Mycobiome of the external ear canal of healthy cows

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Abstract

Malassezia yeasts belong to the normal skin microbiota of a wide range of warm-blooded animals. However, their significance in cattle is still poorly understood. In the present study, the mycobiota of the external ear canal of 20 healthy dairy Holstein cows was assessed by cytology, culture, PCR, and next-generation sequencing. The presence of *Malassezia* was detected in 15 cows by cytology and PCR. The metagenomic analysis revealed that *Ascomycota* was the predominant phylum but *M. pachydermatis* the main species. The *Malassezia* phylotype 131 was detected in low abundance. Nor *M. nana* nor *M. equina* were detected in the samples.

Keywords: *Malassezia*, *M. pachydermatis*, Next-generation sequencing, ear mycobiome, cows.

Lay summary

The mycobiota of the external ear canal of healthy cows was assessed by cytology, culture, PCR, and NGS. The presence of *Malassezia* was detected by cytology and PCR. *Ascomycota* was the main phylum and *M. pachydermatis* the main species. The *Malassezia* phylotype 131 was also detected in the samples.

The yeasts of the genus *Malassezia* belong to the normal skin microbiota of a wide range of warm-blooded animals[1]. Although some *Malassezia* species have been recovered from cattle, their significance in these animals is poorly understood [2-4]. In culture-based studies lipid-dependent species *M. sympodialis*, *M. globosa*, *M. furfur* and *M. slooffiae* are the predominant species in the external ear canal of both healthy and diseased cattle [2,3]. However, when species identification is based on rRNA sequencing, only *M. furfur* [4], *M. equina* [5], *M. pachydermatis* [6], *M. nana* [7,] and *M. slooffiae* [4] have been confirmed. Therefore, molecular methods such as next-generation sequencing (NGS) have been used to study the skin microbiota of different animal species without the need of a culture [8-12].

The aim of this work was to study the *Malassezia* population of the external ear canal of healthy cows using culture and non-culture-based techniques, including NGS. This is the first attempt to study the external ear canal of healthy cows using NGS. For this purpose, a total of 20 dairy Holstein cows from an experimental farm in Monells (Girona, Spain) were sampled. The animals were confined and showed no signs of any disease. Two swabs soaked in wash fluid (0.075 mol/L phosphate-buffered physiological saline, pH 7.9, 0.1% Tween 80) were collected from each external ear canal. Samples were obtained following procedures approved by Ethics Committee on Animal and Human Experimentation from UAB and Generalitat de Catalunya (approval CEEAH 4600). One swab was used for culture and cytological examination. Diff-Quick stained smears were used to assess the presence of *Malassezia* yeast-like cells. The swab was then streaked onto three different media: Sabouraud glucose agar (SGA) (Oxoid), Modified Dixon agar (mDA) [13] and Leeming and Notman agar (LNA) [14] containing 0.05% of chloramphenicol and 0.05% of cycloheximide. The plates were incubated at 32ºC for 20 days. From the other swab, DNA was extracted using the DNeasy PowerSoil Kit (Qiagen, Madrid, Spain)

according to manufacturer's instructions with minor modifications [11]. DNA was stored at -20ºC until used as a template for the PCR and NGS. All samples were selected for the amplification of a highly conserved region within 5.8S ribosomal RNA of *Malassezia* using specific primer pairs designed for *Malassezia* and following the protocol described by Vuran et al[15]. Two samples from two different cows and with a high abundance of *Malassezia* yeast-like cells in the cytology, were selected for metagenomics analysis of fungal 26S rRNA gene. In those samples, the presence of *Malassezia* was also detected by PCR. NGS and data analysis were performed as described previously [11].

The presence of *Malassezia* yeast-like cells was observed in 20 out of the 40 samples (Supplementary Table 1 and Supplementary Table 2) recovered from 15 cows. Two types of cells were observed. The first ones were small, ovoid to globose with a monopolar budding in a relatively narrow base (Figure 1A). The second ones were bigger elongated cells with buds in a monopolar pattern on a broad base (Figure 1B). The two different morphologies observed, suggested the presence of at least two different *Malassezia* species in the samples. However, attempts to recover these microorganisms were unsuccessful as no growth was observed after 20 days of incubation at 32ºC. These results agree with those studies reporting a reduced prevalence of *Malassezia* in healthy cattle and in Holstein cows compared with breeds with long, pendant, and gutter shaped ears [3].

A total of 24 samples from 15 cows had a positive PCR (Supplementary Table 1 and Supplementary Table 2). All samples with a positive cytological examination and four samples with a negative cytological examination had a positive PCR. This could be a result of the higher sensitivity of the PCR method.

