Vegetarian diet and periodontal status: assessment of clinical measures and gingival fluid inflammatory cytokines

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Abstract

Aim: Vegetarian diets are known to reduce inflammation. We investigated the cross-sectional association of gingival fluid inflammatory cytokines with periodontal status in vegetarians and non-vegetarians.

Methods: A total of 54 systemically healthy subjects were enrolled. Dietary intake was assessed by a food frequency questionnaire. A comprehensive periodontal examination was performed. Gingival fluid was collected, samples were assessed for interleukin 1β (IL-1β), interleukin 6 (IL-6), interleukin 8 (IL-8), tumor necrosis factor-alpha (TNF-α) and interleukin 10 (IL-10).

Results: Twenty-eight subjects were categorized as vegetarian and 26 non-vegetarians. Multivariate analyses adjusting for plaque (PI) and demographics showed that bleeding on probing (BOP) and pocket depth (PD) were the only two clinical measures associated with diet. Diet modified the association between PI and BOP ($p<0.01$), vegetarians showed lesser BOP levels than non-vegetarians. Independent of diet, BOP positively associated with IL-1β ($P<0.05$) and TNF-α ($p<0.01$). Diet also modified the association between PI and PD ($p<0.05$), vegetarians showed shallower PD than non-vegetarians. Vegetarians showed comparable IL-10 levels in both shallow and deep pockets. By contrast, non-vegetarians showed decreased IL-10 levels in deep pockets ($p<0.01$). IL-6 and IL-8 showed no associations with diet.

Conclusions: Vegetarian diet may mitigate the gingival inflammatory response to microbial plaque and enhance the gingival anti-inflammatory activity.

Keywords: Cytokine(s); Inflammation; Periodontal disease(s)/periodontitis; Gingival fluid; Diet; Vegetarian.

Introduction

The link between nutrition and periodontal disease is not well established. A recent systematic review reported "only a possible relationship" of certain vitamins and minerals to periodontal disease (Kulkarni *et al*., 2014). However, existing evidence suggests a plausible role for diet, principally the relationship of periodontitis to a number of diet-related diseases and risk factors. Metabolic syndrome, itself a constellation of cardio-metabolic risk factors, is clearly associated with periodontal disease (Nibali *et al*., 2013) and has been found to exacerbate gingival inflammation and alveolar bone loss (Li *et al*., 2015). Overweight and obesity are associated with increased prevalence of periodontitis (Suvan *et al*., 2011). Furthermore, obesity can cause insulin resistance and endothelial dysfunction in gingival tissues (Mizutani *et al*., 2014). Diabetes type-2 is also strongly related to periodontitis incidence and progression (Lalla *et al*., 2000).

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Vegetarian diets have been associated with reducing the risk for a number of chronic diseases such as those just mentioned that are themselves independently linked to periodontal disease. The Adventist Health Studies at Loma Linda University have investigated the possible health benefits of vegetarian diets, the current Adventist Health Study – 2 (AHS-2) being the largest of those studies (Fraser *et al*., 2015). Vegetarian diets have been found to be associated with lower BMI (Tonstad *et al*., 2009); a lower prevalence and incidence of diabetes type 2 (Tonstad *et al*., 2013); lower blood pressure (Fraser *et al*., 2015); lower prevalence of metabolic syndrome (Rizzo *et al*., 2011); and reduced ischemic heart disease mortality (Key *et al*., 1998).

Inflamed gingival tissues are characterized by increased activity of inflammatory biomarkers such as the pro-inflammatory cytokines IL-1β (Baser *et al*., 2009), TNF-α (Baser *et al*., 2009; Ertugrul *et al*., 2013), IL-8 (Ertugrul *et al*., 2013; Giannopoulou *et al*., 2003), IL-6 (Giannopoulou *et al*., 2003) and the anti-inflammatory cytokine IL-10 (Casarin *et al*., 2010; Gamonal *et al*., 2000). The inflammatory cytokines coordinate the gingival inflammatory response to microbial plaque, direct immune cell function and promote the advancement of inflammation closer to the alveolar bone, ultimately resulting in periodontal tissue loss (Kurgan *et al*., 2018).

The AHS-2 studies have shown that a vegetarian diet lowers the systemic inflammatory burden (Jaceldo-Siegl *et al*., 2018). Several foods in a vegetarian diet like fruits , vegetables, fiber and antioxidants tend to decrease inflammatory marker levels in blood (Oliveira *et al*., 2009). Short-term application of a vegan diet reduces C-Reactive Protein (Sutliffe *et al*., 2015). Furthermore, vegetarian diets score less on the Dietary Inflammatory Index (Turner-McGrievy *et al*., 2015). High fiber in vegetarian diets is fermented by colonic bacteria to produce short-chain fatty acids that induce the release of anti-inflammatory IL-10 (den Besten *et al*., 2013; Glick-Bauer *et al*., 2014). The cited studies suggest that vegetarian diets have the potential to alter the gingival inflammatory response to dentogingival microbial plaque.

