Effect of lifestyle factors on *Staphylococcus aureus* gut colonization in Swedish and Italian infants

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Abstract

In recent years, *Staphylococcus aureus* has become a common bowel colonizer in Swedish infants. We aimed to identify host factors that determine such colonization. Stool samples from 100 Italian and 100 Swedish infants were obtained on seven occasions during the first year of life and cultured quantitatively for *S. aureus*. In a subgroup of infants in each cohort, individual strains were identified by random amplified polymorphic DNA analysis. Colonization at each time-point was related to delivery mode, siblings in family and antibiotic treatment. In total, 66% of the Italian and 78% of the Swedish infants had *S. aureus* in their stools on at least one time-point (p 0.08) and 4% of Italian and 27% of Swedish infants were positive on at least six of the seven time-points investigated (p 0.0001). Most infants analysed regarding strain carriage harboured a single strain in their microbiota for several months. The *S. aureus* stool populations in colonized infants decreased from 10^7 to 10^4 colony-forming units/g between 1 week and 1 year of age in both cohorts. In multivariate analysis, the strongest predictor for *S. aureus* colonization was being born in Sweden (OR 3.4 at 1 week of age, p 0.002). Having (an) elder sibling(s) increased colonization at peak phase (OR 1.8 at 6 months, p 0.047). Antibiotic treatment was more prevalent among Italian infants and correlated negatively with *S. aureus* colonization at 6 months of age (OR 0.3, p 0.01). To conclude, *S. aureus* is a more common gut colonizer in Swedish than Italian infants, a fact that could not be attributed to feeding or delivery mode.

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Introduction

Staphylococcus aureus is a versatile pathogen, causing infectious diseases like impetigo, boils, cellulitis, scalded skin syndrome, osteitis and septicaemia, as well as toxin-mediated food poisoning and toxic shock syndrome. In addition, drug-resistant *S. aureus* is an emerging threat. *Staphylococcus aureus* is a typical member of the skin microflora, principally colonizing the anterior nares [1–3]. Nasal carriage has been shown to increase the risk for *S. aureus* infection after surgery [4] and in haemodialysis patients [5]. Hospitalization [6], crowding [7], and immune suppression [8] have been linked to increased nasal *S. aureus* colonization.

More recently, *S. aureus* has become common in the gut flora of Swedish infants [9,10], perhaps as a result of decreased competition with traditional faecal bacteria, whose circulation has decreased in parallel with improved sanitary conditions [11]. As a result, 75% of 49 investigated infants had *S. aureus* in at least one stool culture during the first year of life [10]. Individual *S. aureus* strains usually persist for several months and commonly originate in the parents' skin flora [9]. *Staphylococcus aureus* counts were high in colonized newborn infants (10^7 CFU/g faeces on average), but decreased successively down to 10^4 CFU/g at 1 year of age [9,10]. This demonstrates that *S. aureus* lacks the ability to withstand the competition from the increasingly complex anaerobic microbiota that develops during the first year(s) of life [12]. In contrast, the population sizes of more

"professional" gut bacteria, such as *Escherichia coli*, decrease only moderately over the first year of life [11].

The clinical relevance of bowel carriage and factors promoting such carriage has been little investigated [13]. Two studies show a correlation between antibiotic-associated diarrhoea and intestinal carriage of toxin-producing *S. aureus* [14,15]. To our knowledge there are no studies on either host or bacterial factors that contribute to gut colonization by *S. aureus*. We compare gut colonization in Swedish and Italian infants and relate colonization to lifestyle factors, delivery and feeding modes.

Materials and Methods

Infant cohorts and their characteristics

One hundred healthy, full-term Italian infants born in 2001–2003 at Sandro Pertini's Hospital in Rome and 100 healthy, full-term Swedish infants born in 2000–2003 at the Sahlgrenska University Hospital in Göteborg were studied. The infants participated in the ALLERGYFLORA prospective birth-cohort study designed to investigate the relation between intestinal colonization pattern in infancy and later allergy development [16]. The *S. aureus* colonization pattern of 38 of the Swedish infants has previously been reported [9]. Delivery mode, sex, birth order, parental allergy and presence of pets in the household were recorded using questionnaires. Feeding pattern and antibiotic treatment were recorded by the parents in a diary and the records were collected by telephone interviews at 6 and 12 months of age (Table 1). The Comitato Etico ASL RM/B in Rome and the Medical Ethics Committee of the University of Gothenburg approved the study and written informed consent was obtained from the parents.

