External ear canal mycobiome of some rabbit breeds.

Leyna Díaz[1], Gemma Castellá[1]*, M. Rosa Bragulat[1], Jaume Martorell[2], Andreu Paytuví-Gallart[3], Walter Sanseverino[3], F. Javier Cabañes[1]

[1] Veterinary Mycology Group, Department of Animal Health and Anatomy, Universitat Autònoma de Barcelona, Bellaterra, Catalonia, Spain.

[2] Department of Animal Medicine and Surgery, Universitat Autònoma de Barcelona, Bellaterra, Catalonia, Spain.

[3] Sequentia Biotech SL, Barcelona, Catalonia, Spain.

*Corresponding author:

Gemma Castellá

Grup de Micologia Veterinària

Departament de Sanitat i d'Anatomia Animals

Facultat de Veterinària, Universitat Autònoma de Barcelona

08193 Bellaterra (Barcelona), Spain.

Tel: +34 93 5811089

Fax: +34 93 5812006

e-mail: Gemma.Castella@uab.cat

Abstract

The genus *Malassezia* is part of the normal skin mycobiota of a wide range of warm-blooded animals. In this genus, *M. cuniculi* is the only species described from rabbits. However, *Malassezia* species are rarely studied in lagomorphs. In the present study, the presence of *Malassezia* was assessed in samples from the external ear canal of healthy rabbits of different breeds. Cytological and culture techniques, Sanger sequencing and Next generation sequencing (NGS) were used to describe the ear mycobiota in the samples. Although no growth was observed in the cultured plates, cytological examination revealed the presence of round cells similar to those of *Malassezia* yeasts. For metagenomics analysis the D1/D2 domain of the large subunit of the ribosomal DNA (LSU rDNA) was PCR amplified and the resulting reads were mapped against a custom-made cured database of 26S fungal sequences. NGS analysis revealed that *Basidiomycota* was the most abundant phylum in all the samples followed by *Ascomycota*. *Malassezia* was the most common genus presenting the highest abundance in the external ear canal. *Malassezia* phylotype 131 and *M. cuniculi* were the main sequences detected in the external auditory canal of rabbits. The study included both lop-eared and erect-eared rabbits and no differences were observed in the results when comparing both groups. This is the first attempt to study the external ear canal mycobiome of rabbits of different breeds using NGS.

Keywords: *Malassezia*, *M. cuniculi*, phylotype 131, NGS, ear mycobiome, rabbits.

Lay Summary

In the present study, the presence of *Malassezia* was assessed in samples from the external ear canal of healthy rabbits of different breeds. Cytological and culture techniques, Sanger sequencing and Next generation sequencing (NGS) were used to describe the ear mycobiota in the samples.

1. Introduction

Yeasts of the genus *Malassezia* belong to the normal skin microbiota of a wide range of warm-blooded animal species. The genus *Malassezia* includes, at the present, 18 species that have been mostly isolated from different wild and domestic animals but also from human hosts[1,2,3]. Some *Malassezia* yeasts, appear to have a broad host range, while others are more host-specific with a close adaptation to the cutaneous ecosystem of a single animal species or a group of phylogenetically related animals like *M. cuniculi* which has only been isolated from rabbits[4]. While *M. caprae* and *M. equina* are recovered from ruminants and horses[5], *M. pachydermatis* is the main species in dogs and *M. nana* is commonly isolated from cats[6,7]. In parrots, *M. brasiliensis* and *M. psittaci* have been described[8] and *M. vespertilionis* was recently described in bats[2,9]. However, *Malassezia* species are rarely isolated and studied in lagomorphs[10]. Since the description of *M. cuniculi*[4], a few more studies have been conducted in rabbits[11,12]. At the present, this is the first attempt to study *Malassezia* and the mycobiome in rabbits using advanced molecular techniques like NGS.

Members of the genus *Malassezia* are lipophilic and lipid-dependent yeasts that require specific media with a specific lipid composition for laboratory growth, such as Leeming & Notman agar (LNA)[13] and modified Dixon agar (mDA)[14]. Because of their lack of genes involved in carbohydrate metabolism and the genes encoding for the fatty acid synthase they are unable to synthesize long-chained fatty acids (C14 or C16) *de novo* thus, they rely on the host as an exogenous source of fatty acids[15,16]. They have a predilection for seborrheic skin sites such as the external ear canal, the scalp, the trunk, and the perianal, inguinal and submandibular areas[17]. *Malassezia pachydermatis* is the only member that is able to grow on Sabouraud's glucose agar (SGA) even though they

lack the same genes, is uniquely able to utilize lipid fractions within the peptone component of SGA[16,18,19].

These growth requirements could be the result of a host adaptation phenomenon among *Malassezia* species. In healthy conditions, the distribution of *Malassezia* species, the proportion of colonization and its density may vary according to the animal host and even between anatomic sites on the host. This likely is a result of the cutaneous lipid composition and the competition of different types of microorganisms. Many other factors like environmental conditions, age and gender of the host could influence the distribution of *Malassezia* spp. [10]. In rabbits, a lop-eared morphology is suggested as a predisposing factor for *Malassezia* overgrowth. This morphology causes a stenosis of the ear canal that produces a reduction of the expulsion of cerumen. Therefore, an accumulation of cerumen at the base of the ear occurs [11].

Malassezia species are identified based on morphological, ultrastructural, physiological, and molecular analyses[20]. Members of the genus *Malassezia* share similar morphological and biochemical characteristics, so differentiating between species based solely on phenotypic features could cause ambiguity, it is time-consuming, and it cannot be used to describe new species. Molecular techniques are the most reliable for the identification of *Malassezia*[10]. DNA sequencing has become a widely used method to identify *Malassezia*. In particular, sequencing the domains D1/D2 of the large subunit of the ribosomal DNA (LSU rDNA), internal transcribed spacer region 1 and 2 (ITS), β-tubulin and chitin synthase 2 (CHS2) genes has become a useful method to identify and differentiate species within the genus *Malassezia*[10, 21, 22].

Next generation sequencing (NGS) platforms perform sequencing of millions of small fragments of DNA in parallel, then bioinformatics analyses are needed to piece together these fragments by mapping the individual reads to the reference database. NGS provided a culture-independent method to obtain microorganisms' genome information with high sensitivity at a lower cost. Culturable microorganisms represent only a small fraction of the microbial diversity. NGS represents a useful tool to fully investigate and understand microbial diversity without the need to culture the samples. This method can be used to explore the genetic diversity, population structures and interactions of microbial communities in their ecosystems[23,24,25,26]. Several regions of the fungal rRNA genes have been used in NGS to study fungal taxonomy and diversity, including the small subunit (SSU) and the large-subunit (LSU) rRNA genes and the internal transcribed spacer (ITS)[27,28]. Few metagenomic studies have been conducted in fungi due to the complexity of their genomes, which are generally larger than the prokaryotic genomes because they contain large amounts of non-coding and repetitive DNA, and the lack of validated databases cataloguing enough diversity[29,30].

The aim of this work was to study the external ear canal *Malassezia* population in different rabbit breeds using culture and non-culture based techniques, including NGS, and to identify the presence of non-culturable *Malassezia* yeasts in the skin of these animals.

