

Research Article

In Vitro Assessment of Shiitake Mushroom (*Lentinula edodes*) Extract for Its Antigingivitis Activity

Lena Ciric,¹ Anna Tymon,¹ Egija Zaura,² Peter Lingström,³ Monica Stauder,⁴ Adele Papetti,⁵ Caterina Signoretto,⁶ Jonathan Pratten,¹ Michael Wilson,¹ and David Spratt¹

¹ Department of Microbial Diseases, UCL Eastman Dental Institute, 256 Gray's Inn Road, London WC1X 8LD, UK

² Department of Preventive Dentistry, Academic Centre for Dentistry Amsterdam (ACTA), Gustav Mahlerlaan 3004, 1081 LA Amsterdam, The Netherlands

³ Department of Cariology, Institute of Odontology at Sahlgrenska Academy, University of Gothenburg, 40530 Göteborg, Sweden

⁴ DIPTERIS, University of Genoa, Corso Europa 26, 16132 Genoa, Italy

⁵ Department of Drug Sciences, University of Pavia, Viale Taramelli 12, 27100 Pavia, Italy

⁶ Dipartimento di Patologia-Sezione di Microbiologia, Università di Verona, 37134 Verona, Italy

Correspondence should be addressed to Lena Ciric, l.ciric@ucl.ac.uk

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Gingivitis is a preventable disease characterised by inflammation of the gums due to the buildup of a microbial biofilm at the gingival margin. It is implicated as a precursor to periodontitis, a much more serious problem which includes associated bone loss. Unfortunately, due to poor oral hygiene among the general population, gingivitis is prevalent and results in high treatment costs. Consequently, the option of treating gingivitis using functional foods, which promote oral health, is an attractive one. Medicinal mushrooms, including shiitake, have long been known for their immune system boosting as well as antimicrobial effects; however, they have not been employed in the treatment of oral disease. In the current study, the effectiveness of shiitake mushroom extract was compared to that of the active component in the leading gingivitis mouthwash, containing chlorhexidine, in an artificial mouth model (constant depth film fermenter). The total bacterial numbers as well as numbers of eight key taxa in the oral community were investigated over time using multiplex qPCR. The results indicated that shiitake mushroom extract lowered the numbers of some pathogenic taxa without affecting the taxa associated with health, unlike chlorhexidine which has a limited effect on all taxa.

1. Introduction

Gingivitis is one of the most prevalent infectious diseases of humans, affecting most of the population at some point during their lives [1]. It is easily preventable by the removal of the plaque biofilm but often results in high treatment costs due to poor oral hygiene among the general population. Gingivitis has long been implicated as a potential precursor to periodontitis [2, 3] and is caused by the buildup of the plaque biofilm at the gingival margin. This in turn results in a shift in the resident microbiota as a consequence of environmental changes [4, 5]. The prevalence of *Actinomyces* spp., *Lactobacillus* spp., *Prevotella* spp., and *Fusobacterium nucleatum* is known to increase during gingivitis at the expense of *Streptococcus* spp. [6–9]. This community shift causes inflammation

of the gingiva as part of the immune response [3, 10, 11]. The disease can be prevented and alleviated by the removal of the plaque biofilm and by the use of oral hygiene products such as toothbrushes, toothpastes, and mouthwashes [12]. The constant depth film fermenter (CDFF) has been used previously to model the bacterial community shifts observed during gingivitis and has also been employed to assess the effects of oral hygiene products [13, 14].

Medicinal mushrooms, including *Lentinula edodes* or shiitake, have been used in Asia for centuries and have numerous health benefits. These range from their antioxidant properties, to lowering cholesterol and blood pressure, anti-tumor properties, and antibacterial and libido-enhancing properties [15–18]. The health benefits of shiitake mushrooms are thought to be so great that they have been

incorporated into some foods in order to be delivered to the population, creating functional foods including pork patties, cereals, and cookies [16, 19, 20]. However, shiitake has not as yet been assessed for its oral health benefits.

