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Microbial Safety of Masske: A Traditional Butter from South of Khorasan, Genetic Similarity of Pathogenic Bacteria Indicators

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Background: Masske is a traditional Iranian butter made from yoghurt. The first aim of this study was to isolate and identify the nonlactic pathogenic microflora by culture and molecular methods of identification, and the second purpose was to identify genetic similarity of the isolated bacteria in Masske.
Materials and Methods: In order to detect pathogenic dominant indicator microorganisms, a number of 150 bacterial isolates from three Masske samples, which may comprise the repetitive isolates and could grow on appropriate media for *Staphylococci* and *E.coli*, were classified into 8 groups according to their phenotypic characterization followed by chemical tests. Then 2approximately similar isolates from each group were chosen (total 18 isolates; we selected 3 isolates from 2 groups of eight), and the sequencing of 16S rRNA gene was done for subsequent analysis.
Results: Among 18 bacterial isolates, *Staphylococcus hominis* was the most frequently isolated species during the manufacture of Masske as the presence of this bacterium was confirmed in 14 out of 18 samples. Also, the presence of *Staphylococcus epidermidis* and *Escherichia coli* was identical across the samples (for each one, 2 out of 18).
Conclusion: Our results based on cultural and molecular methods suggest making some improvements to the hygiene of Masske manufacture due to the high population of minor pathogens.

Keywords: Butter, Microbial safety, Pathogenic bacteria, Genetic similarity

1. Background

Traditional dairy products comprise a huge recognized reservoir of phenotypic and genetic microbial diversity that may have many potential biotechnological applications (1-5). Among all dairy products, various types of fermented milk products have an important role throughout the world. Their nature depends on the type of milk used, the pretreatment of the milk, and the conditions of fermentation and subsequent processing. They mainly involve lactic acid bacteria (LAB); however, staphylococci, coliforms, yeasts, and moulds can also occur. These traditional foods have persisted over centuries in different countries (6-8).

Staphylococcal food poisoning (SFP) is one of the most prevalent causes of gastroenteritis worldwide (9). Symptoms of SFP have a rapid onset (from 2 to 6 hours) of abdominal cramps, nausea, and vomiting, sometimes followed by diarrhea (10, 11). Milk and milk products are common vehicles for staphylococcal food poisoning (12-17). They have been frequently implicated in SFP, and often contaminated raw milk is involved (10). These products are highly susceptible to a variety of microorganisms because of their high nutritive value and complex chemical composition. Among *Staphylococci*, coagulase-negative ones have been considered as minor pathogens. These kinds of pathogens were considered to be minor based on the fact that the minor pathogens are not reported quantitatively from each laboratory (18). In addition to the pathogenic *Staphylococcus aureus*, potentially pathogenic coagulase-negative *Staphylococci* (CoNS) have also been identified as the carriers of genes for resistance to macrolides (19, 20).

The CoNS have become the most common bacterial pathogens isolated from milk samples in many countries causing bovine intramammary infections (21-23). They are opportunists and adhere to the metal devices to produce a protective biofilm. The ability to produce biofilm enables CoNS to persist on milking equipment as well as on the milker's hands, which serves as a major source of staphylococcal spread (24, 25). CoNS have traditionally been considered to be normal skin microflora, which as opportunistic bacteria can cause mastitis.

Masske is a dairy product with an attractive appearance, an integrated texture, a pleasant sour nutty aroma, a light yellow color, which is semisolid at room temperature (26-28). Its characteristic flavor serves as a major criterion for its acceptance (28). Masske is made by fermentation of milk fat. It is produced in the northern and eastern regions of Iran: Mazandaran and Khorasan provinces, respectively, as well as in Turkey, Afghanistan, and Balkans Peninsula. In Khorasan, Masske is manufactured in small rural households, and the sale is mainly limited to local markets. It is always produced in a traditional way handed down from the past generations. It can be made from nonpasteurized ewe or goat milk or the mixture of the two (29). The main steps of Masske production are presented in Figure 1. The Fermentation process is completed by both natural microflora and little inoculums, during several days, and the phase changes are fulfilled by tuluming. The Shallaghi and Shiraz are two milk fat byproducts with the lower fat contents that are used to provide drink buttery yoghurt. Due to the lack of using special heating operation to produce this product, it is

probable that pathogenic bacteria could remain in it, so its safety should be checked before use.

