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Detection of anaerobic and aerobic bacteria from commercial tattoo and permanent makeup inks

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ABSTRACT Tattooing and use of permanent makeup (PMU) have dramatically increased over the last decade, with a concomitant increase in ink-related infections. Studies have shown evidence that commercial tattoo and PMU inks are frequently contaminated with pathogenic microorganisms. Considering that tattoo inks are placed into the dermal layer of the skin where anaerobic bacteria can thrive and cause infections in low-oxygen environments, the prevalence of anaerobic and aerobic bacteria should be assessed in tattoo and PMU inks. In this study, we tested 75 tattoo and PMU inks using the analytical methods described in the FDA Bacteriological Analytical Manual Chapter 23 for the detection of both aerobic and anaerobic bacterial contamination, followed by 16S rRNA gene sequencing for microbial identification. Of 75 ink samples, we found 26 contaminated samples with 34 bacterial isolates taxonomically classified into 14 genera and 22 species. Among the 34 bacterial isolates, 19 were identified as possibly pathogenic bacterial strains. Two species, namely *Cutibacterium acnes* (four strains) and *Staphylococcus epidermidis* (two strains) were isolated under anaerobic conditions. Two possibly pathogenic bacterial strains, *Staphylococcus saprophyticus* and *C. acnes*, were isolated together from the same ink samples (n = 2), indicating that tattoo and PMU inks can contain both aerobic (*S. saprophyticus*) and anaerobic bacteria (*C. acnes*). No significant association was found between sterility claims on the ink label and the absence of bacterial contamination. The results indicate that tattoo and PMU inks can also contain anaerobic bacteria.

IMPORTANCE The rising popularity of tattooing and permanent makeup (PMU) has led to increased reports of ink-related infections. This study is the first to investigate the presence of both aerobic and anaerobic bacteria in commercial tattoo and PMU inks under aerobic and anaerobic conditions. Our findings reveal that unopened and sealed tattoo inks can harbor anaerobic bacteria, known to thrive in low-oxygen environments, such as the dermal layer of the skin, alongside aerobic bacteria. This suggests that contaminated tattoo inks could be a source of infection from both types of bacteria. The results emphasize the importance of monitoring these products for both aerobic and anaerobic bacteria, including possibly pathogenic microorganisms.

KEYWORDS tattoo ink, permanent makeup ink, microbial contamination, anaerobic bacteria, aerobic bacteria

Tattooing, along with the use of permanent makeup (PMU), has dramatically increased over the last two decades (1). Approximately 32% of the population in the United States is estimated to have at least one tattoo (https://www.pewresearch.org/short-reads/2023/08/15/32-of-americans-have-a-tattoo-including-22-who-have-more-than-one). As tattooing has become increasingly common, tattoo-related human health risks and adverse events have also increased

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(2–4). Although the most common types of tattoo-associated human complications are often known to be immunologic reactions, including inflammatory reactions and allergic hypersensitivity (5, 6), infectious complications have also been commonly associated with tattoos (5, 7, 8).

Studies showed that approximately 0.5%–6% of tattooed people experienced microbial infectious complications as a result of receiving a tattoo (7, 9, 10). Previously, the sources of infection were mainly associated with issues related to insufficient hygiene practices at the time of tattoo application as well as lack of proper aftercare while healing (7, 9, 10). However, recent findings suggest that tattoo inks themselves have been identified as a potential source of infections (5, 8, 11).

Infectious complications from tattoos can range from mild skin infections to systemic infections, such as life-threatening bacteremia and septic shock (7). Tattooing, which breaches the skin barrier during application, can increase the risk of infection transmission if the tattoo inks used are contaminated with pathogenic bacteria that are embedded deep into the skin throughout the procedure. The low-oxygen environment of the dermal layer of the skin further allows a possibility of infection by anaerobic bacteria. Studies have shown infections related to tattoos caused by anaerobic microorganisms, such as *Clostridium tetani*, the causative agent of tetanus (12, 13).

In our prior studies, we showed that overall, 68 of 197 (35%) unopened and sealed tattoo and PMU inks and diluents from 17 of 26 manufacturers in the U.S. were contaminated with microorganisms (14–16). Some of these products had total bacterial counts as high as 10^8 CFU/g of ink, despite being labeled as sterile (14–16). These results were in line with previous studies reported in multiple European countries, where as much as 80% of tattoo inks were shown to be contaminated with microorganisms, including pathogenic bacteria (17–20). Considering the high portion of tattoo inks contaminated with a variety of bacteria, it is reasonable to question whether tattoo inks are also contaminated with anaerobic bacteria. Currently, little is known about the contamination of tattoo inks with anaerobic bacteria. In this study, we assessed the prevalence of anaerobic and aerobic microbial contaminants in 75 tattoo and PMU inks available on the U.S. market.