Based on the available evidence, it is reasonable to hypothesize that a vegetarian diet would reduce the risk of periodontal disease. The objective of this study was to test the hypothesis that a vegetarian diet would mitigate the gingival inflammatory response to microbial plaque by modifying the expression of five inflammatory markers (pro: IL-1β, IL-6, IL-8, TNF-α, and anti: IL-10) known to be associated with periodontal disease.

Materials and Methods

This cross-sectional study was conducted at Loma Linda University School of Dentistry, Loma Linda, CA from January 2016 to December 2018. The study protocol and consent forms were approved by LLU Institutional Review Board.

Study subject inclusion criteria and recruitment were previously described (Khocht *et al*., 2021). The AHS-2 is a cohort of approximately 96,000 Seventh-day Adventists in the US and Canada, which has been previously described (Fraser *et al*., 2015). Existing AHS-2 participants age 50 years and above within a 50-mile radius of Loma Linda University were selected for possible recruitment. Potential participants were screened by phone for likely eligibility. A final screening for eligibility was done at the in-person visit.

The study included two subject groups, a vegetarian group and a non-vegetarian comparison group. Vegetarians were defined as consuming meat (including red meat, poultry, or fish) never or rarely. Non-vegetarians were defined as consuming non-fish meats 1 time per month or more and all meats combined (fish included) more than 1 time per week. Those with other dietary patterns (e.g. pesco-vegetarians) were excluded. Dietary patterns were initially based on detailed food frequency questionnaires administered between 2002 and 2007 during initial AHS-2 enrollment (Fraser *et al*., 2015). Given the possibility of dietary change, dietary patterns were confirmed by a short questionnaire, and those who no longer met the vegetarian or non-vegetarian dietary pattern definitions were excluded.

Additional exclusion criteria were: history of medical conditions known to impact the periodontium, fewer than 15 teeth present, antibiotic treatment in the past 3 months, dental cleaning in the past 3 months, current use of tobacco products, inability to comprehend or follow instructions in English, and physical limitations interfering with dental examination.

Subject recruitment, screening and enrollment

Since periodontal disease is more prevalent in older age groups, we planned to recruit individuals 50 years and older to maximize the probability of including subjects with periodontitis. A total of 320 potential subjects were selected in a stratified random fashion from the AHS-2 database (balanced on dietary pattern, sex, race, and age). One hundred thirty-six individuals were successfully interviewed by phone to assess eligibility. Fifty-four subjects agreed to participate, fulfilled all entry criteria and were enrolled in the study. Written informed consent was obtained from all subjects.

Assessment of exposure data

For all subjects, a comprehensive medical history interview was performed. Height and weight measurements were recorded and body mass index (BMI) was calculated. Blood pressure was measured in accordance with JNC-7 guidelines. All subjects answered

a questionnaire related to dietary habits, education, income, dental hygiene practice, dental care and past history of cigarette smoking.

Clinical assessment and sample collection

A single experienced dental examiner (LL) performed all dental exams. The examiner was blinded to the patients' dietary habits. The examiner was calibrated and standardized in the use of the clinical evaluation measures employed in the study. The examiner recorded the plaque index (PI) and the gingival index (GI) around all teeth present (Loe, 1967). Third molars, partially erupted teeth and surfaces with large restorations and teeth with crowns were not scored. Caries and missing teeth were also recorded.

A conventional periodontal probe (Michigan-O Probe with Williams markings, Hu-Friedy, Chicago, IL) was used for all probing measurements. Probing depth (PD) was recorded on six sites per tooth, mesiobuccal, buccal, distobuccal, mesiolingual, lingual and distolingual. The position of the gingival margin (GM) to the cementoenamel junction was recorded at the same six sites per tooth. Clinical attachment loss (CAL) was calculated per the formula AL = PD + GM. Interproximal measurements of PD and AL were used to determine periodontal disease status per the Centers for Disease Control criteria (Eke *et al*., 2012).

Gingival fluid sampling and analysis

Gingival crevicular fluid (GCF) was collected by filter paper strips (Oraflow Inc., Smithtown, NY). For the purposes of GCF sampling, non-diseased sites were defined as having PD ≤ 3mm, no bleeding on probing and no loss of attachment; and diseased sites as having PD ≥ 4mm with equivalent or greater attachment loss. In periodontitis subjects 3 interproximal diseased sites and 3 interproximal non-diseased sites were selected. In non- periodontitis subjects 3 interproximal non-diseased sites were selected. All selected sites were chosen at random from teeth in separate quadrants.

The selected sites were isolated, supragingival plaque was removed and the tooth surface air-dried prior to sample collection. The strip was gently inserted into the orifice of the gingival crevice 1 to 2 mm and left for 30 seconds. Sample volume was assessed with Periotron 8000 (Oraflow Inc., Smithtown, NY). The 3 non-diseased samples collected from each subject were pooled together in one microcentrifuge tube; and the 3 diseased samples collected from each periodontitis subject were also pooled together in a second microcentrifuge tube. Samples visibly stained with blood were discarded. Following sample collection, the tubes were sealed, placed on ice and subsequently frozen at -70°C until testing was done.