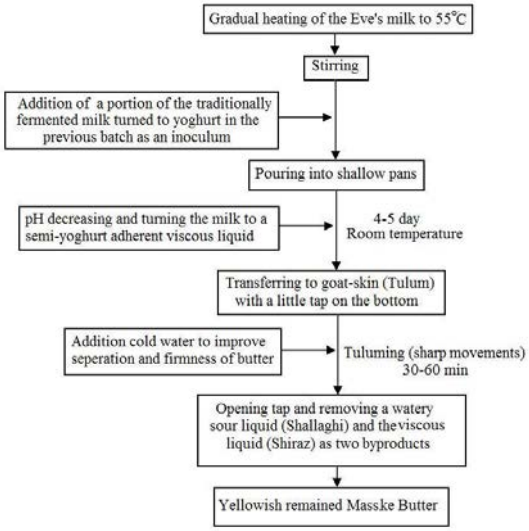

Figure1. Flow scheme of the manufacturing process of Masske butter

2. Objectives

The aim of this study was to identify the predominant species and the genetic similarity of remaining pathogenic bacteria using classical cultural and molecular techniques to provide a phylogenetic tree and genetic similarities matrix.

3. Material and methods

3.1. Sampling of Masske

Three types of Masske designated as BB (butter of Borjuk), BBO (butter of Band Ozbak), and BR (butter of Riab) were collected from local producers in different regions of Gonabad (a southern city of Khorasan province). The samples were intentionally collected from regions where Masske has been traditionally produced in households for centuries. All samples were made from sheep's milk. BB, BBO and BR were collected from the farms situated in the south-western, south-eastern and eastern regions of Gonabad. Samples were taken from the batches of Masske that had been manufactured independently in different periods of the year. The collected samples were kept in sterile bags at 4 °C, and the analyses were performed within the following 24 hr.

3.2. Enumeration and isolation of microbial population

Masske samples, (25 g each), were homogenized in 225 mL of sterile 2% (w/v) tri-sodium citrate dihydrate solution (pH=7.5) then preheated to 40 °C, and then 10-fold serial dilution was made in peptone water (0.1%) (Merck, Germany). A volume of 0.1 mL of appropriate dilutions was spread plated in duplicate on the following media for isolation and enumeration of microbial population (30, 31).

3.3. Total plate counts

Total microbial counts were examined on plate count agar (Merck, Darmstadt, Germany), using the pour-plate technique. Bacteria were enumerated after 24-48 hr of incubation at 30°C (1, 7).

3.4. Coliforms

Coliforms were grown on Violet Red Bile Lactose Agar (VRBLA) (Merck, Darmstadt, Germany), using the pour-plate technique. Bacteria were enumerated after 24-48 hr of incubation at 30 °C (1, 7). In addition, for detection and isolation of *E. coli* specifically, we inoculated 1 mL aliquots from the first three dilutions into 3 Lauryl Sulfate broth (LS) (Merck, Darmstadt, Germany) containing inverted Durham tubes and incubated them at 35 °C. The tubes were examined after 24 hr for gas production. For each gassing LS tube from the Presumptive test, we did the same for EC broth with Durham tubes and incubated EC tubes for 24 hr at 44.5 °C and examined them for gas production. We removed a loop full of EC broth from gas-positive tubes then carried out the same test in Tripton Water medium (Merck, Darmstadt, Germany) and incubated the tubes for 24 hr at 44.5 °C. If the tubes had made a purple ring with the indicator, we could have streaked them for the isolation on Eosin Methylene Blue (EMB) agar (Merck, Darmstadt, Germany) plates and incubated for 24 hr at 35 °C (7,32).

3.5. Staphylococci

Dilutions were grown on Baird–Parker agar (B–PA; Merck) supplemented with egg yolk tellurite solution (Merck). All colonies, with or without egg yolk clearing, were isolated after 24 hr of incubation at 37 °C (1, 7, 32).