Culture and identification of *S. aureus* in the bowel flora

Rectal swabs were obtained 3 days after delivery and cultured semi-quantitatively under aerobic conditions, while faecal samples collected at 1, 2, 4 and 8 weeks and at 6 and 12 months of age were transported under anaerobic conditions and cultured quantitatively within 24 h on a range of selective and non-selective media [11]. Identical methods and reagents were used in the two laboratories to collect, store and cultivate the samples and to identify major commensal gut bacteria. Staphylococci were isolated on *Staphylococcus* medium (no. 110; Scharlau, Barcelona, Spain) prepared in-house in the two laboratories using identical ingredients from the same manufacturers. The plates were incubated aerobically at 37°C for 2 days. Colonies of different morphology, size, colour and texture were picked and enumerated separately, subcultured on blood agar, Gram-stained and tested for catalase and coagulase production. Coagulase-positive isolates were regarded as *S. aureus* and frozen at −70°C until further analysis. The level of detection was 330 ($10^{2.52}$) CFU/g faeces.

The staff were trained together to achieve uniform performance and inter-laboratory variation was monitored regularly by sending portions of freshly collected faecal samples in anaerobic transport bags (AnaeroGen Compact; Oxoid Ltd, Basingstoke, UK). Nine samples were simultaneously analysed in the two laboratories. *Staphylococcus aureus* was recovered from the same four samples in both laboratories, with median population counts of log 4.9 (4.3–5.9) CFU/g faeces in the Italian laboratory and log 5.4 (range 4.1–6.2) CFU/g faeces in the Swedish laboratory. The inter-laboratory variability was in the same range as the intra-laboratory variability, checked by dividing a faecal sample into three portions that were analysed independently by three technicians.

TABLE 1. Description of the Italian and Swedish cohorts

	Italy (n = 100)	Sweden (n = 100)
Vaginally delivered	61	85***
Male sex	49	52
Allergy in at least one parent	56	83***
Elder siblings	46	57
Pet(s) in household	25	12*
Antibiotic treatment (0–12 months)	63	22***
Exclusive breastfeeding		
From day 1	81	100***
At 2 months	76	88
At 4 months	61	73
Partial breast-feeding at 6 months	61	72

*p <0.05; **p <0.01; ***p <0.001.

Strain typing

Staphylococcus aureus isolates from 25 Italian and 25 Swedish culture-positive infants chosen at random were analysed for strain identity using random amplified polymorphic DNA (RAPD) as described previously [10]. Each strain was analysed for *in vitro* production of staphylococcal enterotoxins (SE) A, B, C and D and toxic shock syndrome toxin-1 (TSST-1) (SET-RPLA and TST-RPLA kits; Oxoid).

Statistics

Frequencies were compared using the Chi-square or Fisher's exact tests, and bacterial population numbers were compared by Mann–Whitney *U*-test (GraphPad prism version 3.0). Multiple logistic regression analyses were used to assess the independent effects of country and lifestyle factors on *S. aureus* colonization at each time-point. The OR and p values for the effect of each explanatory variable on the outcome (colonization) were calculated (spss software package version 16.0).

Results

S. aureus colonization in Italian and Swedish infants

The proportion of Italian and Swedish infants yielding a stool culture positive for *S. aureus* as a function of age is shown in Fig. 1(a). Few infants were positive for *S. aureus* in the 3-day sample, but thereafter colonization rate increased rapidly in both cohorts. Colonization reached a maximum at 2 months of age in the Swedish cohort and at 6 months of age in the Italian cohort, whereafter it decreased significantly in both cohorts (p 0.03 and p 0.01, respectively) (Fig. 1a). The *S. aureus* was significantly more often retrieved from Swedish than Italian infants at all time-points between 1 and 8 weeks of age (Fig. 1a). In all, 78% of the Swedish and 66% of the Italian infants had *S. aureus* in at least one stool sample during the first year of life (p 0.08).

