Community Composition of Bacteria Isolated from Swiss Banknotes Varies Depending on Collection Environment

Running title: Variable Monetary Microbiome in Switzerland

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Abstract:

Humans interact constantly with surfaces and associated microbial communities in the environment. The factors shaping the composition of these communities are poorly understood: some proposed explanations emphasize the influence of local habitat conditions (niche-based explanations), while others point to geographic structure and the distance among sampled locations (dispersal-based explanations). However, the relative roles of these different drivers for microbial community assembly on human-associated surfaces are not clear. Here, we used a combination of sampling, sequencing (16S rRNA) and culturing to show that the composition of banknote-associated bacterial communities varies depending on the local collection environment. Using banknotes collected from various locations and types of shops across Switzerland, we found taxonomic diversity dominated by families such as *Pseudomonadaceae*, *Staphylococcaceae* and *Streptococcaceae*, but with banknote samples from particular types of shops (especially butcher shops) having distinct community structure. By contrast, we found no evidence of geographic structure: similarity of community composition did not decrease with increasing distance among sampled locations. These results show that microbial communities associated with banknotes, one of the most commonly encountered and exchanged human-associated surfaces, can reflect the local environmental conditions (in this case, the type of shop), and the signal for this type of variation was stronger than that for geographic structure among the locations sampled here.

Keywords:

microbiome, banknote, community assembly, 16S rRNA

Introduction

Humans encounter microbial communities constantly and we now know that they contribute, among other things, to food and drink quality (Wareing, 2016; Reese *et al.*, 2020), soil fertility (Fierer, 2017) and human health (Libertucci and Young, 2019). Understanding variation of microbial community structure (relative abundances of different taxa) is therefore an important challenge. As for other organisms and in the wider field of community ecology, the contributions of different drivers of microbisl community assembly remain unclear (Chesson, 2000; Ebach, 2015). Selection imposed by local habitat conditions and biotic interactions (niche-based explanations invoking deterministic factors) (Macarthur and Levins, 1967; Diamond, 1975) is expected to result in communities matched to their local environments. Other possible drivers include more stochastic processes, ecological drift or dispersal limitation (Hubbell, 2011), which may result in geographic structuring of communities independent from the effects of local habitat conditions. There is evidence from microbes and other taxa supporting both types of explanation (Webb; Finlay, 2002; Fargione *et al.*, 2003; Whitaker *et al.*, 2003a; Horner-Devine *et al.*, 2004; Zhou *et al.*, 2013), and it is increasingly recognised that multiple drivers are in play (Chase, 2003; Chase and Myers, 2011; HilleRisLambers *et al.*, 2012). However, we still lack a clear picture of their relative roles (Chase, 2010; Hanson *et al.*, 2012; Stegen *et al.*, 2015; Zhou and Ning, 2017). This problem is especially acute for microbial communities on surfaces that humans interact with in daily life. For example, a recent global study of microbial communities on metro and transit systems identified city-specific community composition linked to local conditions, albeit with a shared core microbiota across locations (Danko *et al.* 2021). However, it is not yet clear whether such biogeographical structuring is also evident on other human-associated surfaces, or how strongly such microbiomes reflect local habitat conditions (selection) as opposed to other predictors such as geographic distance among sites.

One of the most important human-associated surfaces is cash money: few everyday objects are as frequently, widely and actively exchanged among humans as banknotes. Paper-based money has been in use for hundreds of years and is now commonplace around the world. Despite the advent of credit cards and other cashless payment instruments, banknotes and coins are still the most common payment option for purchases at the physical point of sale and for person-to-person payments in the EU and Switzerland (Gehring *et al.*; Central Bank, 2020). The rough surface of most banknotes lends itself well to the attachment of microorganisms (Jalali *et al.*, 2015; Vriesekoop *et al.*, 2016), something first investigated in the late 19th century (Schaarschmidt, 1884). Since then, tools such as metagenomic sequencing have enabled more detailed description of microorganisms on money, including characterization of communities on dollar bills in New York City (Maritz *et al.*, 2017), identification of pathogens and antibiotic resistance genes on Rupees in New Delhi (Jalali *et al.*, 2015), and monitoring survival of influenza viruses on banknotes (Thomas *et al.*, 2008). However, to our knowledge it remains poorly understood whether banknotes collected in different places harbour microbial communities that reflect their local environment, which would indicate a role for selection (Hanson *et al.*, 2012; Zhou and Ning, 2017), and/or show biogeographic structuring (e.g., increasing dissimilarity with geographic distance).

Here, we aimed to quantify variation of the bacterial microbiome associated with banknotes originating from different locations across a country, and to test whether this variation can be explained by geographic distances among locations, or other characteristics of the local sampling environments. We did this by collecting banknotes from each of ten locations in Switzerland (Fig. 1A), visiting five different types of shops in each location. We hypothesized community composition would vary among banknotes collected from different types of shops (if there is an effect of local habitat selection on the monetary microbiome), and/or that banknotes collected from more distant locations would harbour more dissimilar communities

(if there is biogeographic structuring in general). We studied the samples isolated from the 50 banknotes by (1) 16S rRNA sequencing to determine community composition, including viable and non-viable, culturable and non-culturable bacteria, and (2) plating on chromogenic agar to detect viable, culturable bacteria. Our results show that the composition of bacterial communities isolated from banknotes – determined by both sequencing and by plating – varies depending on the local environment (shop type) in which banknotes are collected. By contrast, we find no evidence for geographic structuring of bacterial community composition across banknotes sampled in Switzerland.

Materials and Methods

Sample Collection

We collected banknotes from ten locations in Switzerland. In choosing locations, we adhered to several criteria: (1) countrywide distribution of sampled locations (Fig. 1A; (2) fewer than 10'000 inhabitants per location (because we expected any signal for geographic structuring would be stronger among smaller towns); (3) all of the targeted shop types (bakery, butcher, florist, kiosk/newsagent, and pharmacy) present at each location; (4) no significant commuter traffic between locations (according to *Erwerbstätige nach Wohn- und Arbeitsgemeinde, 2014 und 2018* by the Federal Office of Statistics; again we expected geographic structuring of microbial communities would be stronger among less connected towns). The chosen locations were (Fig. 1A): Biasca (BIA), Brienz BE (BRI), Frick (FRI), Klosters-Serneus (KLO), Langnau am Albis (LAN), Orbe (ORB), Porrentruy (POR), Rorschach (ROR), Stein am Rhein (STE), Visp (VIS). We chose the five shop types in an attempt to represent different environments, products and clientele. More details on locations and shops can be found in supplementary files SuppFile_01 and SuppFile_02, respectivley.

106 On arrival in a location, we collected the banknotes from the different shops as follows:
107 donning sterile gloves before entering the shop; choosing an item for less than CHF 10; paying
108 the item with a CHF 20 banknote; folding the CHF 10 banknote received as change in half
109 twice and collecting it in a sterile Nasco Whirl-Pak® sample bag (product nr. 11701973; Fisher
110 Scientific AG, Reinach, Switzerland); storing the sample bag in a paper envelope. Once we
111 had the five banknotes of a given location, we transported the banknotes back to the laboratory
112 and did the initial processing as described below. All samples were collected by the same
113 person between March 12th and March 26th, 2021. Samples from different locations were
114 collected on different days. One sample (butcher-BIA) was classified as "butcher" even though
115 it came from a supermarket with a served meat counter (both butchers in Biasca (BIA) were
116 closed on collection day); this is discussed further below. We contacted all shop owners via
117 email to inform them about our project and gave them the option to have the banknote collected
118 in their shop removed from the study. No shop owner vetoed inclusion of the banknote
119 collected in their shop in the study.
120
121 **Initial Sample Processing and Plating**
122 We performed initial processing for all banknotes on the day of collection as follows: after
123 removing the banknote from the sample bag and placing it on a sterile surface, we swabbed the
124 front of the banknote (not exposed to the bag surface) along the width from top to bottom
125 (Maritz *et al.*, 2017), using a sterile cotton swab (product nr. 80.628; Sarstedt, Nürnberg,
126 Germany) moistened with phosphate-buffered saline (PBS; product nr. P4417-100TAB;
127 Sigma-Aldrich, Merck KgaA, Germany). Turning the banknote 90 degrees, we repeated the
128 procedure. With the same swab, we swabbed the back of the banknote according to the same
129 protocol. We cut the tip of the swab with sterile cissors and placed it in an 2mL Eppendorf tube
130 filled with 950µL PBS. We repeated the procedure with the other four banknotes, sterilizing

the surface between samples. As a negative control, we swabbed the interior of a sample bag and followed the protocol just described. We vortexed all Eppendorf tubes for 5min at the highest amplitude and removed the swab tips with sterile tweezers, squeezing out the remaining liquid.

