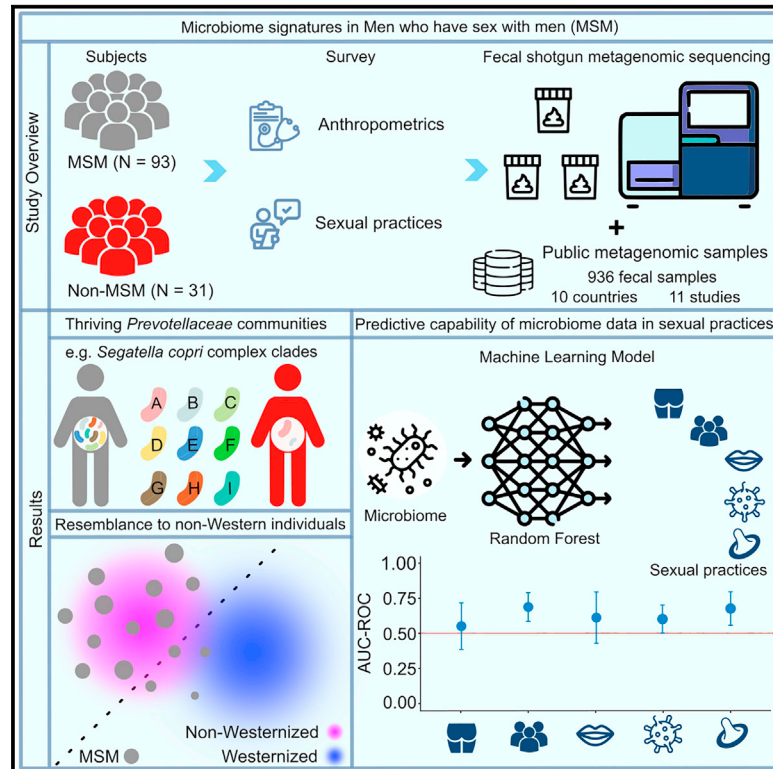**Article**

Establishment of a non-Westernized gut microbiota in men who have sex with men is associated with sexual practices

Graphical abstract

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In brief

Huang et al. analyze the gut microbiota composition of men who have sex with men (MSM) using shotgun metagenomics. Characterization of microbiome architecture identifies an enrichment of *Prevotellaceae* including the *Segatella copri* complex in MSM. Integration of questionnaire-collected data reveals associations for sexual practices in shaping community structure and their similarity to non-Westernized communities.

Highlights

- Men who have sex with men (MSM) harbor a distinctive gut microbiota

- Dominant members include the *Segatella copri* complex and other *Prevotellaceae* species

- The MSM gut microbiome resembles that of non-Westernized populations

- Sexual practices are associated with a non-Westernized gut microbiome in MSM

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Establishment of a non-Westernized gut microbiota in men who have sex with men is associated with sexual practices

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SUMMARY

The human gut microbiota is influenced by various factors, including health status and environmental conditions, yet considerable inter-individual differences remain unexplained. Previous studies identified that the gut microbiota of men who have sex with men (MSM) is distinct from that of non-MSM. Here, we reveal through species-level microbiota analysis using shotgun metagenomics that the gut microbiota of many MSM with Western origin resembles gut microbial communities of non-Westernized populations. Specifically, MSM gut microbiomes are frequently dominated by members of the *Prevotellaceae* family, including co-colonization of species from the *Segatella copri* complex and unknown *Prevotellaceae* members. Questionnaire-based analysis exploring inter-individual differences in MSM links specific sexual practices to microbiota composition. Moreover, machine learning identifies microbial features associated with sexual activities in MSM. Together, this study shows associations of sexual activities with gut microbiome alterations in MSM, which may have a large impact on population-based microbiota studies.