MATERIALS AND METHODS

Sampling and storage of tattoo and PMU inks

A total of 75 tattoo and PMU inks were purchased from 14 tattoo ink manufacturers (Table 1). Six bottles of each individual ink, having the same lot number, were purchased. All ink samples were confirmed to be intact with sealed packaging and were photographed, stored in the storage cabinets, and recorded on the chain-of-custody log. The information from the samples, such as brand and product (color) name, safety data sheets, ingredients, sterility claims, expiration dates, and locations of the manufacturer, was recorded.

Bacteriological analysis of tattoo and PMU ink samples

The prevalence of anaerobes (including facultative anaerobes) and aerobes were analyzed based on the methods of the FDA Bacteriological Analytical Manual (BAM) Chapter 23 (https://www.fda.gov/food/laboratory-methods-food/bam-methods-cosmetics). Briefly, pre-reduced Anaerobe Agar (Thermo Fisher Scientific, Lenexa, KS, USA), 5% defibrinated sheep Blood Agar (Thermo Fisher Scientific, Lenexa, KS, USA), and Modified Letheen Agar (MLA, Thermo Fisher Scientific, Lenexa, KS, USA) were used as growth media. Sample dilution and plating were performed as described for aerobic plate counts. Briefly, samples were decimally diluted in MLB from 10^{-1} to 10^{-3}. Using a new sterile pipet to transfer, 1.0 mL of the current dilution was transferred into 9 mL of fresh MLB to make the next decimal dilution. Dilutions were mixed thoroughly, and all plating was performed in duplicate. An aliquot of 1 mL of 10^{-1} dilution (0.5 mL on each of the

TABLE 1 Summary of ink samples used in this study

	Tattoo ink	PMU[a] ink	Total
No. of samples	40	35	75
No. of brands	7	7	14
Sterility claim			
Yes	35	14	49
NA[b]	5	21	26
Country of origin			
Domestic (USA)	40	13	53
Imported	0	22	22

[a]PMU, permanent makeup.
[b]NA, sterility information not available.

two plates) was plated on MLA, Blood Agar, and Anaerobe Agar to yield a final dilution of 10^{-1}. In addition, from each diluted (10^{-1} to 10^{-3}) solution, 0.1 mL was transferred onto MLA, Blood Agar, and Anaerobe Agar plates to yield final dilutions 10^{-2} to 10^{-4}. Anaerobe Agar plates were incubated in anaerobic chamber AS-580 (Anaerobe systems, Morgan Hill, CA, USA), and Blood Agar plates were incubated in a 5% carbon dioxide microaerophilic atmosphere. The plates were incubated for 48 h before counting and continuously incubated for 2 more days if no colonies appeared at 48 h. Anaerobic Agar plates were pre-reduced in an anaerobic atmosphere overnight before plating experiments. Inoculated Anaerobe Agar plates were initially incubated in an anaerobic atmosphere for 2 days at 35±2°C, then the plates were incubated for up to 10 days for the detection of slow-growing bacteria. MLA plates were aerobically incubated for 2 days at 35±2°C. Isolates recovered from the anaerobic agar plates were sub-cultured under both microaerophilic conditions with 5% CO_2 and anaerobic conditions to confirm their strict anaerobic nature. Positive controls with bacterial species, *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 27853), *Klebsiella pneumoniae* (ATCC 13883), *Bacillus cereus* (ATCC 11778), and *Clostridium sporogenes* (ATCC 3584), were used for aerobic and anaerobic conditions, respectively. Negative controls without bacterial cultures (air plates) were used with each medium and anaerobic and aerobic conditions.

Identification of bacterial isolates

The taxonomy of the colonies from agar plates was identified using 16S rRNA gene sequencing as previously described (21). Briefly, a colony was used for the PCR amplification with the 16S rRNA gene primers 27F and 1492R. The amplified PCR product was purified with ExoSAP-IT (USB Corporation, Cleveland, OH, USA) as suggested by the manufacturer. DNA samples were sequenced at the University of Arkansas for Medical Sciences in Little Rock, AR (https://medicine.uams.edu/mbim/research-cores/dna-sequencing-core-facility/). The DNA sequences of 16S rRNA genes were analyzed for the identification of bacterial species using NCBI BLASTN and the rRNA/ITS database.

Bioinformatics and statistical analysis

The analysis of the co-occurrence network was performed as previously described (15, 16, 22). Briefly, using in-house Python scripts, a species-sample matrix (SSM) was generated. Next, a co-occurrence matrix was generated from the SSM. The co-occurrence relationship was presented when two bacterial species were identified from one tattoo and PMU sample. In a species-centric co-occurrence network (SCN), the bacterial species were presented as nodes and their relationships between bacterial species were presented as edges (i.e., connection degree) weighted by their occurrence counts and frequency of co-occurrence. Network analysis and visualization were performed using Gephi 0.9.2 (https://gephi.org/).

The chi-square test and Fisher exact test were used to explore the statistical significance of the relationship between two categorical variables in this study. In all

tests of significance, $P < 0.05$ indicated a statistically significant relationship between the two variables. The basic format for reporting a chi-square test result was used: χ^2 (degrees of freedom, N = sample size) = χ^2 statistic value, $P = P$ value.

