NRC Research Press

ARTICLE

Microbiota formed on attached stainless steel coupons correlates with the natural biofilm of the sink surface in domestic kitchens

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Abstract: Stainless steel coupons are frequently used in biofilm studies in the laboratory, as this material is commonly used in the food industry. The coupons are attached to different surfaces to create a "natural" biofilm to be studied further in laboratory trials. However, little has been done to investigate how well the microbiota on such coupons represents the surrounding environment. The microbiota on sink wall surfaces and on new stainless steel coupons attached to the sink wall for 3 months in 8 domestic kitchen sinks was investigated by next-generation sequencing (MiSeq) of the 16S rRNA gene derived from DNA and RNA (cDNA), and by plating and identification of colonies. The mean number of colony-forming units was about 10-fold higher for coupons than sink surfaces, and more variation in bacterial counts between kitchens was seen on sink surfaces than coupons. The microbiota in the majority of biofilms was dominated by *Moraxellaceae* (genus *Moraxella*/*Enhydrobacter*) and *Micrococcaceae* (genus *Kocuria*). The results demonstrated that the variation in the microbiota was mainly due to differences between kitchens (38.2%), followed by the different nucleic acid template (DNA vs RNA) (10.8%), and that only 5.1% of the variation was a result of differences between coupons and sink surfaces. The microbiota variation between sink surfaces and coupons was smaller for samples based on their RNA than on their DNA. Overall, our results suggest that new stainless steel coupons are suited to model the dominating part of the natural microbiota of the surrounding environment and, furthermore, are suitable for different downstream studies.

Key words: microbiota, stainless steel coupons, sink surface, domestic kitchens.

Résumé : Les études sur les biofilms utilisent couramment des coupons en acier inoxydable en laboratoire puisque ce matériau est répandu dans l'industrie alimentaire. Ces coupons sont attachés à diverses surfaces afin de créer un biofilm « naturel » aux fins d'études plus approfondies en laboratoire. Or, on s'est peu demandé à quel point la microflore retrouvée sur de tels coupons est représentative de l'environnement avoisinant. On a examiné la microflore de nouveaux coupons d'acier inoxydable attachés à la paroi de l'évier pendant trois mois, ainsi que celle des parois de l'évier comme telles, au moyen du séquençage de prochaine génération (MiSeq) du gène de l'ARNr 16S issu d'ADN et d'ARN (ADNc), et par ensemencement et identification des colonies. Le nombre moyen d'unités formant colonies était environ 10 fois plus élevé sur les coupons que sur les surfaces de l'évier, et on a observé davantage de variations des numérations bactériennes selon les cuisines dans le contexte des surfaces d'éviers que des coupons. La microflore de la majorité des biofilms était dominée par les *Moraxellaceae* (genre *Moraxella*/*Enhydrobacter*) et les *Micrococcaceae* (genre *Kocuria*). Les résultats ont démontré que la variation de la microflore était principalement attribuable aux différences entre les cuisines (38,2 %), suivie par les différentes matrices (ADN ou ARN) (10,8 %) et que seulement 5,1 % de la variation découlait des différences entre les coupons et les surfaces d'évier. La variation entre les coupons et les surfaces d'évier était inférieure lorsque les échantillons étaient issus d'ARN, comparativement à l'ADN. Dans l'ensemble, nos résultats indiquent que les nouveaux coupons d'acier inoxydable conviennent à la modélisation de la portion dominante de la microflore naturelle de l'environnement avoisinant, et se prêteraient ainsi à une variété d'études ultérieures. [Traduit par la Rédaction]

Mots-clés : microflore, coupons d'acier inoxydable, surface d'évier, cuisines domestiques.

Received 27 August 2015. Revision received 19 October 2015. Accepted 9 November 2015.

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Introduction

Studying microorganisms directly in situ is challenging for a number of reasons: it is not possible to transport the surfaces to a laboratory for further analysis or perform certain analyses directly; it is not safe to introduce pathogens outside the laboratory; and it is difficult to compare different treatments, conditions, or surfaces in a systematic and standardized way. Therefore, most studies on biofilms are conducted using either bacteria collected from environmental biofilms or laboratory strains in laboratory models (Giaouris et al. 2015). Biofilms produced at the laboratory are more or less relevant for the environments they are meant to mimic with respect to a range of factors such as materials, microbiota, temperatures, nutrients, sanitation regimes, and the dynamics for all these factors. To achieve a more realistic biofilm, some studies use the addition of food residues or organic soiling in the biofilm formation (Chaitiemwong et al. 2014; Kuda et al. 2015). Another approach to make the models more realistic is to place coupons at the study site and allow a natural biofilm to evolve. The biofilm or attached bacteria can then be investigated in different downstream studies, e.g., cleaning and disinfectant studies and (or) examination of the survival and (or) establishment of potential pathogens. Ideally, this will allow studies on biofilms that are more relevant than those produced using laboratory models. The approach has been used to compare hygienic properties of different materials (Guobjornsdottir et al. 2005), to identify microbiota in food production factories (Hood and Zottola 1997; Mettler and Carpentier 1998; Gunduz and Tuncel 2006), and to detect biofilm formation (Holah et al. 1989; Gibson et al. 1995), and recently, we used this approach to study the effect of kitchen cleaning methods (Røssvoll et al. 2015). However, little has been done to evaluate how well the microbiota developed by this approach reflects the microbiota developed in situ, where the surfaces can be of a different quality and (or) condition than the coupons used.

Next-generation sequencing (NGS) technology using the 16S rRNA gene as a taxonomic marker is often used to study complex microbial communities. NGS generates enormous amounts of data, helping to reveal a more complete picture of the microbiota than do traditional plating-based analyses, which are dependent on growth conditions such as nutrients, atmosphere, and temperature. However, one drawback of DNA-based microbiota analysis is that it does not discriminate between dead and viable bacteria, and this may limit the applicability when studying matrixes with a high proportion of dead bacteria. In many studies the relative amount of dead bacteria is considered insignificant, but when working with biofilms subjected to different environmental stresses, this may not be the case. Using DNA will give a good overview of the complete microbiota of the biofilm (dead and active) while the use of RNA, in principle, will estimate the current in situ activity of a community, because cellular rRNA concentration is generally well correlated with growth rate and activity (Poulsen et al. 1993; Bremer and Dennis 1996). rRNA are also thought to degrade only under certain stress conditions (starvation, stationary phase, or following a nutritional downshift) or when an RNA molecule is defective (Deutscher 2003). A biofilm can fulfill several of these criteria. However, a study of *Pseudomonas aeruginosa* biofilms has shown that cells in the bottom portion of the biofilms maintained a high abundance of ribosomal RNAs as well as mRNA for genes associated with ribosome hibernation factors (Williamson et al. 2012).

