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Insights into the fecal microbiota of captive Iberian lynx (*Lynx pardinus*)

José Alberto Núñez-Díaz,[1] M. Carmen Balebona,[1] Eva M. Alcaide,[2] Irene Zorrilla,[2] Miguel Ángel Moriñigo[1]*

[1]Universidad de Málaga, Andalucía Tech, Departmento de Microbiología, Campus de Teatinos, Málaga, Spain.
[2]Division of Sustainability and Urban Environment Analysis and Diagnostic Center of Wildlife-C.A.D. Environment Agency and Water Andalusia, Málaga, Spain

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Summary. The Iberian lynx (*Lynx pardinus*) is an endangered species restricted to several areas of Spain and Portugal. Its low genetic diversity likely provokes immune depression and high susceptibility to infectious diseases. The intestinal microbiota is closely related to host health and nutrition. In order to contribute to the knowledge of the Iberian lynx intestinal microbiota, fecal microbiota of captive specimens from two breeding centers ("La Olivilla" and "El Acebuche"), located in Southern Spain, were studied by Denaturing Gradient Gel Electrophoresis (DGGE). Results grouped microbiota in two main clusters (I and III) which included DGGE patterns of 19 out of 36 specimens, cluster I being the most frequent in "La Olivilla" (50%) and cluster III in "El Acebuche" (55.55 %) specimens. Bacteroidetes, Firmicutes and Proteobacteria phyla were identified. Segregation of clusters I and III was attributed to different microorganism presence (*Pseudomonas koreensis*, *Pseudomonas migulae*, *Carnobacterium* sp., *Arthrobacter*, *Robinsoniella peorensis* and *Ornithinibacillus* sp.) and ability to use different carbon sources. Biolog EcoPlates® results indicate high functional diversity of fecal microbiota, it being higher in cluster III. The great impact of intestinal microbiota on host health supports the importance of its microbial composition understanding. This study is the first report of captive Iberian lynx fecal microbiota composition. [**Int Microbiol** 20(1): 31-41 (2017)]

Keywords: Iberian lynx (*Lynx pardinus*) · fecal microbiota · biodiversity

Introduction

The Iberian lynx (*Lynx pardinus*) is listed as endangered by the World Conservation Union (IUCN). At present, it is settled in Doñana-Aljarafe and Sierra Morena areas in Southern Spain and it is being reintroduced in other areas of Spain and Portugal [47] (Iberlince project web). Studies related to phylogeny, histopathology and immunohistochemistry indicate low genetic diversity [1,10] and frequently depressed immune system [45], with high susceptibility to infectious diseases [34]. The precarious situation of the wild Iberian lynx led to the establishment of the Iberian Lynx *Lynx pardinus* Conservation Breeding Program [54], that follows a multidisciplinary approach encompassing management and applied re-

*Corresponding author: Miguel Ángel Moriñigo
E-mail: morinigo@uma.es

search strategies in different areas. One of these areas is related to health and veterinary aspects, and the establishment of preventive disease protocols for the captive populations is one of the Program's main lines of action [54].

Intestinal microbiota of mammals play a very important role mainly due to facilitating the absorption of ingested food by degradation of macromolecules [41] and protecting against potential pathogens [9]. In this way, research has convincingly demonstrated that the microbiota is crucial in order to prime and orchestrate innate and adaptive host immune responses [22]. This microbiota constitutes a complex ecosystem consisting of a big amount and diversity of bacterial species and the quantity and types of bacteria are closely linked to environmental variables. Understanding the gut microbial community structure is critical to identify and perform the potential fitness-related traits for the host [56]. In this context, knowledge of the captive Iberian lynx intestinal microbiota can be an useful tool in future preventive strategies against the disease. Several studies have characterized the gut microbiota of felines, such as cats [25], but in the case of the Iberian lynx, only one study that analyses distal gut microbial biodiversity of one specimen has been carried out [3]. For this reason, further studies with a higher number of animals are necessary to increase the understanding of the Iberian lynx intestinal microbiota.

The objective of this study was to assess the composition of the predominant microbial groups in the fecal microbiota of Iberian lynx specimens from two breeding centers where they are confined, "El Acebuche" and "La Olivilla" located in Doñana and Sierra Morena areas, respectively. These two breeding centers account for more than 50% of the captive Iberian lynx population.

Materials and methods

Fecal samples of Iberian lynx. A total of 36 fecal samples of captive Iberian lynx specimens were kindly supplied by Centro de Análisis y Diagnóstico de la Fauna Silvestre de Andalucía (Málaga-Spain) within the context of the routine program of coprologic analysis of this species. Fecal samples were collected in November 2011 and within 3 h after defecation from 36 captive Iberian lynx specimens kept in the breeding centers of "El Acebuche" (10 males and 8 females) (15 ºC, average temperature) and "La Olivilla" (6 males and 12 females) (12 ºC, average temperature), located in Doñana and Sierra Morena areas (Andalucía, Southern Spain), respectively. Diet of Iberian lynx specimens consisted of rabbit obtained from the same supplier (Las Lomas, Cádiz, Spain) along the study period.

After collection, samples were immediately frozen at –20ºC until further analysis. Fecal samples came from apparently healthy specimens and none of them had a history of antibiotic use.

Analysis of fecal microbiota. A portion of 50 mg of the center of feces was obtained in aseptic conditions. Total DNA was extracted from each sample as described by Martínez et al. [37]. DNA was quantified using a spectrophotometer (NanoDrop 1000, Thermo Scientific).