To screen for viable and culturable cells, we plated a 50µL aliquot of each sample on a separate Chromatic™ MH agar plate (product nr. LF-611618; Apteq AG, Cham, Switzerland). Chromatic™ MH agar is a chromogenic agar that allows for the identification of bacteria from environmental and clinical isolates by differential colouring of colonies from different species. We incubated plates at 37°C for 24h and stored the remaining samples at -20°C until further processing as described below. Note that plating on chromogenic agar provides a relatively coarse-grained identification and only for certain taxa, but we employed it here only to test (1) for the presence of viable and culturable (i.e., colony-forming) cells and (2) whether the putative species identities of resulting colonies were consistent with taxa identified by sequence analysis. More details on colony morphology and colour can be found in supplementary file SuppFile_03.

Genetic Analysis and Bioinformatics

We used the DNeasy® PowerLyzer® PowerSoil® Kit (product nr. 12855-100; Qiagen, Hilden, Germany) to extract bacterial DNA from thawed samples, according to the manufacturer's protocol with slight adaptations. In brief: For each sample, we added 700µL to a PowerBead tube (positive control: 700µL PBS plus *Escherichia coli* colony from agar plate; negative control: from empty sample bag, see above) and centrifuged all PowerBead tubes at 13'000rpm for 10min at 4°C. Discarding the supernatant, we dissolved the cell pellet by adding 750µL PowerBead solution and 60µL Solution C1, and inverting the tubes 10 times. After incubating the tubes at 65°C for 10min followed by incubation at 95°C for 10min, we performed bead

beating with a Bead Ruptor 24 (Omni International, Inc, Kennesaw (Georgia), United States of America (USA)) with the following settings: cycle nr = 2, strength = 5.5, time = 45s, interval = 0. After transferring the supernatant to clean 2mL collection tubes, we added 250µL Solution C2, vortexed the tubes for 5s at the highest amplitude and incubated them at 4°C for 5min. After centrifugation at 13'000rpm for 1min at 4°C, we transferred 600µL supernatant into clean 2mL collection tubes and added 200µL Solution C3. We vortexed tubes briefly at the highest amplitude and incubated them at 4°C for 5min, followed by centrifugation at 13'000 rpm for 1min at 4°C. Avoiding the pellet, we transferred 750µL supernatant into clean 2mL collection tubes, added 1200µL well-mixed Solution C4 and vortexed tubes for 5s at the highest amplitude. We loaded 675µL sample onto MB spin Columns, centrifuged them at 13'000rpm for 1min at 4°C and discarded the flow-through. We repeated this until the entire sample was processed. After adding 500µL Solution C5, we centrifuged samples at 13'000rpm for 1min at 4°C and discarded the flow-through, followed by centrifugation at 13'000rpm at 4°C for 30s. We placed the MB Spin Columns into clean 2mL collection tubes and added 70µL Solution C6 to the centre of the filter membrane. Following incubation at 65°C for 5min and centrifugation at 13'000rpm at 4°C for 1min, we discarded the MB Spin Columns and stored samples at -20°C. The 50 samples were processed in three batches (consisting of randomly selected samples) on different days, with a positive and negative control for each batch. After sequencing, we did not detect an effect of extraction batch on either read counts per sample (ANOVA: $F_{2,47} = 2.521$, $p = 0.091$) or community composition (PERMANOVA: $F_{2,41} = 1.711$, $p > 0.1$).

We quantified the obtained DNA using the Quant-iT^{TM} dsDNA BR Assay Kit (product nr. Q33130; Thermo Fisher Scientific, Waltham, USA) in the Spark® 10M multimode microplate reader (Tecan Group Ltd, Männedorf, Switzerland). We sequenced at the Genetic Diversity Centre, Zurich, Switzerland, using the MiSeq Reagent kit v3 (600 cycle PE) (Illumina, San

Diego, USA) after library preparation with the Nextera XT 96 Index kit v2, Set D (Illumina, San Diego, USA). The sequences of the four sets of primers used during library preparation (limited cycle PCR) are listed in supplementary table 1.

If not noted otherwise, raw sequence data were processed with USEARCH (v11.0.667_i86linux64; Edgar (2010)). After end trimming reads (if needed) and merging them into amplicons (commands: *filter_phix*, *filter_lowc* (threshold 25), *fastx_truncate* (trim R1/R2: 25/50), *fastx_syncpairs*, *fastq_mergepairs* (min. overlap: 30; min. identity: 60%; min. merged length: 100; min. merged quality: 8)), we trimmed reads for primers (command: *search_pcr* (amplicon size range 100-600; number of mismatches: 2; coverage: full-length (no end gaps)). We used PRINSEQ-lite 0.20.4 to check quality and size filter (parameters: size range: 150-550; GC range: 30-70; min. Q mean: 20; number of Ns: 1; low complexity: dust / 30). We clustered operational taxonomic units (OTUs) at 97% using the UPARSE-OTU algorithm (number of OTUs: 2378), and used UNOISE3 algorithm to perform denoising (error-correction) of amplicon sequence variants (zero-radius OTUs, number of ZOTUs: 4464), and to cluster ZOTUs at different identity levels (number of ZOTUs 99%: 3148; number of ZOTUs 98%: 2513; number of ZOTUs 97%: 2039). OTU annotation was carried out using SINTAX (reference: SILVA_128_16S_utax_work.fa; tax filter: 0.75).

After removing positive and negative controls and mitochondrial and chloroplast sequences, and before filtering, denoised sequencing data (ZOTUs) consisted of 50 samples with 14’900’848 raw reads in total, ranging from four to 6’752’341 raw reads per sample (mean number of raw reads: 266’086.57; median number of raw reads 158’243). We filtered data by removing samples with low number of raw reads (<1000) and ZOTUs with total abundance lower than 0.1% of total depth, ending up with 42 samples with 10’136’186 filtered reads in total, ranging from 138 filtered reads per sample for butcher-BRI to 6’167’513 filtered reads per sample for florist-BRI (mean number of filtered reads = 241’337.76; median number of

206 filtered reads = 88'192; Fig. S1). We had at least three samples per location and at least six
207 samples per shop type.

208 The sequencing data have been deposited in the European Nucleotide Archive under the
209 study accession number PRJEB45390 (https://www.ebi.ac.uk/ena; data will be made
210 accessible upon publication of the study).

211

212 **Statistics**

213 We used denoised sequences, i.e. ZOTUs, and conducted all statistical analysis in R (version
214 4.1.2).

215 To quantify within-sample diversity, we used the alpha() function in the microbiome
216 package (version 1.16.0) on filtered data to calculate several different diversity indices
217 (Shannon diversity index; Gini-Simpson index; observed richness; Chao1 index). The Shannon
218 diversity index *H* takes into account both richness and evenness; the Gini-Simpson index gives
219 the probabilty that two random draws from the same sample (with replacement) are not from
220 the same type (here, the same family); the observed richness counts the number of unique
221 species (ZOTUs) present in the sample; Chao1 index is a non-parametric estimator of species
222 richness that assumes Poisson distribution of the data (Chao, 1984; Chao and Bunge, 2002).
223 We used analysis of variance (ANOVA) to test whether within-sample diversity varied among
224 locations or shop types (aov() function in the stats package (R version 4.1.3)).

225 Before analysing between-sample diversity and to account for different library sizes (Weiss
226 *et al.*, 2017), we normalized filtered data with a regularized-logarithm (rlog() function in the
227 DESeq2 package (version 1.34.0)), after comparing the two available transformations offered
228 in DESeq2 package, regularized-logarithm transformation and variance-stabilizing
229 transformation. We chose the regularized-logarithm transformation because it (1) is

recommended for smaller datasets and for cases with large ranges of sequencing depths across samples (Love *et al.*, 2014); (2) it performed better in the meanSdPlot generated.

As a first test of whether samples from different locations and shop types differed in terms of bacterial community structure, we used permutational multivariate analysis of variance (PERMANOVA; Anderson, 2001; Zapala and Schork, 2006) with the adonis2() function in the vegan package (version 2.5-7), with filtered-and-normalized data and using Bray-Curtis dissimilarity (vegdist() function) to measure differences among sampled communities. Next, to visualise any clustering among samples in terms of community composition, we performed ordination of filtered-and-normalized data with the ordinate() function in the phyloseq package (version 1.38.0), with non-parametric multidmensional scaling (*method* = "NMDS") and Bray-Curtis dissimilarity (*distance* = "bray") as input parameters. We used NMDS as the ordination method because we obtained a stress value of 0.11 for our data with NMDS ordination; stress values <0.2 generally indicate good fit (Clarke, 1993). We visualised ordination results with the plot_ordination() function in the phyloseq package (version 1.38.0). We used a third type of analysis as an additional test for geographic structuring of microbial community composition, by conducting a Mantel test. This allowed us to account explicitly for the physical distance between individual sampling locations (shops), rather than testing for average differences among locations (as above in PERMANOVA). We did this using the mantel.test() function in the ape package (version 5.5), with the vegdist() function in the vegan package (version 2.5-7) for Bray-Curtis dissimilarity of filtered-and-normalized data. We used the geodist() function in the geodist package (version 0.0.7) to calculate geographical distance (in metres) between individual sampling locations. We used the same functions when repeating the analysis for sample sets from individual shop types; here and elsewhere, we accounted for multiple testing using the Holm–Bonferroni method (sequential Bonferroni).