In recent years, high-throughput culture-independent quantitative methods have revolutionised the investigation of bacterial community structure. These methods are now being employed in the study of microbial communities involved in both oral health and disease [21–23]. In the present study, a set of assays developed previously was used to monitor the bacterial community structure changes within an *in vitro* gingivitis model and to assess the effect of shiitake mushroom extract and chlorhexidine, the leading agent used in the treatment of gum disease [24], on these communities.

2. Materials and Methods

2.1. Saliva Collection. Healthy individuals with good oral hygiene were asked to expectorate into a sterile centrifuge tube up to a volume of 2 mL. Saliva was collected from 20 individuals. The saliva samples were homogenised into pooled saliva, and glycerol was added to a final concentration of 10% v/v. The pooled saliva was dispensed into 1 mL aliquots and stored at -80°C .

2.2. CDFG Gingivitis Model. *In vitro* biofilms, representative of plaque that forms at the gingival margin, were cultured using a CDFG. The environmental conditions within the CDFG were modified in order to mimic those found during gingivitis, as described previously [14]. Briefly, the CDFG was inoculated by 1 mL of pooled saliva sample added to 500 mL artificial saliva medium [25] over 8 hours. The biofilms were cultured at 36°C for one week. The CDFG was kept under microaerophilic conditions using a gas mixture (2% O_2 ; 3% CO_2 ; 95% N_2 at $200 \times 10^5 \text{ Pa}$) pumped into the chamber through a filtered inlet at a rate of $200 \text{ cm}^3 \text{ min}^{-1}$. Artificial saliva medium and artificial gingival crevicular fluid [26] were pumped into the chamber throughout the experiment at a flow rate of $0.72 \text{ litres day}^{-1}$ and $0.072 \text{ litres day}^{-1}$, respectively.

No antimicrobials were pumped into the CDFG during the no treatment control (NTC) experiments. During the chlorhexidine (CHX) and mushroom extract (MUSH) experiments, 0.12% chlorhexidine and 2x low molecular weight shiitake mushroom extract were pumped into the CDFG from 80 h and every 12 hours thereafter to mimic the use of a mouthwash twice daily. Each pulse was pumped in at a rate of 2 mL min^{-1} for 5 minutes.

One pan, containing five disks, was removed aseptically every 24 hours. The biomass of two disks was collected as described previously [14] in duplicate. DNA extractions were then performed on the biomass collected.

2.3. Low Molecular Weight Shiitake Mushroom Extract Preparation. The 2x low molecular weight mushroom extract was prepared as described by Daglia et al. [27].

2.4. DNA Extraction Method. Total nucleic acids were extracted from all samples according to a previously described

protocol [28] using a bead-beating phenol: chloroform: isoamyl alcohol (25:24:1) extraction followed by a 30% PEG 6000 precipitation and 70% ethanol wash. This method was found to be the least biased towards the extraction of nucleic acids from Gram-negative organisms.

2.5. qPCR Method. Three triplex qPCR assays were designed to enumerate four organisms associated with gingivitis (*Actinomyces naeslundii*, *Fusobacterium nucleatum*, *Lactobacillus casei*, and *Prevotella intermedia*), three organisms associated with oral health (*Streptococcus sanguinis*, *Neisseria subflava*, and *Veillonella dispar*), one organism strongly implicated in dental caries (*Streptococcus mutans*), and all organisms as described previously [23]. The detection limits for each of the single taxa were 20 cells and the number rose to 600 cells for the universal assay.

2.6. Statistics. Data were normalised by transformation using \log_{10} . ANOVA analysis was used to test whether changes between the treatments were significant (significant $P \leq 0.005$; and slightly significant $P < 0.01$).

3. Results

3.1. Saliva Community. The microbial community present in the pooled saliva used as the inoculum for the CDFG was analysed using qPCR. The numbers of each of the taxa analysed are shown in Figure 1. The mean ($n = 3$) total number of organisms per millilitre of pooled saliva was found to be $1.01 (\pm 0.41) \times 10^9$ (standard deviation is shown in brackets). The specific taxa being investigated made up 3.75×10^8 of the organisms or 37.24% of the total. Of these taxa, the most numerous were *V. dispar* (22.8%), followed by *N. subflava* (12.1%) and *F. nucleatum* (1.8%), and the least is *L. casei* (0.05%). Very low variation was observed between the three saliva samples which were profiled.