2. Materials and methods

2.1. Animals, sampling, and culture media used

A total of 60 rabbits were sampled including pet and farmed rabbits (Table 1, Supplementary Table 1). All pet rabbits (n= 44) presented to the Exotic Pets Service of

the Veterinary Teaching Hospital of the Universitat Autònoma de Barcelona (UAB, Cerdanyola del Vallès, Spain) from 2017-2019, were considered for the study regardless of the presenting reason for consultation. Farmed rabbits (n=16) were housed on the Experimental Farm Service of the UAB. In all cases, the criterion for inclusion was the absence of history and clinical signs of otitis externa, confirmed by otoscopic examination. Both lop-eared breeds such as Belier and Toy, and erect-eared breeds such as Rex and New Zealand were included in this study.

Samples were collected from the external ear canals of both ears by using a swab soaked in the wash fluid i.e., 0.075 mol/l phosphate-buffered physiological saline, pH 7.9 containing 0.1% Tween 80. Samples were obtained following procedures approved by Ethics Committee on Animal and Human Experimentation from UAB and Generalitat de Catalunya (approval CEEAH 4600).

Two samples from each ear were taken by gently inserting a sterile cotton swab into the external part of the ear canal and rotating fully for 30 seconds. One swab was used for *Malassezia* culture and cytological examination. Briefly, one side of the swab was streaked onto the following media: SGA (Oxoid), mDA (36g malt extract, 6g peptone, 20g desiccated ox-bile, 10ml Tween 40, 2ml glycerol, 2ml oleic acid and 12 g agar per litre, pH 6.0)[14] and LNA (10g peptone, 5g glucose, 0.1g yeast extract, 4g desiccated ox-bile, 1ml glycerol, 0.5g glycerol monostearate, 0.5ml Tween 60, 10ml whole-fat cow's milk and 12g agar per litre, pH 6.2)[13]. All media contained 0.05% of chloramphenicol and 0.05% of cycloheximide. Plates were incubated at 32ºC and examined daily for 20 days. For the cytological examination smears from the cotton swabs were stained with

Diff-Quick stain and the presence of typical *Malassezia* cells was microscopically determined. The other swab was maintained at -20ºC and used for PCR and NGS.

2.2. DNA extraction

Six ear samples cytologically positive for *Malassezia* yeasts like cells and from different pet rabbit breeds were selected for Sanger sequencing studies (Supplementary Table 1). We used Sanger sequencing in these few samples to obtain the complete sequence of the D1D2 and ITS regions in order to get a good identification with BLAST and a suitable sequence for inferring accurate phylogenetic relationships. DNA was extracted from swabs using the DNeasy PowerSoil Kit (Qiagen, Madrid, Spain) according to manufacturer's instructions with two modifications. At the first lysis step, the swab tip was cut and placed in the bead tube, and an incubation step at 65° C for 10 minutes following addition of Solution C1. The final elution step was performed on 50 µL of C6 instead of 100 µL to obtain a higher DNA concentration. A sterile swab was processed under the same conditions as the external ear canal samples in order to control cross-contamination. DNA was stored at −20°C until used as template in the PCR for Sanger sequencing and NGS.

2.3. Sanger sequencing

The variable D1/D2 region of the 26S rRNA gene and the ITS-5.8S rRNA gene were amplified and sequenced, using the primers and the protocols described previously[21]. Sequence alignments were carried out using MUSCLE implemented in MEGA X software[31]. Maximum likelihood analysis of the individual genes was conducted using MEGA 6 software with 1000 bootstrap replicates. A suitable substitution model was determined for each gene. The initial tree for heuristic search was obtained by applying

171 the Neighbour-Joining method to a matrix of pairwise distances estimated using the
172 Maximum Composite Likelihood (MCL) approach. Clades that were supported by
173 bootstrap values (bs) of ≥70% were regarded as strongly supported. Sequences of
174 *Cryptococcus neoformans* CBS 132 were selected as outgroup for the tree construction.
175
176 **2.4. NGS and data analysis**
177 A total of 4 ear samples were subjected to metagenomics NGS of fungal 26S rRNA genes.
178 Three samples were previously selected for Sanger sequencing and a sample of a farmed
179 rabbit cytologically positive to *Malassezia* yeasts like cells was also included
180 (Supplementary Table 1). The samples covered animals from four different breeds: New
181 Zealand (farmed rabbit), Rex, Belier and Toy (pet rabbits). All the animals showed no
182 clinical signs and were free from otitis externa. Both lop (Belier and Toy) and erect-eared
183 (Rex, New Zealand) were included. DNA was extracted as previously described and a
184 positive PCR for D1D2 region was obtained before library preparation.
185
186 Quality control was performed at IGA Technology. DNA concentration was evaluated by
187 using a Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA). Amplicon-seq libraries of
188 D1/D2 regions of the fungal 26S rRNA gene were obtained from each sample by
189 following 16S Metagenomic Sequencing Library Preparation protocol with minor
190 modifications. Briefly, a composite pair of primers: the forward primer (NL1) 5′-
191 GCATATCAATAAGCGGAGGAAAAG-3′ and the reverse primer (NL4) 5′-
192 GGTCCGTGTTTCAAGACGG-3′[32] containing Illumina overhang sequences necessary
193 for the compatibility with Illumina index and sequencing adapters were used for the first
194 PCR amplification under the following conditions: 95°C for 3 minutes; 28 cycles of: 95°C
195 for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds; 72°C for 5 minutes; hold at

4°C. Upon the clean-up, the second PCR was performed under the following conditions: 95°C for 3 minutes; 9 cycles of: 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds; 72°C for 5 minutes; hold at 4°C. Relevant flow-cell binding domains and unique indices (NexteraXT Index Kit, FC-131-1001/FC-131-1002) were integrated to the amplicon target. Libraries were normalized by Qubit 2.0 Fluorometer, pooled and sequenced on MiSeq using paired 300-bp reads, and MiSeq v3 reagents (Illumina, San Diego). Sequence reads were analysed in the cloud bioinformatics platform GAIA (metagenomics.sequentiabiotech.com)[33]. Sequencing quality was assessed using FastQC (bioinformatics.babraham.ac.uk/projects/fastqc/) and BBduk (jgi.doe.gov/data-and-tools/bbtools/), setting a minim length of 35bp and a minimum Phred-quality score of 25.

The resulting high-quality reads were mapped against a custom-made cured database of 26S fungal sequences from NCBI. To have the dataset as complete as possible for the analysis, all the sequences were downloaded from GenBank with the queries "28S rRNA" or "28S ribosomal RNA" not "uncultured" not "fungal endophyte" not "fungal sp." Also, our dataset included a sequence for every *Malassezia* species even the non-culturable ones. The database included only fungal sequences due to the aim of this study. For taxonomic classification, the mapping-based approach against the database with the BWA mapper[34] is followed by an in house Lowest Common Ancestor (LCA) algorithm. The minimum identity thresholds applied to classify the reads into different taxonomic levels were species (99%), genus (98%), family (96%), order (94%), class (92%), and phylum (90%) following the limits proposed by GAIA software. Those taxa with an abundance below 0.01% considering its mean across the different samples within an experimental group were filtered out before further analysis. DESeq2 (v1.26)[35] was used to carry out differential abundance analyses.

GAIA also assesses the diversity within (alpha-diversity) and between (beta-diversity) samples. Alpha-diversity measures the richness (number of OTUs) and evenness (the relative abundance of OTUs) in samples. On the other hand, beta-diversity measures the distance or dissimilarity between pairs of samples. Alpha and beta diversities are calculated using the R package phyloseq[36].

3. Results

3.1. Culture and Cytological examination

The presence of spherical *Malassezia* yeast like cells was identified on 33 out of the 60 rabbits (Table 1, Supplementary Table 1). The cells observed were spherical with buds in a monopolar pattern on a narrowed base that in some cases were elongated but remained more narrow than the bud (Figure 1). No growth was observed in any of the culture media used after 20 days of incubation at 32ºC (Supplementary Table 1).