INTRODUCTION

The human microbiota is shaped by diverse factors including geographical and ethnic origin, socioeconomic status, genetics, health status, diet, and medications; yet, much of the interindividual variability of the human gut microbiota is currently unexplained.[1,2] This suggests that so-far-unknown factors not routinely recorded in large population-based cross-sectional studies may affect the gut microbiota. One of these factors that has been considered is sexual orientation, as men who have sex with men (MSM) have been characterized by a distinctive gut microbiota, characterized by the depletion of *Bacteroides* and the expansion of the genus *Segatella/Prevotella*.[3–5] Notably, earlier studies had suggested HIV infection as a strong driver of gut microbiota alterations toward a *Segatella/Prevotella*-rich gut microbiome,[6] but subsequent studies revealed that these alterations can be explained by an enrichment of

men who have sex with men (MSM) in HIV-positive cohorts.[3,4,7] While MSM exhibit a *Segatella/Prevotella*-rich gut microbiome independently of HIV status, more recent studies have revealed that HIV infection and HIV disease progression have been associated with independent changes in the gut microbiota.[8,9]

The gut microbiome signature of MSM is reminiscent of the initially reported human *Prevotella* enterotype, which has been associated with inflammatory diseases and insulin resistance in Westernized (Wes) populations.[10–12] However, the increased prevalence and/or increased relative abundance of *Segatella/Prevotella* spp. was also associated with health-promoting diets, i.e., a plant-based diet, and beneficial outcomes after dietary interventions.[13–17] Moreover, conflicting results were reported for potential detrimental roles of *Segatella/Prevotella* spp. on human health, with associations varying between studies.[18,19] Interestingly, in a global context, the expansion of the genus *Segatella/Prevotella* and the depletion of *Bacteroides* were also observed

Table 1. Subject characteristics at study entry (N = 124)

Anthropometric and clinical parameter	MSM (%)	Non-MSM (%)	p
Age			1.0000
<45 years	45 (48)	14 (45)	–
>45 years	48 (52)	17 (55)	–
HIV infection			1.0000
Positive	79 (85)	26 (84)	–
Negative	14 (15)	5 (16)	–
Ethnicity			0.0065
White	92 (99)	26 (84)	–
Others	1 (1)	5 (16)	–
Antibiotics treatment in the last 6 months			1.0000
Yes	26 (28)	5 (16)	–
No	66 (71)	26 (84)	–
N/A	1 (1)	–	–
BMI classification			0.4212
Normal	48 (52)	12 (39)	–
Overweight	37 (40)	11 (35)	–
Obese class I	5 (5)	5 (16)	–
Obese class II	1 (1)	1 (3)	–
Obese class III	1 (1)	0 (0)	–
Underweight	1 (1)	2 (7)	–

Differences between MSM (n = 93) and non-MSM (n = 31) for each parameter were evaluated using Fisher's exact test with correction of Benjamini-Hochberg method. Values are given as counts with percentages in parentheses. The cohort consists of 119 male and 5 female subjects.

N/A, not available; BMI classification, body mass index class based on WHO recommendations. See also Table S1.

in non-Westernized (non-Wes) healthy individuals following traditional lifestyles and typically consuming high-fiber and low-fat diets.[18,20,21]

Shotgun metagenome sequencing and advances in bioinformatic analyses have recently uncovered that the family *Prevotellaceae*, the genera *Prevotella* and *Segatella*, and its most prevalent species in humans, *Segatella copri* (formerly *Prevotella copri*), display a much larger taxonomic and functional diversity compared to what has been initially suggested based on *16S rRNA* gene sequencing studies.[5,18] Specifically, through combining cultivation- and metagenomics-based approaches, we recently revealed that the species *S. copri* can be subdivided into at least 13 different clades, which represent different species with distinct functional profiles.[22]

Here, we performed shotgun metagenomc sequencing for a cohort of 124 individuals including MSM and non-MSM and characterized their gut microbiota at the species level. Moreover, we studied the MSM microbiota alteration in the global context using publicly available datasets representing typical Wes and non-Wes populations and those from China but in urbanized communities. With the analysis of clinical and self-reported data together, we were able to identify an association for sexual activities to MSM microbiome composition and diversification.

These data and analyses provide an insight into a previously under-appreciated role of sexual practices in shaping the human gut microbiota.

