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Compositional shifts within the denture-associated bacteriome in pneumonia – an analytical cross-sectional study

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Abstract

Introduction. Bacterial pneumonia is a common cause of morbidity and mortality in elderly individuals. While the incidence of edentulism is falling, approximately 19% of the UK population wear a full or partial removable denture. Despite advances in denture biomaterials, the majority of dentures are fabricated using polymethyl-methacrylate. Growing evidence suggests that colonization of the oral cavity by putative respiratory pathogens predisposes individuals to respiratory infection, by translocation of these microorganisms along the respiratory tract.

Hypothesis/Gap Statement. We hypothesized that denture surfaces provide a susceptible colonization site for putative respiratory pathogens, and thus could increase pneumonia risk in susceptible individuals.

Aim. This study aimed to characterize the bacterial community composition of denture-wearers in respiratory health compared with individuals with a confirmed diagnosis of pneumonia.

Methodology. This was an analytical cross-sectional study, comparing frail elderly individuals without respiratory infection (n=35) to hospitalized patients with pneumonia (n=26). The primary outcome was the relative abundance of putative respiratory pathogens identified by 16S rRNA metataxonomic sequencing, with quantitative PCR used to identified *Streptococcus pneumoniae*.

Results. There was a statistically significant increase in the overall relative abundance of putative respiratory pathogens (P<0.0001), with a greater than 20-fold increase in the bioburden of these microorganisms. In keeping with these findings, there were significant shifts in bacterial community diversity (Chao index, P=0.0003) and richness (Inverse Simpson index P<0.0001) in the denture-associated microbiota of pneumonia patients compared with control subjects.

Conclusion. Within the limitations of this study, our evidence supports the role of denture acrylic biomaterials as a potential colonization site for putative respiratory pathogens, which may lead to an increased risk of pneumonia in susceptible individuals. These findings support prior observational studies which have found denture-wearers to be at increased risk of respiratory infection. Further research is needed to confirm the sequence of colonization and translocation to examine potential causal relationships.

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Keywords: 16S rRNA; metataxonomic sequencing; bacterial pneumonia; denture; bacteriome.

Abbreviations: DMFT, decayed, missing and filled teeth; LDA, linear discriminant analysis; OTU, operational taxonomic unit; PRP, putative respiratory pathogen; qPCR, quantitative PCR; VAP, ventilator-associated pneumonia.

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The 16S rRNA gene sequences, associated datasets and metadata generated for this study are available through the NCBI SRA (accession number PRJNA971933) Open Science Framework online repository: https://osf.io/mknsu/

Two supplementary tables are available with the online version of this article.

DATA SUMMARY

The authors confirm all supporting data, code and protocols have been provided within the article or through supplementary data files.

INTRODUCTION

Lower respiratory tract infections, including pneumonia, are the fourth leading cause of death worldwide, and the most common cause of death due to infectious disease [1]. Globally, pneumonia has a bimodal distribution of incidence, affecting the very young and elderly. However, in the UK, much of Europe and the USA, pneumonia demonstrates a predilection for the elderly, with a ten-fold increase in pneumonia cases in patients over 65 years of age [2] and 85% of pneumonia-related deaths occurring in individuals over the age of 60 years [3].

The term pneumonia describes a clinical phenotype of acute inflammation in the lower respiratory tract [4] which does not necessarily reflect an infectious aetiology. However, most pneumonias occur secondary to microbial infection, which may be viral, bacterial, fungal or polymicrobial [5]. In the UK and much of Europe, pneumonia is most frequently bacterial in aetiology [6]. The diagnosis of pneumonia is challenging due to the non-specific clinical signs and symptoms associated with the disease. Determining a microbial aetiology is confounded by difficulties obtaining a representative sample, free from contaminating microorganisms originating in uninfected regions of the respiratory tissues or oropharynx, and the inability to distinguish microbes colonizing the respiratory tissues from infective species [7].

A burgeoning body of research has revealed an association between changes in oral microbial communities and respiratory infection in susceptible individuals [8–11]. This is most clearly supported in ventilator-associated pneumonia (VAP) which can affect mechanically ventilated intensive care patients. Here, an increase in the relative abundance of putative respiratory pathogens (PRPs) in dental plaque occurs following intubation of patients in intensive care, with subsequent reversal of this community perturbation following extubation [12, 13]. Further, a recent systematic review found evidence supporting the effectiveness of oral care to reduce VAP, although the effect size was modest and the overall quality of evidence available was low [14]. Similarly, a number of researchers have recovered PRPs from denture surfaces [15–17] while enhanced oral care, including denture care, has been found to reduce the incidence of pneumonia among long-term care facility residents [18]. The presence of an endotracheal tube offers a direct conduit to the lungs and necessitates open mouth posture, facilitating the acquisition of exogenous microorganisms; this bypasses the host immune system and enables translocation to the respiratory tissues [19], That a similar relationship appears to exist between the denture-associated oral microbiota and respiratory infection suggests that the presence of an artificial biomaterial surface may itself promote colonization by PRPs, forming a reservoir that can seed infection of the respiratory tissues in susceptible individuals.

Despite indirect evidence suggesting that the oral microbial communities of denture-wearing individuals may contribute to pneumonia risk, direct support for a mechanistic role for denture biomaterial surfaces in promoting respiratory infection is lacking. This study therefore aimed to compare the community composition of denture-associated oral bacteria in patients with a clinical diagnosis of pneumonia with respiratorily healthy care home residents. We hypothesized that there would be an increase in the abundance of putative respiratory pathogens, and the specific pathogens *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Streptococcus pneumoniae* on denture surfaces of individuals with clinically diagnosed bacterial pneumonia (based on clinical, radiographic findings in accordance with the British Thoracic Society guidelines, 2009 [20]), compared with other oral sites and respiratorily healthy participants.

