

Population Biology of Intestinal *Enterococcus* Isolates from Hospitalized and Nonhospitalized Individuals in Different Age Groups

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The diversity of enterococcal populations from fecal samples from hospitalized ($n = 133$) and nonhospitalized individuals ($n = 173$) of different age groups (group I, ages 0 to 19 years; group II, ages 20 to 59 years; group III, ages ≥ 60 years) was analyzed. Enterococci were recovered at similar rates from hospitalized and nonhospitalized persons (77.44% to 79.77%) of all age groups (75.0% to 82.61%). *Enterococcus faecalis* and *Enterococcus faecium* were predominant, although seven other *Enterococcus* species were identified. *E. faecalis* and *E. faecium* (including ampicillin-resistant *E. faecium*) colonization rates in nonhospitalized persons were age independent. For inpatients, *E. faecalis* colonization rates were age independent, but *E. faecium* colonization rates (particularly the rates of ampicillin-resistant *E. faecium* colonization) significantly increased with age. The population structure of *E. faecium* and *E. faecalis* was determined by superimposing goeBURST and Bayesian analysis of the population structure (BAPS). Most *E. faecium* sequence types (STs; 150 isolates belonging to 75 STs) were linked to BAPS groups 1 (22.0%), 2 (31.3%), and 3 (36.7%). A positive association between hospital isolates and BAPS subgroups 2.1a and 3.3a (which included major ampicillin-resistant *E. faecium* human lineages) and between community-based ampicillin-resistant *E. faecium* isolates and BAPS subgroups 1.2 and 3.3b was found. Most *E. faecalis* isolates (130 isolates belonging to 58 STs) were grouped into 3 BAPS groups, BAPS groups 1 (36.9%), 2 (40.0%), and 3 (23.1%), with each one comprising widespread lineages. No positive associations with age or hospitalization were established. The diversity and dynamics of enterococcal populations in the fecal microbiota of healthy humans are largely unexplored, with the available knowledge being fragmented and contradictory. The study offers a novel and comprehensive analysis of enterococcal population landscapes and suggests that *E. faecium* populations from hospitalized patients and from community-based individuals differ, with a predominance of certain clonal lineages, often in association with elderly individuals, occurring in the hospital setting.

Enterococci are relatively minor constituents of the human gastrointestinal microbiota (less than 1%) but are able to cause a wide diversity of infections, mostly in patients with underlying diseases (1, 2). High-density colonization by antibiotic-resistant enterococci increases the risk of bacteremia and transmission; however, the population structure and ecological and evolutionary forces influencing the population dynamics of gut colonizers largely remain unknown (3–5). Next-generation sequencing has provided a wealth of data about the influence of characteristics of the host (age, diet, health status, and antibiotic treatment) on the diversity and population frequency of different bacterial groups, including enterococci (6–10). However, the information provided by current metagenomic analysis, based on 16S rRNA (11, 12), or by the traditional culture-based studies (1, 13, 14) precludes any possible analysis of enterococci at the subspecies level. Furthermore, the available information about the frequency and diversity of enterococcal species in the fecal microbiota by host age is fragmented and contradictory (1, 15).

Different methods based on multilocus sequence typing (MLST), comparative genomic hybridization, and whole-genome sequencing revealed intraspecies diversity for *Enterococcus faecalis* and *Enterococcus faecium*, which are the predominant enterococcal species colonizing the human gastrointestinal tract (16–25). *E. faecium* has a population structure that has split into two major phylogenomic clusters, designated clade B, which includes community-based human isolates, and clade A, which comprises iso-

lates from humans and animals, with a clade A1 being enriched with isolates from hospitalized patients. Specifically, strains belonging to the sequence type (ST) 17 (ST17), ST18, and ST78 lineages, which are found within clade A1, are often resistant to antibiotics and are the most frequently associated with the hospital environment (22, 26, 27). *E. faecalis*, on the other hand, seems to lack such a clear clade structure, probably because this species occupies a larger variety of ecological niches, thus having access to a more heterogeneous spectrum of alleles than *E. faecium* (28). As a result, no clear genotypic differences are observed between hospital and community isolates (25, 28, 29), even though

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some clonal complexes (CCs) are more prevalent either among hospitalized patients, e.g., CC6-ST6, CC9-ST9, CC28-ST87, and CC40-ST40, or among community healthy volunteers, e.g., ST16 and CC58 (30–33). Recombination, which was previously detected in enterococci (17, 34, 35), may have a considerable impact on patterns of evolutionary descent, as displayed by sequence-based gene trees or even by popular allele-based population snapshots provided by eBURST analysis. This may obscure the genetic relatedness of strains and clones and, as such, interfere with epidemiological and clinical investigations, in particular, when strains are assigned to specific CCs. In addition, knowledge about the population structure of enterococcal species is biased by an overrepresentation of contemporary multidrug-resistant (MDR) clinical isolates belonging to a few high-risk clonal complexes often associated with nosocomial outbreaks and frequently associated with elderly individuals (36–38). Studies analyzing early isolates have documented a more diverse enterococcal population able to cause disease acquired either nosocomially or in the community and often associated with nonelderly adults and children. Isolates causing infections or colonizing these populations have less frequently been analyzed at the molecular level (33, 39–41).

The objective of this study was to assess for the first time the population structure of enterococci in the feces of both hospitalized and nonhospitalized individuals within different age groups. In addition, Bayesian analysis of the population structure (BAPS), a nonphylogenetic method able to find the best partition of a set of isolates into subpopulations, was applied, broadening former results obtained for *E. faecium* and providing the first analysis to probabilistically assign *E. faecalis* strains to evolutionary groups.

MATERIALS AND METHODS

Bacterial samples. Three hundred six fecal samples were collected between April 2009 and April 2011 at the Ramón y Cajal University Hospital (HRyC) and its community care area of influence. HRyC is a tertiary care public hospital with 1,155 beds that provides specialized attention to a population of about 600,000 inhabitants in the northern area of Madrid, Spain, who primarily attend 20 primary health centers (PHCs) of the Madrid Health Service (SERMAS). This study was conducted according to applicable government regulations and approved studies by institutional research policies (e.g., reference CEIC-106/09 [A. M. Sánchez-Díaz, C. Cuartero, J. D. Rodríguez, S. Lozano, J. M. Alonso, M. J. Rodríguez-Domínguez, A. P. Tedi, R. del Campo, J. López, R. Cantón, and P. Ruiz-Garbajosa, unpublished data]).

The samples analyzed were recovered from 173 patients with nonsevere diseases that attended a PHC or had a consultation at HRyC (and for whom no hospitalization was registered in the 6 months prior to sample collection) and from 133 hospitalized patients admitted to HRyC. The fecal samples were submitted to HRyC for stool culture with or without a specific request for *Clostridium difficile* or parasite detection and were anonymously processed so that the patients' demographic information was kept confidential. Hospitalized patients were mostly located in medical wards (78.2%), surgical wards (8.3%), and intensive care units (ICUs; 9.8%). All but 20 samples from hospitalized patients were collected after more than 48 h of hospital admission. However, these 20 patients had a history of several recent previous hospitalizations (see Tables S1 and S2 in the supplemental material).

Samples were also classified into three age groups according to the host's age. These three groups are designated with roman numerals as group I (young people 0 to 19 years old, $n = 92$ [30%], 57 nonhospitalized persons and 35 hospitalized patients), group II (adults 20 to 59 years old, $n = 108$ [35%], 62 nonhospitalized persons and 46 hospitalized patients), and group III (elderly individuals ≥ 60 years old, $n = 106$ [35%], 54 nonhospitalized persons and 52 hospitalized patients). Only one sample

per patient was analyzed (see Tables S1 and S2 in the supplemental material).