The core microbiome is defined as the species present above a certain threshold and shared among a set of samples (Salonen *et al.*, 2012; Shade and Handelsman, 2012). We determined the core microbiome, based on ZOTUs, for each shop type as follows: Using filtered-and-normalized relative abundance data (transform() function in the microbiome package (version 1.16.0; "compositional" as input for *transform* argument) and subsetting data by shop type, we applied the core_members() function in the microbiome package (version 1.16.0; *detection* = 0.001 and *prevalence* = 0.5) to filter out core ZOTUs for each shop type. Then, as a fourth analysis of how microbiome structure varies among shop types/locations, we drew a Venn diagram with the venn() function in the eulerr package (version 6.1.0) and determined ZOTU identity with the format_to_besthit() function in the microbiomeutilities package (version 1.00.16), followed by identification of shared ZOTUs with reduce() and intersect() functions (base package, R version 4.1.2).

To test for an association between the abundance of viable-and-culturable bacteria (presence/absence of colonies and number of colonies per plate, after plating samples on chromatic agar) and shop type and location we conducted hurdle regression for count data via maximum likelihood (hurdle() function in pscl package (version 1.5.5), with formula *number.colonies ~ location* or *number.colonies ~ shop.type* and negative binomial regression (with log link) as count model). As our data contained several samples with zero viable cells we adjusted the count table with a dummy variable (dummy species present at count 1 in all samples; Clarke *et al.*, 2006) to perform further analysis, being aware that we cannot be sure that all empty samples had zero viable cells for the same reason. We used the adjusted count table to perform PERMANOVA as described above.

The map of Switzerland with locations (Fig. 1A) was made with the mun.plot() function in RSwissMaps package (version 0.1.0.1) with the following arguments (where relevant): *bfs_id* = dt$bfs_nr, *year* = 2016, *boundaries* = "c". Other packages and functions used in data

processing and drawing figures: cowplot (version 1.1.1); dplyr (version 1.0.7); ggplot2 (version 3.3.5); ggpubr (version 0.4.0); microbial (version 0.0.20); ochRe (version 1.0.0); phyloseq.extended (version 0.1.1.6); phylosmith (version 1.0.6); ranacapa (version 0.1.0); yarrr (version 0.1.5); VennDiagram (version 1.7.0); vsn (version 3.62.0).

Results

Communities Isolated from Banknotes Include *Pseudomonadaceae*, *Streptococcaceae*, and *Staphylococcaceae*

We detected some families of bacteria (Fig. 1B) in every banknote sample: *Pseudomonadaceae* and *Staphylococcaceae* were present in all samples (42/42, 100%; Table S2). These are both diverse families that include species closely associated with humans, e.g. as part of the human skin, respiratory tract or gut microbiome (Cogen *et al.*, 2008; Fierer *et al.*, 2008; Murphy and Parameswaran, 2009; Kim *et al.*, 2013; Fourquin-Gomez *et al.*, 2014; Robert-Pillot *et al.*, 2014; Rock and Donnenberg, 2014; Delanghe *et al.*, 2021; Skowron *et al.*, 2021), but have also been isolated from animal hosts and soil and aquatic environments (Cousin, 1999; Madhaiyan *et al.*, 2020). Other families were detected in significant abundance, but only in subsets of samples, such as *Vibrionaceae* and *Lactobacillaceae* (in 21 and 30 samples out of 42 respectively). The same taxa resurfaced when we calculated the core microbiome for each shop type (taxa found in >50% of samples per shop type at abundance >0.1%). We identified seven core ZOTUs shared between all shop types, attributed to the genera: *Corynebacterium*, *Propionibacterium*, *Pseudomonas*, *Streptococcus* (n = 2) and *Staphylococcus* (n = 2) (Fig. S2; detection threshold = 0.001, prevalence threshold = 0.5). Thus, our sequence data revealed diverse microbial communities associated with Swiss banknotes, but with the relative abundances of individual taxa varying among samples.

Within-sample diversity estimated by Shannon diversity index (Fig. 2) did not vary significantly with either geographic location or shop type (ANOVA: effect of shop type – $F_{4,37}$ = 0.844, p = 0.506; effect of location – $F_{9,32}$ = 1.013, p = 0.45). We obtained the same qualitative result with an alternative diversity index (Gini-Simpson index), and the two diversity indices were highly correlated with each other (linear regression Gini-Simpson ~ Shannon: adjusted R^2 = 0.851). Similarly, within-sample species richness (taken as observed richness (Fig. 2B) or Chao1 index) did not vary significantly among locations or shop types (ANOVA: $p > 0.1$ for shop type and location effects for both measures) and was correlated with Shannon diversity (adjusted R^2 = 0.629 for observed richness and R^2 = 0.478 for Chao1). Thus, within-sample diversity did not vary significantly among shop types or geographic locations.

Community Composition Was Specific to Shop Type, but Not Location

Bacterial community composition was more similar among banknotes collected from the same shop type than from different shop types (PERMANOVA: effect of shop type – $F_{4,41}$ = 1.321, p = 0.03). Visualising the similarities/differences among samples by ordination analysis (non-metric multidimensional scaling NMDS) further supported variation of community composition among shop types (Fig. 3). In particular, several samples from butcher shops clustered together in the top-right corner of the ordination plot (Fig. 3). Note one sample classified as "butcher" but separate from this cluster (butcher-BIA) was collected from a supermarket with a served meat counter but separate cash desk (see Methods). Consistent with the variation among shop types being primarily driven by the distinctiveness of butcher shop samples, if we excluded butcher samples from the analysis we no longer found significant variation among shop types (PERMANOVA: effect of shop type, excluding butcher samples – $F_{3,32}$ = 0.949, p = 0.584); this was not the case when re-running the analysis excluding sample

sets from all other shop types, except for florist samples which resulted in marginal non-significance (PERMANOVA: effect of shop type, without florist samples – $F_{3,32} = 1.287$, $p = 0.056$).

Looking at the ZOTUs making up the core microbiome for each shop type again supported butcher samples as being distinctive: the set of banknotes from butchers (n = 9) had the greatest number of core ZOTUs, both in total and unique to a single shop type (31 core ZOTUs in total, 13 unique core ZOTUs; Fig. S2). The 13 core ZOTUs unique to butchers belonged to the following genera: *Photobacterium* (n = 3; family *Vibrionaceae*), *Streptococcus* (n = 2; family *Streptococcaceae*), *Brochothrix* (n = 1; family *Listeriaceae*), *Corynebacterium* (n = 1; family *Corynebacteriaceae*), *Kocuria* (n = 1; family *Micrococcaceae*), *Lactobacillus* (n = 1; family *Lactobacillaceae*), *Lactococcus* (n = 1; family *Staphylococcaceae*), *Novosphingobium* (n = 1; family *Sphingomonadaaceae*), *Staphylococcus* (n = 1; family *Staphylococcaceae*), *Veillonella* (n = 1; family *Veillonellaceae*). This is consistent with our family-level analysis (Fig. 1), where some of the families to which these butcher-associated genera belong were over-represented among butcher samples (e.g., *Vibrionaceae*, *Lactobacillaceae*).

A comparison between core ZOTUs unique to butcher samples and NMDS loadings (Fig. 3) provides further information about the taxa that drive the among-shop-type variation: the *Veillonella* core ZOTU and one *Streptococcus* core ZOTU unique to butchers were among the ten ZOTUs with the highest positive loadings for NMDS1; the *Lactococcus* and three *Photobacterium* core ZOTUs unique to butchers were among the ten ZOTUs with the highest positive loadings for NMDS2 (again consistent with families *Vibrionaceae* and *Lactobacillaceae* being abundant and over-represented in butcher samples in Fig. 1). Note that the observed variation in community composition among shop types was not explained by variable numbers of reads among samples from different shop types (Fig. S1; effect of shop type in ANOVA – $F_{4,45} = 0.792$, $p > 0.5$).