RESULTS

Increased diversity and altered gut microbiome architecture at the species level in MSM

Insights into the gut microbiome structure of MSM have been mostly generated using *16S rRNA* gene sequencing,[3,4,23,24] which lacks taxonomic precision. Therefore, we performed SMS from fecal samples of a total of 124 subjects (MSM n = 93, non-MSM n = 31; Tables 1 and S1), resulting in, on average, 25 M (with a minimum of 14 M) quality-controlled paired-end reads per sample (average: 6.3 Gbp/sample; Figure S1A; Table S1). Microbial community composition with relative abundance quantification was analyzed using a reference-based approach, namely MetaPhlAn 4.[25] MetaPhlAn 4 identifies species-level genome bins (SGBs), which are clusters of genomes spanning the typical diversity of bacterial species and can include previously cultured (known SGBs [kSGBs]) or can be composed of solely unknown metagenome-assembled genomes (unknown SGBs [uSGBs]).[21] Within the 124 subjects, we identified a total of 1,675 SGBs including 1,124 kSGBs and 551 uSGBs.

Comparison of alpha diversity using the Shannon index revealed a higher microbiome diversity in MSM than in non-MSM (p = 0.028, Wilcoxon rank-sum test; adjusted p value [p_{adj}] = 0.017, adjusted by fixed-effect linear models for confounding effects including age, ethnicity, antibiotics use, BMI, and HIV infection (see STAR Methods; Figure 1A). To study the differences in the microbiome of MSM and non-MSM, we calculated beta diversity using the Bray-Curtis distance, performed a permutational multivariate analysis of variance (PERMANOVA) controlled for confounders (age, ethnicity, antibiotics use, BMI, and HIV infection), and confirmed a species-level microbiome differentiation between MSM and non-MSM (PERMANOVA, R2 = 0.042 and p_{adj} < 0.001; Figure 1B). Additional analysis for subjects stratified according to major covariate groups such as age, BMI, HIV status, or recent antibiotic intake confirmed our observation (Figure S1B). Examining the effect of immunological parameters such as the counts and frequency of CD4$^+$ T cells as well as the CD4/CD8 T cell ratio in 103 HIV-infected individuals further confirmed that the association of the gut microbiome change with MSM was significant, independently of HIV infection (Table 2; Figure S1C). Taken together, our species-level microbiome diversity analysis identified a significantly altered microbiome structure in MSM compared to non-MSM, which is in line with earlier *16S rRNA* gene sequencing-based microbiome studies.[3,24,26] These results corroborate that sexual practices or lifestyle are relevant factors affecting the gut microbiome community composition, regardless of previously reported variables.[3,27–30]

Prevotellaceae-dominated communities in MSM comprise *Segatella copri* complex members and unknown species

A high *Segatella/Prevotella* abundance has been reported as the most distinct feature of the gut microbiome of MSM in Wes populations.[3,4,32] We next aimed to explore *Segatella/Prevotella*

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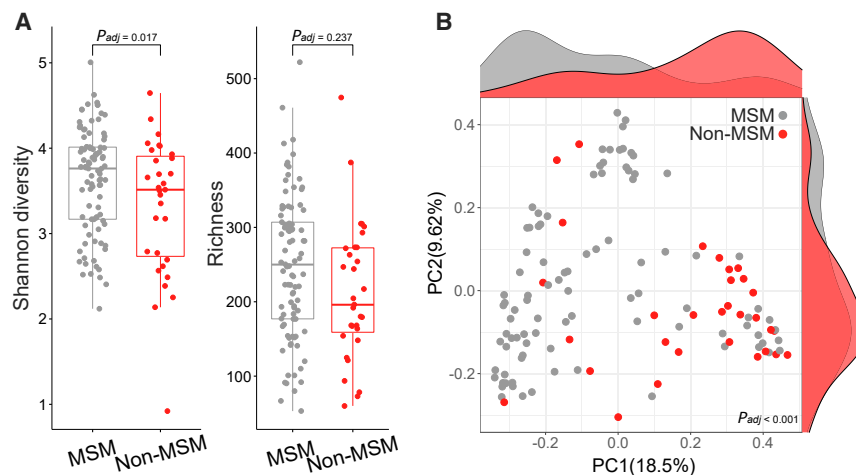## Article

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Figure 1. Gut microbiome community architecture in MSM and non-MSM

(A) Alpha diversity analysis of MSM compared to non-MSM (adjusted by fixed-effect linear models,[31] Shannon diversity p_{adj} = 0.017, richness p_{adj} = 0.237).

(B) Principal-coordinate analysis (PCoA) based on microbial abundance profiled using MetaPhlAn 4[25] between MSM and non-MSM samples (PERMANOVA, R2 = 0.042 and p_{adj} < 0.001). Marginal density plots show distributions of samples along each axis. MSM N = 93, non-MSM N = 31.