Sample processing. About 0.5 g of each fecal sample was suspended in 1 ml of saline solution, plated on plain *m-Enterococcus* agar (Difco, Detroit, MI, USA) or *m-Enterococcus* agar supplemented with either ampicillin (10 $\mu\text{g/ml}$) or vancomycin (6 $\mu\text{g/ml}$), and incubated for 48 h at 37°C. For each sample, one colony per morphology type and plate was selected (28) for further studies. In order to enhance the recovery of minority populations of ampicillin-resistant enterococci and vancomycin-resistant enterococci (VRE), 0.1 ml of the original suspension of each sample was preenriched in brain heart infusion (BHI) broth (Difco, Detroit, MI, USA) supplemented with 2 $\mu\text{g/ml}$ of ampicillin or 2 $\mu\text{g/ml}$ of vancomycin, incubated for 24 h at 37°C, and subsequently plated on *m-Enterococcus* agar (Difco, Detroit, MI, USA) containing ampicillin (10 $\mu\text{g/ml}$) or vancomycin (6 $\mu\text{g/ml}$), respectively.

Identification, antibiotic susceptibility, and virulence traits. Bacterial identification was performed by the amplification of species-specific genes, *D*-alanine-*D*-alanine ligase (*ddl*) for *E. faecalis* and *aac(6')*-*Ii* for *E. faecium*, as previously described (42, 43), and by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (Bruker, Daltonics, Bremen, Germany). Susceptibility to ampicillin, vancomycin, teicoplanin, streptomycin, gentamicin, ciprofloxacin, levofloxacin, erythromycin, tetracycline, and chloramphenicol (Oxoid, Basingstoke, United Kingdom) was determined by the disc diffusion method according to CLSI guidelines (44).

The presence of putative virulence genes encoding the *E. faecium* enterococcal surface protein (*esp*), glycosyl hydrolase (*hyl_{E. faecium}*), and collagen-binding adhesin (*acm*) and the *E. faecalis* enterococcal surface protein (*esp*), hyaluronidase (*hyl_{E. faecalis}*), cytolysin/hemolysin (*cylA*), gelatinase (*gelE*), and aggregation substance (*asa1*) was investigated by PCR and sequencing, as described before (45, 46).

Clonal relatedness. The clonal relationship among isolates of each enterococcal species was established by pulsed-field gel electrophoresis (PFGE) and MLST as previously described (16, 47), and the relationships are detailed in Tables S1 and S2 in the supplemental material. Clusters of related STs for *E. faecalis* (differing in no more than two of the seven MLST loci) were considered to belong to the same CC using the goeBURST algorithm (48, 49). CCs were defined on the basis of goeBURST analysis of the 524 STs present in the *E. faecalis* MLST database (<http://efaecalis.mlst.net/>).

Analysis of population structure. A BAPS software was used to probabilistically assign *E. faecalis* and *E. faecium* STs to nonoverlapping evolutionary groups (27, 50). BAPS clustering was performed with the second-order Markov model and the standard MLST data input option in a hierarchical manner. For *E. faecium*, the major clusters identified at the first stage were reanalyzed after excluding the remaining data. The rationale for this approach is to increase the statistical power to detect the more fine-scale genetic structure of a population when analyzing particular lineages separately from the remaining population. In all BAPS analyses, 10 runs of the estimation algorithm were performed using *a priori* upper bounds (10 to 30 for the major group analysis and 2 to 10 for the subgroup analysis) for the number of clusters over the interval, and in each case the runs converged to a nearly identical partition of the data in question, indicating a high level of peakedness of the posterior distribution (estimated $P = 1.000$).

The accuracy of BAPS for establishing the *E. faecium* population structure was determined using different sample sizes and discarding the inclusion of *E. faecalis* as the outgroup (see Fig. S1 to S3 in the supplemental material) (27). Correlation analysis was performed for each of the comparisons mentioned above using Microsoft Excel 2010 software. This study constitutes the first application of BAPS to investigate *E. faecalis* population and evolutionary genetics by the same approach that was previously used for *E. faecium* (27).

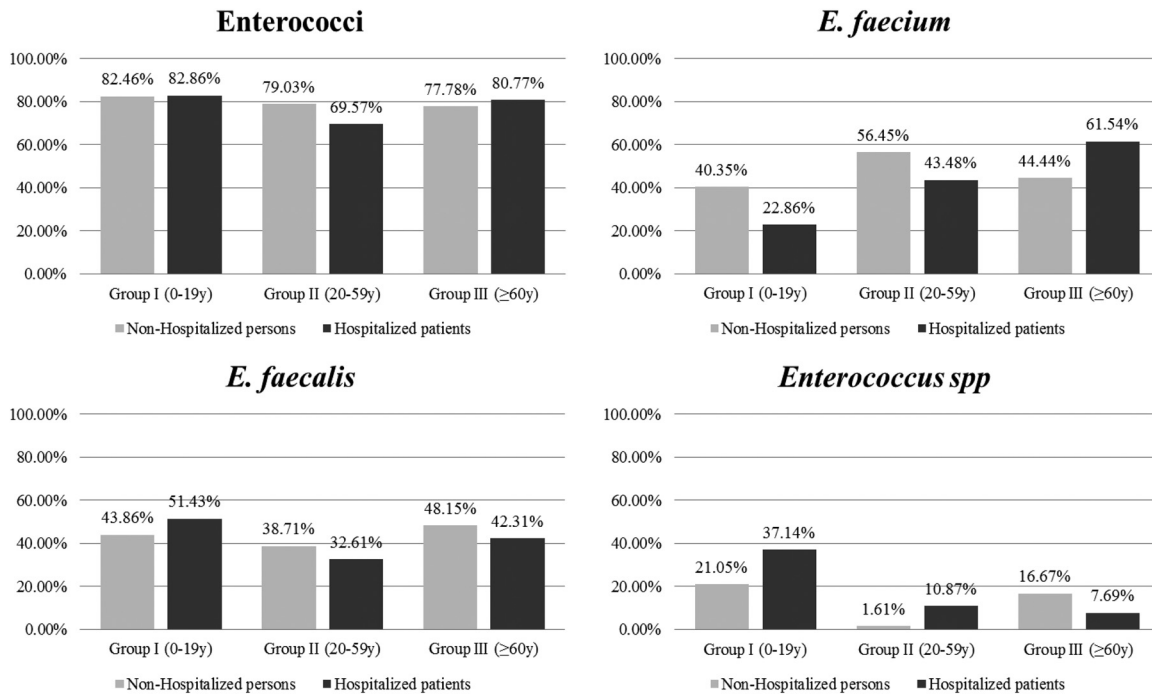


FIG 1 Proportion of nonhospitalized and hospitalized individuals colonized with enterococci by age group. The *E. faecalis*/*E. faecium* colonization ratios in nonhospitalized persons by age group were as follows: group I (0 to 19 years old), 1.08; group II (20 to 59 years old), 0.68; and group III (≥60 years old), 1.08. The *E. faecalis*/*E. faecium* colonization ratios in hospitalized patients were as follows: group I (0 to 19 years old), 2.25; group II (20 to 59 years old), 0.75; and group III (≥60 years old), 0.68.

Statistical analysis. The statistical significance of the results was calculated by the chi-square test; *P* values of <0.05 were considered statically significant.

The STATA generalized estimating equations (GEE) model (which takes into account clone-related data) (51) was used for calculating odd ratios (ORs) and 95% confidence intervals (CIs) related to the colonization isolates. The analyses were done by comparison with major BAPS subgroup 3.3a (BAPS 3.3a) for *E. faecium* and BAPS group 1 (BAPS 1) for *E. faecalis*.

For the analysis of all isolates available in MLST databases, ORs between BAPS groups and different sources (hospitalized patients, nonhospitalized persons, and animals) were calculated. Environmental, food, and other sources were also considered, but due to the low number of isolates in these categories, OR analysis was not performed.