3.6. Phenotypic characterization of isolates

We isolated 150 bacterial isolates from three Masske samples. Fifteen isolates had been grown on VRBA and EMB agar and also made turbid with gas in LSB and EC media tubes. We classified these 15 isolates into two groups according to the shapes of their colonies (all these isolates were indole/ methyl red positive). The rest of the grown isolates on BPA were classified into six different groups according to their colony appearance, catalase test, resistance to 10 and 15% NaCl, and temperature resistance of 15 and 45 ºC.

3.7. Molecular identification of microbial isolates

Eighty colonies from the BP and EMB agar plates were purified by subculturing on the same media, and the pure cultures were stored frozen at −80 °C until the analyses were done. Cultures were recovered in the corresponding media, and the isolated colonies were suspended in 100µl milliQ water. Five to six glasses of beads were added to the microtubes and left in the refrigerator at -20 °C for 5min. Then the tubes were mixed vigorously by vortex, and the microbial suspensions were transferred to the new tubes and heated for 20min at 70 °C. After centrifugation for 10 min at 13,000 g, 50 µl of supernatants (cell free extracts) were transferred into new tubes for the next steps, and 50 µl of new milliQ water was added to the rest (remaining pellet). The mentioned steps were repeated for the pellets, and 50 µl of supernatants was added to the previous supernatants and again centrifuged for 10min at 13,000 g. Then 50 µl of upper liquid parts of supernatants was used as a source of DNA template to amplify a segment of the 16S rRNA gene by the polymerase chain reaction (PCR) technique. For those isolates that didn't respond to the above protocol, the DNA extraction kits were used (Roche applied Science, USA). In this method, isolates were grown in liquid media, centrifuged, resuspended in PBS, and mixed with an enzymatic solution (10mg.mL^{-1} lysozyme pH=8, 10mM Tris-HCl). Next steps were done based on the manufacturer's protocol (1, 24, and31).

3.8. Amplification of 16S rRNA Genes

PCR amplifications were carried out in a 25 µl volume using a Thermal Lab cycler (Sensequest, Germany). The reaction mixture contained 2.5µl 10×PCR buffer, 2 µl dNTPs, 0.2 µl Taq DNA polymerase (1U) (Takara, Japan), 1.2 µl $MgCl_2$ (50mM), 1.25 µl of mixture of primers (5pM), 18 µl ddH_2O, and 1.5 µl of DNA template (1-3,33).The PCR primers used, 27FYM (5´-AGAGTTTGATYMTGGCTCAG-3´) and 1492R (5´-GGTTACCTTGTTACGACTT-3´)(1), were based on conserved regions of the 16S rRNA gene using the following cycling program: initial denaturation at 95 °C for 5 min, 33 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 30 s, elongation at 72 °C for 2 min; and final elongation at 72 °C for 10 min. PCR products were quantified by electrophoresis on 1.5% (wv^{-1}) agarose gel in 1X TBE buffer and visualized by Green DNA viewer staining and visualized by gel documentation (31, 33-35). Amplicons were sequenced by Macrogen Advancing Thought Genome Co., Korea. Homology comparisons were performed using the Basic Local Alignment Search Tool (BLAST), available online at the National Centre for Biotechnology Information (NCBI) homepage (http://www.ncbi.nlm.nih.gov) (36).

3.9. Bioinformatic analysis

Alignment was done through ClustalW algorithm with its default parameters. Subsequently, with the help of the MEGA software (37) version 5, the phylogenetic tree was calculated based on the Neighbour-joining (38) procedure with the Maximum Composite Likelihood model (39). The validity of the tree was also verified based on the random sampling (Bootstrap) method with a thousand repetitions (40).

3.10. Statistical analysis

Standard deviation and average comparisons of $log.cfug^{-1}$ counted colonies on four media of PCA, BPA, VRBA, and EMB were conducted by MINITAB software version 16 and analyzed based on Turkey test at the level of confidence of 95%.

