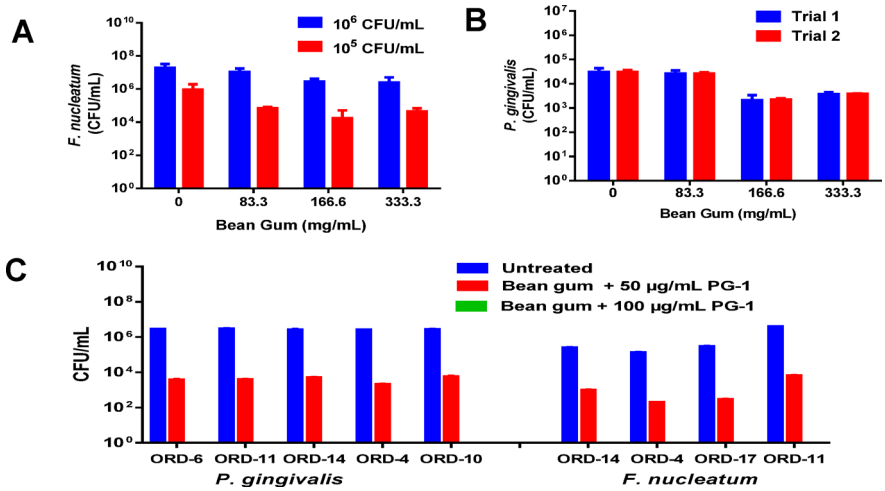

Fig. 4. Optimization of bean gum and protegrin-1 concentrations for growth inhibition of Fn and Pg. Reduction in total viable counts (CFU/mL) of (**A**) Fn (ATCC 25586) and (**B**) Pg (ATCC 33277) for different initial cell densities treated with bean gum conc. (83.3, 166.6 and 133.3 mg/mL) with respect to untreated controls (without bean gum) at 37 °C for 1 h. (**C**) Reduction in total viable counts of Fn and Pg in oral rinse samples treated with bean gum (166.6 mg/mL) along with protegrin-1 (50 and 100 µg/mL) at 37 °C for 60 min. Non-cancer oral rinse samples used in this study were obtained from OralDNA Labs. The colony-forming units for samples treated with bean gum + 100 µg/mL protegrin-1 were completely inhibited (i.e., zero), therefore the bar is not visible on the graph.

Effect of bean gum + protegrin-1 treatment on Pg in HNSCC patients

Saliva from non-cancer control patients showed 10^3–10^5 CFU/ml of Pg (Supplementary Table S1) while cancer patients showed 1000-fold higher counts (10^6–10^8 CFU/mL) compared to non-cancer controls (Fig. 5A; Supplementary Table S2). Non-cancer oral rinse samples also showed significantly reduced (1000-fold less) counts of Pg (10^2–10^3/mL) than cancer patients (10^4–10^6/mL) (Fig. 5B; Supplementary Table S3). Oral rinse samples showed comparatively lower counts of 10^4 – 10^6 CFU/mL of Pg than saliva samples (Fig. 5A, B). Within 72 h of anerobic incubation both saliva and oral rinse samples of cancer patients showed white to grey colonies of Pg on P. GING agar (Fig. 5C, D). The frequency of Pg in saliva and oral rinse samples was 87.5% and 83.3% respectively. Periodontal disease was not examined or recorded in cancer patients in the medical clinic. Therefore, an increase in P. gingivalis due to periodontal disease couldn't be ruled out. We observed a significant 4 to 6 log reduction (99.97–100%) (****p-value < 0.0001) in Pg (Fig. 5) with 166.6 mg/mL bean gum and 100 µg/mL protegrin – 1 in all saliva and oral rinse samples from cancer patients ($n = 39$) (Supplementary Table S2, S4).

Effect of bean gum + protegrin-1 treatment on Fn in HNSCC patients

Saliva samples of non-cancer controls exhibited 10^3 – 10^4 CFU/mL for Fn (Fig. 6A; Supplementary Table S5) while cancer patients showed total viable counts as high as 10^7/mL (Supplementary Table S6). Similarly, non-cancer oral rinse samples showed significantly lower counts (10^3 – 10^4 CFU/mL) than that of cancer patients' samples (10^6 CFU/mL, Fig. 6B; Supplementary Table S7). We observed a significant 4 to 6 log reduction which is approximately 99.97–100% kill (**** p-value < 0.0001) for Fn (Fig. 6A, B) with bean gum + protegrin-1 in both oral rinse and saliva clinical sample cohorts ($n = 40$) (Supplementary Table S6, S8). Fn formed grey to blue colonies on CVE agar within 72 h of anaerobic incubation for saliva and oral rinse samples (Fig. 6C, D). The frequency of Fn in saliva and oral rinse samples analyzed was 100% and 80% respectively.

