



Fungal allergic sensitisation in young rural Zimbabwean children: Gut mycobiome and seroreactivity characteristics

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SUMMARY

Background: The prevalence of allergic diseases has increased over the last few decades, with sensitisation to fungal allergens and gut microbiome dysbiosis implicated in this trend. The fungal community in the gut (mycobiome) has yet to be characterised and related to fungal allergic sensitisation. Thus, we characterised the gut mycobiome and related it to fungal sensitisation and seroreactivity among Zimbabwean children. We further determined the effect of host age, sex, *Schistosoma haematobium* infection and mycobiome composition on fungal sensitisation and seroreactivity.

Methods: Using shotgun metagenomic sequencing, we characterised the gut microbiome of stool samples of 116 preschool aged children (PSAC) (≤ 5 years old, 57 (49.1%) male and 59 (50.9%) female). Sensitisation to common fungi in Zimbabwe was assessed using skin prick tests (SPTs). Allergen-specific IgM, IgA, IgG, IgE and IgG4 antibodies were quantified by ELISA. We analysed the relationship between fungal genera and SPT reactivity by ANOVA; fungal genera and IgE antibody reactivity by linear regression; variation in mycobiome abundance with host and environmental factors by PERMANOVA; SPT reactivity and host and environmental factors by logistic regression; seroreactivity and host and environmental factors by ANOVA.

Results: The mycobiome formed $<1\%$ of the sequenced gut microbiome and 228 fungal genera were identified. The most abundant genera detected were *Protomyces*, *Taphrina*, and *Aspergillus*. *S. haematobium* infection had a significant effect on fungal genera. Prevalence of SPT sensitisation to ≥ 1 fungal species was 96%, and individuals were frequently sensitised to *Saccharomyces cerevisiae*. Antibodies were detected in 100% of the population. There was no relationship between mycobiome abundance and IgE titres or IgE/IgG4 ratios for each fungal species; no significant differences between SPT reactivity and abundance of fungal species except for *S. cerevisiae*; and fungal seroreactivity did not significantly differ with age. There were some sex ($m > f$ for, *Epicoccum nigrum* and *Penicillium chrysogenum*) and SPT reactivity –related differences in seroreactivity.

Conclusion: This is the first comprehensive characterisation of gut mycobiome and fungal allergic sensitisation of rural children in Zimbabwe. Although reported allergic disease is low there is a high percentage of sensitisation. Further studies with larger populations are required to understand the role of the mycobiome in allergic diseases.

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1. Introduction

The prevalence of allergic diseases in childhood has increased over the last few decades (Wong et al., 2012). Sensitisation to allergens occurring in the first few years of life is suggested to be a major risk factor for developing allergic diseases (Holt et al., 2010). These allergic conditions have complex etiologies that involve both genetic and environmental factors (Ober and Yao, 2011). The major factors contributing to increases in allergic diseases are proposed to be related to changes in lifestyle and environmental exposure. Among these, it has been proposed that increases in allergic diseases may be related to gut microbiome dysbiosis (Huang et al., 2017), as well as the immunological effects of a decline in exposure to infectious diseases in more developed and urban environments (Mpairwe et al., 2008).

To date, allergic sensitisation and inflammation studies report that an alteration in the microbiome (Fujimura et al., 2016; Arrieta et al., 2018) is associated with the development or exacerbation of allergic conditions such as asthma (Noverr et al., 2004, 2005; Wheeler et al., 2016; Arrieta et al., 2018). However, these studies have yielded variable findings, due to the heterogeneity of study populations, differences in profiling and study designs, variable definitions of allergy and varying sample sizes (Zhao et al., 2019). Furthermore, few allergy studies have specifically examined the interaction between gut mycobiome and allergen sensitisation (Fujimura et al., 2016), highlighting a need for more research in this area. Investigating the gut mycobiome in individuals with fungal sensitisation may identify associations between the gut mycobial composition and sensitivity or tolerance to fungal allergens. Additionally, a number of unculturable fungal species whose influence on fungal sensitised people is yet unknown may be identified.

Data from previous studies suggests that microbial dysbiosis occurs early in life, preceding the onset of sensitisation. The dysbiosis results in differences in gut microbial composition which have been associated with allergy skin prick test (SPT) response (Balenga et al., 2015), specific IgE levels, (Adlerberth et al., 2007) and allergy status (Thompson-Chagoyan et al., 2010). However, much of this previous work focused on the bacterial microbiome and relied on culture methods, excluding the large majority of organisms that cannot be cultured (Bunyavanich et al., 2016).

Thus, in this descriptive study we characterise the gut mycobiome using shotgun metagenomics and relate this to fungal sensitisation and fungal seroreactivity among Zimbabwean preschool-aged children (PSAC). We further assess whether seroreactivity varies with age, sex, *Schistosoma haematobium* infection status, gut mycobial composition or fungal sensitisation in children who were born in and were permanent residents of the study area. Thus, had comparable dietary and environmental exposure patterns as gathered from questionnaires administered at the time of recruitment of the main study.

2. Methodology

2.1. Ethical approval and consent

This study was part of a larger paediatric schistosomiasis study in children aged 5 years and below. Ethical and institutional approval for this study was granted by the Medical Research Council of Zimbabwe (MRCZ/A/1964) and University of Edinburgh. Permission to conduct the study in the province was obtained from the Mashonaland Central Provincial Medical Director. Before enrolling in the study, all participants and their parents/guardians were informed of the study aims, and procedures in their local language, Shona. Enrolment and participation was voluntary with written informed consent being obtained from the participants' parents/guardians. Participants were free to withdraw from the study at any time with no further obligation.

2.2. Study design, population and site

This cross-sectional study was conducted in Shamva district, one of the seven districts in the Mashonaland Central province of Zimbabwe. It was part of a larger research project, the Paediatric schistosomiasis study, where the overall health impact of paediatric schistosomiasis in children aged 5 years and below was investigated. Within this broader framework, the structure and diversity of the gut microbiome and resistome was characterised in two complementary studies; the first study investigated the association between schistosome infection and the gut microbiome dysbiosis and resistome in PSAC (Osakunor et al., 2020). Whereas, the second study, which is the current study investigates the relationship between gut mycobiome and fungal sensitisation and seroreactivity.

At baseline, the study enrolled children aged 6 months to 5 years who met the following inclusion criteria. The children had to, i) be lifelong residents of the study area, ii) no history of recent major illness/ surgery and iii) guardian/career had given consent for them to participate in the study. The samples used by Osakunor et al. (2020), and subsequently the present study, had to meet further criteria of; iv) consent for stool samples to be used for microbiome characterisation. Following these inclusion criteria, 116 stool samples from 1 to 5 year olds (57 males (49.1%), 59 (50.9%) females) children were included in the study.

To be included in the current study, children who fulfilled the inclusion criteria described above had to meet the additional criteria of; v) consent for serum samples to be used for serological assays, vi) availability of socio-demographic data and; vii) consent to perform skin prick testing (SPT) using allergen extracts. Following these inclusion criteria, the same 116 children were included in the current cross-sectional study.

2.3. Sample collection, processing and DNA extraction

Urine and stool samples were collected from all the participants to screen for schistosomiasis and soil-transmitted helminths as previously described by our group (Osakunor et al., 2020). For the characterisation of the gut microbiome, a small sample of each stool was transferred into a 2 mL cryovial tube and DNA was extracted using the QIAamp DNA Stool Mini Kit (QIAGEN) according to the manufacturer's instructions. To evaluate DNA purity, each sample was quantified at the University of Edinburgh using the Qubit fluorometer (Thermo Fisher Scientific) prior to shipment for DNA sequencing. For serological assays, up to 5 mL of venous blood was collected from each participant into serum separator blood collection tubes (BD Vacutainer®) and serum was separated for analysis.

2.4. Next-generation sequencing

Stool samples for next-generation sequencing (NGS) were prepared as previously described (Osakunor et al., 2020). Sequencing quality control and trimming of the reads was conducted using FASTQC and BBduk2 [BBMap—Bushnell B.—<https://sourceforge.net/projects/bbmap/>] respectively. The trimmed reads were used as input to align direct to reference sequence databases downloaded via NCBI GenBank clade specific assembly_summary.txt files (<ftp://ftp.ncbi.nlm.nih.gov/genomes/genbank>) using k-mer alignment (KMA) (Osakunor et al., 2020). The primary alignment obtained for mapped sequences was used to assign a putative taxonomy, based on the taxonID obtained. TaxonIDs and associated taxonomy classifications were obtained from downloaded reference microbial genomes from NCBI (<ftp://ftp.ncbi.nih.gov/pub/taxonomy/taxdump.tar.gz>), these were assigned to all taxonomic levels.

To obtain information about the abundances of features in the datasets relative to each other, datasets were treated as compositional (Gloor et al., 2017) and prior to transformations, a pseudo-count of half the smallest non-zero abundance per feature was added to each feature

for all the normalised abundance matrices. Microbiota abundance data tables with counts and number of populations (taxa members), were centred log ratio (clr) transformed (Calle, 2019). The clr matrices were used for all downstream analyses.