GCF samples were analyzed for IL-1β, IL-6, IL-8, IL-10 and TNF-α. At the time of analysis, the three strips in each vial were immersed in 1 ml of elution buffer composed of PBS containing 0.1% Triton X-100 and 0.1% bovine serum albumin (BSA) (Aboyoussef *et al*., 1998). The elution process was carried out overnight under refrigeration (Aboyoussef *et al*., 1998). A set of controls for each biomarker (in which a known concentration of each biomarker was added to the filter papers) was used to mimic the elution of the investigated biological factors from the filter paper strips to calculate the percent recovery. The percentage recovery for each of the biomarkers was determined to be at least 98%. The standard curves were generated in the same elution buffer, thus maintaining matrix integrity.

The levels of IL-1β, IL-6, IL-8, IL-10 and TNF-α were determined simultaneously using a Luminex multiplexed panel with magnetic beads (EMD Millipore, Billerica, Massachusetts) coated with specific antibodies (Milliplex MAP Kit). Samples were run in duplicate, according to the manufacturer's instructions. Fluorescence was read using a Luminex 200 instrument (Millipore). Analytes were normalized to total protein concentration, which was determined by Bradford assay (using elution buffer as a baseline), and data expressed in pg/ml.

Statistical analysis

Power analysis, based on estimated attachment levels, indicated that a minimum sample size of 24 subjects per group is required for 90% power to detect a clinically meaningful difference of approximately 1 mm (0.5 standard deviation) in periodontal attachment levels in a t-test at alpha of 0.05.

The distributions of clinical periodontal measures and biomarker levels were positively skewed. We used non-parametric tests for data analyses. Significant differences between dietary pattern groups for continuous measures were determined using the Wilcoxon Rank-Sum test and for categorical variables using Pearson's Chi-squared test.

Rank regression analysis (Kloke *et al*., 2012) was used to assess the relationship between clinical periodontal measures and diet, PI, GCF biomarkers and their interactions. Plausible confounders (age, sex, race and BMI) were selected a priori based on the existing literature and included as covariates in the regression models, but only retained if they showed significant association ($p<0.05$) with the outcome. In the regression models, PI was centered. We first investigated the effects of diet and PI on clinical measures, then we introduced plausible confounders, and finally investigated the effect of inflammatory cytokines. A type 1 error rate of 5% was used.

Results

Twenty-eight subjects were categorized as vegetarian and 26 non-vegetarians. Vegetarians consumed higher amounts of fiber rich plant foods (fruits, vegetables, grains and nuts) and lesser amounts of dairy products (Appendix A1). Table 1a summarizes the demographic, blood pressure and BMI data by diet type. Age, sex, race, income, weight, waist circumference, BMI and blood pressure were comparable between the 2 groups. All subjects reported practicing oral hygiene at least once per day. Table 1b also summarizes the clinical oral/periodontal measures by diet type. Univariate analysis showed statistically similar levels of number of missing teeth, number of dental restorations, PI, GI, GCF volume, bleeding on probing, PD, AL and the proportion of subjects with periodontitis between vegetarians and non-vegetarians. Table 1c shows mean cytokine levels by diet type. Again, univariate analysis showed gingival fluid IL-1β, IL-6, IL-8, IL-10, and TNF-α were comparable between the 2 groups. Despite lack of statistical differences between the groups, non-vegetarians tended to show higher levels of BOP and inflammatory cytokines.

Next, a series of regression analyses were undertaken to investigate the effect of diet on periodontal clinical measures while adjusting for PI, demographics, BMI and GCF cytokines. BOP and PD were the only two clinical measures associated with diet.

Table 2A summarizes the regression analyses predicting BOP. Model 1 showed that when adjusting for PI, diet independently influenced BOP ($p<0.05$). Figure 1 illustrates the differences in BOP levels between the groups; vegetarians showed lesser BOP levels than non-vegetarians ($p<0.05$).

Table 1. Demographics, periodontal measures and GCF cytokines (univariate analysis).

a. Demographics			
	Vegetarian n = 28	**Non-vegetarian n = 26**	**p-value**
Age	70.21 (1.74)	68.61 (1.80)	0.45
Male %	43	50	0.59
Black %	32	42	0.43
Income (%=>75K)	59	54	0.76
Weight (lbs.)	167.36 (6.51)	171.72 (6.63)	0.49
Waist (cm)	95.62 (2.85)	100.34 (2.92)	0.32
BMI	25.55 (0.7)	26.13 (0.81)	0.62
Systolic BP	142.63 (3.71)	144.41 (3.93)	0.62
Diastolic BP	79.70 (2.14)	83.87 (2.27)	0.21
b. Periodontal Clinical Measures			
Missing Teeth	2.03 (0.48)	2.53 (0.5)	0.45
Restorations	9.89 (1.08)	9.68 (1.14)	0.84
Decay (clinically visible)	0	0	
Plaque Index (PI)	0.55 (0.06)	0.55 (0.06)	0.76
Gingival Index (GI)	0.54 (0.05)	0.64 (0.05)	0.15
Gingival bleeding BOP %	6.14 (1.70)	7.38 (1.76)	0.31
Pocket depth (PD)mm	1.97 (0.05)	1.92 (0.06)	0.62
Clinical Attachment Loss (CAL) mm	1.28 (0.16)	1.25 (0.17)	0.95
GCF volume (ul)	2.09 (0.18)	1.76 (0.19)	0.21
CDC-Periodontitis Status			
Healthy %	35.7	34.62	
Slight %	7.14	15.38	0.67
Moderate %	50	38.46	
Advanced %	7.14	11.54	
c. Gingival Fluid Cytokines			
IL-1β pg/ml	6.98 (1.71)	7.23 (3.10)	0.21
IL-6 pg/ml	0.59 (0.19)	0.83 (0.32)	0.79
IL-8 pg/ml	205.68 (55.83)	219.38 (55.94)	0.77
IL-10 pg/ml	3.08 (0.78)	3.66 (0.78)	0.50
TNF-α pg/ml	0.55 (0.17)	1.49 (0.61)	0.78