In order to compare DGGE patterns of the intestinal microbiota of Iberian lynx, the DNA was amplified using 16S rDNA bacterial domain-specific primers 309F (5′-ATCCCTACGGGAGGCWGCAG-3′) and 677R-CGGGG GGATMTCTACGCATTTCACCGCTAC-3′) [30]. PCR was carried out in a 50 µl reaction mixture that included 1 µl (10 µM) of each primer, 200 µM dNTPs, 5 µl 10X DreamTaq Green Buffer (with $MgCl_2$ 20 mM), 37 µl H_2O Milli-Q, 5 µl of DNA template and 1 U DreamTaq Polymerase (Life-Technologies, Eugene, Oregon, USA). The standard cycling conditions were 94 ºC for 2 min, followed by 35 cycles at 94 ºC for 30 s, 56 ºC for 30 s and 68 ºC for 30 s and a final step at 68 °C for 5 min. Amplicons obtained (400 bp size) were electrophoresed on a 1% agarose gel (w/v) to check for product size and quantity. Later, amplicons were separated by denaturing gradient gel electrophoresis (DGGE) according Muyzer et al. [42] using a Dcode TM system (Bio-Rad). Electrophoresis was performed in an 8% polyacrylamide gel (37.5:1 acrylamide-bisacrylamide) using a 30 to 55% denaturing gradient (urea and formamide) for separation of PCR products. Electrophoresis was carried out for 5 hours at 150 V in 0.5 X TAE buffer (20 mM Tris acetate [pH 7.4], 10 mM sodium acetate, 0.5 mM Na_2-EDTA) at 60 °C constant temperature. Gels were subsequently stained with $AgNO_3$ [48]. The number and intensity of DGGE bands were calculated from the densitometric curves of the gel using the software FPQuest 4.0 (Applied Maths BVBA). A matrix of similarity between DGGE profiles was determined based on Pearson similarity coefficient. Clustering of DGGE patterns was achieved by construction of dendrograms using the unweighted pair group method with arithmetic averages (UPGMA). In order to determine the structural diversity of the microbial community corresponding to the DGGE banding pattern, three indices were calculated: (1) Shannon index (H′) was calculated according to the function $H' = -\Sigma\left[p_i * \ln p_i\right]$, where $p_i = n_i / N$, n_i is the peak surface of each band, and N is the sum of all the peak surfaces of all bands; (2) Simpson index (D) was calculated following the function $D = \Sigma p_i^2$, where $p_i = n_i / N$, n_i is the peak surface of each band, and N is the sum of all the peak surfaces of all bands; and (3) Range-weighted richness (Rr) was calculated according to the function $Rr = \left(N^2 * D_g\right)$, where N represents the total number of bands in the pattern, and D_g the denaturing gradient comprised between the first and the last band of the pattern [38].

Predominant gel bands were retrieved for sequencing with sterile pipette tips, placed in 100 µl of double distilled Milli-Q water and incubated at 4 ºC overnight. DNA was amplified using universal primers, 309F and 677R without GC-clamp (5′-ATMTCTACGCATTTCACCGCTAC-3′). PCR was performed in a 50 µl reaction mixture containing 1 µl (10 µM) of each primer, 200 µM dNTPs, 5 µl 10X DreamTaq Green Buffer (with $MgCl_2$ 20 mM), 32 µl H_2O Milli-Q, 10 µl of DNA template and 1U DreamTaq Polymerase (Life-Technologies, Eugene, Oregon, USA). The standard cycling conditions were 95 ºC for 2 min, followed by 45 cycles at 95 ºC for 30 s, 64 ºC for 30 s and 72 ºC for 60 s and a final step at 72 ºC for 5 min. The amplicons were used for sequence analysis (Macrogen Korea sequencing) and sequences were compared with those from the National Center for Biotechnology Information (NCBI) using the BLAST sequence algorithm [4].

Functional metabolic diversity of the fecal microbiota. Functional diversity of fecal microbiota of Iberian lynx specimens included in clusters I and III was assessed by determining the community catabolic profiles (CLCPs) using Biolog EcoPlate® (Biolog, Hayward, CA, USA). Biolog EcoPlate® assays 31 carbon sources grouped by chemical class (carbohydrates, polymers, carboxylic acids, amino acids and amines) and incorporates

a control assay without a carbon source. All tests are carried out in triplicate. Fecal samples (500 mg) of each specimen from clusters I and III were suspended in 50 ml PBS (pH 7.2) vortexed and allowed to rest for 15 min at 4ºC. Then, 150 µl supernatant aliquots were dispensed into each of the 96 wells of the Biolog Ecoplates plates and incubation was performed at 37 ºC in the dark. Activity measures were carried out at 590 nm with a microplate reader (Multiskan Ascent, Thermo Electron, Milford, MA, USA) every 24 h up to 144 h. To determine the level of functional diversity the following indices were calculated: (a) Shannon's index as was applied to assess the substrate utilization pattern according to the following expression: $H' = -\Sigma\left[p_i * \ln p_i\right]$, where $p_i = n_i / N$, n_i is the is the value of OD_{590} of each well, and N is the sum of OD_{590} for all metabolic substrates. (b) Substrate richness (S) corresponding to the number of different substrates used.

Statistical analysis. Significance of differences in Shannon's index (H'), Simpson's index (D) and range-weighted richness (Rr) was determined after performing t-Student test with the software SPSS Statistics 17.0 (IBM). Contribution of each bacterial species identified from sequencing of DGGE bands to similarity percentages (SIMPER) between clusters I and III was determined by using PAST software.

Results

Clustering analysis was applied to fecal microbiota DGGE bands of Iberian lynx specimens, and the dendrogram obtained showed a high group similarity percentage. Cluster analysis based on Pearson coefficient of similarity between band patterns allowed the identification of clearly defined clusters (I to V) (Fig. 1). Distribution of the specimens included in these clusters did not respond to variables such as gender. Clusters I and III grouped 19 out of 36 specimens sampled, cluster I being the most frequent DGGE pattern detected in lynx from "La Olivilla" (50%). On the contrary, none DGGE pattern of this cluster was detected in specimens from "El Acebuche". On the other hand, the DGGE patterns grouped in cluster III showed high values of similarity (about 76%) and included 55.55% of the specimens from "El Acebuche", whilst only 5.55% DGGE patterns of animals from "La Olivilla" were included in this cluster.

The number of DGGE bands was high in all clusters and the ecological index values calculated from the bands and densitometric curves of the gel are summarized in Table 1. Significant differences ($p < 0.05$) were not detected. Shannon diversity index (H') calculated for all lynx specimen samples showed medium-high values (3.05–3.46), whereas low Simpson's index values (0.05–0.09) were observed indicating low dominance of some microbial species (D). Range-weighted richness (Rr) showed high values (>30) indicating an habitable environment.

The bacterial species identified based on the bands sequenced from DGGE gels of fecal samples grouped in clusters are summarized in Table 2. Phyla detected in all the samples included Actinobacteria (0.96% to 5.86%), Bacteroidetes (0.11% to 6.03%), Firmicutes (from 33.58% to 42.38%) and Proteobacteria, specifically γ-Proteobacteria, (ranging from 54.95% to 61.76%).

In order to break down the contribution of each bacterial group to the observed dissimilarity between clusters I and III fecal microbiota samples, similarity percentage analysis (SIMPER) was carried out. In this way, most important species responsible for the observed pattern of dissimilarity are summarized in Table 3. Almost 50% of the differences in bacterial communities detected in samples grouped in cluster I and III were attributed to *Pseudomonas koreensis* (13.9%), *Pseudomonas migulae* (8.9%), *Carnobacterium* sp (6.85%), *Arthrobacter* (5.6%), *Robinsoniella peorensis* (5.6%) and *Ornithinibacillus* sp. (5.3%).