2.5. Skin prick tests (SPTs)

SPTs were conducted using six different fungal allergen extracts (Stallergenes Greer, France): *Alternaria alternata*, *Cladosporium herbarum*, *Epicoecum nigrum*, *Penicillium chrysogenum*, *Rhizopus nigricans* and *Saccharomyces cerevisiae* were chosen based on the Zimbabwean sensitisation profile (Westritschnig et al., 2003). Briefly, drops of each allergen extract were placed on the forearm 2 cm apart and pricked using a calibrated lancet that introduces approximately 1 µg/mL of allergen into the dermis. Histamine dihydrochloride (10 mg/mL) was used as a positive control and a saline solution as a negative control. Reactions were considered valid if the histamine wheal diameter was greater than the negative control. Results were read at 15 min. The largest diameter of the wheal for each allergen extract was measured and a wheal of ≥ 3 mm scored positive.

2.6. Serology assays

2.6.1. Fungus-specific responses

Humoral responses (IgA, IgG, IgM, IgE and IgG4) to fungal antigens were assessed using commercial extracts of *Aspergillus fumigatus*, *A. alternata*, *C. herbarum*, *E. nigrum*, *P. chrysogenum*, *R. nigricans* and *S. cerevisiae* (Stallergenes Greer, USA) in a standard indirect enzyme-linked immunosorbent assay (ELISA). In brief, ELISA plates were coated overnight at 4 °C with 50 µL/well of fungal antigen diluted in PBS at 5 µg/mL. Serum samples and secondary antibodies (anti-human horse-radish peroxidase conjugated) were diluted according to the nominal antigen. The substrate 3,3',5,5'-tetramethylbenzidine (TMB) peroxidase was used for the colorimetric reaction and the absorbance was read at 450 nm.

Optimum dilutions of antigens, sera and secondary antibodies were determined by several serial dilutions, based on protocols previously developed by others. To minimize variations across plates, positive and negative controls were repeatedly run on each plate and plotted to detect any outliers (in which case assays were repeated). The positive controls were pools of sera from six individuals presenting with the highest skin-prick reactivity while negative controls were pools of sera from six individuals with the lowest skin-prick reactivity. To account for background variation, a blank well (containing no sera) was included on each plate and this absorbance reading was subtracted from all other plate-readings. All the samples and the blank were run in duplicate on each plate and reported values are means of duplicates.

2.7. Data analysis

Statistical analyses were performed using SPSS v22 (IBM Corp.) and various Bioconductor packages within the R environment v3.6.1. Data visualization was performed within the R environment and GraphPad Prism v7.02 (GraphPad Software, Inc.). To test whether sample-related metadata predicted within-group dispersion of the microbiome, the Euclidean distances were calculated using R. The effect of such metadata on sample dissimilarities were determined using permutational multivariate analysis of variance (PERMANOVA; adonis2 function in the vegan package) using $p < 0.05$ as the significance threshold. A false discovery rate (FDR (Benjamini–Hochberg FDR)) correction was applied to counteract multiple testing (Benjamini and Hochberg, 1995). Bar plots from normalised, zero-corrected abundance matrices were used to give an overview of the microbiota gene abundances across all samples.

To determine whether age, sex, *S. haematobium* infection status had an effect on SPT reactivity binary logistic regression was performed. To test whether serological reactivity to fungi varies with age, sex, *S.*

Table 1

Demographic characteristics of study population.

Demographic categories		Frequency	Percentage
Gender	Female	59	50.9
	Male	57	49.1
Age group (years)	≤ 3	52	44.8
	4	33	28.4
	5	31	26.7
	Negative	98	84.5
<i>S. haematobium</i> infection status	Positive	18	15.5
Nutritional and growth factors			
	Breastfed (months)		
	< 6	1	1.1
	≥ 6	89	98.9
Solid food introduction (months)	< 6	79	77.5
	≥ 6	23	22.5
Malnourished (WHA)	Yes	4	3.7
	No	103	96.3
Stunted (HAZ)	Yes	16	14.7
	No	93	85.3
Total		116	100

haematobium infection status as well as gut microbiome structure, analysis of variance (ANOVA) was used. Differences were considered to be significant at $p < 0.05$. The experimental data are presented as the mean \pm standard error of the group means. Due to the skewed nature of the fungus-specific antibody responses values were square root-transformed. To characterise patterns of the different fungus-specific antibodies, all titres (IgM, IgA, IgG, IgG4 and IgE), were reduced into axes to facilitate interpretation of patterns and differences among groups using non-metric multidimensional scaling (NMDS). NMDS was run in R using a Bray Curtis distance method and 1–6 dimensions were trialled. The stress scores were recorded to determine the number of axes needed. The stress score produced is a goodness of fit statistic based on the differences between the actual distances and their predicted values. A stress score > 0.1 is 'poor', < 0.1 is 'fair' and < 0.05 is 'good' (Kruskal, 1964). Pearson's correlations were used to determine the relationship between the original variables and the axis and only antibodies with an $r^2 < -0.5$ or > 0.5 were considered adequately reflected by the axis.

NMDS scores were compared by sex, age group, SPT reactivity and *S. haematobium* infection.

Role of funding

The funders played no part in the design of the study, collection, analysis and interpretation of data, and in writing the manuscript.

3. Results

3.1. Population characteristics

Table 1 details the demographic characteristics of the study population. The mean age of the 116 participants was 3.7 ± 1.1 years. Out of the 116 participants majority were female (50.9%, $n = 59$). In regards to the children's dietary habits and nutritional status, the majority were breastfed for six or more months. Children were introduced to solid foods between 1 and 24 months after birth. The main component of the diet was traditional maize flour porridge. Anthropometric measurements, adjusted for age, were used to assess nutritional status (MOH Malawi, 2016; Osakunor et al., 2018). Table 1 shows the children's demographics characteristics.

3.2. Taxonomic composition of the microbiome

Using some of the data analysed and recently published by our research group (Osakunor et al., 2020), the relative abundance was calculated for each microbial community in all samples. An average of 45.1% of read pairs were mapped to specific reference sequences in the

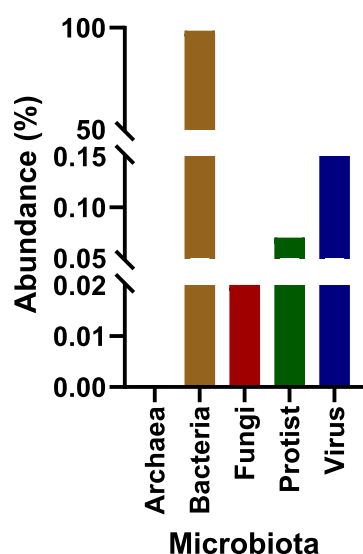


Fig. 1. Composition of the gut microbial communities.

genomic database (Osakunor et al., 2020), similar to values reported in other studies (Afshinnkoo et al., 2015). The mycobiome made up less than 1% of the sequenced gut microbiome as shown in Fig. 1. However, it showed high diversity (Fig. 2). In the 116 stool samples analysed, 228 fungal genera (from six unique phyla) were detected.

3.2.1. Relative abundance fungal phyla and genera in the gut microbiome

Abundance was calculated for each microbial taxon across all samples. The most prevalent phyla were Ascomycota (genera: *Protomyces* (present in 100% of the samples), *Taphrina* (98%), *Aspergillus* (91%),

Saccharomyces (91%)), Microsporidia (*Enterocytozoon* (100%)), and Zoopagomycota (*Entomophthora* (100%)) Fig. 2a-b. These phyla dominated the microbiome. Charts were generated using normalised, zero-corrected abundance matrices. “Other” represents abundance data for all other taxa in the abundance data set.

Fig. 1: Overview of fungal microbiota abundance and composition. Stacked bar charts show the most abundant fungal (a) phyla and (b) genera respectively per sample, proportional to the total microbiota within each sample.

3.3. Variation in the mycobiome and association with participant metadata

To initially examine variability and patterns in the data set, principal

Table 2
Summary of sample metadata and association with gut mycobiome.

Variable	n	p-value	Explained sum of squares	Total sum of squares	FDR
Gender	116	0.370	85.8	9673.3	0.726
Age (years)	116	0.098	123.0	9636.1	0.343
Malnourished (WHA) yes/no	107	0.830	59.5	9148.0	0.830
Stunted (HAZ) yes/no	109	0.624	71.5	9229.7	0.728
Breast-fed (months)	90	0.415	75.2	6986.5	0.726
Solid food introduction (months)	102	0.602	73.6	8794.3	0.728
<i>S.haematobium</i> status	116	0.001	339.1	9281.2	0.007

Classification of nutritional status was based on a cut off <-2 Z scores (MOH Malawi, 2016). WHA, weight-for height Z scores; HAZ, height-for-age Z scores; p-value-unadjusted p-value; FDR- adjusted p-value (FDR-corrected).