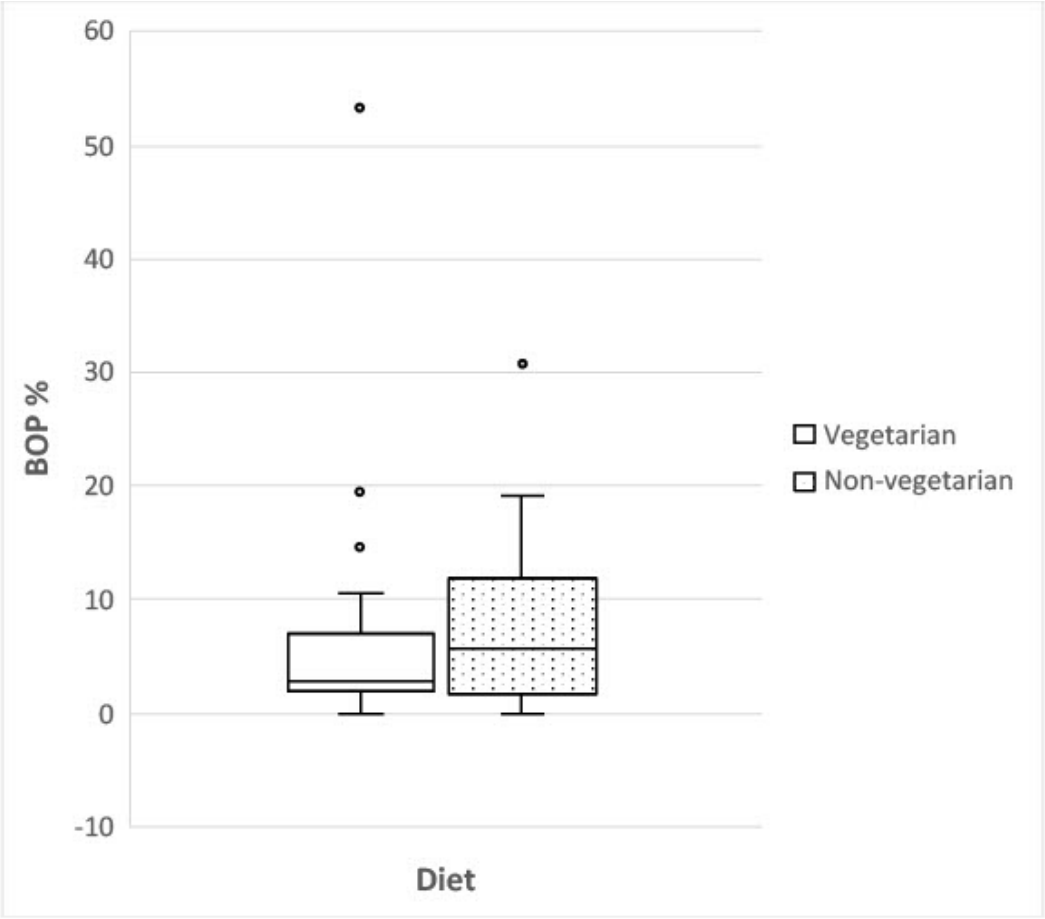

Figure 1. Box plots of BOP percentage by dietary type. The bottom and top sides of each box represent the lower and upper quartiles, respectively. The line inside the box represents the median. The bottom and top whiskers represent the minimum and maximum values, respectively. Outliers are presented by dots. Wilcoxon rank-sum test of unadjusted data was non-significant (Table 1). Rank analysis of covariance adjusting for PI indicated higher BOP levels in non-vegetarians, p = 0.03.

As is expected, PI is positively associated with BOP ($p<0.05$). Furthermore, a diet X PI interaction indicated that diet modified the association between PI and BOP ($p<0.01$), or equivalently, that the dietary effect differs according to the value of PI. Figure 2a illustrates the PI-BOP slope differences between the groups; non-vegetarians showed a steeper slope than vegetarians which suggests a subdued inflammatory response to plaque accumulation in the vegetarian group. Out of the five GCF cytokines investigated, only IL-1β and TNF-α showed direct associations with BOP. Models 2 and 3 introduced the GCF cytokines IL-1β and TNF-α respectively. Model 2 showed that GCF IL-1β positively influenced BOP ($p<0.05$) without affecting the PI X diet interaction noted in model 1. Model 3 showed that GCF TNF-α also positively influenced BOP ($p<0.01$) without affecting the PI X diet interaction noted in model 1. These findings suggest that the effects of GCF IL-1β and TNF-α on BOP are independent of the dietary pattern.

