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Study on microbial communities in domestic kitchen sponges: Evidence of *Cronobacter sakazakii* and Extended Spectrum Beta Lactamase (ESBL) producing bacteria

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Abstract

Domestic environment, in particular, kitchen setting is a well-established source of microbial contamination. Kitchen sponges represent an important vehicle of microbial transmission and maintenance of spoilage bacteria and pathogenic strains responsible for food borne diseases. The aim of this study was to evaluate the microbial communities of 100 'in-use' kitchen sponges, improving the knowledge on their role in cross-contamination in domestic environment and transmission of ESBL-producing strains. Sponges were processed for: aerobic mesophilic bacteria (AMB), *Enterobacteriaceae* (EB), yeasts and molds (YM), coagulase-positive staphylococci (CPS), micrococci (MCC), anaerobic sulfite reducing bacteria (ASR), and for the detection of *Listeria monocytogenes*, *Salmonella* spp. and *Yersinia enterocolitica*. A total of 309 enterobacteria strains were identified and then processed for ESBL (Extended Spectrum Beta Lactamase) phenotypical expression. A high contamination level of kitchen sponges was observed (mean value AMB 8.25±1.1; EB 5.89±1.2; YM 5.57±1.1; MCC 4.82±0.1 log CFU/g). Identified enterobacteria strains revealed several opportunistic and pathogenic agents such as *Enterobacter cloacae* (28%), *Citrobacter freundii* (23.3%), *Cronobacter sakazakii (14.6%)* and other strains in lower percentage. *Listeria monocytogenes* was found in only one sponge (1%). A total of 69 (22.3%) enterobacteria resulted ESBL+, with the following prevalence: *P. rettgeri* (50%), *L. adenocarboxilata* (30%), *K. pneumoniae* (25%), *K. oxytoca* (25%), *C. sakazakii* (20%), *E. cloacae* (20.7%), *C. freundii* (20.1%). Results confirm the potential role of kitchen sponges as vehicle for food-borne pathogens such as, *C. sakazakii* for the first time, infectious agents and spoilage microorganisms. The observed high contamination level and the presence of several ESBLs opportunistic pathogens, stresses the necessity to improve a proper education of the consumers on the effective treatment to reduce their microbial loads.

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Introduction

In the last few years, numerous studies indicate that several food-borne diseases are related to domestic infection sources (EFSA, 2015). Improper food handling and un-hygienic practices are considered the major factors in foodborne illness episodes. Cross-contamination in household kitchens represent another important domestic source of infection (Azevedo *et al*., 2014). However, consumers risk perception of food borne illness in home environment is reported as very low (European Commission, 2016). Kitchens are included among the most contaminated domestic environments, even more of some bath's areas (toilette seat, door knob, light switch, toilet handle) or commonly used objects such as pen, keys, cellular phone, keyboards, etc. (Donofrio *et al*., 2012). Furthermore, 37.3% of food borne outbreaks in EU in 2014, founded their infection sources in home environments (EFSA, 2015). This evidence is confirmed by the frequent isolation of food-borne pathogens in tools, cloths, towels, sponges and kitchen surfaces (Mattick *et al*., 2003). Among these, dish sponges were the most contaminated item in the household, and deserve great attention, for their potential role as vehicle for foodborne pathogens (Donofrio *et al*., 2012). Their role as microbiological hot spots in domestic settings was recently well established (Cardinale *et al*., 2017). They are, indeed, frequently used not only to clean dishes and cookware, but also, different surfaces or even refrigerators shelves, increasing the risk of cross-contamination (Catellani *et al*., 2014). Thanks to their large surface/volume ratio, their constant humidity and the nutrients for bacterial growth contained, sponges are, indeed, an ideal habitat for microorganisms (Cardinale *et al*., 2017). Several authors have investigated the microbiological quality of sponges used in domestic kitchens, reporting a high-level contamination and the frequent isolation of pathogens, such as *Salmonella* spp., *Staphylococcus aureus, Campylobacter* spp. and *Listeria monocytogenes* (Hilton and Austin, 2000).

