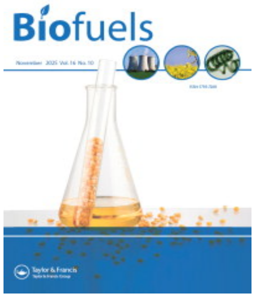

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# Comparison of microbial contaminants in conventional, synthetic and sustainable aviation fuels

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**ABSTRACT**
Microbial contamination in aviation fuel systems can foul sensors, blocking filters, degrade fuel and cause corrosion, requiring regular testing. The shift from fossil fuel sources to synthetic fuels may influence the composition of contaminating microbial communities. This study examines how different aviation fuels influence the microbiome, including sustainable aviation fuels (SAFs), which have been proposed as means of reducing aviation's carbon footprint, as well as diversifying the fuel supply chain. In this study we examined changes in the microbiomes of two communities when cultured with different fuels (conventional kerosene, synthetic and sustainable fuel blends). Microbial growth and community composition was determined by weighing microbial biomass, quantitative PCR and high-throughput DNA sequencing. Microbial communities grew in all fuels tested, but was somewhat reduced in microcosms supplied with Farnesane or Gevo. Although there were numerical differences in the relative abundance of community members, there was no major change in community membership or in the dominant contaminants across the studied fuels. This indicates that culturing with synthetic aviation fuels did not result in significant alterations in the fuel microbiome. However, contaminating microbiomes are known to be highly diverse and this potential issue should be monitored closely by the fuel industry.

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Sustainable aviation fuels (SAFs); synthetic blending components (SBCs); microbial contamination; high-throughput DNA sequencing

## Introduction

The global aviation industry has grown steadily over the past decades and is a key driver of global economic development [1]. However, this industry, like many others that rely on traditional petrochemical fuel stocks, is facing multiple challenges [2]. These include increased global demand for energy, increasingly limited petrochemical feedstocks and the pressing need to reduce global carbon dioxide ($CO_2$) emissions to limit climate change [3].

The aviation industry is estimated to contribute approximately 2.4% of the global $CO_2$ emissions, with this figure due to increase because of the increasing reliance on air transportation for commercial and personal travel [4,5]. Many stakeholders have supported the creation of targets to reduce $CO_2$ emissions by the aviation industry. One of the key strategies to achieve this goal is the use of Sustainable Aviation Fuels (SAFs). Conventional aviation fuels are produced by refining liquid fossil fuel sources. Synthetic aviation fuels, such as those produced from methane (Gas-to-liquid GTL), can be made from fossil or biological sources. The ASTM International has approved a range of non-fossil-fuel-based jet fuel pathways with Sustainable Aviation Fuels (SAFs) being derived from non-fossil fuel sources, but also requiring a lifecycle reduction in carbon emissions, no deforestation and no competition for needed food production [6]. Therefore, not all biologically-derived fuels (biofuels) will meet the criteria to be considered a SAF. Current SAFs include fuels derived from hydroprocessed esters and fatty acids (HEFA) from

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biologically-sourced oils or fats, non-HEFA pathways derived from wastes, and those utilising low carbon power sources. The International Air Transport Association (IATA) states that SAFs have the highest potential to reduce aviation $CO_2$ emissions, with a typical 80% reduction when compared with conventional fuels and alternative aviation fuels such as GTL [4,7].

The introduction of new fuels will impact many areas of aviation fuel systems including supply, storage, usage and certification. SAFs and synthetic fuels are derived from non-conventional feedstocks, utilise a variety of processing technologies and, as such, could differ substantially in terms of the types and proportions of hydrocarbons available to support the growth of fungi, yeasts and bacteria commonly found in fuel systems [4,8]. The microbiome of fuel is of importance not just to the aviation industry, but also to the wider fuel community, as microbial contamination can result in fuel spoilage, microbially-induced corrosion, equipment malfunction or failure, all of which represent significant threats to the safety and security of these systems [9,10]. Currently, eight different Synthetic Blending Components (SBCs) deriving from various renewable feedstocks have been approved for blending with conventional jet fuel to produce SAFs [11]. In this study, we explore the impact of three different sustainable blends on the growth of microbial contaminants.

The use of new fuels could lead to an alteration in the composition of the microbiome in fuel, fuel-associated water and sessile biofilms, subsequently requiring an adjustment of monitoring and testing regimes for the microbial contamination in these systems. The aviation industry currently uses different methodologies to detect microbial contamination during aircraft maintenance operations, and it is important to ensure that these methods remain accurate and reliable. Current testing regimes include both culture-dependent and culture-independent methods. Culture-based methods include IP385 [12] that utilises growth on nutrient media [13] and thixotropic gels [14]. Culture-independent methods include the monitoring of adenosine triphosphate (ATP) produced by microorganisms [15,16] or detecting antigenic material using lateral flow devices [17]. Each method has advantages and disadvantages and assesses the level of contamination using different metrics such as enzymatic reactions, antibody/antigen interactions or assessment of growth. As such, alterations in the microbiome composition due to the introduction of synthetic fuels and SAFs has the potential to influence the performance of the tests. Therefore, as a first step, understanding the impact of these fuels on microbial community composition is vital.

In this study, we explore the impact of conventional, synthetic fuels and SAFs on microbial community composition, using biomass measurements and culture-independent techniques. There is a small body of literature exploring the microbiome of conventional Jet A-1 aviation fuel [10,18–20] and biodiesels [21,22], but few exploring the impact of synthetic fuels. Ruiz et al. [23] used metagenomics and culture-based methods to study the effect of different fuels and blends (conventional diesels and jet fuels, renewable jet fuel (camelina-derived, hydro processed HRJ-5) and renewable diesels (algal-derived, hydro processed F76 and catalytic hydrothermal conversion CHCD)) on bacterial communities derived from seawater although these systems tended to go anaerobic and are more representative of marine sediment. Lobato et al. [24] studied how two fungal isolates grew when supplied with conventional jet fuel, farnesane or a blend. This study is, to the best of our knowledge, one of the first that examines the impact of a range of potential or commercially-available SAFs and synthetic aviation fuels on the growth of mixed microbial communities as assessed by deep sequencing approaches and biomass measurements. Further, it presents an analysis of the most dominant microbial contaminants across the tested fuels, discussing the effectiveness of current fuel microbiological tests.