METHODS

Participant recruitment and sample collection

Ethical approval for this study was obtained from the Wales REC 6; reference 16/WA/0317. All participants provided written consent for this study. This was a cross-sectional study which conforms with STROBE guidelines for human observational studies.

Participants were recruited from private long-term residential/nursing care facilities in Cardiff, or from the respiratory/geriatric wards in University Hospital Wales and University Hospital Llandough, Wales, UK. Recruitment was undertaken between March 2017 and March 2018. Participants were excluded from either group if they lacked capacity to provide consent; were receiving palliative end-of-life care; had taken part in another study in the preceding 6 months; were severely immunosuppressed/immunocompromised; or had a diagnosis of oro-pharyngeal or lung malignancy. Care home residents were excluded if they had a history of respiratory infection in the previous 30 days. Hospitalized patients were included only if there was a confirmed diagnosis of pneumonia supported by radiographic signs. Where this information was not readily available, a diagnosis was sought from the treating respiratory physician.

For each participant, a brief dental history and examination was undertaken, including denture cleaning habits, oral mucosal inflammation (assessed by Newton's index [21]) and a record of decayed, missing and filled teeth [22] as a surrogate marker of

previous oral disease burden. Imprint cultures were taken from the dorsal tongue, denture-bearing palatal mucosa and denture-fit surface of each participant, and transferred sequentially to Mannitol Salt agar (Lab M) and Pseudomonas agar (Lab M) for 60 s each. Sterile cotton swabs were taken from the same sites using a standardized technique and transferred to Amies transport medium.

Agar plates were incubated aerobically at 37 °C for 24–72 h until distinct colonies could be identified. Cultured microorganisms were characterized by routine histological staining and biochemical testing to differentiate *Staphylococcus aureus* and *P. aeruginosa*. The antimicrobial susceptibility of *Staphylococcus aureus* and *P. aeruginosa* isolates was tested according to the EUCAST disc-diffusion method [23].

Bacterial DNA extraction from oral and denture samples, and detection of *Streptococcus pneumoniae* by qPCR and 16S rRNA gene sequencing

Microbial swabs were aseptically transferred to 10 ml bijou bottles containing 1 ml of 0.9% PBS by cutting the swab neck with flame-heated scissors. DNA extraction was performed using the a PuraGene kit (Qiagen) using the protocol for Gram-positive bacteria, with a final elution volume of 20 µl. The following modifications were added to this protocol: bijou bottles containing microbial swabs were vortexed at high speed for 1 min, and the resultant cell suspension was transferred by pipetting to a 1.5 ml microcentrifuge tube on ice. Cell suspensions were centrifuged for 1 min at 5000 *g* and the supernatant was discarded by pouring. The resultant cell pellet was then resuspended in 1 ml of PuraGene cell suspension solution (Qiagen).

Due to the challenges associated with speciating *Streptococcus pneumoniae* by 16S rRNA gene sequencing, a species-specific TaqMan assay which targeted the autolysin-encoding gene *lytA* was used for detection of this microorganism by quantitative (q)PCR. The primers used in this assay were: forward primer sequence: ACGCAATCTAGCAGATGAAGCA, reverse primer sequence: TCGTGCGTTTTAATTCCAGCT, probe sequence: YY-TGCCGAAAACGCTTGATACAGGGAG-BHQ1.

This assay had previously been published as part of a multiplex diagnostic assay [24]. The sensitivity and specificity of the assay in single-plex use were confirmed by a standard curve, using reference strains *Streptococcus pneumoniae* ATCC 49619, *Streptococcus gordonii* ATCC 10558 and *Streptococcus sanguinis* ATCC 7863 in 10-fold serial dilutions to a lower limit of approximately 10 cells ml^{-1}. PCR was undertaken in triplicate using a QuantStudio 6 Flex instrument (Applied Biosystems).

Library preparation and sequencing was undertaken by Research and Testing Laboratories (RTL) using the Illumina Miseq 28 f and 519 r primers to overlap the V1–V3 hypervariable regions of the 16S rRNA gene. A two-step amplification process was used with a preamplification step employing the Illiumina i5 and i7 primers initially. Sequencing parameters and primer sequences used can be found in the supporting information.

16S rRNA gene sequence pre-processing

Sequencing data were provided as paired FASTQ files for each sample. Generation of 16S rRNA gene sequences was undertaken using the open-source software MOTHUR [25]. The Illumina MiSeq standard operating procedure was followed throughout. Paired-end reads were first assembled with the make.contigs command. This command combines the data from the paired FASTQ files and provides a quality score for each file. Each contig was then filtered using the screen.seqs command, using the parameters: maxn=0, maxambig=0, maxhomop=5, maxlength=605. Reads were subsampled to 675, which resulted in the exclusion of three samples (two from pneumonia patients, one from a care home resident).

Rare operational taxonomic units (OTUs) (<10 reads) were excluded from further analysis and any OTUs with less than 98% coverage or 97% sequence identity to a known bacterial species were categorized to genus level only. After manual scanning, OTUs that would not be expected to occur in the oral cavity were re-examined using the NCBI BLASTn database.