RESULTS

Prevalence and diversity of enterococcal species in human fecal samples. Enterococci were recovered by culture from 78.8% of the individuals analyzed (*n* = 241/306) and at similar rates between

hospitalized and nonhospitalized individuals (77.4% versus 79.8%) and among all age groups (75.0 to 82.6%). The enterococci recovered corresponded to three of the five groups of enterococci previously described by Facklam et al. (52) on the basis of phenotypic and genotypic characteristics, which used to be designated by roman numerals (1). The rate at which individuals were colonized by different species varied in each age group, with *E. faecalis* and *E. faecium* being the predominant species identified (Fig. 1 and 2). Among nonhospitalized persons, *E. faecalis* and *E. faecium* colonization rates were age independent (*E. faecalis*/*E. faecium* ratios, 1.14, 0.71, 1.12 for age groups I, II, and III, respectively). The *E. faecalis* colonization rate among hospitalized patients was also age independent, but the *E. faecium* colonization rate and particularly the ampicillin-resistant *E. faecium* colonization rate significantly (*P* < 0.01) increased with age (*E. faecalis*/*E. faecium* ratios, 1.90, 0.71, and 0.65 for age groups I, II, and III, respectively) (Fig. 1 and 3). Besides *E. faecium* and *E. faecalis*, both classified

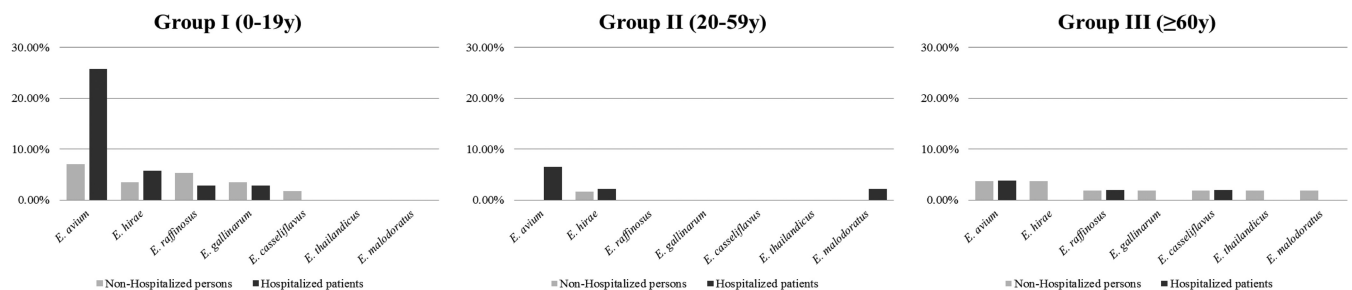


FIG 2 Proportion of nonhospitalized and hospitalized individuals colonized by different *Enterococcus* spp. by age group.

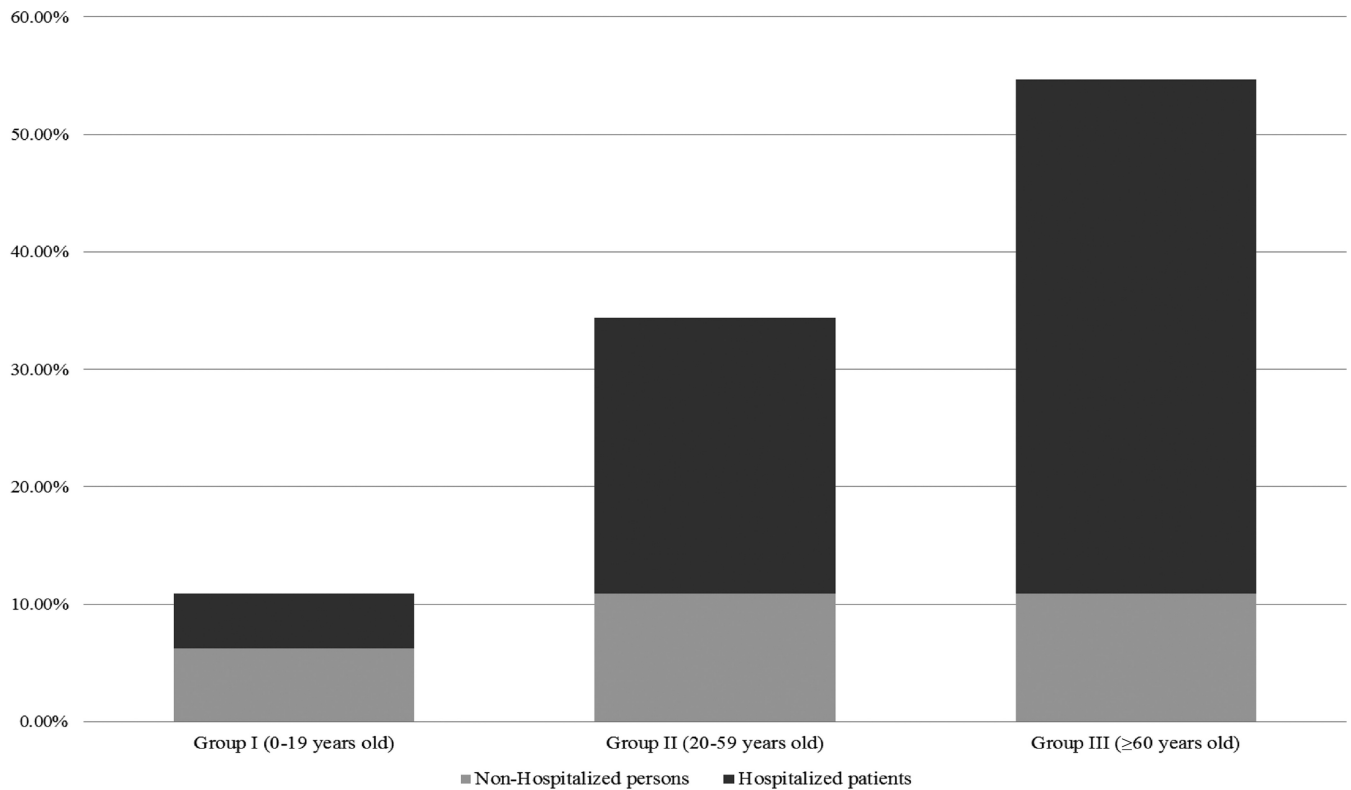


FIG 3 Proportion of nonhospitalized and hospitalized individuals colonized by ampicillin-resistant *E. faecium* by age group. The proportions of hospitalized/nonhospitalized individuals colonized by ampicillin-resistant *E. faecium* were as follows for the different age groups: group I (0 to 19 years old), 0.75; group II (20 to 59 years old), 2.14; and group III (≥ 60 years old), 4.

within enterococcal group II described by Facklam et al. (52), other species within enterococcal groups I (20 *E. avium*, 7 *E. raffinosus*, 2 *E. malodoratus* isolates), II (4 *E. casseliflavus* isolates, 3 *E. gallinarum* isolates, 1 *E. thailandicus* isolate), and group III (8 *E. hirae* isolates) were identified (Fig. 2).

Colonization by more than one enterococcal species was a frequent event. The simultaneous recovery of both *E. faecalis* and *E. faecium* (13.7%, $n = 42/306$) was increasingly observed as age increased ($P < 0.01$) (for age groups I, II, and III, 6.5% [$n = 6/92$], 13.9% [$n = 15/108$], and 19.8% [$n = 21/106$], respectively), suggesting that the increased rate of *E. faecium* colonization in hospitalized patients in age groups II and III did not interfere with the *E. faecalis* colonization rate, although it might have influenced the *E. faecalis* clonal composition. Low rates of cocolonization by *E. faecalis* and *Enterococcus* spp. (5.23%, 16/306), *E. faecium* and *Enterococcus* spp. (4.25%, 13/306), or *E. faecalis*, *E. faecium*, and other enterococcal species (0.65%, 2/306) were also detected.