## Methods

### *Inocula*

Starting inocula were established from bottom water samples taken from contaminated fuel storage tanks. Community 1 (C1) came from the UK whilst community 2 (C2) came from the Netherlands. These were diluted 1:3 with Bushnell Haas medium (Sigma-Aldrich Ltd, UK), overlaid with conventional Jet A-1 fuel and incubated for 3 months at 25 °C. The medium provided all of the elements required for microbial growth except for a carbon source, which came from the fuel. One hundred μL of each of these

**Table 1.** Fuel types used in this study.

| | |
|---|---|
| **Conventional** | |
| Jet A-1 Hydro-treated (A) and (B) | Oxygen and other heteroatoms removed<br>A was supplied by Shell, B came from directly from an airport |
| Jet A-1 Merox treated | Mercaptans removed |
| **Synthetic** | |
| Fischer–Tropsch isoparaffinic (GTL) | Iso-paraffinic; gas-to-liquid kerosene |
| **Sustainable Aviation Fuel (SAF) or Synthetic Blending Component (SBC)** | |
| Distilled Farnesane | Single component (sesquiterpene compound) |
| Gevo Jet (Alcohol-to-Jet) | Renewable bio jet |
| Hydrotreated renewable jet fuel (camelina silva) | Isoparaffin-rich; camelina-derived |

communities was used to inoculate microcosms in sterile jars containing 70 mL of Bushnell Haas Broth (Sigma-Aldrich, UK) and 30 mL of Jet A-1.

## *Microcosms*

Eight aviation fuels were selected for this study, including conventional Jet A-1 fuels, synthetic fuels and SAFs. These fuels were provided to the University of Sheffield by Royal Dutch Shell PLC and are described in Table 1. Fuels were filtered through Attaplagus clay to remove oxidised compounds and the antioxidant 2,6-di-tert-butyl-4 (dimethylaminomethyl)phenol added at 24 mg $L^{-1}$ (Sigma Aldrich, UK). Fuels were sterilised using a 0.22 μm nitrocellulose filter (Merck Millipore, USA) whilst Bushnell Haas medium was autoclaved.

Microcosms were set up in 20 mL sterile glass Universal tubes using 7 mL of Bushnell Haas medium and 3 mL of the selected fuel. These were inoculated with 100 μL of community C1 or C2. Four biological replicates were produced for each condition. Microcosms were incubated at 25 °C and sampled destructively after 2 and 4 weeks.

Fuels were characterised by GC-MS. Fuels were diluted 1:10 in hexane and analysed using a Clarus SQ 8 T GC-MS (Perkin Elmer) operating in electron impact mode. Experimental conditions were as follows: Elite-5MS (Perkin Elmer) column with dimensions 30 m, 0.25 mm ID, 0.25 μm film thickness. Helium was used as the carrier gas at a constant flow rate of 1 mL $min^{-1}$. Injection volume was 2 μL with a split ratio of 1:50. Initial oven temperature was held at 40 °C for 3 min then ramped at 5 °C per minute to 150 °C, then ramped at 40 °C per min to a final temperature of 280 °C. The final temperature was held for 2 min. Data was acquired over a mass range of 40 to 500 Da. GC-MS traces are shown in Supplementary Figure 1.

## *Microbial biomass*

Microbial biomass after 4 weeks of growth was determined by filtering 1 mL of the aqueous phase of each microcosm through a pre-weighed 0.22 μm nitrocellulose filter (Merck Millipore, USA) and re-weighing using a Ohaus Pioneer PX224 Analytical Balance (Ohaus United States). Before re-weighing, filters were washed with heptane 99% (Sigma-Aldrich, UK) to remove excess fuel, placed in a Envair Coalsafe ELITE Fume Cupboard (SLS, Nottingham UK) for 2 h to allow heptane to evaporate, and then dried for 24 h at 40 °C.

## *DNA extraction*

For DNA extraction, 2 mL of the aqueous phase was collected on a 0.22 μm nitrocellulose filter (Merck Millipore, USA). The filter was the transferred into a 2 mL microcentrifuge tube containing acid-washed glass beads (≤106 μm, Sigma-Aldrich, UK) and 150 μL of 1 M sorbitol, 500 mM EDTA pH 8.0 and 10 mM 2-mercaptoethanol. The samples were then agitated using a Genie 2 Vortex with microtube adaptor for 5 min. Fifty units of lyticase from *Arthrobacter luteus,* free of DNA contaminants (Sigma-Aldrich, UK) were added to each sample and incubated in a water bath for 30 min at 30 °C. The lyticase treatment step combined with mechanical lysis increases the DNA yield from fungal cells [25]. DNA was extracted using DNeasy PowerSoil Pro Kit (Qiagen) according to the manufacturer's instructions with an additional thermal shock step added during the mechanical agitation stage: 60 s agitation followed by 60 s on ice, repeated 5 times. The DNA was then eluted in 60 µL 10 mM Tris buffer (DNeasy PowerSoil Pro Kit elution solution). DNA was quantified using a Qubit™ dsDNA HS Assay Kit and a Qubit Fluorometer (ThermoFisher Scientific).