353 We found no evidence that samples collected in different locations (towns) harboured
354 different bacterial microbiomes (PERMANOVA: effect of location – $F_{9,41} = 1.173$, $p = 0.067$;
355 Fig. 3). In a second test for geographic structuring of the sampled communities (Mantel test),
356 we quantified the association between pairwise similarity among samples in terms of
357 community composition and the geographic distances between sampling locations. This
358 revealed no significant association between the two distance measures, neither in the full
359 dataset (n = 42; Mantel test – $z = 39'978'433$, $p = 0.751$), nor when performed separately for
360 each shop type (Mantel test, five tests total with p-values corrected for multiple testing – p_{bakery}
361 $= 1$, $p_{butcher} = 1$; $p_{florist} = 0.315$; $p_{kiosk} = 1$; $p_{pharmacy} = 1$). As for shop type above, there was no
362 association between number of reads and location (ANOVA: effect of location – $F_{9,40} = 0.836$,
363 $p > 0.5$). Thus, unlike for the variation among shop types observed above, we found no
364 evidence that the differences in bacterial community composition among samples increased
365 with geographic distance.
366
367 **Cultivation of Samples on Agar Supports Conclusions Drawn from Sequencing Data**
368 When we plated aliquots of samples on Chromatic™ MH agar plates, we detected a total of
369 157 viable colonies across 24 of 50 plates (48%). The number of colonies per plate varied
370 among samples (Fig. 4), and was highest on average for butcher samples (count model in hurdle
371 regression model for shop type: butcher: $z = 3.44$, $p < 0.001$; all other shop types: $p > 0.05$;
372 Fig. 4). There was no significant variation of the number of colonies per plate among
373 geographic locations (count model in hurdle regression model for location: $p > 0.05$ for all
374 locations). Several plates produced no colonies, but presence/absence of viable colonies was
375 not predicted by either shop type or location (zero hurdle model in hurdle regression model for
376 shop type and for location: $p > 0.05$ for all shop types and all locations).

The identity of colonies (as determined by colony morphology and colour on Chromatic™ MH agar plates, see Methods) corresponded to key taxa identified by sequencing: *E. coli* = *Enterobacteriaceae*; *Staphylococcus aureus* = *Staphlyococcaceae*; *Pseudomonas spp* = *Pseudomonadaceae*; *Klebsiella spp.*, *Enterobacter spp.*, *Serratia spp.* = *Enterobacteriaceae* (Fig. 1B Fig. 4). The exception to this trend was *Enterococcus faecalis* (3/157 colonies or 2%), which belongs to the *Enterococcaceae* family. This was not among the most abundant families in our sequence data (Fig. 1B), and we only found ZOTUs categorized as *Enterococcaceae* prior to filtering the data, indicating it was present but at lower abundances than suggested by colony formation data. For most of the other key taxa identified by sequence data that we did not find on agar plates, prior information about these taxa suggests the growth conditions (Chromatic™ MH agar; 37°C; 24h aerobic incubation) are not conducive to colony formation. For example, *Corynebacteriaceae, Lactobacillaceae*, (environmental) *Moraxellaceae*, and *Vibrionaceae* have growth optima <37°C (De Angelis and Gobbetti, 2011; Vinet and Zhedanov, 2011; Lonvaud-Funel, 2014; Tauch and Sandbote, 2014; Baron *et al.*, 2020). In addition, several of the key taxa (e.g. *Brevibacteriaceae, Corynebacteriaceae Halomonadaceae*, *Lactobacillaceae*, *Moraxellaceae*, *Vibrionaceae)* may have required incubation times >24h (Vinet and Zhedanov, 2011; Argandoña *et al.*, 2012a; Forquin and Weimer, 2014; Tauch and Sandbote, 2014; De Angelis and Gobbetti, 2016; Baron *et al.*, 2020), or different media composition such as higher salt or nutrient content (*Halomonadaceae* and *Corynebacteriaceae*, respectively, (Argandoña *et al.*, 2012b; Tauch and Sandbote, 2014)). Therefore, the absence of these taxa from our cultured samples is probably because they do not grow well in these conditions, and does not necessarily contradict our finding them in the sequence data.

As above for our community-level sequence data, the relative abundances of different taxa inferred from colony counts on chromatic agar varied among different shop types

(PERMANOVA with zero-adjusted Bray-Curtis dissimilarity (Clarke *et al.*, 2006): effect of shop type – $F_{4,49} = 2.507$, $p = 0.003$) but not geographic locations (effect of location – $F_{9,41} = 0.080$, $p = 0.701$). Thus, banknotes yielded viable colonies and taxonomic identification on chromatic agar, albeit relatively coarse-grained compared to our sequence data, supporting similar conclusions (both in terms of which taxa were present and variation of community composition with collection environment but not geographic location).

DISCUSSION

We found that Swiss banknotes taken directly from circulation harbour a variety of bacterial DNA, with the key taxa (Fig. 1B) including families found before in humans and human-associated environments, e.g. the human skin, gut or respiratory tract microbiome, or human food production (Cogen *et al.*, 2008; Fierer *et al.*, 2008; Murphy and Parameswaran, 2009; Oren, 2011; Fourquin-Gomez *et al.*, 2014; Delanghe *et al.*, 2021; Skowron *et al.*, 2021). Our finding that community composition varied depending on the shop type in which each banknote was collected is surprising: this suggests money has a microbiome reflecting its local environment, even though we assume banknotes are only transiently exposed to each habitat/shop type. By extension, we expect individual banknotes to have a dynamic microbiome that changes during their "lifecycle" as they pass from one habitat to another. By contrast, we found no evidence for geographic structuring, unlike some previous studies with microbes in other contexts (Whitaker *et al.*, 2003b; Horner-Devine *et al.*, 2004), but consistent with other observations of relatively weak biogeographic structuring in microbes (Hillebrand *et al.*, 2001; Finlay, 2002; Zhou *et al.*, 2008). Our plating results corresponded well with conclusions from sequencing, indicating that at least some taxa are present as viable, culturable cells. Together these observations suggest, on the scale investigated here, deterministic assembly processes (e.g., selection imposed by habitat heterogeneity) are more important

drivers of banknote-associated bacterial community composition than other processes associated with geographic distance among sites, such as dispersal limitation (Chase, 2010).

The taxa we identified as being relatively more abundant on banknotes collected from butcher shops (*Brevibacteriaceae*, *Halomonadaceae*, *Lactobacillaceae*, *Vibrionaceae*) are known to include bacteria previously identified in "meaty" contexts. *Halomonadaceae* and *Brevibacteriaceae* have previously been found in high-salinity environments and salty foods such as poultry skin or Chinese pork cured with salt (Oren, 2011; Fourquin-Gomez *et al.*, 2014). *Brevibacteriaceae* and *Vibrionaceae* are frequently found in samples from butchers, slaughterhouses, abattoirs (Sierra *et al.*, 1995; Połka *et al.*, 2015; Fuertes-Perez *et al.*, 2019; Dourou *et al.*, 2021). Representatives of the *Lactobacillaceae* family are key species used as starter cultures in production of, and have been isolated from, cured and fermented meat (Deibel *et al.*, 1961; Samelis *et al.*, 1994; Cocolin *et al.*, 2011). It is important to note that members of the same families have also been isolated from a wide range of other environments including humans, plants, animals and foods (Walter, 2008; Oren, 2011; Felis and Pot, 2014; Forquin and Weimer, 2014; Franz *et al.*, 2014; Leisner and Pot, 2014; Pot *et al.*, 2014; Takemura *et al.*, 2014)(Oren, 2011) . Therefore, while our results are consistent with past work finding these taxa in "meaty" environments, they can also occur elsewhere. It is interesting in this context that the sample butcher-BIA – classified as "butcher" but in fact originating from a supermarket till due to the respective location's two butchers being closed on collection day – was relatively impoverished for the taxa linked above to the other butcher samples (butcher-BIA: 0.45% *Brevibacteriaceae*, 0.00% *Halomonadaceae*, 0.07% *Lactobacillaceae*, 0.00% *Vibrionaceae*).