In this study we aimed to investigate how the microbiota developing on stainless steel coupons placed in domestic kitchen sinks (stainless steel of various ages and conditions) for 3 months compared with the natural microbiota of the kitchen sink surfaces. The samples were collected in a parallel study previously published (Røssvoll et al. 2015). Domestic sinks were chosen as a suitable environment, since their material (stainless steel) is comparable to that of the coupons and since they are heavily exposed to and colonized by bacteria (e.g., from raw produce, water, and skin microbes). The microbiota from the coupons and the sink surfaces was compared on the basis of their DNA (live and dead bacteria) and RNA (potentially active bacteria). In addition, we used traditional plating followed by 16S rRNA gene sequencing of isolates to see how well the microbiota identified by RNA reflected what could be cultivated.

Materials and methods

Experimental design

The experimental setup of the steel coupons in the domestic kitchen sinks (stainless steel) has previously been described (Røssvoll et al. 2015). Briefly, 8 volunteers attached 3 new stainless steel coupons (AISI 304, 2B; Norsk Stål AS, Nesbru, Norway) in their kitchen sinks in January 2013. The kitchen sinks were all of stainless steel but of various age and quality. The surface of the stainless steel coupon was 2 cm × 6 cm. The volunteers were instructed to use their kitchen sinks as normal but to avoid the use of disinfectants and direct scrubbing of the coupons. The coupons were left in the kitchen sinks for 3 months. In April 2013 the volunteers were instructed to sample an area the size of a coupon (2 cm × 6 cm) beside each of 3 different coupons in their kitchen sink. All volunteers were provided with equipment and detailed instructions of how to swab the specific areas in their kitchen sink surface (Hedin et al. 2010). They were also instructed on how to remove the coupons in their sinks with gloved hands to avoid contamination, and to place each coupon in a prelabeled 50 mL tube for transportation to the laboratory. The swabs and the coupons were sampled in the morning by the volunteers, brought to the laboratory, and analyzed within an hour.

Surface sampling and cultivation methods

The swabbing and plating were as described previously (Røssvoll et al. 2015). Briefly, 2 swabs were used for each coupon–sink area and both swabs were put in the same tube with 3 mL of Dey–Engley (D/E) neutralizing broth (BD Difco, New Jersey, USA), and serial 10-fold dilutions were prepared in PBS and spiral plated on tryptic soy agar (TSA; Oxoid, Basingstoke, UK). The plates were incubated at 25 °C for 3 days before determination of colony-forming units (CFU) and isolation of single colonies. A total of 20 colonies (or less at low cell numbers) were picked at random from plates from each kitchen, resulting in up to 60 colonies picked per kitchen. The colonies were restreaked on TSA and incubated at 25 °C for 3 days before being prepared for sequencing.

DNA and RNA extraction and cDNA synthesis

The leftover material (swabs in D/E neutralizing broth) used for plating (~2 mL per coupon–sink surface) was used to extract DNA and RNA. The neutralizing broth originating from the swabs from 3 coupons per kitchen were mixed and then split into 2 samples: 1 for DNA extraction and 1 for RNA extraction. The same was done for the 3 sink surface areas. For DNA extraction, the samples were centrifuged at 13 000*g* for 5 min and then frozen at –20 °C for 1–2 weeks before extraction using the QiaAmp Stool kit (Qiagen, Valencia, California). Briefly, the bacterial pellet was resuspended in 500 μL of ASL buffer (stool lysis buffer, Qiagen), transferred to Lysis Matrix E (MP Biomedicals, Solon, Ohio, USA) tubes, and lysed in a FastPrep bead beater (MP Biomedicals) for 40 s at 6 m/s. The samples were centrifuged briefly before adding an additional 500 μL of ASL buffer and were then vortexed. Afterwards, the samples were incubated at 70 °C for 5 min and centrifuged at 14 000*g* for 5 min before transferring to new tubes, adding 400 μL of ASL buffer, and following the manufacturer's protocol.

The samples for RNA extraction were added to tubes containing RNA Protect (Qiagen), vortexed for 5 s, incubated 5 min at room temperature, centrifuged at 5000*g* for 10 min, the supernatant was decanted, and the pellets was kept at –80 °C until extraction using the RNeasy mini kit (Qiagen) and an on-column DNase digestion (Qiagen). Briefly, 700 μL of buffer RTL (lysis buffer, Qiagen) (with 40 μL of 1 mol/L DTT/mL RTL) was added to the pellet, vortexed 5–10 s, and then transferred to Lysis Matrix E (MP Biomedicals) tubes, and lysed as described above. The samples were centrifuged at 14 000*g* for 5 min before adding ethanol and following the Qiagen protocol from this point. The RNA was measured using nanodrop (NanoDrop Technologies, Inc., Wilmington, Delaware, USA) and stored at –80 °C until cDNA synthesis. The cDNA synthesis was performed using SuperScript™ III reverse transcriptase (Invitrogen, Life Technologies Ltd., Paisley, UK) as recommended by the manufacturer, with and without enzyme (negative control).

PCR and sequencing of colonies

PCR and sequencing was performed as described previously (Røssvoll et al. 2015). Briefly, universal primers (Nadkarni et al. 2002) were used for 16S rRNA gene amplification (V3–V4) and sequencing. DNA was isolated by lysing single colonies using a microwave oven (Sharp Microwave oven R-5000E). The microwave lysis was performed by applying a small amount of the colony on the bottom of the PCR well followed by microwave treatment for 1 min at maximum power. Amplification was performed using 0.25 μmol/L (each) primer, 10 μL of Qiagen multipleks PCR kit (2×) (Qiagen, Oslo, Norway) to a total volume of 20 μL. The cycling conditions, PCR purification, and sequencing were performed as described previously by Omer et al. (2015). The taxonomy was identified using the RDP (Ribosomal Database Project) SeqMatch (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp). The thresholds used in the RDP search were as follows: both type and non-type strains, both uncultured and isolates, only good sequences >1200 nt and KNN = 1.