Table 2B summarizes the regression analyses for PD. Model 1 showed a weak diet X PI interaction effect on PD ($p<0.05$). In model 2, adjusting for sex significantly strengthened the diet X PI interaction effect on PD ($p<0.01$). Figure 2b illustrates the PI-PD slope differences between the groups; again, non-vegetarians showed a steeper slope than vegetarians which further suggests a subdued inflammatory response to plaque accumulation in the vegetarian group. Out of the five GCF cytokines investigated, only IL-10 showed a direct association with PD. Introducing GCF IL-10 to model 3 did not affect the diet X PI interaction effect noted in model 2. Regardless of diet, IL-10 levels inversely associated with PD ($p<0.01$). Post-hoc analyses indicated that in non-vegetarians, IL-10 levels are significantly lower in deep than shallow pockets ($p<0.01$); conversely vegetarians maintained comparable IL-10 levels in both shallow and deep pockets (Figure 3).These findings suggest that vegetarians have a stronger anti-inflammatory capability.

Although IL-6 and IL-8 showed significant direct zero-order associations with BOP and PD (data not shown), in the adjusted regression models both lost their significance.

Table 2. Multivariate analysis:

A. Rank regression models relating BOP with diet, PI, and inflammatory cytokines.
Bleeding on Probing is dependent variable

Predictors	**Model 1** R Sq. = 0.40	**Model 2** R Sq. = 0.41	**Model 3** R Sq. = 0.51
Diet	2.35 *(1.07)	2.61* (1.13)	2.07* (0.99)
Cent. PI	4.35* (1.92)	3.42 (2.05)	4.36* (1.72)
Diet X Cent. PI	11.34** (3.43)	12.13** (3.60)	10.08** (3.09)
GCF IL-1β		0.10* (0.05)	
GCF TNF-α			0.79** (0.22)

Data presented as Estimate (Std. Error).
*P < 0.05. **P < 0.01.
In these models, age, sex, race, BMI, IL-6, IL-8, and IL-10 were noncontributory and removed from analyses.
Additional details are presented in Appendix A2.

B. Rank regression models relating PD with diet, PI, sex and IL-10.
Pocket Depth is dependent variable

Predictors	**Model 1** R Sq. = 0.17	**Model 2** R Sq. = 0.23	**Model 3** R Sq. = 0.35
Diet	0.04 (0.07)	0.05 (0.07))	0.05 (0.7)
Cent. PI	0.16 (0.13)	0.04 (0.14)	0.11 (0.12)
Diet X Cent. PI	0.42 (0.24)*	0.61** (0.25)	0.47** (0.22)
Sex		0.18** (0.08)	0.11 (0.07)
IL-10			-0.02*** (0.008)

Data presented as Estimate (Std. Error).
* P < 0.1, **P < 0.05. ***P < 0.01.
In these models, age, race, BMI, IL-1β, IL-6, IL-8, and TNF-α were noncontributory and removed from analyses.
Additional details are presented in Appendix A3.