Ampicillin resistance (22.2%, $n = 68/306$) was detected among *E. faecium* (94.1%, $n = 64/68$) and *E. raffinosus* (5.9%, $n = 4/68$) isolates. Ampicillin-resistant *E. faecium* isolates were more significantly associated with hospitalized patients (44.7%, $n = 46/103$) than with nonhospitalized individuals (13.0%, $n = 18/138$). A low number of individuals colonized with VRE, all of which were identified to be vancomycin-resistant *E. faecalis* isolates, was also detected (1.6%, $n = 5/306$, consisting of 2 nonhospitalized and 3 hospitalized individuals of different ages) (see Table S2 in the supplemental material).

The population structure of the *E. faecium* and *E. faecalis* iso-

lates is detailed below. For other enterococci, isolates of the same species exhibited different PFGE types, with the exception of some *E. avium* isolates (data not shown). All these species were resistant to quinupristin-dalfopristin, were often resistant to erythromycin (*E. avium*, *E. hirae*, *E. raffinosus*, *E. gallinarum*, *E. casseliflavus*) and tetracycline (*E. raffinosus*), and eventually became resistant to levofloxacin (*E. raffinosus*, *E. gallinarum*) and high concentrations of streptomycin (*E. avium*, *E. raffinosus*, *E. gallinarum*) and gentamicin (*E. avium*, *E. raffinosus*).

BAPS analysis of *E. faecium* population structure. A BAPS analysis was used to infer the population structure of the *E. faecium* isolates according to previous findings that demonstrated that eBURST analysis is not sufficient to reliably delineate the patterns of recent evolutionary descent of *E. faecium* (27, 53). The analysis was repeated by taking into account the significant enlargement of the MLST database (<http://efaecium.mlst.net/>), in which the number of STs increased from 492 to 837 in the 2 years since the time of publication of the original 2012 study (27).

A hierarchical BAPS clustering analysis of the currently available 837 *E. faecium* STs representing 2,402 isolates of different origins yielded 8 BAPS groups. The majority of STs grouped in BAPS 1, BAPS 2, BAPS 3, and BAPS 7 (15.1%, 39.7%, 31.5%, and 8.5%, respectively), while BAPS 4, BAPS 5, BAPS 6, and BAPS 8 were much more infrequently detected (1.3%, 1.9%, 0.8% and 1.2%, respectively). BAPS nested analysis subdivided BAPS 1 into six subgroups (BAPSs 1.1 to 1.6) and BAPS 2 (BAPSs 2.1a, 2.1b, 2.3a, 2.3b), BAPS 3 (BAPSs 3.1, 3.2, 3.3a, 3.3b), and BAPS 7 (BAPSs 7.1 to 7.4) into four subgroups each (Table 1). The origi-

TABLE 1 *E. faecium* BAPS analysis data

BAPS group	BAPS subgroup	No. of STs	% STs	No. of isolates
BAPS 1	1.1	9	1.08	11
	1.2	61	7.29	100
	1.3	12	1.43	16
	1.4	2	0.24	2
	1.5	36	4.30	41
	1.6	6	0.72	6
	Subtotal		126	15.05
BAPS 2	2.1a	88	10.51	577
	2.1b	133	15.89	321
	2.3a	78	9.32	135
	2.3b	33	3.94	49
	Subtotal		332	39.67
BAPS 3	3.1	72	8.60	122
	3.2	28	3.35	59
	3.3a	107	12.78	679
	3.3b	57	6.81	92
	Subtotal		264	31.54
BAPS 4		11	1.31	11
BAPS 5		16	1.91	19
BAPS 6		7	0.84	9
BAPS 7	7.1	54	6.45	120
	7.2	6	0.72	6
	7.3	10	1.19	14
	7.4	1	0.12	2
	Subtotal		71	8.48
BAPS 8		10	1.19	11
Total		837		2,402

nal BAPS subgroups 2.1, 2.3, and 3.3 described by Willems et al. (27) have now been split into two subgroups each, and here these are arbitrarily designated BAPSs 2.1a and 2.1b, BAPSs 2.3a and 2.3b, and BAPSs 3.3a and 3.3b, for backwards compatibility (see Fig. S3 in the supplemental material).

Next, we analyzed the congruence between the BAPS grouping of the 492 STs using the BAPS assignment previously described by Willems et al. (27) and the BAPS grouping from this study. A correlation coefficient of 0.5958 indicates some discrepancies between the partitioning of the 492 STs. These discrepancies, probably related to the presence of an *E. faecalis* outgroup in the BAPS analysis of Willems et al. (27), are mostly due to STs that moved from BAPS 2 (15 STs), BAPS 3 (25 STs), and BAPS 5 (1 ST) in the 2012 study (27) to BAPS 7 in our study (see Fig. S1 in the supplemental material). Subsequently, the 492 *E. faecium* STs included in the work of Willems et al. (27) were compared to the BAPS grouping of the same 492 STs obtained using the extended *E. faecium* MLST database of 837 STs in order to infer the influence of the sample size on BAPS assignment. The correlation coefficient analysis revealed almost perfect correlations for the classification of BAPS groups (0.9996) and BAPS subgroups (0.9988) on the basis of 492 and 837 STs (see Fig. S2 and S3 in the supplemental material) and that only a small number of changes in BAPS assignment (44/837 STs, 5.2%) occurred at either the group or the subgroup level when the number of STs analyzed was significantly increased. This further indicates that, for *E. faecium*, BAPS analysis is both reproducible and robust and may accurately describe the *E. faecium* population structure.

Since the extended data set of 837 STs slightly changed the BAPS grouping of STs, we decided to recalculate the ORs to assess the significance of the association between the BAPS groups and the origins of the isolates (see Table S3 in the supplemental material). This revealed that isolates from hospitalized individuals were positively associated with BAPSs 2.1a and 3.3a and negatively associated with all other BAPS groups. Conversely, isolates of all BAPS groups from nonhospitalized individuals were negatively associated with BAPSs 2.1a and 3.3a but positively associated with BAPS 1.2 and BAPS 3.3b (Fig. 4).

Isolates of animal origin were negatively associated with BAPS 3.3a and BAPS 1.2 but showed a positive association with BAPSs

