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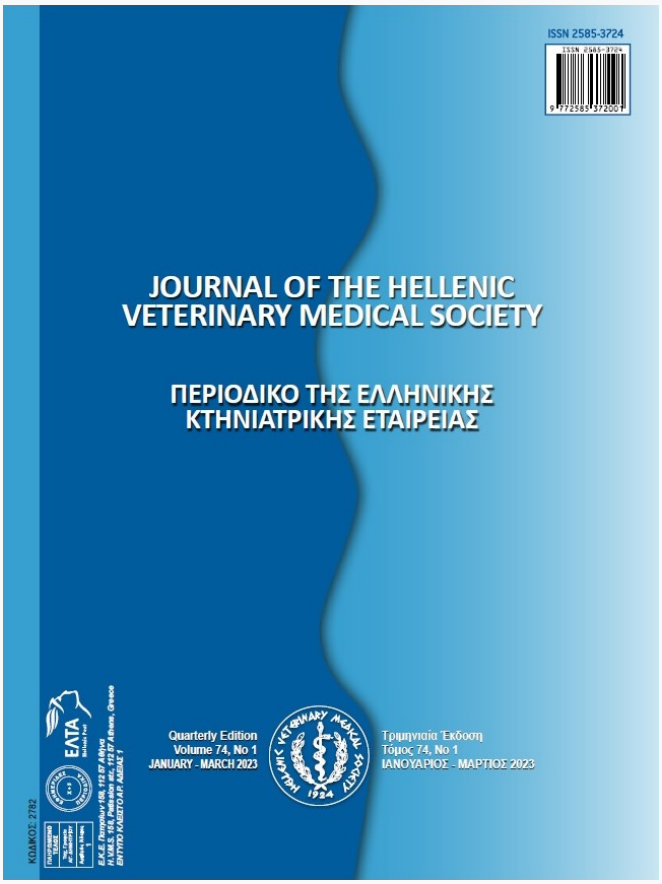

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Research article
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Annealing temperatures affect 16S rRNA gene-amplicon Illumina sequencing-based bacterial community analysis of canine skin

N. Apostolopoulos[1*], N. Thom[1], R. Bagwe[2], R.R. Müller[3], C. Ewers[4], P.S. Glaeser[2]

[1]*Department of Dermatology, Small Animal Clinic - Internal Medicine, Justus Liebig University, Giessen, Germany*

[2]*Institute for Applied Microbiology, Justus Liebig University Giessen, Giessen, Germany*

[3] *Bioinformatics and Systems Biology, Justus-Liebig-Universität, Giessen, Germany*

[4]*Institute for Hygiene and Infectious Diseases of Animals, Giessen, Germany*

ABSTRACT: Nowadays, 16 S rRNA-based analysis of the bacterial community structure of dog skin samples, is widely used. Among others, the 16S rRNA gene amplicon Illumina sequencing technique is well established and routinely applied to get a first insight in to the bacterial community diversity and taxonomic composition. However, as it is a molecular-based technique, bias due to methodology is possible and should be minimized. In this study, we tested the effects of annealing temperature (50°C vs 55°C) on the 16S rRNA gene amplicon analysis of the bacterial microbiota of skin and ear canal samples from a German shepherd dog.Although beta diversity was not affected by the higher annealing temperature, alpha diversity values showed a shift [overall diversity (Shannon) and evenness were increased, whereas dominance (D), number of taxa (S), richness (Chao 1) and the total numbers of individuals (N) were reduced, with higher annealing temperature]. The biological relevance of this finding remains unclear. Thus, our results underline the importance of optimal annealing temperature in order to minimize bias, as well as the necessity of further similar studies with a larger sample size.

Keywords: German shepherd dog, bias, microbiome, proportionality, Phi-coefficient

Corresponding Author:
Neoklis Apostolopoulos, Department of Dermatology, Small Animal Clinic - Internal Medicine, Justus Liebig University, Giessen, Germany
E-mail address: neo-vet@hotmail.com