Finally, metabolic analysis of fecal microbiota included in clusters I and III showed ability to use 24 and 30 out of 31 carbon sources assayed, respectively (Fig. 2). Catabolic profiles were determined and Shannon diversity values obtained indicate significantly lower ($p < 0.05$) number of substrates used by microbiota of the cluster I compared to fecal microbiota of cluster III after 24, 48 and 72 h incubation (Table 4).

Discussion

Understanding the gut microbial community structure is critical to identify and establish the potential fitness-related traits for the host [56]. In this study, DGGE methodology was used to describe the microbial composition of feces of captive Iberian lynx. The limitations of DGGE in microbial analysis have been previously described [43], nevertheless substantial information about species composition can be obtained from very complex microbial communities such as the gut microbiota [31]. Changes in DGGE band abundance as reflected by bacterial diversity can indicate ecological shifts in the characteristics of bacterial communities [53]. It is commonly accepted that the intensity of the DGGE band is directly related to the density of the corresponding 16S rDNA. For this reason, Pearson similarity coefficient, that considers both position and band intensity, was used in this study to carry out the clustering analysis of the DGGE patterns. Although the same predominant DGGE bands were present in all DGGE pat-

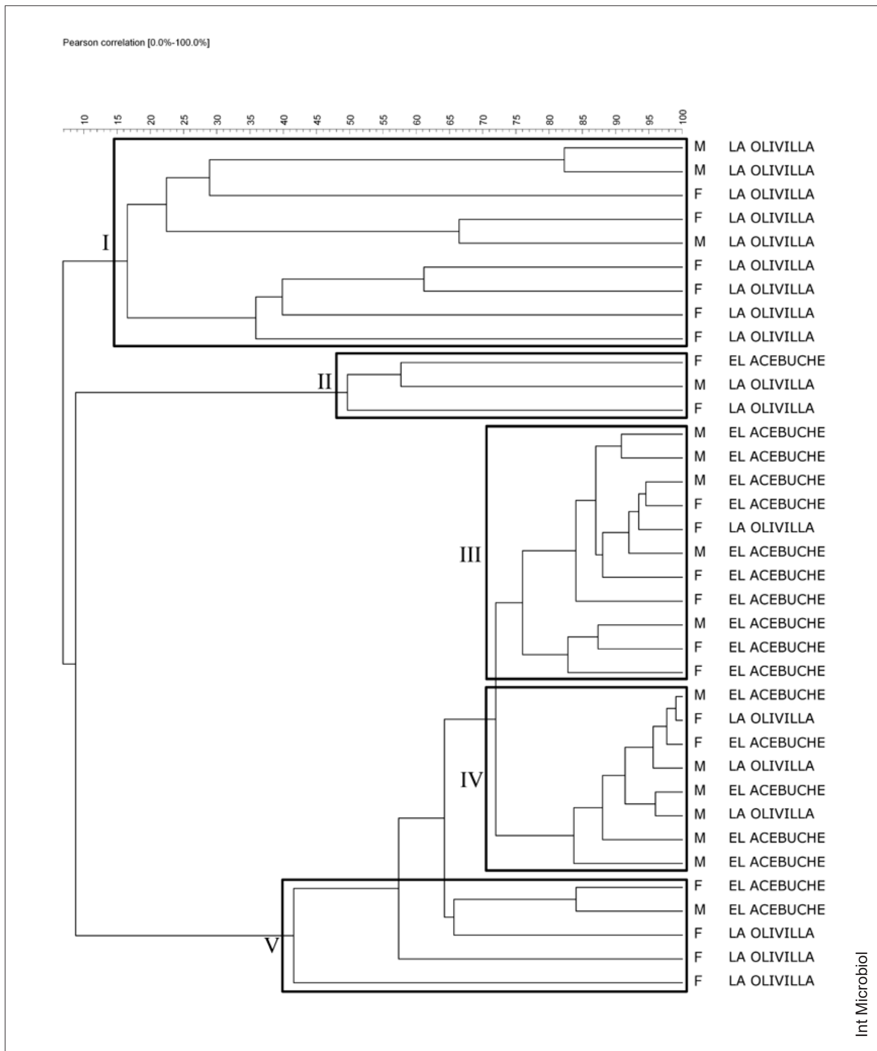

Fig. 1. Clustering analysis based on pair wise similarity index of DGGE patterns obtained from fecal samples of Iberian lynx specimens from “El Acebuche” and “La Olivilla” centers.

terns, clearly defined clusters were detected. Clusters I and III included profiles of 19 out of 36 specimens, these clusters displaying the most frequently DGGE patterns detected in lynx specimens from “La Olivilla” and “El Acebuche”, respectively. Cluster III included 55.55% of the specimens from “El Acebuche”, percentage clearly higher than that (5.55%)

Table 1. Percentage of captive Iberian lynx specimens included in the clusters described in this study and Shannon diversity index (H′), Simpson dominance index (D) and range weighted richness (Rr) values obtained for the microbiota of the different clusters. Data of the ecological parameters are expressed as mean ± standard deviation. Significant differences ($p < 0.05$) were not detected.

	CLUSTER				
	I	II	III	IV	V
Specimens from “El Acebuche” (%)	0	5.55	55.55	27.70	11.11
Specimens from “La Olivilla” (%)	50	11	5.55	16.67	16.67
Ecological parameters					
Shannon index (H’)	3.05 ± 0.11	3.13 ± 0.13	3.17 ± 0.07	3.46 ± 0.41	3.19 ± 0.09
Simpson index (D)	0.06 ± 0.02	0.05 ± 0.01	0.05 ± 0.01	0.09 ± 0.03	0.05 ± 0,00
Range-weighted richness (Rr)	67.18 ± 20.33	61.37 ± 21.58	89.39 ± 31.80	52.06 ± 34.45	83.48 ± 45.45

Table 2. Nearest-match identification of 16S rDNA sequences corresponding to microbiota DNA recovered from the DGGE bands of each cluster.