RESULTS

Bacterial isolation

Of the 75 ink samples, 34 bacterial isolates were recovered from 26 ink samples. As shown in Table 2, the 34 bacterial isolates were clustered into three groups based on their growth patterns under the three different growth conditions (i.e., no oxygen, low oxygen, and atmospheric oxygen). Group 1, consisting of obligate anaerobes (i.e., bacteria growing only on oxygen-free anaerobic agar in the anaerobic chamber) contained six isolates. Group 2 has seven isolates showing a common growth pattern of some facultative anaerobes and aerobes. By contrast, Group 3, with a typical growth pattern of obligate aerobic bacteria (i.e., growing only on aerobic medium MLA), contained 21 isolates.

Bacterial identification

The 34 bacterial isolates were taxonomically identified using their 16S rRNA gene sequences. They belonged to a phylogenetically diverse group of 14 genera and 22 species (Tables 3 and 4). The six isolates showing anaerobic growth patterns (Group 1) were the obligate anaerobic *Cutibacterium acnes* (four isolates) and the facultative anaerobic *Staphylococcus epidermidis* (two isolates). None of the six isolates grew in MLA agar in aerobic conditions (Table 3). By contrast, the isolates of both Group 2 and 3 were identified as aerobes based on oxygen requirement though the seven isolates, such as *Pseudomonas putida*, *Staphylococcus saprophyticus*, and *Stenotrophomonas maltophilia*, were isolated in the facultative anaerobic and aerobic culture conditions (Tables 3 and 4). The 16S rRNA gene sequence-based taxonomical identification of the isolates agrees with their phenotypic growth patterns.

Overall, *Staphylococcus* spp. (eight isolates) were the most prevalent taxa identified in the samples, followed by *C. acnes* (four isolates), *Sphingomonas* spp. (four isolates), *Bacillus* spp. (three isolates), *P. putida* (three isolates), *S. maltophilia* (three isolates), and *Streptomyces* spp. (two isolates) (Fig. 1). Among the 22 bacterial species, eight species are possibly pathogenic (Tables 3 and 4 and Fig. 1). They included strains of *S. saprophyticus*, *Staphylococcus xylosus*, *S. epidermidis*, *Staphylococcus warneri*, *C. acnes*, *S. maltophilia*, *Nocardiopsis dassonvillei,* and *P. putida* (25–32).

Occurrence and co-occurrence of bacterial contaminants

Figure 1 shows an SCN describing the occurrence and co-existence patterns of 34 bacterial isolates at the species level. The 34 bacterial isolates were mapped to produce the SCN with 22 nodes (bacterial species) and seven edges (co-occurrence relationship). Among the 22 bacterial species, two possibly pathogenic bacteria, *C. acnes* and *S. saprophyticus,* showed a relatively high occurrence and strong co-occurrence (Fig. 1). In the SCN, *S. saprophyticus* is a hub species with the highest degree of connection

TABLE 2 Classification of 34 bacterial isolates from 26 ink samples based on their growth under three different conditions (growth media and oxygen levels)

Group	Bacterial growth based on medium and growth condition[a]			No. of isolates[b]
	Anaerobe agar (no oxygen)	Blood agar (low oxygen)	MLA (atmospheric oxygen)	
1	+	–	–	6
2	–	+	+	7 (1)
3	–	–	+	21 (15)

[a]MLA, modified letheen agar; +, bacterial growth; –, no bacterial growth.
[b]Number in parentheses indicates growth observed after broth enrichment step.