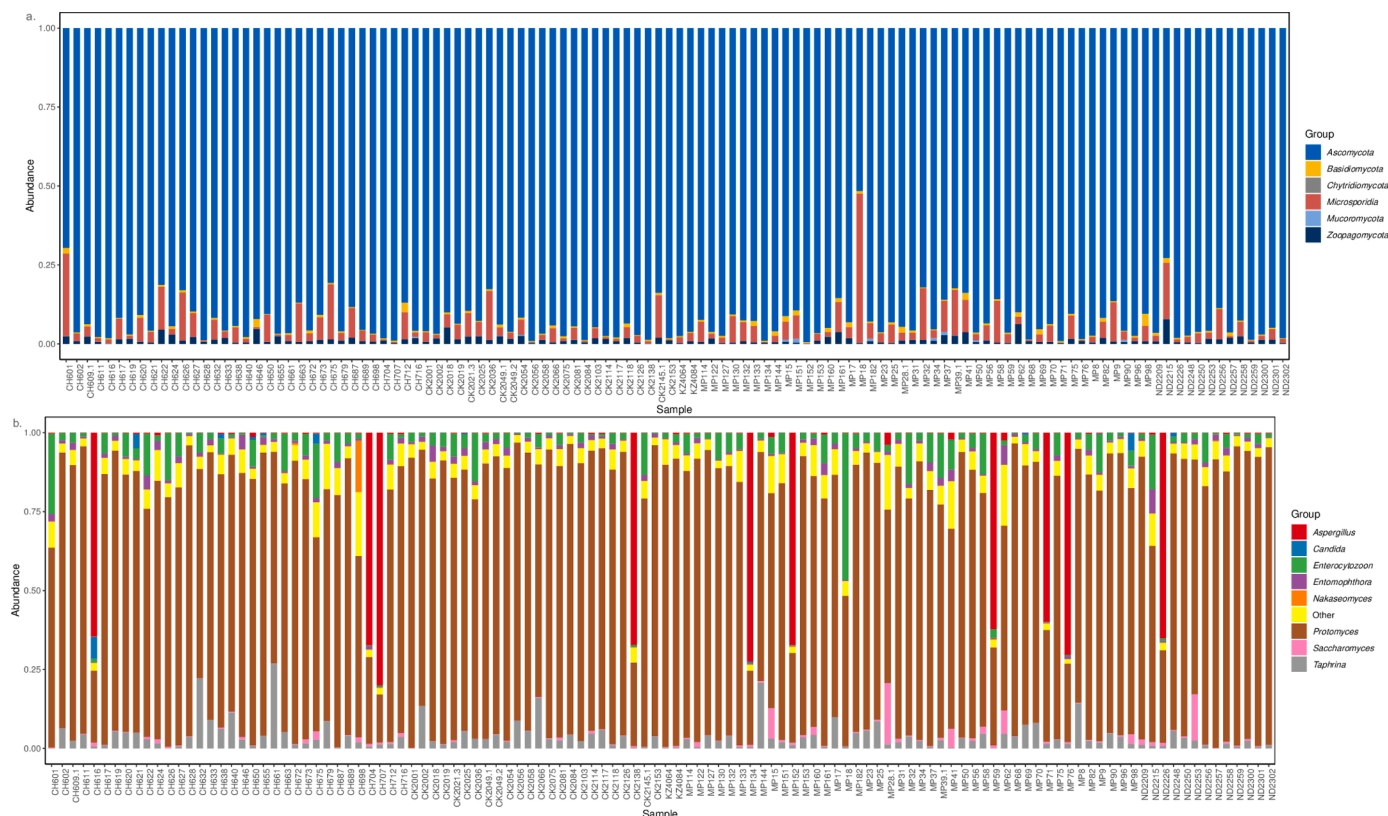


Fig. 2. Overview of fungal microbiota abundance and composition. Stacked bar charts show the most abundant fungal (a) phyla and (b) genera respectively per sample, proportional to the total microbiota within each sample.

Table 3

Prevalence of fungal sensitisation based on skin prick testing (SPT) of the study population.

Total	48(96.0)	ONE-FS* 7(14.6)	TWO-FS* 9(18.6)	MFS* 32(66.7)
<i>A.alternata</i>	16 (33.3)	0	0	16(50.0)
<i>C.herbarum</i>	28(58.3)	3 (42.9)	1(11.1)	24(75.0)
<i>E.nigrum</i>	27(56.25)	1(14.3)	5(55.6)	21(65.6)
<i>P.chrysogenum</i>	28(58.3)	0	4(44.4)	24(75.0)
<i>R.nigricans</i>	24(50.0)	0	4(44.4)	20(62.5)
<i>S.cerevisiae</i>	29(60.4)	2(28.6)	4(44.4)	23(71.9)

* ONE-FS: one positive SPT to a single fungal species; TWO-FS: two positive SPT to fungi; MFS: more than two positive SPT to fungi (multiple fungi sensitization).

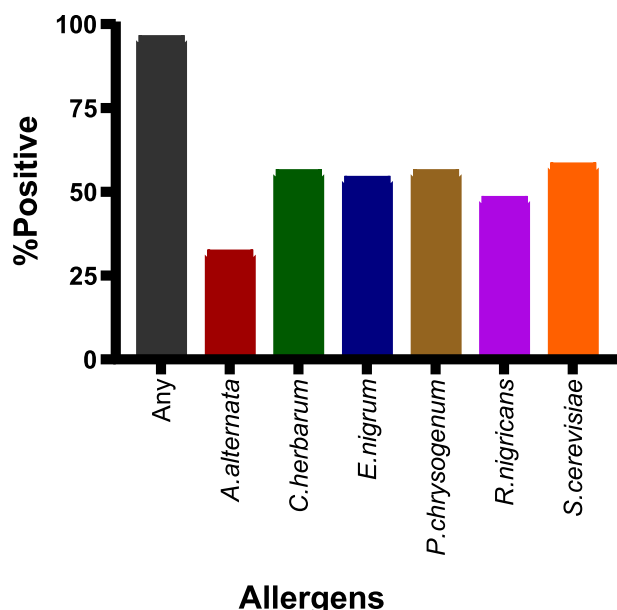


Fig. 3. Prevalence of fungal sensitisation based on skin prick testing (SPT) of the study population; Prevalence of positive SPT to different fungal species tested.

component analysis was used and the model showed homogeneity with no distinct clustering according to metadata as previously reported by our research group (Osakunor et al., 2020). The cluster dendrograms are shown in Supplementary Figure S1.

PERMANOVA analysis showed a significant effect of *S.haematobium* infection status (FDR= 0.007) on fungi genera, across samples, however, no such effects were observed for age, sex, nutritional and growth variables and feeding. Summary output for the analysis is shown in Table 2.

Of the 116 children with a characterised microbiome, 71 were included in the subsequent analysis. The decrease in sample size was due to loss of SPT follow-up or inadequate serum sample volumes. The age range for the final samples was 2–5 years, with a mean age of 3.98 years.

3.4. Skin prick reactivity profiles

Of the 71 children included in the serological study, only 50 had SPTs performed, as other participants withdrew due to aversion to the procedure. Forty-eight study participants (96%) were SPT-positive to at least one fungal source. Ages ranged from 3 to 5 years, with a female/male ratio of 1.5. Table 3 summarises the prevalence of different patterns of fungal sensitization. Majority of the participants were sensitised to two (TWO-FS=18.6%) or multiple fungi (MFS = 66.7%). *S.cerevisiae*, *C.herbarum*, and *E.nigrum* were positive in 85.7% of the participants. All participants sensitized to *A.alternata* were poly-sensitized. Fig. 3

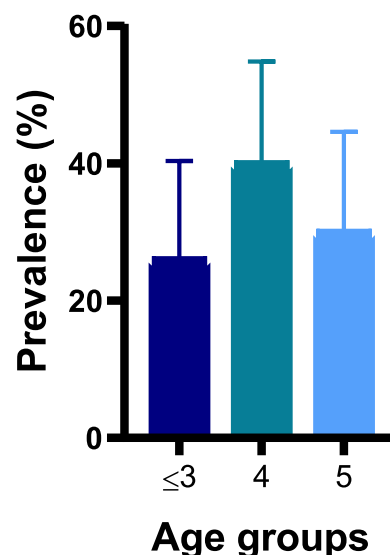


Fig. 4. Prevalence (%) of positive SPTs for the different age groups with 95% CI.

summarises the sensitisation pattern observed in the study population.

The prevalence of positive SPTs decreased with age (26% for the <3 years group, 40% for the 4 years group, and 30% at 5 years; Fig. 4.

3.4.1. Effects of age, sex and *S.haematobium* infection on SPT reactivity

A binary logistic regression was performed to ascertain the effects of age, sex, and *S.haematobium* infection on SPT reactivity. In all cases, the predictor variables (age, sex, and *S.haematobium* infection) had no significant effect on SPT reactivity ($p > 0.05$) (see Supplementary Table S1).

3.5. Antibody profiles for fungus-specific immune responses

Overall, fungal seroprevalence was 100% (95% Confidence Interval (CI) 94.94–100). IgE antibodies were detected in 85.9% (95% CI 75.6–93.0) of the study population whereas IgG4 antibodies were detected in 32.4% (95% CI 21.8– 44.6). Ages ranged from 2 to 5 years, with a mean age of 3.98 years with a female/male ratio of 1.2. Figs. 5 and 6 summarise the frequency distribution of children producing each antibody (with 95% CI) and mean antibody levels (absorbance) against each fungal species respectively.

The response to allergens induces different levels of Immunoglobulin classes, depending on the specific allergen. IgM and IgG, which are produced predominantly in the first exposure to an allergen, (Scott-Taylor et al., 2018) had higher mean antibody responses for all fungal species except *A.fumigatus*, where IgM titre was low. In all cases where IgM and IgG responses were high, IgE titres were lower or in some cases, not detected altogether (*R.nigricans*, *P.chrysogenum*, and *E.nigrum*) as shown in Fig. 6.

3.5.1. Characterisation of fungus-specific antibodies

To characterise patterns of the different fungus-specific antibodies, all antibodies produced by the participants were reduced into NMDS axes using the vegan package in R v3.6.1 (metaMDS) which follows the ordination with a rotation via principal components analysis. This is a useful feature because it ensures that NMDS axis 1 (NMDS1) reflects the principal source of variation (McCune and Grace, 2002). Prior to analysis all antibodies were square-root transformed for each participant to reduce the influence of outlier values.