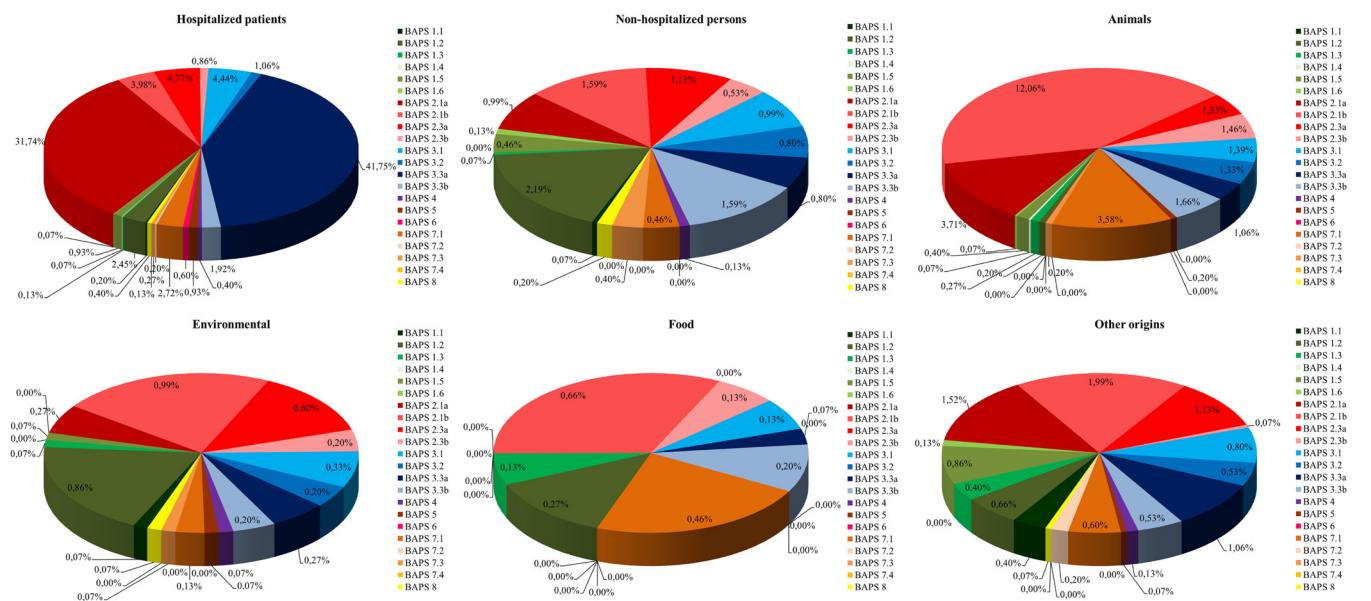


FIG 4 *E. faecium* BAPS group distribution by isolate origin. This distribution by isolate origin was calculated by inclusion of all isolates present in the *E. faecium* MLST database (<http://efaecium.mlst.net/>) in August 2013.

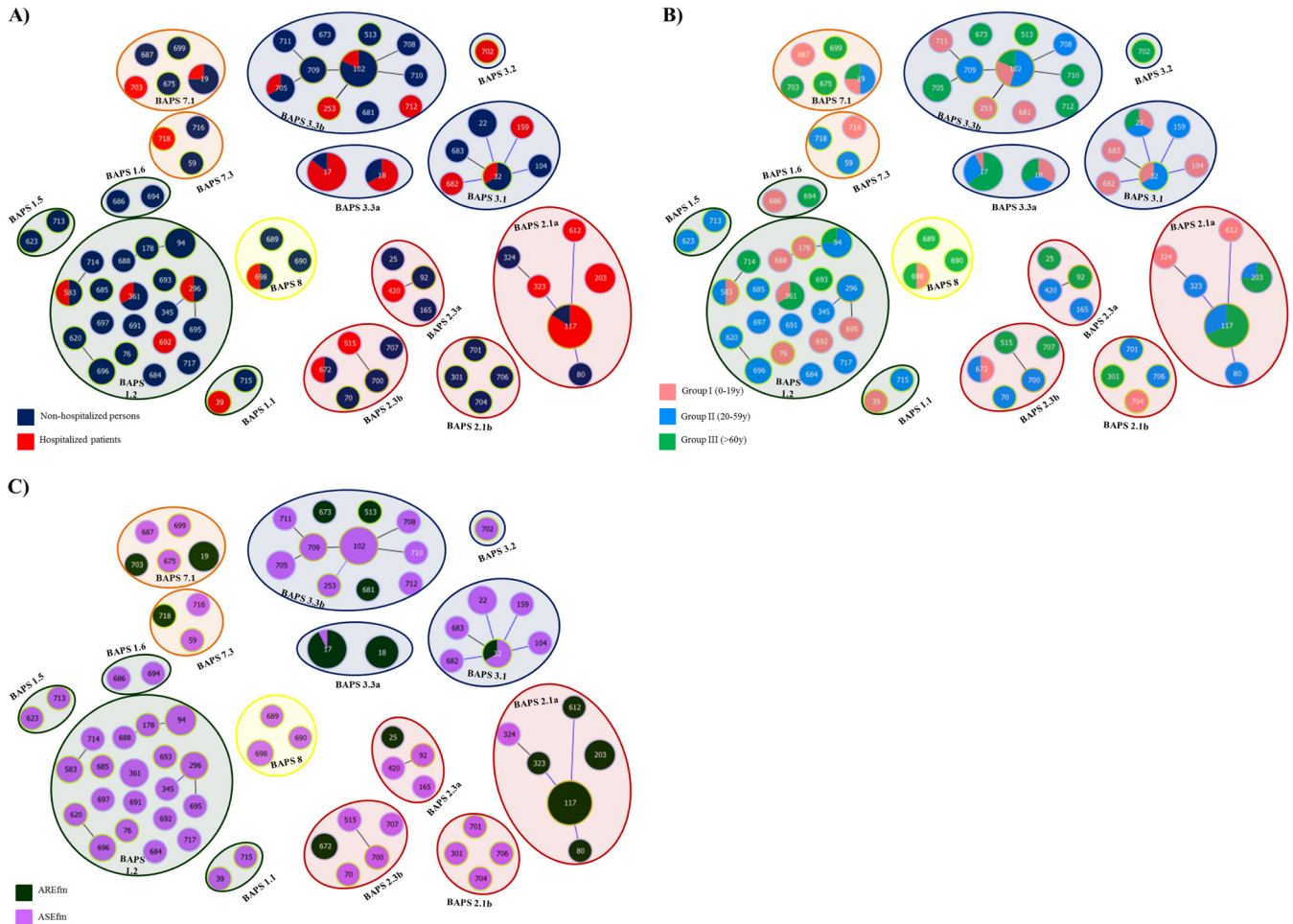


FIG 5 Population structure of *E. faecium* colonization by origin (A), by age group (B), and by susceptibility to ampicillin (C). AREfm, ampicillin-resistant *E. faecium*; ASEfm, ampicillin-susceptible *E. faecium*.

1.5, 2.1a, 2.1b, 2.3a, 2.3b, 3.1, 3.2, and 7.1 (Fig. 4; see also Table S3 in the supplemental material).

Genotypic relatedness of *E. faecium* isolates colonizing different age groups. The 150 *E. faecium* isolates obtained from 142 samples in this study corresponded to 75 distinct STs. Forty-seven STs, representing 62.7% of the studied isolates, were STs first reported here (see Table S1 in the supplemental material). The remaining ones corresponded to globally spread STs, like ST78 ($n = 34$, 7 STs), ST17 ($n = 14$, 1 ST), and ST18 ($n = 6$, 1 ST), and also ST102 ($n = 20$, 7 STs), ST22 ($n = 13$, 9 STs), ST94 ($n = 12$, 7 STs),

ST9 (2, 2 STs), and ST5 (1 ST), which were previously detected among community-based isolates (see Table S1 in the supplemental material). The 75 STs were partitioned into BAPS 1 (24 STs, 22.0% of isolates), BAPS 2 (19 STs, 31.3% of isolates), BAPS 3 (20 STs, 36.7% of isolates), BAPS 7 (8 STs, 7.3% of isolates), and BAPS 8 (3 STs, 2.7% of isolates) (Fig. 5).

STs classified as BAPS 1 mainly corresponded to subgroup 1.2 ($n = 27$ [81.2%], 19 STs). The proportion of isolates with STs that grouped in BAPS 1 steadily decreased with age (Fig. 5 and 6), but isolates of this group were still prevalent among the adults of

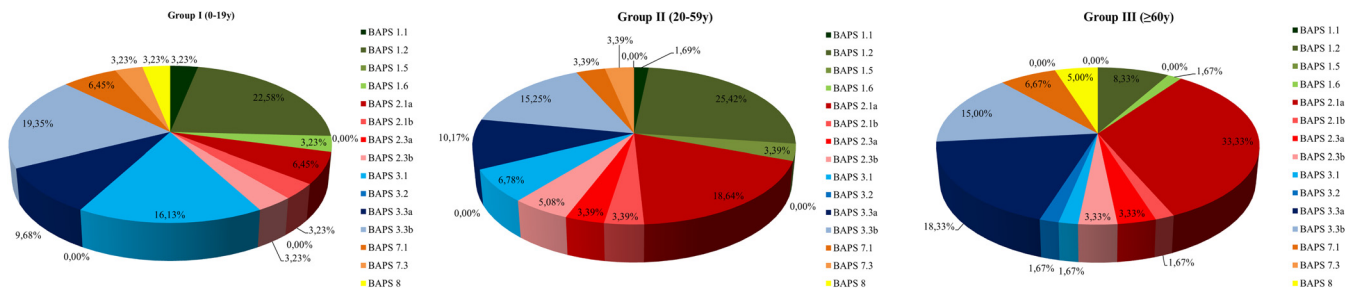


FIG 6 *E. faecium* BAPS group distribution in human colonization by age group.