Phylum / Band number	Closest relative	Similarity (%)	Genbank Number	I	II	III	IV	V
Actinobacteria								
23	*Arthrobacter* sp. IARI-L-29	99	JF343188	5.86	2.11	2.16	0.96	4.10
Bacteroidetes								
17	*Bacteroides xylanisolvens* strain EBA22-11	97	JF298887	3.79	0.11	1.92	5.29	6.03
Firmicutes								
4	*Bacillus psychrodurans* strain CBG_LBI34	97	JF909578	2.55	3.24	6.70	7.90	4.46
10	*Carnobacterium maltaromaticum*	98	AB680942	1.76	3.78	3.94	4.20	2.59
18	Uncultured *Carnobacterium* sp. clone CTL-18	95	JQ798984	10.26	12.03	6.25	7.97	10.86
25	*Clostridium hiranonis* strain 45	100	JF693906	3.82	2.17	1.35	0.79	3.17
6	*Kurthia zopfii*	99	AB680230	1.15	4.32	4.41	2.86	1.75
5	*Ornithinibacillus* sp. XJSL10-7	98	GQ903476	1.29	1.18	5.98	3.61	2.01
15	*Robinsoniella peoriensis* strain PPC31	97	NR_041882	6.25	9.19	2.62	3.08	2.49
8	*Sporosarcina ureae* strain KNUC424	98	JQ071513	2.14	3.03	4.27	5.78	2.93
14	Uncultured bacterium clone TuCw28	90	DQ071463	4.36	3.44	3.08	2.66	3.52
Proteobacteria								
26	*Agarivorans* sp. B29	97	AB542196	2.50	3.50	1.07	0.62	2.52
9	*Escherichia coli* strain G5B128	98	GU646167	1.52	3.36	5.76	5.24	2.11
21	*Escherichia coli* strain DP170	100	JF895181	2.39	2.98	2.04	1.38	5.68
22	*Escherichia coli* strain ADB-2	99	JX094849	4.09	3.37	1.16	1.69	2.86
13	*Escherichia fergusonii* strain G30	98	HQ259962	2.24	3.63	3.31	1.23	4.37
16	*Escherichia fergusonii* strain M5	99	HQ259946	2.60	2.65	4.19	0.85	2.21
7	*Escherichia vulneris* strain M3	100	HQ259947	1.07	2.56	4.44	3.38	3.14
1	*Proteus mirabilis* strain FFL2	99	JN222368	1.80	2.93	4.74	4.23	3.87
2	*Pseudomonas brenneri* strain KOPRI 25949	100	HQ825070	2.52	3.27	5.19	5.98	4.40
27	*Pseudomonas koreensis* strain J9B-42	99	HQ238778	10.74	2.49	0.94	0.26	3.09
19	*Pseudomonas migulae* strain A74	97	JN390959	11.81	5.30	6.36	11.50	6.27
12	*Pseudomonas psychrophila* strain Ibu-08	98	JQ782895	2.01	6.42	3.63	2.71	2.71
20	*Pseudomonas tolaasii* strain B44	98	EU169145	2.99	3.32	3.36	2.02	3.61
3	*Shigella flexneri*	99	AB639118	2.04	6.56	5.78	7.39	4.46
11	*Shigella sonnei* strain DY89	99	HQ591457	4.29	5.99	3.43	5.46	2.67
24	Uncultured bacterium clone Tutokochoana-138	96	JQ283151	2.13	3.43	1.94	1.01	1.70

detected in specimens from “La Olivilla”, for which the most frequently DGGE pattern detected corresponded to cluster I. On the contrary, cluster I did not group specimens from “El Acebuche”. The percentage of similarity showed by the DGGE patterns of cluster III was higher than that of profiles included in cluster I, showing higher homogeneity in microbiota composition of specimens grouped in cluster I.

Ecosystem function and stability are influenced by species and functional group richness [7] biodiversity being essential in the protection of ecosystems against declines in their functionality [58]. In this context, the number of DGGE bands was high in all clusters and ecological index values calculated from the band patterns did not show significant differences. Shannon diversity index (H’) showed medium-high values corresponding to the typical profile of intestinal environment [14], with high evenness of the individuals among the bacterial species. Low Simpson’s index (D) values indicate low dominance of some species, whereas range-weighted richness (Rr) values obtained are among those classified as high according to the criteria proposed by Marzorati et al. [38]. These

Table 3. SIMPER analysis indicating the contribution of each bacterial group to total dissimilarity among microbiota of captive Iberian lynx specimens included in clusters I and III. Bacterial groups contributing ≥ 5% to total dissimilarity are marked in bold

Phylum/Band number	Closest relative	Dissimilarity (%)
Actinobacteria		
23	*Arthrobacter* sp. IARI-L-29	**5.59**
Bacteroidetes		
17	*Bacteroides xylanisolvens* strain EBA22-11	2.99
Firmicutes		
4	*Bacillus psychrodurans* strain CBG_LBI34	4.44
10	*Carnobacterium maltaromaticum*	2.24
18	Uncultured *Carnobacterium* sp. clone CTL-18	**6.85**
25	*Clostridium hiranonis* strain 45	3.73
6	*Kurthia zopfii*	3.65
5	*Ornithinibacillus* sp. XJSL10-7	**5.33**
15	*Robinsoniella peoriensis* strain PPC31	**5.57**
8	*Sporosarcina ureae* strain KNUC424	2.11
14	Uncultured bacterium clone TuCw28	2.40
Proteobacteria		
26	*Agarivorans* sp. B29	2.21
9	*Escherichia coli* strain G5B128	4.76
21	*Escherichia coli* strain DP170	0.90
22	*Escherichia coli* strain ADB-2	4.32
13	*Escherichia fergusonii* strain G30	0.82
16	*Escherichia fergusonii* strain M5	1.37
7	*Escherichia vulneris* strain M3	3.81
1	*Proteus mirabilis* strain FFL2	3.15
2	*Pseudomonas brenneri* strain KOPRI 25949	2.69
27	*Pseudomonas koreensis* strain J9B-42	**13.87**
19	*Pseudomonas migulae* strain A74	**8.88**
12	*Pseudomonas psychrophila* strain Ibu-08	1.52
20	*Pseudomonas tolaasii* strain B44	0.16
3	*Shigella flexneri*	4.04
11	*Shigella sonnei* strain DY89	1.88
24	Uncultured bacterium clone Tutokochoana-138	0.65

range-weighted richness indicate increased genetic variability of fecal microbiota [13]. Based on these results the intestinal tract of Iberian lynx could be considered as a high habitable environment, hosting species phylogenetically different. This trait could protect this ecosystem against changes and declines in their functioning, because many species provide guarantees that some will maintain functioning even if others fail [58].