Whereas 23% of the Swedish infants harboured *S. aureus* on at least six of the seven culture occasions, this was only true for 4% of the Italian infants (p 0.0001). Similarly, 51% of the Swedish infants were positive on at least four occasions compared with 27% of the Italian infants (p 0.008).

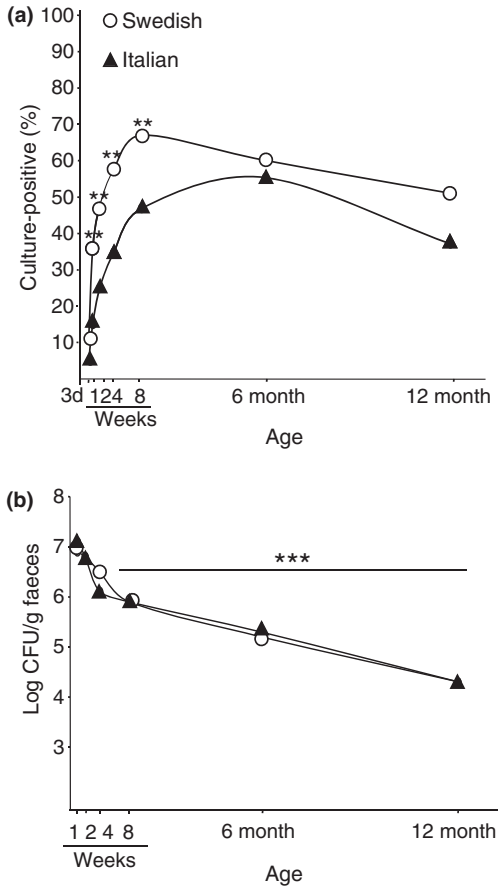

FIG. 1. (a) Intestinal colonization frequency of *Staphylococcus aureus* in 100 Italian infants (filled triangles) and 100 Swedish infants (open circles) followed during the first year of life. The 3-day sample consisted of a rectal swab culture, the rest of the samples were quantitative stool cultures with a limit of detection of 330 colony-forming units (CFU)/g faeces (2.52 log units). The difference in colonization rate between Swedish and Italian infants was significant at the level: **p <0.01. (b) *S. aureus* population counts in stool samples collected at different time-points from culture-positive Italian (n = 64) and Swedish (n = 78) infants. In both populations, *S. aureus* stool counts decreased significantly (***p <0.001) between 2 and 12 months of age in culture-positive individuals, while there was no significant difference between Italian and Swedish infants at any time-point.

Persistence of individual strains

We have previously shown that Swedish infants who were positive for *S. aureus* in repeated cultures usually harboured a single strain that persisted over several months in the microbiota. Here we performed RAPD analysis on a subsample of 25 Italian and 25 Swedish culture-positive infants to determine whether *S. aureus* isolates found in repeated cultures belonged to the same strain or not. In infants harbouring the same strain on at least two time-points (21/25 in both cohorts), the strains persisted for an average of 6.9 and 8.1 months in the Italian and Swedish cohorts, respectively (p 0.34). Averages of 1.6 and 1.3 strains were detected per infant (p 0.14).

In vitro production of the superantigenic toxins SEA to SED and TSST-1 was assessed in the strains identified in the 25 Italian and Swedish infants (Table 2). Production of more than one toxin, most commonly SEA and TSST-1, occurred in nine of 39 strains colonizing Italian infants compared with two of 33 strains from Swedish infants. SEC production characterized most Swedish toxin-positive strains.

