# Evaluation of Subgingival Microbiome After Switching from Cigarettes to Nicotine Pouches

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## ABSTRACT

Completely switching from cigarette smoking to oral tobacco-derived nicotine products, like nicotine pouches, presents a potential harm reduction opportunity for adult smokers who are unable or unwilling to quit using tobacco. The objective of this study was to compare subgingival microbiome profiles in adult smokers who switched from cigarette smoking (CS) to using on!® nicotine pouches (NPs) in a single-center, randomized, open-label, parallel-group study. Adult smokers with moderate gingivitis were randomly assigned into NP (n=88) and CS (n=61) groups at baseline. Subgingival plaque was collected by curettes from two separate sites with the highest gingival inflammation determined by the modified gingival index and bleeding at baseline. Sampling was repeated at 12- and 24 weeks from the same sites. The V1-V3 regions of 16S rDNA were sequenced using an Illumina platform. Alpha (Shannon and Simpson indices) and beta diversities, differential abundance with bias control, and LefSe analyses were determined. In NP users, no statistically significant changes were observed in alpha and beta diversities at both 12- and 24 weeks compared to baseline. However, using LefSe analysis, health-associated species, e.g., *Rothia aeria*, *Streptococcus cristatus*, *Kingella oralis*, species of *Neisseria* and *Haemophilus*, became more predominant while relative abundances of putative pathogens, e.g., *Fusobacterium*, *Saccharibacteria TM7* and *Prevotella spp.* declined at both time points. In the continued smoking group, no statistically significant changes were observed in alpha or beta diversities at 12- or 24 weeks compared to baseline. In these smokers, using differential abundance analysis, one *Capnocytophaga* species was statistically higher (p<0.05) at 24 weeks compared to baseline while some, albeit low abundance, differences using LefSe were observed over time.

## BACKGROUND

Oral disease from the use of tobacco products is a significant public health concern. A consistent association between cigarette smoking and elevated oral disease (e.g. oral cancer) risks has been demonstrated relative to non-tobacco use.[1] Subgingival microflora of smokers with gingivitis is preceded by an increase in the abundance of periodontal pathogens.[2] Combustible tobacco products confer significant risk due to harmful constituents such as carcinogens, respiratory toxicants, cardiovascular toxicants, and reproductive or developmental toxicants.[3] Next-generation oral tobacco products, such as nicotine pouches, do not contain tobacco leaf and thus many of the tobacco-related toxicants are substantially reduced (>95%) or eliminated, which may offer harm reduction opportunities for adult smokers.

## STUDY PRODUCT

The test products used in this study were pouch products containing tobacco-derived nicotine and flavors. These nicotine pouch products are currently marketed under the brand name on!®. The on!® nicotine pouches are innovative oral tobacco products that do not contain cut, ground, powdered, or leaf tobacco – a point of differentiation compared to smokeless tobacco (ST) products currently commercially marketed in the US. The on!® nicotine pouches are intended for adult smokers and ST users (including dual users of cigarettes and ST products).

*on!® Nicotine Pouches (2, 4, & 8 mg, NICOTINE, MINT FLAVOR)*

### Table 1. Study Products

| Study Group | Description | Mode of Use |
|---|---|---|
| Test | on!® Nicotine Pouches (2, 4, & 8 mg nicotine, mint flavor, manufactured by Helix Innovations, LLC) | Ad libitum |
| Control | Combustible Cigarette (subject's usual brand cigarette, self-supplied) | Ad libitum |