3.2. Gingivitis CDFG Plaque Biofilm Communities. The data regarding the numbers of individual taxa analysed and the total number of bacteria present over the course of the treatment experiments is shown in Table 1.

3.2.1. No Treatment Control (NTC). There was little change in total numbers of organisms present over time, the numbers increased from around 10^7 at the start to around 10^8 cells per disk up to 72 hours and remaining at this level throughout the experiment. Numbers of *L. casei*, *P. intermedia*, and *A. naeslundii* were very low throughout. Other taxa increased over time (mainly between the 72 h sampling point and the 96 h point) by 3 \log_{10} , for example, *F. nucleatum* (from 0.007% to 5.399%), *V. dispar* (from 0.072% to 13.093%), and *N. subflava* (from 0.022% to 78.446%). *S. sanguinis* increased by 0.5 \log_{10} (0.139% to 0.379%). *S. mutans* was not detected at any time points.

3.2.2. Chlorhexidine Treatment (CHX). As with the NTC experiment, the total numbers of organisms remained broadly steady over the experiment. Numbers of *L. casei*, and *P. intermedia*, and *A. naeslundii* were found in similar levels

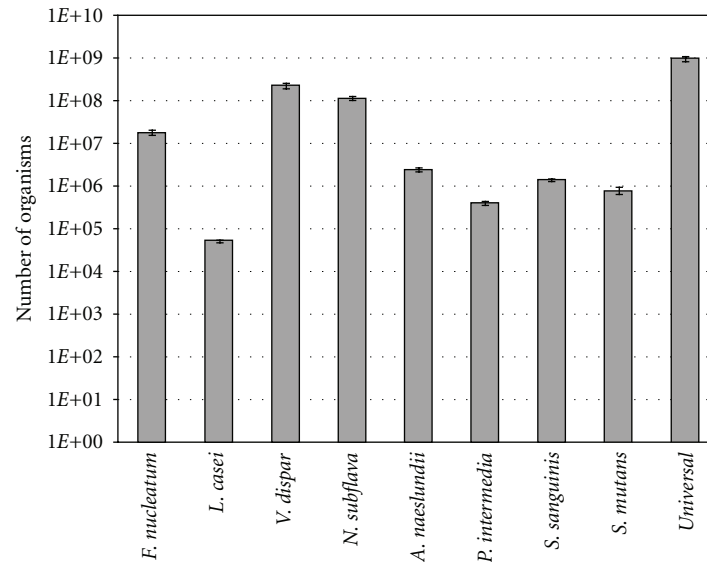


FIGURE 1: Numbers of each of the taxa investigated in pooled saliva. Error bars represent the standard deviation ($n = 3$).

as with the NTC experiment. However, numbers of *N. subflava* only rose by 1 log₁₀ throughout the experiment (from 3.838% to 10.919%), number of *V. dispar* and *S. sanguinis* remained similar (1.590% to 0.774%; 0.360% to 0.275%, resp.), and *F. nucleatum* decreased by around 1 log₁₀ (from 0.026% to 0.001%). *S. mutans* was not detected at any time points.

3.2.3. Mushroom Treatment (MUSH). Total numbers of organisms were found to be around 10⁸ cells per disk for the duration of the experiment. Numbers of *L. casei*, *P. intermedia*, and *A. naeslundii* were very low throughout. However, numbers of *N. subflava* rose by 3 log₁₀ (from 0.011% to 54.374%) throughout the experiment, number of *V. dispar* rose by 6 log₁₀ (0.000002% to 8.556%) throughout the experiment, and *F. nucleatum* remained steady (0.00001% to 0.00009%). *S. sanguinis* numbers rose by 2 log₁₀ (0.001% to 1.841%) throughout the experiment. *S. mutans* was not detected at any time points.