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INTRODUCTION

Next-generation sequencing (NGS) studies are routinely used to investigate bacterial community structure, diversity, and taxonomy,overcoming limitations of culture-dependent studies.However,several factors can lead toa bias of results. Briefly, bias can be introduced for example due to primer mismatch, primer binding energy, 1:1 ratio mixture of genes,-chimera formation, and DNA extraction and storage (Kanagawa, 2003; Cardona et al., 2012; Rubin et al., 2013; Knight et al., 2018; Pollock et al., 2018).The annealing temperature is a very important factor for the PCR, affecting the primer binding specificity to the target sequence (Markoulatos et al., 2002), and choosing the correct annealing temperature is critical especially if mismatched primers are used (Ishii and Fukui, 2001). Few studies are reporting the effect of some of those bias factors on 16S rRNA gene-based analyses, reporting higher preferential amplification with increased annealing temperature (Hongoh et al., 2003; Sipos et al., 2007).In a recent16S rRNA gene amplicon-pyrosequencing-based study, using chicken caecal samples, it could be shown that annealing temperature had a minor effect in species richness and evenness as well as in microbial community structure(Sergeant et al., 2012). Nowadays, another 16S rRNA gene amplicon-based technique using the Illumina technology is well established and routinely used (Caporaso et al., 2011). Besides a study evaluating ifPCR product pooling and repeating experimental procedures (sample preparation) and template concentration affect the bacterial profile(Kennedy et al., 2014), there is a lack of studies evaluating if annealing temperature affects the 16S rRNA gene amplicon-based Illumina sequencing.Therefore, we selected DNA samples of our previously published dataset (Apostolopoulos et al., 2021)to evaluate the effect of higher annealing temperature on the analysis of the skin and ear canal microbiota of a German shepherd dog.

MATERIALS AND METHODS

Sampling

Four body sites [axilla (A), interdigital area (Int), groin (L), and ear canal (O)]of a healthy German shepherd dog (GSD) were sampled at the small animal clinic of the Justus-Liebig University Giessen in Germany. The owner of the dog was informed of the procedures and signed a consent form to participate in the study. The Animal Welfare Committee of the Justus-Liebig University of Giessen was informed about the study protocol and especially the sampling method was discussed. An ethical approval by the responsible authority was not required, because neither pain, harm nor damage was caused by gently rolling a cotton swab on skin. We used 70% ethylene oxide sterilized forensic swabs with transport tube, polystyrene stem material and viscose swab material (Forensic Swab, Nr 80.629, Sarstedt AG & Co. KG, Nümbrecht, Germany). The swabs were rubbed 40 times on the target body site, rotating one-quarter of the swab´s site for 10 times. Subsequently, the swabs were immediately transported without transport medium at 8°C to the Institute of Applied Microbiology, University Giessen, for storage (at -20°C) and further processing.

DNA extraction and 16S rRNA gene amplicon sequencing

DNA was extracted using the NucleoSpin® 96 Soil kit (96-well extraction system, Macherey Nagel AG, Oesingen, Switzerland), as previously described (Apostolopoulos et al., 2021). DNA was quantified spectrophotometrically using a NanoDrop spectrophotometer (Thermo Scientific, Germering, Germany).The 16S rRNA gene sequences of Bacteria were amplified for Illumina amplicon sequencing (LGC Genomics, Berlin, Germany) using a nested PCR approach. This method was used due to the results from previous studies, where only a low amount of microbial DNA was detected on human skin analysed by a single PCR approach (Zeeuwen et al., 2012; Li et al., 2014).The PCRs included 1-10 ng extracted DNA (total volume 1µl), 15 pmol of each forward primer and reverse primer in 20 µL volume of 1 x MyTaq buffer containing 1.5 units MyTaq DNA polymerase (Bioline, Luckenwalde, Germany), 2 µl of BioStabII PCR Enhancer (Sigma-Aldrich, St. Louis, United States of America) and 0.2 µl DNase (Arctic Zymes, Tromsø, Norway). For each sample, the forward and reverse primers had the same 10-nt barcode sequence. The first PCR (20 cycles) with the primer system 341F(5′-CCTACGGGAGGCAGCAG-3′) and1061R(5′-CRRCACGAGCTGACGAC-3′) (variable regions (V): V3-V6)(Ong et al., 2013),had the following conditions: 2 min 96°C pre-denaturation; 96°C for 15 seconds (s), 50°C for 30 s, 70°C for 90 s. For the nested PCR,1 µl PCR product of the first round was used with PCR primer system 515F(5′-GTGYCAGCMGCCGCGGTAA-3′)-Y and 926R(5′-CCGYCAATTYMTTTRAGTTT-3′)-jed (V4-V5) (Parada et al., 2016), whereas the conditions were