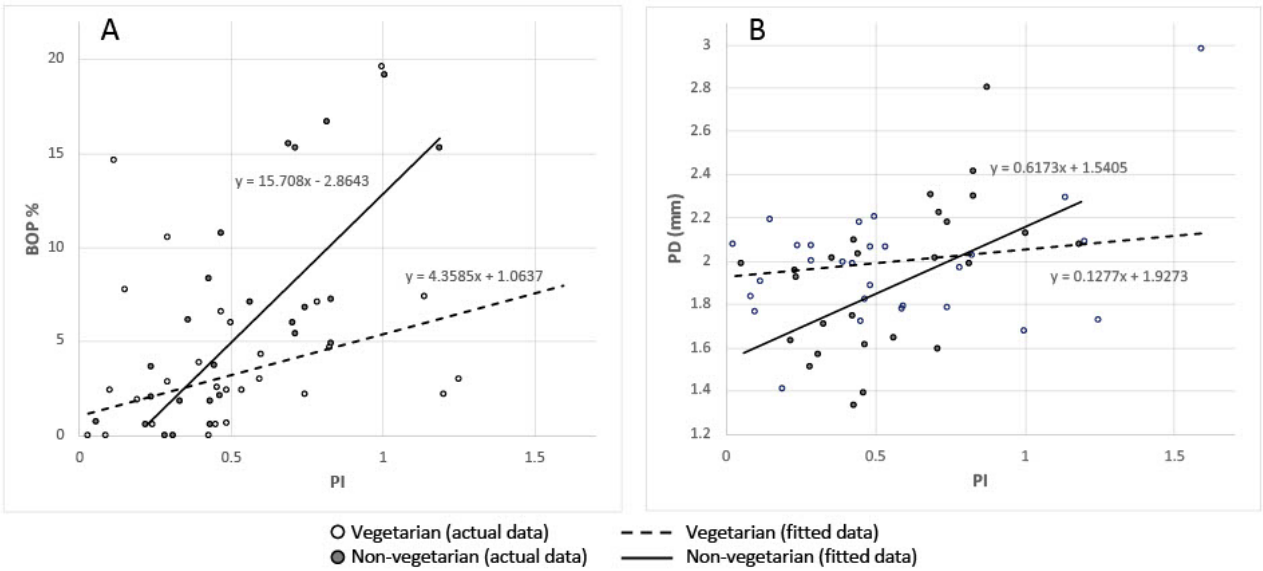

Figure 2. A. Scatter plot (actual data) and regression lines (fitted data) of BOP% versus PI by dietary group. Fitted data generated from rank regression analysis, table 2a, model 1. Slope equations are indicated. Outliers not presented. Pairwise slope difference test: Difference (vegetarian - non-vegetarian) = -11.35, p = 0.002. B. Scatter plot (actual data) and regression lines (fitted data) of PD versus PI by dietary group. Fitted data generated from rank regression analysis, table 3a, model 1. Slope equations are indicated. Pairwise slope difference test: Difference (vegetarian - non-vegetarian) = -0.49, p = 0.08.

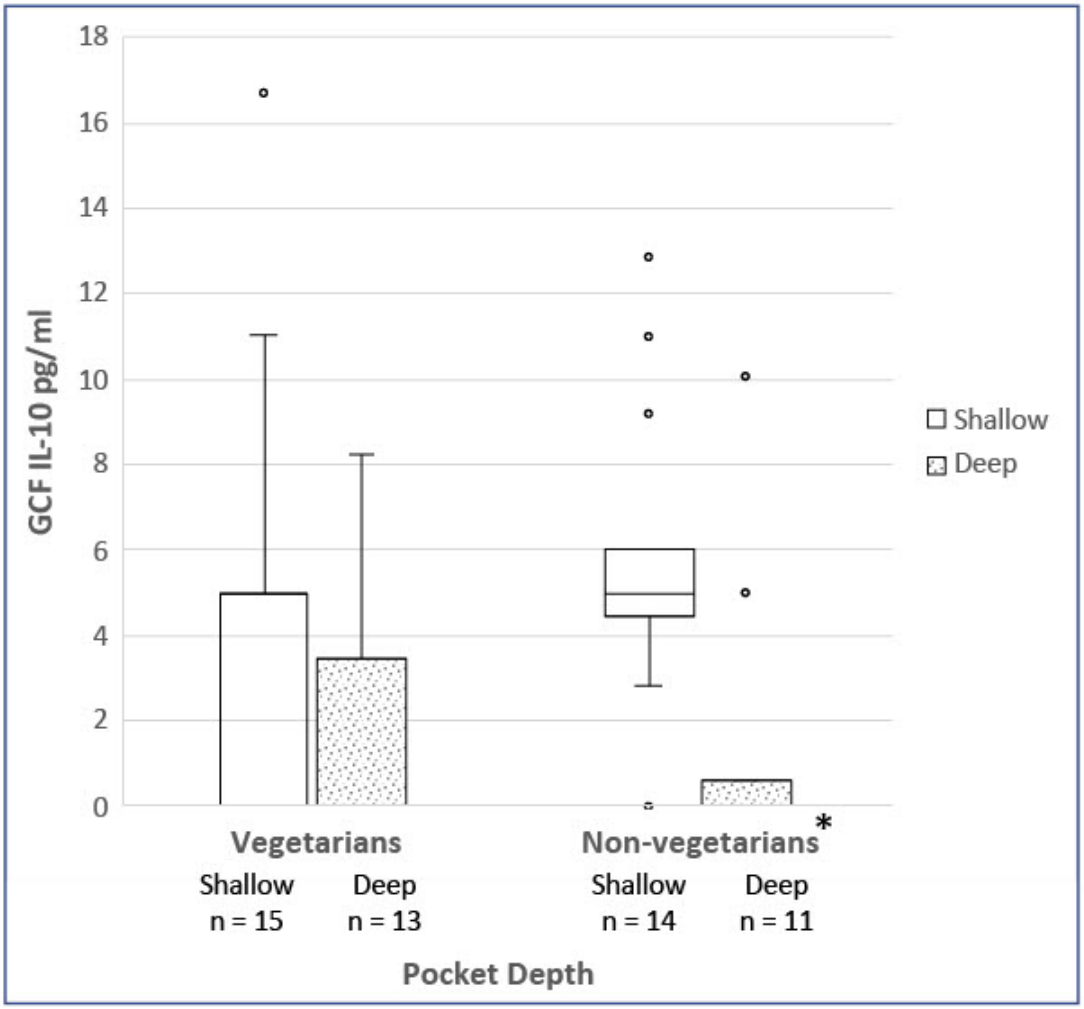

Figure 3. Box plots of GCF IL-10 levels in shallow and deep pockets by diet. The bottom and top sides of each box represent the lower and upper quartiles, respectively. The line inside the box represents the median. The bottom and top whiskers represent the minimum and maximum values, respectively. Outliers are presented by dots. In vegetarians with shallow pockets the median is at the same level of the upper quartile. In vegetarians and non-vegetarians with deep pockets the median is at the same level of the lower quartile. In non-vegetarians with both shallow and deep pockets, the upper whisker outside the box does not appear because the maximum value and the upper quartile are at the same level.
The significance of the difference for IL-10 levels between shallow (below median, <3mm) and deep (above median, ≥3mm) pockets for each group was determined using the Wilcoxon rank-sum test. In non-vegetarians, IL-10 levels significantly differed between shallow and deep pockets, *p<0.01. Rank analysis of covariance adjusting for PI and sex confirmed the univariate findings.