## OBJECTIVES

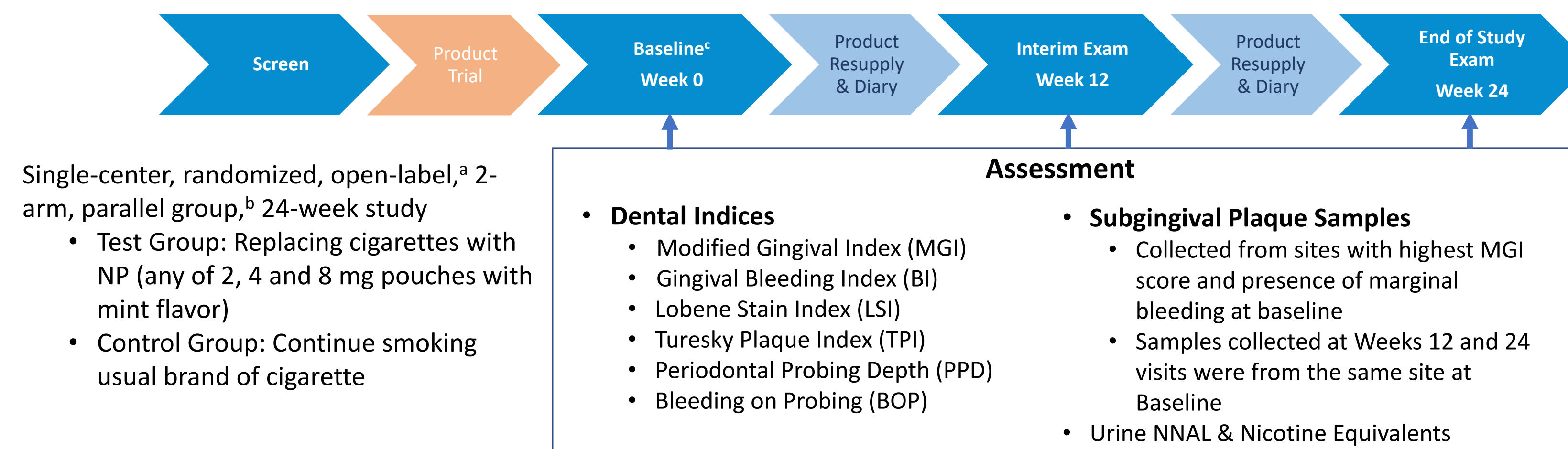The objective of this study was to compare subgingival microbiome profiles in adult smokers who switched from CS to using on!® NPs in a single-center, randomized, open-label, parallel-group study.

## DISCLOSURES

## METHODS

### Study Design

Screen → Product Trial → Baseline[c] Week 0 → Product Resupply & Diary → Interim Exam Week 12 → Product Resupply & Diary → End of Study Exam Week 24

**Assessment**

Single-center, randomized, open-label,[a] 2-arm, parallel group,[b] 24-week study
- Test Group: Replacing cigarettes with NP (any of 2, 4 and 8 mg pouches with mint flavor)
- Control Group: Continue smoking usual brand of cigarette

**Dental Indices**
- Modified Gingival Index (MGI)
- Gingival Bleeding Index (BI)
- Lobene Stain Index (LSI)
- Turesky Plaque Index (TPI)
- Periodontal Probing Depth (PPD)
- Bleeding on Probing (BOP)

**Subgingival Plaque Samples**
- Collected from sites with highest MGI score and presence of marginal bleeding at baseline
- Samples collected at Weeks 12 and 24 visits were from the same site at Baseline
- Urine NNAL & Nicotine Equivalents

[a] Examiner blind
[b] Test:Control = 3:2; Stratified by Age, Gender and Bleeding Index
[c] Complete dental scaling and polishing at Baseline

### Inclusion Criteria
- Between the ages of 23 and 65 years
- Mean MGI score of ≥1.75 and BI ≥10 Bleeding sites at the Screening and Baseline visits
- Currently smoke at least 10 combustible cigarettes per day
- Willingness to replace cigarette smoking for 24 weeks with the mint NP

### Exclusion Criteria
- History of any type of malignant tumors
- BMI >40 kg/m² or less than 18 kg/m² at screening
- Uncontrolled Diabetes defined as HbA1c >8.0%
- Exhibit ≥30% of teeth with stage II – IV periodontitis at Screening or Baseline visits
- More than 3 teeth with PPD >5mm
- Any health condition requiring prophylactic antibiotics prior to dental exams
- History of periodontal treatment within 6 months or surgery within 3 years

### Subgingival Plaque Microbiome Analysis

DNA isolation + sequencing of V1-V3 regions (16S rRNA NGS using an Illumina platform)
- Alpha (Shannon and Simpson Indices) diversity[4]
- Beta diversity[5]
- Differential abundance with bias control[5]
- LefSe analyses[7]