3.2. Sanger sequencing

D1/D2 region was successfully amplified and sequenced for five swabs (Supplementary Table 1) resulting in a product of 603 bp. The sequences were nearly identical showing only a base pair difference.

A BLAST search against the NCBI database revealed that this sequence had a percent identity of 100% to an uncultured Basidiomycota clone 131[37] with a query coverage of 74%, and 91% to the sequence GU733708 belonging to *M. cuniculi* CBS 11721 type strain, which was the closest match. Our sequences differed from the sequence of *M. cuniculi* at 56-57 positions (dissimilarity 9%).

ITS-5.8S rRNA was successfully amplified and sequenced for five swabs (Supplementary Table 1), resulting in a product of 807 base pairs. These sequences obtained were identical. A search on GenBank database revealed that this sequence had a percent identity of 80.74% to *M. cuniculi* CBS11721 type material (NR_137752).

The sequences generate have been deposited at the GenBank database under accession numbers MT812469, MT812503, MT812504.

Maximum likelihood analysis of the D1/D2 and ITS-5.8S rRNA sequences are shown in Fig. 2 and Fig. 3, respectively. With both genes, the sequences obtained clustered close to *M. cuniculi* CBS11721.

3.3. NGS data analysis

All samples were correctly sequenced, and the generated fastq files reported an average value of 52,886 reads passing filter. The number of generated sequences reads of each sample is described in Table 2. The raw sequencing data is available at the NCBI database, SRA accession PRJNA649860.

To characterize the diversity of species in each sample we used the Shannon diversity index (Table 2) which increases as both the richness and the evenness of the community increase. We obtained an average of 120 species and a diversity of 2.43. Sample from Rex rabbit was the most diverse among the group and sample from New Zealand rabbit showed the least diversity.

We investigated the taxonomic compositions of all samples at various taxonomic levels. Fungi from three different phyla were identified (Figure 4). The most abundant fungal phyla across all samples was *Basidiomycota* with a median relative abundance of 56.29% (range, 43.77-83.49 %) followed by *Ascomycota* (median= 17.32% ; range, 2.68-27.91%) and *Mucoromycota* (median= 0.25%; range, 0.09-0.38 %).

When analysed at class (Figure 5), order (Figure 6) and family level (Figure 7), *Malasseziomycetes* (51.89%), *Malasseziales* (51.77%), and *Malasseziaceae* (51.39%) represented the highest abundance. As shown in Figure 8, within the phylum *Basidiomycota*, the most common genus was *Malassezia* (30.56-78.63%; median=48.55%), followed by *Filobasidium* (0.04-0.79%; median=0.37%) and *Vishniacozyma* (0.06-1.75%; median=0.21%) in much smaller percentage. Within the phylum *Ascomycota* the three most common genera were *Cladosporium* (0.65-7.39 %; median=3.25%), *Fusarium* (0.00-1.25 %; median=0.55%) and *Alternaria* (0.21-0.98 %; median=0.40%). Within the phylum *Mucoromycota* not enough reads were classified at the level of genera.

Different fungal taxa were identified in the samples as shown in Figure 9. *Malassezia* phylotype 131 was detected in all samples and it was the predominant taxa (median= 41.45%; range, 26.08-69.13%). This study identified other *Malassezia* species (Figure 10). *Malassezia cuniculi* was also detected from all samples but in lower abundance (median=0.02%; range, 0.01-0.03%). *Malassezia pachydermatis* and *M. restricta* were only present in two samples corresponding to the Rex and New Zealand rabbits. An average of 10.09% of the *Malassezia* sequences were not identified to species level. Within this percentage, the majority of sequences were marked as unknown because a

296 match with enough coverage and identity was not found in our database for them. Within
297 this group of unknown, 7.20% were identified to genus level, 2.50% to family level, and
298 0.36% and 0.16% to order and class level, respectively. A small percentage of the
299 sequences (0.036%) were considered ambiguous because their sequence matched with
300 two different *Malassezia* species sequences.
301
302 Neither *Malassezia* phylotype 131 nor *M. cuniculi* were present in the sterile cotton swab
303 used as control. In this swab, *M. globosa, M. pachydermatis* and *M. restricta* were
304 detected at percentage between 0.52-1.94%.
305
306 No significant differences were observed in the amount of *Malassezia* sequences detected
307 between lop and erect-eared rabbits.
308
309 **4. Discussion**
310 The presence of *Malassezia* in the samples was determined by both cytological
311 examination and Sanger sequencing. On direct microscopic exam the presence of round
312 yeast cells with unipolar budding was detected in more than 50% of the samples. The
313 morphology of the yeast cells observed suggested a *Malassezia* species different from *M.*
314 *pachydermatis* and similar to *M. cuniculi*. When attempted to recover this organism in
315 culture, no growth was observed in any of the culture media used including LNA from
316 which *M. cuniculi* was isolated for the first time[4]. Some *Malassezia* species are fastidious
317 yeasts that have specific nutritional requirements to grow. For example, *M. cuniculi* is
318 only able to grow after 7 days of incubation on LNA and grows better at 37-40ºC. At
319 32ºC the colonies are smaller than the ones cultured between 37-40ºC[4].
320

In this study the sequences D1/D2 of the 26S rRNA gene and the ITS rRNA genes of the samples were sequenced by Sanger sequencing. The main sequence obtained was coincident with that of *Malassezia* phylotype 131, a non-described *Malassezia* yeast detected in the external auditory ear canal of humans[37]. This phylotype has not yet been cultured, and would explain the lack of growth observed in our study.

The phylogenetic trees inferred from the maximum likelihood analysis of both the D1D2 and the ITS sequences obtained in this study show that *Malassezia* phylotype 131 clustered close but with a significant distance to *M. cuniculi*. These results agree with the study conducted by Zhang *et al.* (2012) in which the phylotype 131 was described.

The D1/D2 region of the fungal 26S rRNA gene was selected in this study for the metagenomics analysis of the samples instead of the widely extended ITS region. The 26S rRNA gene is part of the LSU that has been used extensively for fungal phylogeny and taxonomic placement[38]. The ITS region is considered the universal barcode for fungi[39]. However, the extent of the ITS sequence length variability among the different fungal species and genera does not allow for robust sequence alignment[40] and may lead to preferential amplification and sequencing. Also, an incorrect estimation of the abundance of population may occur[28]. Thus, in this study the LSU region was considered the most reliable because this region provides a molecular marker placement of new fungal lineages or for analysis of fungal lineages[40,41,42], especially in *Malassezia* genus[21]. A study comparing NGS results using both ITS and 26S as targeted genes, Mota-Gutiérrez and co-workers suggested that 26S as a target gene showed a greater biodiversity in biological samples compared with the universal primer ITS[43]. Currently the LSU has shown to work better in species discrimination for yeasts than for