otherwise the same as before. The same procedure was repeated for target DNA from the same samples(with the WH abbreviation at the end of the sample name) using an annealing temperature of 55°C. Gel electrophoresis determined the DNA concentration of amplicons of interest. About 20 ng amplicon DNA of each sample was pooled carrying different barcodes. The amplicon pool was purified with one volume Agencourt AMPure XP beads to remove primer dimer and other small mispriming products, followed by an additional purification on MiniElute columns (Qiagen, Hilden, Germany). About 100 ng of the purified amplicon pool DNA was used to construct Illumina libraries using the Ovation Rapid DR Multiplex System 1-96 (NuGEN, San Carlos, United States of America). Illumina libraries were pooled and size selected by preparative gel electrophoresis. High-throughput 2 x 300 bppaired-end 16S rRNA gene amplicon sequencing was performed on Illumina MiSeq using V3 Chemistry (Illumina, San Diego, United States of America).

Data analysis

The NGS analysis pipeline (www.arb-silva.de/ngs) of the SILVA rRNA gene database (SILVAngs 1.3) (Quast et al., 2013), was used for sequence analysis as previously described (Apostolopoulos et al., 2021). This process resulted in quantitative information (number of individual reads per taxonomic path), despite the PCR limitations, possible sequencing technique biases and multiple rRNA operons. Reads without any BLAST hits or reads with weak BLAST hits, where the function "(% sequence identity + % alignment coverage)/2" did not exceed the value of 93, remained unclassified and were assigned in the virtual taxonomical group "no relative" in the taxonomic fingerprint and Krona charts (Ondov et al., 2011), as previously reported (Ionescu et al., 2012; Klindworth et al., 2013). Taxonomic fingerprint and Krona charts were provided by SILVAngs(Ondov et al., 2011; Quast et al., 2013). The program PAST 4.04 (www.nhm.uio.no/english/research/infrastructure/past) was used to perform alpha and beta diversity analysis(Oyvind Hammer et al., 2001). The analysis was performed at the genus level. Alphadiversity was measured by calculating the following diversity indices: Shannon (H; overall α-diversity), Chao 1 (richness; number of taxa corrected by the presence of singleton), dominance (D), evenness (Buzas and Gibson´s evenness: exp(H)/S), number of taxa (S), total numbers of individuals (N)(Harper, 1999)and rarefaction curve analyses considering the number of taxonomic paths and the number of reads per taxonomic path to evaluate richness in different sample sizes (Krebs, 1989). Boxplots and paired line plots of the alphadiversity indices were further evaluated in SigmaPlot 13 (Systat Software, Inc., San Jose, United States of America). The phylogenetic composition of the bacterial communities was studied based on relative abundance data analysed by non-metric multidimensional scaling (NMDS;(Taguchi and Oono, 2005)) using Bray-Curtis similarity matrix (Bray and Curtis, 1957). Due to the small sample size, significance was not tested. The Phi coefficient described by Lovell et al (Lovell et al., 2015) was applied to all relative abundances of all taxa in order to evaluate proportionality between taxa among the different annealing temperatures. This method requiresnon-zero values. Taxa with zero reads in all samples were removed because they are uninformative, and do not contribute to the differentially abundant Taxa. The zero values that only occurred in single samples were treated in two different ways. In the first approach, these zero values were randomly (and without duplicates) replaced by randomly non-duplicate generated values between 10^(−8) and 2.10^(−8), similarly to another study (Gorvitovskaia et al., 2016). Those values were then used on PAST program and phi coefficients were calculated. The second approach treated the remaining zero values in a Bayesian context (Friedman and Alm, 2012; Fernandes et al., 2013) using the ALDEx2 R package (Fernandes et al., 2013; Fernandes et al., 2014). Phi coefficient values were finally calculated with those data using the ALDEX2 packages as described by Gloor et al. (2016)(Gloor and Reid, 2016).Regarding interpretation of proportionality, the closest to zero the phi values are, the stronger is the proportionality (Lovell et al., 2015). The cut-off phi value for very strong proportionality was <0.01(Gorvitovskaia et al., 2016).

RESULTS

Analysis of the phylogenetic composition of the skin microbiota

Overall, 240,113 paired-end sequences with an average sequence length of 373 nucleotides (nt) were obtained. In total 240,018 sequences were further analysed, because 95 sequences did not pass the SILVAngs pipeline quality control and were thus removed. 12,638 unique reads (5.26% of the finally analysed sequences) were assigned to operational taxonomic units (OTUs). Furthermore, 72,919 (30.37%)

sequences (reads with 98% identical sequence to each other; defined as "clustered") and 154,461 (64.33%) sequences (reads100% identical to each other; defined as"replicates") were assigned to OTUs. OTUs were classified in the SILVA database with taxonomic paths with maximum resolution at the genus level (phylogenetic groups for unnamed genera)(Supplementary file; Table 1).Sequences corresponding to *Archaea* (n=58), mitochondria (n=6,193), and chloroplasts (n=27), were not further evaluated. Additionally, sequences that did not match any of the known taxa (sequence similarity <93% to the next known taxon) were classified as "no relative"(n=73 sequences) and were also not considered any further. Finally, 233,667 sequences (assigned to the domain *Bacteria*) were included in further analyses and were set to 100%.