These findings induce much more concern, considering that common dishwashing soaps or chemical compounds do not reduce significantly microbial load in kitchen sponges (Sharma *et al*., 2009). Moreover, multi-drug resistance bacteria were common in household's environment, and no significant differences were noted between biocide users and non-users; as well as the frequency of pathogen recovery (Marshall *et al*., 2012). Among this, ESBLs (Extended Spectrum Beta Lactamase) are a group of evolving enzymes that are able to hydrolyze extended spectrum cephalosporin. ESBL producing enterobacteria are a wide range of resistant strains that have gained much more importance in public health in the recent years. Infections due to ESBL producers range from uncomplicated urinary tract infections to life-threatening sepsis representing a serious challenge for clinical treatments. It is well established the role of animals and derived products as a source of diffusion of resistant strains (Beninati *et al*., 2015; EFSA, 2011) For all these reasons, the aim of the present study was to improve the knowledge on the microorganisms frequently involved "in-use" kitchen sponges' colonization, and their role in maintaining and diffuse ESBL-producing organisms in domestic environment.

Materials and Methods

Samples collection and macroscopic evaluation

A total number of 100 sponges from domestic kitchens was collected within 24 hours from the last use and transported to our laboratory in sterile sampling bags. All processed sponges were characterized by a rough thin layer (scrub pad) covering a spongy synthetic material and by the following dimension 120 ± 20 mm (h) × 80 ± 10 mm (w) × 20 ± 10 mm (d). Samples were also macroscopically evaluated by 3 members of the staff of Laboratory of Animal Origin (Department of Veterinary Sciences, Messina, Italy) and classified by an increasing scoring from 1 to 3 for the following parameters: i) consumption (1: like new; 2: normal consumption; 3: excessive consumption); ii) dirt (1: like new; 2: slight color change; 3: intensive blackening) and iii) presence of food debris/extraneous particles (1: from 0 to 2; 2: from 3 to 5; 3: more than 5). For each sample, the days of actual domestic use were recorded.

Microbiological analysis

For quantitative determinations, a total amount of 3±0.5 g from each sponge was collected and transferred to a stomacher bag and buffered peptone water (Biolife, Milan, Italy) was added with a ratio of 1:9 (w/v). The suspension was then homogenized for 60 s at 230 rpm in a peristaltic homogenizer (Stomacher 400 Circulator, Seward, UK) and 1:10 serial dilutions of the homogenate in BPW were prepared. Each sample was then processed for the following determinations: i) aerobic mesophilic bacteria count (AMB) according to UNI EN ISO 4833-1: 2013; ii) *Enterobacteriaceae* count (EB) according to UNI EN ISO 21528-2: 2017; iii) yeasts and molds count (YM) according to UNI EN ISO 21527-2: 2008; iv) Coagulase-positive staphylococci (CPS) and Micrococci (MCC) count according to UNI EN ISO 6888-1:2004; v) *Salmonella* spp. detection according to UNI EN ISO 6579-1: 2017; vi) *Listeria monocytogenes* detection according to UNI EN ISO 11290-1: 2017; vii) detection of presumptive pathogenic *Yersinia enterocolitica* according to UNI EN ISO 10273:2003; viii) anaerobic sulfite reducing bacteria count (ASR) on sulfite-polymyxin-sulfadiazine (SPS) agar (Biolife, Milano, Italy) incubated under anaerobic conditions at 37°C for 24h.

Enterobacteria strains identification

A total of 309 confirmed enterobacteria colonies, collected from the required five colonies from the highest dilution for each sample according to ISO UNI EN ISO 21528-2: 2017, were sub cultured on Tryptic Soy Agar (TSA) plates with 5% sheep blood (Biolife, Milano, Italy), then incubated at 37°C for 24h. Isolated colonies were then identified by Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS). Single cells were selected and directly transferred as a thin film on the 48-well sample plate and overlaid with 1 µL of matrix solution (saturated solution of alfa-cyano-4-hydroxycinnamic acid in 50% acetonitrile, and 2.5% tri-fluoracetic acid). *E. coli* ATCC 8739 was used as standard and loaded in specific control wells. After the crystallization of the matrix and microbial material, the metal plate was introduced in the mass spectrometer Vitek MS, (bioMérieux, Firenze, Italy) and was bombarded with brief laser pulses. MALDI-TOF generates unique MS signatures (spectra) for microorganisms, that were transferred into the AgnosTec-SARA-MIS software (Spectral Archive and Microbial Identification System) (bioMérieux, Firenze, Italy) were they were compared to the database containing the reference spectra of common bacteria.