See also Figure S1 and Table S1.

communities at the species level, specifically whether *Prevotellaceae* expansion is driven by a single species or rather a diversification of the family. Therefore, we compared the richness, abundance, and prevalence of all species belonging to the *Prevotellaceae* family between MSM and non-MSM. Notably, the average number of *Prevotellaceae* species was three times higher in the microbiome of MSM compared to non-MSM (Wilcoxon rank-sum test p = 1.65e−06; covariates adjusted by fixed-effect linear model p_{adj} = 1.63e−04; Figure 2A). For comparing *Prevotellaceae* community diversity, we estimated the Shannon index specifically for members of *Prevotellaceae* family, confirming a higher alpha diversity in the MSM gut microbiome compared to that in non-MSM (Wilcoxon rank-sum test p = 1.35e−06; covariates adjusted by fixed-effect linear model p_{adj} = 2.69e−06; Figure 2A). We also observed a significant separation between MSM and non-MSM samples based on a *Prevotellaceae*-centric beta diversity analysis (covariate-controlled PERMANOVA p_{adj} < 0.001; Figure 2B). Of 47 *Prevotellaceae* species identified in this study (kSGBs n = 28, uSGBs n= 19), when only considering species with a minimum relative abundance of 0.1 at the 90th percentile of all samples, 16 were differentially enriched for MSM compared to non-MSM (Bonferroni-corrected Fisher's exact test false discovery rate [FDR] < 0.05; Figures 2C and S2; Table S2). Unknown *Prevotellaceae* members (uSGBs) were similarly overrepresented in the gut microbiome of MSM displaying an elevated abundance and prevalence compared to non-MSM (Figures 2C and S2). Strikingly, members belonging to the *S. copri* species complex were the most common and abundant *Prevotellaceae* members in MSM, with 70% of MSM carrying at least two *S. copri* clades compared to only about 30% of non-MSM (Figures 2C and 2D). Moreover, the co-occurrence of strains from multiple *S. copri* clades, a feature previously observed in typical non-Wes microbiomes,[18] was consistently higher in MSM than in non-MSM (Wilcoxon rank-sum test p = 0.001; Figure 2D).

The MSM gut microbiota resembles a microbial diversity present in non-Wes microbiomes

To further compare the MSM microbiota structure in the global context, we utilized publicly available and curated shotgun meta-genomic datasets from 936 healthy adult individuals representing typical Wes (n = 93) and non-Wes (n = 481) populations across nine countries as well as those from a recently Wes (or urbanized) population in China (China [urban], n = 66)[18,21,33–41] (Table S3; see STAR Methods). These samples were profiled for quantitative taxonomic composition using MetaPhlAn 4 in the same way as samples sequenced in this study (see STAR Methods). The family *Bacteroidaceae* is ubiquitous in the gut microbiome of Wes populations, with *Bacteroides* being the most abundant genus,[14,42] while *Prevotellaceae/Segatella/Prevotella* represent a major part in non-Wes populations.[18,21,43–46] In MSM, both *Bacteroidaceae* and *Bacteroides* were significantly lower in abundance compared to both the Wes and urban Chinese populations (Bonferroni-corrected Fisher's exact test, to Wes population, FDRs = 6.33e−13 and = 8.33e−25, respectively; to urban Chinese population, FDRs = 0.02 and = 3.51e−09, respectively) but were significantly more abundant relative to non-Wes samples (FDRs = 6.73e−11 and 9.19e−10, respectively, Figure 3A). In contrast, *Prevotellaceae* and *Segatella/Prevotella* abundances were significantly higher in MSM compared to both Wes subjects (FDRs = 7.15e−19 and 4.46e−18) and urban Chinese subjects (FDRs = 3.75e−07 and 1.32e−05) but were not different from non-Wes subjects (FDRs = 0.25 and 0.52; Figure 3A). We further extended the comparison from *Prevotellaceae* to the whole microbiome community, which revealed that overall MSM microbiomes were characterized by signatures that are distinct from both Wes and non-Wes samples (PERMANOVA p < 0.001; Figure 3B). Expectedly, urban Chinese samples clustered with those from Wes populations, suggesting that modern lifestyles exerted a converging effect of shaping the gut microbiome composition in geographically different populations. However, with only a few MSM scattering between the non-Wes and Wes samples in principal-coordinate analysis (n = 12 overlapped with hyperplane), a large proportion of MSM samples clustered with the non-Wes samples (n = 54 falling on the non-Wes side), and a smaller number of samples grouped with the Wes samples (n = 27 falling on the Wes side, Figure 3B). This reveals that most MSM microbiomes bear remarkable similarity to those of the non-Wes subjects, particularly for *Prevotellaceae/Segatella/Prevotella* accumulation and *Bacteroidaceae/Bacteroides* depletion, compared to the Wes subjects. Accordingly, of the top 50 abundant species in each population, MSM and non-Wes