Effect of higher annealing temperature on the bacterial community composition (beta diversity)

Bacterial community composition was visualized in a non-metric multidimensional scaling (NMDS) plot (Figure1a). NMDS plots did not show prominent differences in the bacterial community profiles obtained by the use of two different annealing temperatures, 50°C and 55°C.All phi values of the "zero replaced" data (Supplementary file; Table 2)were >0.01 (Supplementary file; Table 3).Similarly, all phi values using the ALDEx2 package were all >0.01 (Supplementary file; Table 4). Therefore, no strong proportionality among taxa due to higher annealing temperature was documented.

SILVAngs identified 10main phyla (Figure1b; cluster number 1) belonging to the domain *Bacteria*. Based on taxonomic fingerprint (Figure1b) Actinobacteria and Proteobacteria were the two most dominant phyla, and a higher annealing temperature resulted in lower OTUs per path (smaller size of the symbol; Figure1b).Gaining a deeper insight into those two phyla, minor changes in the composition at the class level were revealed (Supplementary Figure).

Effect of higher annealing temperature on the alphadiversity in dog skin microbiota

Shifts in alpha diversity were documented. An increase in the Shannon index values and evenness of the bacterial community were determined at the higher (55°C) annealing temperature (Figure 2a, Supplementary file; Table 5). This finding was supported by respective rarefaction curves showing that the 55°C