ESBL phenotypic expression

Screening test

To evaluate the presence of presumptive ESBL producing bacteria, all 309 strains were tested with a chromogenic media, containing a mixture of antimicrobic and chromogenic substances that allow the growth of ESBL producing strains with a specific color (Chromatic™ ESBL, Liofilchem, France). Considering that only few species (*E. coli*, *Klebsiella* spp., *Enterobacter* spp., *Serratia* spp., *Proteus* spp.) have been checked by the producer for the specific coloration expressed we confirmed for ESBL also the strains that showed a not colored growth. Each sample was spread on the surface of a dried media plate and incubated for 24h at 37°C.

Confirmation test: combination disk assay

To confirm phenotypic expression of ESBL producing strains, the combination disk assay was carried out. As proposed by Luzzaro *et al.* (2007), the combination assay consists in testing a β-lattamic alone and in the presence of an inhibitor of the β-lactamase, in order to evaluate the recovery activity of the antibiotic in the presence of the inhibitor. At this purpose, each sample that resulted positive for the screening test, was sub cultured in Brain Heart Infusion Broth (BHIB, Biolife, Italy) and incubated at 37°C for 24h. The overnight cultures were spread on the surface of Mueller-Hinton agar plates (MH, Biolife, Italy). Then antibiotic disk (6 mm Ø) of Cefotaxime (CTX) 30 µg, Cefotaxime + Clavulanic acid (CTL) 40 µg, Ceftazidime (CAZ) 30 µg and Ceftazidime + Clavulanic acid (CAL) 40 µg (Liofilchem, France) were placed on the inoculated medium. After incubation at 37°C for 24 h, the inhibition diameters were calculated. For each tested antibiotic, the ESBL production was considered positive if the inhibitory diameter was ≥5 mm, compared to the β-lactam tested alone according to CLSI method (2016).

Data analysis

Least squares linear and multiple regression analysis were performed to estimate the influence of microbiological parameters loads on AMB count and coefficients of determination (R^2 and adjusted R^2) were calculated to estimate the strength of the relationship between our models and the observed data. Spearman's rank correlation coefficient (r_s) was estimated to verify relationships between macroscopic scoring and microbiological parameters. F-test was performed in order to estimate significative associations. Significance level was assumed as $P<0.05$. Microbial loads were converted in log CFU/g to facilitate the expression of results.

Results

Macroscopic evaluation scoring

Results are showed in Table 1. Considering consumption level, 40.0% of samples revealed the maximum score 3 conditions, showing in some cases the lack of the superficial scrub pad and/or the spongy tissue. For dirt scoring, 34.0% of

Table 1. Sample distribution on macroscopic observations scoring.

Parameter	Score, %		
	1	2	3
Consumption	17.0	43.0	40.0
Dirt	14.0	52.0	34.0
Food debris/Extraneous materials	55.0	30.0	15.0

Consumption: (1) like new; (2) normal consumption; (3) excessive consumption. Dirt: (1) like new; (2) slight color change; (3) intensive blackening. Food debris/extraneous particles: (1) from 0 to 2 units; (2) from 3 to 5 units; (3) more than 5 units.

processed sponges achieved the higher score 3. Those samples resulted, indeed, very soiled and/or with an intensive blackening of the spongy material. Some samples presented an overall low level of dirt/consumption but a consistent presence or foreign particles, which were minimally represented by papers fragments or organic material and largely by human or pets' hairs. Spearman's Rank correlation coefficient r_s=0.77 attests a strong and significative (P<0.05) relationships between consumption and dirt. Nevertheless, only 15.0% of samples showed more than five extraneous particles and/or food debris. No significative relationship (P>0.05) was reported between this parameter and the previous macroscopic observation. Considering the period of actual utilization, 30% of sponges were used from 5 to 15 days, another 30% from 15 to 30 days and the last 40% for a period greater than 30 days. It is interesting to highlight that no significative relationships (P>0.05) were observed between the period of utilization and the other macroscopic observations.