Statistical analyses

No power calculation was undertaken for this pilot study, due to lack of available data to inform estimates. The primary outcome was the frequency of detection/culture of *Streptococcus pneumoniae, P. aeruginosa* and *Staphylococcus aureus*. Secondary outcomes included the relative abundance of putative respiratory pathogens, and diversity and species richness of the bacterial communities for each oral site. Statistical analysis was conducted using R [26], SPSS 21, Graphpad Prism 8.0 and Microsoft Excel. Simple descriptive summary statistics were generated for participant demographic data and oral health measures. Age, Charlson Index [27], Denture Hygiene Score, Newton's Classification and DMFT (decayed, missing, filled teeth) scores were treated as continuous variables. The remaining data were analysed as categorical variables. Distribution of data was assessed by visual inspection of histograms, the Kolgomorov–Smirnov test of normality (alpha set to $P<0.05$) and inspection of Q–Q plots. To assess differences between participant cohorts at baseline, the Kruskal–Wallis test was undertaken on non-parametric data, while one-way ANOVA was used to analyse normally distributed continuous data. Categorical variables were analysed using the chi-squared (χ^2) goodness of fit test. Missing data were excluded from analyses.

Alpha diversity was measured by the Chao2 and Inverse Simpson Indices. Alpha diversity indices were compared using the Kruskal–Wallis test and Median K-tests.

PRP species were assigned to 10 groupings: enterococci, *Acinetobacter* spp., *Enterobacteriaceae*, *Haemophilus* spp., *Klebsiella* spp., *P. aeruginosa*, *Serratia* spp., *Staphylococcus aureus*, *Escherichia coli* and *Streptococcus pneumoniae*. The percentage relative abundance of PRP species was calculated and analysed using the Two-Stage Linear Step-up Procedure of Benjamini *et al.* [28] to control the false discovery rate, with Q-value set at 0.05. Fold differences between participant cohorts' PRP relative abundance were calculated for each oral site.

Percentage relative abundance was converted to decimal data, and Linear discriminant analysis of Effect Size (LEfSe) as conducted using the open access galaxy module [29].

RESULTS

Participant demographics and clinical characteristics

We recruited a total of 66 denture-wearing individuals from long-term residential care facilities (*n*=35) and hospital wards (*n*=26) between March 2017 and March 2018. Participants were pre-screened by care facility and hospital staff based on the provided inclusion/exclusion criteria and their professional assessment of whether it would be appropriate to approach the individuals under their care. Of the 133 potential participants approached, 61 were recruited for the study. Reasons for non-participation were: not currently wearing dentures (*n*=24), unable to provide informed consent (*n*=20), declined to consent (*n*=11), unclear diagnosis in hospital patients (*n*=6), immunocompromised or palliative care (*n*=6), and recent respiratory infection in care home residents (*n*=5). Participant demographic information is summarized in Table 1. Pneumonia patients were significantly younger than care home residents (mean difference 4 years, *P*=0.0006). All pneumonia patients received antibiotic therapy (intravenous amoxicillin and clarithromycin, *n*=15; other, *n*=11), while only four of the 35 care home residents included had received antibiotics in the preceding 6 months. All participants were deemed to have a safe swallow with normal oral intake at the time of assessment. Otherwise, there were no significant differences observed between participant cohorts.

Table 1. Summary participant information

	Care home residents (*n*=35)			Pneumonia patients (*n*=26)		
Mean age (years, SD)	88 (7.6)			84 (8.2)		
Gender (% male)	15			15		
Antibiotics in last 90 days (%)	15			100		
Smoking history (%)	15 current smokers 54 ex-smokers 31 never smoked			8 current smokers 65 ex-smokers 27 never smoked		
Mean Charlson Comorbidity Index* (SD)	5.5 (0.97)			5.1 (2.11)		
Mean DMFT score† (SD)	Decayed	Missing	Filled	Decayed	Missing	Filled
	1.6 (2.00)	25.0 (5.33)	2.3 (4.38)	1.8 (1.48)	24.3 (5.77)	3.6 (3.06)
Complete or partial denture (%)	62.9 complete 27.1 partial (10 no denture in one arch)			59.6 complete 15.4 partial (25 no denture in one arch)		
Acrylic or cobalt chromium denture (%)	91.4 acrylic 8.6 cobalt chromium			92.3 acrylic 7.7 cobalt chromium		
Mean Denture Cleanliness Index‡ (SD)	1.8 (1.11)			1.6 (1.19)		
Mean Newton Index§ (SD)	0.9 (0.53)			1.1 (0.80)		

*The Charlson Comorbidity Index scores a number of physiological measures and diseases to provide an estimate of 10 year survival. The maximum score (highest mortality risk) is 33. A score of ≥7 indicates a predicted 10 year survival rate of 0%.
†The DMFT score is indicative of decayed, missing, filled teeth. Absence of a tooth, or the presence of any dental restoration or caries scores 1 point. The maximum score is 28. Wisdom teeth were not included in this score.
‡The Denture Cleanliness Index scores denture cleanliness from 0 (pristine denture surfaces) to 4 (damaged dentures).
§The Newton Index scores palatal inflammation from 0 (normal, healthy mucosa) to 3 (grossly erythematous, swollen mucosa).