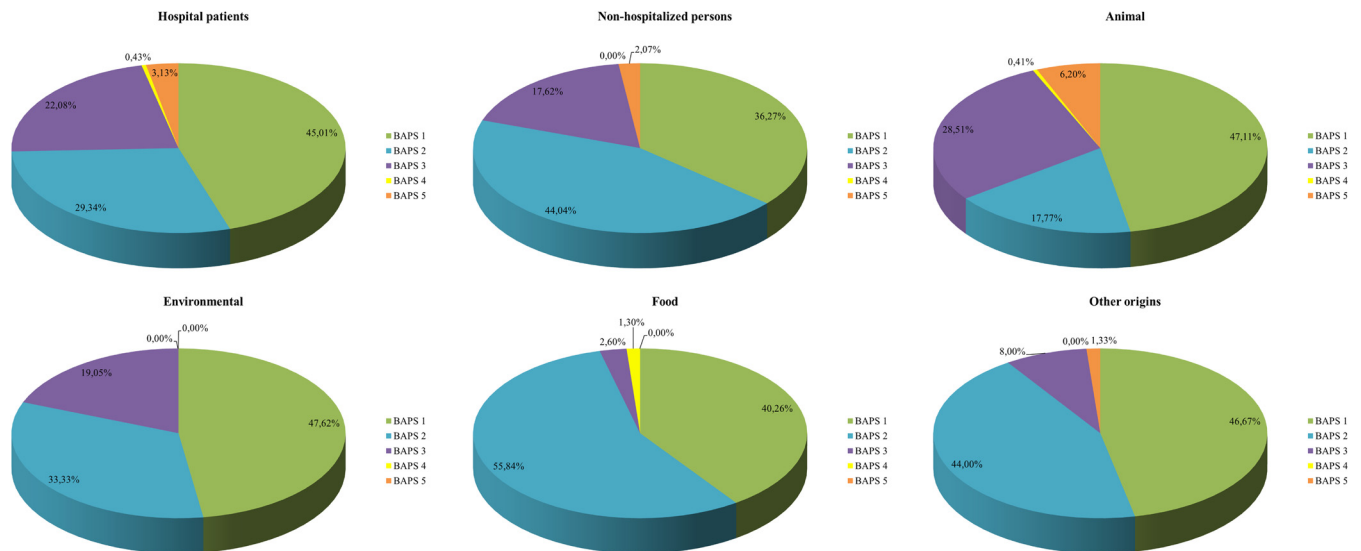


FIG 7 *E. faecalis* BAPS group distribution by origin. This analysis of the distribution by origin included all isolates present in the *E. faecalis* MLST database (<http://efaecalis.mlst.net/>) in August 2013.

group II (15/27). All strains within BAPS 1 were ampicillin susceptible and were mainly recovered from nonhospitalized persons (23/27, $P < 0.01$).

Within BAPS 2, subgroup 2.1a was predominant (70.2%, 33/47) and increasingly detected with age, constituting the predominant group in elderly patients (Fig. 5 and 6). Most isolates were recovered in hospitals, exhibited ampicillin resistance, and harbored genes encoding adhesive surface protein (Esp) and collagen-adhesin (Acm) (27/33 and 31/33, respectively), which are associated with colonization and pathogenicity (see Table S1 in the supplemental material). STs contained within BAPS 2.1a were ST117 ($n = 25$), ST203 ($n = 4$), ST80 ($n = 1$), ST323 ($n = 1$), ST324 ($n = 1$), and ST612 ($n = 1$). *E. faecium* ST117 (strain showing PFGE type CEFm1), apart from predominantly being a colonizing clone, was also frequently associated with severe infections in patients in HRyC (54). The other BAPS 2 subgroups, namely, BAPS 2.3b ($n = 6$ [12.8%], 5 STs), BAPS 2.3a ($n = 4$ [8.5%], 4 STs), and BAPS 2.1b ($n = 4$ [8.5%], 4 STs) were detected in both hospitalized and nonhospitalized individuals (Fig. 5 and 6).

BAPS 3 was represented by subgroups 3.1, 3.2, 3.3a, and 3.3b. Most isolates in BAPS 3.1 (6 STs, 18.2% of isolates) were ampicillin-susceptible *E. faecium* isolates (9/10) from nonhospitalized individuals (7/10) ($P < 0.01$) in age groups I and II (Fig. 5 and 6; see also Table S1 in the supplemental material). Isolates in BAPS 3.3a and BAPS 3.3b, previously described to be BAPS 3.3, differed in their susceptibility to ampicillin. BAPS 3.3a ($n = 20$ [36.4%], 14 ST17 and 6 ST18 isolates) comprised ampicillin-resistant *E. faecium* isolates (19/20, $P < 0.01$) that contained *hyl* from *E. faecium* (16/20) and that were predominantly recovered from hospitalized patients (16/20, $P < 0.01$). Conversely, BAPS 3.3b (11 STs, 43.6% of isolates) was significantly associated with ampicillin-susceptible *E. faecium* isolates (21/24, $P < 0.01$), mostly from nonhospitalized persons (18/24, $P < 0.01$).

BAPS 7.1 was the most predominant subgroup within BAPS 7 and comprised 5 STs representing 8 isolates. Finally, 3 STs comprising BAPS 8 and representing 4 isolates (2 ST698 isolates, 1 ST689 isolate, and 1 ST690 isolate), all of which were ampicillin-

susceptible *E. faecium* isolates (of which 3 were *esp*⁺), were recovered from nonhospitalized persons (see Table S1 in the supplemental material).

Differences in the rates of recovery of ampicillin-resistant enterococci (56.3%, 36/64) but not in the rates of recovery of vancomycin-resistant enterococci (100%, 5/5) were noticed when samples were cultured without or with selective enrichment. Ampicillin-resistant *E. faecium* isolates that were cultured only after enrichment mostly belonged to BAPS 2.1a ($n = 14$; 9 ST117 isolates, 3 ST203 isolates, 1 ST80 isolate, and 1 ST323 isolate) and BAPS 3.3a ($n = 8$; 6 ST17 and 2 ST18 isolates), and the majority of these isolates were recovered from hospitalized patients. Isolates of other BAPS groups were also found and are described in Table S1 in the supplemental material.

BAPS analysis of *E. faecalis* population structure. Previous studies based on MLST have suggested that recombination may play an important role in the diversification of *E. faecalis* (17, 20, 21). As methods to infer evolutionary descent are highly influenced by recombination, we analyzed the *E. faecalis* population structure using Bayesian-based population genetic modeling implemented in BAPS software, in addition to goeBURST analysis. The sample included 1,310 isolates corresponding to 523 STs available in a public database (<http://efaecalis.mlst.net/>).

A maximum likelihood-based phylogenetic reconstruction of STs using concatenated MLST gene sequences placed ST80 far apart from all other STs. When ST80 (amounting to only 1 isolate from the MLST database) was excluded from the analysis to better observe differences among tree features, practically all clades showed low bootstrap support, which supports previous analyses indicating that recombination may obscure the phylogenetic signal in nucleotide-based phylogenetic reconstructions in *E. faecalis*. A hierarchical BAPS clustering analysis subdivided the *E. faecalis* population into 5 BAPS groups (Fig. 7). Most of the STs and isolates were distributed among BAPSs 1, 2, and 3 (44.7%, 27.5%, and 20.6%, respectively), while BAPSs 4 (1.0%) and 5 (6.1%) represented only a small fraction of the STs analyzed (see Table S4 in the supplemental material).