346 filamentous fungi[44]. A study conducted by Vu *et al.* demonstrated that while ITS worked
347 better in species discrimination in *Ascomycota*[44], the LSU outperformed in
348 *Basidiomycota*. Finally, a study conducted by Hoggard *et al.* comparing the results
349 obtained in NGS using three different genes (ITS, LSU and SSU) demonstrated that
350 *Malassezia* spp. are markedly under-represented using ITS[45].
351
352 It has been demonstrated by previous authors that strains of yeasts species show less than
353 1% of dissimilarity in LSU regions[40,41,42]. Therefore, an identity threshold of 99% for
354 taxonomic classification at the level of species was applied in this study.
355
356 *Basidiomycota* was the main phylum identified in the ear samples of healthy rabbits,
357 followed by *Ascomycota*. Within *Ascomycota*, *Clasdosporium*, *Fusarium* and *Alternaria*
358 were the three most common fungal genera detected. These genera are commonly isolated
359 from environmental samples and they are considered transient mycobiota. Within
360 *Basidiomycota, Malassezia* was the main genus present in the samples from healthy
361 rabbits as it is a common member of the mycobiota of the skin and the external ear canal
362 of rabbits. These results differ from those obtained in studies of the ear mycobiota of
363 healthy dogs and cats[46,47]. In the ear canal of both dogs and cats the main phylum
364 identified was *Ascomycota*. Within the *Basidiomycota, Cryptococcus* was the main genus
365 in dogs and cats, followed by *Malassezia* in dogs[46,47]. Two comparative studies of the ear
366 mycobiota of healthy and dogs with otitis externa conducted by Korbelik *et al.* and
367 Bradley *et al.* agreed with the results obtained by previous authors [48,49]. *Ascomycota* was
368 the main phylum followed by *Basidiomycota* in healthy dogs. The samples from allergic
369 dogs and dogs with otitis showed less fungal diversity and richness in both studies[46,48]. In
370 dogs with affected ears *Basidiomycota* was the most abundant phylum and *M.*

371 *pachydermatis* the most abundant species[48,49]. Neither *M. cuniculi* nor phylotype 131
372 were detected in the samples of dogs and cats [46,47,48,49]. In those studies, the ITS region
373 was selected to be amplified and the Findley *et al.* and the UNITE fungi databases were
374 used[50].
375
376 In humans, *M. slooffiae* and *M. restricta* were the predominant *Malassezia* species in the
377 external ear canal[37]. Therefore, *Malassezia* was the predominant genus in the external ear
378 canal of humans and thus *Basidiomycota* the main phylum. *Malassezia cuniculi* was not
379 detected in the samples of humans[37].
380
381 The presence of a small amount of *M. pachydermatis* and *M. restricta* was detected in
382 two of the samples corresponding with the New Zealand and Rex breeds. Both *M.*
383 *pachydermatis* and *M. restricta* were also detected in the sterile swab used as negative
384 control. The inclusion of control is indispensable in multiple steps of NGS-based studies
385 due to the greater detection ratio compared to traditional technics[51,52]. In our study a sterile
386 swab was included as a negative control and processed in parallel with the other samples.
387 Controls must be treated identically to other samples during the whole process. Currently
388 there is no consensus in how to handle the sequences recovered from the negative
389 controls. One approach could be to simply eliminate from all the samples any OTUs that
390 appeared in negative controls. This approach could eliminate some of the most abundant
391 OTUs. To avoid eliminating OTUs that could be relevant to the study another approach
392 could be to subtract the number of sequences of each OTU present in the negative control
393 from the abundance of that OTU found in the samples[51]. In this case, neither *M.*
394 *pachydermatis* nor *M. restricta* would be present in the rabbit samples. The presence of
395 these OTUs in the negative control could be the result of contamination during the

manipulation of the samples (sampling, storage, DNA extraction, PCR…) but also it could be the result of primer cross-contamination at any stages from oligonucleotide manufacturing to PCR[51].

Although the ear morphology has been suggested as a factor that could affect the diversity of the ear mycobiota, our study did not identify a significant difference in the abundance of *Malassezia* between lop and ear-erected rabbits. This lack of significant difference could be as a result of the fairly small sample size in our study. However, our results agree with the findings of previous studies of the increased predisposition of lop-eared rabbit to aural and dental problems and of the external auditory meatus in healthy domestic rabbits[11,12]. *Malassezia* being part of the normal microbiota of the ear canal of rabbits, as it is in other species such as dogs or cats, could explain this lack of statistical difference between lop and erect-eared rabbits[11,12,53].

Conclusions

Although no growth was observed in any of the culture media used, the presence of *Malassezia* fungal taxa was detected in more than a half of the external ear canal of the rabbits by cytology. *Malassezia* was also detected by Sanger sequencing and NGS in all selected samples. Regarding NGS, the use of the LSU as a target gene allowed us the description of fungal diversity and the taxonomic classification of several *Malassezia* species. *Malassezia* phylotype 131 and *M. cuniculi* were detected in all the samples. Among all the fungal taxa detected in this study, *Malassezia* phylotype 131 showed the highest abundance in all the samples. Further studies would be needed to isolate and characterize the *Malassezia* phylotype 131 and to know its role in the external ear canal mycobiome of rabbits and human beings.

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Conflict of Interest Statement

Walter Sanseverino and Andreu Paytuví-Gallart were employed by company Sequentia Biotech S.L. All other authors declare no competing interests.

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574 **Figure captions**

575 **Figure 1.** Diff-Quick stain of a smear from an otic swab of a rabbit showing the presence
576 of spherical yeasts cells, possibly of *Malassezia* phylotype 131 (2-2.5 μm in diameter).

577 **Figure 2.** Molecular phylogenetic tree inferred from maximum likelihood analysis of
578 D1D2 sequences of members of the genus *Malassezia*. Bootstrap values > 70% in 1000
579 replications are shown at the nodes. Sequences of *Ustilago maydis* ATCC MYA-4924
580 and *Cryptococcus neoformans* CBS 132 were selected as outgroup for the tree
581 construction.

582 **Figure 3.** Molecular phylogenetic tree inferred from maximum likelihood analysis of ITS
583 sequences of members of the genus *Malassezia*. Bootstrap values > 70% in 1000
584 replications are shown at the nodes. Sequence of *Cryptococcus neoformans* CBS 132 was
585 selected as outgroup for the tree construction.

586 **Figure 4.** Average relative abundance of fungal phyla across the different rabbit breeds.

587 **Figure 5.** Average relative abundance of fungal classes across the different rabbit breeds.

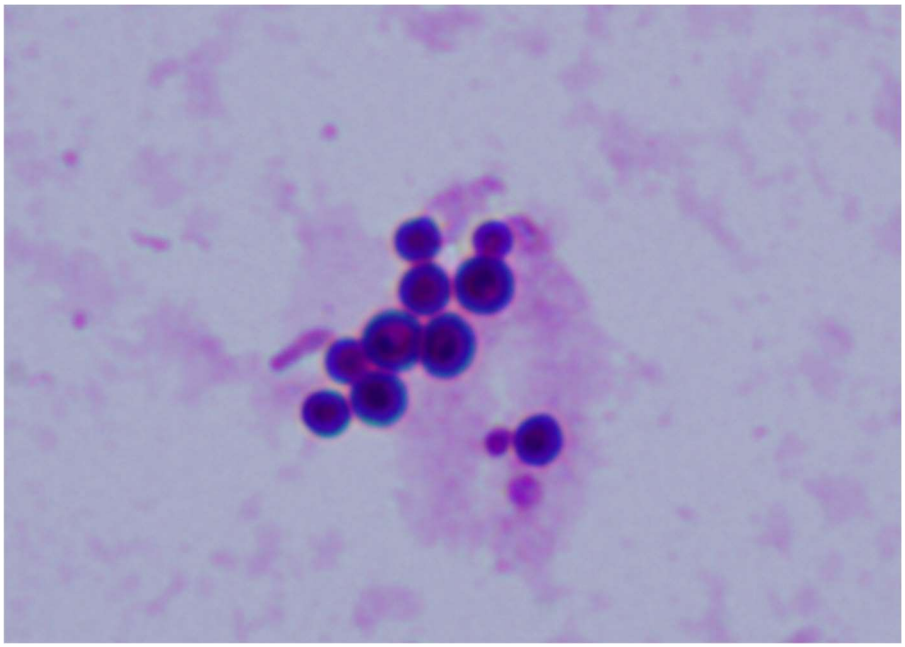
588 **Figure 6.** Average relative abundance of fungal orders across the different rabbit breeds.