Biofilm microbiota study (NGS)

DNA and RNA (cDNA) from sink surface and coupon samples (described above) were used as templates for the NGS (MiSeq, Illumina Inc., San Diego, California, USA) analysis. A portion of the 16S rRNA gene spanning the variable region 4 (V4) was amplified using the barcoded, universal primer set (515F and 806R) (Caporaso et al. 2012). PCR mixture and thermal cycling conditions were the same as described by Caporaso et al. (2012). In addition to the experimental samples, the MiSeq run also contained a control library made from PhiX Control v3, which in this run accounted for 50% of reads. The library quantification and sequencing was performed by the Norwegian Sequencing Centre (http://www.sequencing.uio.no/). The sample pool was quantified using the Invitrogen Qubit, diluted to 2 nmol/L, and the MiSeq Protocol provided by Illumina was then followed.

The total number of sequences was 18 162 924. The forward and reverse reads were joined using the QIIME toolkit (Caporaso et al. 2010*b*) (version 1.7.0), and the barcodes corresponding to the reads that failed to assemble were removed. The total number of sequences that joined was 10 517 341, with an mean join length of 49.18. The sequences were then demultiplexed in QIIME allowing zero barcode errors and a quality score of 30 (Q30) resulting in 6 187 913 sequences with a median sequence length of 253 bp. The mean number of sequence per sample was 193 372 (min 160 167; max 226 801). Reads were assigned to their respective bacterial ID using 2-step open-reference operational taxonomic unit (OTU) picking workflow (Rideout et al. 2014). Briefly, after sequences were demultiplexed and quality filtered, reads were first clustered with a reference database (the Greengenes database (gg_13_5)) preclustered at 97% identity. Second, reads that did not group with any sequences in the reference collection were clustered de novo. Cluster-

ing at 97% identity was carried out using the UCLUST algorithm (Edgar 2010). Reads that did not match a reference sequence were discarded. Representative sequences were chosen for each OTU (cluster centroids) and aligned against the Greengenes core set with PyNAST (Caporaso et al. 2010*a*). Chimeric sequences were removed in QIIME using ChimeraSlayer. Singletons were removed resulting in 5 955 225 sequences. In total, 5661 OTUs passed the filter. Of these, 48% were 'novel' (i.e., not found in the Greengenes database (gg_13_5).

Statistical analyses

The α diversity (observed species) in all kitchens was calculated in QIIME by 100.000 rarefactions, and differences between groups were tested using paired *t* tests (Minitab® (Minitab 16.1.1, 2010 (Minitab Ltd., Coventry, UK)). The differences between mean bacterial counts were also tested using paired *t* tests (Minitab®).

The differences in microbiota were analyzed by principal component analysis (PCA) and 50–50 MANOVA (Langsrud 2002). 50–50 MANOVA is a method for multivariate analysis of variance (ANOVA) with a high number of collinear responses and was used to focus on partitioning the variation due to differences between kitchens, sink surface vs coupon, and DNA vs RNA, and on identifying the bacterial groups that are significantly different. All analyses were performed at the genus level (level 6 by taxa table from QIIME). 50–50 MANOVA was calculated in MATLAB (Release 2013b, The MathWorks, Inc., Natick, Massachusetts, USA) and the taxa were scaled to unit variance to remove abundance effects from the analysis.

Results

Total bacterial counts of coupons and sink surface swabs

The mean bacterial counts were significantly ($p < 0.001$) higher for coupons than for corresponding samples taken from the sink surface, with mean CFU of log 6.0 (±0.4) and log 4.8 (±0.8), respectively (Fig. 1). The bacterial counts on the coupons from all kitchens were similar (not significantly different between kitchens). On the other hand, the bacterial counts on the sink surface were significantly different ($p < 0.05$) among the different kitchens, and kitchen No. 1 had the highest CFU count. Figure 1 shows the mean CFU ($\log_{10}$) for coupons and sink surfaces (both 12 cm²) for all kitchens.

Microbiota (NGS)

Overall bacterial composition

The microbiota across all samples (DNA and RNA, coupons, and sink surfaces) was dominated by the phylum *Proteobacteria* (mean 54%), followed by the phyla *Actinobacteria* (34%), *Firmicutes* (8%), *Bacteroidetes* (2%), *Cyanobacteria* (1%), and *Fusobacteria* (0.2%). Most *Proteobacteria* belonged to the class *Gammaproteobacteria* (44%).

Fig. 1. Bar chart showing the mean colony-forming units (CFU) ($\log_{10}$) for coupons and sink surface samples (both 12 cm²) for 8 kitchens (Nos. 1–8). Each bar represents 3 replicates per kitchen (only 2 replicates for kitchen Nos. 1 and 8). The error bars are standard error of mean.

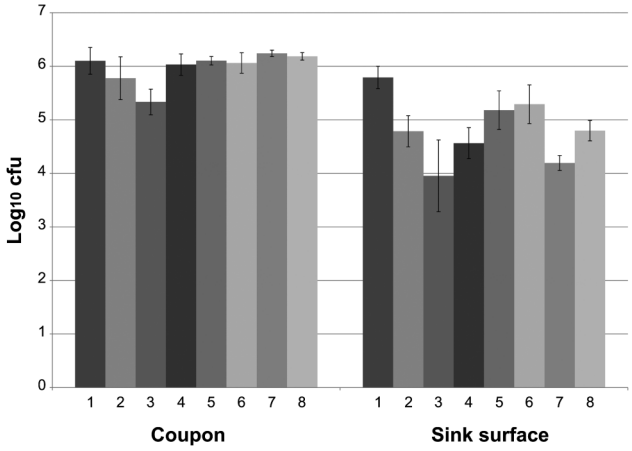


Figure 2 shows the mean relative abundances (percentage) of the dominant bacterial taxa (phylum and family level) for DNA (coupon and sink surface) and RNA (coupon and sink surface).

Overall the biofilm samples were dominated by 2 families: *Moraxellaceae* (genus *Moraxella*/*Enhydrobacter*) and *Micrococcaceae* (genus *Kocuria*). However, there were variations among the kitchens, and kitchens No. 7 and 8 had a different dominating bacterial population. The samples from the sink surface in kitchen No. 7 had a more diverse microbiota than the samples from the other kitchens and had high relative values of the families *Staphylococcaceae* and *Streptococcaceae* in addition to *Moraxellaceae* and *Micrococcaceae*, while the sink surface in kitchen No. 8 (DNA) was dominated by *Enterobacteriaceae*. Table 1 shows the distribution of taxa down to family level (represented above 5% in 1 or more samples) for all samples.