NMDS1, which accounted for the most variation within the data, was positively correlated with *A.fumigatus* (*A.f*) –IgG4; *E.nigrum* (*E.n*)–IgG; *P.chrysogenum* (*P.c*) – IgG and IgG4, whilst negatively correlated with *A.*

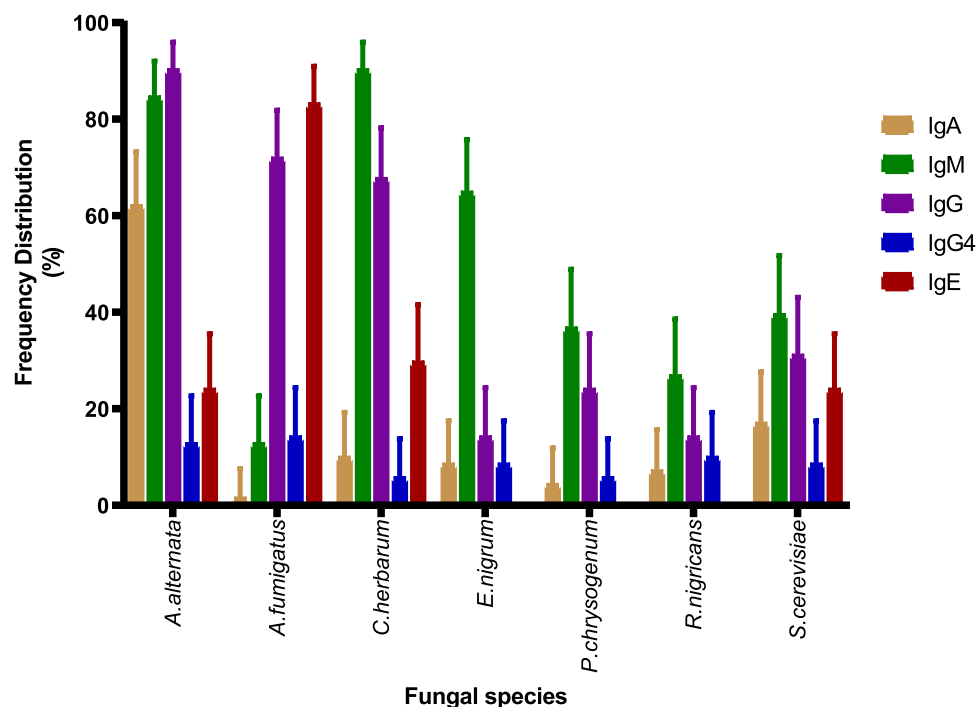


Fig. 5. Frequency distribution of children producing each antibody against each fungal species with 95% CI.

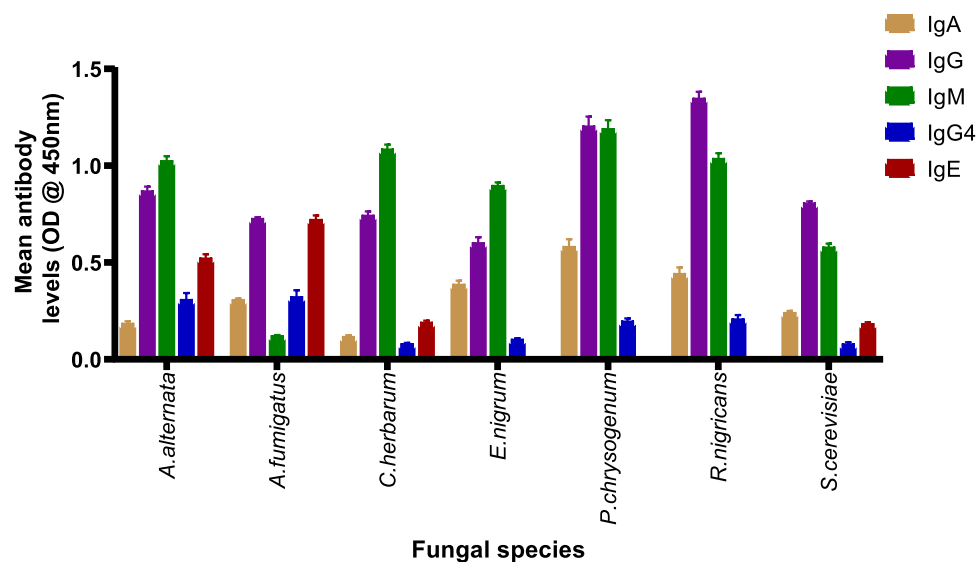


Fig. 6. Mean antibody production with SE bars for each antibody for each fungal species.

alternata (*A.a*)-IgG4 and IgE; *C. herbarum* (*C.h*) - IgM and IgG; *S. cerevisiae* (*S.c*)- IgM, IgA and IgG; *P. chrysogenum* (*P.c*)-IgM; *R. nigricans* (*R.n*)-IgA, IgG and IgG4. NMDS2 was positively correlated with *A. fumigatus* -IgA and IgE; *P. chrysogenum*- IgA; *S. cerevisiae*- IgE and IgG4, whilst negatively correlated with *C. herbarum*- IgG4; *E. nigrum*- IgM and IgA; *R. nigricans* -IgM. NMDS3 was positively correlated with *E. nigrum*-IgG4 and IgM; *P. chrysogenum*- IgA, *A. alternata*-IgG whilst negatively correlated with *A. fumigatus* -IgM and *S. cerevisiae*- IgA Fig. 7. Summary of the NMDS scores are shown in Supplementary Table S2.

As IgM and IgG are often associated with first exposure to allergen, *C. herbarum*, *P. chrysogenum* (IgM), *R. nigricans* and *S. cerevisiae* were negatively associated with allergen exposure. Whereas *A. alternata*, *P. chrysogenum* (IgG) and *E. nigrum* were positively associated with allergen exposure. As IgE is associated with allergic sensitisation, *A. fumigatus* and

S. cerevisiae were associated with allergic sensitisation.

3.5.2. Serological reactivity to fungi varies with sex, *S. haematobium* infection and SPT reactivity

To determine whether SPT reactivity influenced the antibody profiles within our study population and to identify whether age, sex as well *S. haematobium* infection status affected the antibody profiles identified, three factorial ANOVA models were used: assessing the influence of age and SPT status on NMDS scores, sex and SPT status on NMDS scores as well as *S. haematobium* infection status and SPT status on NMDS score.

When examining the effects of age followed by SPT status, our results showed that age had no significant effect on NMDS scores for all the fungal species included in the analysis (Figs. 8–11(a, d and g) and Figs. S2–S3).

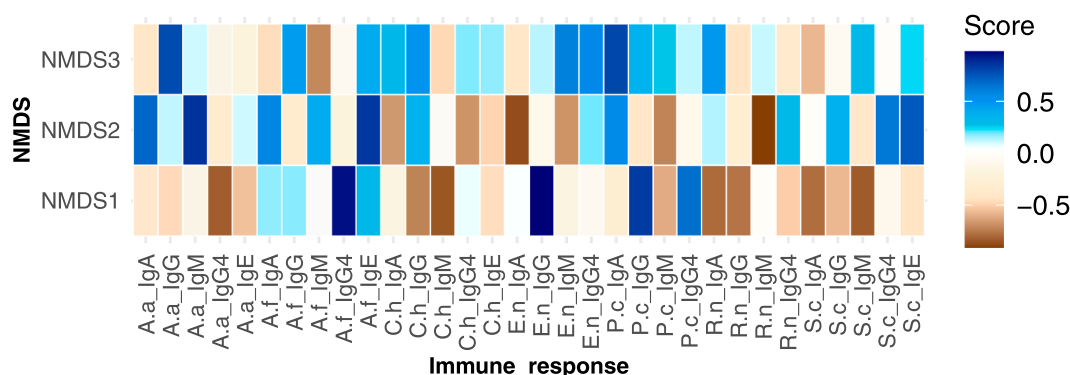


Fig. 7. The NMDS scores of the original variables for each axis calculated after NMDS of fungal-specific antibodies. Those variables which were significantly positively correlated score >0.5 onto an axis are shown in dark blue and those variables which were significantly negatively correlated (score <-0.5) are shown in dark brown. There is a colour gradient, whereby the darker the colour, the higher the score (if >0) or lower the loading score (if <0).