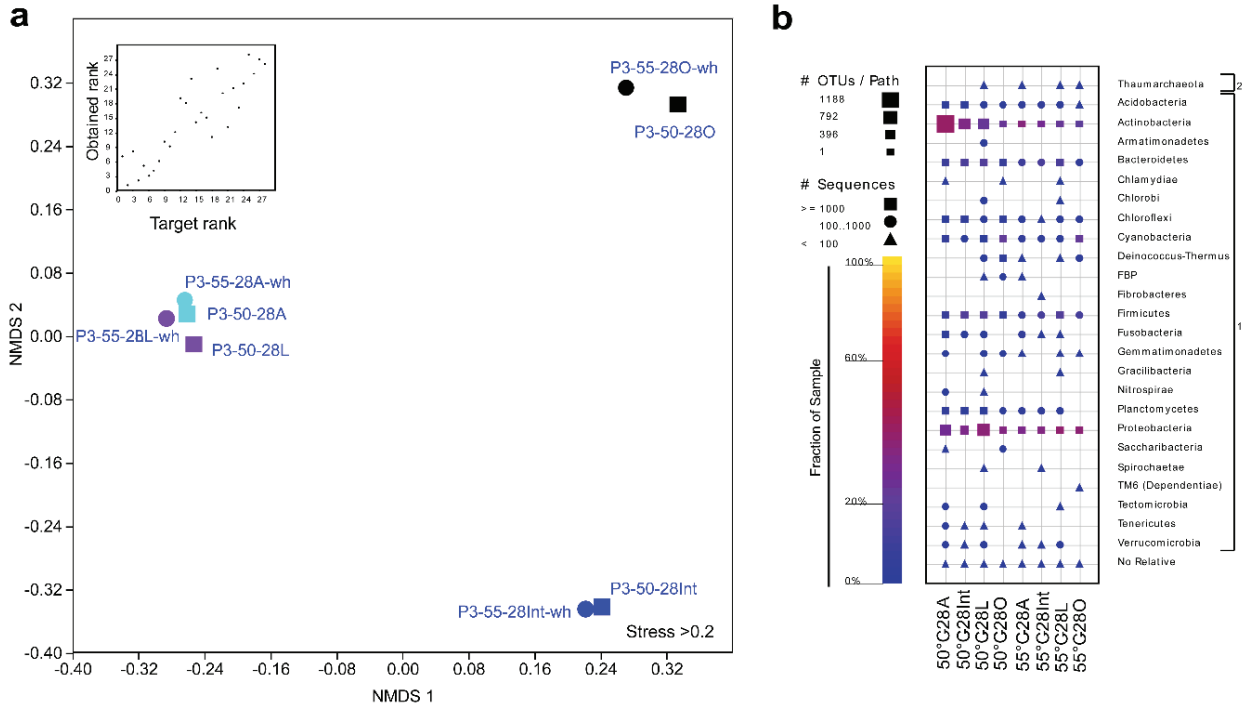

Figure 1. a) Comparative analysis of the skin microbiota composition of relative abundance patterns at each annealing temperature (square: 50°C vs circle: 55°C) for each body site (Axilla; A, Interdigital area; I, Groin; L, Ear canal; O: each site has a different color), performed by NMDS analysis based on a Bray-Curtis similarity matrix. b) Taxonomic Fingerprint (operational taxonomic units (OTUs) per path, sequences and % fraction of sample) at phylum level, obtained from the SILVAngs pipeline, for four body sites (Axilla; A, Interdigital area; I, Groin; L, Ear canal; O) using two different annealing temperatures (50°C vs 55°C).

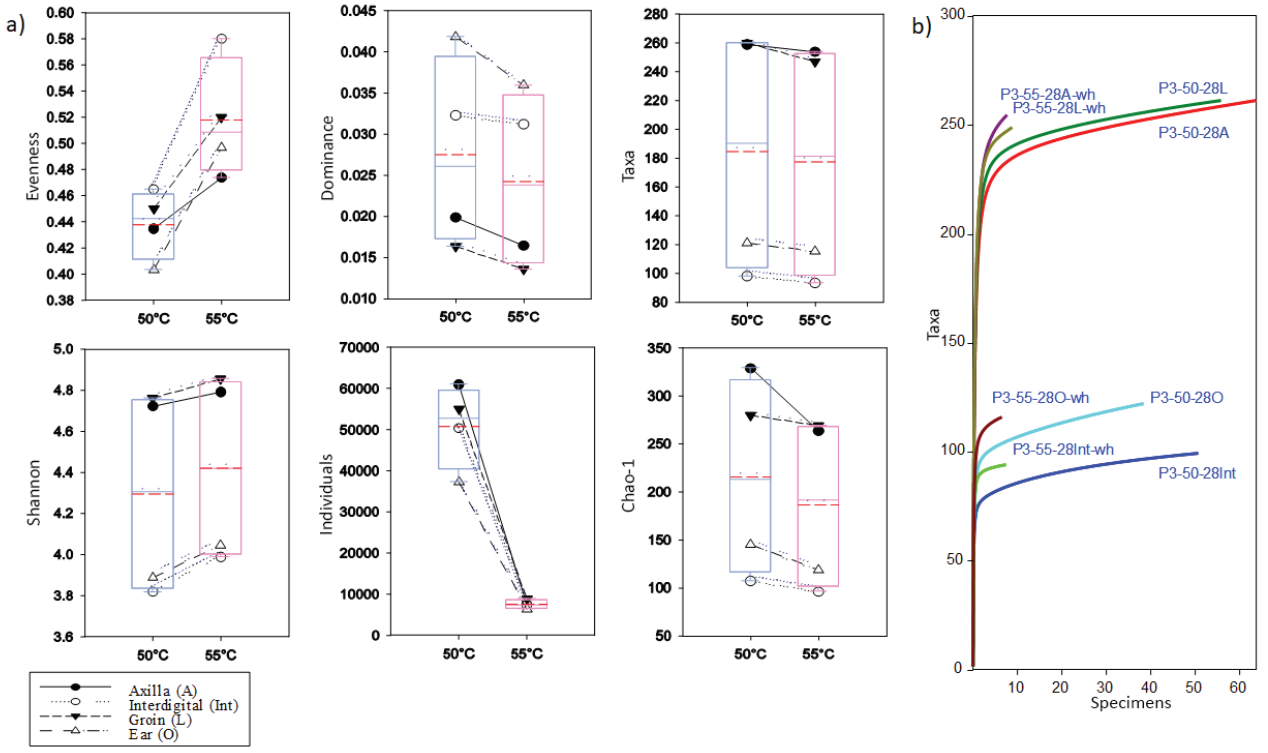

Figure 2. a) Box-plots of alpha diversity indices (taxa (S), individuals (N), dominance (D), Shannon (H), evenness (exp(H)/S), Chao 1) and paired line plots of all body sites (Axilla; A, Interdigital area; I, Groin; L, Ear canal; O) at two different annealing temperatures (50°C vs 55°C), based on 16S rRNA gene amplicon Illumina sequencing of microbial communities. Each line corresponds to a body site. b) Rarefaction curves (specimens versus taxa) based on Illumina 16S rRNA gene amplicon sequencing of microbial communities from four body sites (Axilla; A, Interdigital area; I, Groin; L, Ear canal; O) using two different annealing temperatures (50°C vs 55°C).