589 **Figure 7.** Average relative abundance of fungal families across the different rabbit
590 breeds.

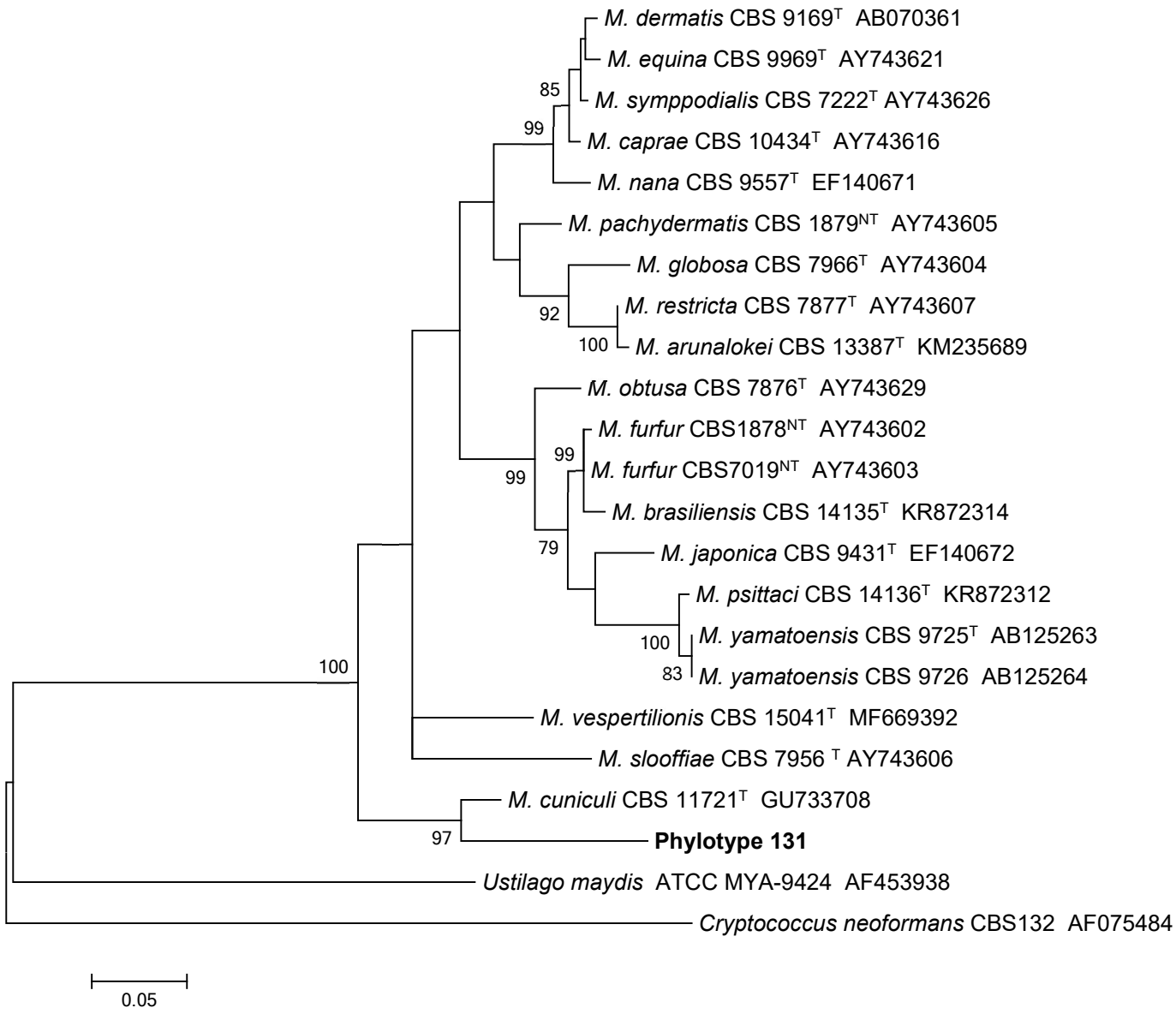
591 **Figure 8.** Average relative abundance of fungal genera across the different rabbit breeds.

592 **Figure 9.** Average relative abundance of fungal species across the different rabbit breeds.

593 **Figure 10.** Average relative abundance of *Malassezia* taxa across the different rabbit
594 breeds. (**A**) Taxa with a percentage greater than 1%. (**B**) Taxa with a percentage lower
595 than 1%.

596 

597 **Figure 1.**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M. caprae CBS 10434T AY743616
M. nana CBS 9557T EF140671
M. pachydermatis CBS 1879NT AY743605
M. globosa CBS 7966T AY743604
M. restricta CBS 7877T AY743607
M. arunalokei CBS 13387T KM235689
M. obtusa CBS 7876T AY743629
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M. brasiliensis CBS 14135T KR872314
M. japonica CBS 9431T EF140672
M. psittaci CBS 14136T KR872312
M. yamatoensis CBS 9725T AB125263
M. yamatoensis CBS 9726 AB125264
M. vespertilionis CBS 15041T MF669392
M. slooffiae CBS 7956 T AY743606
M. cuniculi CBS 11721T GU733708
Phylotype 131
Ustilago maydis ATCC MYA-9424 AF453938
Cryptococcus neoformans CBS132 AF075484
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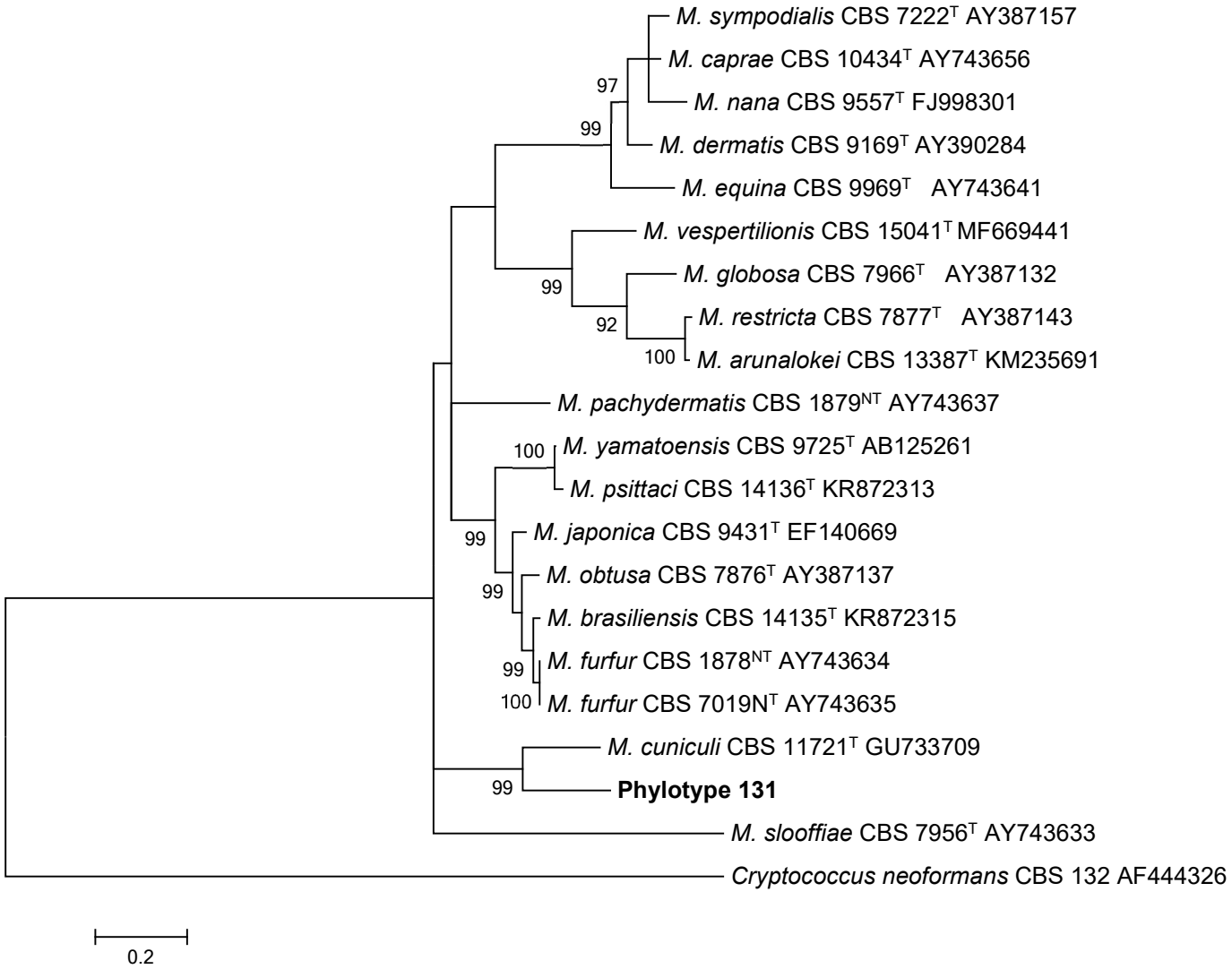
598 **Figure 2.**