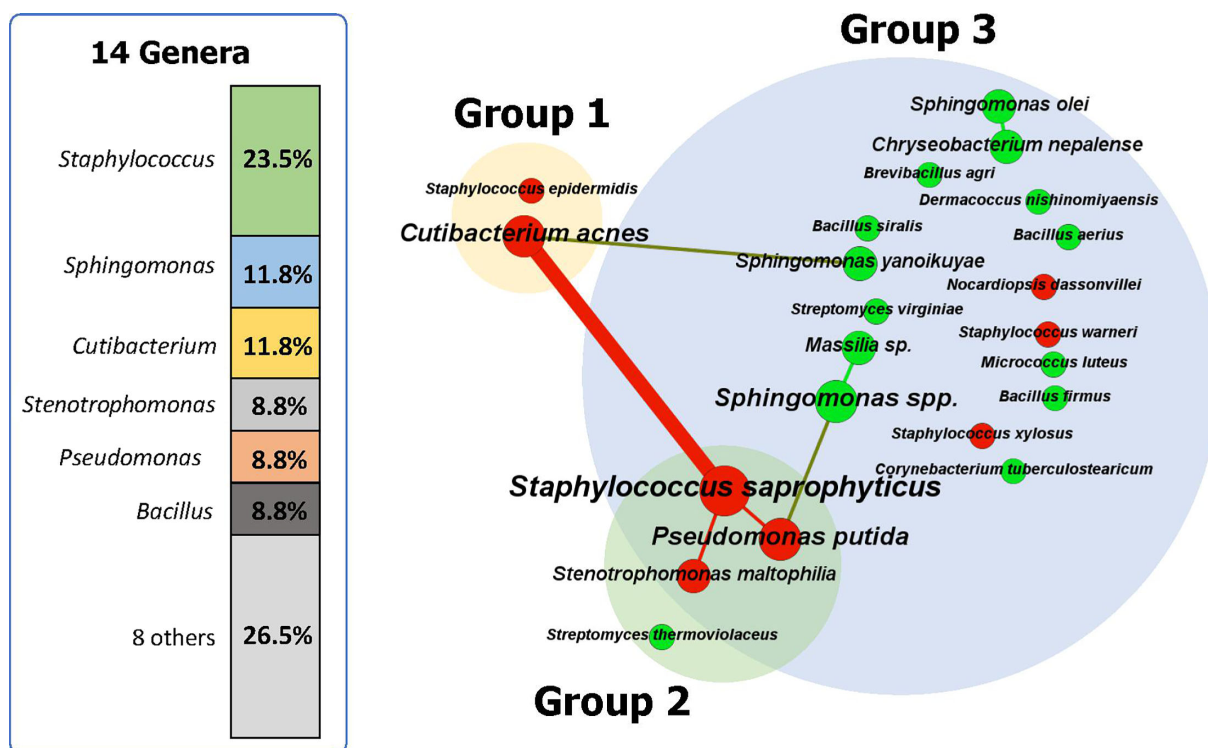

FIG 1 A species-centric co-occurrence network (SCN) of 34 isolates from 26 contaminated ink samples. In the SCN, the bacterial species are presented as nodes, and their co-occurrence relationships were presented as edges (i.e., connection degree). Node size and edge width were weighted by their occurrence counts and frequency of co-occurrence, respectively. The possibly pathogenic species (n=8) and non-pathogenic species (n=14) are colored in red and green, respectively.

(three connections with three potential pathogenic bacteria, *P. putida*, *S. maltophilia*, and *C. acnes*). The obligate anaerobe, *C. acnes* (Group 1), showed co-occurrences with both obligate aerobe, *Sphingomonas yanoikuyae* (Group 3), and facultative anaerobe, *S. saprophyticus* (Group 2 growing in both aerobic and facultatively anaerobic conditions). Such co-occurrences among the isolates of the three groups indicate that PMU inks can be contaminated with both aerobic and anaerobic bacteria together. The average degree (the average number of edges, or connections, that each node in a network has) of the SCN is 0.63, indicating that most bacterial contaminants have few connections (i.e., the majority of contaminated ink samples exhibited the presence of a bacterial strain).

Bacterial contamination prevalence among tattoo and PMU inks

Overall, 26 of 75 tattoo and PMU inks (35%) from 10 of 14 manufacturers were contaminated (Table 4). Based on the occurrence and co-occurrence of bacterial contaminants with different oxygen dependencies, the 26 contaminated ink samples were categorized into five categories (Table 5). Only one PMU ink sample (E-04) was contaminated by obligate anaerobic *C. acnes* (category 1). While two PMU ink samples (C-05 and C-07) were contaminated by both obligate anaerobic and facultative anaerobic bacterial strains (category 2), one PMU ink sample (D-02) was contaminated by both obligate anaerobic and aerobic bacterial strains (category 3) (Tables 4 and 5). Two PMU ink samples (A-01 and I-03) were contaminated by facultative anaerobic *S. epidermidis* (category 4), and the rest (20 ink samples) belong to category 5, contaminated by aerobic bacteria (Tables 4 and 5). All nine contaminated tattoo ink samples fall into category 5, where all bacteria are aerobic.

All contaminated ink samples showed <250 CFU/g, except for eight ink samples, including five PMU inks (C-01, C-07, D-02, D-03, and E-03) and three tattoo inks (H-03,

TABLE 3 Identification and phenotypic features of 34 bacterial isolates

Group	Identification	No. of strains	Oxygen requirement[a]	Potential pathogenicity[b]
1	*Cutibacterium acnes*	4	Anaerobe	+
	Staphylococcus epidermidis	2	Facultative anaerobe	+
2	*Pseudomonas putida*	1	Aerobe	+
	Staphylococcus saprophyticus	3	Facultative anaerobe	+
	Stenotrophomonas maltophilia	2	Aerobe	+
	Streptomyces thermoviolaceus	1	Aerobe	–
3	*Bacillus aerius*	1	Aerobe	–
	Bacillus firmus	1	Aerobe	–
	Bacillus siralis	1	Aerobe	–
	Brevibacillus agri	1	Aerobe	–
	Chryseobacterium nepalense	1	Aerobe	–
	Corynebacterium tuberculostearicum	1	Aerobe	–
	Dermacoccus nishinomiyaensis	1	Aerobe	–
	Massilia sp.	1	Aerobe	–
	Micrococcus luteus	1	Aerobe	–
	Nocardiopsis dassonvillei	2	Aerobe	+
	Pseudomonas putida	1	Aerobe	+
	Sphingomonas olei	1	Aerobe	–
	Sphingomonas spp.	2	Aerobe	–
	Sphingomonas yanoikuyae	1	Aerobe	–
	Staphylococcus saprophyticus	1	Facultative anaerobe	+
	Staphylococcus warneri	1	Aerobe	+
	Staphylococcus xylosus	1	Aerobe	+
	Stenotrophomonas maltophilia	1	Aerobe	+
	Streptomyces virginiae	1	Aerobe	–