### Quantification of prokaryotic and eukaryotic DNA (real-time quantitative PCR)

Quantitative PCR (q-PCR) was used to determine the quantity of eukaryotic and prokaryotic DNA. The V1–V3 region of the 16S rRNA gene was amplified with the primer pair 28 F/519R [26,27]. The internal transcribed spacer (ITS) region was targeted using the ITS1/ITS2 primer pair [28]. q-PCR was performed in a final reaction volume of 10 µL containing 1 µL of DNA extract, 5 µL SensiFAST™ SYBR® No-ROX Kit (Bioline, United Kingdom) and 40 nM primers. Amplifications were performed in a Biorad CFX96 Touch Real-Time PCR System (California, United States) with 3 min initial denaturation at 95 °C, then 40 cycles at 95 °C for 0.5 s, 60 °C for 30 s, followed by 95 °C for 0.5 s, with a final melt curve from 65 °C for 0.5 s to 95 °C in 0.5 °C increments.

Standard curves were prepared using DNA from *Pseudomonas aeruginosa* (NCIMB 9571) or *Candida tropicalis* AL-6981-X (ATCC 48138). The *P. aeruginosa* genome contains 4 copies of the 16S rRNA gene [29] and the *C. tropicalis* genome 3 copies of the ITS regions [30].

### High-throughput DNA sequencing

PCR reactions were performed using 2 µL of DNA, 20 µL of Phusion Flash High-Fidelity PCR master mix (Thermo Scientific), 40 nM of either 16S or ITS primers in a final volume of 40 µL. The primers had Illumina adapters added:

forward: 5′TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3′
reverse: 5′GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-3′.

Amplification conditions were 98 °C for 1 min, 35 cycles of 94 °C for 10 s, 60 °C for 15 s and 72 °C for 30 s with a final extension at 72 °C for 7 min. Amplicons were checked by electrophoresis through 1.5% (w/v) agarose gels. Not all samples contained detectable fungal DNA, so sequencing of ITS amplicons from these samples was not performed. Amplicons were sent to RTL Genomics (Lubbock Texas USA) for purification, MiSeq index attachment and Illumina MiSeq sequencing.

### Bioinformatics

Sequencing data were provided as Fastq files with Illumina primers and indices removed. Data were processed using dada2 [31] version 1.16.0. Taxonomies were assigned by comparison to the SILVA non-redundant 16S rRNA data set (99%, version 138) and UNITE ITS database (release 8.2) [32].

For the 16S rRNA sequences, the amplification primers were removed, and sequences filtered for quality (maxEE = 2 and 5 for forward and reverse sequences, respectively). Errors were corrected and then sequence pairs merged. Chimeric sequences were identified using the 'consensus' method and removed. Pairs were assessed for error rates, corrections applied and then merged as appropriate. Chimeric sequences were removed using the consensus method and sequences less than 450 bp long removed. A phylogenetic tree was created using the optimised Neighbour-Joining method using the R package 'phangorn' (version 2.6.3) [33]. Fungal sequences were processed in a similar way except only the forward sequences were analysed (as there was insufficient overlap between the forward and reverse reads on the more variable length ITS amplicons) and no phylogenetic tree was created as ITS sequences are not phylogenetically informative. Fungal sequences longer than 250 bp were retained.

Further analysis was performed in R using the phyloseq package [34]. Samples were filtered for read depth (minimum of 5000 for bacteria and 1000 for fungi). Dominant ASVs were selected with an abundance greater than 1% (bacteria) or 3% (fungi) in any sample. Statistical analysis of sequences that differed significantly between samples were performed using generalized linear models implemented using DESeq2 [35].

### Statistical analysis

All additional statistical analyses were performed in R [36]. Linear models were calculated and contrasts were compared using the package 'emmeans' [37] with compact letter displays calculated using the package 'multcomp' [38].

## Results

### Biomass accumulation

The dry weight of samples after 4 weeks of growth is shown in Figure 1A. (The biomass after 2 weeks' growth was too low to measure reliably and would have benefited from a greater volume being filtered). Microbial growth was evident in all microcosms containing fuel – there was no growth in the controls containing BH medium without fuel. There was no significant difference (Kruskal-Wallis test, df $= 15$, $p = 0.672$) in total biomass between fuel types or the two starting communities.

DNA was extracted from each microcosm after 2 and 4 weeks, and the amount of bacterial and fungal DNA quantified using q-PCR against 16S rRNA and ITS genes respectively (Figure 1B). Bacterial 16S rRNA genes were much more abundant (mean 5 x $10^7$ copies $mL^{-1}$) compared to eukaryotic ITS genes (mean 41 copies $mL^{-1}$). For eukaryotic ITS genes, there was no significant difference between fuel types, or week of harvest, but community 2 samples contained a mean of 81 ITS copies $mL^{-1}$ compared to 22 ITS copies $mL^{-1}$ in community 1 ($p < 0.001$). In contrast, there was no difference in 16S copies $mL^{-1}$ between communities ($p = 0.17$), a small difference between week 2 and week 4 ($p = 0.029$) and differences between fuels ($p = 0.0028$). Post-hoc analysis showed that 16S rRNA copies $mL^{-1}$ were lower in microcosms containing Farnesane and Gevo blend than the other fuels. These data were not corrected for potential differences in rRNA or ITS copy number in the target organisms as uncertainties in the true copy numbers expected in diverse microbial populations do not improve the reliability of metataxonomic surveys [39]. These differences are likely to be more marked in fungal communities [40] but the overall fungal abundance was very low in these samples.

### High-throughput sequencing of microbial communities

High-throughput sequencing of 16S rRNA and ITS amplicons was used to investigate the impact of different fuel types on microbial community composition. For the bacterial 16S rRNA amplicons, high quality sequence data was obtained for the majority of samples. However, for the ITS amplicons, very few sequences were obtained for community 1, reflecting the low biomass of eukaryotic microbes in these samples, and some samples contained few amplifiable ITS sequences for community 2. Therefore, results are presented for all bacterial samples, but only a limited number of Community 2 for fungal samples.