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600 **Figure 3.**

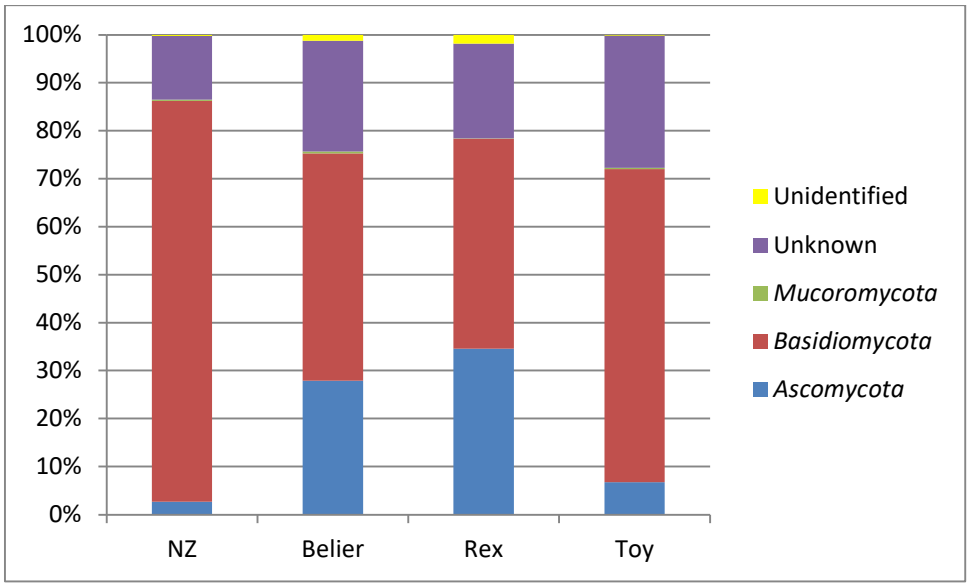
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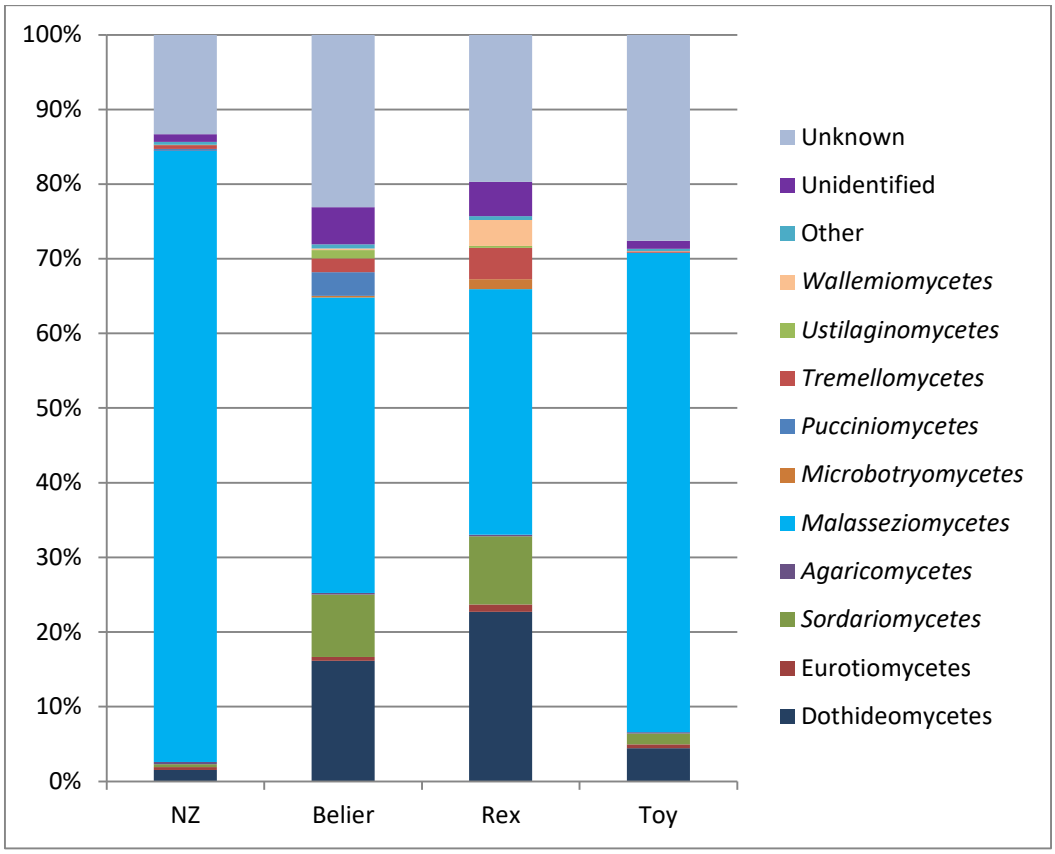
Figure 4.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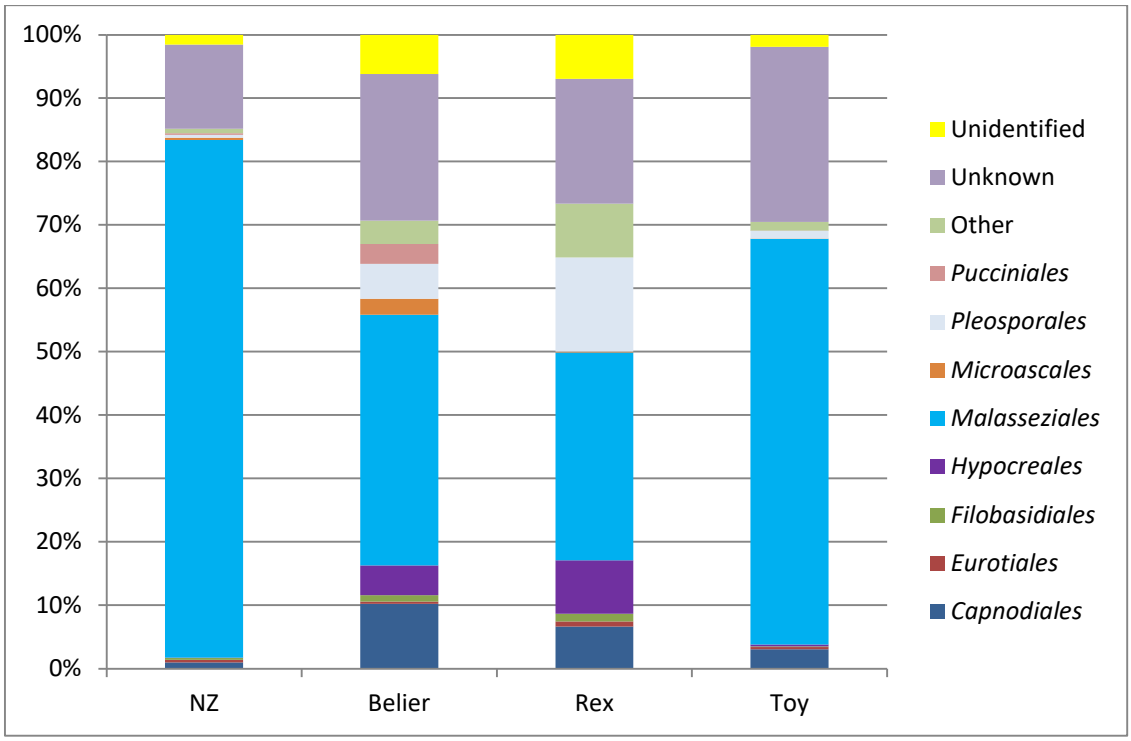
Malasseziomycetes
Agaricomycetes
Sordariomycetes
Eurotiomycetes
Dothideomycetes