4. Result

4.1. Enumeration of bacteria

According to Figure 2, the numbers of hygienic-indicator populations were notable throughout all the Masske samples, reaching their highest levels at sample BBO for total counts (7.6 $log.cfug^{-1}$), sample BB for coliforms (5.6 $log.cfug^{-1}$) and for *E.coli* (3.3 $log.cfug^{-1}$), and sample BR for staphylococci (6.5 $log.cfug^{-1}$). The results were in agreement with Alegria and colleagues (2009) about the total aerobic counts and coliforms of the curd and 3 day and 7 day ripened cheese for coliforms and Staphylococci respectively (1). Our results were also in agreement with Sengül's study (2006), in five of the fifteen samples (41). The 16s rRNA sequencing showed the genus, species, and strains of the 18 isolates susceptible for being *Staphylococci* and *Escherichia*. As we expected, all the 18 isolates belonged to these two genera. The results indicated that 14 isolates belonged to *Staphylococcus hominis*, 2 to *Staphylococcus epidermidis,* and 2 to *E. coli* (Table1). The results also indicated that two *E. coli* isolates were obtained from sheep's milk and not from butter; it may be because of the process of pH reduction in Masske production that kills some gram-negative bacteria such as *E. coli*. On the other hand, raw milk is a good rich nutrient resource for them.

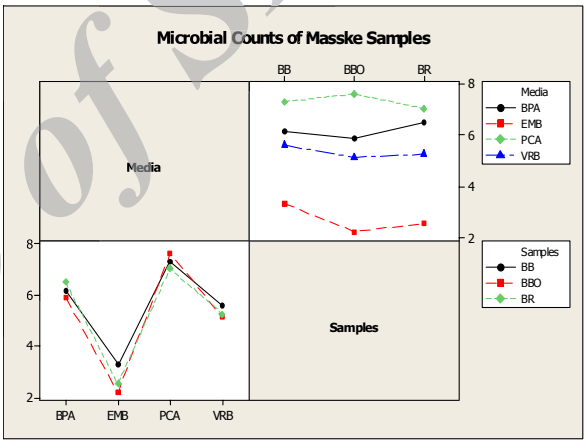

Figure2. Intractionpragh of microbial counts of Masske samples.

Table1. Majority of microorganisms identified from Masske samples isolated in three different culture media.

No. of isolate	Name of isolate	Name in matrix	Name of bacteria in 16s rRNA sequencing	%iIdentity	Accession No.
1	B176	GIV1	*Staphylococcus hominis* strain *77 (BC26)*	99	(KF254627.1)
2	B60	GIV2	*Staphylococcus hominis* strain *77 (BC26)*	99	(KF254627.1)
3	B27	GII1	*Staphylococcus hominis* strain *HACF7*	99	(KC581939.1)
4	B95	GIV3	*Staphylococcus hominis* strain *77 (BC26)*	99	(KF254627.1)
5	B14	GII2	*Staphylococcus hominis* strain *HACF7*	99	(KC581939.1)
6	B213	GI1	*Staphylococcus hominis* strain *A8DI*	99	(KC898305.1)
7	B189	GIV4	*Staphylococcus hominis* strain *77 (BC26)*	100	(KF254627.1)
8	B216	GIII1	*Staphylococcus hominis* strain *AW14*	100	(JX281775.1)
9	B224	GIV5	*Staphylococcus hominis* strain *77 (BC26)*	100	(KF254627.1)
10	B212	GII3	*Staphylococcus hominis* strain *HACF7*	99	(KC581939.1)
11	B61	GIII2	*Staphylococcus hominis* strain *AW14*	98	(JX281775.1)
12	B16	GIV6	*Staphylococcus hominis* strain *77 (BC26)*	99	(KF254627.1)
13	B231	GIV7	*Staphylococcus hominis* strain *77 (BC26)*	98	(KF254627.1)
14	B64	GIV8	*Staphylococcus hominis* strain *77 (BC26)*	100	(KF254627.1)
15	B117	S.e(R)	*Staphylococcus epidermidis* strain *WIF14*	94	(HM480310.1)
16	M97	S.e1	*Staphylococcus epidermidis* strain *258 (P37A)*	100	(KF254632.1)
17	M66	E1	*Escherichia coli* strain *KVP104*	98	(JX290084.1)
18	M247	E2	*Escherichia coli* strain *BAB-538*	100	(KF535120.1)

4.2. Identification of isolates

We isolated 150 bacterial isolates from three Masske samples. Fifteen isolates had been grown on VRBA and EMB agar and also made turbid with gas in LSB and EC media tubes. We classified these 15 isolates to two groups according to the shapes of their colonies (all these isolates were indole and methyl red positive). The rest of the isolates on BPA were classified into six different groups according to their colony appearance, catalase test, resistance to 10 and 15% NaCl, and temperature resistance of 15 and 45 ºC.