At a broader level, the taxa we identified across all shop types are in line with previous research into monetary microbiomes. For example, in a metagenomic analysis of dollar bills from New York City, Maritz *et al.* (2017) found representatives of several of the families that

were dominant in our dataset (including *Staphylococcaceae*, *Micrococcaceae*, *Streptococcaceae*, *Corynebacteriaceae*, and *Moraxellaceae*). Similarly, an analysis of Chinese RMB banknotes and US dollar bills (Lin *et al.*, 2021) revealed the most abundant taxa on the family level were *Moraxellaceae*, *Pseudomonadaceae* and *Enterobacteriaceae* (all abundant in our data set). Other studies of monetary microbiomes identified similarly overlapping sets of taxa, including analysis of Indian Rupee banknotes (*Actinobacteria*, *Bacteriodetes*, *Firmicutes*, and *Proteobacteria*; no lower-taxa resolution available) (Jalali *et al.*, 2015), banknotes from Hong Kong (*Moraxellaceae*, *Micrococcaceae*, *Enterobacteriaceae, Vibrionaceae*, *Corynebacteriaceae*, *Pseudomonadaceae*, among others) (Heshiki *et al.*, 2017) and from Brazil (*Enterobacteriaceae*, *Staphylococcaceae*, *Lactobacillaceae*, *Moraxellaceae*, and *Corynebacteriaceae*, among others) (da Fonseca *et al.*, 2015). Our results go beyond past work on monetary microbiomes by showing that the relative abundances of different taxa (families and ZOTUs) vary among collection environments (shop types), but not with geographic distance within a European country.

A key implication of our results is that even temporary exposure of banknotes to certain environments is reflected by the community composition of colonizing bacteria. This is surprising because we assume the residence time of individual banknotes in a given shop is short (hours, days or weeks). It also suggests high variability in temporal beta diversity in the monetary microbiome. We found several taxa (ZOTUs) that were unique to particular shop types, and at the family level it was also visible that some taxa were overrepresented in butcher samples. Despite this, analogous to the core microbiome Danko *et al.* (2021) found in urban mass transit systems, we found something akin to a core microbiome across our samples in that two families (*Pseudomonadaceae, Staphylococcaceae*) were present in every sample.

Another important implication of our results is that many of the key taxa of the monetary microbiome in Switzerland are also present as viable cells (culturable on agar). This raises an

interesting question about colonization dynamics in the monetary microbiome. If viable microbes are present on banknotes, are they growing and interacting on the banknote itself? We can speculate the rough surface of most banknotes not only allows attachment of bacteria, but also human skin cells, secretions and remnants of food or products during handling. Potentially, this could support bacterial growth after colonization. If the local microenvironment influences the relative growth/death rates of different taxa, this could even shape community composition and contribute to habitat-specific community composition as we observed here. Alternatively, community composition may be determined primarily at the colonization step, with different shop types harbouring different microbiomes in general (due to local habitat selection), which are "sampled" by banknotes passing through the shop. One avenue for future work aiming to tease apart these different potential drivers of the monetary microbiome would be to experimentally colonize banknotes and track community abundance and composition over time (longitudinal sampling of individual banknotes).

We emphasize strongly that our results do not address or make any predictions regarding food hygiene or potential health hazards posed to humans. We were solely interested in the microbial community composition on banknotes and how this relates to the sampling environment. Finding distinct microbiomes in different shop types does not necessarily translate to variable infection risk or indicate variable hygiene standards, only that the local monetary microbiome reflects the local commercial environment. One limitation of our study is that the SARS-CoV2 pandemic has led to changes in customers' payment behaviour (Kraenzlin *et al.*, 2020). This might influence the transfer of microbiota to banknotes, because of increased hygienic awareness in customers and shop staff. For example, we observed in one case a banknote being disinfected by shop staff (the sample extracted from the banknote from this shop was removed in the initial filtering step due to low read counts, see Methods). However if anything we would expect any effect of pandemic-related hygiene measures to

make our conclusions conservative: when hygiene standards are less stringent we might expect even more exchange of microbiota between the local environment and banknotes. A further limitation of our study is the spatial scale and number of shop types. While our study design revealed significant variation among shop types, the lack of geographic structure across Switzerland may differ from that across larger spatial scales or across different currency types.

In summary, we found that the bacterial communities associated with Swiss banknotes can vary depending on the environment in which the banknotes were collected and that, by contrast, geographic distance between sampled locations does not predict variation of the associated microbial communities. Our results also show that the most abundant taxa are not only present in the form of cell debris and genetic material, but as viable cells. This raises the question of banknotes potentially being involved in transmission of bacteria between people and the environment, and/or of genes, such as antibiotic resistance genes, among bacteria co-occurring on the same banknotes. Therefore, a key avenue for future work building on the link between microbial community structure on banknotes and the local environment (shop type) identified here is to ask whether this translates to variable infection risk or horizontal gene transfer.

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Data Accessibility and Benefit-Sharing Statement

The sequencing data have been deposited in the European Nucleotide Archive under the study accession number PRJEB45390 (https://www.ebi.ac.uk/ena; data will be made accessible upon publication of the study).

Other data presented in this study will be made available at the Dryad Digital Repository: http://dx.doi.org/...: to be completed after manuscript is accepted for publication.

Benefits Generated: Benefits from this research accrue from the sharing of our data and results on public databases as described above.

Author Contributions

AMB, ARH	designed research.
AMB	performed research.
AMB, ARH	analyzed data.
AMB, ARH	wrote paper.

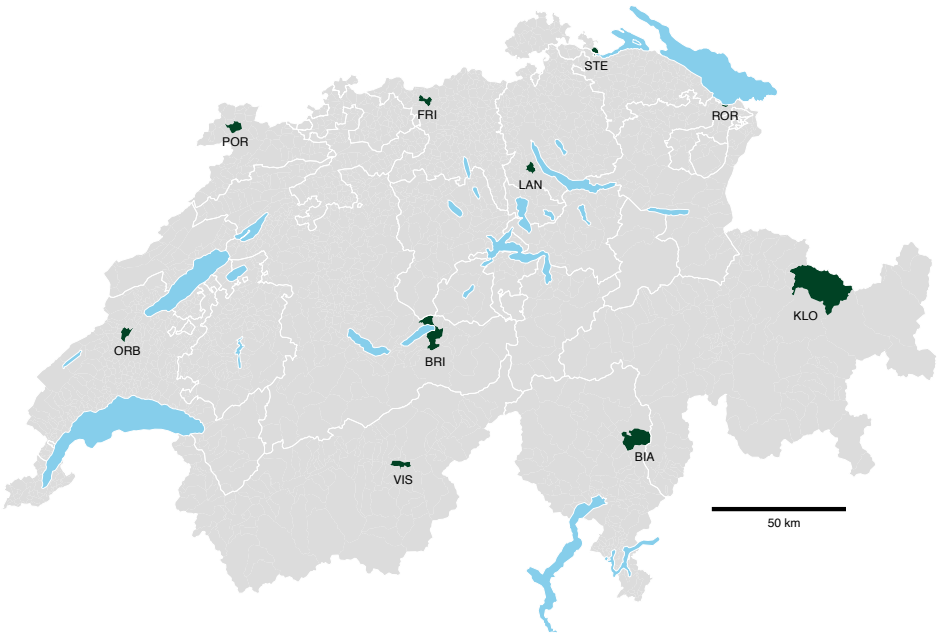
Tables

Supplementary Table 1. Sequences of primers used during library preparation. These primers were used to amplify the V3-V4 regions of the 16S rRNA gene during the first course of limited cycle PCR.

forward primers	
340F_nex0	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGCCTACGGGNGGCWGCAG
340F_nex1	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGNGCCTACGGGNGGCWGCAG
340F_nex2	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGNNGCCTACGGGNGGCWGCAG
340F_nex3	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGNNNGCCTACGGGNGGCWGCAG
reverse primers	
805R_nex0	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTGACTACHVGGGTATCTAATCC
805R_nex1	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGNTGACTACHVGGGTATCTAATCC
805R_nex2	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGNNTGACTACHVGGGTATCTAATCC
805R_nex3	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGNNNTGACTACHVGGGTATCTAATCC