However, it is interesting to determine the predominant microbial species present in the feces and gut of Iberian lynx. In this sense, Alcaide et al. [3] using high-throughput sequencing techniques reported the absolute dominance of phylogenetic lineages of Firmicutes, Bacteroidetes besides Fusobacteria, whereas members of Proteobacteria and Actinobacteria were also identified in relatively abundant quantities in the distal gut of Iberian lynx. In our study, sequencing of the predominant DGGE bands of all clusters showed that the prevailing phyla are Firmicutes, Proteobacteria, specifically γ-Proteobacteria class, Bacteroidetes and Actinobacteria, whereas Fusobacteria were not detected as predominant DGGE bands.

In the present work, predominant DGGE bands related to

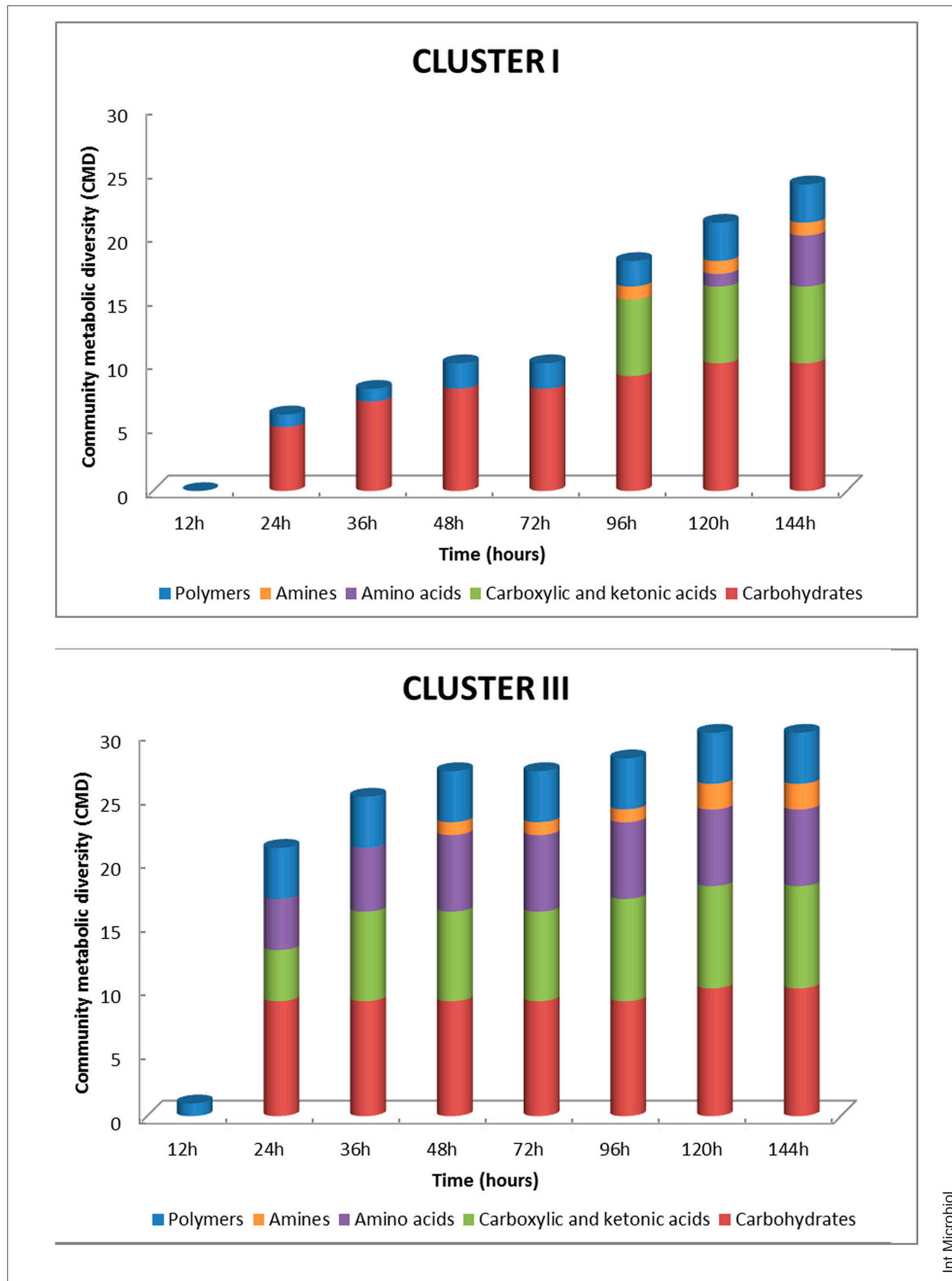

Fig. 2. Community metabolic diversity of fecal microbiota of Iberian lynx specimens included in clusters I and III based on the utilization of different carbon sources. Assayed substrates were grouped by chemical class (carbohydrates, carboxylic acids, polymers, amino acids and amines).

classes Clostridia and especially Bacilli were sequenced, similarly to Alcaide et al. [3] who reported these two classes as two of the predominant in the fecal microbiota of Iberian lynx. We also sequenced DGGE bands related to Lachnospiraceae family, specifically *Robinsoniella* genus. Similarly, Alcaide et al. [3] also sequenced Lachnospiraceae as one of the largest clostridia families detected in fecal samples of Iberian lynx. However, other bacterial groups such as the family Ruminococcaceae were sequenced by Alcaide et al. [3] but not detected in our study. Differences between these two stud-

Table 4. Shannon diversity values based on microbiota metabolic abilities of fecal suspensions of captive Iberian lynx specimens included in clusters I and III. Functional diversity was determined with Biolog EcoPlate® at different incubation periods. Data are expressed as mean ± standard deviation. Asterisk denote significant differences (p<0.05) between values of the same row.

Time of incubation (h)	Cluster I	Cluster III	t-Student value	p value
24 h	2,64 ± 0.07	2.97 ± 0.07*	2.22	0.0024
48 h	2.48 ± 0.09	3.20 ± 0.05*	2.22	< 0.0001
72 h	2.58 ± 0.09	3.25 ± 0.04*	2.22	< 0.0001
96 h	2,81 ± 0.08	3.31 ± 0.04*	2.22	< 0.0001
120 h	2.81 ± 0.08	3.39 ± 0.04*	2.22	< 0.0001
144 h	2.86 ± 0.08	3.38 ± 0.03*	2.22	< 0.0001

ies could be attributed to differential sensitivity of the technology used. In this way, DGGE band intensity is directly related to the density of the corresponding 16S rDNA, and it is commonly accepted that only those microbial groups representing ≥1% in terms of relative proportion are displayed in the DGGE patterns [18].