Group I colonies were nonpigmented, flat (gray or grayish white), smooth, mucoid or slimy, which could grow strongly in the presence of 10% NaCl and 45 ºC but not 15 ºC.

Group II Colonies were black, opaque, and 1mm diameter, which could grow only in 45 ºC.

Group III colonies were yellowish-orange, watery, and bigger than group II, which could grow weakly in the presence of 10% NaCl, however, in 45 ºC they were noticeable. No growth was observed in 15 ºC and 15% NaCl. Group IV colonies were catalase negative and were omitted from other tests. Group V colonies were the same as Group III but couldn't grow at any percentage of NaCl and temperatures of 15 and 45 ºC. Group VI colonies were black but grew weakly in the presence of 10% NaCl and 45 ºC.

4.3. Cladograms

According to genetic similarity that was seen throughout the cladogram, *S. hominis* were classified into four groups and the other three species to only one group. According to Figure 2, the four groups of Staphylococci had varied similarity (45-100%). We selected a reference (marked with "R" letter) species for each group for the best comparison. As we see in Figure 2 and 3, we had four critical points where bootstraps had arisen up to 99-100% because of the importance of these points. We saw that the genetic distances from strains started from these points, so the groups were classified according to them. These genetic distances showed that two *E. coli* strains had more differences between themselves, rather than the Staphylococci. Moreover, the cladogram marked three points where the genetic differences had increased between four groups of *Staphylococci* (14-16, 36, 42).

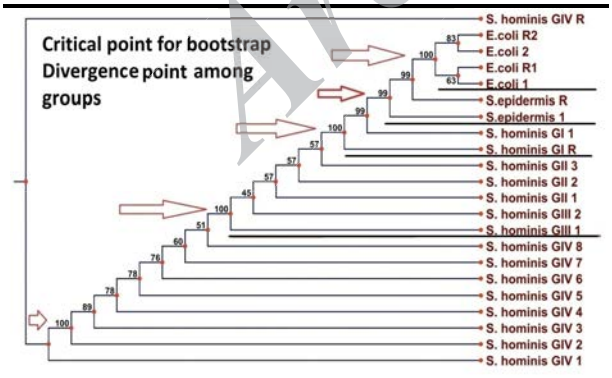

Figure3. Genetic dendrogram of *Staphylococci* and *Escherichia* isolated from Masske based on bootstrap

4.4. Similarity-distance matrix

The matrix shows that (the numbers above main diameter represent genetic distances and the numbers below talk the number of nucleotide coverage) among *Staphylococci* groups, GI, GII, and GIII differences were minute and in the range of 0 to 20%, and the nucleotide coverage (nc) reached up to 670-673 (Figure 4). For *S. epidermidis,* there was no difference and nc was 673. On the other hand, genetic distance between the first three groups of *S.hominis* and the fourth group (GIV) was high (72%) and nc was reduced to 359-360. Unexpectedly, the difference between the first three groups (GI, GII and GIII) and *E. coli* was 68%, which was less than the difference between the first three groups and the fourth group of *S.hominis* (GIV) (72%). Maybe it is because of the different origin of *S.hominis GIV*. Finally, *S.hominis GIV* and *E.coli* had the most distance (186%) and the lowest nc (269).The results also show that the most correlations belonged to strains of *E.coli* (855-850). These results are partially in agreement with Hebremedhin et al., (2008) (43).

The results also show that the Staphylococci in Group IV have a little Cognation relationship with the other Staphylococci strains indicators in dairy products and plant resources; however, according to the reference strain that was obtained from feces (36). This Cognation relationship is very close to strains isolated from animal feces. So it is necessary to make a suitable decision to eliminate the existing pathogens without harming the nutritional values of product.

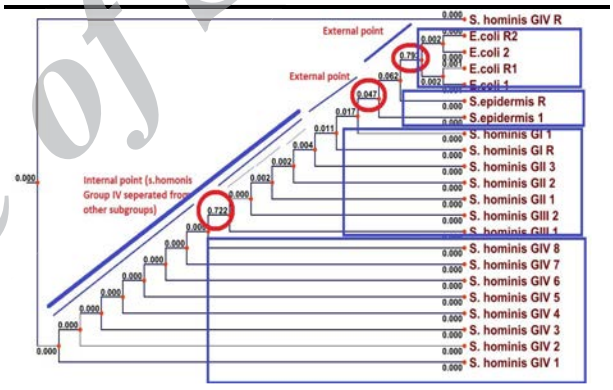

Figure4. Genetic dendrogram of *Staphylococci* and *Escherichia* isolated from Masske based on genetic distances.