Table 2. Immunological measures for HIV-infected subjects (N = 103)

Immunological measures	MSM	Non-MSM	p
CD4 cell counts	680.92 ± 282.36 (92–1,640)	683.19 ± 267.24 (149–1,310)	0.74
CD4/CD8 ratio	0.86 ± 0.50 (0.2–2.24)	0.92 ± 0.42 (0.08–1.77)	0.73
CD3/CD4 (%)	31.58 ± 8.95 (15.1–54)	32.85 ± 10.13 (6.82–49.8)	0.73

Differences between MSM (N = 77) and non-MSM (N = 26) with HIV infection were evaluated for each measure using Wilcoxon rank-sum test with correction of Benjamini-Hochberg method. Values are given as mean ± standard deviation with ranges in parentheses. All HIV-infected individuals were treated with antiretroviral therapy. See also Table S1.

individuals shared 33 species, compared to only 15 that were shared between MSM and Wes individuals as well as 15 between MSM and urban Chinese individuals (Figure 3C). Of note, 92 out of 93 MSM in this study have European ancestry, and all have long resided in Germany, a typical Wes country.

Yet, there were other microbial traits likely to differentiate MSM from both the Wes and non-Wes individuals. Specifically, we found 216 and 187 species enriched in MSM samples relative to Wes or non-Wes samples, respectively (Table S3). In addition to multiple *S. copri* complex species and *Prevotella* members such as *Prevotella pectinovora* (FDR = 6.2e−28, to Wes), microbes enriched in MSM relative to both the Wes and non-Wes individuals included *Megasphaera elsdenii* (FDRs = 3.6e−05 and 1.7e−04, to Wes and non-Wes, respectively), *Megasphaera hexanoica* (FDRs = 8.7e−17 and 1.8e−19), and *Paraprevotella clara* (FDRs = 3.4e−12 and 9.1e−41; Figure 3D; Table S3). Interestingly, these species were previously identified to be preferentially transferred during fecal microbiota transplantation (FMT), suggesting they are engraftment-amenable genera.[48] Intriguingly, a subtype of eukaryotic parasite, *Blastocystis* subtype 1 (FDR = 4.2e−03), which was reported as a widespread colonizer in non-Wes populations,[49] was also found enriched in MSM relative to Wes subjects (Figure 3D; Table S3). On the other hand, 94 species were more abundant in Wes and 357 in non-Wes samples compared to MSM (Table S3). Wes-population-enriched species included *Akkermansia muciniphila* (FDR = 3.3e−03), *Streptococcus infantis* (FDR = 1.7e−02), and *Erysipelatoclostridium ramosum* (FDR = 5.0e−04) (Figure 3D; Table S3). Non-Wes-population-enriched species were represented by *Klebsiella pneumoniae* (FDR = 5.9e−14), *Veillonella parvula* (FDR = 1.7e−04), *Veillonella tobetsuensis* (FDR = 2.7e−05), and *Haemophilus parainfluenzae* (FDR = 3.5e−12) (Figure 3D; Table S3). Notably, these species belong to the least likely engrafted genera during FMT.[50]

Assessing engraftment rate and horizontal transmissibility of MSM-enriched species