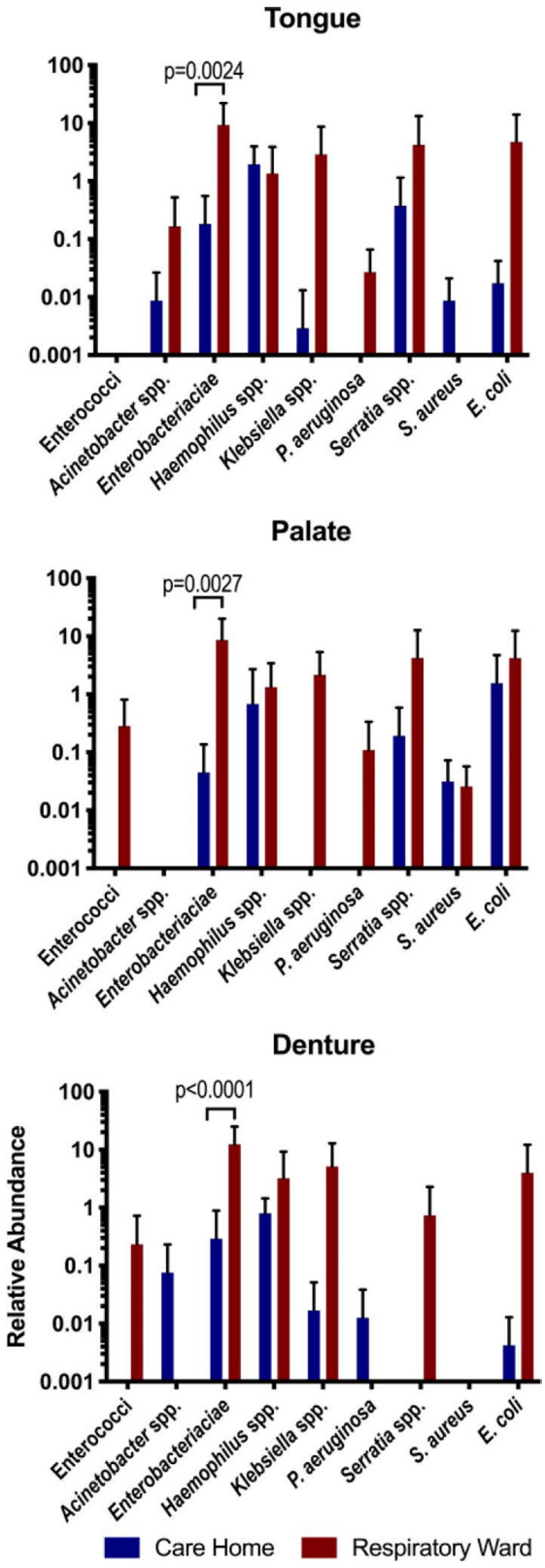

Fig. 1. Relative abundance (%) of putative respiratory pathogens from each oral site. OTUs are grouped at either genus or species level to collate bacteria associated with respiratory infection at the lowest discriminatory phylogenetic level. Note that the *y*-axis features a $\log_{10}$ scale. Mean values are shown, and error bars represent 95% confidence intervals.

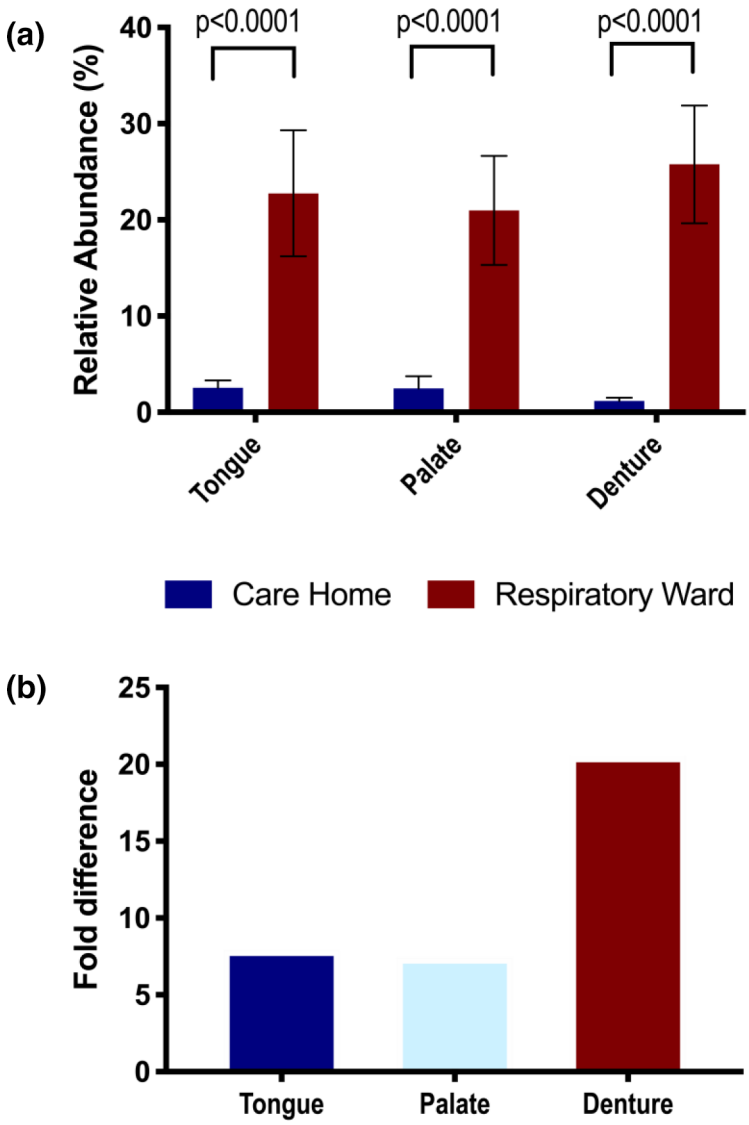

Fig. 2. (a) Cumulative relative abundance (%) of PRPs identified in care home residents compared to respiratory ward patients. Mean values are shown. Error bars represent 95% confidence intervals. (b) Fold difference of PRP cumulative relative abundance in respiratory ward patients, normalized to care home residents. Fold difference was calculated using mean values reported in (a).