## RESULTS

### Study Subjects

Randomized (n=155)
- Test Group (n=92)
  - on!® Switcher Subject (OSS)[a]: n=12 @ Week 12, n=10 @ Week 24
  - Dual Use Subject (DUS)[b]: n=31 @ Week 12, n=26 @ Week 24
- Control Group (n=63)
  - CS: n=52 @ Week 12, n=45 @ Week 24

[a] OSS: Used NP, Smoked ≤10% and NNAL ≤25% of the baseline
[b] DUS: Used NP, Smoked >10% or NNAL >25% of the baseline

### Differential Abundance with Bias Control
**W12 vs Baseline (Test Subgroup OSS)**

| | Species | Adjusted p-value |
|---|---|---|
| ↑ | Solobacterium moorei (SP106) | <0.001 |
| ↑ | Streptococcus oralis_subsp._dentisani_clade 058 (SP14) | <0.001 |
| ↑ | Neisseria flavescens (SP209) | <0.001 |
| ↑ | Actinomyces israelii (SP256) | <0.001 |
| ↑ | Rothia aeria (SP269) | <0.001 |
| ↑ | Fretibacterium fastidiosum (SP345) | <0.001 |
| ↓ | Peptidiphaga sp._HMT 183 (SP428) | <0.001 |
| ↓ | Prevotella oulorum (SP57) | <0.001 |
| ↓ | Alloprevotella tannerae (SP63) | <0.001 |
| ↓ | Capnocytophaga sp._HMT_380 (SP75) | <0.001 |
| | Kingella oralis (SP253) | NS |
| | Streptococcus cristatus (SP214) | NS |
| | Haemophilus parainfluenzae (SP83) | NS |
| | Neisseria sicca (SP215) | NS |
| | Fusobacterium nucleatum (SP17) | NS |
| | Capnocytophaga endodontalis (SP159) | NS |
| | Campylobacter concisus (SP88) | NS |
| | Capnocytophaga gingivalis (SP6) | NS |
| | Capnocytophaga sputigena (SP24) | NS |

**More Abundant**

Health-associated species, e.g., *Rothia*, *Neisseria*, and spp. of *Streptococcus* more abundant (green arrow) at Week 12 as compared to baseline (p<0.001)

*Kingella oralis*, was also more abundant (p=0.08)