TABLE 2. *In vitro* toxin production by *Staphylococcus aureus* strains isolated from Italian and Swedish infants

	***S. aureus* strains**			
	Italian cohort		**Swedish cohort**	
	n	**%**	***n***	**%**
Enterotoxin A	7	18	2	6
Enterotoxin B	5	13	0	0
Enterotoxin C	6	15	13	39
Toxic shock syndrome toxin-1	8	21	6	18
None of the above	18	46	13	39
Total	39		33	

Strains from 25 culture-positive Italian infants and 25 culture-positive Swedish infants were analysed for toxin production (SEA-D, TSST-1) by latex agglutination of overnight cultures. None of the strains produced enterotoxin D (SED).

S. aureus population levels in colonized infants

Population counts of *S. aureus* in culture-positive infants are shown in Fig. 1(b). In both cohorts, *S. aureus* averaged 10^7 CFU/g faeces in 1-week-old infants, but declined progressively to 10^4 CFU/g 12 months of age ($p < 0.0001$ for both cohorts). At no time-point did *S. aureus* stool counts differ significantly between culture-positive Swedish and Italian infants (p 0.2 at 4 weeks).

Lifestyle factors and colonization by *S. aureus*

We investigated the effect of feeding and delivery mode, antibiotic treatment and pets and siblings in the household on *S. aureus* colonization in the bowel. Several of these factors were unequally distributed between the Italian and Swedish cohorts (Table 1). Italian infants not only received antibiotics more often than Swedish infants (Table 1), but the types of antibiotic given to the Italian infants were also more often those that theoretically would be active against most *S. aureus* strains. Hence, cephalosporins, ampicillin with the β-lactamase inhibitor clavulanic acid, or other broad-spectrum antibiotics were more commonly given to Italian infants, whereas Swedish infants mostly received penicillin V, to which most *S. aureus* are resistant today (Table 3).

Univariate analysis. The effect of lifestyle factors on colonization by *S. aureus* was first analysed univariately for the Italian and Swedish cohorts separately. In the Swedish cohort, colonization by *S. aureus* was more prevalent in infants delivered vaginally (52% vs 20% at 2 weeks of age, p 0.027 and 71% vs 40% at 8 weeks, p 0.035). In the Italian cohort, elder siblings correlated positively with *S. aureus* colonization (60% vs 35%, p 0.02 by 2 months of age).

At 6 and 12 months of age, Italian infants who had received antibiotics in the two previous months less frequently harboured *S. aureus* compared with non-treated infants (29% vs 64%; p 0.004 and 23% vs 43%; p 0.09). Swedish infants with a family history of allergy were significantly less often colonized by *S. aureus* at 3 days of age than infants without such a history (7% vs 29%; p 0.025).

The effect of breastfeeding was investigated comparing infants who were exclusively breastfed at 1 or 2 months of age, with infants who were only partially breastfed or were not breastfed at the same time-points. At 6 and 12 months of age, infants receiving some breast-milk were compared with completely weaned infants. No significant differences were found in either cohort.

Multivariate analysis. Logistic regression analyses were performed with *S. aureus* colonization as dependent variable. Country of residence and the factors used in univariate analysis were the independent variables. As colonization was strongly age-dependent, separate models were made for each time-point.

Being born in Sweden was the strongest predictor for intestinal colonization by *S. aureus* with OR between 2.1 and 3.4 over the first 2 months (Table 4). Having elder siblings was also associated with an increased rate of *S. aureus* colonization, but the effect was seen mainly in the peak colonization phase (2–6 months). Antibiotic treatment strongly decreased *S. aureus* colonization in the late phase (OR 0.3 at 6 months, p 0.01, Table 4).

Delivery mode, exclusive or partial breastfeeding, pets in the household or a history of family allergy had no significant effects on *S. aureus* colonization rate.

TABLE 3. Frequency of antibiotic treatment during the first 12 months of life in the Italian and Swedish cohorts

	Number of infants treated with antibiotics					
	0–6 months			6–12 months		
	Swedish	Italian	p	Swedish	Italian	p
Any antibiotics	7	28	0.0001	20	45	0.0003
Penicillin V	6	0	0.03	14	0	0.0001
Ampicillin/Amoxicillin	0	12	0.0003	2	19	0.0001
Amoxicillin + Clavulanic acid	0	7	0.01	3	14	0.009
Cephalosporins	0	7	0.01	3	13	0.02
Macrolides	2	10	0.03	2	13	0.005
Trimethoprime/ Sulphametoxazole	1	0		2	2	
Nitrofurantoin	0	0		1	0	

Treatment with antibiotics during the first year of life was recorded by the parents in a diary. One hundred infants in each cohort were examined.