Effect of bean gum+ protegrin-1 against HNSCC associated pathogens

In this project, bacteria with minimal information on their mechanism of initiation of HNSCC (binding proteins, host responses) are considered cancer associated pathogens. Non-cancer participants (control) saliva samples showed 10^4–10^7 CFU/mL whereas all cancer patients showed 1000-fold higher counts (10^6 – 10^9 /

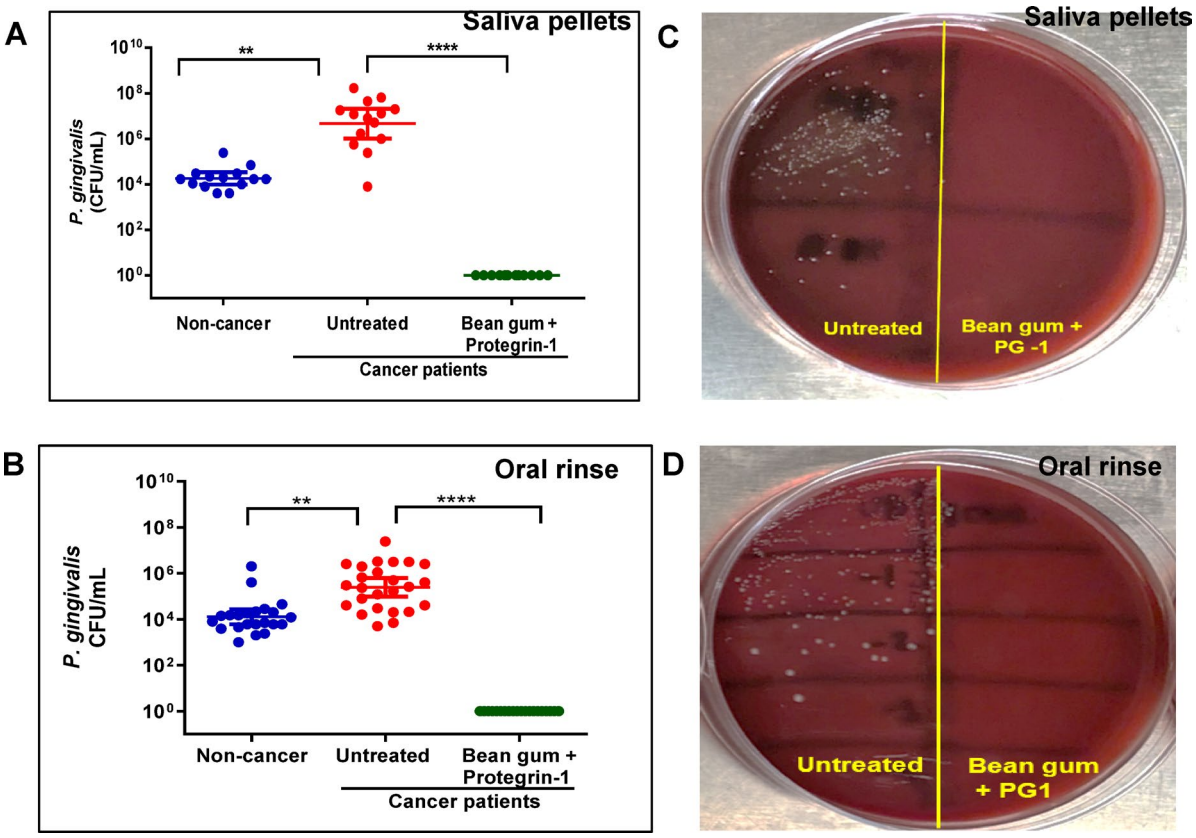

Fig. 5. Effect of bean gum+PG1 on Pg in saliva and oral rinse samples of HNSCC patients. Total viable counts of Pg in (**A**) Saliva non cancer ($n = 14$), untreated cancer ($n = 16$) and treated cancer samples ($n = 16$) (**B**) Oral rinse non-cancer ($n = 22$), untreated cancer ($n = 30$), and treated cancer samples ($n = 30$). Significant reduction in Pg counts observed after 1 h of bean gum + PG1 treatment (166.6 mg/mL + 100 µg/mL) at 37 °C (****$p < 0.0001$; One way ANOVA). (**C**, **D**) Morphology of Pg after anaerobic incubation for 72 h at 37 °C on PGING agar plate. Bean gum extract used in these assays was prepared from the bean gum tablet stored for 33 months, 23 days at USP controlled temperature (20–25 °C).