3.3. Comparison of Taxa Numbers between Treatments. *P. intermedia*, *L. casei*, and *A. naeslundii* numbers were found in low numbers during all three of the treatments with no significant differences between treatments at any time points. The numbers of *N. subflava* cells appeared to be lower during the CHX treatment from 72 h; however, no significant difference between treatments was found until the 168 h time point (NTC, $P = 0.010$; MUSH, $P = 0.004$) (Figure 2). *V. dispar* cell numbers were found to be significantly lower during the CHX treatment at 96 h, 120 h, and 168 h ($P \leq 0.003$, $P \leq 0.021$, and $P = 0.001$, resp.) (Figure 2).

S. sanguinis numbers were significantly higher during the MUSH treatment than during the CHX treatment at time points 48, 96, 120, and 168 hours ($P = 0.047$, $P = 0.052$, $P = 0.032$, and $P = 0.021$, resp.) (Figure 2). Numbers of *F. nucleatum* were found to be significantly lowered by

the MUSH and CHX treatments from 96 hours onwards ($P \leq 0.030$) (Figure 2). Finally, examining the universal assay cell numbers, the CHX experiment counts are significantly lower than those in the MUSH experiment at 48, 96, 120, and 168 hours ($P = 0.010$, $P = 0.019$, $P = 0.044$, and $P = 0.022$, resp.) (Figure 2).

4. Discussion

4.1. Saliva Community. The bacterial community found in salivary fluid is composed of the amalgamation of the communities found around the mouth. The predominant taxa were found to be *V. dispar*, *N. subflava*, *F. nucleatum*, *A. naeslundii*, and *S. sanguinis*. These taxa have all been associated with healthy dental plaque biofilms in previous culture independent studies [29, 30]. The tongue community in healthy subjects has previously been found to comprise mostly *Streptococcus* spp., *Veillonella* spp., and *Actinomyces* spp. [31, 32]. A recent study looking into the unculturable microbiota of the tongue has also identified the above genera, along with a *Lysobacter*-type species as the predominant organism found on the tongue [33].

Two studies using culture-independent molecular methods have shown that the dominant phyla most commonly found in saliva were Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, and Fusobacteria, respectively [22, 34]. The multitriplex qPCR method showed a similar picture: the Firmicutes were the dominant organisms, followed by Proteobacteria, Fusobacteria, Actinobacteria, and Bacteroidetes.

4.2. CDFF Plaque Biofilm Communities. Whilst the universal assay confirmed total cells numbers in the biofilms to be high in all of the CDFF experiments, some of the taxa investigated were only detected in low levels including *P. intermedia*, *L. casei*, *S. mutans*, and *A. naeslundii*. While *Actinomyces* spp. are known to be one of the early colonizers in the formation

TABLE 1: Numbers of each of the taxa investigated in biofilms grown in the gingivitis CDFF over one week under various treatments: Control ($n = 2$), Chlorhexidine ($n = 4$), and LMW mushroom ($n = 2$) pulsing.