712 **Figures**

A



B

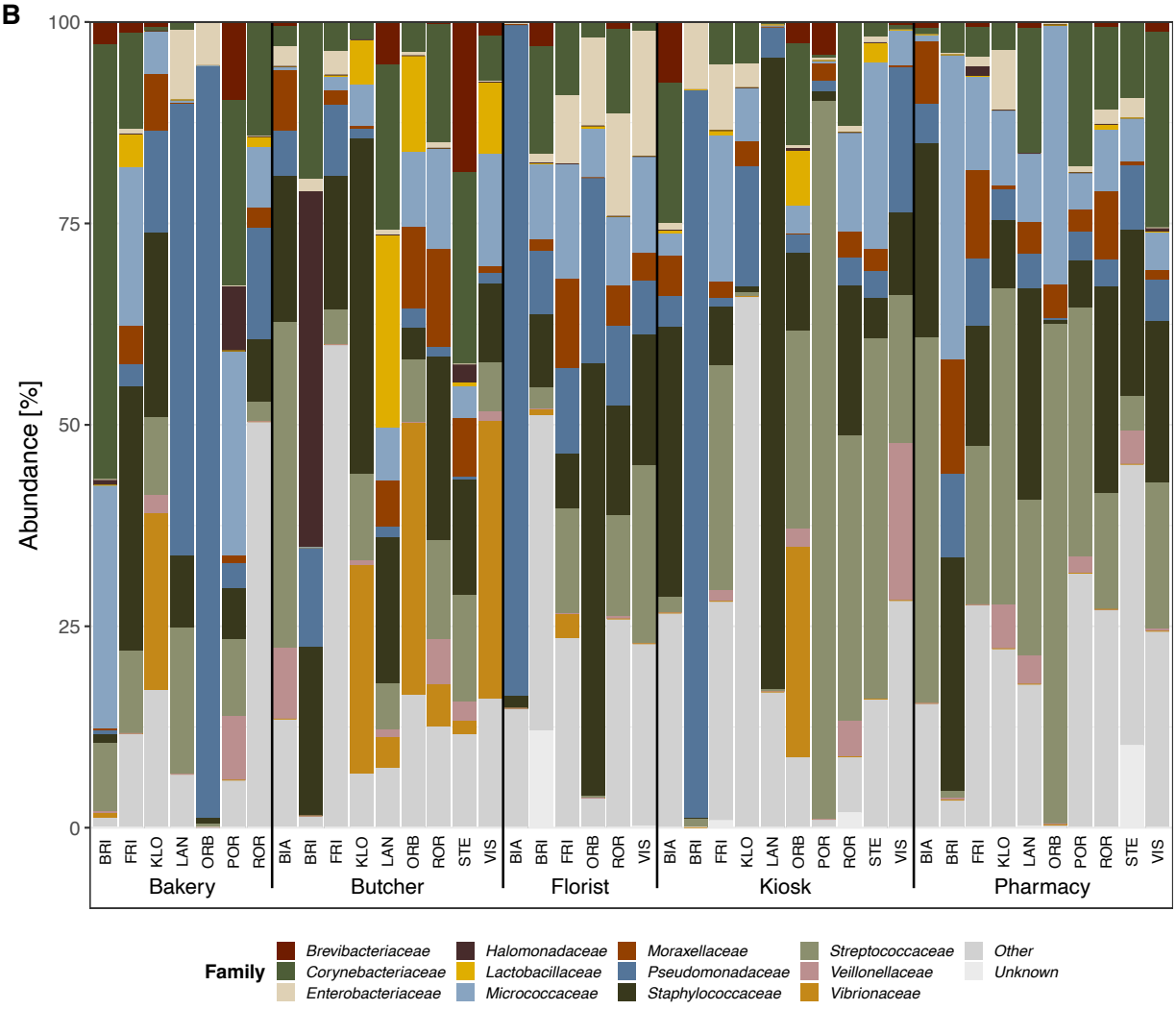


Figure 1. Bacterial community composition across sampling locations and shop types. Panel A: Distribution of sampling locations across Switzerland. The map shows the 10 municipalities, highlighted in dark green, in which banknotes were collected. Panel B: Relative abundances of the main bacterial families identified by amplicon sequencing from banknotes sampled in each shop (lower *x*-axis) and location (upper *x*-axis, indicated by three-letter abbreviations). *Unknown* contains non-assigned ZOTUs; *Other* contains assigned ZOTUs not belonging to the most abundant families. Abbreviations in both panels: BIA = Biasca, BRI = Brienz BE, FRI = Frick, KLO = Klosters-Serneus, LAN = Langnau am Albis, ORB = Orbe, POR = Porrentruy, ROR = Rorschach, STE = Stein am Rhein, VIS = Visp.

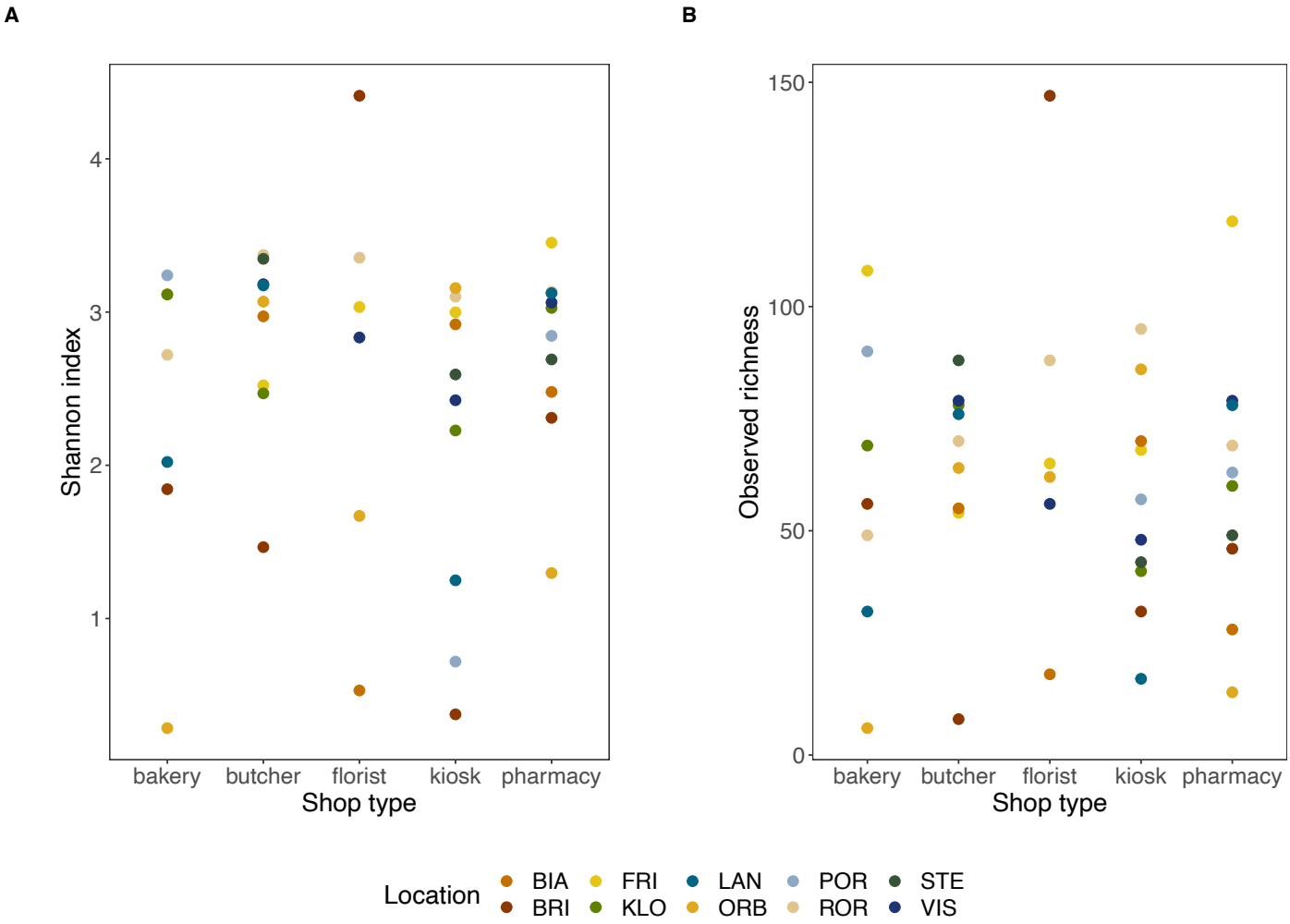
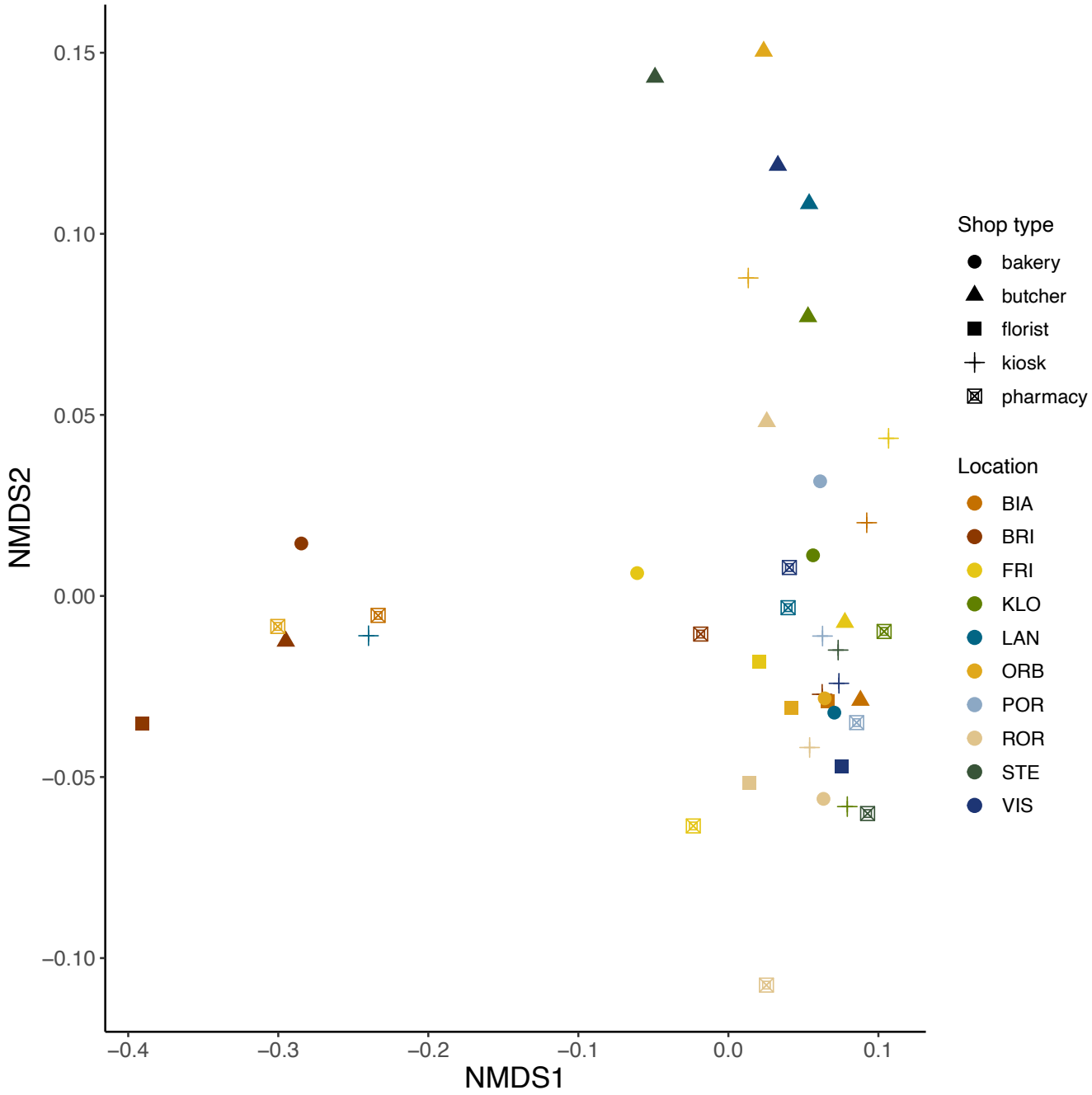