Bacterial diversity within samples

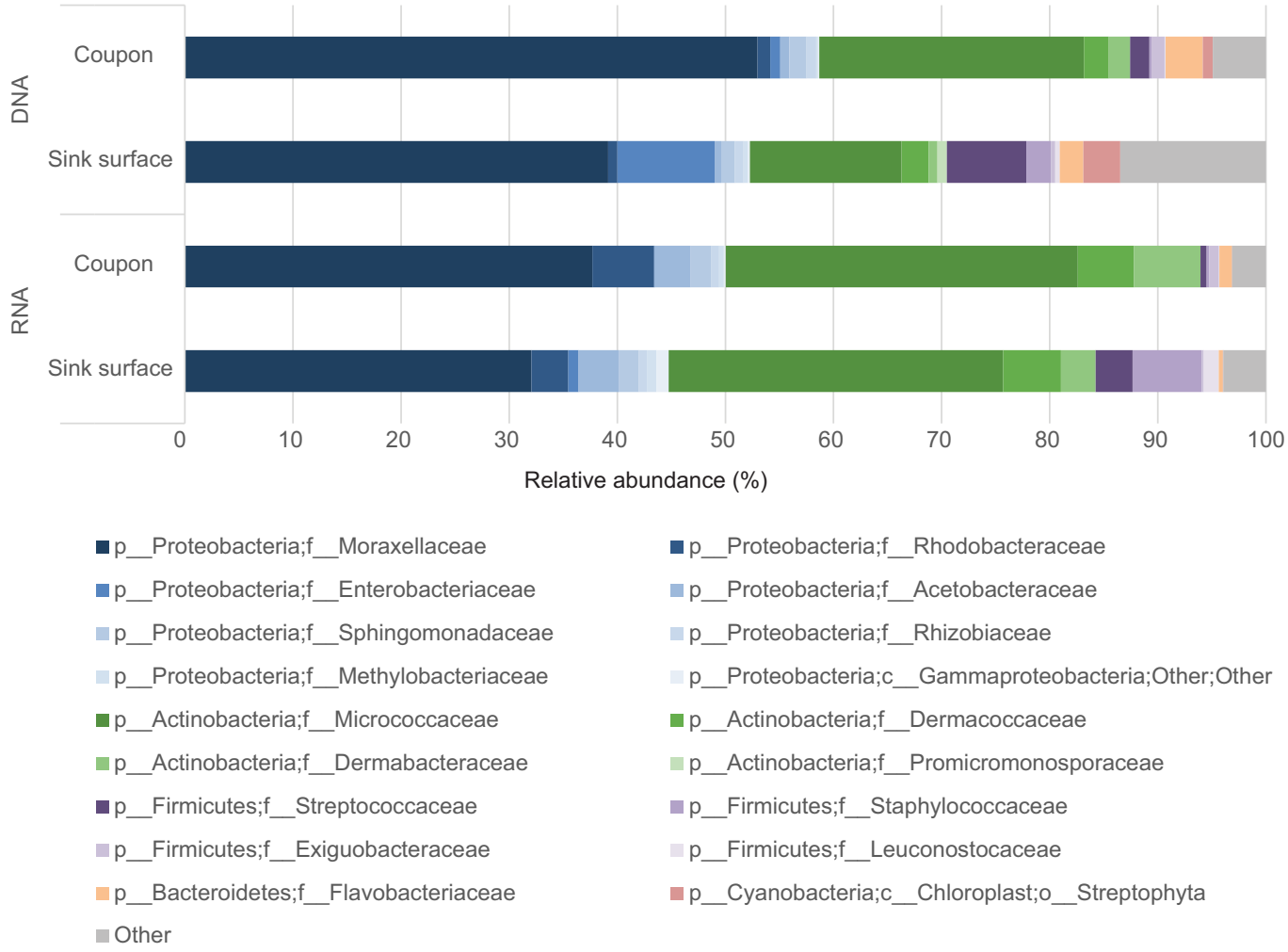
To investigate the bacterial diversity within the different samples, an α diversity analysis was performed (QIIME). This analysis revealed a tendency (not significant at 5% level) for higher diversity (observed species) in samples identified on the basis of DNA than RNA, and for sink samples than coupon samples.

Bacterial diversity between samples

To investigate the variation in bacterial composition between the samples, a β diversity analysis (weighted and unweighted unifrac) was performed (QIIME) (Fig. S1[1]). This analysis revealed that it was mainly the bacteria of low abundance that were responsible for the difference between the experimental variables (kitchens, RNA and

[1]Supplementary data are available with the article through the journal Web site at http://nrcresearchpress.com/doi/suppl/10.1139/cjm-2015-0562.

Fig. 2. Mean relative abundances (percentage) of the dominant bacterial taxa (phylum and family levels) for DNA (sink surface and coupon) and RNA (sink surface and coupon). Taxa represented above 5% in 1 or more samples are shown.



DNA, coupons and sink surface), and that the dominating microbiota was similar for most biofilms. Therefore, further statistical analysis was performed on standardized variables (to give equal weight to all OTUs regardless of abundance). This analysis revealed some significant differences in the bacterial composition between the experimental variables (kitchen (variable A), sink surface or coupon (variable B), and DNA or RNA (variable C)) (see Table 2).

The differences between kitchens accounted for the largest variation in the data, both with regard to main effect (38.2%) and interaction with sink surface or coupon (21.8%). The interaction means that there was a significant difference between sink surface and coupon but that the bacteria causing the difference were not the same for all kitchens. The variation due to differences between coupons or sink surface (5%) and DNA or RNA (11%) was small in comparison. Even if these effects were statistically significant, this indicates that the coupon was in practice quite representative for the sink surface and that the main results were similar on the basis of analyses for both RNA and DNA. The differences, however small, are illustrated in principal component (PC) plots in Fig. 3. From the scores plot (Fig. 3A), it is clear that there was a separation between RNA-based samples (green) and DNA-based samples (blue) along PC1 (explains 17% of the variance). Note also that the variation in microbiota in sink surfaces (outlined area) was larger than in coupons (filled area) and that this variation was larger in DNA- than RNA-based samples. This indicates that there was a systematic difference between sink surface and coupon for DNA but not for RNA. The loadings plot (Fig. 3B) shows the significant bacteria (determined from 50–50 MANOVA) as filled circles, and the circle size is proportional to abundance. The taxa of the bacteria significantly different in 1 or more sample categories are listed in the table in Fig. 3. From this, we can see that relative proportions of *Acinetobacter*, *Dermacoccus*, *Dermabacteraceae*, *Chryseobacterium*, *Streptophyta*, *Actinomycetales*, and *Comamonadaceae* were significantly different in the microbiota identified based on DNA than based on RNA, where the order *Actinomycetales* (including *Dermacoccus* and *Dermabacteraceae*) had a higher abundance in samples identified on the basis of RNA. There