Control							
	24 h	48 h	72 h	96 h	120 h	144 h	168 h
<i>F. nucleatum</i>	4.68 (± 0.71) $\times 10^3$	1.38 (± 0.11) $\times 10^3$	5.39 (± 0.41) $\times 10^3$	5.07 (± 0.05) $\times 10^5$	2.99 (± 1.82) $\times 10^6$	3.31 (± 0.87) $\times 10^6$	6.26 (± 0.30) $\times 10^6$
<i>L. casei</i>	6.20 (± 4.81) $\times 10^2$	7.80 (± 4.53) $\times 10^2$	7.80 (± 4.53) $\times 10^2$	8.20 (± 9.05) $\times 10^2$	9.10 (± 9.76) $\times 10^2$	9.80 (± 9.33) $\times 10^2$	9.00 (± 10.7) $\times 10^2$
<i>V. dispar</i>	4.91 (± 4.13) $\times 10^4$	2.29 (± 2.18) $\times 10^5$	2.65 (± 1.41) $\times 10^6$	7.83 (± 3.74) $\times 10^6$	8.94 (± 7.24) $\times 10^6$	8.28 (± 3.96) $\times 10^6$	1.52 (± 0.26) $\times 10^7$
<i>N. subflava</i>	1.52 (± 0.72) $\times 10^4$	2.43 (± 1.80) $\times 10^5$	2.49 (± 1.70) $\times 10^7$	3.69 (± 1.79) $\times 10^7$	6.49 (± 5.97) $\times 10^7$	5.51 (± 4.14) $\times 10^7$	9.10 (± 3.74) $\times 10^7$
<i>A. naeslundii</i>	6.00 (± 8.49) $\times 10^1$	4.00 (± 5.66) $\times 10^1$	1.00 (± 1.41) $\times 10^1$	2.80 (± 1.70) $\times 10^2$	1.60 (± 0.28) $\times 10^2$	1.40 (± 0.57) $\times 10^2$	2.40 (± 3.11) $\times 10^2$
<i>P. intermedia</i>	5.00 (± 1.41) $\times 10^1$	5.00 (± 1.41) $\times 10^1$	6.00 (± 0.00) $\times 10^1$	4.00 (± 2.83) $\times 10^1$	4.00 (± 2.83) $\times 10^1$	3.00 (± 1.41) $\times 10^1$	5.00 (± 1.41) $\times 10^1$
<i>S. sanguinis</i>	9.49 (± 1.65) $\times 10^4$	8.05 (± 1.41) $\times 10^4$	1.39 (± 0.59) $\times 10^6$	5.63 (± 0.99) $\times 10^5$	3.15 (± 1.63) $\times 10^5$	5.18 (± 3.05) $\times 10^5$	4.40 (± 1.71) $\times 10^5$
Universal	6.81 (± 2.59) $\times 10^7$	3.48 (± 2.22) $\times 10^7$	1.56 (± 1.02) $\times 10^8$	1.72 (± 1.08) $\times 10^8$	1.51 (± 1.28) $\times 10^8$	1.30 (± 0.83) $\times 10^8$	1.61 (± 0.01) $\times 10^8$
Chlorhexidine							
	24 h	48 h	72 h	96 h	120 h	144 h	168 h
<i>F. nucleatum</i>	5.28 (± 8.28) $\times 10^3$	8.25 (± 9.77) $\times 10^2$	2.05 (± 4.10) $\times 10^2$	6.40 (± 7.18) $\times 10^2$	6.00 (± 6.37) $\times 10^2$	2.80 (± 5.20) $\times 10^2$	5.05 (± 4.71) $\times 10^2$
<i>L. casei</i>	3.60 (± 5.80) $\times 10^2$	2.60 (± 3.81) $\times 10^2$	6.50 (± 13.0) $\times 10^1$	3.20 (± 5.13) $\times 10^2$	2.60 (± 3.59) $\times 10^2$	6.00 (± 10.7) $\times 10^1$	3.15 (± 3.00) $\times 10^2$
<i>V. dispar</i>	3.28 (± 5.02) $\times 10^5$	4.05 (± 5.69) $\times 10^5$	3.53 (± 3.35) $\times 10^5$	4.86 (± 2.24) $\times 10^5$	2.08 (± 1.93) $\times 10^5$	7.02 (± 7.24) $\times 10^5$	5.65 (± 2.77) $\times 10^5$
<i>N. subflava</i>	7.91 (± 10.0) $\times 10^5$	5.98 (± 8.67) $\times 10^5$	4.80 (± 4.91) $\times 10^6$	9.34 (± 12.1) $\times 10^6$	6.95 (± 7.91) $\times 10^6$	1.08 (± 1.21) $\times 10^7$	7.97 (± 4.94) $\times 10^6$