Microbiological results

Table 2 summarized microbiological results. The 15.0% of samples presented AMB loads ranging from 5.0 to <7.0 log CFU/g, while the remaining 85.0% reported values from 7.0 to <10.0 log CFU/g (Figure 1). The poor hygienic condition was also assessed by the detection of EB in all the samples. EB count showed a wide distribution range (Figure 1) from 3.0 to <6.0 log CFU/g in 50% of samples and the remaining 50% from 6.0 to <9.0 log CFU/g. MCC and YM were isolated in all the samples with loads ranging from 2.0 to <8.0 log CFU/g and from 3.0 to <8.0 log CFU/g respectively (Figure 1). CPS and ASR bacteria were occasionally isolated in 11% samples with mean value of 3.29±0.4 and 1.68±0.8 CFU/g respectively. Multiple regression analysis revealed a significative (P<0.05) influence of EB, MCC and YM on AMB loads. Linear regression test revealed that each of these parameters was significative (P<0.05) related to AMB. No significative (P>0.05) association was observed among CPS *vs* AMB and ASR *vs* AMB.

The association between AMB and EB, MCC and YM have been expressed in statistical models by fitting a linear equation to observed data, in which AMB and the other parameters were considered as dependent and independent variable respectively. According to our statistical models, EB have a considerable influence on AMB (45%) (Figure 2A). MCC loads influenced

Table 2. Microbiological loads and prevalence in kitchen sponges.

Parameter	log CFU/g	Prevalence,%
Aerobic mesophilic bacteria count	8.25±1.1	100
Enterobacteriaceae count	5.89±1.2	100
Micrococci	4.82±1.1	100
Yeasts and Molds count	5.57±1.2	100
Coagulase-positive staphylococci	3.29±0.4	11.0
Anaerobic sulfite reducing bacteria count	1.68±0.8	11.0
Listeria monocytogenes	+	1.0
*Salmonell*a spp.	-	0
Yersinia enterocolitica	-	0

+ detected; - not detected.

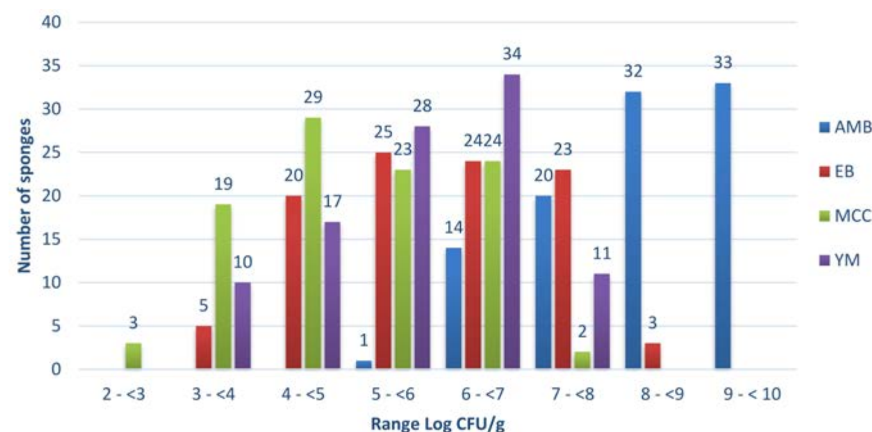

Figure 1. Distribution of Aerobic Mesophilic Bacteria (AMB), *Enterobacteriacea*e (EB), Micrococci (MCC), Yeast and Mold (YM) loads in 100 kitchen sponges.