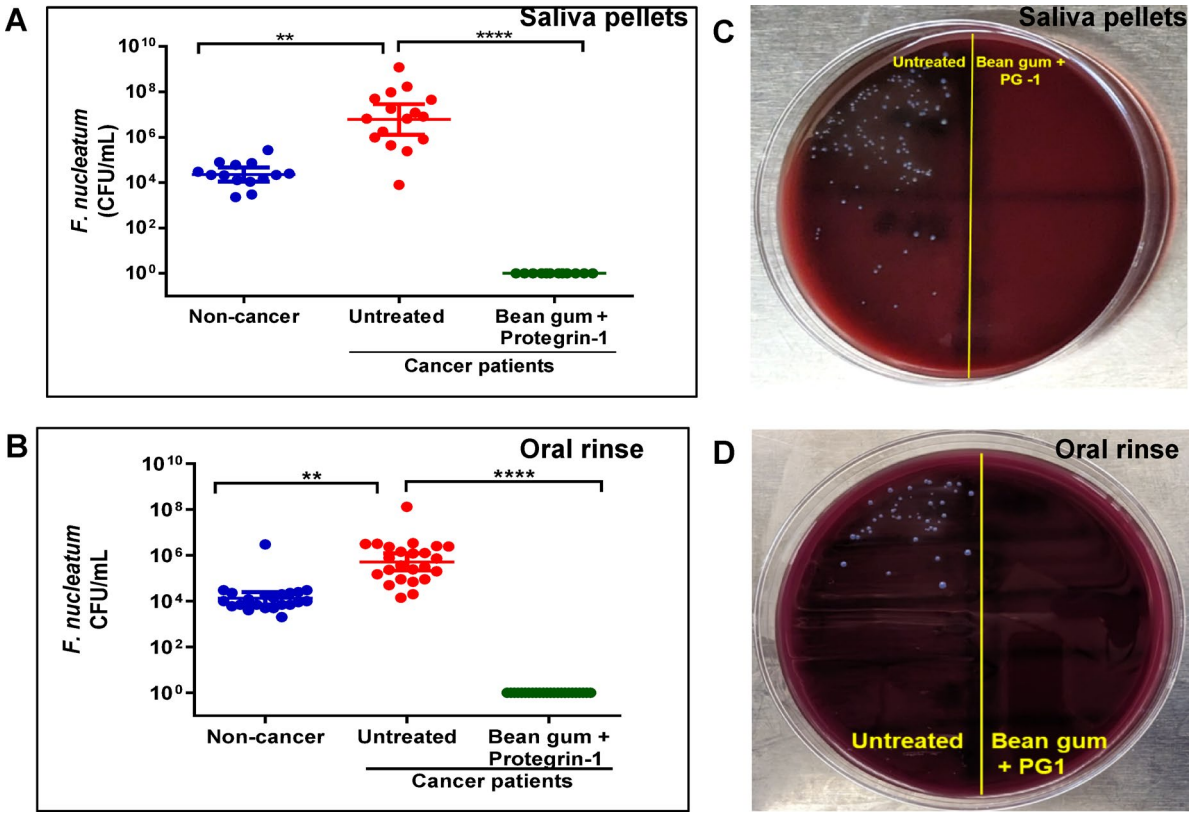

Fig. 6. Effect of bean gum+PG1 on Fn in saliva and oral rinse samples of HNSCC patients. Total viable counts of Fn in (**A**) Saliva non cancer ($n = 14$), untreated cancer ($n = 16$) and treated cancer samples ($n = 16$) (**B**) Oral rinse non-cancer ($n = 22$), untreated cancer ($n = 30$), and treated cancer samples ($n = 30$).Significant reduction in Fn counts observed after 1 h of bean gum + PG1 treatment (166.6 mg/mL + 100 µg/mL) at 37 °C (****$p < 0.0001$; One way ANOVA). (**C**, **D**) Morphology of Fn after anaerobic incubation for 72 h at 37 °C on Crystal Violet Erythromycin (CVE) Agar plate. Bean gum extract used in these assays was prepared from bean gum tablet stored for 33 months, 23 days at USP controlled temperature (20–25 °C).

mL) of *Streptococci* spp. on StrepA agar plates (Supplementary Table S9, S10). Non-cancer oral rinse samples showed 10^5–10^7/mL while cancer patient oral rinse samples showed 10^7 – 10^9/mL total *Streptococci* counts. Both saliva and oral rinse cancer samples exhibited 100% frequency of Streptococci with 100-fold higher counts than their respective non-cancer controls (Supplementary Table S11, S12). Treatment with bean gum + protegrin-1 resulted in 1-2 log or no substantial reduction of *Streptococci* spp. in saliva pellets and oral rinse due to protection conferred by secreted capsule polysaccharides[60,61] (Fig. 7A, B; Supplementary Table S10, S12). We observed an overall low incidence of *Candida albicans* 12.5% in saliva pellets and 10% in oral rinse samples of cancer patients (n=46) while none of the samples from non-cancer cohort ($n = 36$) including saliva pellets and oral rinse showed presence of *Candida albicans* (Fig. 7C, D; Supplementary Table S13, S14, S15, S16). The presence of *Leptotrichia buccalis* was also observed in most of the oral rinse samples of cancer (n= 30) in the range of 10^3 – 10^6 CFU/mL with 97% of frequency. Cancer patients' oral rinse samples showed 100-fold more counts of *Leptotrichia buccalis* as compared to non-cancer participants (Supplementary S17, S18). Bean gum+ protegrin-1 treatment was effective against *Leptotrichia buccalis* exhibiting >99% inhibition (Fig. 7E, F; Supplementary Table S18). We did not observe growth of *Campylobacter* spp. in oral rinse samples of non-cancer participants and cancer patients (Supplementary Table S19, S20).