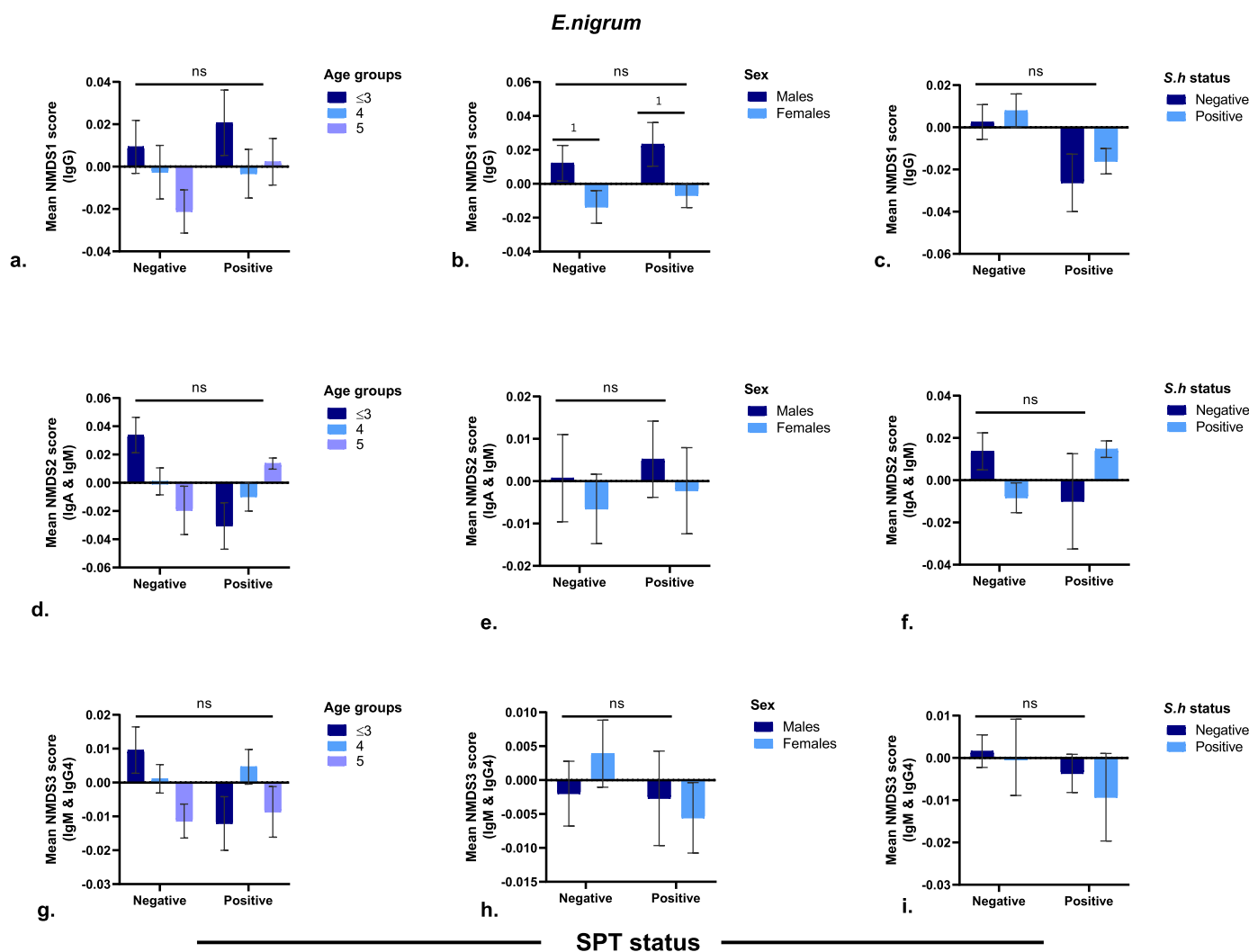


Fig. 8. The mean NMDS scores for each axes, by age and SPT reactivity (a, d & g), by sex and SPT reactivity (b, e & h), and *S.haematobium* infection status and SPT reactivity (c, f & i) for the NMDS of antibody responses. Significance between the main effects, age and SPT reactivity, sex and SPT reactivity as well as *S.haematobium* infection status and SPT reactivity, are indicated on the graph using *, $p < 0.05$ =*, $p > 0.05$ = ns. Standard error of mean is indicated on the graphs for each group.

When examining the effects of sex and SPT status, our results showed that sex had a significant effect on NMDS score for NMDS1 (comprising of IgG) for *E. nigrum* and (IgG, IgG4 and IgM) for *P. chrysogenum* with males having a significantly higher NMDS score compared to females (ANOVA, $F(1, 46) = 8.035$, $p = 0.007$; Fig. 8b and $F(1, 46) = 4.398$, $p =$

0.042; Fig. 9b respectively).

For *C. herbarum*, SPT status had a significant effect on NMDS score for NMDS1 (IgG and IgM) with SPT-positive individuals having significantly higher NMDS score compared to SPT-negative (ANOVA, $F(1, 46) = 4.727$, $p = 0.035$; Fig. 10b). There was a statistically significant

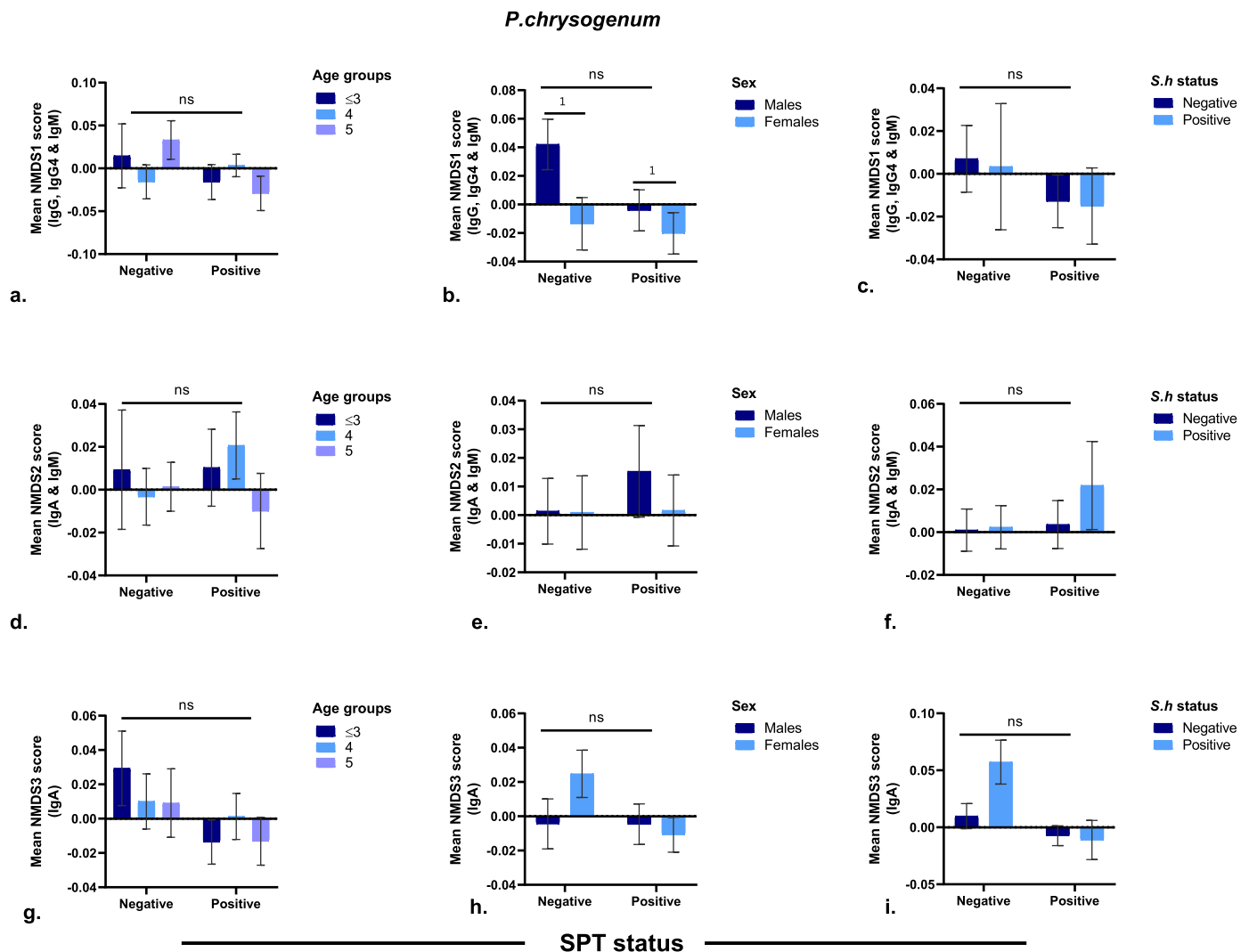


Fig. 9. The mean NMDS scores for each axes, by age and SPT reactivity (a, d & g), by sex and SPT reactivity (b, e & h), and *S.haematobium* infection status and SPT reactivity (c, f & i) for the NMDS of antibody responses. Significance between the main effects, age and SPT reactivity, sex and SPT reactivity as well as *S.haematobium* infection status and SPT reactivity, are indicated on the graph using *, $p < 0.05$; ns, $p > 0.05$. Standard error of mean is indicated on the graphs for each group.

interaction between sex and SPT status on NMDS2 for *C.herbarum* ($F(1, 46) = 5245$, $p = 0.027$). A pairwise comparisons was conducted and revealed that there was a statistically significant difference in mean NMDS2 (IgG4) scores between males and females who were SPT positive ($F(1, 46) = 4.305$, $p = 0.044$; Fig. 10e).

When examining the effects of *S.haematobium* infection status and SPT status our results showed that SPT status had a significant effect on NMDS score for NMDS3 (IgA) for *S.cerevisiae* with SPT-negative individuals having significantly higher NMDS score compared to SPT-positive ($F(1, 46) = 4.330$, $p = 0.043$; Fig. 11i).

3.6. Association between gut mycobial abundance with SPT reactivity and fungal-specific antibody responses

There was no association between mycobium abundance and SPT reactivity or fungal-specific antibody responses as determined by PERMANOVA analysis, Tables 4 and 5 respectively.

3.6.1. Association between of fungal abundance and SPT reactivity with skin prick reactivity, and IGE response and IGE/ IGG4 ratio to specific fungal species

We further assessed the association of abundance of specific fungal genera with fungal SPT reactivity and antigen-specific IgE responses by

ANOVA and linear regression analysis, respectively.