Community composition and Principal Components Analysis are shown in Figure 2. Generalised linear models were used to identify statistically significant differences between bacteria.

The initial inocula of both communities were dominated by bacteria in the Family Pseudomonadaceae, with contributions from Comamonadaceae, Xanthomonadaceae and Aquaspirillaceae in community 1 and Enterobacteriaceae in community 2. The fungal inoculum in community 2 was composed mainly of the Genera *Amorphotheca resinae* and *Cadophora melinii*. The number of sequences obtained from fungal communities was low for many samples, hence a comprehensive analysis of the impact of different fuel types on fungal growth was not possible, but in most samples, *A. resinae* and *C. melinii* remained dominant with additional contributions from *Hawksworthiomyces taylorii*.

When incubated with different fuel types, the complexity of the bacterial communities tended to fall after 2 week's incubation, with Pseudomonadaceae dominant, and then recover (Supplementary Figure 2). With community 1, microcosms containing Farnesane and Gevo behaved differently from the rest of

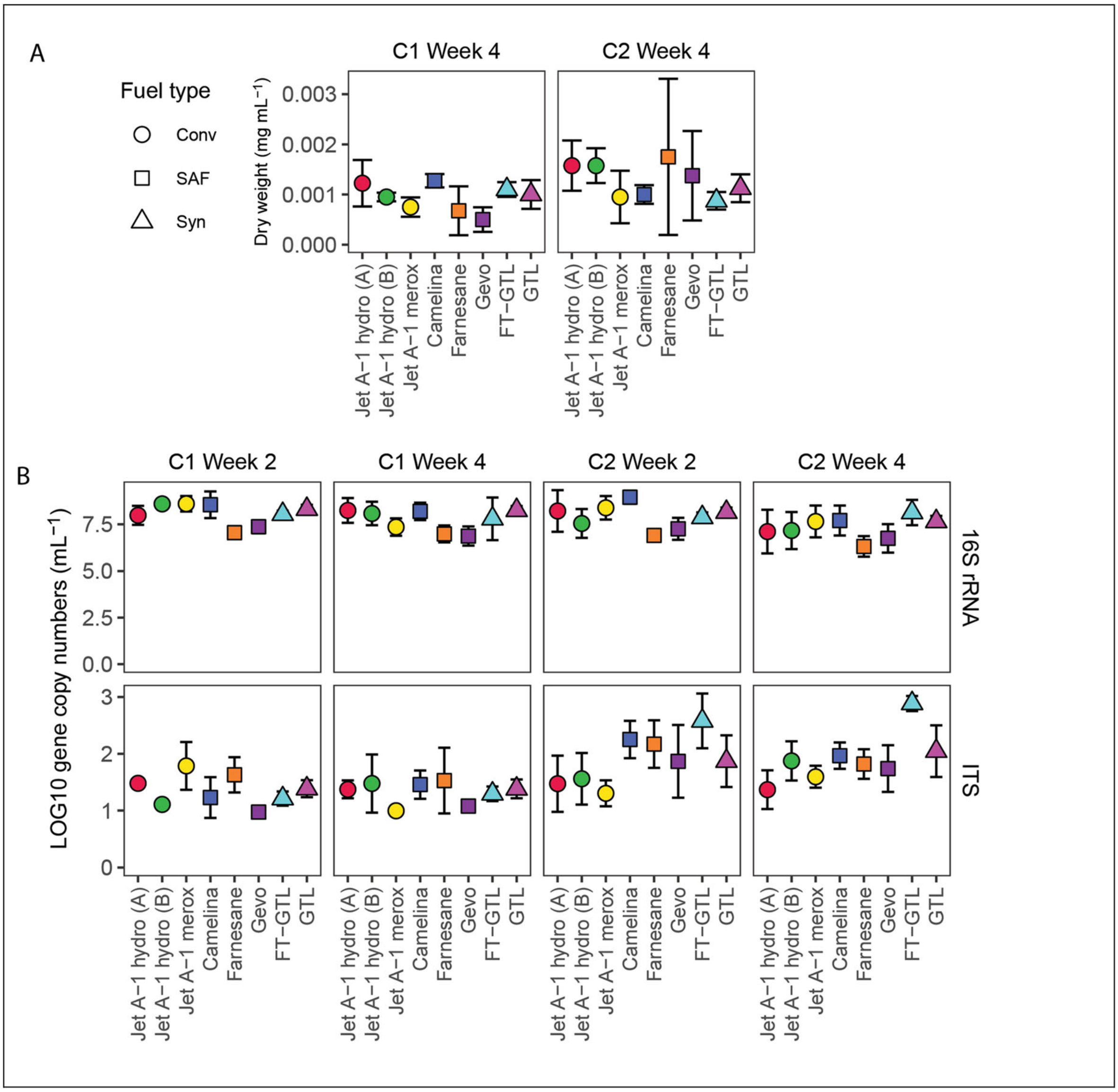

**Figure 1.** A. Dry weight of biomass after 4 week's growth on different fuels inoculated with community 1 (C1) and 2 (C2). B. Bacterial (16S rRNA) and fungal (ITS) and gene copy numbers mL$^{-1}$ of microcosms containing different fuels, inoculated with two communities and harvested after 2 or 4 weeks of growth. Results are the mean +/− standard error. Fuel types are indicated by different shapes (Conv – conventional fuel, SAF – Sustainable Aviation Fuel, Syn -Synthetic aviation fuel.

the fuels, with a greater relative abundance of Comamonadaceae present at both week 2 and 4 and a corresponding decrease in the relative abundance of the Pseudomonadaceae. In community 2, microcosms containing Farnesane and Gevo showed an increase in the relative abundance of Enterobacteriaceae (although this was not statistically significant as there was variation in the responses of the replicate microcosms) with a reduction in the relative abundance of Pseudomonadaceae. The other major ASVs showed little, or no, significant changes between fuel types or were of low relative abundance.