Table 1. Relative abundance (percentage) of the dominant bacterial taxa (family level) across all samples (all taxa represented

	Actinobacteria				*Bacteroidetes*	*Cyanobacteria*	*Firmicutes*			
	Actinobacteria				*Flavobacteria*	*Chloroplast*	*Bacilli*			
	Actinomycetales				*Flavobacteriales*	*Streptophyta*	*Bacillales*	*Exiguobacterales*	*Lactobacillales*	
Kitchen No.	*Dermabacteraceae*	*Dermacoccaceae*	*Micrococcaceae*	*Promicromonosporaceae*	*Flavobacteriaceae*	—	*Staphylococcaceae*	*Exiguobacteraceae*	*Leuconostocaceae*	*Streptococcaceae*
DNA										
Sink										
1	0.3	3.1	33.6		1.8	0.4		2.8		3.7
2	0.1	0.1	3.1		4.2	4.3	0.7	0.4	0.5	3.5
3	3.5	1.0	9.8	0.1	1.0	0.1			0.3	4.7
4	0.1	11.9	5.8	6.7	6.4	7.2	0.3		0.5	2.9
5	0.2	0.3	17.3		2.3	5.5	1.3		0.2	4.6
6	1.7	0.5	12.6		0.6	8.0	1.0			12.8
7	0.1	0.3	10.1		0.8	1.0	14.3		0.2	24.3
8	0.5	2.9	19.9		0.4	0.7	0.5		1.5	2.4
Coupon										
1	1.1	3.4	8.7		4.7	0.2		5.9		3.3
2	1.2		10.7		9.3	0.6		3.4		0.4
3	4.4	3.5	25.7		0.4	0.1	0.1			0.1
4	0.5	5.3	15.3		5.7	2.8	0.1		0.1	2.0
5	0.1	0.4	23.5		2.2	2.4	0.3		0.1	1.1
6	4.6	1.0	45.0		0.9	1.0	0.3			2.9
7	0.7	0.5	27.6		0.9	0.1	1.0			4.1
8	3.2	4.4	39.2		3.2	0.1			0.3	0.5
RNA										
Sink										
1	1.3	8.0	56.8		0.2			1.2		0.7
2	1.2	0.2	17.2		0.2	0.2	2.3	0.4	0.4	0.6
3	18.1	2.1	18.2		0.2		0.1			0.1
4	1.1	24.1	15.2		1.9		0.8		1.1	4.4
5	0.1	0.5	33.6		0.1		1.9			1.8
6	2.0	0.5	3.0		0.3		2.4		0.1	2.9
7	0.8	1.0	26.8				41.8		0.2	14.7
8	1.1	6.4	49.5		0.1		1.8		9.2	2.1
Coupon										
1	4.6	12.4	15.2		1.5			5.2		0.8
2	7.0	0.1	27.5		2.2		0.1	2.0		0.1
3	13.9	5.0	31.6							
4	2.2	11.2	30.4		3.2		0.1		0.1	1.6
5	1.2	0.7	27.9		0.7		0.4			0.4
6	7.4	1.6	51.0		0.3		0.6			0.5
7	2.6	1.1	49.4				0.5			1.2
8	10.4	9.4	27.5		1.4				0.2	0.1

Note: The 2 overall dominating families are highlighted in gray.

were 3 bacterial taxa significantly different between coupons and sink surfaces (*Streptococcus*, *Chryseobacterium*, and *Exiguobacterium*), where *Streptococcus* had a higher abundance in sink surface samples.

Bacterial taxa from isolates plated from coupons

To get a more comprehensive overview of the viable population (bacteria growing aerobically on TSA) of the microbiota on the coupons, the identities of randomly selected isolates were determined (Table 3).

Results from NGS analysis based on RNA was used for comparisons with the microbiota determined from identification of plated bacteria as the former should in principle reflect the active part of the population. Both methods resulted in the same dominating families and genera; *Micrococcaceae* (genus *Kocuria*) and *Moraxellaceae* (genus *Moraxella*/*Enhydrobacter*). Bacteria belonging to the genera *Rhodococcus* (family *Nocardiaceae*), *Microbacterium* (family *Micrococcaceae*), and *Brevundimonas* (family *Caulobacteraceae*) were isolated from some coupons, but these genera were not found using NGS. NGS detected *Dermacoccaceae* (0.1%–12.4%) and *Rhodobacteriaceae* (0.1%–15.9%) from most coupons, but these families were not represented among the cultivated isolates.

above 5% in 1 or more samples).

Proteobacteria								
Alphaproteobacteria					*Gammaproteobacteria*			
Rhizobiales		*Rhodobacterales*	*Rhodospirillales*	*Sphingomonadales*	*Enterobacteriales*	Other	*Pseudomonadales*	
Methylo-bacteriaceae	*Rhizo-biaceae*	*Rhodo-bacteraceae*	*Aceto-bacteraceae*	*Sphingo-monadaceae*	*Entero-bacteriaceae*	Other	*Morax-ellaceae*	Other
	0.1	0.1	0.1	0.3	0.3		51.2	2.0
0.3	0.3	0.1	0.4	3.5	12.6	1.0	54.6	10.3
		0.6	0.3	0.5			73.2	4.9
0.1	2.1	0.4	0.4	1.1	1.1	0.3	33.3	19.5
2.1	3.1	5.5	2.7	3.8	0.7		39.0	11.4
0.1		0.1	0.7	0.1	0.4		50.6	10.8
0.5	0.8	0.1	0.1	0.7	4.4		5.8	36.4
			0.1	0.2	52.4	0.1	5.6	12.7
	1.1	0.6	0.2	0.9	0.7		62.9	6.4
0.2	1.3	0.5	0.3	2.6	0.3	0.6	60.4	7.8
0.2		1.3	0.7	0.5	1.0		59.2	2.7
	1.4	0.2	0.8	1.2	1.0		58.5	4.9
0.3	0.5	4.0	1.1	1.1	0.1		58.7	4.0
0.3	0.4	0.3	2.8	1.2	0.1		34.7	4.4
0.1	1.6			3.7	0.4	0.2	54.6	4.4
0.2	0.8	2.4	0.7	1.8	3.8	0.1	34.7	4.7
	0.1	0.4	0.6	0.2	0.1		28.1	2.2
0.6	0.1	0.6	8.4	10.1	0.4	8.5	44.5	4.2
0.1		3.4	9.4	0.5	0.1	0.1	46.7	0.9
0.5	5.2	4.5	2.2	1.2		0.4	31.1	6.2
5.3	0.5	17.7	7.8	0.9		0.1	27.7	2.0
0.1		0.1	1.6	0.1	0.1		58.3	1.5
0.3		0.1		1.2			4.2	8.7
0.1		0.5	0.2	0.3	6.9		15.9	5.9
	1.2	3.0	1.3	0.8	0.2		47.6	6.0
1.0	1.1	5.4	1.4	4.0	0.1	0.9	40.4	6.8
		8.4	5.1	0.5	0.1	0.1	34.2	1.1
0.1	1.5	0.8	3.5	0.4	0.1	0.1	41.9	2.8
0.8	0.3	10.7	3.3	0.5			51.2	1.7
0.3	0.5	1.1	8.5	0.8			24.4	3.0
0.6	0.7	0.1	0.2	7.6		0.2	34.9	0.9
0.7	0.7	15.9	2.7	0.9	0.1	0.1	27.2	2.6