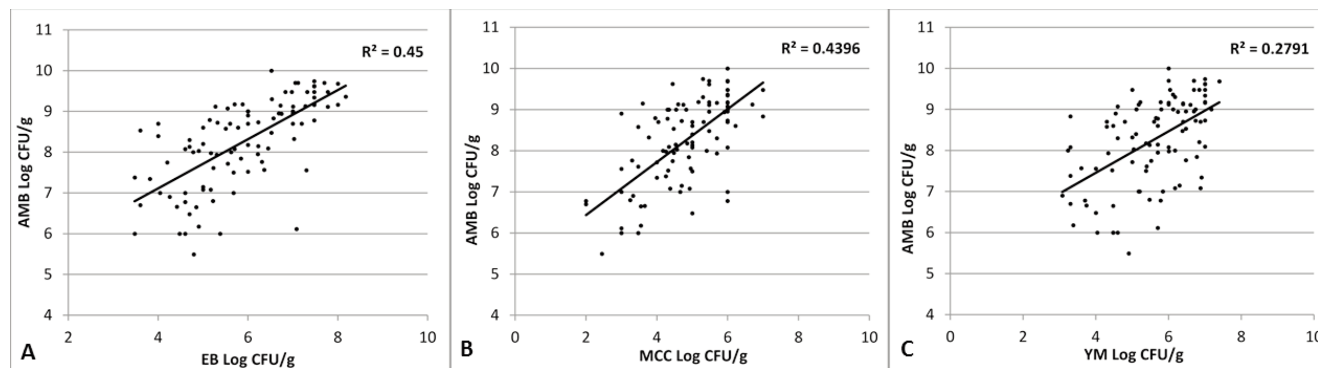

Figure 2. Linear regression scatter plots, trend lines and R2 values, relating: A) *Enterobacteriaceae* count (EB) to Aerobic Mesophilic Bacteria count (AMB); B) Micrococci count (MCC) to Aerobic Mesophilic Bacteria count (AMB); C) Yeast and Mold count (YM) to Aerobic Mesophilic Bacteria count (AMB).

AMB variance for 44.0% (Figure 2B), while YM gave a less contribution (28.0%) (Figure 2C), but were constantly detected and can be considered as characterizing microbiota within the already mentioned parameters. No significative (P>0.05) relationships were detected between microbiological loads (AMB and EB) and macroscopic scoring including the period of usage.

Listeria monocytogenes was detected only in one sample characterized by: AMB 9.7 log CFU/g; EB 7.1 log CFU/g; MCC 5.5 log CFU/g; YM 6.7 log CFU/g; ASR 3.6 log CFU/g. Moreover, the sample with *Listeria monocytogenes* was classified as score 3 for dirt and 2 both for consumption and presence of particles. No *Salmonella* spp. and *Yersinia enterocolitica* were found.

Enterobacteria identification

The identification of enterobacteria, which, as reported, are the predominant flora in kitchen sponges, showed a limited variability of strains identified. On the 309 strains, in fact, 87 were identified as *Enterobacter cloacae* (28.2%), 72 as *Citrobacter freundii* (23.3%), 48 as *Klebsiella oxytoca* (15.5%), 45 as *Cronobacter sakazakii* (14.6%), 30 as *Leclercia adecarboxylata* (9.7%), 12 as *Klebsiella pneumoniae* (3.9%), 6 as *Providencia rettgeri* (1.9%), 6 as *Serratia liquefaciens* (1.9%) and finally 3 as *Acinetobacter johsonii* (1.0 %) (Figure 3).

ESBL phenotypical expression

A total of 69 (22.3%) among 309 tested strains resulted positive for ESBL phenotypical test. The chromogenic media employed revealed a high rate (78%) of false-positive ESBL strains, probably due to an over-production of cephalosporinases or chromosomal penicillases, by some *Enterobacteriaceae*. Moreover, strains belonging to genus *Citrobacter*, *Leclercia*, *Acinetobacter* and *Providencia* showed growth with variable colorations (green, blue, pink) for the same strain, whether or not they were confirmed as ESBL producers. The confirmation test reported the following prevalence of resistance: *P. rettgeri* (50%); *L. adenocarboxilata* (30%); *K. pneumoniae* (25%); *K. oxytoca* (25%); *E. cloacae* (20.7%); *C. freundii* (20.1%); *C. sakazakii* (20%) (Figure 3). No ESBLs were reported among *S. liquefaciens* and *A. johsonii.*

Discussion

The macroscopic results reported in our sponges confirm the low consumers risk perception of food borne illness related to cross-contamination in home environment (European Commission, 2006). The presence of foreign particles such as human or pets' hairs, in fact, underlines the promiscuous use of sponges in real-life domestic conditions, like the cleaning of pet's bowls or other tools and surfaces non-related to kitchen environment. The microbiological data of this study confirms a great level of sponge contamination and poor hygienic conditions, as observed by other authors (Azevedo *et al.*, 2014; Mattick *et al*., 2003; Cardinale *et al*., 2017). The role of kitchen cleaning tools in bacterial transfer to surfaces was, already, confirmed by Hilton and Austin (2000) on cloths contamination. In that study, no significant differences between the microbial load of wet cloths, dry cloths, those used for short and prolonged periods and cloths used for different activities were observed. Our results confirm, as just previously reported, that in kitchen sponges' bacterial colonization and subsequent replication occur rapidly and high loads were maintained all over their period of use (Cardinale *et al.*, 2017).