Culture isolation and antimicrobial susceptibility testing of target microorganisms

Both *Staphylococcus aureus* and *P. aeruginosa*, two pathogens frequently associated with respiratory infection and a range of healthcare-associated infections, were recovered from the oral cavities of individuals in both cohorts (Table S1, available in the online version of this article). There was no statistically significant difference between recovery rates between patients with pneumonia and respiratorily healthy individuals.

Cultured isolates of *Staphylococcus aureus* and *P. aeruginosa* were tested for susceptibility to a range of relevant antimicrobials (Table S2). Resistance rates varied between different antimicrobials, with no clear trend for isolated microbes from pneumonia patients to exhibit increased resistance to β-lactams, although there was greater resistance of *Staphylococcus aureus* isolates to macrolides.

Analysis of metataxonomic sequencing data

Analysis of metataxonomic sequencing data revealed an increased relative abundance of *Enterobacteriaceae* in all oral sites of patients with pneumonia. Although there was a trend of increased relative abundance of most PRP species, this did not reach the threshold of statistical significance (Fig. 1). However, when the cumulative relative abundance of all PRPs was assessed, there

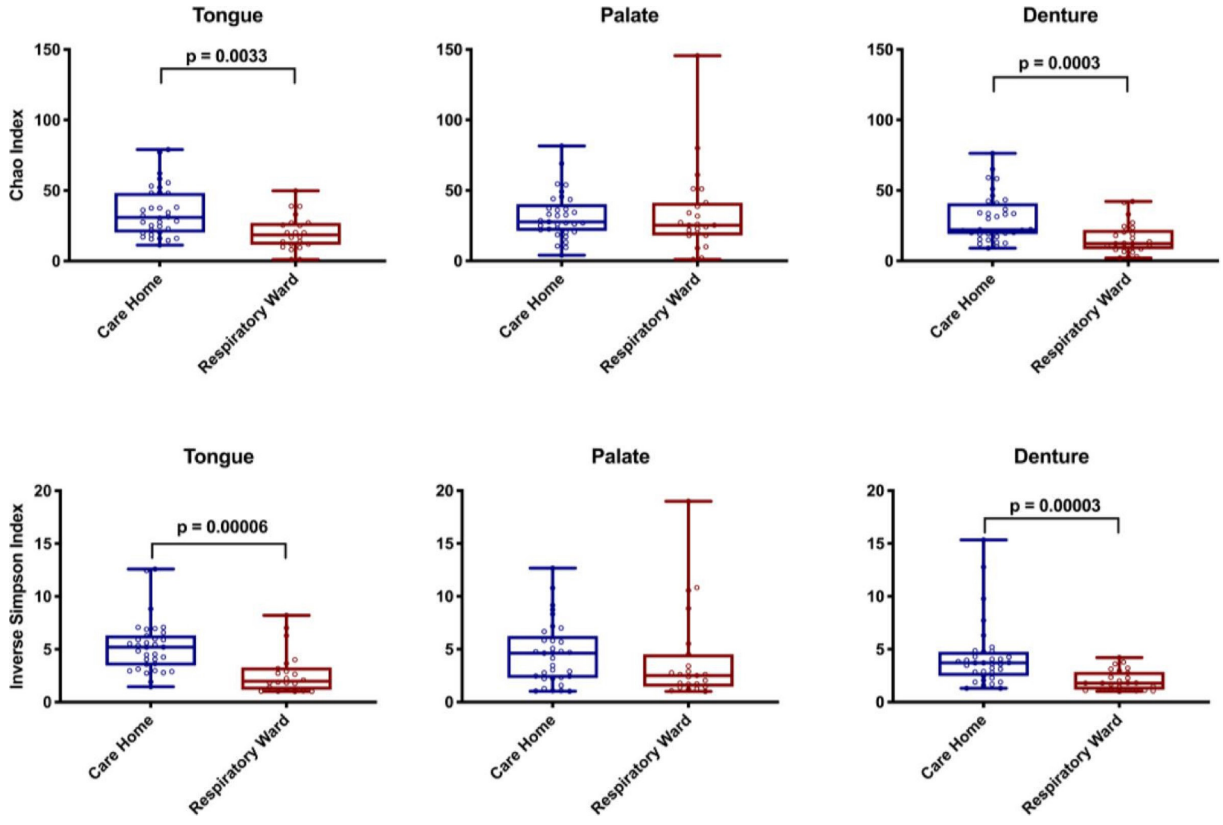

Fig. 3. Chao (upper panel) and Inverse Simpson (lower panel) indices for oral sites in each participant cohort. Individual data points with representative box plots are shown.

was a significant increase noted in pathogenic bioburden compared with respiratorily healthy care home residents (Fig. 2a). Calculation of the fold-difference in the cumulative relative abundance of PRPs showed that the increase in pathogenic bioburden was especially elevated in denture samples, with a greater than 20-fold increase in PRPs (Fig. 2b).

In keeping with these findings, there were significant compositional shifts in the microbial communities, with a decrease in species richness and beta diversity in bacterial communities (Fig. 3) measured by Chao2 and Inverse Simpson indices, respectively. Decreased community diversity and species richness were observed in dorsal tongue and denture samples only.

Further exploration of bacterial community composition by linear discriminant analysis (LDA) confirmed an increased bioburden of PRP species with a concomitant reduction in typical oral commensals in pneumonia patients (Fig. 4).