Discussion

In the present study we investigated how the microbiota developing on stainless steel coupons placed in domestic kitchen sinks (stainless steel) compared with the natural microbiota of the kitchen sink surfaces. In addition, we compared the microbiota identified on the basis DNA with that identified on the basis of RNA to get a picture of the total (live and dead) microbiota and the potentially active microbiota, and also to see how well the microbiota identified on the basis of RNA reflected the microbiota that could be plated.

The results showed that the bacterial composition of coupons correlated well with the sink surface, with the best correlation resulting from microbiota based on RNA. The plating results showed higher bacterial counts on coupons than sink swabs. For some coupons, a visible fouling was observed at the lower parts, and that water attached to the fouling. This could produce a more humid environment with higher survival and growth of bacteria compared with the sink surface. The biofilms on the coupons were also younger (3 months) than those on the sink walls, and one cannot exclude the possibility

Table 2. Explained variance due to the different experimental variables (50–50 MANOVA).

Source	df	Explained variance (%)	No. of significant bacteria
Kitchen (A)	7	38.2***	9
Sink surface or coupon (B)	1	5.1***	3
DNA or RNA (C)	1	10.8***	7
A×B	7	21.8***	5
A×C	7	13.5 (ns)	0
B×C	1	2.3 (ns)	0
Error	7	8.2	

Note: The analysis is done on the 35 most abundant bacteria on genus level, and variables were standardized to remove abundance effects prior to the analysis. ***, significant $p < 0.001$; ns, not significant at 5% level.

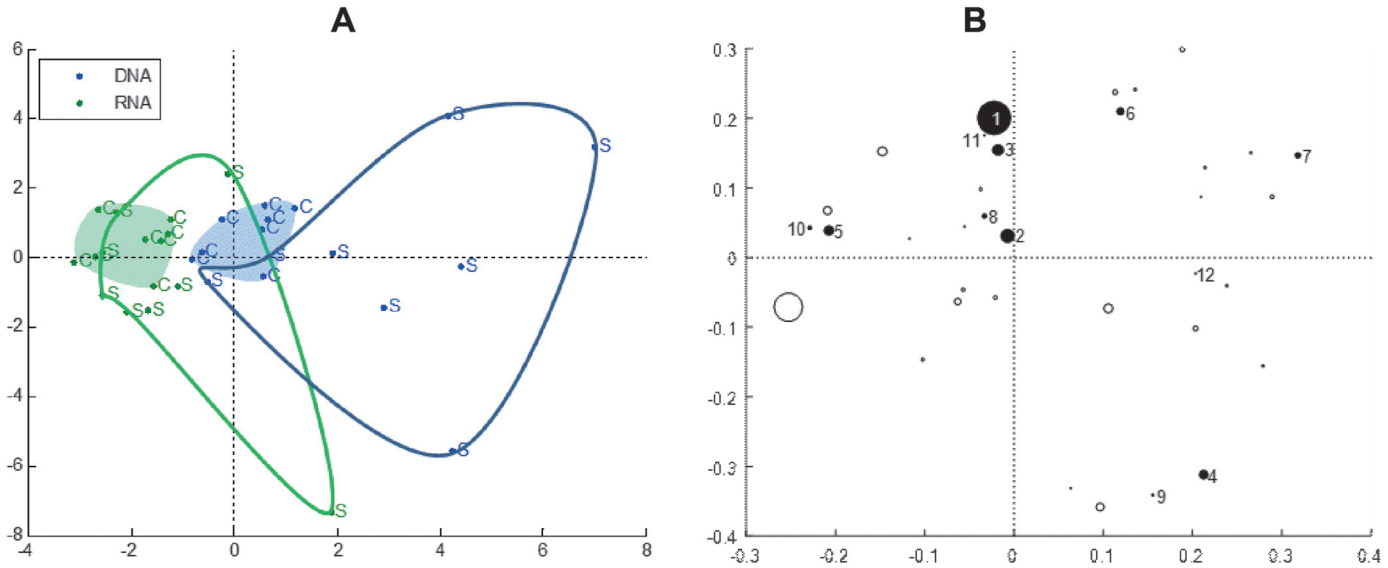
that in the quantitative analysis, a higher proportion of cells were detached from the coupons than from the sink surfaces, as it is known that mature biofilms are difficult to remove and require increased mechanical force, e.g., brushing rather than wiping. Further studies are needed to find the optimum attachment time and sampling method. Also, the chemical composition of the biofilm was not assessed, and thus structural and chemical differences between biofilms of the sink surface and coupons cannot be excluded. However, the selective pressure, for example long periods of drying (during the working day and night), was still quite similar for coupons and the sink surface, as the dominating microbiota was not systematically different.

Overall, the majority of the biofilms were dominated by *Moraxellaceae* (genus *Moraxella*/*Enhydrobacter*) and *Micrococcaceae* (genus *Kocuria*). This is in accordance with what has been found by others, although there are variations between studies. The microbiota in domestic kitchen sinks has been studied in some detail by Flores et al. (2013), where sink samples from 4 kitchens were investigated together with over 80 other kitchen surfaces. In comparison with the other surfaces, Flores et al. (2013) found the least diverse communities associated with metallic surfaces in and around sinks, which were dominated by biofilm-forming Gram-negative bacteria, including known biofilm-formation organisms like *Sphingomonadaceae*. They also found *Moraxellaceae* to be the dominating family in sink basin and sink backsplash. In another study on common household surfaces, Saha et al. (2014) found that *Kocuria* spp. were among the most frequently recovered isolates and the most frequently recovered isolates from kitchen sinks, and Stellato et al. (2015) found *Kocuria* spp. in all sink samples belonging to the pre-processing zones in a cooking center for hospital foodservice.