[a]The oxygen requirements of the isolates in this study were determined based on the oxygen requirements of known reference species. The oxygen requirements of three bacterial isolates belonging to genera *Massilia* and *Sphingomonas*, but not assigned to a specific species, are based on their respective genera.
[b]+, potential pathogen; -, no potential pathogen. The potential pathogenicity of the isolates was derived from reference species known to be pathogenic within their respective genera (23, 24). The potential pathogenicity of three bacterial isolates belonging to the genera *Massilia* and *Sphingomonas*, but not assigned to a specific species, is based on their respective genera.

N-02, and N-06), which showed >800 CFU/g (Table 4). An obligate anaerobic *C. acnes* and a facultative anaerobic *S. epidermidis* showed <250 CFU/g in the PMU ink samples. By contrast, the facultative anaerobic *S. saprophyticus* and aerobic *S. yanoikuyae* in the PMU ink samples belonging to categories 2 and 3 showed 2.5×10^3 CFU/g (Table 4).

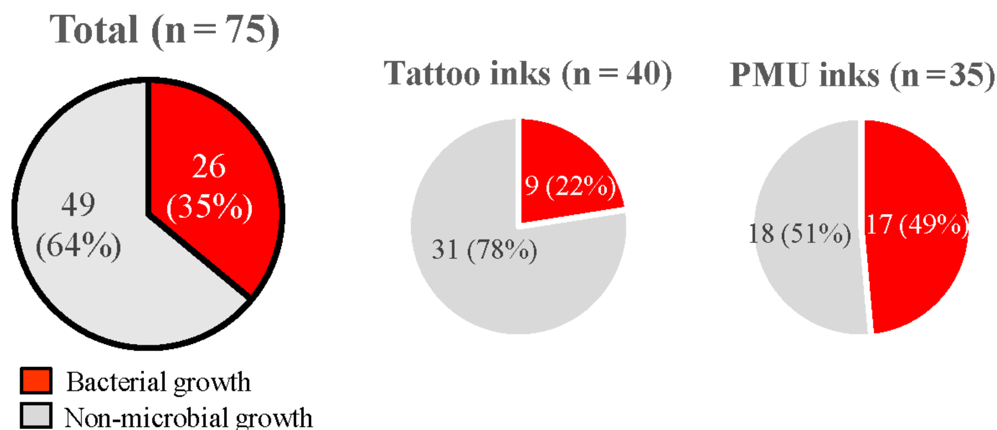

FIG 2 Comparison of the detection of microbial contamination in different tattoo and permanent makeup (PMU) ink samples.

Comparison of microbial contamination of tattoo and PMU inks

PMU inks showed a higher rate of microbial contamination (17 of 35 PMU inks, 49%) than tattoo inks (9 of 40 tattoo inks, 18%) (χ^2 [(1, N = 75]) =5.6, P < 0.05)] (Tables 4 and 5 and Fig. 2).

Sterility claims and microbial contamination

Nine manufacturers labeled their ink products (49 inks) as sterile, whereas the other five manufacturer labels did not contain such claims (26 inks, Tables 1 and 4). Among the nine manufacturers with claims of sterility, only products from three manufacturers, J, L, and M, did not have any microbial growth (Table 4). Of 49 inks with sterility claims on the product label, 16 inks (32.7%) contained bacterial contaminants, whereas 10 of 26 inks (38.5%) without sterility claims on the labels were contaminated with bacteria (Fig. 3). No significant association was found between a claim of sterility and absence of bacterial contamination (χ^2 [(1, N = 75]) =0.2, P = 0.61)].

Bacterial contamination of domestic and imported PMU inks

A total of 35 PMU inks (22 imported and 13 domestic inks) were surveyed in this study. Of the imported inks, 13 of 22 (59%) were contaminated with microorganisms compared with 4 of 13 (31%) domestic inks. Among the imported inks, six (from four imported manufacturers) were contaminated with anaerobes, including facultative anaerobes, whereas nine (from three imported manufacturers) were contaminated with aerobes. Conversely, among the domestic inks, one ink was contaminated with anaerobes, and three inks (from two manufacturers) were contaminated with aerobes. However, the difference in microbial contamination between imported and domestic inks was not statistically significant (Fisher exact test, P = 0.72).

DISCUSSION

This is the first microbiological survey of commercial tattoo and PMU inks that examined bacterial contamination under anaerobic conditions. The results of this study showed that unopened and sealed bottles of tattoo and PMU inks were contaminated with anaerobic and aerobic bacteria, indicating that contaminated tattoo inks can be a source of infection not only with aerobic but also anaerobic bacteria. In addition, from a methodological standpoint, this study confirmed that the current BAM Chapter 23 methods can detect anaerobic bacterial contaminants in tattoo and PMU ink products.