Our results showed the presence of *Escherichia* genus in the intestine of Iberian lynx, coinciding with those observed by Gonçalves et al. [19], who demonstrated the presence of β–lactamase-producing *E. coli* strains in feces of Iberian lynx. Microorganisms identified as *Shigella sonnei* and *Shigella flexneri* were also present in fecal samples analyzed, species described as disease agents of bacillary dysentery affecting humans and primates [12]. The results obtained in our study should be considered for future research related with potential inflammatory diseases affecting to Iberian lynx, as increased counts of mucosa-associated Enterobacteriaceae have been found in duodenal biopsies of cats with intestinal bowel disease (IBD) [23]. Similarly, dogs with IBD showed lower abundance of Bacteroidetes but higher abundance of Proteobacteria in comparison with healthy specimens [51].

In the present work, the presence of Bacteroidetes, in clusters II and III showed the lowest band intensity values. On the other hand, changes in Actinobacteria were mainly due to variations in *Arthrobacter* genus, which was more abundant in specimens grouped in clusters I and III. However, the level of Actinobacteria and Bacteroidetes in fecal and intestinal microbiota of cats has been reported that is affected by different conditions [15].

The main difference observed between the fecal microbiota of animals from the two breeding centers studied was the frequency of detection of DGGE patterns corresponding to clusters I and III. In order to analyze the contribution of each bacterial group to the observed dissimilarity between clusters I and III, SIMPER analysis was carried out. The most important species responsible for the dissimilarity between clusters I and III were *Pseudomonas koreensis*, *Pseudomonas migulae*, *Carnobacterium* sp, *Arthrobacter*, *Robinsoniella peorensis* and *Ornithinibacillus* sp. Some *P. koreensis* strains have been described as spoilage bacteria from meat products [11], and have been isolated from eye lesions in fish [49], but others have also exhibited plant growth promoting traits [6] and ability to produce lipases and biosurfactant substances [5]. *Pseudomonas migulae* includes strains isolated from soil [33], water [55] and food [17]. Some strains have shown potential effects on plant growth [52] and ability to inhibit foodborne pathogen growth such as *E. coli* and *Salmonella* virulent strains [24,57].

On the other hand, *Carnobacterium* genus consists of species, mainly *C. divergens* and *C. maltaromaticum*, commonly found in the environment [28], foods [8] and intestinal tract of insects [50] and fish [2]. This microbial group is able to catabolize multiple carbohydrates [32] some species, such as *C. maltaromaticum*, showing chitinase activity [32] and being able to grow in meat at low temperature [26]. In addition, some *Carnobacterium* strains have the ability to produce bacteriocins [20] capable to inhibit pathogenic microorganisms [16] and to improve fish immune response [29]. These events have led to propose *Carnobacterium* strains as probiotics in aquaculture [44].

Ornithinibacillus species include aerobic, rod-shaped, motile and endospore-forming bacteria that have been isolated from several environments such as sludge compost [35], clams [57], Artic terrains [60], pasteurized milk [39] and hu-

man blood [27]. On the other hand, the genus *Arthrobacter* includes a heterogeneous group of obligate aerobic species commonly found in soil, but also isolated from human clinical specimens [36].

The anaerobic and spore-forming genus *Robinsoniella* has been identified as the most abundant member of Firmicutes in wild mice [56], suggesting an important biological role [56]. In this context, changes in the level of this genus have been associated with colitic process in mice [59] and in nonalcoholic fatty liver disease patients [46]. The presence of enterotypes in several mammalian hosts or distinct clusters characterized by the abundance of signature bacterial genera has been proposed [21,40]. Wang et al. [56] described two clusters in the intestinal microbiome of wild mice with differences in bacterial taxons such as members of genus *Robinsonella*. It would be very suggestive to think that the results obtained in our study support the existence of enterotypes in the microbiome of Iberian lynx, although further studies are needed to confirm this findings.

Fecal microbiota of clusters I and III were able to use 24 and 30 out of 31 different carbon sources (carbohydrates, polymers, carboxylic acids, amino acids and amines), respectively. These results reflect high functional diversity of fecal microbiota, as they are able to metabolize most different substrates assayed in Biolog EcoPlates®. However, this high functional diversity was not equal along the incubation time. Thus, the fecal microbiota of cluster III showed ability to use higher number of carbon substrates earlier than cluster I, especially amino acids and carboxylic acids, at 24, 48 and 72 h. These differences could be related to the fact that Biolog EcoPlates® aerobic incubation carried out shows functional diversity of aerobes and facultative anaerobes only. In this way, the intensities of the DGGE bands corresponding to anaerobes such as Bacteroidetes and Firmicutes such as *Bacteroides xylanisolvens*, *Clostridium hiraronis* and *Robinsoniella peorensis* were higher in fecal microbiota from cluster I than in cluster III. This different microbiota composition could explain the delay in substrate metabolization observed. However, it is necessary consider that the environmental conditions of "El Acebuche" and "La Olivilla" are not identical. Thus, although time span between defecation and freezing of the feces was short, different environmental conditions in these areas could have some effect, which may be considered for future research.

This study is the first report comparing the composition of bacterial fecal microbiota of Iberian lynx specimens from two breeding centers of captive Iberian lynx where they are confined. Results obtained show that the microbiota present in the feces could indicate that the intestinal tract of Iberian lynx is an environment very habitable and with high biodiversity of microbial species. Changes in the gut microbiota are associated with far reaching consequences on host health and development. For this reason, the understanding of the gut microbiota may lead to targeted interventions for health promotion, disease prevention and management through tools such as diet and functional foods (with pre and probiotics), contributing for the future conservation of the Iberian lynx.