Discussion

Survival rates of untreated recurrent or metastatic oral cancer after surgery with risk-adjusted adjuvant therapies continue to be low, worsened by high abundance of Pg and Fn infections[35–38]. Current treatments focus on dividing cancer cells or reducing tumors, but minimal attention is paid to reduce carcinogenic microbes that initiate cancer or cause recurrence. Indeed, radiation therapy kills dividing salivary gland cells, decreases saliva production, reduces commensal bacteria, increases pathogenic microbes, with long lasting impact far beyond treatment duration[38,62]. Therefore, in this ex vivo clinical study, we evaluated efficacy of the bean gum + protegrin-1 in cancer patient samples.

Bean powder contains FRIL and is Generally Regarded as Safe (GRAS notice No GRN 000879, 2020), issued based on toxicology reports submitted[63]. The dose of Lablab bean powder in the bean chewing gum is 79 mg, when compared to 500 g ingested in human clinical toxicology evaluation[64]. This is 6,330-fold less than what

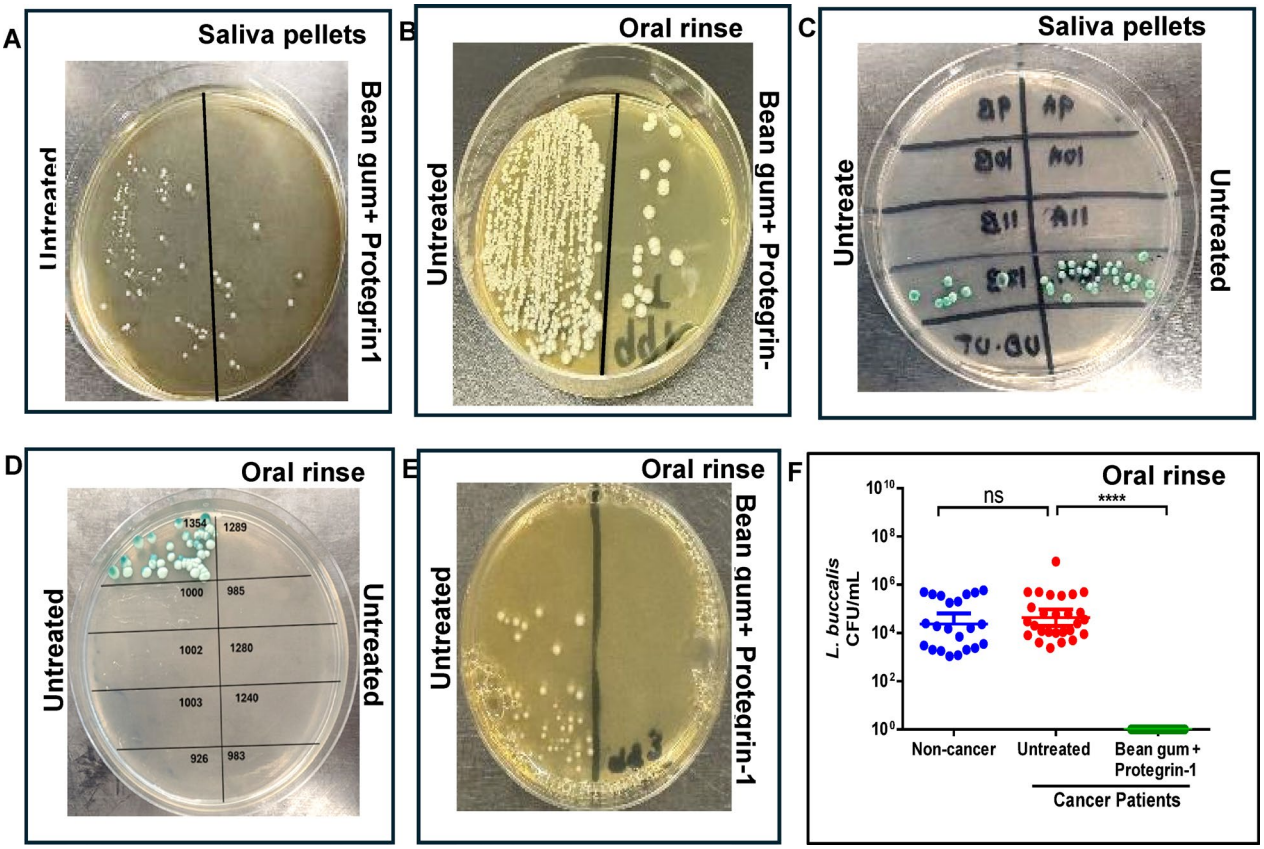

Fig. 7. HNSCC associated microbes in saliva (n= 14 non-cancer; n = 16 cancer samples) and oral rinse (n=22 non cancer, n=30 cancer samples) of HNSCC patients and non-cancer cohort. (**A**, **B**) Colony morphology of Streptococci on StrepA agar before and after treatment with bean gum + protegrin-1 for saliva pellets and oral rinse samples of HNSCC patients. (**C**, **D**) Colony morphology of *Candida albicans* on CHROMagar™ Candida agar from saliva pellets and HNSCC patients. (**E**) Colony morphology of *Leptotrichia buccalis* cultured from HNSCC oral rinse samples on brucella agar after incubation at 37 °C for 72 h. (**F**) Colony counts of *Leptotrichia buccalis* before and after treatment with bean gum + PG1 at 37 °C (****$p < 0.0001$; One way ANOVA). Bean gum extract used in these assays was prepared from bean gum tablet stored for 33 months, 23 days at USP controlled temperature (20–25 °C).

would be consumed as food. Chewing gum has evolved over 9000 years, transitioning from chewing resin and sap directly from trees to medicated gums containing aspirin or nicotine or vitamins[3]. We have recently reported preparation and characterization of clinical grade chewing gum tablets, efficacy in neutralization of H1N1, H3N2, HSV-1, HSV-2 > 95% (1,000 copies of virus per ml, typical viral load observed in the oral cavity). FRIL in the bean chewing gum is stable and maintains antiviral efficacy for up to 823 days when stored at ambient temperature[57]. Composition of the chewing gum with antiviral protein has been submitted to FDA (IND 154897) and approved for evaluation of Coronavirus infection and transmission (NCT05433181).

Several lines of evidence support antiviral and antibacterial efficacy of bean gum+protegrin-1 in the current study. Single bean gum application decreased HPV viral load in untreated salivary samples 86–95% and results were highly significant (p-value < 0.0001). However, only the viral aggregation effect of FRIL is measured by ELISA but not total blockage of HPV at the endosomes by FRIL in host cells[6,7]. The dosage of FRIL to completely neutralize several oral viruses like H1N1, H3N2, HSV-1, HSV-2 present in a single gum tablet is > 40-fold higher in each gum treatment based on plaque reduction assays that measures both