**Less Abundant**

*Fusobacterium nucleatum*, a putative pathogen, and *Solobacterium moorei*, often associated with halitosis, were reduced (red arrow) at Week 12 (p<0.001)
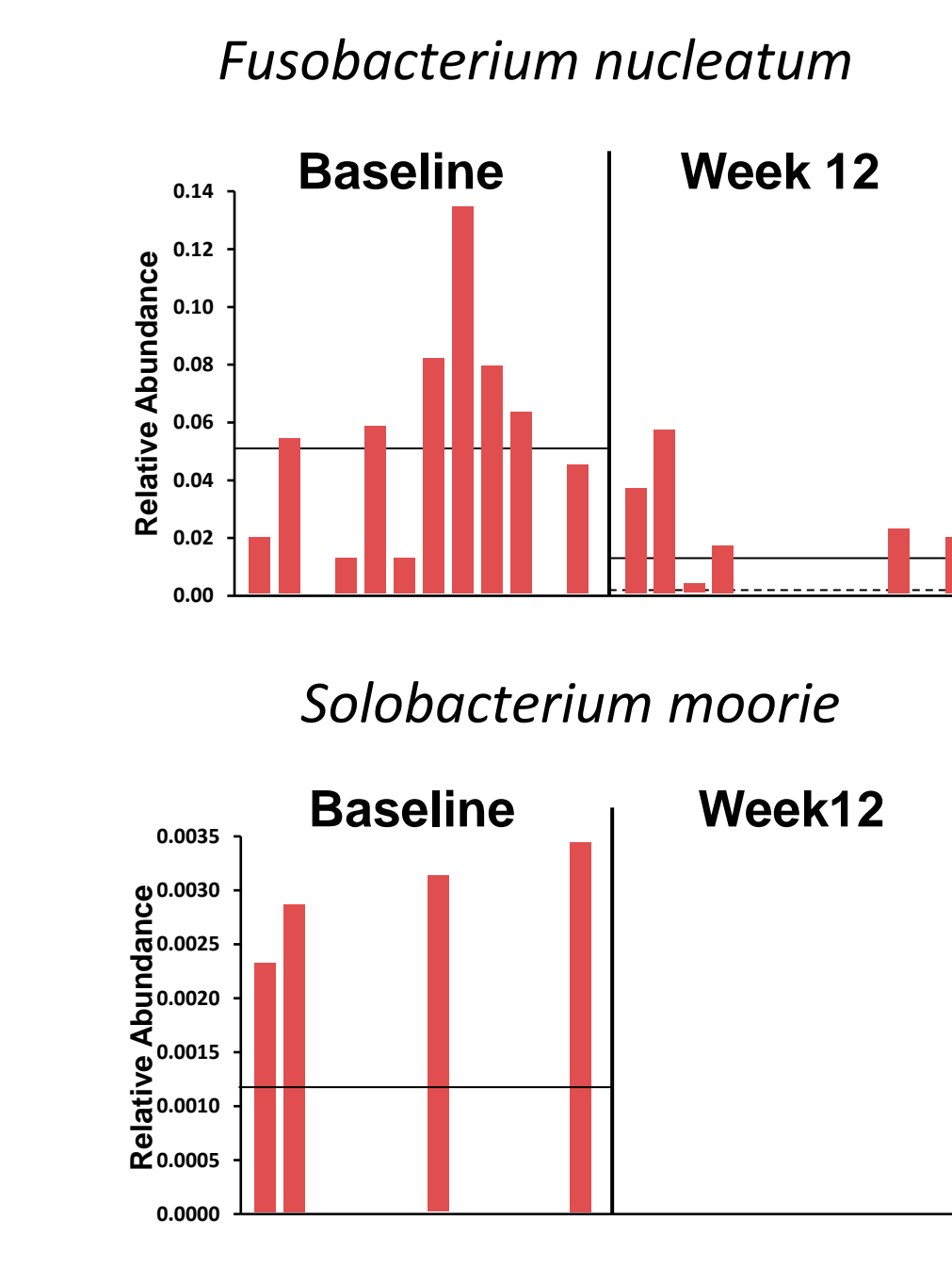
### Alpha Diversities
(Test Subgroup OSS)

Observed / Shannon / Simpson / Observed / Shannon / Simpson (B, W12, B, W24)

### Beta Diversities
(Test Subgroup OSS)

Week 12 / Week 24

No significant differences between Baseline (B) and Week 12 (W12), Baseline and Week 24 (W24) for Test, OSS, DUS groups and between Test and Control groups