OR calculations revealed that isolates from hospitalized patients were not significantly associated with any of the BAPS groups, while BAPS 2 was positively associated with isolates from nonhospitalized persons (OR = 1.8507, $P < 0.01$) and negatively associated with animal isolates (OR = 0.4659, $P < 0.01$) (Fig. 7; see also Table S5 in the supplemental material). Although signals of microevolutionary hospital specialization within the different BAPS groups were not found, some STs were enriched in isolates from hospitalized patients: ST6 (107/123), ST64 (12/18), ST9 (22/25), ST28 (16/17), ST87 (15/16), ST49 (4/4), ST88 (4/4), and ST159 (4/4). Furthermore, isolates from animals were frequently found to be ST58 (8/8), ST82 (25/27), and ST174 (11/11).

We also analyzed the *E. faecalis* population for traces of significant admixtures, as recombination is the driving force of admixture dynamics and it might influence the evolvability of specific amplified lineages. Admixtures were significantly present in some STs from animal and community-based hosts. However, additional analyses revealed that admixtures were not significantly found in STs that were unique or shared between hosts, STs from hospital or nonhospital origin, STs from human and nonhuman origin, or STs that represented antibiotic-resistant isolates (data not shown). The combination of these results suggests that the majority of *E. faecalis* isolates seem to belong to a single recombining population that exchanges alleles regardless of the genetic background (BAPS groups), ecological origin (isolation source, hospital or nonhospital, human or nonhuman), or antibiotic resistance phenotype.

The influence of the sample size (and, therefore, the underlying diversity) in the accuracy of BAPS for establishing the *E. faecalis* population structure was assessed using two data sets (see Fig. S4 in the supplemental material). The first data set consisted of 433 STs available in the MLST database (<http://efaecalis.mlst.net/>) before, including the new *E. faecalis* STs found in this study. The second data set included 523 STs available in the MLST database at the end of 2013. In both analyses, ST80 was excluded. The negative correlation coefficient of -0.6439 obtained when comparing ST assignments to BAPS groups of the set with 433 STs and those to BAPS groups of the set with 523 STs was due to the split of BAPS 1 and BAPS 2 and the existence of three more BAPS groups when using the second, larger data set (see Fig. S4 in the supplemental material). These results indicate that in *E. faecalis*, BAPS analysis is highly influenced by the sample size, as larger samples contain a higher diversity of strains of different spatial-temporal origins.

Genotypic relatedness of *E. faecalis* isolates colonizing different age groups. The 130 *E. faecalis* isolates identified in this study represented 58 STs (see Table S2 in the supplemental material) that were partitioned into *E. faecalis* BAPS 1 (36.9%), BAPS 2 (40.0%), and BAPS 3 (23.1%). OR calculations revealed that none of the three BAPS groups were significantly associated with a particular source or age group, as all the BAPS groups contained isolates from both hospitalized and nonhospitalized individuals of all ages in more or less equal numbers (Fig. 8).

Within BAPS 1 ($n = 48/130$ [36.9%], 20 STs), ST6 ($n = 16$) was predominant and mainly comprised isolates from hospitalized patients (13/16) and elderly individuals (11/13) (Fig. 8; see also Table S2 in the supplemental material). All were multidrug resistant (MDR), showing high levels of resistance to gentamicin or streptomycin and also to erythromycin (100% of isolates), tetracycline (93.8%, 15/16), and levofloxacin (87.5%, 14/16) and exhibiting a highly similar PFGE profile (the ST6-H10 profile) iden-

tical to that of the widespread international mid-Atlantic clone, which also causes bacteremia in HRyC (55). The 5 vancomycin-resistant *E. faecalis* isolates (*vanA*; data not shown) found in this study were also ST6-H10. Putative virulence factors *asa1* (100%) and *gelE* (81.3%) were identified in most ST6 isolates, while *cylA* (56.3%) and *esp* (37.5%) were less frequently identified. Other STs were represented by a very small number of isolates, which were usually susceptible to antibiotics and which had a highly variable presence of virulence factors.

Within BAPS 2 ($n = 52/130$ [40.0%], 26 STs), ST40 isolates ($n = 15$) were predominant. These isolates were recovered from both nonhospitalized and hospitalized individuals of different ages, often harbored *gelE* (88.2%) and, less frequently, *asa1* (41.2%) and *esp* (47.1%), and were resistant to tetracycline (70.6%) and erythromycin (47.1%). Similar to the findings for BAPS 1, other STs were represented by a single isolate or very few isolates that often contained *esp* (see Table S2 in the supplemental material). Among them were STs that were identified over several decades to be ST55, ST30, or ST19 (31, 33).

Finally, BAPS 3 ($n = 30/130$, 12 STs) was predominantly comprised of ST16 and ST179, previously classified to be CC16 by goeBURST analysis (7 ST16 and 11 ST179 isolates). These STs also included isolates from both nonhospitalized and hospitalized individuals of different ages that often harbored *asa1* (99.4%), *esp* (77.8%), or *gelE* (61.1%) and that were often resistant to different antibiotics (see Table S2 in the supplemental material). Other STs classified before as CC28 by goeBURST analysis (1 isolate each of ST333, ST518, and ST519) and recovered from adults or elderly hospitalized patients were also enriched in isolates that harbored putative virulence factors (all harbored *asa1*, *gelE*, *esp*, and *cylA*) and that were also MDR (with all isolates showing high levels of resistance to gentamicin and streptomycin, tetracycline, erythromycin, and levofloxacin) (see Table S2 in the supplemental material).

DISCUSSION

This study describes a consistently high rate of recovery of enterococci from feces from both hospitalized and nonhospitalized individuals and individuals in different age groups, similar to the findings reported in other studies, in which the rates of recovery ranged from 71% to 80% (1, 56, 57). These equilibrated constant rates of colonization indicate a major resiliency of the genus *Enterococcus* under heterogeneous conditions imposed by age, changing environments, and highly variable host niches. Previous studies (6, 58–60) have described changes in the rates of recovery of the genus *Enterococcus* in fecal microbiota with aging, which was not confirmed in our work, and a consistent predominance of the species *E. faecalis* in the fecal flora of young and elderly individuals, which is essentially consistent with our findings, with the important exception of the growing predominance of *E. faecium* in elderly individuals, particularly hospitalized elderly patients. Other studies yielded contradictory information about the frequency and diversity of *E. faecium* and other enterococcal species in the fecal microbiota (1, 15). Shifts in the prevalence of *Enterococcus* populations might result from fluctuating changes in the environmental conditions over time as a result of changes in diet (10) or changes in health status or antibiotic treatment (1, 5, 61–64), all of which delineate particular selective landscapes in hospitals (58, 61). Aging interacts with these conditions, and age-dependent enterococcal colonization dynamics have also been

