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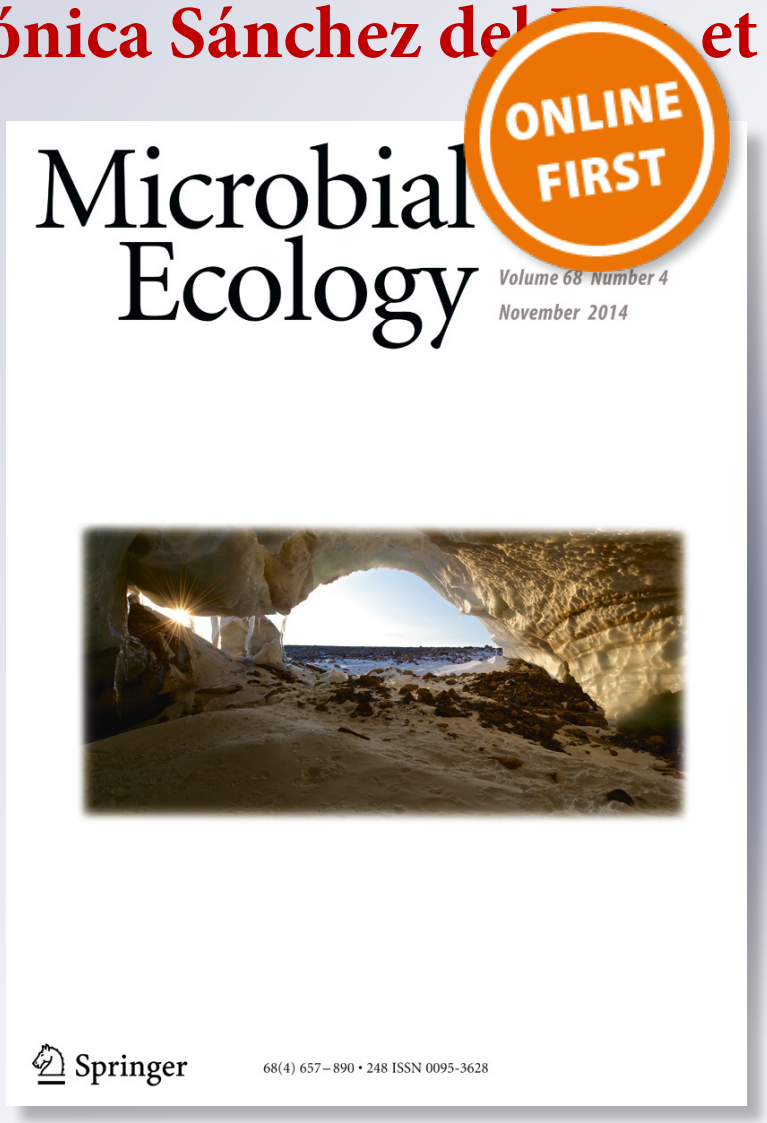

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Estimation of Cultivable Bacterial Diversity in the Cloacae and Pharynx in Eurasian Griffon Vultures (*Gyps fulvus*)

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Abstract In this work, we describe the biodiversity of cloacal and pharynx culture-based bacteria (commensal and pathogenic), in 75 Eurasian griffon vultures (*Gyps fulvus*) from two geographic areas. We address the question of whether the cultivable microbiota of vultures is organised into assemblages occurring by chance. In addition, we assess bacterial diversity in both anatomic regions and geographic areas. Bacterial diversity was represented by 26 Gram-negative and 20 Gram-positive genera. The most common genera were *Escherichia*, *Enterococcus*, *Staphylococcus*, *Clostridium* and *Lactococcus. Escherichia coli* and *Enterococcus faecalis* were the most common species in cloacal and pharyngeal samples. *Staphylococcus* and *Erysipelothrix* were isolated from the pharynx and *Salmonella* and *Corynebacterium* from the cloacae, and no *Campylobacter* was isolated from the cloacal swabs. Ten cloacal swabs were positive for *Salmonella*, of which five isolates were *Salmonella enterica* serotype 4,(5),12:i:-, one isolate was *S. enterica* serotype Derby, three isolates were *S. enterica* serotype 61:k:1,5,7 and one isolate was *S. enterica* serotype Infantis. The null modelling approach revealed that the commensal bacteria of vultures are not structured in assemblages. On the other hand, differences in bacterial genus and species richness between cloacal and pharyngeal samples or between geographic areas were clear, with the pharynx in vultures from both geographic areas being richer. The results of this study indicate also that vultures can serve as a reservoir of certain pathogenic zoonotic bacteria. The dissemination of these zoonotic pathogens in wildlife could be prevented by periodic sanitary surveys.

Keywords Griffon vultures · Cloacae · Pharynx · Bacteria richness · Species evenness

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Introduction

Exploration of diversity of commensal microbiota is a field of growing interest in microbial ecology. Similar to other vertebrates, the gastrointestinal tract of birds is colonised by microorganisms that play a main role in bird nutrition physiology [54], body growth [37] and protection to pathogen infection [8]. Specific studies have evidenced the importance of the age [53] or genetics and rearing conditions in the nest [43] on microbiota composition. Recent studies evidence that local variations have more influence on shaping gut microbiota than genetics or rearing conditions [32], probably due to changes in diet composition. However, and despite the potential ecological and evolutionary importance of commensal microbiota, factors shaping the composition of bacterial communities in wild bird species remain poorly understood.

One interesting avian model for exploring such variations in microbiota composition is the scavengers. Scavenging birds feed regularly on carcasses of animals that have succumbed to infectious diseases. It is broadly accepted that vultures display high resistance to pathogens lethal to other animals [40], probably thanks to the protection provided by commensal

microbiota [56]. But, little information exists about the role of host- or environmental-related factors on microbiota diversity and composition. Moreover, there is a lack of knowledge of the community structure of intestinal and respiratory microbiota in wild birds, and most published research is descriptive [3, 5, 48]. As a result, and despite their important role as reservoirs of enterobacterial pathogens [56], the composition and structure of commensal bacteria are poorly known in most of scavenger birds.