### LefSe Health Associated Species
(Week 12)

- Health-associated species (HAS) were more abundant in on!® switchers (OSS) compared to continued smokers (CS) at Weeks 12 and 24 (data not shown).
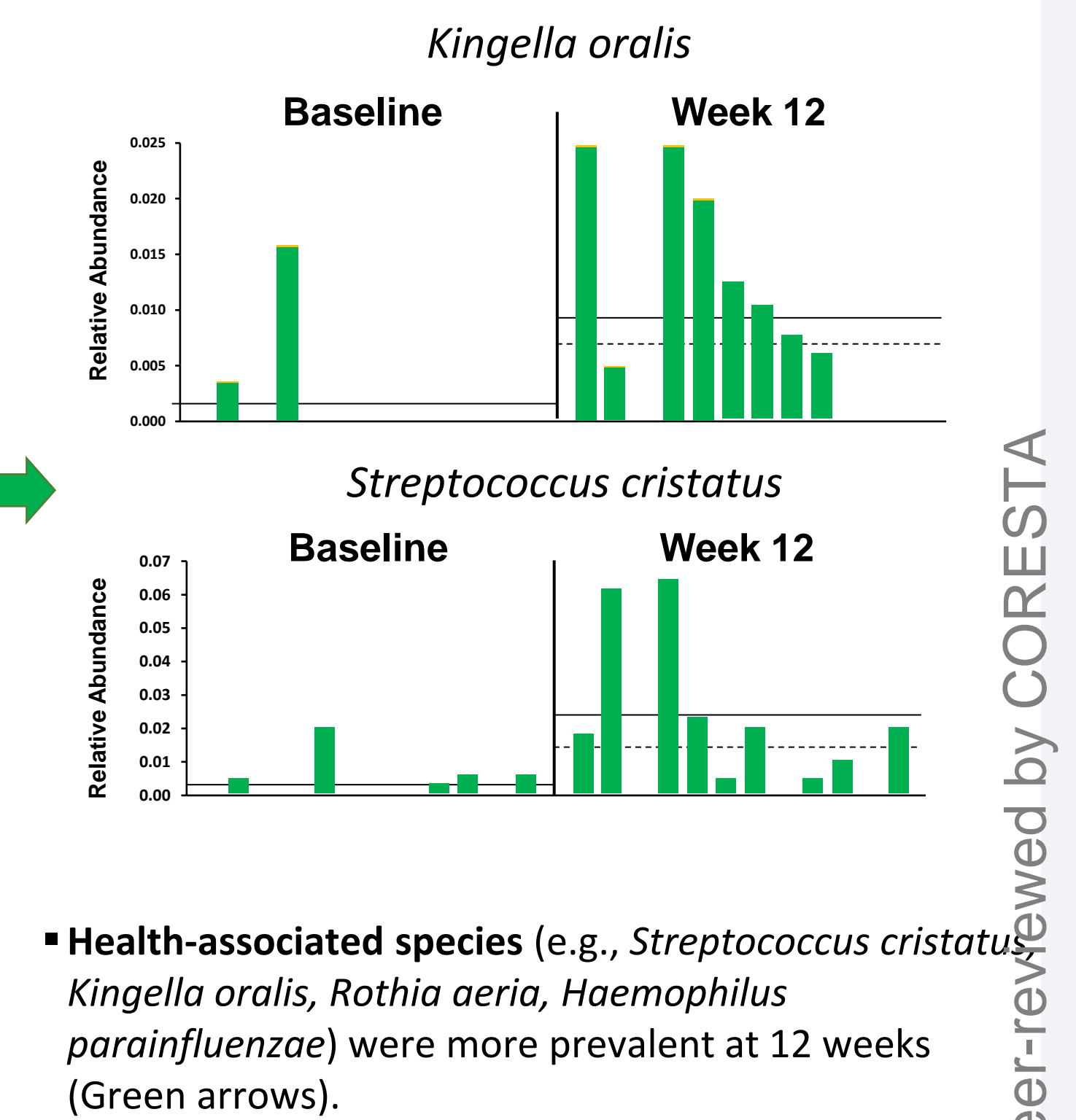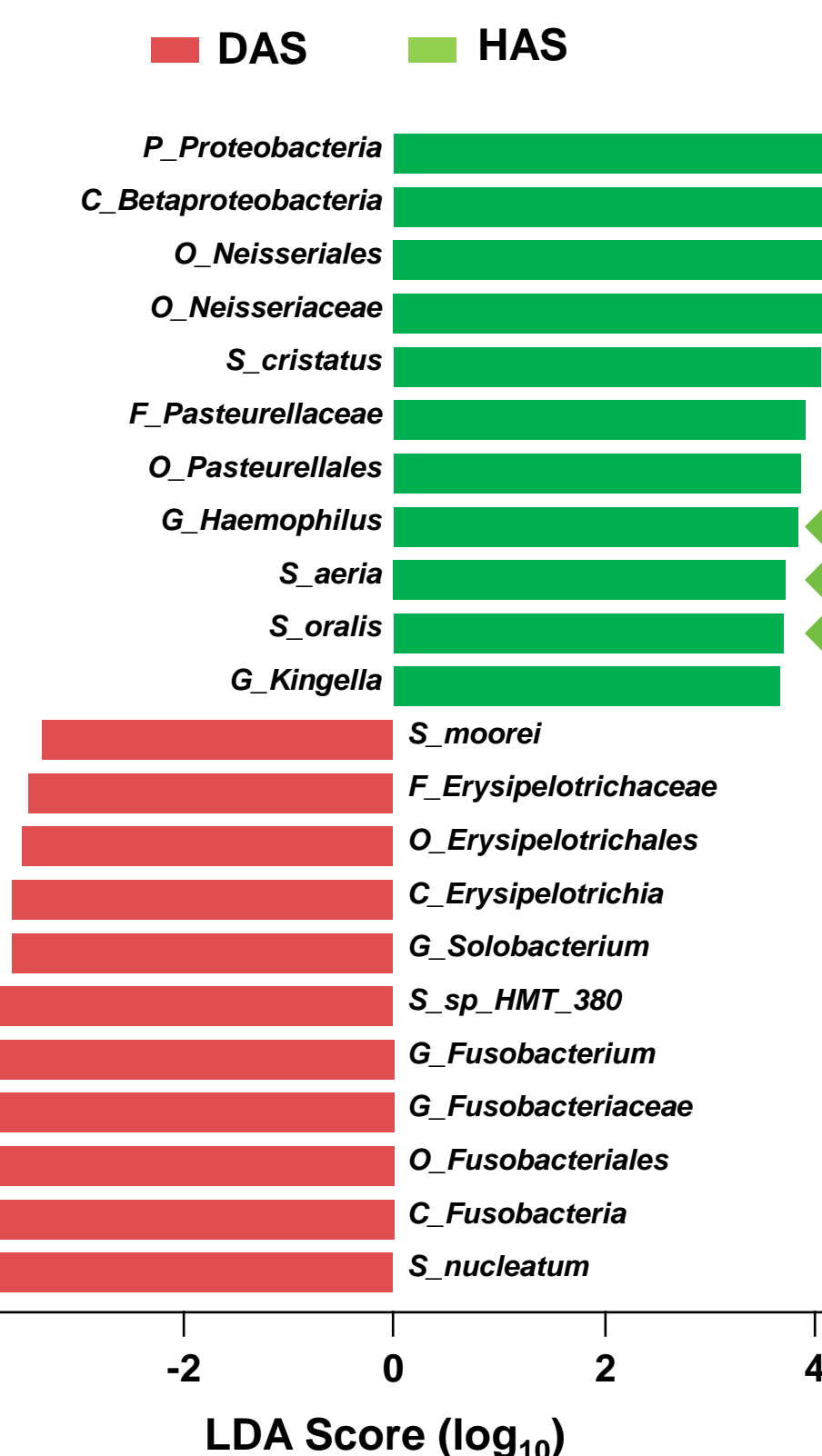
*Cornebacterium spp.* (Baseline / Week 12)
*Kingella oralis*
*Streptococcus spp.* (OSS / CS)
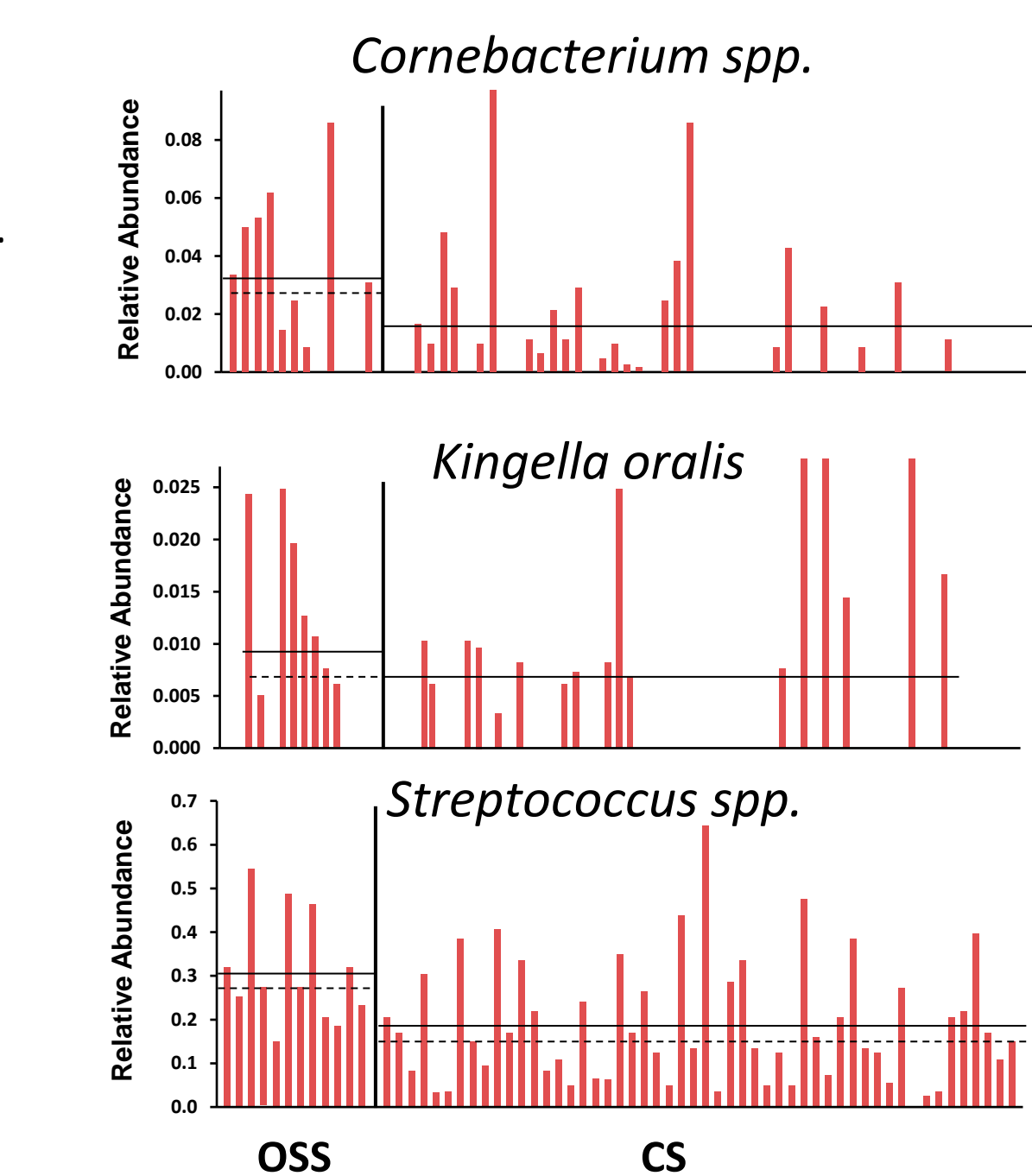
### LefSe at Species Level
(Week 12)