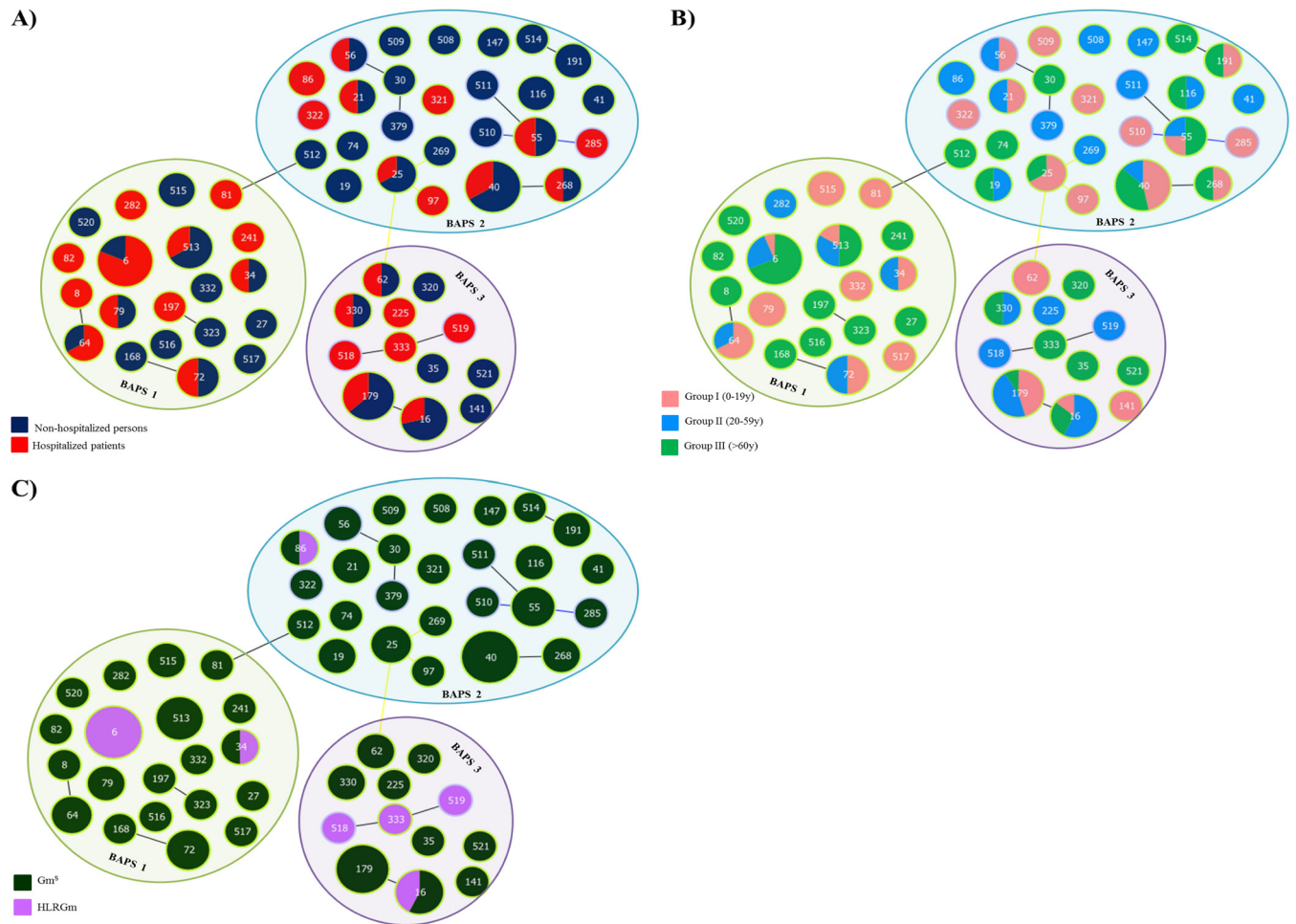


FIG 8 Population structure of *E. faecalis* colonization by origin (A), by age group (B), and by susceptibility to gentamicin (C). Gm^S, gentamicin susceptible; HLRGm, high-level resistance to gentamicin.

demonstrated for chickens and calves (1, 65), probably as an interaction with antibiotic consumption (1, 66, 67).

Considering the currently available diversity of known genotypes, the superimposing of goeBURST analysis of the clonal relationship among multiple isolates with BAPS allowed the detection of a low number of presumptive evolutionarily and functionally heterogeneous clades for the *E. faecium* species (22, 25–27). BAPS 1 *E. faecium* isolates, associated with the clade B phylogenetic lineage (a clade with isolates with pathways of complex carbohydrate utilization linked to host diet and with a majority of ampicillin-susceptible *E. faecium* strains), were highly represented in the different age groups, although their incidence was slightly reduced in the elderly (26, 27). Conversely, BAPS 2.1a and BAPS 3.3a (containing most of the ampicillin-resistant *E. faecium* strains), associated with clade A1 (26, 27) and mostly found in elderly hospitalized patients, represented *E. faecium* strains that are spreading in hospitals and causing clinical infections. The rates of these populations in the nosocomial and the community settings might be underestimated, as we have demonstrated here that if you do not pre-enrich the sample, some of the clones that are even more widespread might escape screening, probably due to low colonization densities. The observed population structure of *E. faecium* indicates a certain specialization of subpopulations for

the colonization of particular age groups, which is usually associated with several other host-associated factors and also differences in the selectable characteristics harbored by the isolates, such as antibiotic resistance genes. Interestingly, some groups evolve independently from the acquisition of ampicillin resistance, suggesting a certain genetic isolation, which seems to be the case for different lineages within BAPS 3.3b, BAPS 1, and BAPS 2. These results further confirm a population structure comprised of ecotypes representing specialization in different hosts (16, 68).

E. faecalis populations showed a considerable level of genetic diversity. Because of that and in contrast to the findings for *E. faecium*, no BAPS groups were significantly associated with aging, hospital exposure, or host species, and with the exception of BAPS 2, none of the BAPS groups showed a positive association with nonhospitalized individuals. The wide recovery of certain STs (e.g., ST6, ST16, ST40, or ST55) able to colonize hospitalized and nonhospitalized humans (this study) and also animals (30, 31, 69) may be related to the more generalist lifestyle of this enterococcal species, which weakens the possibility of the recognition of ecotypes associated with a particular environment, at least by using the same approach that was so useful with *E. faecium*. However, despite possible limitations in the methods available for analysis of the *E. faecalis* population structure, it is now clear that

certain multihost *E. faecalis* subpopulations, such as ST6 or ST16, have developed different strategies of adaptation to harsh and fluctuating habitats (31, 33). Among these are the lack of loci for cluster regulatory interspaced short palindromic repeats (CRISPR; a bacterial defense system against foreign DNA that facilitates the acquisition of foreign DNA, such as antibiotic resistance and virulence genes) (70) and the frequent acquisition of phages (71).

Other enterococcal species have largely been recognized to be part of the human fecal microbiota (1), and this was confirmed in our study. The inverse parallel trends in the frequencies of populations of these species and the frequency of *E. faecium* are of particular interest. The dynamics of colonization by these species might reflect differences in the functional requirements of the host with age and deserve further analysis.

This study provides a novel, integrated, and comprehensive image of the landscape of *Enterococcus* populations in a balanced amount of nonhospitalized and hospitalized individuals of different ages and suggests that a number of enterococcal lineages might be predominant in certain age groups and/or hospital environments. However, a number of clones are spread in different types of individuals and their prevalence is reduced in others in a kind of source-sink dynamics (72–74), with frequent cases of coexistence and the preservation of rare clonal populations being found. This suggests a frequency-dependent evolution of enterococcal populations which prevents the extinction of different genotypes that do not play equivalent ecological roles (75–78).

The work also illustrates the high degree of plasticity of *E. faecium* and *E. faecalis* genomes, as reflected by admixture analysis (27; this study), which showed variable intraclonal PFGE patterns (31, 63) (see Tables S1 and S2 in the supplemental material) and recombination of large fragments of the chromosome (79–83; our unpublished results). The consequences of such a high degree of variability have scarcely been explored from a population-based perspective. However, it can be expected that genome plasticity would contribute to the variation and selection of genes from a common intraspecies genetic pool needed for adaptation to environments imposing different stress conditions. Future progress in understanding enterococcal population biology will require a global analysis combining many ecological features, population dynamics, and population genetics (78, 84, 85).

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