Figure 5.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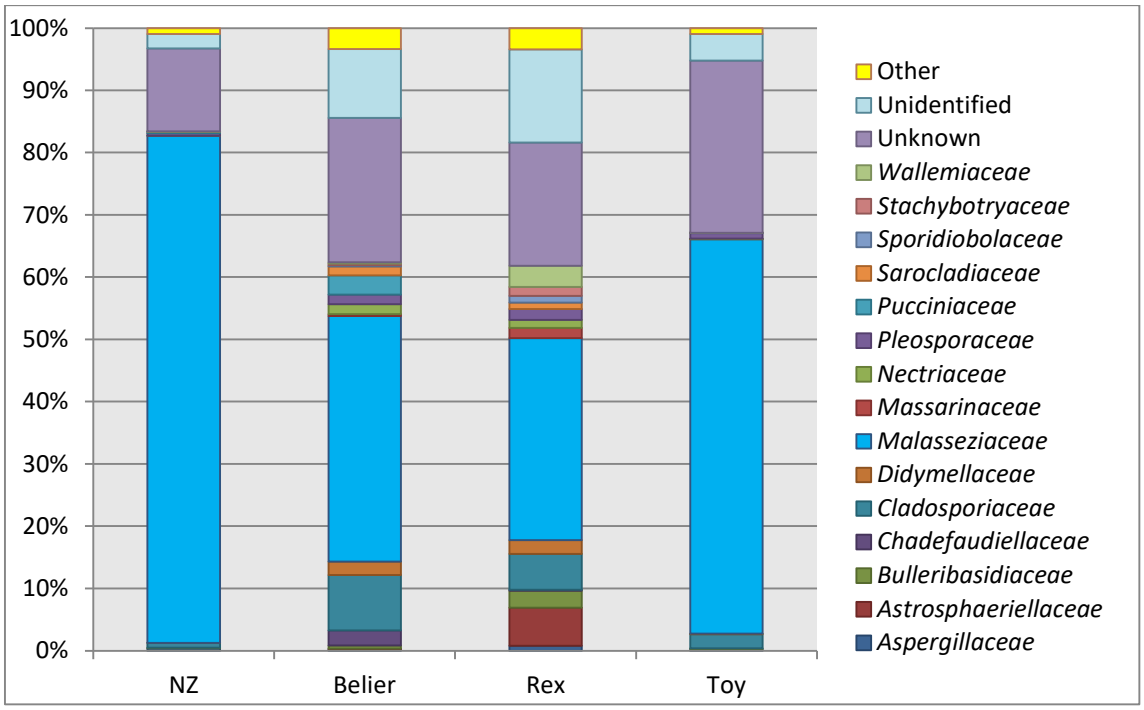
Eurotiales
Capnodiales

Figure 6.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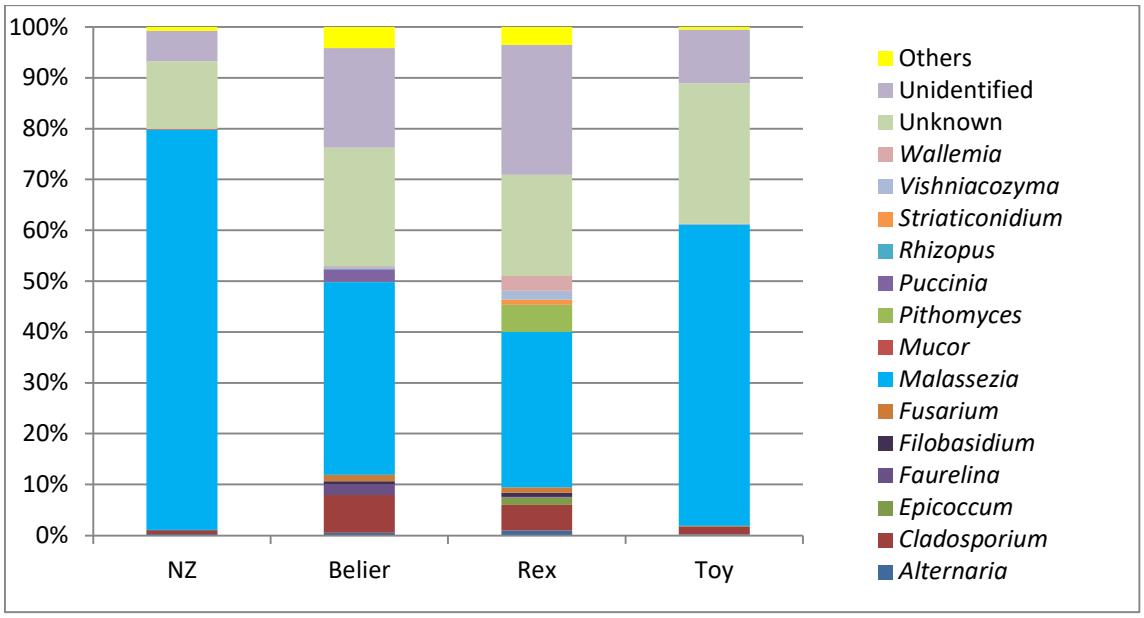
Sporidiobolaceae
Sarocladiaceae
Pucciniaceae
Pleosporaceae
Nectriaceae
Massarinaceae
Malasseziaceae
Didymellaceae
Cladosporiaceae
Chadefaudiellaceae
Bulleribasidiaceae
Astrosphaeriellaceae
Aspergillaceae

Figure 7.