Microcosms containing these fuels were separate from the conventional fuels in the Principal Components Analysis (See Supplementary Table 1 for statistical analysis). In community 2, microcosms containing Farenesane and Gevo also differed from the other fuels, although members of the Enterobacteriaceae increased in relative abundance. These patterns were still evident at week 4 although members of the Aquaspirilliaceae and Burkholderiaceae increased in relative abundance. For fungal communities, most samples were dominated by *Amorphotheca, Aspergillus* and *Cadophora spp.*

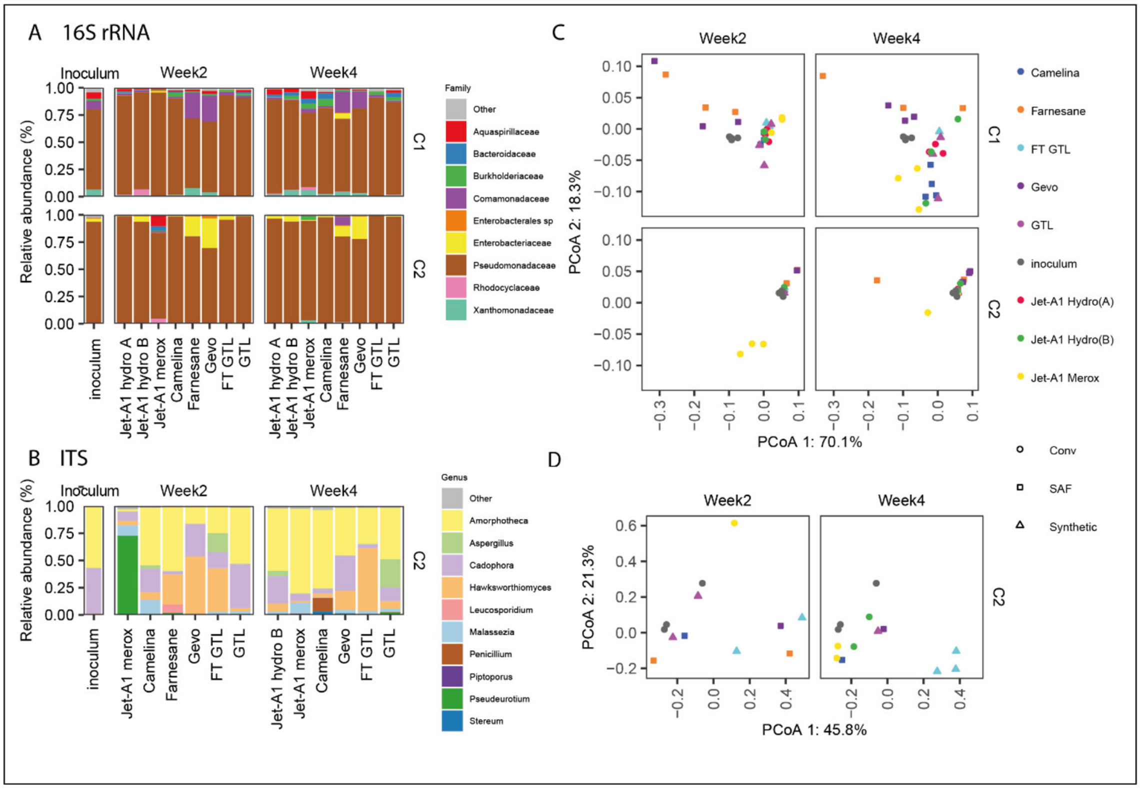

**Figure 2.** Relative abundance of (A) bacterial ASVs at the Family and (B) fungal ASVs at the Genus level. Replicates have been merged and ASVs whose relative abundance was less than 2% have been grouped as 'Other'. Taxa ending in 'sp' could not be classified at the level indicated – the lowest taxonomic group that could be assigned is shown. Principal components analysis of samples based on (C) 16S rRNA sequences and the weighted Unifrac distance measures and (D) ITS sequences based on weighted Bray distance measures. Each point is an individual replicate. Fuel types are indicated by different shapes (Conv – conventional fuel, SAF – Sustainable Aviation Fuel, Synthetic aviation fuel. The % variance explained by each component is indicated on the axes.

**Table 2.** Coverage metric (90% detection limit) for inocula and microcosm samples (mean +/− SE).

| | Community 1 | | Community 2 | |
|---|---|---|---|---|
| Inoculum | 22.5 ± 0.6 | | 4.7 ± 0.3 | |
| Conventional | Week 2 | Week 4 | Week 2 | Week 4 |
| JetA1 hydro(A) | 8.0 ± 0.6 | 11.3 ± 2.7 | 2.0 ± 0.0 | 2.0 ± 0.0 |
| JetA1_hydro(B) | 8.0 ± 2.0 | 10.3 ± 4.3 | 4.0 ± 0.0 | 3.0 ± 1.0 |
| JetA1 merox | 3.3 ± 1.3 | 19.7 ± 4.2 | 15.3 ± 1.2 | 4.0 ± 2.0 |
| Synthetic | | | | |
| FT GTL | 6.0 ± 0.6 | 6.0 ± 0.0 | 2.3 ± 0.3 | 2.0 ± 0.0 |
| GTL | 10.7 ± 3.3 | 13.0 ± 0.6 | 2.0 ± 0.0 | 2.0 ± 0.0 |
| SAF | | | | |
| Camelina | 8.7 ± 2.0 | 11.2 ± 1.5 | 2.0 ± 0.0 | 2.7 ± 0.7 |
| Farnesane | 16.3 ± 3.3 | 10.3 ± 3.4 | 5.0 ± 0.0 | 8.7 ± 4.2 |
| Gevo | 18.7 ± 3.3 | 13.3 ± 2.2 | 7.0 ± 0.0 | 4.7 ± 0.9 |