The Eurasian griffon vulture (*Gyps fulvus*, Accipitridae) is one of the seven species in this genus of Old World vultures found over a wide geographic range [13]. It is the largest vulture in the Mediterranean countries, including Spain, Croatia, Albania, Turkey and Italy. During the 1980s and 1990s, the vulture population underwent a sharp increase in Spain [45]. The feeding habits of Eurasian griffon vultures are based almost exclusively on carrion, preferentially of mammals, with natural wild species having been largely replaced by domestic livestock. Livestock carrion from intensive farming is discarded for elimination in some places. As a result, avian scavengers concentrate at these supplementary feeding points, which are a source of superabundant and predictable food. In Spain, the consumption of carcasses and other waste disposal at these supplementary feeding points may have indirect effects on the commensal bacteria in vultures. Moreover, domestic livestock may be sick possibly leading to the acquisition of pathogenic bacteria by vultures. However, only limited studies have investigated the presence of pathogenic bacteria in the respiratory tract or gut of vultures. *Salmonella* was present in 15 % of guts from turkey vultures, *Cathartes aura* [56]. *Mycoplasma* species are commonly isolated from the respiratory tract of vultures [42, 51]. The purpose of this study was to further investigate the biodiversity of cultivable bacteria, including pathogenic organisms, in Eurasian griffon vultures from two geographic regions. We first examined whether the commensal microbiota in vultures is structured in assemblages or, on the contrary, occurs by chance. In addition, we explored the importance of the anatomic region (cloacae vs pharynx) and the geographic area on the bacterial richness and evenness in this scavenger.

Material and Methods

Sampling Procedure

Cloacal or pharyngeal samples from 75 apparently healthy Eurasian griffon vultures (*G. fulvus*) were collected and submitted under refrigeration (4 °C) to the VISAVET Health Surveillance Centre of the Universidad Complutense (Madrid, Spain) for bacteriological analysis within 48 h after animals were captured. Eurasian griffon vultures were located in two areas representative of the habitat used by these animals in Spain, one in Catalonia (north east of Spain; n=29) and the other in Navarra (north of Spain; n=46). Catalonia vultures are fed with carcasses from livestock, mainly cattle and pigs. Vultures from Navarra are fed mainly with pigs and sheep. Vultures were sampled over a period of 8 months (October 2010 to May 2011). Captured vultures were carefully handled to prevent potential injury and to alleviate distress. Samples were collected with sterile swabs using Amies medium (Sterile R, Meus s.r.l.) and Amies with charcoal medium (Copan Italia SpA) and were analysed within 8 days after collection.

Microbiological Isolation and Identification

In order to maintain a uniform criterion for isolation of bacterial isolates that would be further identified, all isolations were carried out by the same microbiologist. Cloacae and pharynx samples were plated onto the non-selective medium Columbia sheep blood agar (bioMérieux) and incubated aerobically and anaerobically at 37 °C for 48 h. This agar is a highly nutritious non-selective media widely used for the isolation and cultivation of non-fastidious and fastidious microorganisms from a variety of clinical and nonclinical materials. The bacterial growth density was semi-quantitatively estimated by successively streaking a loop of each sample onto the four quadrants in which agar plates were divided. Growth density is scored as 4+ (heavy growth) if growth carried over to the fourth quadrant, 3+ (moderate) if growth carried over to the third quadrant, 2+ (few or light) if growth occurred the second quadrant and 1+ (rare) if growth only occurred in the first quadrant. For each agar plate, only one representative colony of the most abundant morphologically distinct colonies (4+ and 3+) were randomly selected and subcultured on a separate Columbia agar plate for further biochemical identification. Preliminary tests such as Gram staining and catalase and oxidase production were performed for all isolates according to standard procedures [49]. Isolates were further biochemically identified using ad hoc multi-substrate identification systems (API® Strips, BioMérieux Clinical Diagnostics, Spain): rapid ID 32 Strep, API 32 Staph, API 20 NE, API 20 E and API Coryne. Isolates were identified as a particular species only if identification scores in the multi-substrate identification systems were excellent, very good or good (99.9–99.0 % ID); otherwise, identification was made only at the genus level.

For lactobacilli, samples of both the cloacae and pharynx were cultured onto Lactobacillus-MRS Agar (Difco). Incubation was carried out at 37 °C for 7 days under anaerobic conditions. For *Campylobacter* culture, charcoal cloacal swabs were directly streaked on modified charcoal cefoperazone deoxycholate agar (mCCDA, Oxoid) and incubated at 42 °C for 48 h under microaerobic atmospheric conditions (Genbag microaerobic atmosphere generator,

BioMérieux), following the protocol recommended by the European Commission for avian samples [17].

For *Salmonella*, the method recommended by the International Organization for Standardization [36] was used. Thus, cloacal samples were enriched in buffered peptone water at 37 °C for 18 h. After incubation, 100 μl divided into three drops was cultured on modified semi-solid Rappaport-Vassiliadis (MSRV) (Difco). MSRV was incubated at 41.5 °C for 24–48 h. When suspected growth was detected, MSRV was subcultured on xylose lysine desoxycholate agar (XLD, bioMérieux) and chrom IDTM Salmonella agar (SM2, bioMérieux) for 24 h at 37 °C. Presumptive colonies detected on XLD and SMID according to manufacturer instructions were cultured on Columbia 5 % sheep blood agar (bioMérieux), and one isolate per animal was biochemically (Enterotube TM II, BD BBL TM) and serologically confirmed as *Salmonella* spp. following the Kauffmann–White scheme [29].

Microbiota Community and Diversity Using Null Models

Null model analysis was used to study whether or not cultivable microbiota in Eurasian vultures occurred by chance. Data were organised as presence/absence matrices in which each row represented a pathogen species and each column represented an individual vulture. In a presence/absence matrix, "1" indicates that a species is present at a particular site or host, and "0" indicates that a species is absent [24]. A total of four matrices were created separately for each combination of anatomical region (pharynx and cloacae) and population (Navarra and Catalonia).