Discussion

This cross-sectional study showed that vegetarian diet favorably modified the association of plaque accumulation with clinical measures of periodontal disease. Vegetarians showed significantly weaker associations between microbial plaque accumulation with both gingival bleeding and pocket depth than non-vegetarians. The attenuated gingival response in vegetarians may be attributed to a combination of factors associated with diets rich in fruits and vegetables such as decreased cellular immunity (Tong *et al*., 2019), antioxidant activity (Liu, 2004) and inhibition of pro-inflammatory signal transduction pathways (Bolat *et al*., 2020). Furthermore, a vegetarian diet may promote a subgingival microbiota associated with periodontal health (Khocht *et al*., 2021).

In this health-conscious population of Seventh-day Adventists, subjects in both groups showed minimal loss of periodontal clinical attachment levels despite their advanced age, suggesting that health focused behavior is a valuable preventive measure against periodontal disease. The unadjusted clinical periodontal parameters between the vegetarians and non-vegetarians were statistically comparable (Table 1). However, adjusting for oral hygiene showed that vegetarians had lesser gingival bleeding and probing depth (Table 2).

Although oral hygiene in both groups was equally good, it seems that variations in plaque scores among subjects masked the differences in gingival condition between vegetarians and non-vegetarians.

Similar to the clinical parameters, the GCF cytokine measures were also statistically comparable between the two groups. Low plaque scores and minimal sites with gingival inflammation in both groups may explain the lack of statistically significant differences in gingival fluid cytokine levels between groups. Despite lack of statistical significance, all cytokine measures tended to be somewhat higher in the non-vegetarians.

IL-1β is a proinflammatory cytokine with angiogenic activity that contributes to blood vessel formation in inflamed tissues (Carmi *et al*., 2013). IL-1β levels tend to be elevated with gingival inflammation and increased probing depth (Baser *et al*., 2009). In this study, regardless of dietary type, IL-1β positively associated with gingival bleeding. Gingival epithelial cells provide an initial barrier to microbial irritants in the dentogingival region (Schroeder *et al*., 1997). Gingival epithelial cells have been shown to produce IL-1β in response to microbial pathogens (Uchida *et al*., 2001). Bacterial molecular patterns acting through pattern recognition receptors on epithelial cells induce the expression of IL-1β as an initial alert signal. Our data suggests that the expression of IL-1β in the gingival tissues plays an important role in the induction of the subepithelial vascular changes associated with gingival bleeding.

TNF-α is an important pro-inflammatory cytokine that is produced by activated macrophages and other inflammatory cells (Zhang *et al*., 2001). TNF-α is elevated in GCF of individuals with gingival inflammation (Baser *et al*., 2009; Ertugrul *et al*., 2013) and facilitates the advancement of the inflammatory front deeper into the gingival connective tissue. Our data showed a significant association between TNF-α and gingival bleeding. TNF-α is known to induce angiogenic actions through the expression of platelet-derived growth factor B (PDGFB) and vascular endothelial cell growth factor receptor-2 (VEGFR-2) (Sainson *et al*., 2008). Since the GCF levels of TNF-α and IL-1β were significantly correlated with one another, when included together in the same model they were not able to independently predict gingival bleeding (data not presented).

IL-10 is an anti-inflammatory cytokine that inhibits activation and effector function of immune cells. IL-10 levels tend to inversely associate with pocket depth (Gamonal *et al*., 2000). This study confirmed the inverse association between IL-10 GCF levels and pocket depth. Furthermore, our data showed that diet modified the association between IL-10 and pocket depth. Vegetarians showed higher IL-10 levels in deep pockets than non-vegetarians. Although the deep pocket environment tends to be dominated by proinflammatory activity that may overwhelm immune regulatory function (Khocht *et al*., 2017), vegetarians maintained adequate IL-10 activity in deep pockets. Which suggests that a vegetarian diet is associated with a stronger anti-inflammatory activity to microbial irritants than a non-vegetarian diet.

Vegetarian diet is rich in fiber which is fermented in the gut and their products exert an anti-inflammatory effect on gut epithelium and immunoregulatory cells (Torquati *et al*., 2019). In mouse models, commensal gut bacteria have been shown to affect host IL-10 responses (Lee *et al*., 2010). In humans, intake of dietary fiber is associated with increased IL-10 levels in blood (Kohl *et al*., 2009). Furthermore, omega-3 fatty acids are readily available in a wide variety of plant foods including walnuts, flaxseeds, chia seeds, hemp seeds, and others (Welch *et al*., 2010). Eicosapentaenoic acid (EPA) synthesized from omega-3 fatty acids express mediators that have anti-inflammatory effects such as PGE-3 which promotes production of IL-10 (Foitzik *et al*., 2002).