Two samples were correctly sequenced by NGS, and the number of generated sequence reads is described in Supplementary Table 3. The raw sequencing data is available at the NCBI database, SRA accession PRJNA1072328. The taxonomic composition of the samples was investigated at various levels by NGS. *Ascomycota* with a median of abundance of 55.78% was the main phyla followed by *Basidiomycota* (12.55%) (Supplementary Figure 1). However, at the level of genus (Figure 2), *Malassezia,* was the main genus with a median of abundance of 5.72%, followed by *Cladosporium* (5.17%) and *Aspergillus* (4.48%). *Malassezia pachydermatis* (1.15%) (Supplementary Figure 2) was the predominant species, followed by *Pichia kundriavzevii* (1.10%) and *Aspergillus sydowii* (1.04%). Metagenomic studies in healthy dogs and cats, revealed that the predominant phylum was *Ascomycota* and *Cladosporium* the main genus [9,10,12]. Although in healthy cows the predominant phylum was *Ascomycota,* the main genus was *Malassezia* as it occurs in healthy rabbits [11] and healthy humans [16]. In non-metagenomic studies, *Malassezia* has been isolated from the external ear canal of healthy cattle and cattle with otitis externa. Moreover, *Malassezia* was the most frequently recovered fungal genus in both healthy and diseased cattle [2-4]. The significant prevalence of *Malassezia* in the external ear canal of healthy cattle suggests that these yeasts may be a member of the microbiota of the ear [2,3]. However, in other studies [2,3] *M. sympodialis*, *M. slooffiae*, *M. furfur* or *M. globosa* were the most common ones whereas in our study was *M. pachydermatis*. In our study, *Malassezia sympodialis* (0.36%), *M. restricta* (0.04%) and *M. globosa* (0.03%) were identified in both samples (Supplementary Figure 3). Those species are part of the normal skin microbiota of humans [17] and their presence in our samples could be result of human manipulation of the animals. Besides, small amounts of *M. restricta* and *M. globosa* sequences were detected in the negative control and thus, their presence could be result of cross-contamination during the procedure [11, 18].

Moreover, those species have been previously cited from cattle, but their identification was not confirmed by rRNA sequencing methods [2,3]. Besides, other *Malassezia* species have been isolated from cattle such as *M. nana* from the external ear canal of Gyr [3,7] and *M. equina* from cows' skin [5]. In our study nor *M. nana* nor *M. equina* sequences were detected. The *Malassezia* phylotype 131 was identified in both samples with low abundance. This phylotype was firstly described in humans [8] and has also been reported to be the main taxa in rabbits [11]. An average of 6.17% of the *Malassezia* sequences could not be identified to the species level. All those sequences belonged to the class *Malasseziomycetes* and could represent new taxa yet to be described.

Even though no growth was obtained in any of the culture media used, the presence of *Malassezia* was detected in the samples by cytology and PCR. The NGS analysis using the LSU as target gene, allowed the identification of several *Malassezia* species and the study of fungal diversity. Further studies would be necessary to elucidate the possible role of *Malassezia* in the etiopathology of otitis in these animals.

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Conflict of interest

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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193

194 **Figure captions**

195 **Figure 1** Diff-Quick stain of a smear from an otic swab of a dairy Holstein cow from an
196 experimental farm in Monells showing the presence of (A) ovoid to globose yeast cells
197 with a monopolar budding in a narrow base, possibly of the genus *Malassezia* and (B)
198 Elongated yeast cells with a bud in a monopolar pattern on a broad base, possibly of the
199 genus *Malassezia*.

200

201 **Figure 2** Relative abundance of fungal genera across the two external ear canal samples
202 from two Holstein cows.

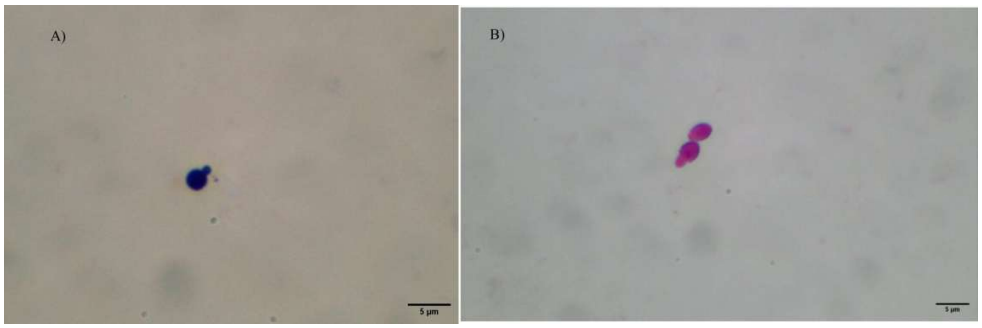


Figure 1 Diff-Quick stain of a smear from an otic swab of a dairy Holstein cow from an experimental farm in Monells showing the presence of (A) ovoid to globose yeast cells with a monopolar budding in a narrow base, possibly of the genus *Malassezia* and (B) Elongated yeast cells with a bud in a monopolar pattern on a broad base, possibly of the genus *Malassezia*.