viral aggregation and endosome blockage after entering human cells[6,57]. Therefore, chewing 3–4 gum tablets per day in the clinic is anticipated to be more than sufficient to neutralize HPV. Unlike most oral microbiome studies that rely on PCR and DNA sequencing to quantify abundance, our study evaluated microbial density by directly culturing them in suitable growth media. Pg and Fn were 100-1000-fold higher in saliva and oral rinse cancer samples. Oral rinse samples showed lower frequency of both Pg and Fn than saliva due to sample dilution. Despite the difference in sample types, a single dose of bean gum+ protegrin-1 reduced Pg and Fn > 99% with minimal impact on non-pathogenic commensal bacteria. Notably, clinical samples containing *Streptococci* spp. were not killed completely by bean gum+protegrin-1, indicating a targeted elimination of anaerobic bacteria associated with HNSCC, unlike radiation therapy that reduces commensal and increases pathogenic bacteria[62]. Resistance of certain Streptococcus species is conferred by their protection through capsules[60,61] or biofilm[3,10,65] secreted by them. We have previously shown that enzymatic digestion of biofilm is needed to kill S. mutans protected

by biofilm[10,65]. Therefore, killing of pathogenic Streptococcal species would require additional treatments while nonpathogenic commensal Streptococci are not affected by protegrin treatment because they are protected by capsules. We observed an overall low incidence of *Candida albicans* 12.5% in saliva pellets and 10% in oral rinse samples of cancer patients (n = 46) which could be treated by antifungal gum containing enzymes to degrade the cell wall[66,67]. *Leptotrichia buccalis* and *Campylobacter* spp. have been associated with the development of premalignant oral disorders like oral leukoplakia and oral lichen planus by producing harmful metabolites[68]. These bacteria can contribute to oral dysbiosis of the oral microbiome, leading to chronic inflammation and cancer formation[69,70].

HPV and anaerobic bacteria are not only present outside the cell but are also present within tumor cells. HPV viral genome initially exists in episomal form but subsequently integrates into the host genome causing genome instability and genic alteration which facilitate mutation and hyperproliferation[23,24]. However, FRIL enters host cells and blocks viruses at the endosome level, thereby blocking virus integration into the host cell genome[7]. Similarly, Pg and Fn are present within tumor cells[25–30]. We have previously shown that protegrin enters human head and neck squamous cell carcinoma cells[10]. Therefore, prolonged local delivery of FRIL+ protegrin-1 via chewing gum should not only decrease HPV, Pg and Fn on the surface but could also help control them after their entry into cancer cells.

There are several limitations to the current study. This is an ex vivo study using saliva or oral rinse samples collected from HNSCC patients and this is not a human clinical study to monitor long-term changes in oral microbiome. Future clinical studies will provide such valuable data. Current study evaluated saliva or oral rinse samples from cancer patients, but future studies will be conducted in tumor cells, especially to evaluate penetration of FRIL and protegrin into tumor cells and kill HPV or pathogenic bacteria.