Figure 2. Within-sample taxonomic diversity of samples from banknotes collected in various shop types across Switzerland. Panel A: The Shannon diversity index *H* takes into account both richness (number of species present) and evenness (how evenly do present species occur). Panel B: Observed richness gives the number of unique ZOTUs present in a sample. Colours indicate locations (abbreviations: BIA = Biasca, BRI = Brienz BE, FRI = Frick, KLO = Klosters-Serneus, LAN = Langnau am Albis, ORB = Orbe, POR = Porrentruy, ROR = Rorschach, STE = Stein am Rhein, VIS = Visp).



729 **Figure 3. Ordination analysis.** NMDS ordination based on Bray-Curtis dissimilarity between
730 the bacterial communities isolated from banknotes (stress = 0.11). Symbols represent different
731 shop types and are coloured according to location. Abbreviations: BIA = Biasca, BRI = Brienz
732 BE, FRI = Frick, KLO = Klosters-Serneus, LAN = Langnau am Albis, ORB = Orbe, POR =
733 Porrentruy, ROR = Rorschach, STE = Stein am Rhein, VIS = Visp.

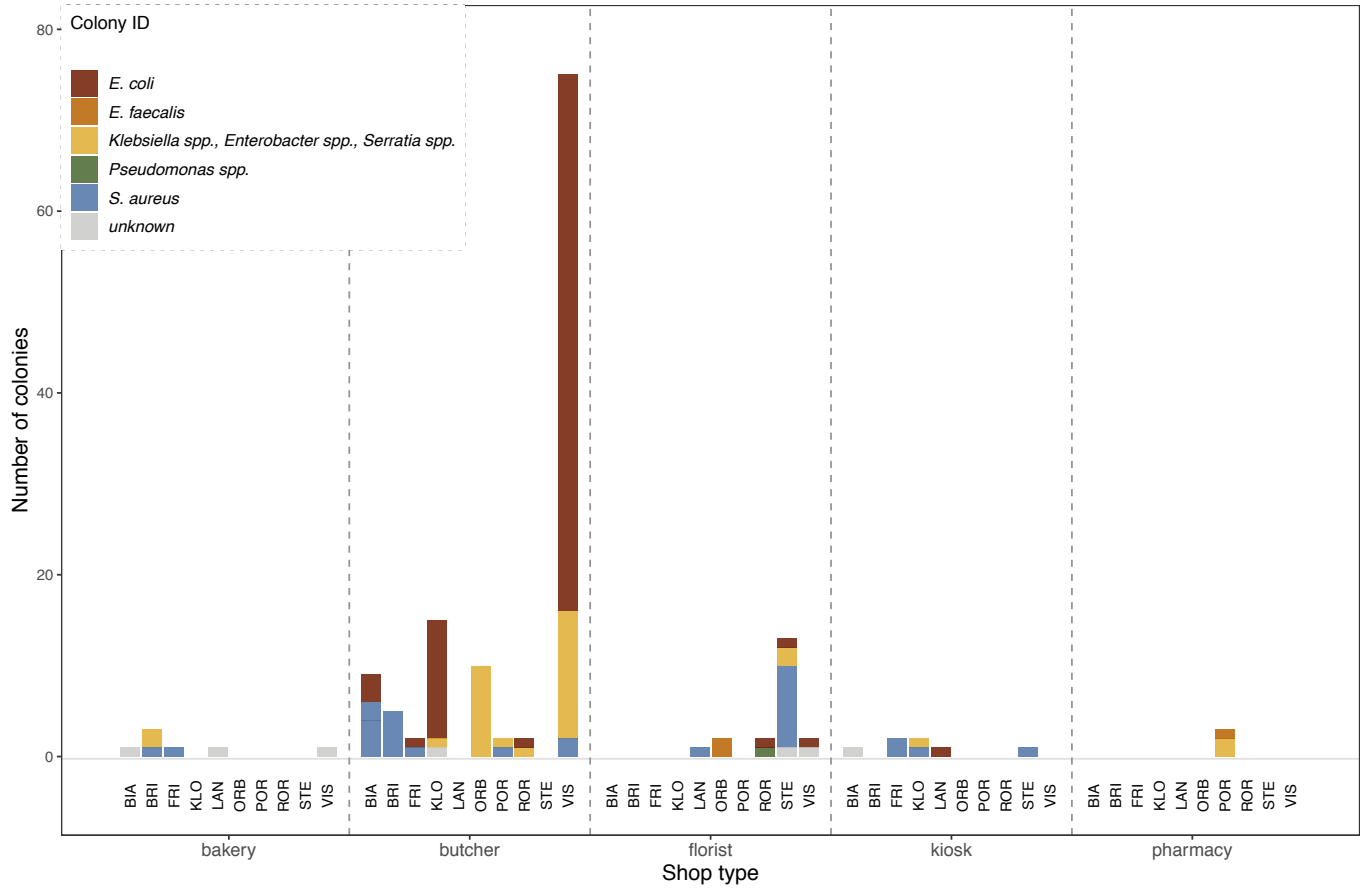
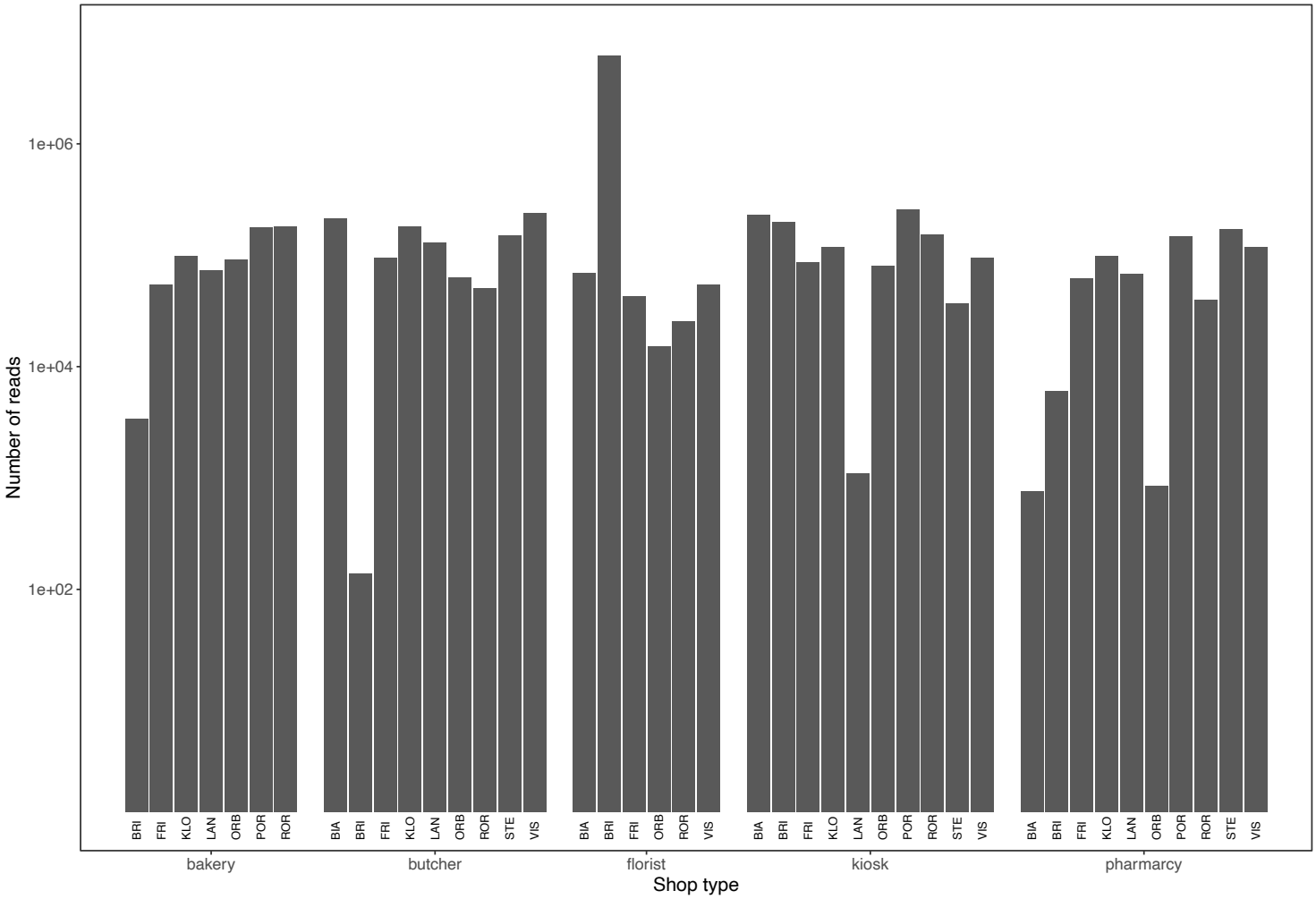
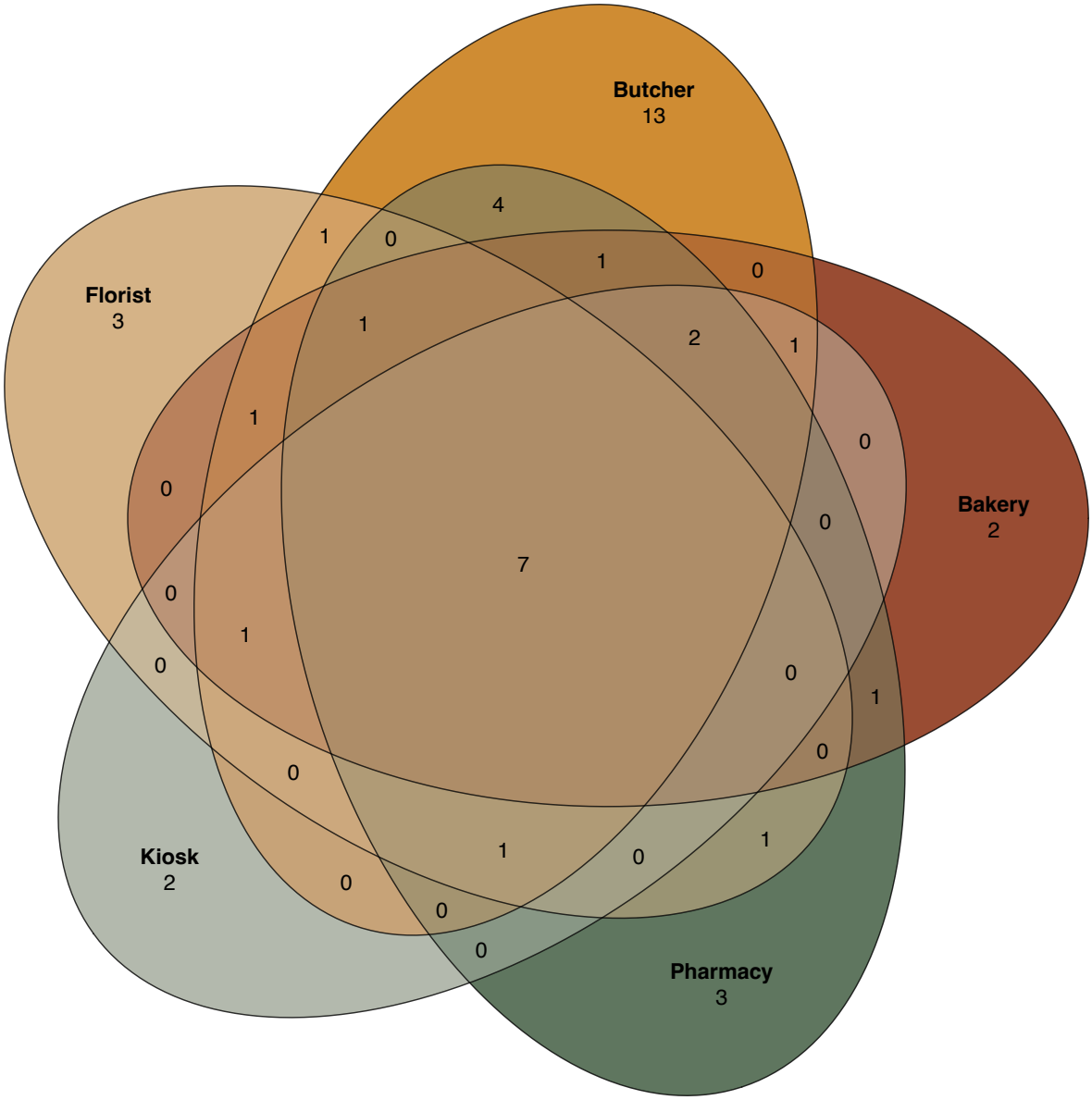


Figure 4. Number and identity of colonies isolated from banknotes collected in various shop types across Switzerland. Each composite bar gives the number and identity of colonies detected in samples isolated from banknotes. The bar colour indicates species identity (based on morphology and colour on Chromatic™ MH agar, see Methods). Colonies classified as "unknown" had uncertain identity (regular-shaped, small