190x62mm (300 x 300 DPI)

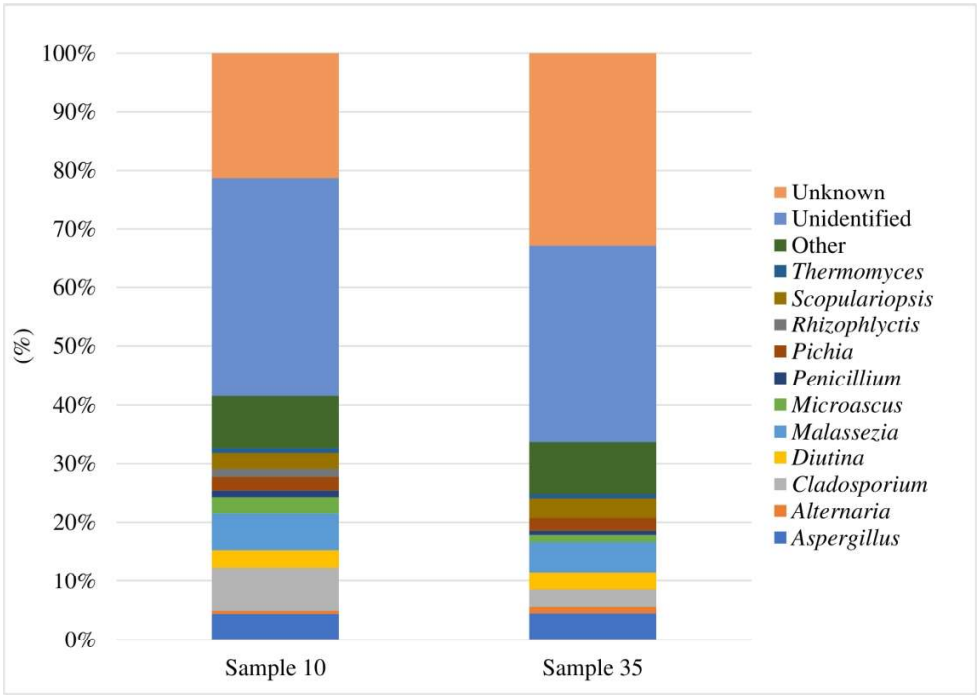


Figure 2 Relative abundance of fungal genera across the two external ear canal samples from two Holstein cows.

173x122mm (300 x 300 DPI)

Supplementary Table 1. Results obtained in samples from the external ear canal of Holstein cows including cytological examination and PCR.

SAMPLE	COW	CYTOLOGY	PCR
1	1 (R)	+/a	+
2	1 (L)	+/a	+
3	2 (R)	-	+
4	2 (L)	+/a	+
5	3 (R)	+/a	+
6	3 (L)	+/a	+
7	4 (R)	+/a	+
8	4 (L)	-	+
9	5 (R)	+a	+
10*	5 (L)	+/b	+
11	6 (R)	+/a,b	+
12	6 (L)	-	+
13	7 (R)	-	-
14	7 (L)	-	-
15	8 (R)	-	-
16	8 (L)	-	-
17	9 (R)	+/a	+
18	9 (L)	-	-
19	10 (R)	-	-
20	10 (L)	-	-
21	11 (R)	-	-
22	11 (L)	+/a	+
23	12 (R)	+/a	+
24	12 (L)	-	-
25	13 (R)	-	-
26	13 (L)	-	-
27	14 (R)	-	-
28	14 (L)	+/b	+
29	15 (R)	+/a,b	+
30	15 (L)	-	-
31	16 (R)	-	-
32	16 (L)	-	-
33	17 (R)	+/a	+
34	17 (L)	-	-
35*	18 (R)	+/a	+
36	18 (L)	+/a	+
37	19 (R)	+/a	+
38	19 (L)	+/a	+
39	20 (R)	-	+
40	20 (L)	+/a	+

(R) Right external ear canal, (L) Left external ear canal, (+) Positive, (-) Negative, (a) Small ovoid to globose *Malassezia* yeast-like cells, (b) Elongated *Malassezia* yeast-like cells, (*) Samples subjected to next-generation sequencing.