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References

1. Abascal F, Corvelo A, Cruz F, Villanueva-Cañas JL, Vlasova A, Marcet-Houben M, Martínez-Cruz B, Cheng JY, et al. (2016) Extreme genomic erosion after recurrent demographic bottlenecks in the highly endangered Iberian lynx. Genome Biol 17:251
2. Al-Hisnawi A, Ringø E, Davies SJ, Waines P, Bradley G, Merrifield DL (2015) First report on the autochthonous gut microbiota of brown trout (*Salmo trutta* Linnaeus). Aquac Res 46:2962-2971
3. Alcaide M, Messina E, Ritcher M, Bargiela R, Peplies J, Huws SA, Newbold CJ, Golyshin PN, et al. (2012) Gene sets for utilization of primary and secondary nutrition supplies in the distal gut of endangered Iberian lynx. PLoS One 7:e51521
4. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403-410
5. Anbu P (2014) Characterization of an extracellular lipase by *Pseudomonas koreensis* bk-l07 isolated from soil. Prep Biochem Biotech 44:266-280
6. Babu AG, Shea PJ, Sudhakar D, Jung IB, Oh BT (2015) Potential use of *Pseudomonas koreensis* AGB-1 in association with *Miscanthus sinensis* to remediate heavy metal(loid)-contaminated mining site soil. J Environ Manage 151:160-166
7. Bell T, Newman JA, Silverman BW, Turner SL, Lilley AK (2005) The contribution of species richness and composition to bacterial services. Nature 436:1157-1160
8. Cailliez-Grimal C, Edima HC, Revol-Junelles AM, Millière JB (2007) *Carnobacterium maltaromaticum*: the only *Carnobacterium* species in French ripened soft cheeses as revealed by polymerase chain reaction detection. J Dairy Sci 90:1133-1138
9. Candela M, Biagi E, Maccaferri S, Turroni S, Brigidi P (2012) Intestinal microbiota is a plastic factor responding to environmental changes.

Trends Microbiol 20:385-391
10. Casas-Marce M, Soriano L, López-Bao JV, Godoy JA (2013) Genetics at the verge of extinction: insights from the Iberian lynx. Mol Ecol 22:5503-5515
11. Chen QS, Li HH, Ouyang Q, Zhao JW (2014) Identification of spoilage bacteria using a simple colorimetric sensor array. Sensor Actuat B-Chem 205:1-8
12. Clemens J, Kotloff K, Kay B, 1999. Generic protocol to estimate the burden of Shigella diarrhea and dysenteric mortality. World Health Organization: Departament of Vaccines and Biologicals, Geneva.
13. De Schryver P, Sinha AK, Kunwar PS, Baruah K, Verstraete W, Boon N, Gudrum De Boeck G, Bossier P (2016) Poly-β hydroxybutyrate (PHB) increases growth performance and intestinal bacterial range-weighted richness in juvenile European sea bass, *Dicentrarchus labrax*. Appl Microbiol Biotechnol 186:1535-1541
14. Dethlefsen L, Huse S, Sogin ML, Relman DA (2008) The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16S rRNA sequencing. PLoS One 6:e280
15. Deusch O, O'Flynn C, Colyer A, Morris P, Allaway D, Jones PG, Swanson KS (2014) Deep Illumina-based shotgun sequencing reveals dietary effects on the structure and function of the fecal microbiome of growing kittens. PLoS One 9:-e101021
16. Drider D, Firmland G, Héchard Y, McMullen LM, Prévost H (2006) The continuing story of class IIa bacteriocins. Microbiol Mol Biol Rev 70:564-582
17. Franzetti L, Scarpellini M (2007) Characterisation of *Pseudomonas* spp. isolated from foods. Ann Microbiol 57:39-47
18. Fromin N, Hamelin J, Tarnawski S, Roesti D, Jourdain-Miserez K, Forestier N, Teyssier-Cuvelle S, Gillet F, et al. (2002) Statistical analysis of denaturing gel electrophoresis (DGGE) fingerprinting patterns. Environ Microbiol 4:634-644
19. Gonçalves A, Igrejas G, Radhouani H, Estepa V, Alcaide E, Zorrilla I, Serra R, Torres C, Poeta P (2011) Detection of extended-spectrum beta-lactamase-producing *Escherichia coli* isolates in faecal samples of Iberian lynx. Lett Appl Microbiol 54:73-77
20. Gursky LJ, Martin NI, Derksen DJ, van Belkum MJ, Kaur K, Vederas JC, Stiles ME, McMullen LM (2006) Production of piscicolin 126 by *Carnobacterium maltaromaticum* UAL26 is controlled by temperature and induction peptide concentration. Arch Microbiol 186:317-325
21. Hildebrand F, Nguyen TL, Brinkman B, Yunta RG, Cauwe B, Vandenabeele P, Raes J (2013) Inflammation-associated enterotypes, host genotype, cage and inter-individual effects drive gut microbiota variation in common laboratory mice. Genome Biol 14:-R4
22. Hooper LV, Littman DR, Macpherson AJ (2012) Interactions between the microbiota and immune system. Science 336:1268-1273
23. Janeczko S, Atwater D, Bogel E, Greiter-Wilke A, Gerold A, Baumgart M, Bender H, McDonough PL, et al. (2008) The relationship of mucosal bacteria to duodenal histopathology, cytokine mRNA, and clinical disease activity in cats with inflammatory bowel disease. Vet Microbiol 128:178-193
24. Jay-Russell MT, Hake AF, Bengson Y, Thiptara A, Nguyen T (2014) Prevalence and characterization of *Escherichia coli* and *Salmonella* strains isolated from stray dog and coyote feces in a major leafy greens production region at the United States-Mexico border. PLoS One 9:e113433
25. Jia J, Frantz N, Khoo C, Gibson GR, Rastall RA, McCartney AL (2011) Investigation of the faecal microbiota of kittens: monitoring bacterial succession and effect of diet. FEMS Microbiol Ecol 78:395-404
26. Jones RJ (2004) Observations on the succession dynamics of lactic acid bacteria populations in chill-stored vacuum-packaged beef. Int J Food Microbiol 90:273-282
27. Kämpfer P, False E, Lodders N, Langer S, Busse HJ, Schumann P (2010) *Ornithinibacillus contaminans* sp nov, an endospore-forming species. Int J Syst Evol Microbiol 60:2930-2934
28. Katayama T, Tanaka M, Moriizumi J, Nakamura T, Brouchkov A, Douglas TA, Fukuda M, Tomita F, et al. (2007) Phylogenetic analysis of bacteria preserved in a permafrost ice wedge for 25,000 years. Appl Environ Microb 73:2360-2363
29. Kim DH, Austin B (2006) Innate immune responses in rainbow trout (*Oncorhynchus mykiss*, Walbaum) induced by probiotics. Fish Shellfish Immunol 21:513-524
30. Klijn N, Weerkamp AH, de Vos WM (1991) Identification of mesophilic lactic acid bacteria by using polymerase chain reaction-amplified variable regions of 16S rRNA and specific DNA probes. Appl Environ Microb 57:3390-3393
31. Laukens D, Brinkman BM, Raes J, de Vos WM, Vandenabeele P (2016) Heterogeneity of the gut microbiome in mice: guidelines for optimizing experimental design. FEMS Microbiol Rev 40:117-132
32. Leisner JJ, Laursen BG, Prévost H, Drider D, Dalgaard P (2007) *Carnobacterium*: positive and negative effects in the environment and in foods. FEMS Microbiol Rev 31:592-613
33. Li X, Kot W, Wang D, Zheng S, Wang G, Hansen LH, Rensing C (2015) Draft Genome Sequence of Se(IV)-Reducing Bacterium *Pseudomonas migulae* ES3-33. Genome Announc 3:-e00406-15
34. López G, López-Parra M, Garrote G, Fernández L, del Rey-Wamba T, Arenas-Rojas R, García-Tardío M, Ruíz G, et al. (2014) Evaluating mortality rates and causalities in a critically endangered felid across its whole distribution range. Eur J Wild Res 60:359-366
35. Lu Q, Yuan H, Li J, Zhao Y, Zhou S (2015) *Ornithinibacillus composti* sp npv., isolated from sludge compost and emended description of the genus *Ornithinibacillus*. A Van Leeuw J Microb 107:813-819
36. Mages IS, Frodl R, Bernard KA, Funke G (2008) Identities of *Arthrobacter* spp. and *Arthrobacter*-like bacteria encountered in human clinical specimens. J Clin Microbiol 46:2980-2986
37. Martínez G, Shaw EM, Carrillo M, Zanuy S (1998) Protein salting-out mathod applied to genomic DNA isolation from fish whole blood. Biotechniques 24:138-139
38. Marzorati M, Wittebolle L, Boon N, Daffonchio D, Verstraete W (2008) How to get more out of molecular fingerprints: practical tools for microbial ecology. Environ Microbiol 10:1571-1581
39. Mayr R, Busse HJ, Worliczek HL, Ehling-Schulz M (2006) *Ornithinibacillus* gen nov, with the species *Ornithinibacillus bavariensis* sp nov and *Ornithinibacillus californiensis* sp nov. Int J Syst Evol Microbiol 56:1383-1389
40. Moeller AH, Degnan PH, Pusey AE, Wilson ML, Hahn BH, Ochman H (2012) Chimpanzees and humans harbour compositionally similar gut enterotypes. Nat Commun 3:-1179
41. Montagne L, Arturo-Schaan R, MelaLe-Floch N, Guerra L, Le Gall M (2010) Effect of sanitary conditions and dietary fibre on the adaptation of gut microbiota after weaning. Livest Sci 133:113-116
42. Muyzer G, de Waal EC, Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl Environ Microb 59:695-700
43. Muyzer G, Smalla K (1998) Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. A Van Leeuw J Microb 73:127-141
44. Nayak SK (2010) Probiotics and immunity: A fish perspective. Fish Shellfish Immunol 29:2-14