The detection of *Listeria monocytogenes,* also if only in one sample, confirms the potential role of kitchen sponges as food-borne pathogen vehicle, considering its ability in adhesion and surviving on different surfaces (Kilonzo-Nthenge *et al*., 2012; Mattick *et al*., 2003). Among food-borne pathogen bacteria deserve particular attention the presence in 16 sponges (16%) of *Cronobacter sakazakii* (previously *Enterobacter sakazakii*). This occurrence confirms, as reported by Kilonzo-Nthenge *et al*. (2012), that it can colonize several kitchen districts. To date and based on the recently paper of Cardinale *et al.* (2017) on the microbiome analysis on used kitchen sponges this is the first evidence of *Cronobacter sakazakii* presences among the bacteria involved in kitchen sponges' colonization. These bacteria, frequently isolated from environment and humans and animals' intestinal tract, can cause necrotizing enterocolitis, bacteremia, and meningitis in children and infants, with a 40-80% mortality rate. It can also cause diarrhea and urinary tract infections in people of all ages, especially in YOPI subjects (young, old, pregnant, immunosuppressed) (Healy *et al*., 2010). *C. sakazakii* has been found in several foods, but mostly powdered infant formula has been linked to disease outbreaks (Adekunte *et al*., 2010). Its presence in this food is related to contamination in raw ingredients, during the manufacturing process or during the preparation/reconstitution process. Contamination also may occur through blenders, feeding bottles and utensils used to cleanse feeding bottles (Adekunte *et al*., 2010). This occurrence deserves particular attention considering that kitchen sponges are commonly used for baby tools cleaning (baby bottles, children's cutlery, bowls ecc.). This study confirms the high stress, environmental resistance of *C. sakazakii* and also reports the diffusion of ESBL+ strains in household settings (Abdel-Galil *et al*., 2015). In regards of other *Enterobacteriaceae* identified in our kitchen sponges, the higher prevalence reported for *Enterobacter cloacae,* is probably related to its high diffusion and resistance. Moreover, these bacteria, with *Citrobacter freundii*, *Klebsiella oxytoca* and *Klebsiella pneumoniae* have been reported as important opportunistic and multiresistant bacterial pathogens for humans during the last three decades in hospital wards (Mezzatesta *et al.*, 2012; Munoz-Price *et al*., 2013; Whalen *et al*., 2007). *L. adecarboxylata,* often found in water environ-

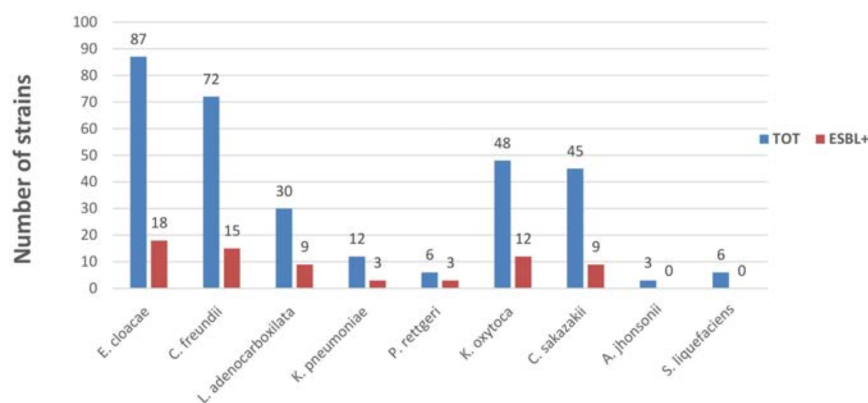

Figure 3. Prevalence of ESBL (Extended Spectrum Beta Lactamase) strains among 309 identified *Enterobacteriaceae.*