<i>A. naeslundii</i>	3.88 (± 3.47) $\times 10^1$	1.15 (± 1.92) $\times 10^1$	0.00 (± 0.00) $\times 10^0$	1.50 (± 3.00) $\times 10^1$	1.00 (± 2.00) $\times 10^1$	1.50 (± 3.00) $\times 10^1$	9.75 (± 12.1) $\times 10^0$
<i>P. intermedia</i>	4.00 (± 4.90) $\times 10^1$	4.00 (± 4.32) $\times 10^1$	2.50 (± 3.79) $\times 10^1$	5.50 (± 5.26) $\times 10^1$	4.50 (± 4.43) $\times 10^1$	2.00 (± 2.83) $\times 10^1$	3.50 (± 3.00) $\times 10^1$
<i>S. sanguinis</i>	7.41 (± 8.43) $\times 10^4$	2.25 (± 2.39) $\times 10^4$	5.75 (± 6.38) $\times 10^4$	1.39 (± 1.52) $\times 10^5$	7.38 (± 7.62) $\times 10^4$	9.35 (± 10.3) $\times 10^4$	2.01 (± 1.69) $\times 10^5$
Universal	2.06 (± 2.57) $\times 10^7$	1.49 (± 0.47) $\times 10^7$	2.99 (± 2.57) $\times 10^7$	5.01 (± 1.91) $\times 10^7$	4.33 (± 2.17) $\times 10^7$	6.75 (± 4.92) $\times 10^7$	7.30 (± 2.85) $\times 10^7$
LMW mushroom							
	24 h	48 h	72 h	96 h	120 h	144 h	168 h
<i>F. nucleatum</i>	3.30 (± 3.82) $\times 10^2$	5.40 (± 2.26) $\times 10^2$	3.40 (± 3.68) $\times 10^2$	3.10 (± 2.12) $\times 10^2$	6.60 (± 9.33) $\times 10^2$	3.40 (± 3.96) $\times 10^2$	2.40 (± 3.11) $\times 10^2$
<i>L. casei</i>	1.05 (± 1.34) $\times 10^1$	5.10 (± 3.54) $\times 10^2$	1.90 (± 1.27) $\times 10^2$	5.00 (± 7.07) $\times 10^1$	5.00 (± 7.07) $\times 10^1$	7.00 (± 9.90) $\times 10^1$	5.00 (± 7.07) $\times 10^{-1}$
<i>V. dispar</i>	8.05 (± 11.2) $\times 10^1$	1.32 (± 0.29) $\times 10^4$	6.73 (± 0.05) $\times 10^6$	6.92 (± 2.18) $\times 10^6$	1.46 (± 0.65) $\times 10^7$	2.43 (± 1.19) $\times 10^7$	2.35 (± 1.09) $\times 10^7$
<i>N. subflava</i>	4.86 (± 1.78) $\times 10^5$	2.20 (± 1.40) $\times 10^7$	5.39 (± 2.25) $\times 10^7$	7.10 (± 3.29) $\times 10^7$	1.26 (± 0.65) $\times 10^8$	1.40 (± 0.83) $\times 10^8$	1.50 (± 0.50) $\times 10^8$
<i>A. naeslundii</i>	0.00 (± 0.00) $\times 10^0$	2.00 (± 2.83) $\times 10^1$	2.20 (± 0.57) $\times 10^2$	3.00 (± 1.41) $\times 10^1$	0.00 (± 0.00) $\times 10^0$	0.00 (± 0.00) $\times 10^0$	0.00 (± 0.00) $\times 10^0$
<i>P. intermedia</i>	1.00 (± 1.41) $\times 10^1$	1.00 (± 1.41) $\times 10^1$	1.00 (± 1.41) $\times 10^1$	2.00 (± 2.83) $\times 10^1$	3.00 (± 4.24) $\times 10^1$	1.00 (± 1.41) $\times 10^1$	2.00 (± 2.83) $\times 10^1$
<i>S. sanguinis</i>	5.99 (± 4.29) $\times 10^4$	8.67 (± 3.87) $\times 10^5$	3.72 (± 0.76) $\times 10^6$	6.36 (± 2.07) $\times 10^6$	6.55 (± 1.97) $\times 10^6$	5.99 (± 2.97) $\times 10^6$	5.06 (± 1.58) $\times 10^6$
Universal	4.60 (± 2.56) $\times 10^7$	8.96 (± 1.11) $\times 10^7$	1.98 (± 0.69) $\times 10^8$	2.88 (± 0.39) $\times 10^8$	3.80 (± 1.04) $\times 10^8$	3.25 (± 0.12) $\times 10^8$	2.75 (± 0.59) $\times 10^8$