C-score and the PIE Hurlbert's [35] indexes were used to determine whether or not cultivable bacteria in griffon vultures are structured into communities. The C-score was used as a co-occurrence index for exploring co-occurrence patterns [50] and the FE algorithm (fixed row-equiprobable column) chosen for analysing the results obtained. The C-score measures the average number of checkerboard units between all possible pairs of species. In a competitively structured community, the observed C-score should be significantly larger than expected by chance (O>E). Otherwise, a C-score smaller than expected by chance (O<E) indicates a randomly assembled community [28]. The observed C-score was calculated for each presence/absence matrix and compared with the expected C-score calculated for 5,000 randomly assembled null matrices by Monte Carlo procedures. In addition, to compare the degree of co-occurrence across data, a standardised effect size (SES) for each matrix was calculated. The SES measures the number of standard deviations that the observed index (C-score) is above or below the mean index of the simulated communities.

The biodiversity of cloacae and pharynx bacterial species was also studied using a null model approach. Data were organised as two vectors of abundances (one by population), each represented by two columns of data (one by anatomic region: pharynx and cloacae). The number of colonies of each bacterium species or genera isolated and identified in the vulture samples was represented in these vector cells.

Since classical diversity indexes can result from various combinations of species richness and evenness, two indicators of diversity were later used to characterise the bacterial diversity of vultures: the species (or genera) richness (number of bacterial species or genera by individual vulture) and the PIE index [35]. The latter calculates the probability of an interspecific encounter (PIE), which is to say that the probability that two randomly sampled bacteria belongs to different bacteria species or genera. This index was selected for several reasons, but mainly because it is easily interpreted as a probability and because the PIE is one of the few indexes that is unbiased by sample size [25, 26].

A single sample unit (one sterile swab by anatomic region of each individual vulture) was used, and thus, an individual sampling strategy to estimate biodiversity was followed. In brief, individual bacterium from a single specified column of data was randomly drawn to create an individual-based rarefaction curve [i.e., species or genera diversity controlled for differences in abundance, (26)]. A total of 36 abundance levels (increasing by 2 units) were specified for building these curves, with 5 being the smallest number of individual bacteria and 75 the greatest. In the case of average species richness in the pharynx, we used the same minimum number of individual bacterium with 60 as the maximum. Subsequently, five randomly chosen individuals were drawn, and both species and genera richness and the PIE for each vector for 5,000 randomly assembled vectors of data by Monte Carlo procedures were calculated. Microbiota assemblages and bacterial diversity were assessed using the software EcoSim 7.72 [27].

Statistical Modeling

After null modelling, a set of generalised additive models (GAMs) [57] to explore the importance of the anatomical region (pharynx and cloacae) and the host population (Catalonia vs Navarra), as explanatory variables, in bacterial diversity in the Eurasian griffon vultures were fitted. In these models, abundance levels were the covariate (smoothed) in all GAMs. A set of linear models (LM) was used for exploring the role of the previously mentioned explanatory variables (e.g., anatomical region and host population) on the PIE Hurlbert's index [35].

In all cases, a model selection procedure based on the information-theoretic approach and the Akaike's information criterion [7] was followed. Subsequently, Akaike weight (*wi*), defined as the relative probability that a given model is the best model among those being compared, was estimated. Once the best model was selected, the general assumptions of GAM and LM following the previously published recommendations [57–59] were confirmed. Moreover, the Fisher's

Table 1 Number (%) of Eurasian griffon vultures with positive detection of the bacterial genus isolated

Bacterial genera	Cloacal samples (%)[a]			Pharynx samples (%)[a]		
	Navarra *n*=46	Catalonia *n*=29	Total *n*=75	Navarra *n*=46	Catalonia *n*=29	Total *n*=75
Gram-negative						
Acinetobacter	0	0	0	2 (4.3)	0	2 (2.2)
Aeromonas	0	2 (6.9)	2 (2.7)	1 (2.2)	2 (6.9)	3 (4.0)
Bifidobacterium	0	0	0	1 (2.2)	0	1 (1.3)
Brevundimonas	0	0	0	0	1 (3.4)	1 (1.3)
Buttiauxella	0	0	0	1 (2.2)	0	1 (1.3)
Chryseomonas	0	0	0	0	1 (3.4)	1 (1.3)
Citrobacter	0	2 (6.9)	2 (2.7)	3 (6.5)	7 (24.1)	10 (13.3)
Enterobacter	0	0	0	5 (10.9)	5 (17.2)	10 (13.3)
Escherichia	21 (45.7)	17 (58.6)	38 (50.7)	12 (26.1)	7 (24.1)	19 (25.3)
Fusobacterium	0	0	0	1 (2.2)	0	1 (1.3)
Hafnia	0	1 (3.4)	1 (1.3)	7 (15.2)	1 (3.4)	8 (10.7)
Klebsiella	0	1 (3.4)	1 (1.3)	2 (4.3)	0	2 (2.7)
Kluyvera	2 (4.3)	0	2 (2.7)	0	0	0
Leclercia	1 (2.2)	0	1 (1.3)	0	0	0
Moellerella	0	0	0	4 (8.7)	1 (3.4)	5 (6.7)
Moraxella	3 (6.5)	0	3 (4.0)	0	0	0
Morganella	0	0	0	1 (2.2)	0	1 (1.3)
Pantoea	0	0	0	0	1 (3.4)	1 (1.3)
Pasteurella	3 (6.5)	0	3 (4.0)	0	1 (3.4)	1 (1.3)
Plesiomonas	2 (4.3)	0	2 (2.7)	0	0	0
Proteus	3 (6.5)	3 (10.3)	6 (8.0)	13 (28.3)	0	13 (13.3)
Pseudomonas	0	0	0	0,0	2 (6.9)	2 (2.7)
Psychrobacter	0	0	0	1 (2.2)	0	1 (1.3)
Salmonella	4 (8.7)	6 (20.6)	10 (13.3)	ND	ND	ND
Serratia	1 (2.2)	0	1 (1.3)	0	1 (3.4)	1 (1.3)
Vibrio	0	1 (3.4)	1 (1.3)	0	0	0
Gram positive						
Abiotrophia	1 (2.2)	0	1 (1.3)	0	0	0
Aerococcus	6 (13.0)	0	6 (8.0)	12 (26.1)	0	12 (16.0)
Alloicococcus	3 (6.5)	0	3 (4.0)	4 (8.7)	0	4 (5.3)
Arthrobacter	3 (6.5)	0	3 (4.0)	5 (10.9)	0	5 (6.7)
Brevibacterium	1 (2.2)	0	1 (1.3)	0	0	0
Cellulomonas	0	0	0	0	1 (3.4)	1 (1.3)
Clostridium	17 (37,0)	6 (20.7)	23 (30.7)	25 (54.3)	0	25 (33.3)
Corynebacterium	3 (6.5)	2 (6.9)	5 (6.7)	1 (2.2)	0	1 (1.3)
Dermobacter	0	1 (3.4)	1 (1.3)	0	0	0
Dermacoccus	1 (2.2)	0	1 (1.3)	1 (2.2)	0	1 (1.3)
Enterococcus	32 (69.6)	27 (93.1)	59 (78.7)	26 (56.5)	28 (96.6)	54 (72.0)
Erysipelothrix	0	0	0	1 (2.2)	0	1 (1.3)
Gemella	1 (2.2)	0	1 (1.3)	2 (4.3)	1 (3.4)	3 (4.0)
Kocuria	2 (4.3)	0	2 (2.7)	0	1 (3.4)	1 (1.3)
Lactococcus	1 (2.2)	5 (17.2)	6 (8.0)	9 (19.6)	7 (24.1)	16 (21.3)
Leuconostoc	1 (2.2)	0	1 (1.3)	1 (2.2)	3 (10.3)	4 (5.3)
Listeria	1 (2.2)	1 (3.4)	2 (2.7)	2 (4.3)	2 (6.9)	4 (5.3)
Micrococcus	4 (8.7)	0	4 (5.3)	4 (8.7)	0	4 (5.3)