DISCUSSION

While there has been mounting interest in exploring artificial biomaterial surfaces in the oral cavity as potential reservoirs of respiratory pathogens, this was the first study to directly explore compositional shifts in the denture-associated oral microbiome correlated with pneumonia status, using contemporary molecular techniques to limit selectivity bias. Not only was an increased bioburden of putative respiratory pathogens in individuals with pneumonia found, there was a concurrent loss of species richness and diversity typically associated with a dysbiotic shift in the microbial community. Importantly, these differences were especially pronounced in denture samples, highlighting the role of dentures as a possible nidus for respiratory infection.

In order to reach, colonize and infect the lungs, bacteria must either pass from an external source through the oral cavity, or intrinsically through the gastrointestinal tract [30]. Thus, the relationship seen between the oral microbiome and pneumonia status may hold diagnostic potential, due to the close anatomical approximation of the oral cavity with the lungs and gastrointestinal tract, and the interface formed with the external environment. Given the poor reliability of sampling the infected lung [7], which must be performed essentially 'blind', the ease of access to the oropharynx for microbial sampling could lead to rapid, reliable identification of potential causative microorganisms, and provide antimicrobial susceptibility profiles to aid diagnosis and treatment of pneumonia [31].

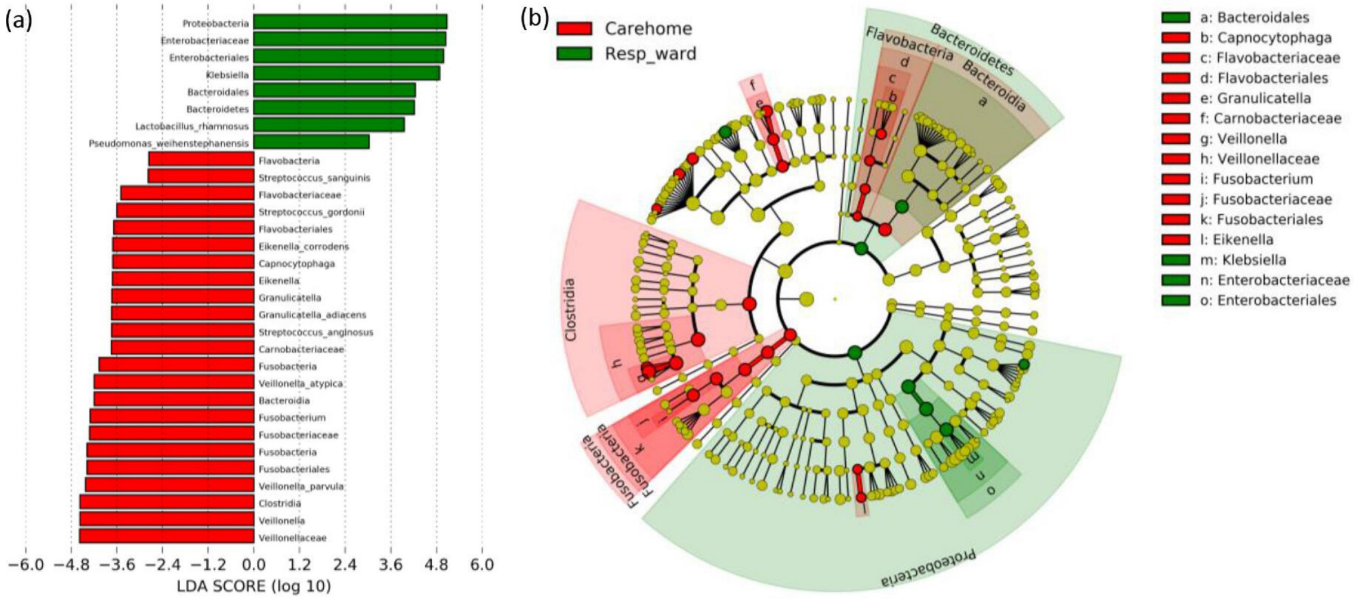

Fig. 4. LEfSe analysis of differential OTU relative abundance between participant cohorts for denture samples. (a) Histogram of significantly different LDA scores for samples. (b) Cladogram representing taxonomic relationships of significantly different abundances between cohort samples. Green indicates taxa with increased abundance in pneumonia patients, and red indicates taxa with increased abundance in samples from care home residents. Yellow circles represent taxa which showed no significant differences between cohorts. The diameter of each circle in the cladogram is proportional to the relative abundance of the taxon represented.

Recruitment of eligible participants was a major challenge encountered during this study as many care home residents were cognitively impaired and thus unable to consent. Similarly, a number of pneumonia patients had cognitive impairment either as a background comorbidity or due to acute delirium. The cross-sectional design of this study was another limitation. As recruited respiratory ward patients had received a diagnosis of pneumonia prior to recruitment, it was not possible to track changes in composition of the oral microbiota from respiratory health to disease. Similarly, there was no follow-up to examine shifts in microbial communities upon resolution of pneumonia. Performance status (e.g. Eastern Cooperative Oncology Group, ECOG [32], other frailty measures such as the G8 assessment [33]) was not evaluated in this study. This would be an important addition to any future research to characterize the degree of frailty in the study population, as this may impact pneumonia risk. It was therefore not possible to determine if changes in the oral microbiome preceded pneumonia onset, a key step in determining causality [34].