5. Discussion

In the present study, the diversity of predominant species of pathogenic indicators in analyzed Masske samples was identified through classical culturing and molecular techniques. This study provided a phylogenetic tree for the evaluation of the genetic diversity and the relationships between those species. The present data illustrated that *S.hominis* was the predominant isolate recovered from the examined Masske samples. The predominance of *S.hominis* among the CoNS was also recorded earlier in sheep's dairy product (24, 25).

In order to evaluate dramatic changes in the microbial community and the microbial genetic biodiversity during the process of Masske production, we should take into consideration a main factor that influences the microbial community during this process.

The microbial growth conditions in terms of chemical factors along with the main compounds present in every sample during Masske production cause some of the microbial species to be predominant; raw milk is a good source of gram negative bacteria (GNB) such as *E. coli* and coliforms that prefer neutral pH of environment to survive and grow. During Masske production, when the milk is turned to yoghurt, the pH decreases vigorously, so most of

these bacteria die. Only the microbial flora that can tolerate this pH decrease can survive. Finally, when the yoghurt is turned to Masske, pH increases slightly, and these surviving microorganisms can grow. On the other hand, the microbial diversity of goat skin and also the human skin that forms the product may influence the Masske final microflora. This microbial population is a reflection of the normal udder and teat skin flora too (44).

The results indicate that although the numbers of Staphylococci were high, strains of *Staphylococcus aureus* were never detected in the BPA counting plates and also in sequencing. The pathogenic microorganisms were *E. coli*, *S. hominis,* and *S. epidermidis*. The high rate of *S.hominis* may be due to the localization of this microorganism inside or/and outside of the udder and also in human skin flora, which have been entered from hands skin during hand-formation process of Masske. Many studies in different regions worldwide have investigated the occurrence of CoNS. The prevalence of intramammary infections with CoNS was reported in Finland (50% of the positive for bacterial growth) (45). In a similar study that was done in Norway, the occurrence of CoNS was 16% (46), while in Germany CoNS were isolated from 9% of the dairy samples in a total of 80 dairy herds (47). In two dairy herds in Canada, CoNS were the most common bacteria (51%) causing intramammary infection (IMI)(48). Although these species of Staphylococci don't have the pathogenicity capacity as *S. aureus,* lately the Centers for Disease Control and Prevention's National Nosocomial infection surveillance system has reported that *S. epidermidis* is responsible for 33.5% of nosocomial blood stream infection. So the importance of coagulase-negative staphylococci (CoNS) has increased and they have become the predominant pathogens isolated from subclinical infections in several countries (47-49) and classified among the pathogens responsible for diverse nosocomial infections. Most of the strains are highly resistant to multiple antibiotics such as penicillin, tetracycline, methicillin, and many more, which makes the infections caused by these bacteria very difficult to treat (50).

6. Conclusion

It could be concluded that CoNS are important minor pathogens in Masske traditional butter. Such species can cause subclinical and nosocomial infections by its consumption. This reflects the environmental hazard such as udder health, raw milk microbial load, sanitary conditions of dairy farms and the people who work there. Further investigations should be carried out on the epidemiology of CoNS-causing disease. More reliable identification methods would be beneficial for the classification of CoNS species isolates based on virulent genes not only 16S rRNA.

Conflict of Interests

The authors declare they have no conflict of interests.