Table 1 (continued)

Bacterial genera	Cloacal samples (%)[a]			Pharynx samples (%)[a]		
	Navarra *n*=46	Catalonia *n*=29	Total *n*=75	Navarra *n*=46	Catalonia *n*=29	Total *n*=75
Staphylococcus	6 (13.0)	0	6 (8.0)	16 (34.8)	1 (3.4)	17 (22.7)
Streptococcus	8 (17.4)	0	8 (10.7)	12 (26.1)	1 (3.4)	13 (17.3)

[a] Different species of a particular genus were isolated from the same sample. In these samples, only one species was considered to calculate the frequency of detection of that genus in vultures

ND not determined

exact test was used to compare isolation rates of the selected bacterial species or genera between anatomical regions or geographic areas. On the other hand, vultures base their diet almost exclusively on carcasses, and thus, strong dietary seasonal sifts are unexpected [15]; hence, the month of sampling was not considered in our model selection. Statistical analyses were performed using the "mgcv" package version 1.7–12 [57] of the statistical software R version 3.1.3 [14].

Moreover, the Fisher's exact test was used to analyse the relationship between the isolation rates of the different bacterial genera or species and samples and between these bacteria and the geographic area using the WinPepi program [version 11.25 (http://www.epi-perspectives.com/content/8/1/1)]. Differences were considered significant at $p<0.05$.

Results

A total of 517 bacterial isolates were obtained from the cloacal and pharyngeal samples (358 isolates were Gram-positive and 159 were Gram-negative, distributed in 20 and 26 genera, respectively). The most frequently isolated Gram-positive organisms were of the genus *Enterococcus* (30.8 % of the total of isolates). Other bacteria, such as *Clostridium* (9.3 % of the total of isolates), *Staphylococcus* (5.8 % of the total of isolates) and *Lactococcus* (4.6 % of the total of isolates), were also frequently isolated. Among Gram-negative bacteria, the most predominant bacterium identified was *Escherichia* (12.0 % of the total of isolates).

We identified 37 genera and 44 species from pharynx samples and 32 genera and 32 species from cloacae samples (Tables 1 and 2). From pharyngeal samples, the most frequent genera identified were *Enterococcus* (72 %), *Escherichia* (25.3 %), *Lactococcus* (21.3 %) and *Staphylococcus* (22.7 %). The first two were also the most frequently isolated from cloacae (78.7 and 50.7 %, respectively) (Table 1). Other genera isolated with rates lower than 10 % are shown in Table 1. *Campylobacter* spp. and *Lactobacillus* spp. were not isolated from the cloacae of any of the vultures examined. *Lactobacillus* spp. was not isolated from the pharynx. Genera that were more frequently isolated from pharynx than from cloacae were *Citrobacter* (13.3 vs 2.7 %; $p>0.05$), *Enterobacter* (13.3 vs 0 %; $p<0.05$), *Hafnia* (10.7 vs 1.3 %; $p>0.05$), *Lactococcus* (21.3 vs 8.0 %; $p>0.05$) and *Staphylococcus* (22.7 vs 8.0 %; $p>0.05$). On the other hand, the genus *Escherichia* was significantly associated with cloacal samples (50.7 vs 25.3 %; $p<0.05$).

Overall, using multi-substrate identification systems and the criteria indicated in the section above, 54 bacterial species were identified from the cloacae and pharynx (Tables 1 and 2). The most frequent species isolated from cloacae were *Enterococcus faecalis* (52.0 %), *Escherichia coli* (49.3 %), *Enterococcus gallinarum* (24.0 %), *Enterococcus faecium* (17.3 %), *Clostridium beijerinckii* (13.3 %), *Salmonella enterica* (13.3 %) and *Enterococcus hirae* (12.0 %). The serotypes of the *Salmonella* isolates were 4,12:i:- (50 %), Derby (10 %), 61:k:1,5,7 (30 %) and Infantis (10 %). The species more frequently isolated from pharynx were *E. coli* (22.7 %), *E. faecalis* (41.3 %), *E. faecium* (17.3 %) and *Aerococcus viridians* (13.3 %) (Table 2). The percentage of isolation from cloacal samples and pharyngeal samples was dependent on the source (pharynx vs cloacae) ($p<0.05$) for *Citrobacter brakii* (1.3 vs 9.3 %), *C. beijerinckii* (13.3 vs 0 %), *E. gallinarum* (24.0 vs 1.3 %), *Enterococcus casseliflavus* (6.7 vs 0 %), *Escherichia fergusonii* (8.0 vs 0 %) and *E. coli* (22.7 vs 49.3 %).