No significant differences were found between species abundance in skin test-positive and skin test-negative children for *A.alternata* and *C. herbarum* (Fig. 12 [1a and b]). In contrast, species abundance of *S.cerevisiae* was significantly higher in children who were skin test-negative (Fig. 12[1c]). No significant correlation was found between the species abundance and IgE reactivity or IgE/Ig4 ratios for *A.alternata*, *C.herbarum* or *S.cerevisiae* (Fig. 12 [2a-f])

4. Discussion

This study is to our knowledge the first comprehensive descriptive study of the relationship between gut mycobium, fungal sensitisation and fungal seroreactivity in rural pre-school aged African children. The key findings were that fungal sensitisation is common and that gut mycobial abundance and diversity is not associated with SPT reactivity or seroreactivity.

In the current study, the mycobium of the children was heterogeneous and comprised less than 1% of the sequenced gut microbiota, which is comparable to previous studies (Qin et al., 2010; Cardinelli et al., 2015). Our dendrograms showed no distinct clustering (Figure S1) reflecting high inter-individual variability and this is similar to what was reported in the Human Microbiome Project (HMP) cohort. In the HMP it

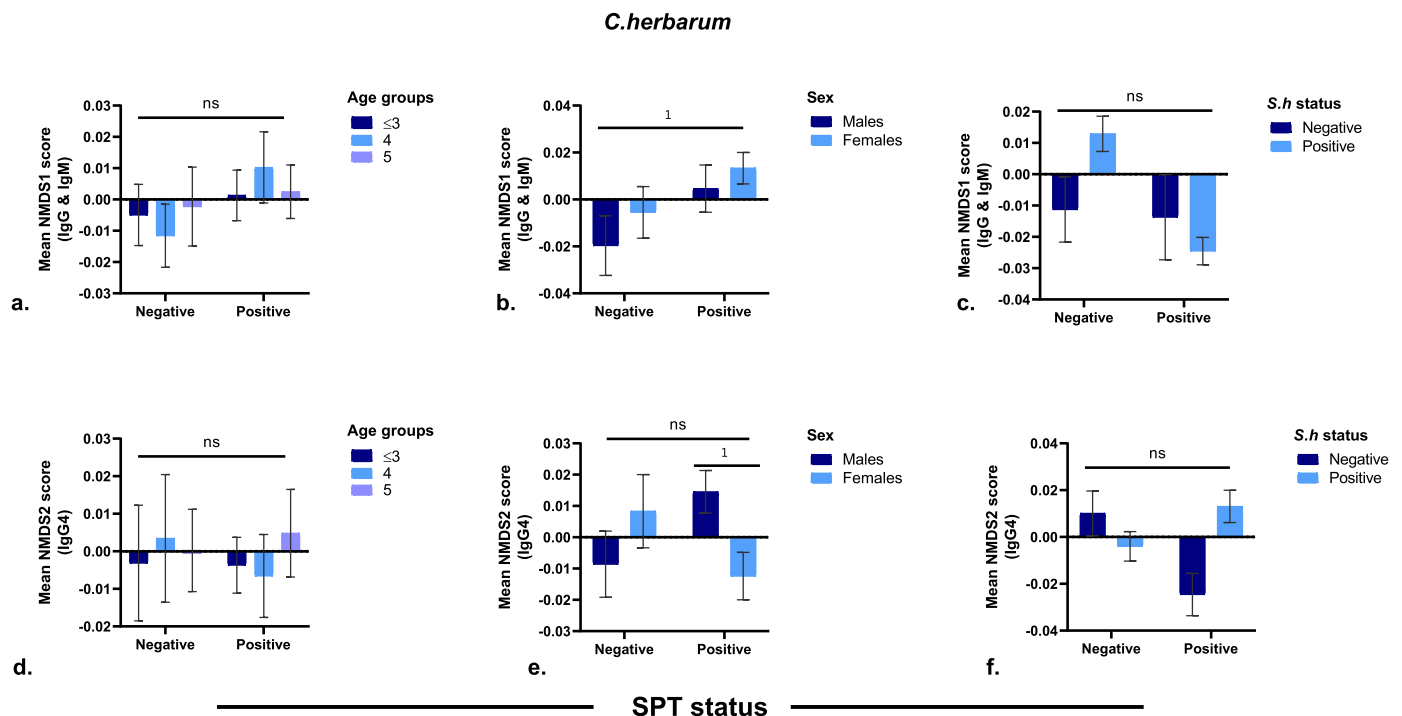


Fig. 10. The mean NMDS scores for each axes, by age and SPT reactivity (a & d), by sex and SPT reactivity (b & e), and *S. haematobium* infection status and SPT reactivity (c & f) for the NMDS of antibody responses. Significance between the main effects, age and SPT reactivity, sex and SPT reactivity as well as *S. haematobium* infection status and SPT reactivity, are indicated on the graph using *, $p < 0.05$; ns, $p > 0.05$. Standard error of mean is indicated on the graphs for each group.

was reported that, in contrast to bacteria, the mycobiome has both high inter- and intra-individual variability (Nash et al., 2017).

Previous studies have shown that diet (Hoffmann et al., 2013) age, (Rodríguez et al., 2015) environmental patterns, (De Filippo et al., 2010) geography (Yatsunenko et al., 2012; Kabwe et al., 2020) and sex (Strati et al., 2016) are determinants of the gut microbial community structure (Conlon and Bird, 2014; Vangay et al., 2018). In the current study, there was a significant association between *S. haematobium* infection status and gut mycobial abundance. However, there were no significant differences observed with age, sex and nutritional and growth variables possibly due to the comparable dietary and environmental exposure as the children were born in and were permanent residents of the study area. Moreover, they were all ≤ 5 years, thus comparable age-range and sex-specific microbiome profiles have been suggested to emerge later after puberty (Markle et al., 2013; Kim et al., 2020).

The most prevalent genera were *Protomyces*, *Aspergillus*, *Saccharomyces*, and *Taphrina*. In the Hoffmann study in USA on diet as a determinant of the gut mycobial community structure, the most abundant fungal genera noted were *Saccharomyces*, *Candida* and *Cladosporium* (Hoffmann et al., 2013) whereas *Pichia*, *Candida*, *Aspergillus* and *Cladosporium*, dominated the South African gut microbiome when geographical location was studied (Kabwe et al., 2020). Our findings are comparable with these previous studies which show that *Aspergillus*, *Candida*, *Malassezia*, *Penicillium*, *Pichia*, and *Saccharomyces* genera are among the most prevalent fungal genera (Hallen-Adams et al., 2015). The differences observed may be due to factors highlighted such as diet, lifestyle or age (Yatsunenko et al., 2012; Hoffmann et al., 2013; Rodríguez et al., 2015; Kabwe et al., 2020).

We found that *Aspergillus* was among the most abundant fungal genera, and an increase in fungus populations such as *Aspergillus* has been associated with increased eosinophil levels and an exaggerated Th2 response (Wheeler et al., 2016), both of which are characteristic of allergic responses. However, it remains to be established if our observations were due to primary changes in the fungal population or were

secondary to changes to other microbial communities such as bacteria (Osakunor et al., 2020). Further exploration of the microbiome using clinically defined allergy cases would be informative.

We investigated whether abundance of specific fungal genera was associated with fungal skin prick reactivity and specific IgE antibody reactivity as well as IgE/ IgG4 ratio. No significant correlation was found between the species abundance and IgE reactivity or IgE/IgG4 ratios. Although we observed no significant differences between children who were SPT negative or positive for abundance of *A. alternata* and *C. herbarum*, we found significant differences for *S. cerevisiae* (Fig. 12). For SPT-negative children with higher species abundance, and failing to elicit an immune response, there is a possibility of tolerance arising from an active control mechanism, or a state of non-responsiveness whereby IgG4 antibodies inhibit IgE receptor-facilitated allergen binding to B cells thereby diminishing SPT reactivity. This has been demonstrated in immunotherapy studies (Wachholz and Durham, 2004; Shamji and Durham, 2011) which have also shown how this could diminish mast cell and basophil activation (Wisniewski et al., 2013).