45. Peña L, García P, Jiménez MA, Benito A, Pérez Alenza MA, Sánchez B (2006) Histopathological and immunohistochemical findings in lymphoid tissues of the endangered Iberian lynx (*Lynx pardinus*). Comp Immunol Microb Infect Dis 29:114-126
46. Raman M, Ahmed I, Gillevet PM, Probert CS, Ratcliffe NM, Smith S, Greenwood R, Sikaroodi M, et al. (2013) Fecal microbiome and volatile compound metabolme in obese humans with nonalcoholic fatty liver disease. Clin Gastroenterol Hepatol 11:868-875
47. Rodríguez A, Calzada J. *Lynx pardinus*. The IUCN Red List of Threatened Species. IUCN - International Union for Conservation of Nature (2015) .
48. Sanguinetti CJ, Dias Neto E, Simpson AJ (1994) Rapid silver staining and recovery of PCR products separated on polyacrylamide gels. Biotechniques 17:914-921
49. Shahi N, Mallik SK (2014) Recovery of *Pseudomonas koreensis* from eye lesions in golden mahseer, *Tor putitora* (Hamilton, 1822) in Uttarakhand, India. J Fish Dis 37:497-500
50. Shannon AL, Attwood G, Hopcroft DH, Christeller JT (2001) Characterization of lactic acid bacteria in the larval midgut of the keratinophagous lepidopteran *Hofmannophila pseudospretella*. Lett Appl Microbiol 32:36-41
51. Suchodolski JS, Xenoulis PG, Paddock CG, Steiner JM, Jergens AE (2010) Molecular analysis of the bacterial microbiota in duodenal biopsies from dogs with idiopathic inflammatory bowel disease. Vet Microbiol 142:394-400
52. Suyal DC, Shukla A, Goel R (2014) Growth promotory potential of the cold adapted diazotroph *Pseudomonas migulae* S10724 against native green gram (*Vigna radiata* (L.) Wilczek). Biotechniques 4:665-668
53. van der Gast CJ, Jefferson B, Reid E, Robinson T, Bailey MJ, Judd ST, Thompson IP (2006) Bacterial diversity is determined by volume in membrane bioreactors. Environ Microbiol 8:1048-1055
54. Vargas A, Sánchez I, Martínez F, Rivas A, Godoy JA, Roldán E, Simón A, Serra R, et al. (2008) The Iberian lynx *Lynx pardinus* Conservation Breeding Program. Int Zoo 42:190-198
55. Verhille S, Baida N, Dabboussi F, Hamze M, Izard D, Leclerc H (1999) *Pseudomonas gessardii* sp nov and *Pseudomonas migulae* sp nov., two new species isolated from natural mineral waters. Int J Syst Evol Microbiol 49:1559-1572
56. Wang J, Linnenbrink M, Künzel S, Fernandes R, Nadeau MJ, Rosenstiel P, Baines JF (2014) Dietary history contributes to enterotype-like clustering and functional metagenomics content in the intestinal microbiome of wild mice. Proc Natl Acad Sci USA 111:2703-2710
57. Whon TW, Roh SW, Shin NR, Kim MS, Kim YO, Bae JW (2011) Genome sequence of strain TW25, a novel member of the genus *Ornithinibacillus* in the family *Bacillaceae*. J Bacteriol 193:2884-2885
58. Witebolle L, Vervaeren H, Verstraete W, Boon N (2008) Quantifying community dynamics of nitrifiers in functionally stable reactors. Appl Environ Microb 74:286-293
59. Wohlgemuth S, Keller S, Kertscher R, Stadion M, Haller D, Kisling S, Jahreis G, Blaut M, Loh G (2011) Intestinal steroid profiles and microbiota composition in colitic mice. Gut Microbes 2:159-166
60 Yukimura K, Nakai R, Kohshima S, Uetake J, Kanda H, Takeshi N (2009) Spore-forming halophilic bacteria isolated from Artci terrains: implications for long-range transportation of microorganisms. Polar Sci 3:163-169