## *Community members common to different fuel types*

Detection of microbial contamination of fuels based on the identification of specific ASVs will be influenced by the relative abundance of core community members. Given the low abundance of fungal ASVs in these samples, analysis is focussed on detection of bacterial ASVs. The 'observed' index indicates the number of ASVs found in each sample (note: dada2 processing removes singletons so rare ASVs will not be counted). The 'coverage' metric calculates the number of different ASVs that would need to be detected to sample a given proportion of the population (Table 2, Sup Figure 2). The starting inocula had observed values for C1 of 80.0 +/− 3.2 and C2 of 31.7 +/− 4.65. After 2- or 4-weeks' growth in microcosms, these values fell to mean values of 46.5 +/− 2.1 and 19.7 +/− 1.9, respectively (averaged across both sampling times and all fuels). Using a detection limit of 90%, the coverage metric for the C1

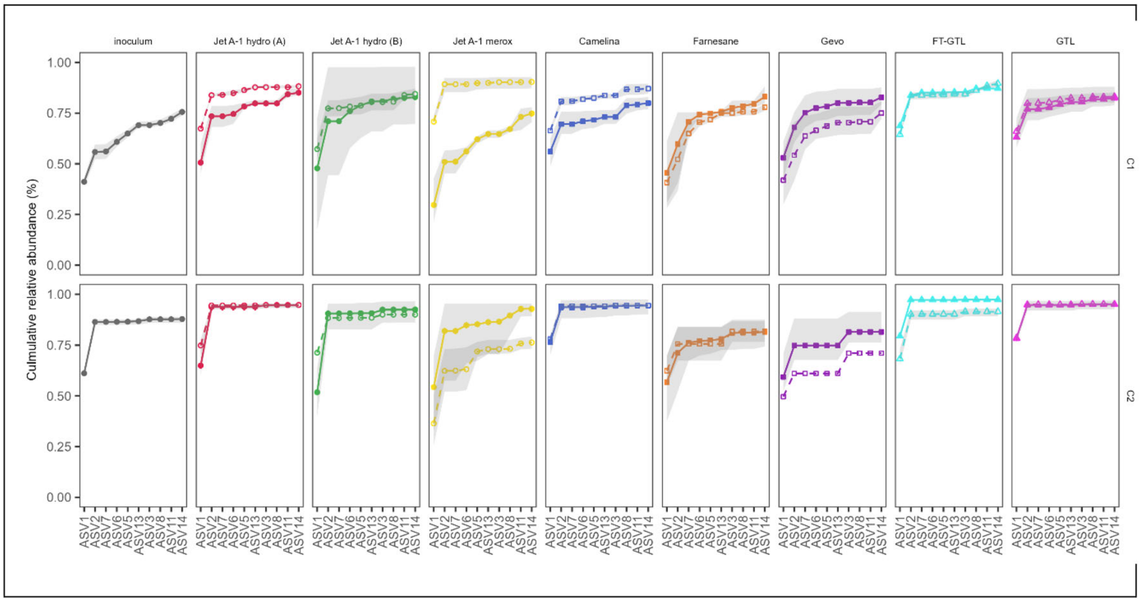

**Figure 3.** Cumulative relative abundance of the top 10 most abundant ASVs, ranked for all fuels. For microcosms, open shapes are week 2, filled shapes week 4. Values are the mean with the standard error shown in grey; Pseudomonadaceae: *Pseudomonas sp.* (ASV1, ASV2, ASV13, ASV11); Comamonadaceae: *Acidovorax sp.* (ASV7, ASV14); Xanthomonadaceae: *Xanthomonas* SN8 (ASV6); Aquaspirillaceae: *Microvirgula aerodenitrificans* (ASV5); Enterobacteriaceae: *Kosakonia sp* (ASV3); Burkholderiaceae: *Cupriavidus sp* (ASV8).

inoculum was 22.5 +/− 0.6 ASVs and for the C2 inoculum, 4.7 +/− 0.3 ASVs. The dominance of Pseudomonads in these samples was evident as these values fell to 2 and 1 ASVs respectively when a 50% coverage rate was considered.

After incubation for 2 weeks, the coverage values of microcosms inoculated with community 1 fell markedly, then increased after 4 weeks (except in Farnesane and Gevo, where it further decreased). The lower diversity of C2 resulted in much lower coverage values at both time points.

The 10 most abundant ASVs, based on their mean relative abundance across all samples, were used to determine the proportion of bacterial ASVs that would be detected in each sample (Figure 3). In these communities, which were dominated by *Pseudomonas spp.*, ASV1 had a mean relative abundance of 59.3% across both time points, communities and all fuels, and a minimum relative abundance of 17.7% in any individual sample.

The greater diversity in C1 compared to C2 was evident in the cumulative abundance curves. In C2, the curves showed sharp inflection points with most fuels indicating the dominance of the *Pseudomonads* ASV1 and ASV2. Exceptions were Jet A-1 Merox, Farnesane and Gevo, where other ASVs made significant contributions to the overall community. With C1, sharp inflections tended to be seen at Week 2 but were less evident at week 4, indicating an initial dominance in ASVs 1 and 2, then other community members becoming established. Again, microcosms containing Jet A-1 Merox, Farnesane and Gevo tended to show a more even community membership than the other fuels tested.