Atopic diseases are common chronic childhood disorders and sensitisation to allergens is recognised as the most important risk factor (Chiu et al., 2014). From our data, a 96% prevalence of SPT reactivity to at least one of the six selected fungal species was found. Grouping the fungi-sensitised individuals according to the pattern of SPT reactivity showed that the largest subgroup of fungi-sensitised individuals was reactive to multiple fungi species which could have been either due to genuine sensitisation to a variety of fungi, or due to cross-reactivity between fungal allergens. Our high rate of SPT reactivity is in contrast to rates reported by other countries, ranging from 3% to 58% (D'Amato et al., 1997; Arbes et al., 2005; Bousquet et al., 2007; Wiszniewska et al., 2013; Fernández-Soto et al., 2018; Kwizera et al., 2019). These results highlight differences between different populations and thus emphasize the need for studies to characterise sensitisation patterns in different regions of the world (Eriksson and Holmen, 1996; Mpairwe et al., 2008). Many studies have suggested that the occurrence and severity of atopic disease symptoms in later childhood are directly related to allergen

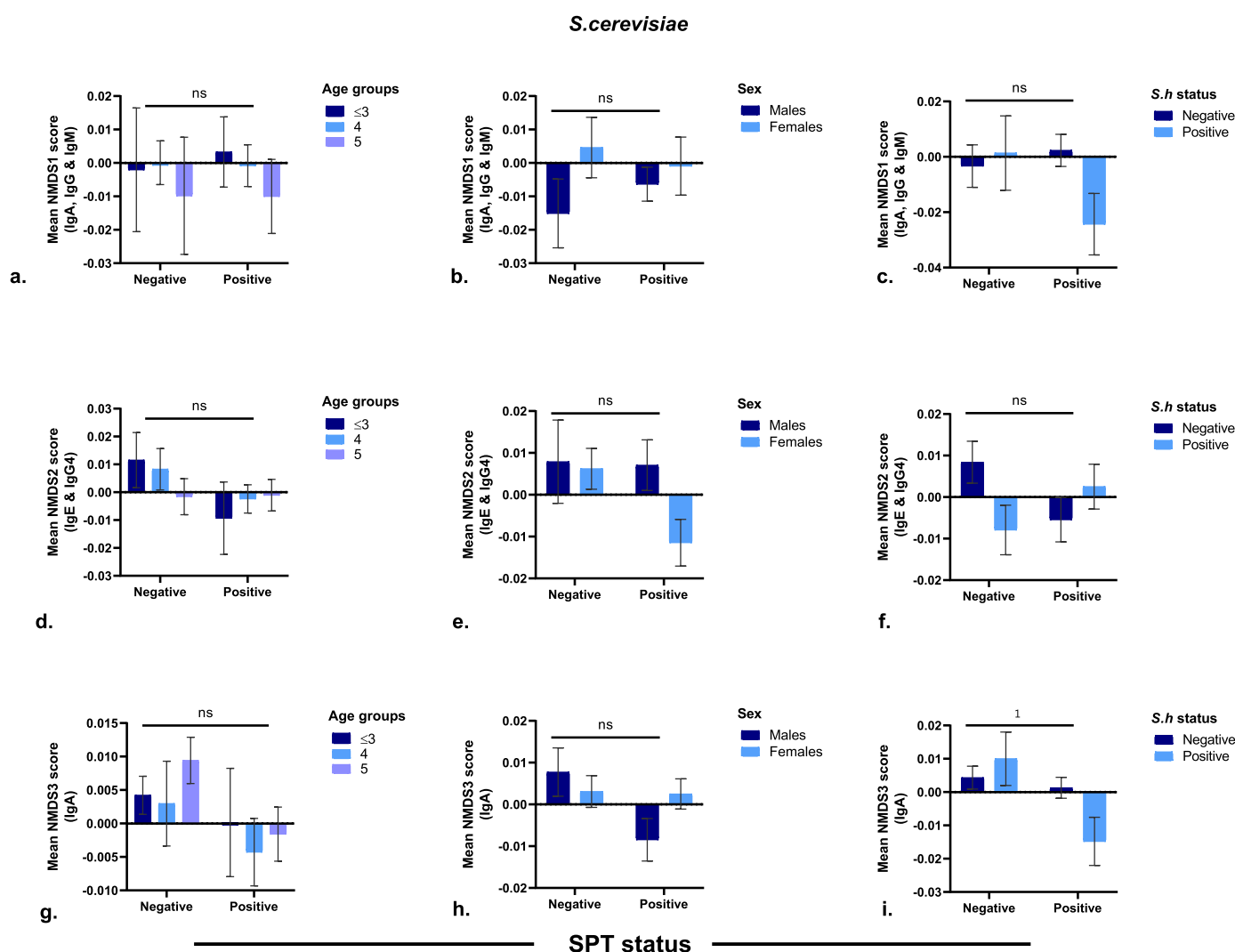


Fig. 11. The mean NMDS scores for each axes, by age and SPT reactivity (a, d & g), by sex and SPT reactivity (b, e & h), and *S.haematobium* infection status and SPT reactivity (c, f & i) for the NMDS of antibody responses. Significance between the main effects, age and SPT reactivity, sex and SPT reactivity as well as *S.haematobium* infection status and SPT reactivity, are indicated on the graph using *, $p < 0.05$; ns, $p > 0.05$. Standard error of mean is indicated on the graphs for each group.

Table 4

Summary of SPT reactivity and association with gut mycobiome.

Variable	n	p-value	Explained sum of squares	Total sum of squares	FDR
<i>A.alternata</i>	50	0.155	117.2	4323.4	0.458
<i>C.herbarum</i>	50	0.944	52.9	4387.8	0.944
<i>S.cerevisiae</i>	50	0.252	109.6	4331.0	0.458
<i>P.chrysogenum</i>	50	0.270	101.8	4338.9	0.458
<i>R.nigricans</i>	50	0.371	92.1	4348.5	0.458
<i>E.nigrum</i>	50	0.382	91.4	4349.2	0.458

p-value-unadjusted p-value; FDR- adjusted p-value (FDR-corrected).

sensitisation in infancy (Kulig et al., 1998; Sun et al., 2014). Thus, knowledge of the sensitisation patterns may provide a means by which to establish allergic diseases management strategy for allergists (Lou et al., 2017).

Empirical studies have indicated sex and age differences in the prevalence of allergy sensitisation and diseases (Govaere et al., 2007; Chen et al., 2008) with males being more atopic in childhood than females (Govaere et al., 2007). However, this trend changes after puberty with allergies becoming more apparent in females (Shah, 2012). In our study, there were no significant effects of sex and age on SPT reactivity; however, prevalence of SPT reactivity decreased with age. There were

no significant effects of *S.haematobium* infection status on SPT reactivity and this might have been due to the small sample size of *S.haematobium* infected people.

We were able to detect IgA, IgG, IgM and IgG4 antibodies to all the fungal allergens but not IgE in participants who were SPT positive for *E. nigrum*, *P.chrysogenum*, *R.nigricans*. This is not surprising because a positive SPT without detectable IgE in the ELISA is indicative of non IgE-mediated response (Denning et al., 2006) and the presence of high titres of allergen-specific IgG antibodies can interfere with IgE reactivity through competition with IgE for binding to the solid phase-bound allergens (Van der Zee et al., 1988). Seroprevalence was high in the

Table 5

Summary of fungal antibody responses and their association with gut mycobiome.

Variable		n	p-value	Explained sum of squares	Total sum of squares	FDR
<i>A.alternata</i>	IgA	71	0.507	72.6	5556.5	0.839
	IgG	71	0.956	47.3	5581.7	0.956
	IgM	71	0.881	54.2	5574.8	0.924
	IgG4	71	0.381	80.2	5548.9	0.839
	IgE	71	0.708	62.5	5566.6	0.888
<i>A.fumigatus</i>	IgA	71	0.471	76.5	5552.5	0.839
	IgG	71	0.740	61.6	5567.5	0.888
	IgM	71	0.830	56.6	5572.4	0.924
	IgG4	71	0.495	74.2	5554.9	0.839
	IgE	71	0.582	69.3	5559.7	0.839
<i>C.herbarum</i>	IgA	71	0.403	79.9	5549.1	0.839
	IgG	71	0.562	70.1	5558.9	0.839
	IgM	71	0.306	85.9	5543.2	0.839
	IgG4	71	0.242	94.3	5534.8	0.839
	IgE	71	0.898	50.7	5578.4	0.924
<i>S.cerevisiae</i>	IgA	71	0.545	71.7	5557.4	0.839
	IgG	71	0.739	61.3	5567.7	0.888
	IgM	71	0.192	97.3	5531.8	0.839
	IgG4	71	0.283	88.5	5540.6	0.839
	IgE	71	0.458	75.9	5553.2	0.839
<i>E.nigrum</i>	IgA	71	0.529	72.2	5556.9	0.839
	IgG	71	0.150	108.0	5521.1	0.839
	IgM	71	0.689	64.0	5565.0	0.888
	IgG4	71	0.168	101.2	5527.9	0.839
<i>P.chrysogenum</i>	IgA	71	0.868	54.6	5574.5	0.924
	IgG	71	0.678	64.5	5564.5	0.888
	IgM	71	0.273	90.3	5538.7	0.839
	IgG4	71	0.236	94.1	5535.0	0.839
<i>R.nigricans</i>	IgA	71	0.151	106.8	5522.3	0.839
	IgG	71	0.472	75.9	5553.2	0.839
	IgM	71	0.838	55.6	5573.5	0.924
	IgG4	71	0.537	70.9	5558.1	0.839

p-value-unadjusted p-value; FDR- adjusted p-value (FDR-corrected).

population and this could have been due cross-reactivity as some of these fungal allergens are known to cross-react with each other resulting in false positives (Cramer et al., 2009; Fukutomi and Taniguchi, 2015).

To characterise patterns of the different fungus-specific antibodies in the participants, NMDS was used to reduce antibody responses into variables. We went on to determine whether skin prick test (SPT) reactivity influenced the antibody profiles within our study population and identify whether age, sex as well *S.haematobium* infection status affected the antibody profiles identified in PSAC. From the analysis, we observed that age had no significant effect on NMDS scores for all fungal species investigated. The absence of an association with age is not surprising since the age range studied is narrow.