The study participants were recruited pragmatically with inclusion criteria that were as open as feasible to ensure participants would be representative of the typical care home and respiratory ward populations. However, exclusion of certain groups such as those with severe cognitive impairment or major comorbidities and immunocompromise means that the study findings may be impacted by this selection bias, limiting the generalizability of the findings. All patients with suspected pneumonia received empirical antibiotic therapy according to local policy, which reflects the British Thoracic Society guidelines on the management of severe community-acquired pneumonia [20]. As only a low proportion of care home residents had received any antimicrobials in the preceding 30 days, differential antibiotic use is a potential confounder for the altered oral microbial composition seen. However, several factors suggest that while antibiotic use may have contributed to reduced community diversity and species richness, the differences cannot be entirely explained by antibiotic use alone. First, it would be expected that denture-associated biofilms would be least affected by antibiotic use compared with other oral sites, as biofilms may confer antimicrobial tolerance to constituent microbes [35]. Moreover, antibiotics must traverse the oral mucosal barrier, diffuse through the palatal microbial biofilm and then penetrate the denture-associated biofilm in sufficient concentration to perturb microbial communities.

It should be noted that *Enterobacteriaceae* are typically not susceptible to macrolide antibiotics such as clarithromycin and are intrinsically resistant to amoxicillin and other beta-lactamases [36]. The aggressive use of these antibiotic regimes in pneumonia patients may act as a selective pressure to suppress growth and survival of normal oral microbes, particularly *Streptococcaceae*, leading to an increased relative abundance of more virulent microorganisms [37]. Nonetheless, the finding that the difference in relative abundance of PRPs between cohorts was most pronounced in denture samples suggests that antibiotic use was unlikely to be the primary contributor to the changes in microbial community composition. Notably, no *Staphylococcus aureus* isolates recovered from respiratory ward patients were resistant to amoxicillin, compared to over one quarter of those from care home

residents. However, macrolide resistance was more than doubled in respiratory ward *Staphylococcus aureus* isolates. There were much higher rates of resistance to the beta-lactam antibiotic piperacillin-tazobactam in *P. aeruginosa* isolates from care home residents compared with pneumonia patients, as was seen for the related cephalosporin ceftazidime. However, resistance of *P. aeruginosa* isolates to ciprofloxacin, a fluoroquinolone antibiotic, was found to be much higher among pneumonia patients than among care home residents. While the low number of both *Staphylococcus aureus* and *P. aeruginosa* isolates recovered precludes any reliable statistical evaluation, the equivocal resistance patterns observed suggest that antibiotic treatment may not have exerted a major selective pressure upon the oral microbiota. This was particularly evident in the case of *Staphylococcus aureus* isolates, where amoxicillin-sensitive strains were isolated from respiratory ward patient samples despite empirical therapy with this agent.

CONCLUSIONS

This study revealed that perturbations within the denture-associated oral microbiome are associated with pneumonia. Having demonstrated an association between a deranged oral microbiome and an increase in the bioburden of putative respiratory pathogens, the premise for a causal association is established. Future research should aim to further disentangle the relationship between oral health, the oral microbiome and pneumonia pathogenesis, as well as assess the impact of effective oral and denture care on modulating the oral microbiome and decreasing pneumonia risk in susceptible individuals.

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Author contributions
J.T. contributed to conception, design, data acquisition and interpretation, drafted and critically revised the manuscript. A.S. contributed to data acquisition and interpretation, and critically revised the manuscript. C.H. contributed to design, data acquisition and interpretation, and critically revised the manuscript. M.J.W. contributed to conception, design, data interpretation and critically appraised the manuscript. J.L. contributed to conception, design and critically appraised the manuscript. M.W. contributed to conception, design and critically appraised the manuscript. D.W.W. contributed to conception, design, data interpretation and critically appraised the manuscript. All authors gave their final approval and agree to be accountable for all aspects of the work.

Conflicts of interest
The author(s) declare that there are no conflicts of interest.

Ethical statement
Ethical approval for this study was obtained from the Wales REC 6; reference 16/WA/0317. All participants gave informed consent for this study.

References

1. Lim SS, Vos T, Flaxman AD, Danaei G, Shibuya K, *et al*. A comparative risk assessment of burden of disease and injury attributable to 67 risk factors and risk factor clusters in 21 regions, 1990–2010: a systematic analysis for the Global Burden of Disease Study 2010. *The Lancet* 2012;380:2224–2260.
2. Welte T, Torres A, Nathwani D. Clinical and economic burden of community-acquired pneumonia among adults in Europe. *Thorax* 2012;67:71–79.
3. Myles PR, Hubbard RB, Gibson JE, Pogson Z, Smith CJP, *et al*. Pneumonia mortality in a UK general practice population cohort. *Eur J Public Health* 2009;19:521–526.
4. McLuckie A. *Respiratory Disease and Its Management*. London: Springer Science & Business Media; 2009.
5. Cillóniz C, Civljak R, Nicolini A, Torres A. Polymicrobial community-acquired pneumonia: an emerging entity. *Respirology* 2016;21:65–75.
6. Torres A, Blasi F, Peetermans WE, Viegi G, Welte T. The aetiology and antibiotic management of community-acquired pneumonia in adults in Europe: a literature review. *Eur J Clin Microbiol Infect Dis* 2014;33:1065–1079.
7. Torres A, Lee N, Cilloniz C, Vila J, Van der Eerden M. Laboratory diagnosis of pneumonia in the molecular age. *Eur Respir J* 2016;48:1764–1778.
8. Azarpazhooh A, Leake JL. Systematic review of the association between respiratory diseases and oral health. *J Periodontol* 2006;77:1465–1482.
9. O'Grady NP, Murray PR, Ames N. Preventing ventilator-associated pneumonia: does the evidence support the practice? *JAMA* 2012;307:2534–2539.
10. Paju S, Scannapieco FA. Oral biofilms, periodontitis, and pulmonary infections. *Oral Dis* 2007;13:508–512.
11. Awano S, Ansai T, Takata Y, Soh I, Akifusa S, *et al*. Oral health and mortality risk from pneumonia in the elderly. *J Dent Res* 2008;87:334–339.
12. Sands KM, Twigg JA, Lewis MAO, Wise MP, Marchesi JR, *et al*. Microbial profiling of dental plaque from mechanically ventilated patients. *J Med Microbiol* 2016;65:147–159.
13. Sands KM, Wilson MJ, Lewis MAO, Wise MP, Palmer N, *et al*. Respiratory pathogen colonization of dental plaque, the lower airways, and endotracheal tube biofilms during mechanical ventilation. *J Crit Care* 2017;37:30–37.
14. HuaF, XieH, Zhang Q, Li C, Worthington HV, *et al*. Oral hygiene care for critically ill patients to prevent ventilator-associated pneumonia. *Cochrane Database Syst Rev* 2020;12:CD008367.
15. Russell SL, Boylan RJ, Kaslick RS, Scannapieco FA, Katz RV. Respiratory pathogen colonization of the dental plaque of institutionalized elders. *Spec Care Dentist* 1999;19:128–134.