The 'core' microbiome refers to microbes common to a set of samples, but its calculation depends on the thresholds used to determine presence and the phylogenetic relationship between community members. The latter is particularly dependent on the method used to identify community members - an Amplified Sequence Variant (ASV) is an exact, error-corrected sequence hence ASVs may differ by a single nucleotide, whereas Operational Taxonomic Units (OTUs) are grouped on the basis of sequence similarity (typically 97%) [41]. As small sequence variations are unlikely to influence microbial detection strategies, the core microbiome was calculated at the Genus level. Supplementary Figure 3 shows the relative abundance of dominant community members present at a relative abundance greater 1%. As the communities were dominated by Pseudomonads, this was the only core Genus identified in all samples and both communities. Other Genera were present in one or more fuel types.

## Discussion

The move towards synthetic fuels and SAFs has been identified by many fuel producers as important for the future of the aviation industry. In this study we examine the impact of such fuels on the growth of microbial communities. Alterations in microbial community composition in response to changes in fuel composition may influence fuel system design, operation and contaminant monitoring to ensure effective microbial control. Previous studies exploring the microbiome of fuels have typically focused on those found in conventional fuels such as commercial Jet A-1 and military JP-8, diesels and biodiesels [10,18,20,21,42,43]. A metagenomics study [23], looked at the effect of conventional (Jet-A, JP-5, JP-8, F-76) and sustainable (hydro-processed renewable jet and diesel and hydrothermal conversion diesel) fuels on seawater bacterial communities, indicating increased growth of Proteobacteria in all studied fuels but also some taxa being more abundant in certain fuel types. Another recent study [24], compared the growth of two fungi in jet fuel, farnesane, and in 10% farnesane blend in jet, concluding no inhibition or promotion of fungal growth by farnesane. To our knowledge, the current study is the first to compare the impact of sustainable, synthetic and conventional aviation fuels on mixed, fuel-derived microbial communities, applying both biomass measurements and a culture-independent approach.

Dry biomass is a useful metric for the analysis of microbial growth under different culture conditions [44,45]. Whilst dry biomass was too low to determine accurately 2 weeks after inoculation, by four weeks, measurable biomass accumulated in all microcosms, with no significant difference between fuel types or starting inocula. qRTPCR analysis showed the communities were dominated by bacteria with a relatively low abundance of fungi. This reflected the composition of the two inocula used. The relative abundances of bacteria and fungi can differ markedly between sample originating from different locations. For example, Gaylarde et al. [46] report that cultivatable bacterial cells typically exceed fungal cell numbers by ∼100-fold whereas [43] report similar numbers of bacterial and fungal colony forming units (CFUs) in 3 out of 4 contaminated fuel samples (with no fungal growth detected in the fourth sample). Using culture-independent qRTPCR, Krohn et al. [22] found very variable bacterial:fungal ratios in an analysis of 1,258 samples; bacteria dominated fuel samples, fungi tended to be more prevalent in biofilm samples and a highly variable ratio found in water samples (bacteria were always numerically dominant, but fungi varied from almost zero to near equivalence). The communities in the current study were numerically dominated by bacteria, but fungi were present and included those typical of contaminated fuel. Microbial communities will depend on composition of the starting inocula. Such 'founder effects' are commonly seen in microcosm studies, but are equally applicable to constructed environments, where microbial populations develop from an initial source of contamination (whether external input or residual biomass that persists in the tank after cleaning). There was a small effect of fuel type, but it is evident that microbial growth occurred in microcosms with all fuels tested (conventional, synthetic and SAF), and therefore operational risks are likely to continue regardless of the fuel type. However, given the diversity of microbial communities found in surveys, different fuel types may more markedly influence microbial communities that have different compositions.

Here, two different starting communities, derived from contaminated field samples, that were maintained as complex communities, were used to inoculate microcosms where the sole carbon source was provided by eight different fuels. High-throughput DNA sequencing analysis showed that the samples contained a range of taxonomic groups (15 bacterial Families, 14 fungal Genera at relative abundances greater than 1%) but that the evenness of the starting inocula were low, with the bacterial communities dominated by *Pseudomonads*. The number of observed bacterial ASVs declined in most microcosm samples, but the evenness ('Simpson' index) increased indicating that a wider range of bacterial ASVs had become numerically important in the microcosms. This is likely to have resulted from an initial 'bottle' effect, often seen when communities are grown in confined environments [47] with later increases resulting from the establishment of slower growing organisms and cross-feeding by primary degraders of other community members [29]. The fungal component of the inoculum was dominated by *Amorphotheca resinae* (previously known as *Hormoconis resinae* and *Cladosporium resinae*) and *Cadophora sp* – both of which are common fuel contaminants. These persisted in the microcosms along with a *Hawksworthiomyces taylorii.* The latter is not a reported fuel degrader, but is a known wood degrader [48], many of which are found as fuel contaminants with capability to degrade aromatic ring structures.

Previous research has demonstrated that differences in fuel type may play a role in the community composition. For example, a study by Brown et al. [42] found that the bacterial communities in Jet A-1 and JP8 samples differed, although multiple reasons may have accounted for these differences including the routine use of fuel system icing inhibitor (FSII) in military JP-8 fuel and that the Jet A-1 fuel samples came from aircraft in long-term storage in hot desert conditions. Therefore, the differences observed could not be attributed to fuel type but, as with all dynamic systems, other factors may be involved such as environmental conditions, additive packages and geographical location. Exploring the impact of different environmental factors in combination with synthetic and SAFs is an area that requires further exploration.