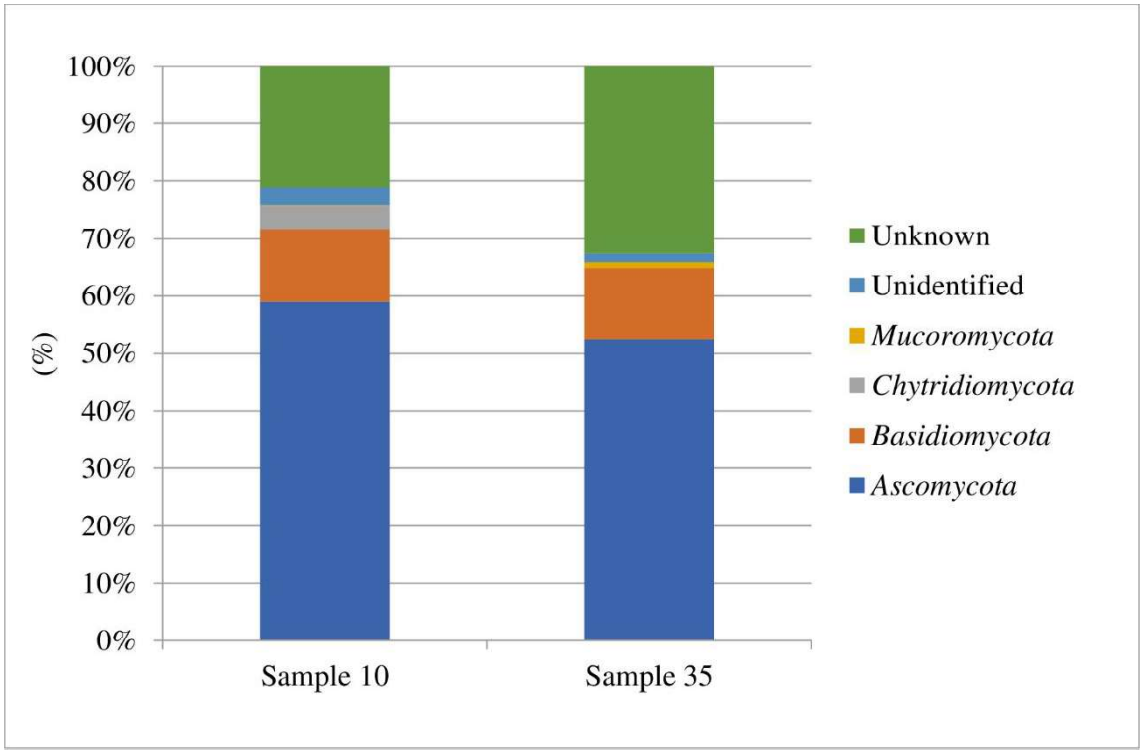
Supplementary Table 2 Crosstab analysis and chi-square test comparing the results of the cytological examination and PCR of samples from the external ear canal of Holstein cows.

Cytological examination	PCR		
	Negative samples	Positive samples	Total
Negative samples	16	4	20
Positive samples	0	20	20
Total	16	24	40