All individuals were colonised by at least one bacterial species (28 % of vultures had 6 bacterial species, 24 % had 5 species, 19 % had 4, 14 % had 3, 10 % had 2 and 15 % had 1 bacterial species). The most common species combination was *E. faecalis* and *E. coli* (7.3 % of vultures). *E. faecalis* and *E. faecium* were presented in 2.3 % of the birds. The null model analysis, however, showed that there was not a statistically significant difference between the observed and expected C-scores, and hence, the microbial community was not aggregated; that is, vultures were not colonised by some specific, non-random combinations of bacterial species (Table 3). These random assemblages occurred in both anatomical regions and vultures from both populations.

According to our model selections, both anatomical region and host population drove species ($W_{AR + Population}=0.99$,

Table 2 Number (%) of Eurasian griffon vultures with positive detection of the bacterial species isolated

Bacterial species[a]	Cloacal samples (%)			Pharynx samples (%)		
	Navarra *n*=46	Catalonia *n*=29	Total *n*=75	Navarra *n*=46	Catalonia *n*=29	Total *n*=75
Gram-negative						
Aeromonas hydrophila	0	0	0	1 (2.2)	1 (3.4)	2 (2.7)
Unknown *Aeromonas* isolates	0	2 (6.9)	2 (2.7)	0	1 (3.4)	1 (1.3)
Brevundimonas vesicularis	0	0	0	0	1 (3.4)	1 (1.3)
Chryseomonas luteola	0	0	0	0	1 (3.4)	1 (1.3)
Citrobacter braakii	0	1 (3.4)	1 (1.3)	2 (4.3)	5 (17.2)	7 (9.3)
Citrobacter youngae	0	0	0	0	1 (3.4)	1 (1.3)
Unknown *Citrobacter* isolates	0	1 (3.4)	1 (2.7)	1 (2.2)	1 (3.4)	2 (2.7)
Enterobacter amnigenus	0	0	0	1 (2.2)	2 (6.9)	3 (4.0)
Enterobacter cloacae	0	0	0	0	1 (3.4)	1 (1.3)
Enterobacter sakazaki	0	0	0	1 (2.2)	0	1 (1.3)
Unknown *Enterobacter* isolates	0	0	0	3 (6.5)	2 (6.9)	5 (6.7)
Escherichia coli	21 (45.7)	15 (57.7)	37 (49.3)	11 (23.9)	6 (20.7)	17 (22.7)
Escherichia fergusonii	3 (6.5)	3 (10.3)	6 (8.0)	0	0	0
Unknown *Escherichia* isolates	0	0	0	1 (2.2)	1 (3.4)	2 (2.7)
Hafnia alvei	0	1 (3.4)	1 (1.3)	7 (15.2)	1 (3.4)	8 (10.7)
Klebsiella pneumoniae	0	1 (3.4)	1 (1.3)	1 (2.2)	0	1 (1.3)
Unknown *Klebsiella* isolate	0	0	0	1 (2.2)	0	1 (1.3)
Moellerella wisconsensis	0	0	0	4 (8.7)	1 (3.4)	5 (6.7)
Morgarella morganii	0	0	0	1 (2.2)	0	1 (1.3)
Plesiomonas shigelloides	2 (4.3)	0	2 (2.7)	0	0	0
Proteus mirabilis	3 (6.5)	0	3 (4.0)	11 (23.9)	0	11 (14.7)
Proteus vulgaris	0	0	0	2 (4.3)	0	2 (2.7)
Unknown *Proteus* isolates	1 (2.2)	3 (10.3)	4 (5.3)	0	0	0
Pseudomonas fluorescens	0	0	0	0	2 (6.9)	2 (2.7)
Salmonella enterica	4 (8.7)	6 (20.7)	10 (13.3)	ND	ND	ND
Serratia liquefaciens	0	0	0	0	1 (3.4)	1 (1.3)
Unknown *Serratia* isolate	1 (2.2)	0	1 (1.3)	0	0	0
Gram positive						
Aerococcus viridans	5 (10.9)	0	5 (6.7)	10 (21.7)	0	10 (13.3)
Unknown *Aerococcus* isolates	1 (2,2)	0	1 (1,3)	2 (4,3)	0	2 (2,7)
Alloicococcus otitis	3 (6.5)	0	3 (4.0)	4 (8.7)	0	4 (5.3)
Clostridium beijerinckii	9 (19.6)	1 (3.4)	10 (13.3)	0	0	0
Unknown *Clostridium* isolates	12 (26.1)	1 (3.4)	13 (17.3)	25 (54.3)	0	25 (33.3)
Corynebacterium jeikeium	1 (2.2)	0	1 (1.3)	0	0	0
Corynebacterium propinquum	1 (2.2)	0	1 (1.3)	1 (2.2)	0	1 (1.3)
Corynebacterium striatum	0	1 (3.4)	1 (1.3)	0	0	0
Unknown *Corynebacterium* isolates	1 (2.2)	1 (3.4)	2 (2.7)	0	0	0
Enterococcus avium	2 (4.3)	1 (3.4)	3 (4.0)	0	0	0
Enterococcus casseliflavus	1 (2.2)	4 (13.8)	5 (6.7)	0	0	0
Enterococcus durans	0	0	0	0	2 (6.9)	2 (2.7)
Enterococcus faecalis	19 (41.3)	20 (69.0)	39 (52.0)	14 (30.4)	17 (58.6)	31 (41.3)
Enterococcus faecium	2 (4.3)	11 (37.9)	13 (17.3)	5 (10.9)	8 (27.6)	13 (17.3)
Enterococcus gallinarum	9 (19.6)	9 (31.2)	18 (24.0)	0	1 (3.4)	1 (1.3)
Enterococcus hirae	5 (10.9)	4 (13.8)	9 (12.0)	4 (8.7)	1 (3.4)	5 (6.7)
Unknown *Enterococcus* isolates	3 (6.5)	0	3 (4.0)	11 (23.9)	6 (20.7)	17 (22.7)
Erysipelothrix rhusiopathiae	0	0	0	1 (2.2)	0	1 (1.3)

Table 2 (continued)