The genus *Enhydrobacter* has been found in widely diverse environments like athletic equipment (Wood et al. 2015), skin (buttocks) (Zeeuwen et al. 2012), toilet samples (Jeon et al. 2013), and a beer bottling plant (Timke et al. 2005). A search in BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) revealed a 100% match to both *Moraxella osloensis* and *Enhydrobacter aerosaccus* for the OTU and isolates representing genus *Enhydrobacter* in our study. Near-full-length 16S rRNA gene sequences of 1 random isolate (classified as genus *Enhydrobacter*) confirmed that our isolates were most similar to these 2 species (99% identity, data not shown). Both *Moraxella osloensis* and *Enhydrobacter aerosaccus* have been found in skin microbiota (Gao et al. 2007; Jeon et al. 2013). *Moraxella osloensis* has also been found in the biofilm of various pipe materials in drinking water distribution systems (Zhu et al. 2014) and is the bacterium responsible for the locker-room smell or shower-curtain odor (Kubota et al. 2012). *Moraxella* spp. was also identified as a part of the microbial population on stainless steel coupons placed in fish and shrimp factories for a 3 month period (Guobjornsdottir et al. 2005), but not as the dominant genus. *Moraxella* is neither associated with food-borne infections or spoilage. The best sequence match for the OTU and isolates representing the genus *Kocuria* was *K. rhizophila* (confirmed by near-full-length 16S rRNA gene sequencing of a few random isolates). Members of the genus *Kocuria* have been isolated from a wide variety of natural sources, including mammalian skin, soil, the rhizosphere, fermented foods, clinical specimens, and freshwater and marine sediments. The genus has also been isolated from other food production environments (Carpentier and Chassaing 2004; Møretrø et al. 2011, 2013). Survival in these environments can be explained by resistance to desiccation, biofilm-forming abilities, and tolerance to chlorine (Leriche et al. 2003; Møretrø et al. 2013). Others have shown that *K. rhizophila* can survive on dry surfaces for several days and has tolerance to high-salt concentrations in growth medium (Kovacs et al. 1999; Kim et al. 2004). *Kocuria* spp. is not considered to be pathogenic, but in a study on bacteria surviving cleaning and disinfection in food processing plants, a *Kocuria varians* strain increased biofilm production in *Listeria monocytogenes* (Carpentier and Chassaing 2004). However, further analyses are needed if one wants to determine if our isolates represent a threat for safety.

As expected, most of the variation in the microbiota was related to different kitchens and not to the sampling site (i.e., coupon or sink surface). This variance is likely to be associated with specific selective characteristics such as physical cleaning regimes, food preparation regimes, and water availability. In a kitchen sink environment, high loads of organic particulate matter such as fats and proteinaceous material represent a source of nutrients for attached and (or) transient microorganisms. The different kitchens would also have been exposed to different sources of bacteria from raw produce, different microbiota of the residents' skin, differences in the faucet water (5 of the 8 kitchens had different water sources (all public water sources); kitchen Nos. 1 and 4 had the same water source, and kitchen Nos. 5, 6, and 8 had

Fig. 3. Overview of results from principal components analysis and 50–50 MANOVA. The scores plot (A) shows the distribution of samples, where labels S and C correspond to sink surface (outlined area) and coupon (filled area), and colors correspond to DNA (blue) and RNA (green). The loadings plot (B) shows the bacteria significantly different between 1 or more sample categories (determined from 50–50 MANOVA) as filled circles, and the circle size is proportional to relative abundance. The corresponding table shows which bacteria were significantly different ($p < 0.05$) between the sample categories: kitchens (A), sink/coupons (B), DNA/RNA (C), and interaction between A×B. One of the dominating operational taxonomic units (affiliated with *Enhydrobacter*) had a statistically significant interaction between sink surface or coupon and kitchen. This means that there was a significant difference in the relative amount of this bacteria between sink surfaces and coupons but that this difference was not systematic, that is the relative amount was sometimes higher in sink surface than coupon and vice versa. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns, not significant.



	Bacterial taxa	Kitchen (A)	Sink/Coupon (B)	DNA/RNA (C)	A×B
1	Proteobacteria;Gammaproteobacteria;Pseudomonadales;Moraxellaceae;Enhydrobacter	*	ns	ns	*
2	Proteobacteria;Gammaproteobacteria;Pseudomonadales;Moraxellaceae;Acinetobacter	***	ns	*	ns
3	Actinobacteria;Actinobacteria;Actinomycetales;Dermacoccaceae;Dermacoccus	*	ns	*	ns
4	Firmicutes;Bacilli;Lactobacillales;Streptococcaceae;Streptococcus	**	**	ns	**
5	Actinobacteria;Actinobacteria;Actinomycetales;Dermabacteraceae;Other	*	ns	*	ns
6	Bacteroidetes;Flavobacteriia;Flavobacteriales;Flavobacteriaceae;Chryseobacterium	**	*	**	ns
7	Cyanobacteria;Chloroplast;Streptophyta	ns	ns	*	ns
8	Firmicutes;Bacilli;Exiguobacterales;Exiguobacteraceae;Exiguobacterium	***	*	ns	*
9	Firmicutes;Bacilli;Lactobacillales;Enterococcaceae;Enterococcus	**	ns	ns	*
10	Actinobacteria;Actinobacteria;Actinomycetales;Other;Other	*	ns	***	ns
11	Proteobacteria;Alphaproteobacteria;Rhizobiales;Other;Other	ns	ns	ns	**
12	Proteobacteria;Betaproteobacteria;Burkholderiales;Comamonadaceae;Other	ns	ns	**	ns

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Table 3. Relative abundance (percentage) of the different bacterial taxa (genus level) characterized from isolates cultured from