annealing temperature samples had a higher slope than the curves from the 50°C in a body site-sample specific manner, indicating the presence of more complex bacterial communities when the annealing temperature is higher (Figure 2b). In contrast, dominance, number of taxa (S), richness (Chao 1) and the total numbers of individuals (N) were reduced with higher annealing temperature (Figure 2a, Supplementary file; Table 5).

DISCUSSION

NMDS plot suggests that increased annealing temperature (55°C) had only a low effect on the bacterial community composition. This is in contrast with another study that showed a minor effect on Principal Component Analysis (PCA) analysis of the bacterial community structure in a 16S rRNA gene amplicon pyrosequencing-based study, using chicken caecal samples (Sergeant et al., 2012). Differences in sample type and preparation, including DNA extraction, or sequencing techniques could be a possible explanation for this disagreement. In this study, non-invasive swabs from different body sites were used, whereas Sergeant et al. (2012) used caecal tissue samples from euthanised chickens in their study(Sergeant et al., 2012). It is well accepted that even at different sites of the gastrointestinal tract the bacterial community composition can vary (Pollock et al., 2018). Furthermore, significant differences have been obtained in microbial composition comparing non-invasive sampling techniques (for example swabs, as used in this study) versus invasive biopsy sampling methodology (Pollock et al., 2018). Also the applied method for DNA extraction can have an effect on microbial community analysis, as different cells or different samples may vary in their lysis properties and in the presence of inhibitors, respectively(Pollock et al., 2018).In this study, NucleoSpin® 96 Soil kit (Macherey Nagel AG) was used for all samples, while in the study by Sergeant et al(Sergeant et al., 2012),the QIAamp DNA Stool MiniKit (Qiagen) was utilized, which might be a possible explanation for the differences between the studies.

One of the most striking results of our study is that the higher annealing temperature (55°C) influences some diversity indices, increasing overall diversity (Shannon) and evenness of the bacterial community.

In previous studies, annealing temperature (Sergeant et al. (2012): 30°C vs 35°C vs40°C vs 45°C vs 50°C vs 55°C, amplifying a standard-length primer pair, F20/R19,and Gohl et al. (2016): 50°C vs 55°C amplifying the V4 region) had a minimal effect on alpha diversity (Sergeant et al., 2012; Gohl et al., 2016) of the amplicon-based data analysis. According to the literature, the lowest possible annealing temperature should be determined experimentally and used to reduce primer bias (Gołębiewski and Tretyn, 2020). Our study indicated a shift on the alpha diversity of the canine skin microbiota. Our data are not feasible to indicate if this shift plays an important role (and its effect-magnitude) when analysing the bacterial microbiota, using a 16S rRNA gene amplicon-based Illumina sequencing technique.

One major limitation of this study due to its nature is the small sample size. Therefore, the results cannot be generalised. However, an important insight into the effect of higher annealing temperature is gained, to design further research. Systematic studies comparing annealing temperatures with more samples as well as mock samples and different master mixes should be applied to evaluate our results and produce standardized protocols.

CONCLUSIONS

Our study suggests that higher annealing temperature can affect amplicon-based microbial data analysis in terms of alpha diversity. The bacterial community composition probably will be not affected. Our findings should be interpreted with caution due to the small sample size and should be evaluated by future studies. Nevertheless, our results provide valuable information for future investigations to reduce bias of microbiota analysis, given the lack of a gold-standard protocol.

CONFLICT OF INTEREST

The authors declare no conflicts of interest relative to this work.

SUPPORTING INFORMATION

Supplementary file S1; Tables 1 - 5

Supplementary Figure: Overview of the composition and relative abundance (%) at the class level for the phyla Actinobacteria and Proteobacteria of all samples (axilla, interdigital skin, groin, ear canal) at two different annealing temperatures (50°C vs 55°C), based on Krona charts obtained from SILVAngs.

The code used in this study for the ALDEx2 R package, as elsewhere described (Friedman and Alm, 2012; Fernandes et al., 2013; Gloor and Reid, 2016), and our analysis: https://doi.org/10.5281/zenodo.4461368

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