16. Sumi Y, Miura H, Michiwaki Y, Nagaosa S, Nagaya M. Colonization of dental plaque by respiratory pathogens in dependent elderly. *Arch Gerontol Geriatr* 2007;44:119–124.
17. O'Donnell LE, Smith K, Williams C, Nile CJ, Lappin DF, *et al*. Dentures are a reservoir for respiratory pathogens. *J Prosthodont* 2016;25:99–104.
18. Yoneyama T, Yoshida M, Ohrui T, Mukaiyama H, Okamoto H, *et al*. Oral care reduces pneumonia in older patients in nursing homes. *J Am Geriatr Soc* 2002;50:430–433.
19. Scannapieco FA, Binkley CJ. Modest reduction in risk for ventilator-associated pneumonia in critically ill patients receiving mechanical ventilation following topical oral chlorhexidine. *J Evid Based Dent Pract* 2012;12:15–17.
20. Lim WS, Baudouin SV, George RC, Hill AT, Jamieson C, *et al*. BTS guidelines for the management of community acquired pneumonia in adults: update 2009. *Thorax* 2009;64 Suppl 3:iii1–55.
21. Newton AV. Denture sore mouth: a possible aetiology. *Br Dent J* 1962;112:357–360.
22. Klein H, Palmer CE, Knutson JW. Studies on dental caries. *Public health rep* 1938;53:751–765.
23. The European Committee on Antimicrobial Susceptibility Testing Disc diffusion manual v9.0. EUCAST; 2021. http://www.eucast.org/ast_of_bacteria/disk_diffusion_methodology/ [accessed 14 February 2021].
24. Gadsby NJ, McHugh MP, Russell CD, Mark H, Conway Morris A, *et al*. Development of two real-time multiplex PCR assays for the detection and quantification of eight key bacterial pathogens in lower respiratory tract infections. *Clin Microbiol Infect* 2015;21:788.
25. R Development Core Team. R: A language and environment for statistical computing. c1999-2013. R Foundation for Stastical Computing; (n.d.). http://lib.stat.cmu.edu/R/CRAN/doc/manuals/r-devel/fullrefman.pdf [accessed 14 February 2021].
26. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, *et al*. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microbiol* 2009;75:7537–7541.
27. Charlson ME, Pompei P, Ales KL, MacKenzie CR. A new method of classifying prognostic comorbidity in longitudinal studies: development and validation. *J Chronic Dis* 1987;40:373–383.
28. Benjamini Y, Krieger A, Yekutieli D. Two staged linear step up FDR controlling procedure. Tel Aviv University, and Department of Statistics, Wharton School, University of Pennsylvania; 2001
29. Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, *et al*. Metagenomic biomarker discovery and explanation. *Genome Biol* 2011;12:R60.
30. Dickson RP, Huffnagle GB, Goldman WE. The lung microbiome: new principles for respiratory bacteriology in health and disease. *PLoS Pathog* 2015;11:e1004923.
31. Holter JC, Müller F, Bjørang O, Samdal HH, Marthinsen JB, *et al*. Etiology of community-acquired pneumonia and diagnostic yields of microbiological methods: a 3-year prospective study in Norway. *BMC Infect Dis* 2015;15:64.
32. Zubrod CG, Schneiderman M, Frei E, Brindley C, Lennard Gold G, *et al*. Appraisal of methods for the study of chemotherapy of cancer in man: Comparative therapeutic trial of nitrogen mustard and triethylene thiophosphoramide. *J Chronic Dis* 1960;11:7–33.
33. Bellera CA, Rainfray M, Mathoulin-Pélissier S, Mertens C, Delva F, *et al*. Screening older cancer patients: first evaluation of the G-8 geriatric screening tool. *Ann Oncol* 2012;23:2166–2172.
34. Hill AB. The environment and disease: association or causation *Proc R Soc Med* 1965;58:295–300.
35. Aggarwal S, Stewart PS, Hozalski RM. Biofilm cohesive strength as a basis for biofilm recalcitrance: are bacterial biofilms overdesigned? *Microbiol Spectr* 2015;8:29–32.
36. Bouza E, Cercenado E. Klebsiella and enterobacter: antibiotic resistance and treatment implications. *Semin Respir Infect* 2002;17:215–230.
37. Sommer MOA, Dantas G. Antibiotics and the resistant microbiome. *Curr Opin Microbiol* 2011;14:556–563.