		Actinobacteria				*Bacteroidetes*	
		Actinobacteria				*Flavobacteria*	*Sphingobacteria*
		Actinomycetales				*Flavobacteriales*	*Sphingobacteriales*
		Nocardiaceae	*Dermabacteraceae*	*Microbacteriaceae*	*Micrococcaceae*	*Flavobacteriaceae*	*Sphingobacteriaceae*
Kitchen No.	Coupon No.	*Rhodococcus*	*Brachybacterium*	*Microbacterium*	*Kocuria*	*Chryseobacterium*	*Sphingobacterium*
1	3 (n = 6)	17		17	17		
	6 (n = 6)	17		67	17		
2	2 (n = 7)		14		29		
	4 (n = 4)		50				
	5 (n = 6)			33	50		
3	2 (n = 7)				43		
	4 (n = 7)				14	14	
	6 (n = 4)			25	50	25	
4	2 (n = 5)				40		
	3 (n = 5)				20		
	5 (n = 6)	17					17
5	2 (n = 7)				29		
	3 (n = 5)				20		
	6 (n = 4)				50		
6	2 (n = 5)		40	20	20		
	4 (n = 4)				100		
	6 (n = 4)		50		25		
7	2 (n = 3)				67		
	4 (n = 6)				83		
	6 (n = 3)						
8	2 (n = 2)		50				
	6 (n = 7)		29		71		

Note: Total number of sequenced isolates was 113. The 2 overall dominating families and (or) genera are highlighted in gray. The "n" is the

the same water source). Flores et al. (2013) identified 3 indicator taxa from raw produce (*Enterobacteriaceae*, *Microbacteriaceae*, and *Bacillales*), 4 from the human skin (*Propionibacteriaceae*, *Corynebacteriaceae*, *Staphylococcaceae*, and *Streptococcaceae*), and 3 from the faucet water samples (*Sphingomonadaceae*, *Methylobacteriaceae*, and *Gallionellaceae*). Two of the indicator taxa from human skin (*Staphylococcaceae* and *Streptococcaceae*) were major taxa in 1 of the kitchens (No. 7) in our study. Further studies are needed to demonstrate the effect of differential usage of the sink.

We found the variation in the microbiota on the coupons to be smaller than the variation on sink surfaces from the different kitchens. One theory could be that all the coupons were new and of the same steel quality, whereas there was greater variation among the sink surfaces attributable to different age, different manufacturer, and different history of usage. More differences were also found between microbiota on coupons and sink surfaces when using results derived from DNA than from RNA, indicating differences in the dead population of cells. This was not surprising, since the dead-cell population will reflect the part of the population not selected for survival and this may be different for a surface exposed to bacteria for years compared with coupons that had been placed in the sink for a 3 month period. RNA was chosen to illustrate the active taxa, since cellular rRNA concentration is generally well correlated with growth rate and activity (Poulsen et al. 1993; Bremer and Dennis 1996). There are several studies that have used rRNA to characterize the growing or active microbes. For example, Blazewicz et al. (2013) found >100 studies that used rRNA for these purposes, including recent studies using rRNA to identify currently active microbes (e.g., Gentile et al. 2006; DeAngelis et al. 2010; Jones and Lennon 2010; Gaidos et al. 2011; Lanzen et al. 2011; Wust et al. 2011; Brettar et al. 2012; Mannisto et al. 2013). However, Blazewicz et al. (2013) argued that there are conflicting patterns between rRNA content and growth rate, indicating that rRNA is not a reliable metric for growth or activity, and suggested instead to employ rRNA abundance data as an index of potential activity that provides the basis for further investigations. Recognizing that the RNA-derived identification of microbiota reflects past, current, and future activities in addition to different life strategies, we cannot conclude that the microbiota illustrated based on RNA reflect the true viable, active bacteria. However, the fact that the microbiota on coupons and sink surfaces correlated better when its identification was derived from RNA than from DNA, and that systematic differences between coupons and sink surfaces were not found in RNA-based samples, indicate that

coupons (partial 16S rRNA gene).

Proteobacteria								
Alphaproteobacteria			*Gammaproteobacteria*					
Caulobacterales	*Rhizobiales*	*Sphingomonadales*	*Enterobacteriales*		*Pseudomonadales*			
Caulobacteraceae	*Rhizobiaceae*	*Sphingomonadaceae*	*Enterobacteriaceae*		*Moraxellaceae*		*Pseudomonadaceae*	
Brevundimonas	*Rhizobium*	*Sphingomonas*	*Enterobacter*	Unclassified_ *Enterobacteriaceae*	*Acinetobacter*	*Moraxella/ Enhydrobacter*	*Pseudomonas*	
50						57		
				25		25		
						17		
		14				43		
	14	14				43		
20						40		
20						60		
					50		17	
14						57		
						80		
						50		
20								
						25		
	33							
						17		
		67				33		
			50					

number of isolates or sequences per coupon.

RNA gave the best picture of the dominating, active microbiota in our study. To investigate this further, we also identified a random selection of isolates plated from the coupons. The plating results showed a high number of cultivable bacteria, and although some differences in the microbiota were observed, the dominating taxa were similar to the NGS result. One must have in mind that only a few isolates were analyzed compared with the high-throughput results from the NGS analysis. The plating results are also likely to be influenced by the use of a single culture agar, which is unlikely to meet the nutritional requirements necessary to maximize the recovery of all the bacteria present. The NGS results could also have been influenced by the choice of PCR primers and PCR conditions. For example, *Microbacterium* (family *Micrococcaceae*) was only detected by plating as also previously reported by Brightwell et al. (2006). Our study clearly shows that both culture-independent and culture-dependent techniques are important to give the best representations of the microbiota in domestic kitchen sinks.

The results presented show that stainless steel coupons are suited to model the active and dominating microbiota of the domestic kitchen sink surface, although the coupons in general had a higher microbial load. Such coupons therefore are suited for further studies, e.g., the effects of hygienic procedures (Røssvoll et al. 2015). The methodology could also be developed for use in other environments and could potentially be used to study the ability of pathogens to attach to a biofilm produced in situ, an experiment that would not be feasible to perform in food-processing environments or in the domestic environment, for example. Sampling of the surrounding surfaces, however, should always be performed as a control. We have also shown that the choice of nucleic acid template will influence the results and that care should be taken with respect to interpretation of bacterial activity.

Acknowledgements

The authors wish to thank The Norwegian Sequencing Centre for their assistance. We thank Janina Berg, Signe Marie Drømtorp, Merete Rusås Jensen, and Anette Wold Åsli for excellent technical assistance. This work was supported by a grant (185143/I10) from the Norwegian Research Council and grants from the Research Funding for Agriculture and Food Industry (project No. 224921/F40).

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