Globally, cancer is the second leading cause of premature death with an estimated cost of $25 trillion between 2020 and 2050[39]. GLOBOCAN epidemiology data reported 98,412 new OPC cases and 48,143 OPC deaths estimated worldwide in 2020[71]. Lip and oral cavity cancer was the 7th leading cancer type in cancer incidence and mortality rate worldwide in adolescents, young adults, middle aged adults, and working adults in 2022[39]. Unfortunately, most recent cancer drugs granted accelerated approval by FDA did not significantly improve overall survival or quality of life within five years[41], underscoring the need for adjuvant therapies to improve survival. Systematic review and meta-analysis reveal that HPV is associated with significant overall survival, confirming that HPV viral load in the oral cavity is an adverse prognostic factor in HNSCC[35]. Increase in HNSCC cases in the United States and globally is linked with HPV-16 and increasing popularity of oral sex[23]. Prognostic impact studies of oral microbiome on survival of malignancies reveal that high abundance of Fn and Pg in cancer tissues is associated with poor overall survival in multivariate analysis[37,38]. Therefore, new approaches are needed to control pathogenic HPV and anaerobic bacteria associated with HNSCC.

Conclusions

In this ex vivo clinical study we quantified pathogenic live bacteria/yeast (by culturing) or virions (by ELISA) and developed affordable therapeutic approaches to reduce their load to attenuate HNSCC initiation or recurrence. Therapeutic combination of antiviral and antimicrobial proteins delivered using chewing gums is very effective in neutralizing HPV, Pg and Fn, with minimal impact on healthy oral microbiome. These results augur well for further clinical evaluation of bean gum+ protegrin-1 as adjuvants with current treatments to reduce carcinogenic microbes that initiate proliferation or promote tumor progression.

Materials and methods

Salivary and oral rinse sample collection

Salivary samples (5 to 6mL) were collected from non-cancer and cancer patients who visited/treated at VAGLAHS, Los Angeles, California, with institutional IRB approval. The samples included both oral cancers of the tongue and oropharyngeal cancers of the pharynx and tonsil. Informed consent approval was obtained from all research non-cancer and cancer patients. Samples were spun at 2,500xg for 20 min at 4 °C to collect cells and supernatants. Aliquots were frozen at -80 °C freezer. Cells (pellet fraction) of salivary samples were used to detect HPV and aggregation assays.

The Institutional Review Board at the University of Kansas Medical Center approved the acquisition of oral rinse samples after reviewing compliance with ethical standards. Samples collected from HNSCC patients and cancer-free participants, were deidentified after obtaining written informed consent. Oral cancer patients or cancer-free participants were asked to rinse their oral cavity with 10 ml sterile isotonic saline for 30 s. The oral rinse samples were collected, aliquoted in 1 ml and stored at -80 °C.

All methods were performed in accordance with the relevant guidelines and regulations.

Bean chewing gum extract Preparation

Bean gum tablets were manufactured by Per Os Biosciences and components, dosage, antiviral efficacy, potency and stability have been reported previously (57). Bean gum tablets were stored in FDA compliant black Uline containers at USP controlled temperature (20 –25 °C). Bean chewing gum extracts were prepared by suspending crushed gum powder of 666 mg in 1 mL extraction buffer (100 mM NaCl, 200 mM Tris-Cl (pH-8.0), 400 mM Sucrose, 2 mM PMSF and 0.5x Protease Inhibitor Cocktail) as described previously[52]. After incubation at 4 °C for 1 h on vortexer (homogenizer) for homogenization of suspension 9 cycles of sonication was done (10 s on, 15 s off, 80% amplitude) using probe sonicator. Sonicated suspension was spun at 750xg for 5 min and collected supernatant used in aggregation assays of HPV in salivary and oral rinse sample of cancer patients. Bean gums used in HPV aggregation assays of salivary and oral rinse cancer samples were stored at ambient temperature up to 657 days. In bean gum extraction procedure ratio of bean gum powder and buffer was selected on the basis

of optimum aggregation results obtained in lab for previous aggregation assays done for several different types of viruses[6,57].