Bacterial species[a]	Cloacal samples (%)			Pharynx samples (%)		
	Navarra *n*=46	Catalonia *n*=29	Total *n*=75	Navarra *n*=46	Catalonia *n*=29	Total *n*=75
Lactococcus garvieae	1 (2.2)	1 (3.4)	2 (2.7)	5 (10.8)	1 (3.4)	6 (8.0)
Lactococcus lactis	0	4 (13.8)	4 (5.3)	4 (8.7)	6 (20.7)	10 (13.3)
Micrococcus luteus	3 (6.5)	0	3 (4.0)	3 (6.5)	0	3 (4.0)
Micrococcus lylae	1 (2.2)	0	1 (1.3)	1 (2.2)	0	1 (1.3)
Staphylococcus aureus	0	0	0	6 (13.0)	0	6 (8.0)
Staphylococcus auricularis	1 (2.2)	0	1 (1.3)	1 (2.2)	0	1 (1.3)
Staphylococcus capitis	0	0	0	2 (4.3)	0	2 (2.7)
Staphylococcus chromogenes	0	0	0	2 (4.3)	0	2 (2.7)
Staphylococcus epidermidis	1 (2.2)	0	1 (1.3)	2 (4.3)	0	2 (2.7)
Staphylococcus equorum	0	0	0	2 (4.3)	0	2 (2.7)
Staphylococcus hominis	0	0	0	1 (2.2)	0	1 (1.3)
Staphylococcus intermedius	1 (2.2)	0	1 (1.3)	0	0	0
Staphylococcus lentus	0	0	0	1 (2.2)	0	1 (1.3)
Staphylococcus sciuri	2 (4.3)	0	2 (2.7)	4 (8.7)	1 (3.4)	5 (6.7)
Staphylococcus schleiferi	1 (2.2)	0	1 (1.3)	0	0	0
Staphylococcus xylosus	1 (2.2)	0	1 (1.3)	1 (2.2)	0	1 (1.3)
Streptococcus acidiminimus	8 (17.4)	0	8 (10.7)	7 (15.2)	0	7 (9.3)
Streptococcus bovis	0	0	0	3 (6.5)	0	3 (4.0)
Streptococcus constellatus	0	0	0	1 (2.2)	0	1 (1.3)
Streptococcus uberis	0	0	0	1 (2.2)	0	1 (1.3)
Unknown *Streptococcus* isolate	0	0	0	0	1 (3.4)	1 (1.3)

[a] Only organisms with excellent, very good or good identification scores in the multi-substrate identification systems used were considered; otherwise, the identification was made only at the genus level

ND not determined

R^2=0.95, Tables 4 and 6) and genus ($W_{AR + Population}$=0.99, R^2=0.94, Tables 5 and 6; Fig. 1) composition. In fact, the pharynx exhibited a higher bacterial diversity both at the species ($\beta_{Pharynx}$=2.81, SE=0.33, t=8.31) and the genus level ($\beta_{Pharynx}$=2.21, SE=0.19, t=11.42) than the cloacae samples. In the same line, the probability of an inter-specific encounter (PIE) depended on both anatomic location and the population of origin ($W_{AR * Population}$=0.99, R^2=0.99 in both species and genus, Tables 4 and 5), with higher probabilities in pharynxes from vultures sampled in Navarra (Table 6) and lower ones in the cloacae from vultures of Catalonia (Table 6).

Table 3 Observed (O) values and those expected by chance (E) of the C-score for positive/negative matrices of bacteria communities of pharynx and cloacae of 75 griffon vultures from northern Spain

	Catalonia				Navarra			
	O	E	p	SES	O	E	p	SES
Pharynx	5.34	5.28	0.34	0.39	11.04	10.91	0.06	1.62
Cloacae	12.22	12.13	0.37	0.26	10.22	10.03	0.14	1.08

The p indicates the p value and the SES the standardised effect size

Table 4 Model selection for investigating the importance of the anatomical region (AR, pharynx vs cloacae) and the host population (Catalonia vs Navarra) in bacterial species richness and evenness isolated in 75 Eurasian griffon vultures

Biological models	K	AICc	Δi	*Wi*
Species bacteria richness				
Abundance+AR+population	8	268.82	0	0.99
Abundance+population	6	317.22	48.39	< 0.001
Species bacterial evenness				
AR * population	5	−730.47	0	0.99
AR+Population	4	−478.84	251.62	< 0.001

K number of parameters, *AICc* corrected Akaike information criterion, *Δi* difference of AICc with respect to the best model, *wi* Akaike weight, *Mo* null model only the intercept and error terms

In bold, models with substantial support for being the best model. Because Δi with respect to the second competitive model was larger than 10, only the first competitive model has been shown in each case. Data (bacterial richness or evenness) were generated by specific individual-based rarefaction curves built from 75 randomly sampled individual bacteria. Bacterial richness was analysed by generalised additive models (GAM) and evenness (e.g., probability of an inter-specific encounter, PIE) by lineal models (LM)

Table 5 Model selection for investigating the importance of the anatomical region (AR, pharynx vs cloacae) and the host population (Catalonia vs Navarra) of bacteria genus richness and evenness isolated in 75 Eurasian griffon vultures

Biological models	K	AIC	Δi	*Wi*
Bacteria genus richness				
Abundance+AR+population	8	460.99	0	0.99
Abundance+population	6	554.33	93.34	< 0.001
Bacteria genus evenness				
AR * population	5	−2778.83	0	0.99
AR+Population	4	−1305.20	1473.64	< 0.001

Bacteria richness was analysed by generalised additive models (GAM) and evenness (e.g., probability of an inter-specific encounter, PIE) by lineal models (LM)