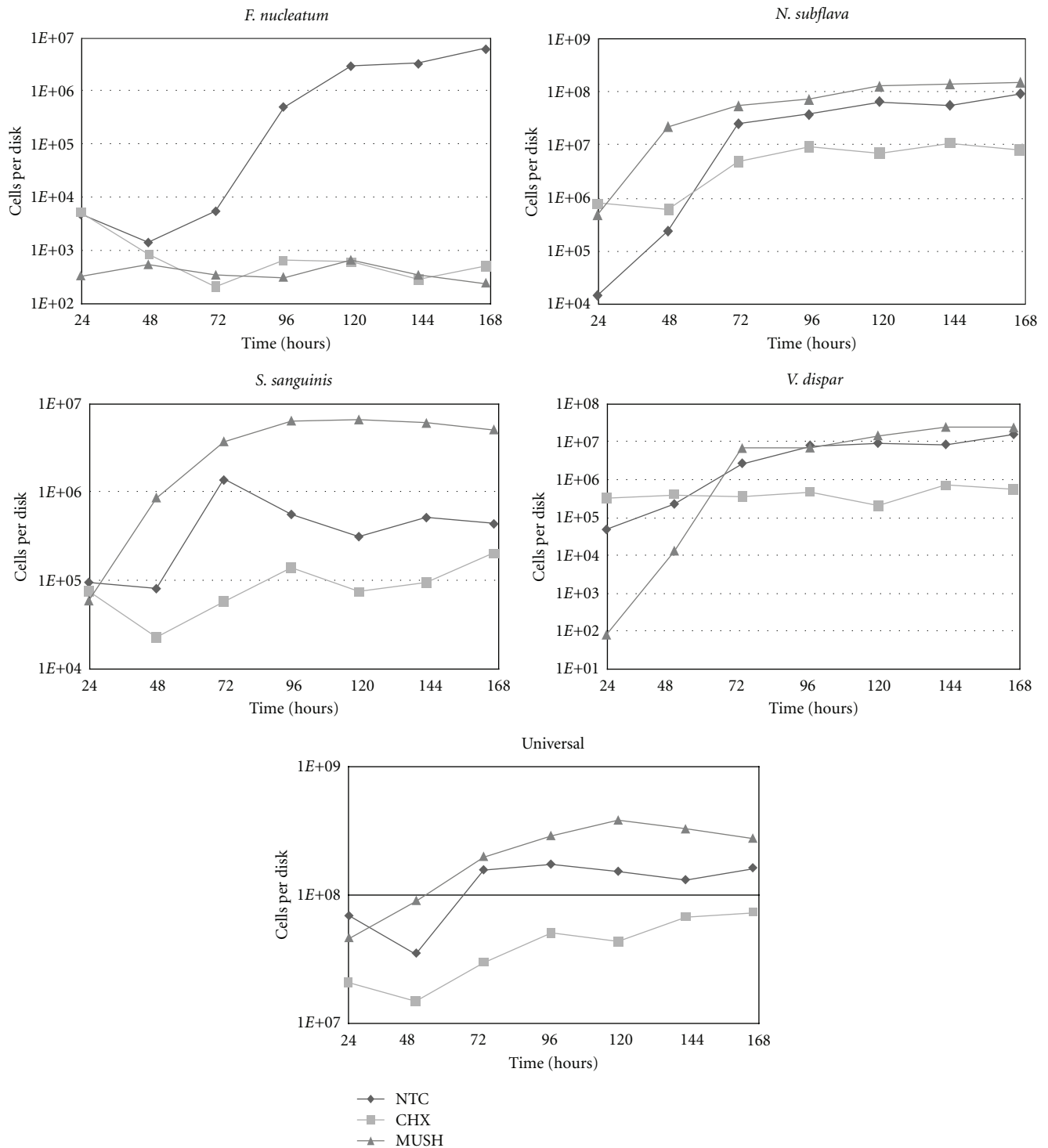


FIGURE 2: *F. nucleatum*, *N. subflava*, *S. sanguinis*, *V. dispar*, and total bacterial cell numbers which displayed significant differences between the different treatments.