HPV detection ELISA assay

In HPV detection ELISA assay, 50 to 100 µL (based on availability) salivary or oral rinse samples of non-cancer and HNSCC patients were coated into wells of microtiter plate (Corning Incorporated, Costar Assay Plate 3590, NY, USA) and incubated for 4 h at RT. For oral rinse samples supernatant and pellet suspension after centrifugation at 21,000xg for 10 min was coated in separate wells. After three washes with PBST (Phosphate-buffered saline + 0.05% Tween-20), blocking was done with 200 µL of 5% non-fat dry milk in PBST overnight at 4 °C. Wells washed three times and incubated with multivalent anti-papillomavirus antibody (Millipore, MA, USA) specific to viral surface protein L1 of HPV type 1, 6, 11, 16, 18 and 31 in 1:100 dilution. After incubating for 2 h at RT, plate was washed three times and incubated with secondary antibody (goat anti-mouse IgG, ads-HRP, Southern Biotech, AL, USA) added and plate incubated for 2 h at RT. After three washes with PBST, 100 µL of substrate (TMB/E Solution, Mllipore, MA, USA) added and incubate for 30–45 min at RT for color development. The reaction was stopped by adding 50 µL of 2 N H_2SO_4, and absorbance measured at 450 and 700 nm on a microplate reader (BioTek, Synergy H1, VT USA). After subtracting the absorbance of respective controls, obtained data was analyzed for the presence of virus in the tested salivary or oral rinse samples. HPV detection ELISA assays used to determine presence of HPV of oral rinse sample in the supernatant or pellet suspension after centrifugation at 21,000xg for 10 min. The detection assays were also used to evaluate and compare the presence of HPV in non-sonicated and sonicated salivary sample of HNSCC patients. Sonication of cancer sample was done by using probe sonicator (2 cycles, 10 s on, 15 s off at 80% amplitude).

HPV aggregation assay and detection of HPV in supernatant fraction

In HPV aggregation assay, supernatant of bean gum extract incubated with salivary or oral rinse samples HPV in presence of 100 mM $MnCl_2 4H_2O$ for 1 h at 37 °C with intermittent mixing. Supernatant of bean gum extract without virus and virus without bean gum supernatant used separately as control. Incubated samples spun at 18,407xg for 10 min, supernatant collected and evaluated for the presence of HPV by the HPV detection ELISA assay using antibody against surface protein L1 of HPV as described previously. During the aggregation assay optimization process, we investigated the effect of centrifugation to precipitate HPV of HNSCC oral rinse samples in presence and absence of bean gum extract. HPV incubated with bean gum extract aggregated and precipitated in pellet fraction after centrifugation at 18,407xg for 10 min. While in absence of bean gum extract, centrifugation of oral rinse HNSCC samples HPV even centrifugation at 21,000xg for 2 h was insufficient to precipitate in the pellet fraction.

Reagents of Microbiological investigations

Commercial Protegrin-1 1 mg (Cat. Number: AS-64819-1) was purchased from Anaspec (Fremont, CA 94555, USA). BD BBL™ Prepared Plated Media: Trypticase™ Soy Agar (TSA II™) with Sheep Blood, purchased from Fisher Scientific and used for assessing aerobic and anaerobic microbial counts in clinical samples. Crystal Violet Erythromycin Agar (CVE -AS 647), P. GING Agar (AS-6422), BBE (AS-144) were purchased from Anaerbe systems (15906 Concord Circle Morgan Hill, CA 95037). Dehydrated media CHROMagar™ Candida, dehydrated culture media, 5000 mL reconstituted and Chromagar™ Strep A 5000Ml (50-194-7651) were purchased from DRG International Inc.

Optimization of bean gum for *P. gingivalis* and *F. nucleatum*

The effects of different concentration bean gum extract was analyzed on pure cultures of *P. gingivalis* (ATCC 33277) and *F. nucleatum* (ATCC 25586). Four to five colonies were picked up from fresh plate of *P. gingivalis* and *F. nucleatum* and transferred to 2 mL BHI broth with Hemin and grown anaerobically at 37 °C until 1.0 OD_{660} is reached. *F. nucleatum* culture suspension was diluted aseptically using sterile PBS and adjusted to get the final counts 10^5 and 10^6 CFU/mL. To the diluted suspension bean gum extract was added to get final concentrations (83.3, 166.6 and 333.3 mg/mL) and mixture was incubated for 60 min at 37 °C. Untreated control was prepared by adding sterile PBS solution instead of bean gum extract. After treatment, suspensions were aseptically inoculated onto plate blood agar plates. Plates were incubated for 72 h. Counts were recorded for all plates of different concentrations. Reduction in the counts for *F. nucleatum* was evaluated with respect to negative control plate without bean gum addition. The effect of bean gum on *P. gingivalis* was evaluated at 10^3 CFU/mL initial cell density as per procedure mentioned for *F. nucleatum*.

Optimization of protegrin-1 concentration for saliva and oral rinse samples

Two different concentrations of protegrin-1 viz.; 50 and 100 µg/mL were used along with 166.6 mg/mL bean gum extract to evaluate antibacterial effect in non-cancer oral rinse controls. One ml of non-cancer control oral rinse sample was thawed from −80 °C. and aliquoted aseptically. Aliquot containing 25 µL oral rinse sample was mixed with 25 µL cocktail of bean gum and protegrin-1 to achieve final concentrations; 166.6 mg/mL bean gum + 50 µg/mL and 166.6 mg/mL bean gum + 100 µg/mL. Untreated control was prepared by adding 25 µL of sterile phosphate buffer saline to 25 µL of oral rinse sample. All samples including untreated controls were incubated at 37 °C for 1 h in shaker incubator. After treatment, 10 µL of samples was aseptically inoculated on Crystal Violet Erythromycin Agar (CVE -AS 647) and P. GING Agar (AS-6422) and incubated for 72 h. Total viable counts were recorded for all plates. Reduction in total viable count was calculated by comparing CFU of treated plates with untreated controls. The dose of bean gum combined with protegrin-1 that demonstrated the maximum reduction in total viable counts for *P. gingivalis* and *F. nucleatum* was chosen for cancer sample analysis.