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Authors' Contribution

Conceived and designed the experiments: Masoud Yavarmanesh, Morteza Khomeiri and Fariba Ghiamati Yazdi. Performed the experiments: Fariba Ghiamati Yazdi. Analyzed the data: Fariba Ghiamati Yazdi, Masoud Yavarmanesh, Morteza Khomeiri and Morteza Mahdavi. Contributed reagents/material/ analysis tools: Fariba Ghiamati Yazdi and Morteza Mahdavi. Wrote the paper: Fariba Ghiamati Yazdi and Morteza Mahdavi. Paper revision: Masoud Yavarmanesh, Morteza Khomeiri, Fariba Ghiamati Yazdi and Morteza Mahdavi.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
S. hominis GII 1	1		0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.02	0.72	0.72	0.72	0.72	0.72	0.72	0.72	0.72	0.69	0.69	0.68	0.69
S. hominis GII 2	2	673		0.00	0.00	0.00	0.00	0.00	0.02	0.02	0.72	0.72	0.72	0.72	0.72	0.72	0.72	0.72	0.72	0.69	0.69	0.68	0.69
S. hominis GII 3	3	673	673		0.00	0.00	0.00	0.00	0.02	0.02	0.72	0.72	0.72	0.72	0.72	0.72	0.72	0.72	0.72	0.69	0.69	0.68	0.69
S. hominis GIII 1	4	672	672	672		0.00	0.00	0.00	0.02	0.02	0.72	0.72	0.72	0.72	0.72	0.72	0.72	0.72	0.72	0.69	0.69	0.68	0.69
S. hominis GIII 2	5	672	672	672	673		0.00	0.00	0.02	0.02	0.72	0.72	0.72	0.72	0.72	0.72	0.72	0.72	0.72	0.69	0.69	0.68	0.69
S. hominis GI R	6	671	671	671	670	670		0.00	0.02	0.02	0.72	0.72	0.72	0.72	0.72	0.72	0.72	0.72	0.72	0.68	0.68	0.69	0.68
S. hominis GI 1	7	671	671	671	670	670	673		0.02	0.02	0.72	0.72	0.72	0.72	0.72	0.72	0.72	0.72	0.72	0.68	0.68	0.69	0.68
S.epidermis 1	8	659	659	659	658	658	661	661		0.00	0.73	0.73	0.73	0.73	0.73	0.73	0.73	0.73	0.73	0.68	0.68	0.68	0.68
S.epidermis R	9	659	659	659	658	658	661	661	673		0.73	0.73	0.73	0.73	0.73	0.73	0.73	0.73	0.73	0.68	0.68	0.68	0.68
S. hominis GIV R	10	361	361	361	361	361	361	361	359	359		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.86	1.86	1.86	1.86
S. hominis GIV 1	11	361	361	361	361	361	361	361	359	359	361		0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.86	1.86	1.86	1.86
S. hominis GIV 2	12	361	361	361	361	361	361	361	359	359	361	361		0.00	0.00	0.00	0.00	0.00	0.00	1.86	1.86	1.86	1.86
S. hominis GIV 3	13	361	361	361	361	361	361	361	359	359	361	361	361		0.00	0.00	0.00	0.00	0.00	1.86	1.86	1.86	1.86
S. hominis GIV 4	14	361	361	361	361	361	361	361	359	359	361	361	361	361		0.00	0.00	0.00	0.00	1.86	1.86	1.86	1.86
S. hominis GIV 5	15	361	361	361	361	361	361	361	359	359	361	361	361	361	361		0.00	0.00	0.00	1.86	1.86	1.86	1.86
S. hominis GIV 6	16	361	361	361	361	361	361	361	359	359	361	361	361	361	361	361		0.00	0.00	1.86	1.86	1.86	1.86
S. hominis GIV 7	17	361	361	361	361	361	361	361	359	359	361	361	361	361	361	361	361		0.00	1.86	1.86	1.86	1.86
S. hominis GIV 8	18	361	361	361	361	361	361	361	359	359	361	361	361	361	361	361	361	361		1.86	1.86	1.86	1.86
E.coli 2	19	474	474	474	474	474	476	476	478	478	269	269	269	269	269	269	269	269	269		0.00	0.01	0.00
E.coli R2	20	474	474	474	474	474	476	476	478	478	269	269	269	269	269	269	269	269	269	860		0.01	0.00
E.coli 1	21	475	475	475	475	475	473	473	475	475	269	269	269	269	269	269	269	269	269	855	855		0.00
E.coli R1	22	473	473	473	473	473	475	475	477	477	269	269	269	269	269	269	269	269	269	857	857	858	

Figure 5. Similarity-distance Matrix of *Staphylococci* and *Escherichia* isolated from Masske based on genetic distances.

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