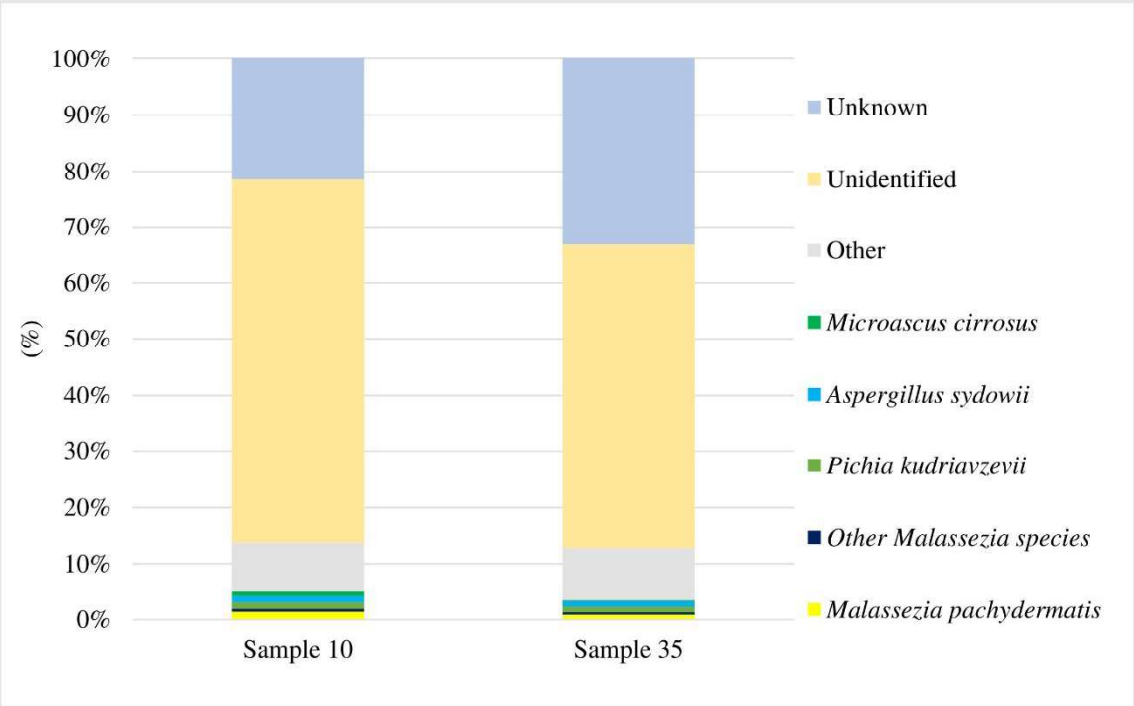
Chi-Square value= 26.667, $p < 0.001$

Supplementary Table 3. Next-generation sequencing reads after filter and biodiversity data obtained from metagenomics analysis of two external ear canal samples from two Holstein cows.

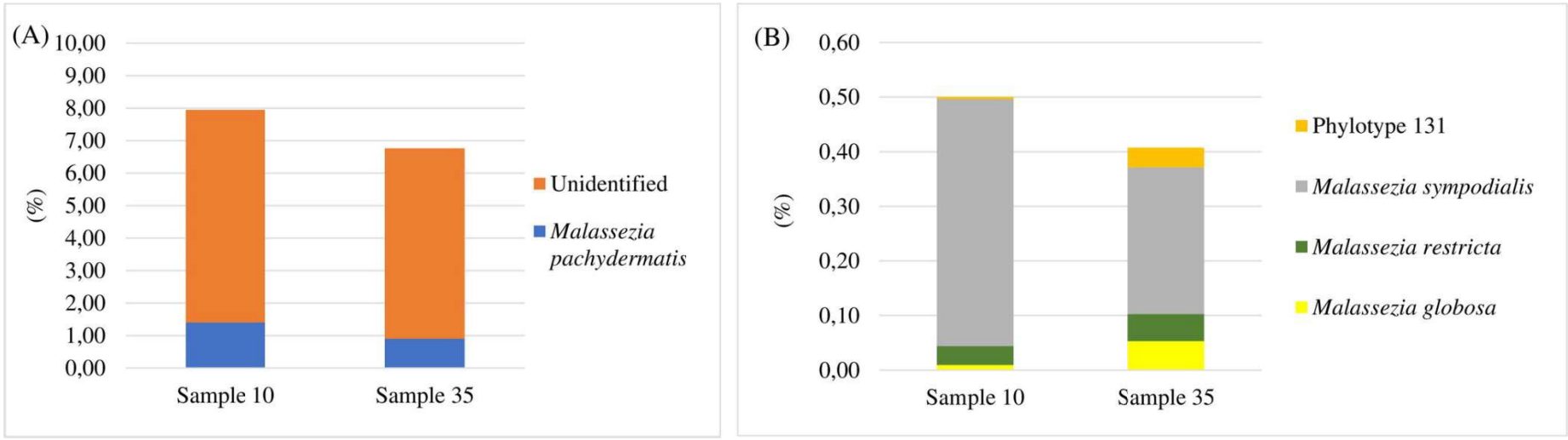
Sample	Nº reads after quality processing	% Reads classified to genus	Shannon species diversity	Nº of species identified
Sample 10	53257	41.55	4.11	330
Sample 35	74511	33.84	3.78	341
Average	63884	37.70	3.94	335.5



Supplementary Figure 1. Relative abundance of fungal phyla across the two external ear canal samples from two Holstein cows.



Supplementary Figure 2. Relative abundance of fungal species across the two external ear canal samples from two Holstein cows.



Supplementary Figure 3. Relative abundance of *Malassezia* taxa across the two external ear canal samples from two Holstein cows. (A) Taxa with a percentage greater than 1%. (B) Taxa with a percentage lower than 1%.