ments, is reported as occasionally pathogenic agent, even in healthy subjects (Keren *et al*., 2014). The pathogenic potential of *Providencia rettgeri* is well known, as well as the diffusion of extended spectrum beta lactamase (ESBL) and metallo-beta lactamase (NDM-1) strains (Tada *et al*., 2014). Finally, *S. liquefaciens* is widely isolated in nature, including river water, mineral water, domestic sewage, fish, minced meat and pasteurized milk or cream (Muscolino *et al*., 2014; Ziino *et al*., 2010). In humans, it has been, rarely, reported as a cause of nosocomial infections, including urinary tract infection, pneumonia, neonatal meningitis, endocarditis and septicemia (Mossad, 2000). As reported, in our study the highest prevalence of ESBLs was assigned to *P. rettgeri* and *L. adenocarboxilata* whose beta-lactamase resistance was already reported for nosocomial infections (Sheng *et al*., 2013). Resistance patterns for *Klebsiella* genus, are well-known and several strains represent the most prevalent clinical isolates in complicated infections (Barrios *et al*., 2017). The situation becomes even more worrying, considering that *Klebsiella* is one of the most widespread opportunistic pathogens in nosocomial infections with fatal prognosis (Tuon *et al*., 2011). *Klebsiella* ESBL-producing strains, often, carry resistance determinants against fluoroquinolones, cotrimoxazole and aminoglycosides. These microorganisms are rapidly evolving in response to the selective pressure created by antibiotics abuse, resulting in a serious clinical and epidemiological problem (Tuon *et al*., 2011). *E. cloacae* and *C. freundii* ESBL strains are more and more frequently implied in multiple resistance patterns against insidious infections (Lagha *et al*., 2016).

The results of this study (Figures 1 and 2) obtained with traditional microbiology methodology on a relevant number of samples (n. 100) reveled difference on *Enterobacteriaceae* abundance respect to the those of Cardinale *et al.* (2017) on the total of 223,741 raw sequences obtained by 454-pyrosequencing of 16S rRNA gene amplicon libraries from 28 sponge samples. In our samples *Enterobacteriaceae* have a considerable influence (45%) on the AMB, while for Cardinale *et al.* (2017) have a cumulative relative abundance of 1.18% only after *Bacteroidaceae, Bdellovibrionaceae, Brevibacteriaceae, Caulobaceriaceae,* and *Comamobacteriaceae* family. Except for *Escherichia*, were in accordance the reported genera *Enterobacter*, *Citrobacte*r and *Leclercia* with our results among those reported in the *Enterobacteriaceae* family (Cardinale *et al.,* 2017).

Conclusions

In conclusion, the high contamination level observed in our samples must be assumed as unacceptable for a kitchen tool, considering the potential role of sponges in cross contamination events. Sponges create a setting for colonization and subsequent microbial replication thanks to favorable factors such as high-level humidity, presence of organic residuals and promiscuous use. Moreover, the presence of ESBL-producing strains in kitchen sponges confirms how the domestic settings are potential transmission pathways, explaining the spread of ESBL *Enterobacteriaceae* from the food chain to humans (Tschudin-Sutter *et al*., 2014).

Microorganisms, in fact, in kitchen cloths and sponges are protected by soil aggregates, surviving thermal or chemical stress of washing (Park *et al*., 2006). For all these reasons in order to reduce microbial loads, it would be necessary change, frequently, kitchen sponges and make, periodically, efficient sanification treatments. Microwave and dishwasher, reaching high temperatures, represent the most effective treatments to reduce, significantly, microbial loads (Park *et al*., 2006; Sharma *et al*., 2009). High temperature in combination with washing is more effective in reducing bacteria in kitchen sponges than using heat alone (Tate, 2006). As reported by Erdoğrul and Erbilir (2005), the regular dish washing liquid was not effective in reduction of bacteria in the house hold in use sponges, as the presence of food residues strongly reduces the product's efficacy, while in the laboratory tests, the regular dish washing liquid was demonstrated to be effective in reduction of bacteria. The necessity of efficient sanification treatments is supported by the detection of pathogenic and ESBL producing strains, confirming kitchen sponges as vehicle of their transmission and permanence in domestic settings.

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