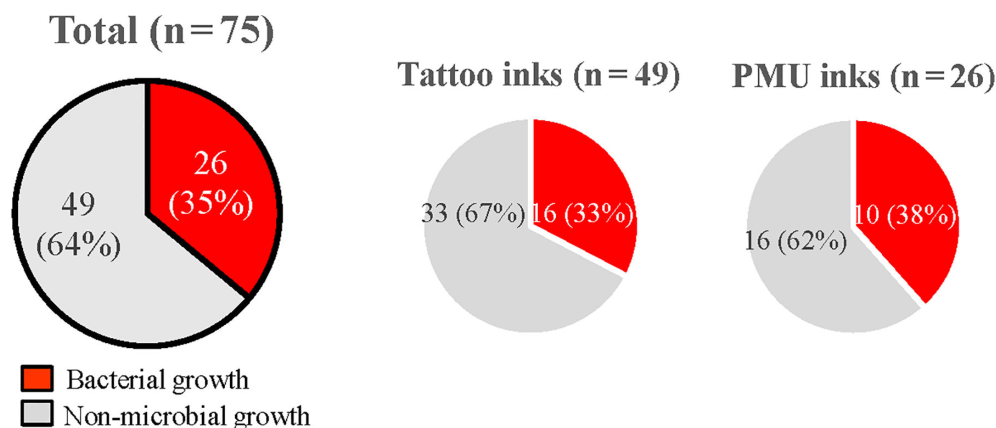

FIG 3 Comparison between inks with and without sterility claims in terms of microbial contamination.

TABLE 4 Detection and identification of bacteria in tattoo or PMU inks[a]

Ink #	Mfr	Sample #	Country of origin	Ink type	Sterility claim[b]	CFU/g			Bacterial identification
						Anaerobic	CO_2 (5%)	Aerobic	
1	A	A-01	Germany	PMU	Y	<250	–	–	*Staphylococcus epidermidis*
2		A-02		PMU	Y	–	–	–	
3		A-03		PMU	Y	–	–	–	
4	B	B-01	USA	PMU	NA	–	–	–	
5		B-02		PMU	NA	–	–	–	
6		B-03		PMU	NA	–	–	–	
7		B-04		PMU	NA	–	–	–	
8		B-05		PMU	NA	–	–	–	
9	C	C-01	France	PMU	Y	–	–	5.0×10^3	*Pseudomonas putida*
						–	2.5×10[d]	–[e]	*Staphylococcus saprophyticus*
10		C-02		PMU	Y	–	–	–	
11		C-03		PMU	Y	–	–	–[e]	*Bacillus aerius*
12		C-04		PMU	Y	–	–	–	
		C-05		PMU	Y	–	<250	<250	*Staphylococcus saprophyticus*
13						<250[c]	–	–	*Cutibacterium acnes*
14		C-06		PMU	Y	–	–	–	
		C-07		PMU	Y	–	2.5×10[d]	2.5×10^3	*Staphylococcus saprophyticus*
15						<250[c]	–	–	*Cutibacterium acnes*
16		C-08		PMU	Y	–	–	–[e]	*Staphylococcus xylosus*
17	D	D-01	China	PMU	NA	–	–	–[e]	*Stenotrophomonas maltophilia*
18		D-02		PMU	NA	<250[c]	–	–	*Cutibacterium acnes*
						–	–	2.5×10^3	*Sphingomonas yanoikuyae*
19		D-03		PMU	NA	–	–	3.0×10^3	*Sphingomonas olei*
						–	–	–[e]	*Chryseobacterium nepalense*
20		D-04		PMU	NA	–	–	–[e]	*Sphingomonas* spp.
						–	–	–[e]	*Massilia* spp.
21		D-05		PMU	NA	–	–	–	
22		D-06		PMU	NA	–	–	–[e]	*Sphingomonas* spp.
						–	<250	–[e]	*Pseudomonas putida*
23	E	E-01	China	PMU	NA	–	–	–	
24		E-02		PMU	NA	–	–	–	
25		E-03		PMU	NA	–	2.5×10^5[d]	2.5×10^5[d]	*Streptomyces thermoviolaceus*
26		E-04		PMU	NA	<250[c]	–	–	*Cutibacterium acnes*
27		E-05		PMU	NA	–	–	–	
28	F	F-01	USA	PMU	Y	–	–	–	
29		F-02		PMU	Y	–	–	–[e]	*Streptomyces virginiae*
30		F-03		PMU	Y	–	–	<250	*Nocardiopsis dassonvillei*
31	G	G-01	USA	Tattoo	NA	–	–	–	
32		G-02		Tattoo	NA	–	–	–	
33		G-03		Tattoo	NA	–	–	–	
34		G-04		Tattoo	NA	–	–	–	
35		G-05		Tattoo	NA	–	–	–[e]	*Staphylococcus warneri*
36	H	H-01	USA	Tattoo	Y	–	–	–[e]	*Staphylococcus saprophyticus*
37							–[e]	–[e]	*Stenotrophomonas maltophilia*
		H-02		Tattoo	Y	–	–	–	
38		H-03		Tattoo	Y	–	800	800	*Stenotrophomonas maltophilia*
39		H-04		Tattoo	Y	–	–	–	
40		H-05		Tattoo	Y	–	–	–	
41	I	I-01	USA	PMU	NA	–	–	–[e]	*Corynebacterium tuberculostearicum*
42		I-02		PMU	NA	–	–	–	
43		I-03		PMU	NA	<250	–	–	*Staphylococcus epidermidis*