size) or a morphology more commonly associated with yeasts/fungi (astral appearance, polychromatic, with spores/fruiting body-like structures). Abbreviations: BIA = Biasca, BRI = Brienz BE, FRI = Frick, KLO = Klosters-Serneus, LAN = Langnau am Albis, ORB = Orbe, POR = Porrentruy, ROR = Rorschach, STE = Stein am Rhein, VIS = Visp.

Supplementary Figures



Supplementary Figure 1. Number of reads per sample. The figure shows the samples and number of reads for each sample retained after filtering. Total reads per sample varied greatly, with 140 reads for the butcher in BRI and 7'152'829 reads for the florist in BRI (~51'092x difference). Note: *y*-axis is log10-transformed. Abbreviations: BIA = Biasca, BRI = Brienz BE, FRI = Frick, KLO = Klosters-Serneus, LAN = Langnau am Albis, ORB = Orbe, POR = Porrentruy, ROR = Rorschach, STE = Stein am Rhein, VIS = Visp.



751

752 **Supplementary Figure 2. Venn diagram of unique and shared core ZOTUs samples from**
753 **banknotes collected in various shop types across Switzerland.** Each ellipse gives the
754 number of ZOTUs present at an abundance of >0.1% in >50% of a set of banknotes attributed
755 to a certain shop type. Numbers in overlapping areas of ellipses indicate shared ZOTUs
756 between sets of banknotes collected in different types of shops. The seven core microbiome
757 ZOTUs present in >50% of every set of banknotes were attributed to the genera: *Escherichia-*
758 *Shigella*, *Pseudomonas*, *Propionibacterium*, *Corynebacterium*, *Streptococcus*, *Streptococcus*,
759 and *Staphylococcus*.