Concerning the host population, the higher species ($\beta_{Navarra}$=0.03, SE=0.0005, t=58.98, Tables 4 and 6) and genus diversity ($\beta_{Navarra}$=2.914, SE=0.19, t=15.42, Tables 5 and 6) was observed in Navarra. In fact, 26 genera and 27 species were identified from the cloacae of vultures from Navarra, and 14 genera and 16 species from the cloacae of vultures from Catalonia. Similar results were observed for the pharyngeal samples (29 genera and 36 species in vultures from Navarra, and 21 genera and 20 species in vultures from Catalonia). Also, the genera and species found in the cloacal and pharyngeal samples of vultures from Navarra and Catalonia were qualitatively quite different. Only 21.6 and 27.1 % of the genera found in the cloacae and pharynx, respectively, were present in the vultures of both geographic areas (Table 1). Similarly, only 38.8 and 39.4 % of the species identified in the present study were isolated in vultures of Navarra and Catalonia (Table 2). Lactococci were isolated more frequently from cloacal samples of animals from Catalonia (17.2 vs 2.2 %; p<0.05). On the other hand, there was a statistically significant association (p<0.05) between the genera *Proteus* (28.3 vs 0 %), *Aerococcus* (26.1 vs 0 %), *Clostridium* (54.3 vs 0 %), *Staphylococcus* (34.8 vs 3.4 %) and *Streptococcus* (26.1 vs 3.4 %) with the pharyngeal samples of vultures from Navarra. This statistical association was also observed between bacterial species. *Aerococcus viridans*, *Streptococcus acidominimus* and *Proteus mirabilis* were isolated more frequently from the pharyngeal samples of vultures from Navarra than those from Catalonia (21.7 vs 0 %, 15.2 vs 0 % and 23.9 vs 0 %, respectively). *E. faecium* was associated with cloacal samples of vultures from Navarra and *S. acidominimus* with the cloacal samples of vultures from Catalonia (Table 1). *S. enterica* serotype 4,(5),12:i:- (n=5) and *S. enterica* serotype Derby (n=1) were isolated only from vultures of Catalonia while *S. enterica* serotype 61:k:1,5,7 (n=3) and *S. enterica* serotype Infantis (n=1) were isolated only from those of Navarra.

Discussion

The aims of this study were to characterise and compare the cloacal and pharyngeal cultivable bacteria of Eurasian griffon vultures. Despite the analysis of microbial communities based on culturing techniques detects only a small fraction of the microbial community [34], it has the advantage of having pure isolates than can be used in further epidemiological or physiological studies. Using this approach, this report demonstrates that their microbial flora is diverse and made up of a variety of aerobic, facultative and anaerobic bacteria. *Escherichia* and *Enterococcus* were the genera, and *E. coli* and *E. faecalis* the species, most frequently found in both cloacal and pharyngeal samples (Tables 1 and 2). These genera and species are considered part of the microbiota of many avian species [33, 38, 48]. Moreover, other genera such as *Clostridium*, *Lactococcus* and *Staphylococcus* were also commonly found in cloacae and pharynx (Table 1). Cloacae and pharynx shared only 47.8 % of the genera identified in the present study. Thus, our overall results indicate that the spectrum of bacteria looks relatively dissimilar in cloacae and pharynx, detecting (a) some bacterial genera and species only at one anatomical site (Tables 1 and 2) and, moreover, (b) statistically significant relationships between some genera with the cloacae or pharynx. These results are similar to those

Table 6 Average richness and mean probability of an inter-specific encounter (PIE, Hurlbert's 1971) of bacteria genera and species cultured from pharynx and cloacae of 175 Eurasian griffon vultures from two populations (Navarra and Catalonia), in northern Spain

		Pharynx		Cloacae	
		Catalonia	Navarra	Catalonia	Navarra
Richness	Genus	14.4±4.9 (3.7–21)	15.4±4.7 (4.3–21.3)	10.2±2.9 (3.5–14)	14.7±4.9 (4.1–21.1)
	Species	11.5±4.9 (1–18.2)	15.3±7.11 (1–24.4)	10.6±3.9 (1–15.1)	14.5±6.11 (1–21.9)
PIE	Genus	0.84±0.002 (0.829–0.840)	0.93±0.001 (0.924–0.928)	0.83±0.002 (0.802–0.812)	0.89±0.001 (0.888–0.892)
	Species	0.88±0.002 (0.87–0.88)	0.95±0.001 (0.952–0.956)	0.87±0.001 0.87–0.88)	0.91±0.008 (0.909–0.912)

These descriptive statistics come from values generated by specific individual-based rarefaction curves based on 60 randomly sampled individual bacteria. Numerical values represent the mean±the standard error. The minimum and maximum values are shown in brackets