(*Continued on next page*)

TABLE 4 Detection and identification of bacteria in tattoo or PMU inks[a] (*Continued*)

Ink #	Mfr	Sample #	Country of origin	Ink type	Sterility claim[b]	CFU/g			Bacterial identification
						Anaerobic	CO_2 (5%)	Aerobic	
44	I-04	PMU	NA	–	–	–			
45	I-05	PMU	NA	–	–	–			
46	J	J-01	USA	Tattoo	Y	–	–	–	
47		J-02		Tattoo	Y	–	–	–	
48		J-03		Tattoo	Y	–	–	–	
49		J-04		Tattoo	Y	–	–	–	
50	K	K-01	USA	Tattoo	Y	–	–	–	
51		K-02		Tattoo	Y	–	–	–	
52		K-03		Tattoo	Y	–	–	–[e]	*Brevibacillus agri*
53		K-04		Tattoo	Y	–	–	–	
54		K-05		Tattoo	Y	–	–	–	
55		K-06		Tattoo	Y	–	–	–	
56		K-07		Tattoo	Y	–	–	–	
57		K-08		Tattoo	Y	–	–	–	
58		K-09		Tattoo	Y	–	–	–	
59		K-10		Tattoo	Y	–	–	–	
60		K-11		Tattoo	Y	–	–	–	
61		K-2		Tattoo	Y	–	–	–	
62	L	L-01	USA	Tattoo	Y	–	–	–	
63		L-02		Tattoo	Y	–	–	–	
64		M-01	USA	Tattoo	Y	–	–	–	
65	M	M-02		Tattoo	Y	–	–	–	
66	N	N-01	USA	Tattoo	Y	–	–	<250	*Micrococcus luteus*
67		N-02		Tattoo	Y	–	–	4.5×10^4	*Pseudomonas putida*
68		N-03		Tattoo	Y	–	–	–	
69		N-04		Tattoo	Y	–	–	<250	*Bacillus firmus*
70		N-05		Tattoo	Y	–	–	–	
71		N-06		Tattoo	Y	–	–	1.2×10^3	*Dermacoccus nishinomiyaensis*
72		N-07		Tattoo	Y	–	–	–	
73		N-08		Tattoo	Y	–	–	–	
74		N-09		Tattoo	Y	–	–	–[e]	*Bacillus siralis*
75		N-10		Tattoo	Y	–	–	–	

[a]PMU, permanent makeup; Mfr, manufacturer; CFU, colony-forming unit.
[b]Y, sterility claimed in labeling; NA, sterility information not available.
[c] Growth was observed after extended time of incubation (7 to 14 days).
[d]Growth was observed from 1:1,000 dilution.
[e]Growth was observed after broth enrichment step.

Anaerobic plate count for tattoo and PMU inks

The anaerobic plate count (BAM Chapter 23 section H.3) method adopts three different bacterial growth media and conditions to detect anaerobes and aerobes. As shown in this study, the different growth media and conditions led to the successful cultivation of a variety of bacterial contaminants, including some obligate anaerobes, from the tattoo and PMU inks. In addition, the cultivation approach provided another opportunity to compare and verify each cultivation's results. In this study, six strains capable of obligate anaerobic growth were isolated. The sensitivity of the anaerobically cultured strains to oxygen was tested by inoculation on aerobic agar plates and cultivation under aerobic conditions. None of the six isolates grew on the MLA agar under aerobic conditions. The 16S rRNA gene sequence-based taxonomical identification of the six isolates further supports their phenotypic growth patterns. This study adopted phenotype-based (culture-based) bacterial isolation and genotype-based identification.

In this study, we used the anaerobic plate count methods from BAM Chapter 23 to recover and identify both anaerobes and aerobes. However, these techniques may not

TABLE 5 Classification of the 26 contaminated ink samples based on the ability to recover bacterial isolates under different oxygen concentrations[a]

Sample category	Anaerobe	Facultative anaerobe	Aerobe	No. of inks	
				Tattoo ink	PMU[b] ink
1	+	–	–	0	1
2	+	+	–	0	2
3	+	–	+	0	1
4	–	+	–	0	2
5	–	–	+	9	11

[a]Please refer to Table 3 for the oxygen requirements of bacterial contaminants in the products, some of which had multiple bacterial contaminants; +, bacterial growth; -, no bacterial growth.
[b]PMU, permanent makeup.

efficiently recover endospores and therefore our enumeration and detection may not have accounted for all endospores present in tattoo inks.