- Disease-associated species (DAS) *Fusobacterium*, *Solobacterium* in OSS were prevalent at baseline and reduced at Weeks 12 and 24 (data not shown).

*Fusobacterium nucleatum* (Baseline / Week 12)
*Solobacterium moorie* (Baseline / Week12)

**Significant Represented Taxa**

DAS / HAS

P_Proteobacteria, C_Betaproteobacteria, O_Neisseriales, F_Neisseriaceae, S_cristatus, F_Pasteurellaceae, O_Pasteurellales, G_Haemophilus, S_aeria, S_oralis, G_Kingella (HAS, green)

S_moorei, F_Erysipelotrichaceae, O_Erysipelotrichales, C_Erysipelotrichia, G_Solobacterium, S_sp_HMT_380, G_Fusobacterium, F_Fusobacteriaceae, O_Fusobacteriales, C_Fusobacteria, S_nucleatum (DAS, red)

LDA Score (log₁₀)

*Kingella oralis* (Baseline / Week 12)
*Streptococcus cristatus* (Baseline / Week 12)

- Health-associated species (e.g., *Streptococcus cristatus*, *Kingella oralis*, *Rothia aeria*, *Haemophilus parainfluenzae*) were more prevalent at 12 weeks (Green arrows).

## CONCLUSIONS

- Completely switching from cigarettes to on!® nicotine pouches may improve the oral microbiome by increasing health-associated species.
- The clinical significance of this change has not been established.
- This beneficial change in the oral microbiome was only observed upon complete switching.
- Adults who continue to smoke cigarettes or use on!® nicotine pouches and smoke cigarettes exhibited higher putative pathogens and halitosis-associated species.

## LIMITATIONS

Due to drop-out and small sample size at the end of study,
- permutation tests (PERMONAVA)—non-parametric permutational analysis and the repeated measure design could not be evaluated
- benefits were seen from switching, but the results cannot be generalized

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