Engraftment probabilities of microbes during FMT are influenced by complex factors such as antibiotic pre-conditioning and microbial properties compared to transmission and engraftment in non-medical settings, e.g., between familial members or part-

ners.[47,50] Hence, to examine if microbial inter-host transfer capability was related to MSM enrichment/depletion, we first assessed the engraftment rate and horizontal transmissibility of microbial species differentially abundant in MSM samples compared to non-MSM samples, utilizing previously quantified gut microbial engraftment rate and household transmissibility data[47,50] (see STAR Methods). Shortly, these recent studies contributed methodological developments to the strain-level profiling of the microbiome, including the establishment of operational species-specific strain boundaries, and then quantified frequency of engraftment of microbial species upon FMT[50] (engraftment rate) and of transmission within households[47] (horizontal transmissibility). Overall, we found that there was no significant difference in both engraftment rate and horizontal transmissibility between MSM and non-MSM samples (Figure S3A; Table S3). Likewise, species associated with MSM and non-MSM appeared to have similar carriage patterns of engraftment-/transmission-related phenotypes including spore formation, aerotolerance, motility, and Gram-staining (Figure S3B; Table S3).

Next, we compared the engraftment rate and horizontal transmissibility of microbial species that were enriched in MSM with those enriched in the Wes and the non-Wes subjects. We observed a slightly higher mean transmissibility in MSM-enriched species when comparing MSM- to Wes-enriched microbes (Figure S3A; Table S3). As for the engraftment rate, a similar trend was observed but without significance (Figure S3A; Table S3). Analyzing engraftment-/transmission-related phenotypes also in this case did not yield a general difference between species enriched in MSM and those enriched in Wes subjects (Figure S3C; Table S3). However, compared to species enriched in MSM, those enriched in the non-Wes subjects were more likely to be Gram-negative—generally more resilient to sanitizers and disinfectants—which were expected to have enhanced gut microbial transmissibility due to longer persistence outside the human body[47,51,52] (FDR = 0.02; Figure S3D; Table S3).

Sexual practices associated with the change of gut microbiome of MSM toward non-Westernization

Sexual practices can increase transmission and incidence of microbial infections, e.g., HIV, viral hepatitis, and syphilis.[53–55] However, the influence of sexual practices on the gut microbiome remains underexplored, especially in MSM.[56,57] Hence, we explored this link in a subset of our cohort (N = 52 MSM) surveyed for sexually transmitted infections (STIs) and sexual practices including receptive anal intercourse (RAI), number of sexual partners (in the last 12 months), oral sex, and condom use (Table S1). Alpha diversity was not generally associated with sexual practices, as only "condom use" reached statistical significance for Shannon diversity (p_{adj} = 0.023) and observed richness (p_{adj} = 0.016) after accounting for confounding factors (Figure S4A). Specifically, individuals who practiced condomless RAI were characterized by a higher alpha diversity of the microbiota than individuals who sometimes or always used condoms (Figure S4A). For beta diversity, a significant difference in the whole microbiome composition was found associated with "oral sex" and "number of partners" (covariate-controlled PERMANOVA p_{adj} < 0.05; Figure S4B).
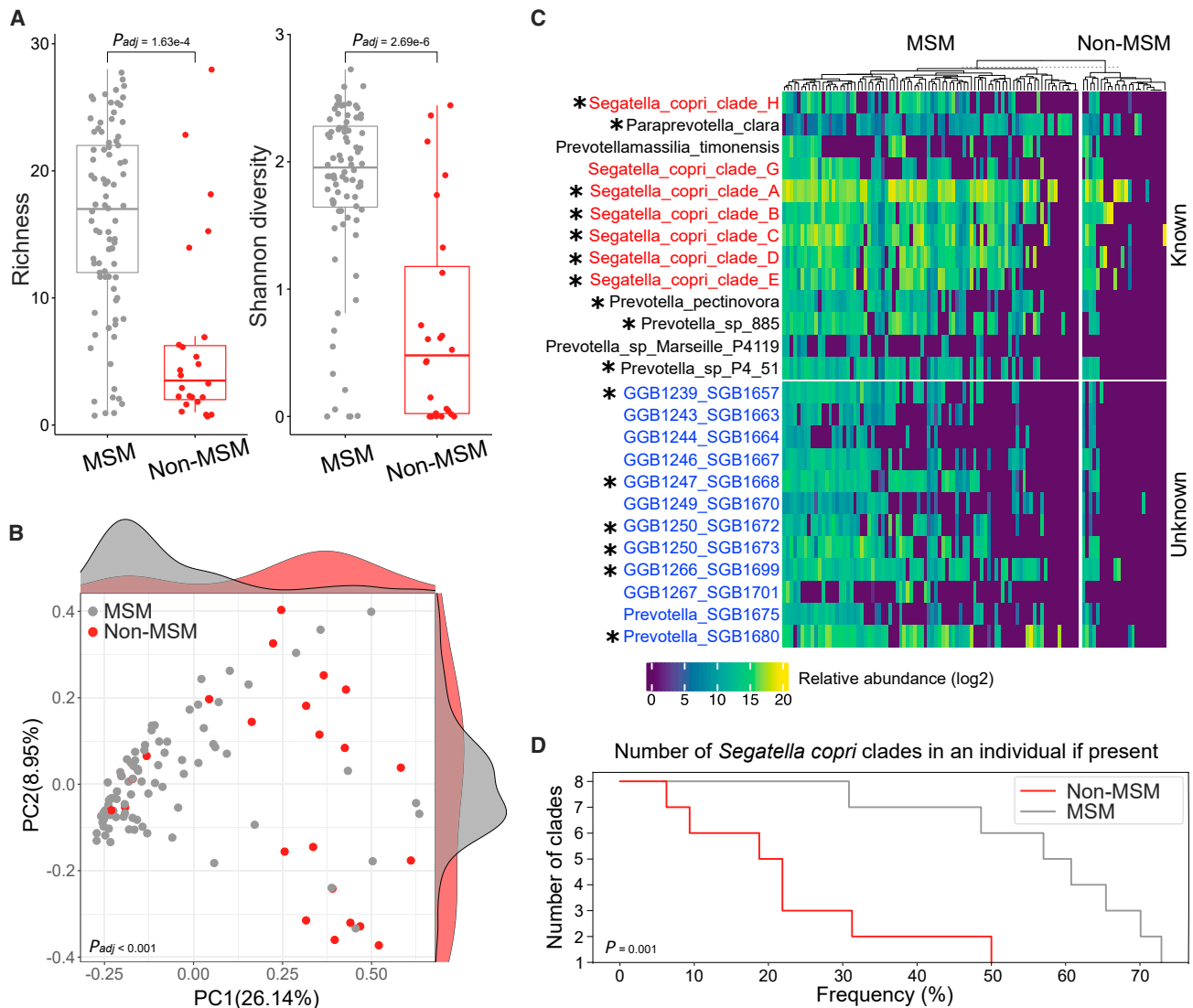
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Figure 2. The *Prevotellaceae* community structure in MSM and non-MSM