Potential inhibition effect of some ingredients

The PMU sample E-03 showed microbiological growth in 1:1,000 dilution but no growth in 1:10 or 1:100 dilution. The isolate was identified as a thermophilic *Streptomyces thermoviolaceus*. The growth pattern of the bacterial contaminant suggests a possible inhibition effect of the ingredient(s) of the PMU ink sample on microbial growth. In BAM Chapter 23, dilution and plating media that partially inactivate preservative systems commonly found in tattoo inks are utilized to minimize the inhibition of microbial contaminants. Previously, multiple potential antimicrobial ingredients have been identified in the tattoo and PMU ink matrices, including formaldehyde, methanol, denatured alcohols, aldehydes, titanium oxide, carbon, iron oxide, turmeric, copper, cadmium red, and tannins (33–35). We did not analyze or confirm the ingredient composition of the E-03 ink sample, but based on its ingredients list on the label, the PMU ink matrix (ink sample E-03) has propylene glycol (PG), a compound that can have bactericidal activity at certain concentrations.

Anaerobic and aerobic bacterial contamination

The six strains growing under anaerobic culture conditions were identified as an obligate anaerobic bacterium, *C. acnes*, and a facultative anaerobic bacterium, *S. epidermidis* (28, 36). To our knowledge, this is the first report regarding the isolation of obligate anaerobic *C. acnes* from PMU inks (Table 1). Strains of *S. epidermidis*, known to be a facultative anaerobe, were isolated from an obligate anaerobic condition but not from blood agar plates incubated in a 5% CO_2 atmosphere, which is more favorable for facultative anaerobic bacteria (32). By contrast, six strains capable of growing in both facultative and aerobic conditions were identified as aerobes.

C. acnes, S. saprophyticus, and *S. epidermidis*

C. acnes is a slow-growing anaerobic bacterium responsible for human diseases, such as acne and implant-associated infection (28). *C. acnes* is also known to contribute to the pathogenesis of staphylococcal skin infection via biofilm formation and break of homeostasis of the skin's microbiome (28). The co-occurrence of *C. acnes* and *S. saprophyticus* was found in a patient with inflammatory cutaneous and osteoarticular conditions (37). In our study, *S. saprophyticus* showed the highest connection degree to other organisms (i.e., a strong co-occurrence pattern). *S. saprophyticus* is a Gram-positive, coagulase-negative, non-hemolytic coccus that is a common cause of uncomplicated urinary tract infections, particularly in young sexually active females (38). By contrast, the facultative anaerobic *S. epidermidis* strains showed no co-occurrence pattern, even with other anaerobic isolates. A systematic analysis of the occurrence and co-occurrence of microbial contaminants could be useful to address fundamental and practical

questions (e.g., microbial contamination sources, degree of microbial complexity, and origins of microbial infection) in terms of microbial contamination and the corresponding infectious complications of tattoo and PMU inks (15, 16).

Sterility claims and microbial contamination

Of 49 inks (33%) labeled "sterile," 16 were still found to be contaminated with microorganisms, a smaller percentage compared with the previous survey (10 of 23 inks, 49%) (14–16). As confirmed in this study, no significant association was found between sterility claims and lack of bacterial contamination. These findings indicated that the actual sterilization process may not be effective to remove all microorganisms, or the label claims may not be accurate. Thus, the effectiveness of the current sterilization methods used in the tattoo ink manufacturing process needs to be evaluated.

Difference in bacterial contamination of tattoo and PMU inks

In this study, PMU inks showed a higher statistically significant percentage of contamination than tattoo inks. All of the tattoo inks surveyed in this study were from manufacturers whose products were previously evaluated by the FDA. By contrast, as revealed in a comparison of the abundance and diversity of the bacterial contaminants, PMU ink samples showed more diverse contamination profiles (14–16). It is worth noting that some PMU inks were imported to the U.S., whereas all tattoo inks were domestically produced. Further work is needed to assess pathogenic bacteria and bioburden, and the effects of microbial contamination on tattoo and PMU inks.

Bacterial contamination of domestic and imported inks

In this study, we have surveyed 75 ink samples from 14 manufacturers, consisting of 10 USA manufacturers and four foreign manufacturers. Although the ink samples of four USA manufacturers showed no microbial contamination, one USA (F) and two foreign manufacturers (C, France, and D, China) had the highest rate of bacterial contamination. Despite all the ink samples from manufacturer C (France) being labeled "sterile," 5 of 8 samples were contaminated with several bacterial strains, including an anaerobic pathogen, *C. acnes*, and an aerobic pathogen, *S. saprophyticus*. The ink samples from the top three manufacturers with the highest bacterial contamination rate were all PMU inks.

In conclusion, the results indicate that commercial tattoo and PMU inks contain both aerobic and anaerobic bacteria and highlight the importance of monitoring these products for the occurrence of pathogenic microorganisms.

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