of dental plaque [35], *A. naeslundii* is only one species representative of this genus. It is likely that the environmental conditions within the CDFE experiments were not optimal for the above taxon, but other members of the genus may have been present. Previous studies have found that *L. casei*, *S. mutans*, and *A. naeslundii* all grow well in biofilms cultured

using saliva and the addition of a carbohydrate such as glucose or sucrose [22, 36]. The lack of glucose or sucrose in the culture media in the present study could account for the low detection rates of these organisms. A previous study has shown that *Prevotella* spp. were detectable in the CDFE inoculum but not during the duration of the experiment

using molecular methods [37], supporting the data from the current study where the pathogen was detected at very low levels throughout.

The organisms found in consistently high numbers from the beginning of all of the experiments were *N. subflava*, *S. sanguinis*, and *V. dispar*. All of these organisms have been shown to be early colonizers during the formation of dental plaque as well as being among the most abundant taxa in the oral cavity [34, 35, 38]. *F. nucleatum* numbers increased at a slightly later stage of plaque biofilm formation once the environmental conditions were optimal [39], as seen in the NTC experiment designed to mimic conditions during gingivitis.

Gingivitis is caused by the buildup of the plaque biofilm at the gingival margin, which in turn results in a shift in the resident microbiota as a consequence of environmental changes [4, 5]. The prevalence of *Actinomyces* spp., *Lactobacillus* spp., *Prevotella* spp., and *F. nucleatum* is known to increase during gingivitis at the expense of *Streptococcus* spp. [6, 7, 9]. It was apparent that numbers of *F. nucleatum* rose over time in the NTC experiment and that *S. sanguinis* numbers declined after an initial peak at 72 h coinciding with the *F. nucleatum* increase.

Looking at the treatment effects, the application of chlorhexidine significantly lowered the numbers of *N. subflava*, *V. dispar*, and *F. nucleatum* compared to NTC. The total cell numbers were also lower during the CHX treatment, no doubt in part due to the lower numbers of the above taxa. Chlorhexidine is considered the gold standard [24] in the treatment of gum disease, and its action has been well studied. Previous studies looking at the effects of chlorhexidine on plaque biofilms *in vitro* have shown an effect on *Veillonella* sp., *Fusobacterium* sp., and *Streptococcus* sp. numbers [22, 25], supported by the current study. The MUSH treatment significantly lowered the numbers of *F. nucleatum*, an oral pathogen, but also resulted in significantly higher numbers of *S. sanguinis*, normally associated with oral health, when compared to the CHX treatment. This increase in *S. sanguinis* numbers despite the gingivitis conditions in the CDFF is an important effect. Furthermore, the MUSH treatment did not have a negative effect on *N. subflava* and *V. dispar*, both organisms associated with oral health [34, 38]. The data presented in the current study are supported by previous research which demonstrated the antimicrobial effects of shiitake mushroom products on a number of Gram-positive and negative organisms including some oral pathogens [17, 40, 41].

In conclusion, the comparison of the different treatments using the CDFF has given a valuable insight into the community dynamics of dental plaque as well as an indication of the efficacy of the treatments. Chlorhexidine was found to be effective at lowering a number of taxa, associated with both health and disease; however, shiitake mushroom extract was shown to be effective at reducing the numbers of the oral pathogen *F. nucleatum*, while having little effect on some of the taxa associated with health. The results imply that the action of shiitake mushroom extract should be investigated further for its beneficial effects on oral health.

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