Discussion

We have previously reported that *S. aureus* has become a member of the commensal gut flora of a significant proportion of Swedish infants [9,10]. Here we demonstrate that 66% of the Italian infants studied also had *S. aureus* in their stools for part of their first year of life. In the subgroups of Italian and Swedish infants analysed regarding strain carriage, most infants harboured strains that persisted in the gut microbiota for several months, confirming the conclusion from our previous study that the phenomenon with gut *S. aureus* colonization is not the result of occasional contamination from the skin [9,10]. Furthermore, *S. aureus* population levels in the stools were initially quite high (about 10^7 CFU/g faeces), numbers that can only be reached if the bacteria are actively dividing in the gut. However, *S. aureus* population numbers soon started to decline and averaged 10^4 CFU/g faeces by 1 year of age. This 1000-fold reduction reflects the

TABLE 4. Effect of country of residence and lifestyle factors on *Staphylococcus aureus* colonization frequency at different time-points

		Effect of variable on *S. aureus* colonization (odds ratio)						
		3 days (*n* = 188)	1 week (*n* = 180)	2 weeks (*n* = 185)	4 weeks (*n* = 186)	8 weeks (*n* = 186)	6 months (*n* = 184)	12 months (*n* = 184)
Born in Sweden	OR	3.0	3.4	2.3	2.3	2.1	0.96	1.8
	p	0.079	**0.002**	**0.02**	**0.01**	**0.03**	0.9	0.08
Atopic heredity	OR	0.36	0.96	1.2	1.1	0.86	0.67	0.55
	p	0.078	0.91	0.63	0.80	0.69	0.26	0.08
Delivered by caesarean section	OR	0.79	0.64	0.71	0.87	1.0	0.7	1.1
	p	0.74	0.31	0.37	0.71	0.99	0.35	0.68
Breastfeeding	OR	NA	NA	NA	0.7[a]	0.84[a]	0.64[b]	1.0[b]
	p				0.42	0.68	0.19	0.98
Siblings	OR	1.3	0.85	1.04	1.3	1.8	1.8	0.95
	p	0.59	0.65	0.89	0.42	0.065	**0.047**	0.87
Pets	OR	1.5	2.2	0.66	0.89	0.75	1.29	1.3
	p	0.60	0.08	0.35	0.76	0.47	0.53	0.51
Antibiotics in previous 2 months	OR	NA	NA	NA	NA	0.78	0.30	0.60
	p					0.75	**0.01**	0.20
R^2		0.03	0.08	0.07	0.05	0.06	0.08	0.04

The likelihood of yielding a stool culture-positive for *S. aureus* at different time-points was modelled as a function of potential explanatory factors by logistic regression. The cohort consisted of 100 Swedish and 100 Italian infants.
NA, not analysed; variable not included in the multivariate analysis because very few infants in either group.
[a]Exclusive breastfeeding (=1) vs partial or no breastfeeding (=0).
[b]Partial or exclusive breastfeeding (=1) vs no breastfeeding (=0).
Significant p values (<0.05) are in bold in the table

poor capacity of *S. aureus* to survive in a complex gut microbiota. *Escherichia coli* and enterococci also decline in numbers over the first year of life, but only by a factor of ten [11], which demonstrates their superior fitness in the bowel flora compared with *S. aureus*, whose normal niche is the skin.

Staphylococcus aureus is a common colonizer of the nose and pharynx. From these sites, *S. aureus* could seed the gut, but the high population levels strongly indicate that *S. aureus* multiplies in the gut of the infant, which is the definition of colonization. We did not investigate skin or nasal carriage of *S. aureus* in the infants studied here. In a previous study, we investigated nasal and gut colonization by *S. aureus* in 35 3-day-old infants. Four of these infants harboured *S. aureus* in the nose only, four in the gut only and two were colonized at both locations [9]. Our preliminary results from an ongoing study indicate that colonization of both the nose and the gut is the most common pattern in infants colonized in the first weeks of life, but a minority are positive in the nose or in the gut only, each pattern being approximately equally common (Lindberg E, Adlerberth I, Rudin A, Hesselmar B, Saalman R, Aberg N, Wold AE, unpublished data).