Surprisingly in this study neither IL-8 nor IL-6 played a major role in relation to diet and clinical periodontal measures. IL-8 is a chemokine primarily released by monocytes/macrophages. Its main function is the recruitment and activation of neutrophils (Wilson *et al*., 1985). Neutrophils and also monocytes recruited by IL-8 release copious inflammatory cytokines such as IL-1β and TNF-α. Our data suggest that IL-8 activities in gingival inflammation are overshadowed by the products of the immune cells it recruits. IL-6 is a multifunctional pro-inflammatory cytokine primarily produced by T cells. Its main role is the terminal differentiation of B-lymphocytes to plasma cells, which are associated with advanced periodontal disease (Giannopoulou *et al*., 2003). In this cohort of individuals with relative periodontal health, IL-6 activities were not readily apparent.

The finding from this study enhance our understanding of the relationship between diet and periodontal health. However, these data are cross-sectional and cannot infer the temporality of association between diet, gingival cytokine activity and clinical outcomes. The small number of subjects with deepened bleeding pockets made it difficult to assess the observed associations between diet and gingival cytokine activity in a truly diseased environment. Also serum levels of the inflammatory cytokines were not assessed. Other health related behaviors that may be clustered together with a particular dietary choice (e.g. perceived value for health, daily life circumstances, exercise habits and others) were not specifically investigated in this pilot study.

In conclusion, the present findings suggest that vegetarian diet may mitigate the gingival inflammatory response to microbial plaque and enhances the gingival anti-inflammatory activity. The implications of dietary pattern in the prevention of periodontal disease may be significant and worthy of further investigation.

Sources of Funding

This research was supported by GRASP small project grant, Loma Linda University to Drs. Khocht and Orlich; and NIH grant # U01CA152939 to Dr. Fraser (NIH support for parent cohort Adventist Health Study-2).

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Appendix A1. Diet consumption comparison.

var	unit	mean_veg	mean_nonveg	t_pval	sig_0.05
Kcal diet	kcal	1876.029008	1745.598902	0.437019127	FALSE
Fiber diet	g	41.64100786	27.50814219	2.14E-04	TRUE
Dairy kcaldiet	kcal	122.3379524	218.5828926	0.04284019	TRUE
Wholegrains kcaldiet	kcal	1876.029008	1745.598902	0.437019127	FALSE
Potatoes kcaldiet	kcal	1876.029008	1745.598902	0.437019127	FALSE
Vegetables kcaldiet	kcal	1876.029008	1745.598902	0.437019127	FALSE
Fruit kcaldiet	kcal	1876.029008	1745.598902	0.437019127	FALSE
Snacks kcaldiet	kcal	1876.029008	1745.598902	0.437019127	FALSE
Butter kcaldiet	kcal	22.83775255	40.42001173	0.202387719	FALSE

Intergroup comparisons:
*Significantly lower than Group MAT at 7 days (p=0.023).

Appendix A2. Detailed results from ranked regression models relating BOP with diet, PI, and inflammatory cytokines.

Effect of Diet on BOP, at Contrasting PI levels (10th/90th percentiles)

	PI = 0.17	PI = 1.01	P value*
Expected value of BOP in Non-vegetarians	0¥ (0 to 3.73)	12.93 (9.67 to 16.16)	
Expected value of BOP in Vegetarians	1.78 (0.27 to -3.25)	5.45 (3.77 to 7.14)	
Difference in BOP Effect§ by Diet	1.78 (-5.05 to 1.28)	7.48 (3.75 to 11.10)	0.0018
P value†	0.23	0.0002	

Data presented as Expected Value (95% CI).
*P value testing significance of the modification of the dietary effect by PI value
†P value testing the significance of a dietary effect given the nominated fixed PI levels.
§ This is (Expected value in non-vegetarians) – (Expected value in vegetarians)
¥ The linear model predicted an out of range slightly negative value which is defaulted to zero.

Effect of IL-1β and TNF-α on BOP, at Contrasting PI levels (10th/90th percentiles)

Comparing 90th to 10th percentiles of IL1β (i.e. 20.392 and 0.268 units) in its effect on BOP, gives a difference of 2.087 units (95% CI 0.23 - 3.94)
Comparing 90th to 10th percentiles of TNF-α (i.e. 0 to 2.256 units) in its effect on BOP, gives a difference of 1.77 units (95% CI 0.78 – 2.77)

Appendix A3. Detailed results from ranked regression models relating PD with diet, PI, sex and IL-10.

Effect of Diet on PD, at Contrasting PI levels (10th/90th percentiles)

	PI = 0.17	PI = 1.01	P value*
Expected value of PD in Non-vegetarians	1.66 (1.43 to 1.88)	2.21 (1.97 to 2.45)	
Expected value of PD in Vegetarians	1.95 (1.84 to 2.07)	1.98 (1.86 to 2.12)	
Difference in PD Effect by Diet§	-0.29 (-0.55 to -0.04)	0.23 (-0.04 to 0.49)	0.0195
P value†	0.026	0.11	

Data presented as Expected Value (95% CI).
* P value testing significance of the modification of the dietary effect by PI value
† P value testing the significance of a dietary effect given the nominated fixed PI levels.
§ "Effect" is (Expected value in non-vegetarians) – (Expected value in vegetarians)