When examining the effects of sex and SPT status, our results showed that sex had a significant effect on NMDS score for NMDS1 (comprising of IgG) for *E.nigrum* and (IgG, IgM and IgG4) for *P. chrysogenum* with males having significantly higher responses compared to females. It has been suggested that the risk of being allergic is greater for males in childhood (Jensen-Jarolim and Untersmayr, 2008), and this may explain our observation of significantly higher antibody responses in males. This gender difference seems to be less pronounced after puberty as girls become more likely to be atopic throughout the reproductive years (Govaere et al., 2007) and this has been suggested to be due to differences in sex hormones during onset of puberty (Paus-Jenssen and Cockcroft, 2003; Chen et al., 2008). However, this cannot explain the differences observed in our study population and therefore the exact pathophysiologic mechanism of gender differences in atopy remains unclear (Kim et al., 2014).

SPT status had a significant effect on NMDS score for NMDS1 (IgG and IgM) for *C.herbarum*, with SPT-positive individuals having significantly higher responses compared to SPT-negative. Our findings are consistent with the suggestions that IgM and then IgG are produced primarily in the first exposure to an allergen and associated with allergic diseases (Scott-Taylor et al., 2018). Therefore, a higher response in

SPT-positive individuals would be expected as observed with *C. herbarum*.

Several epidemiology studies have shown inverse associations of chronic parasitic worm infections with allergy and atopy in regions of high prevalence of such infections (van den Biggelaar et al., 2004; Leonardi-Bee et al., 2006; Mpairwe et al., 2008; Rujeni et al., 2012; Webb et al., 2016). Other studies in schoolchildren have produced conflicting results with regard to the effects of deworming on SPT responses (van den Biggelaar et al., 2004; Cooper et al., 2006). A study in Indonesia found that SPT reactivity increased after 1 year of albendazole treatment, in Gabon SPT reactivity was significantly reduced in children infected with *S.haematobium* and in Ecuador, albendazole treatment had no effect on the prevalence of SPT reactivity (Lynch et al., 1993; van den Biggelaar et al., 2000; Cooper et al., 2006; Staal et al., 2018). In this study, when we examined the effects of *S.haematobium* infection status and SPT status on NMDS scores our results showed that SPT status had a significant effect on NMDS scores for NMDS3 (IgA) for *S.cerevisiae* with SPT-negative individuals having significantly higher IgA response compared to SPT-positive individuals. This observation might be due to the protective role of IgA, which has been suggested to contribute to the maintenance of mucosal tolerance by dampening immune responses and hence is thought to prevent the development of hyperinflammatory responses towards environmental allergens that would otherwise cause allergic inflammation (Gloudemans et al., 2013).

Finally, a substantial number of studies have suggested that SPT-positive individuals sensitised to aeroallergens are more prone to developing allergic disease (Hagy and Settupane, 1976; Bodtger et al., 2003). Important questions our study raises are; what is the long-term consequence of being SPT positive in childhood. Does this predispose to future atopic disease? What is the role of the mycobiome in inhalant allergy sensitisation?

There are some limitations to the present study. Not all study participants with the characterised mycobiome could be included for the

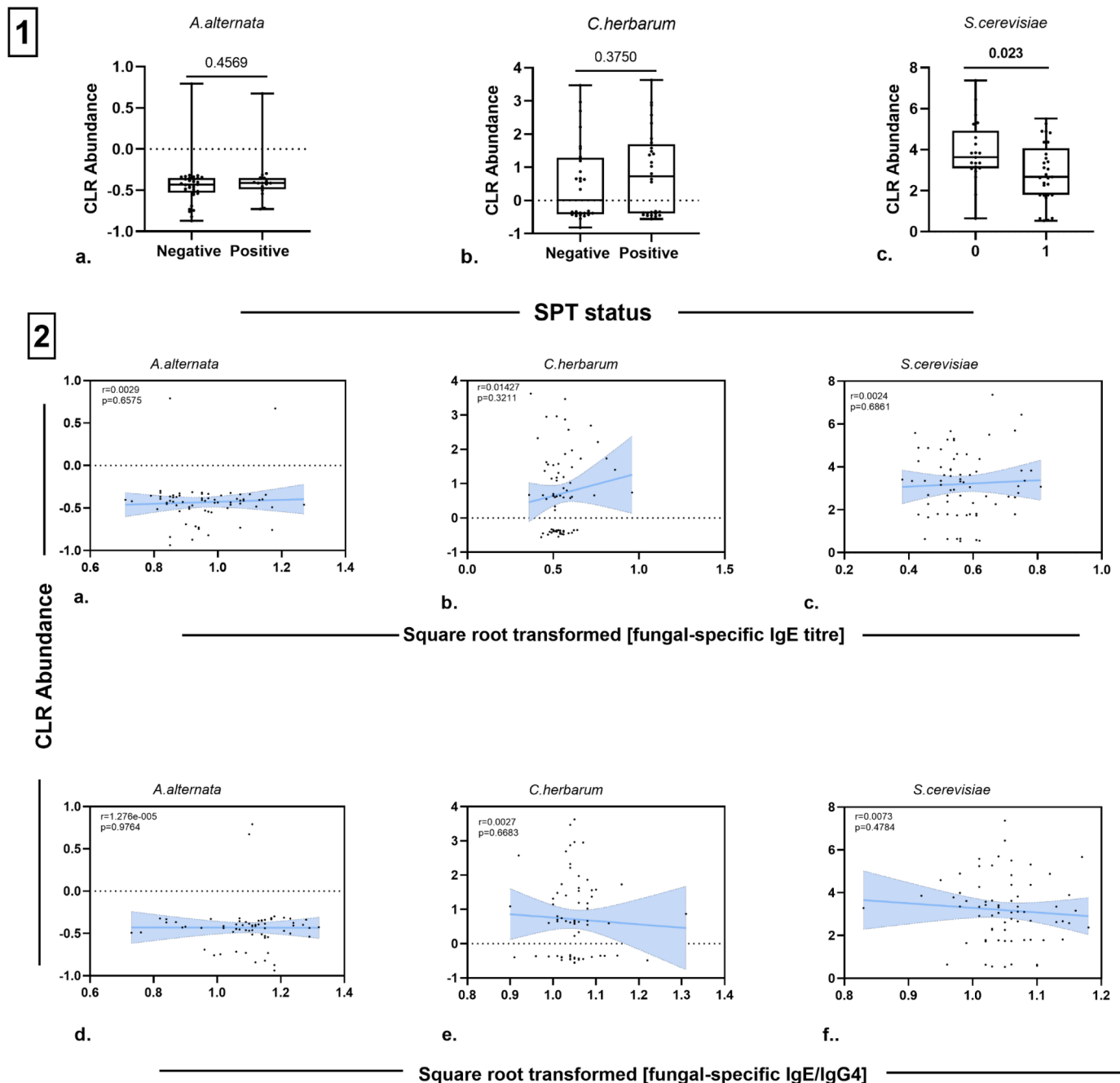


Fig. 12. Relationship between fungal species abundance and [1] skin prick test reactivity, [2] antigen-specific IgE response and IgE/IgG4 ratios Box plots showing the mean abundance of specific fungal genera, grouped by skin prick reactivity [1a-c]. The horizontal box lines represent the first quartile, the median and the third quartile. Whiskers denote the range of data within the first quartile $-1.5 \times$ the interquartile range and the third quartile $+1.5 \times$ the interquartile range. [2a-c] Scatter plots showing linear regression analysis of fungus-specific IgE antibody titres and fungal genera abundance. [2d-e] Scatter plots showing linear regression analysis of fungus-specific IgE/IgG4 ratios and fungal genera abundance Shaded areas indicate the 95% CI. Significant p -value is indicated in bold.

subsequent analysis due to loss of follow up, inadequate sample volumes or participants who withdrew due to aversion to the SPT procedure. The descriptive study design allowed the characterisation of the gut mycobiome and its relationship with SPT and seroreactivity to fungi as well as the determination of effects of host factors on seroreactivity and SPT reactivity of preschool-aged children at a single time point. A longitudinal study would be more useful for evaluating the relationship between the host factors and the development of fungal sensitivity over time as well as assessing its clinical relevance. Furthermore, the lack of participants with clinical symptoms meant the clinical relevance of the SPT could not be ascertained.

A further study evaluating the mycobiome in healthy and confirmed fungal-allergic individuals would be interesting to see whether variations in the mycobiome will be observed depending on allergy status. This study did not evaluate for possible interactions between other biomes such as bacteria, viruses and fungi. Investigating this would help evaluate what interactions occur between these biomes and the effect they may have on the host (Chin et al., 2020). Furthermore, future studies should assess the profile of fungal sensitisation amongst atopic children in other parts of Zimbabwe. These studies will aid in improved awareness of fungal sensitisation, early diagnosis of atopic diseases and implementation of preventative measures in the country.

5. Conclusions

This study provided the first comprehensive characterisation of the gut mycobiome and fungal allergic sensitisation of rural children in Zimbabwe. This indicated a high percentage of sensitization but reported allergic disease is low. Further studies with a larger number of well-characterised patients and controls are needed to understand the role of mycobiome in allergic diseases.

Author contributions

FM, TM & EN conceived the study. FM, TM, EN & LP conducted the fieldwork, LP, conducted the laboratory work and LP & FM conducted the data analysis. FM, SB, MW & EN; Supervision. LP & FM drafted the manuscript and all authors contributed to the final version.

Data sharing

All the individual participant data that underlie the results reported in this manuscript (after de-identification) will be fully available with publication, without restriction through the University of Edinburgh database.

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Declaration of Competing Interest

The authors have declared that no competing interests exist.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.crmicr.2021.100082](https://doi.org/10.1016/j.crmicr.2021.100082).

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