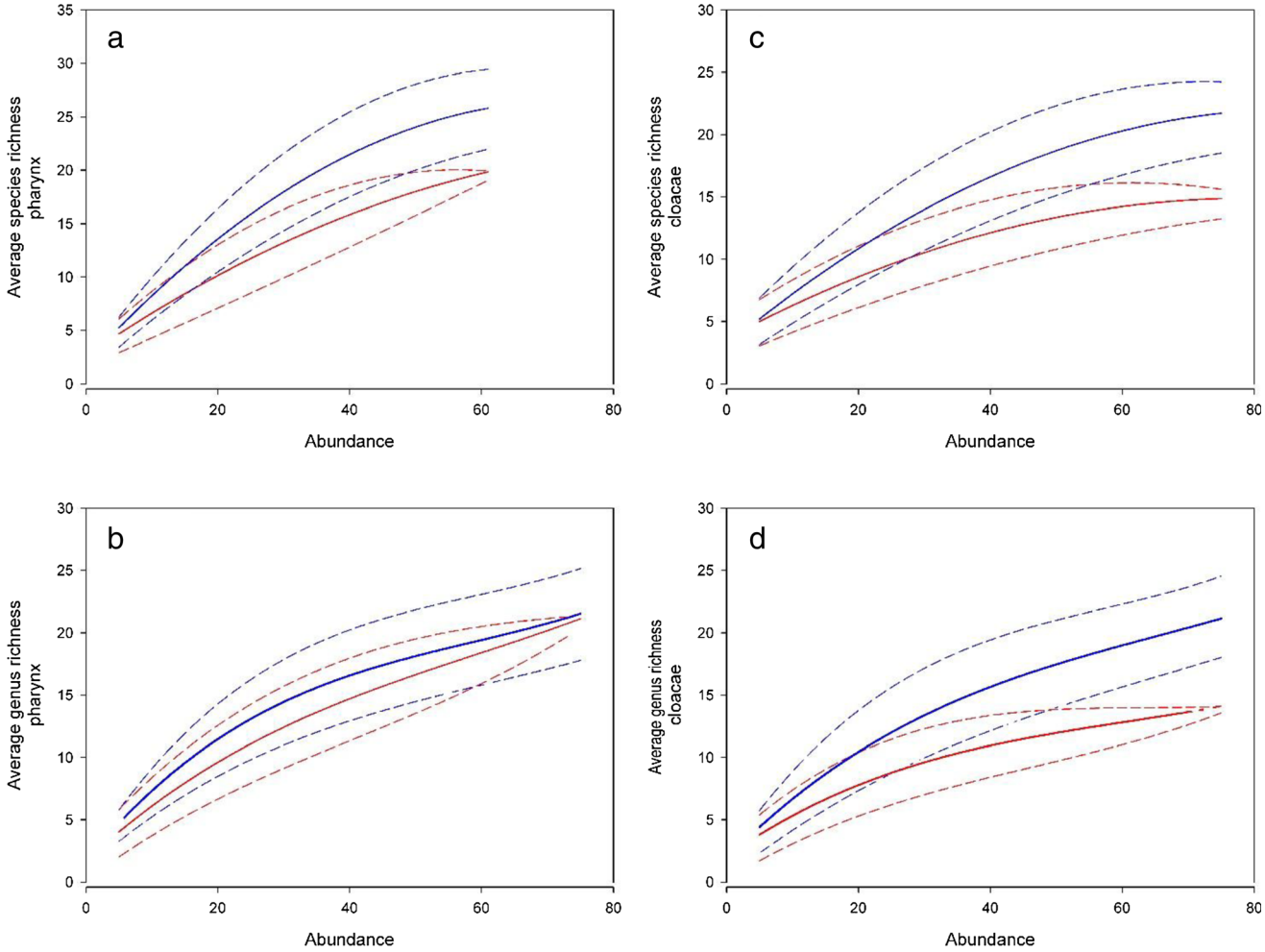

Fig. 1 Individual-based rarefaction curves representing the average cultivable species richness from re-samples of pooled individual bacteria (abundance) isolated from pharynx (**a**) and cloacae (**c**) in Eurasian griffon vultures from two distant geographic areas in northern Spain: Catalonia (*red lines*) and Navarra (*blue lines*). Cultivable genus species richness from pharynx (**b**) and cloacae (**d**) is also shown. Abundance levels for bacteria species in pharynx samples were fixed to 60 individuals and to 75 for the rest of simulations. *Broken lines* represent the 95 % confidence intervals of mean bacterium species richness

found by D'Aloia et al. [9] who showed differences in the cloacae and oropharynx bacteria in different wild birds. We also observed differences in the cultivable bacteria of the vultures based on their geographic origin. The microbiota from cloacae and pharynx exhibited differences both in the diversity and the frequency of isolation of certain species between vultures of Catalonia and Navarra (Table 2). The intestinal microbiota in wild birds can be affected by diet [3, 5]. Therefore, the differences observed in the bacteria of cloacae may be associated with the different diets of vultures in each geographic area; in Catalonia, vultures feed on livestock slaughtered for human consumption that had been under sanitary control, and presumably poor in enterobacterial species, while vultures in Navarra feed on extensively reared pigs and sheep that die from disease or accident. In addition, the consumption of other foods obtained by vultures outside of their supplementary feeding points could also influence the differences observed in the bacteria of cloacae.

Diets rich in meat result in a decrease in the numbers of lactobacilli and an increase in coliform organisms in mice and rats [16]. The fact that vultures are scavengers and their diet is made up mainly of meat could explain the absence of lactobacilli and the high presence of *E. coli* in the samples examined (Tables 1 and 2). However, it may also be possible that, unlike chickens in which lactobacilli are the predominant bacteria of the intestinal microbiota [21, 23], these microorganisms do not constitute part of the microbiota of cloacae in vultures.

With respect to the cultivable bacteria of the pharynx, we did not find any reasonable explanation for the differences observed in the species and genus isolated from vultures from Catalonia and Navarra, nor for the absence of lactobacilli in the pharynx of vultures, given that lactobacilli are present in the respiratory organs of chicken as indigenous microbiota [39].

Wild birds usually carry different *Campylobacter* pathogenic species for humans or livestock species [6, 41]. However, these organisms were not isolated from any of the vultures examined in the current study, indicating that they would not represent a potential source of infection for other animals or humans. On the other hand, different species of *Staphylococcus*, *Corynebacterium*, *Erysipelothrix* and *Salmonella* were isolated from the pharynx and/or cloacae (Table 2). These organisms are potentially pathogenic and associated with mortality and reduced fitness in wild bird populations [41, 52, 55], with different clinical processes in animal and humans [12, 19, 47] or of zoonotic significance [46, 55].

S. enterica is a well-recognised human pathogen [2, 10] and is also an important cause of disease in poultry [4]. Of particular interest is the isolation from wild vultures of different serotypes of *S. enterica* other than Typhimurium, given that *Salmonella* Typhimurium is the serotype most commonly isolated from vultures [18, 44]. *S. enterica* serotype Infantis and *S. enterica* serotype Derby have been isolated previously from humans, chickens and other animals [1, 30, 31]. The monophasic *S. enterica* serotype 4,(5),12:i:- variant is one of the most prevalent serovars associated with human infections worldwide [22] and has also been isolated from livestock [20]. Finally, *S. enterica* serotype 61:k:1,5,7 is frequently isolated and considered to be adapted to sheep [11]. It is interesting to note that the three isolates of this serotype were isolated only from vultures in Navarra, where sheep are fed upon. Vultures might acquire *Salmonella* by scavenging on contaminated livestock carcasses or from human food scraps [44].

In conclusion, the cultivable bacteria of cloacae and pharynx of the vultures examined in this study consisted mainly of *Escherichia*, lactococci, enterococci, staphylococci and clostridia. Particularly, *E. coli* and *E. faecalis* were the predominant species isolated from both cloacae and pharynx. Vultures can also serve as a reservoir of pathogenic bacteria such as *Staphylococcus* and *Erysipelothrix* in the pharynx, and *Corynebacterium* and *Salmonella* in the cloacae. The transmission of these bacterial pathogens to other animal species may spread and amplify their presence in wildlife.

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