The composition of jet fuel is defined by specific criteria based on operational capability rather than chemical composition [49]. The fuels in this study had very different compositions ranging from complex mixtures of paraffins, iso-paraffins, cyclo-paraffins, olefins and aromatics found in conventional fuels, through simpler synthetic fuels such as GTL which lack aromatics, and SBCs/SAFs such as Farnesane or alcohol-to-jet (Gevo), composed of one or two main molecular species (see Supplementary Figure 1). Therefore, microbial community composition may be influenced by degradation capabilities. However, the enzymatic systems employed by microorganisms can often degrade a range of similar moieties. For example, alkanes of different chain lengths can be degraded *via* oxidation to fatty acids that then enter the $\beta$-oxidation pathway, with each round reducing the alkyl chain length by 2 carbon groups [50–52]. Also, different isolates of the same species may show different preferences or capabilities for fuel components. For example, different isolates of *C. resinae* shows different abilities to grow on n-alkanes in the range $C_9$ to $C_{18}$ [53,54]. Striebich et al. [55] showed that *Pseudomonas*, *Acinetobacter* and *Yarrowia* preferred $C_{10}$-$C_{22}$ *n*-alkanes, whereas *Marinobacter* preferred lighter or branched chain alkanes. These preferences may influence the precise microbial composition, but generalist degraders are likely to be favoured over specialists. Additionally, current SAF fuels are typically blended with conventional fuels in proportions no greater than 50%, thus it may be that the overall fuel composition is not altered sufficiently to cause significant shifts in the bacterial communities when compared to conventional aviation fuels. While aviation fuels composed of up to 100% SAF have been trialled successfully, 100% drop-in blends are not yet allowed based on ASTM International, who set global standards for aviation fuels [49]. As such it will be important to continue monitoring potential shifts in the microbiome that may be caused by various feedstocks, production processes, and varying blending ratios. Although beyond the scope of this project, alterations in fuel composition may require the use of additives such as lubricity improvers, which may act as carbon sources, or metal atom deactivators which can include heteroatoms such as N. These may also influence microbial growth and community composition.

The detection of microbial contamination in fuel systems needs to target the dominant community members, as small community differences are generally not a concern. In some studies e.g. Krohn et al. [22] diverse microorganisms have been identified in both floating and sessile phases, including those that are slow or difficult to grow in culture. Therefore, the detection of key indicator species is an efficient way to determine contamination in fuel systems providing these are representative of the diversity found in field samples and different operational conditions. Here, *Pseudomonadaceae,* a Family that contains species such *Pseudomonas*, *Moraxella*, and *Acinetobacter,* were dominant in both starting inocula. These organisms are frequently found in fuel systems and have been linked to operational problems [56]. The dominance of *Pseudomonadaceae* has been noted in previous fuel studies with [57] examining 54 fuel and fuel-associated water samples, noting high levels of abundance of *Pseudomonas* spp. in 21% of the cultured isolates. In other studies, however, different groups became dominant including *Marinobacter, Burkholderia and Halomonas* [10]. Unlike our results, a large diversity of genera has been found in field samples from aircraft tanks and previously less studied genera such as *Methylobacterium* sp. were identified by transcriptome analysis as the most active in sessile biofilm swabs [22].

Analysis of the relative proportions of the most abundant bacterial ASVs (across all fuels) (Figure 3) showed that detection of these would identify the majority of microbes in the different microcosms, although the cumulative abundance curves were less steep for Jet A-1 Merox, Farnesane and Gevo fuels reflecting the greater diversity in these samples. Analysis of fungal ASVs was limited by their low abundance in these studies, but inspection of the fungal community C2 showed that *Hormoconis resinae* was present in all samples, suggesting that it is as a good 'indicator' species for the detection of fungal contamination in SAFs as it is in conventional Jet A-1 [46]. Other eukaryotes were detected with these often associated with fuel contamination or as environmental organisms associated with wood degradation. Other studies have observed uncultured and unknown organisms [22] and a greater diversity of organisms are likely to be identified in future through high-throughput DNA sequencing [19].

Comparisons (Figure 2) showed that, numerically, microbial composition was affected by fuel type. After 2 week's growth, there was little difference between fuel types, except for Jet A-1 Merox, where the relative abundance of the Pseudomonadaceae and, to a lesser extent, Aquaspirillaceae and Enterobacteriaceae, differed. After 4 week's growth, there were small differences in community composition between fuel types in microcosms inoculated with C1, but with C2 the relative abundances of Pseudomonadaceae often differed, particularly in Farnesane, Gevo and Camelina fuels.

While within this study it was more difficult to compare the profiles of the different fungal communities, it appears that the profiles were all relatively similar to those in conventional aviation fuels. In samples collected from aircraft fuel tanks [22], fuel and water samples were always dominated by bacteria whereas biofilms sometimes dominated by fungi. Therefore, current testing regimes should be able to assess fungal material in planktonic phases, but caution is required when considering biofilms.

## Conclusions

Changing fuel types and the feedstocks used to produce aviation fuels may, in the future, influence the type of microbial contamination that is observed in fuel and fuel systems. In this series of experiments, the relative abundance of specific bacterial microorganisms did vary somewhat between fuel types, but overall biomass accumulation was similar and detection of the most abundant bacterial ASVs would be effective at detecting the majority of microbial contamination in all fuel types. This would indicate that current testing regimes are likely to be effective with new fuel compositions. However, whilst the starting inocula tested did contain many organisms typically found in contaminated aviation systems, they were dominated by *Pseudomonads*, and the composition of the starting inocula was likely a driving factor in the communities observed in the microcosms. In future, comparisons of widely differing communities, such as those reported by Krohn et al. [22], would be beneficial to ensure that the findings of this study were generally applicable across the range of contaminants found in aviation systems. Reductions in community diversity, or strong founder effects that strongly influenced community structure, might also lead to the risk that dominant ASVs might not be detected by a specific test method. Using a range of test methods based on different detection approaches (direct microbial detection e.g. antigen or other affinity methods, metabolites such as ATP or culture-based methods) would provide the most robust approach for detection of microbial contamination. In addition, this study has focussed on microorganisms in the planktonic growth phase – the phase that is generally sampled to detect microbial contamination. However, microorganisms often grow as attached communities which may differ markedly, hence these should also be the subject of further investigation.

## Disclosure statement

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## Data availability statement

The data for this study have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB51365.

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