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615 **Figure 8.**

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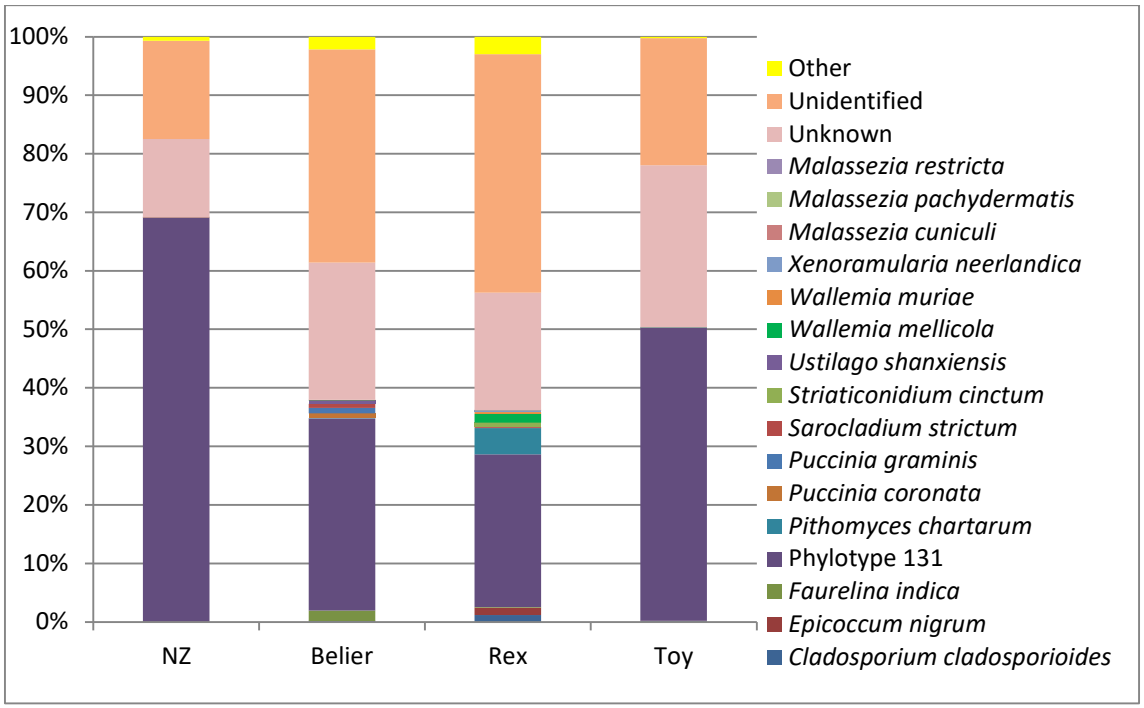

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618 **Figure 9.**

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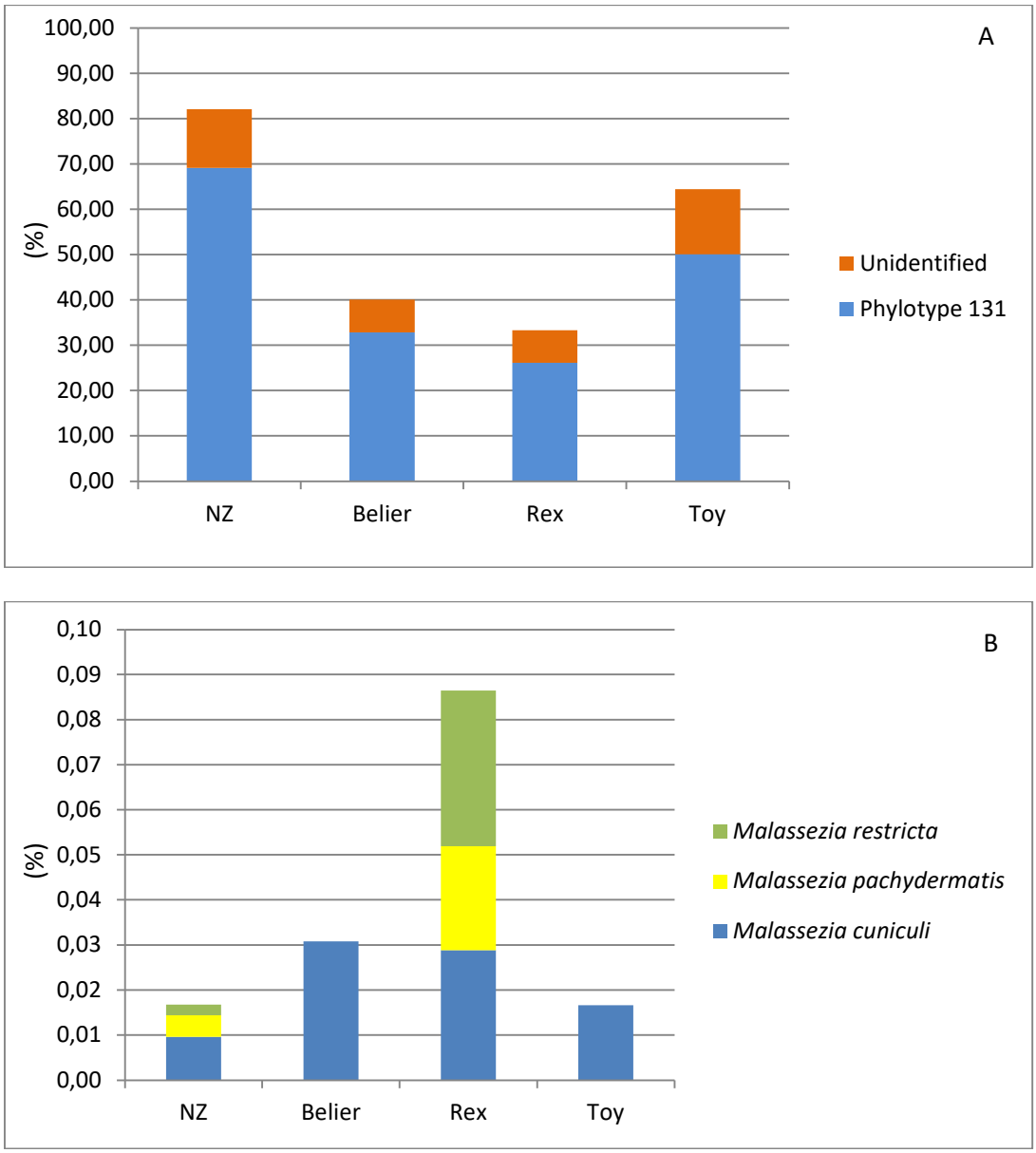
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Malassezia restricta
Malassezia pachydermatis
Malassezia cuniculi

Figure 10.

626 **Table 1. Animals per breed included in this study and results of the cytological examination.**
627

Pet rabbits (n=44)			Farmed rabbits (n=16)		
Breed	nº of animals	positive cytology	Breed	nº of animals	positive cytology
New Zealand	8	6	New Zealand	13	13
Belier	10	5	Belier	-	-
Rex	3	2	Rex	3	1
Toy	21	5	Toy	-	-
Mixed breed	2	1	Mixed breed	-	-
Total	44	19		16	14

628

629 **Table 2. NGS reads after filter and biodiversity data obtained from metagenomics analysis.**

Sample	Number reads after quality processing	% reads classified to genus	Shannon species diversity	Number of species identified
New Zealand	41,913	80.67	1.20	116
Belier	46,162	57.24	2.82	184
Rex	52,886	54.55	3.26	176
Toy	54,285	61.79	1.59	120
Average	52,886	59.51	2.43	120

630