Bean gum + protegrin – 1 treatment for saliva and oral rinse

Frozen saliva samples of cancer and non-cancer controls were shipped to School of dental medicine, University of Pennsylvania through an overnight shipment. Upon arrival, all samples were checked and preserved at – 80 °C until use. For microbiological assessment, saliva sample and oral rinse sample were removed from the – 80 °C freezer and thawed immediately in pre-disinfected laminar air flow workstation. A 100 µL of sample was divided into two sets 50 µL each aseptically (one aliquot for microbial assessment and the other for HPV assessment). The remaining samples were stored at – 80 °C freezer for future investigations. A 50 µL of sample was serially diluted using sterile phosphate buffered saline (PBS). Appropriate dilution was taken for treatment using bean gum and protegrin-1. The same method was followed for oral rinse samples. An equal amount of saliva and oral rinse sample was divided into 2 Eppendorf tubes. One tube was used as untreated control and the other tube was used for treatment. Total reaction mixture of 50 µL was prepared containing Protegrin-1 at 100 µg/mL and bean gum extract 166.6 mg/mL for treatment. To the untreated control tube, equal amount of sterile PBS was added. Both tubes were incubated at 37 °C shaker, for 1 h. After incubation, 10 µl of sample was inoculated on 2 blood agar plates (aerobic and anaerobic incubation), 1 Crystal Violet Erythromycin (CVE) agar plate (detection of *F. nucleatum*), 1 P.GING Agar (detection of *P. gingivalis*), 1 Candida CHROMagar™ (detection of *Candida albicans* and non-albicans spp.), 1 CHROMagar™ Strep A Agar (detection of Streptococcus spp.), 1 Brucella agar, 1 CHROMagar™ Campylobacter and lastly 1 Bile Esculin (BBE) Agar (detection of Bacteroides spp.). Plates of Candida, CHROMagar™, Strep A and blood agar were incubated aerobically at 37 °C for 72 h. While P. GING, CVE, CHROMagar™ Campylobacter and BBE along with corresponding blood agar plates were incubated in anaerobic chamber at 37 °C for 72 h[58,59]. Colony counts for all plates were noted after 72 h and reduction in CFU/mL was calculated to evaluate efficacy of the treatment. The same protocol was followed for all samples.

Statistical analysis and reproducibility

Data analysis was performed using GraphPad prism using student -T test with Welch's correction and ANOVA. To ensure reproducibility of the results, all experiments were performed in replicates as outlined in the respective methods.

Data availability

All data generated and statistical analysis are included in the written text, figures in the main text and in supplementary tables.

Code availability

No unique code is generated or used in this study.

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Author contributions

HD conceived the project on neutralization of pathogenic oral viruses and bacteria using bean gum and protegrin-1, designed experiments, interpreted data, wrote several versions of this manuscript and obtained funding. RS, GW, SN prepared Figure 1 on mechanistic studies from published results and wrote that section. RS performed HPV ELISA experiments analyzed, and interpreted data presented in Figs. 2 and 3 and wrote corresponding sections. GW and SN performed Pg, Fn *,* non-targeted microbial investigations, analyzed, and interpreted data presented in Figs. 4, 5, 6 and 7 and wrote corresponding sections. AB and SMT provided oral rinse samples and edited the manuscript. RS, GW, SN and SMT wrote methods section for respective experiments. ESS, MBW and SKB provided saliva samples from cancer patients and edited the manuscript. All authors reviewed this manuscript.

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Declarations

Competing interests

HD is a patentee in plant-based oral and topical drug delivery and was previously funded by Novo Nordisk, Bayer, Shire and Takeda. List of awarded and published patents are available in Google Scholar and Scholar GPS links below. However, there is no specific financial conflict related to this manuscript. All other authors declare that they have no competing interests. Scholar GPS: https://scholargps.com/scholars/82094026790000/henry-daniellGoogle Scholar: http://scholar.google.com/citations?user=7sow4jwAAAAJhl=en.

Ethical approval and consent to participate

Salivary samples from non-cancer and oral cancer patients were collected after obtaining approval from the IRB committee of the Veterans Administration of Greater Los Angeles Healthcare System (VAGLAHS), Los Angeles, California (IRB study #1615727). Informed consent approval was obtained from all cancer and non-cancer volunteers. The IRB at the University of Kansas School of Medicine approved the study (IRB# STUDY00001732). Informed consent was obtained from patients prior to commencement of all study activities. Samples collected from HNSCC patients and cancer-free participants were deidentified after obtaining written consent.

Additional information

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