Italian infants were less often colonized by *S. aureus* than were Swedish infants at all time-points before 6 months of age and more Swedish than Italian infants yielded *S. aureus* in several consecutive samples. In multivariate analysis, Swedish residence was the strongest determinant of *S. aureus* colonization with OR varying between 2.1 and 3.4 over the first 2 months of life. Most *S. aureus* strains colonizing Swedish infants derive from their parents' skin or nasal flora [9]. Whether nasal carriage was more common in the Swedish than in the Italian parents in the present study is not known because no sampling of family members was performed. However, previous studies indicate similar figures of nasal *S. aureus* carriage in Sweden and Italy [17,18]. Other possible explanations for the high rate of *S. aureus* gut colonization in Swedish compared with Italian infants include more contact leading to transmission in Swedish compared with Italian families, or reduced obstacles to expansion of *S. aureus* in the gut microbiota of Swedish compared with Italian infants. Having elder siblings was positively associated with *S. aureus* colonization at 6 months of age, indicating transmission between children in a family, as previously shown between parent(s) and child in Swedish families [9].

We saw no difference in colonization by *S. aureus* related to breastfeeding, which contrasts with an earlier Swedish study where *S. aureus* colonization was more prevalent in breastfed infants [19]. However, as practically all infants were breastfed for at least 2 months, we could only study the effect of breastfeeding after 2 months of age, when this factor may be less important. The dramatically increased prevalence of breastfeeding in the last decades, e.g. a tripled incidence in Sweden between 1975 and 1995 [20] could be one factor behind the increased *S. aureus* colonization rate.

Antibiotic treatment was negatively associated with *S. aureus* colonization rate at 6 months of age, an effect only seen in Italian infants who often received antibiotics such as cephalosporins and macrolides that could be active against *S. aureus* in the gut flora. Swedish infants received fewer antibiotics and were usually given drugs with a narrow spectrum (penicillin V) without activity against *S. aureus*.

We identified colonization at the strain level in a subsample of 25 culture-positive Swedish infants and 25 culture-positive Italian infants. The pattern was similar to what we reported for a Swedish cohort studied previously [10], i.e. persistence of a single strain for many months being the most common colonization pattern with occasional occurrence of a second strain during shorter time span. At least half of the strains could produce one or more of the classical superantigenic toxins *in vitro*, usually SEA, SEC and/or TSST-1, which is similar to what we previously reported for another cohort of Swedish infants [10]. Whether *S. aureus* superantigens are produced *in situ* in the gut of the colonized infants is not known, but we have earlier reported that colonization by toxin-producing *S. aureus* is associated with accelerated maturation of the serum IgA system [21]. Furthermore, colonization by *S. aureus* is associated with increased levels of the immunoregulatory molecule soluble CD14 in serum [22] and protection from food allergy [22]. The superantigens of *S. aureus* are the strongest T-cell stimulators known, activating 10–30% of all T cells [23], and protein A, produced by almost all *S. aureus*, is a B-cell mitogen [24]. Hence, we believe that *S. aureus* gut colonization might have strong immune stimulating and important immunoregulatory effects. Despite the fact that *S. aureus* toxins are a cause of food poisoning, infants colonized by toxin-producing strains had no more gastrointestinal symptoms than other infants [10].

During the 1970s and 1980s *S. aureus* was rare in the infantile intestinal microflora [19,25] and its increase may be the result of reduced or delayed colonization by traditional faecal bacteria in the last decades and to changes in lifestyle. As *S. aureus* is a versatile pathogen and also commonly carries antibiotic resistance elements, its emergence as a common gut colonizer in infants should be recognized as a phenomenon of potential clinical importance.

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Transparency Declaration

The authors declare no conflict of interest.

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