(A) Richness and Shannon diversity of *Prevotellaceae* species members in MSM and non-MSM (adjusted by fixed-effect linear models,[31] richness p_{adj} = 1.63e−4, Shannon diversity p_{adj} = 2.69e−6).

(B) PCoA based on log2-transformed abundances of *Prevotellaceae* members of MSM and non-MSM (PERMANOVA, R2 = 0.034 and p_{adj} < 0.001). Marginal density plots show distributions of samples along each axis.

(C) Log2-transformed abundances of *Prevotellaceae* members with a minimum abundance of 0.1 at 90th percentile in all subjects. *S. copri* complex clades are highlighted in red, and uSGBs are denoted in blue. Asterisk signs indicate species differentially enriched in MSM relative to non-MSM.

(D) Percentage of individuals harboring multiple *S. copri* complex clades (p = 0.001; Wilcoxon rank-sum test). Only samples carrying *Prevotellaceae* members were considered (MSM n = 84, non-MSM n = 24).

See also Figure S2 and Table S2.

As summary metrics like alpha/beta diversity and richness may not integratively reflect microbial features related to sexual practices, a random-forest-based machine learning approach was next utilized to evaluate the prediction power provided by microbiota-derived information for sexual practices. In our case, we aimed to estimate how much accuracy can be achieved to classify a sample into the corresponding categories of sexual practices (e.g., having >3 or 0–3 partners) solely based on the gut microbiome taxonomic composition. Notably, micro-

biome-based prediction capability was observed for all sexual practices (Figure 4A), and it reached the highest performance in identifying individuals who have either >3 or 0–3 partners (area under the receiver operating characteristic curve 0.70) (Figure 4B). Intriguingly, the number of sexual partners was the only factor linked to the relative abundance of members belonging to both *Prevotellaceae* and *Segatella/Prevotella*, with statistically higher abundances in those who had >3 sexual partners during the last 12 months (Figure S4C).
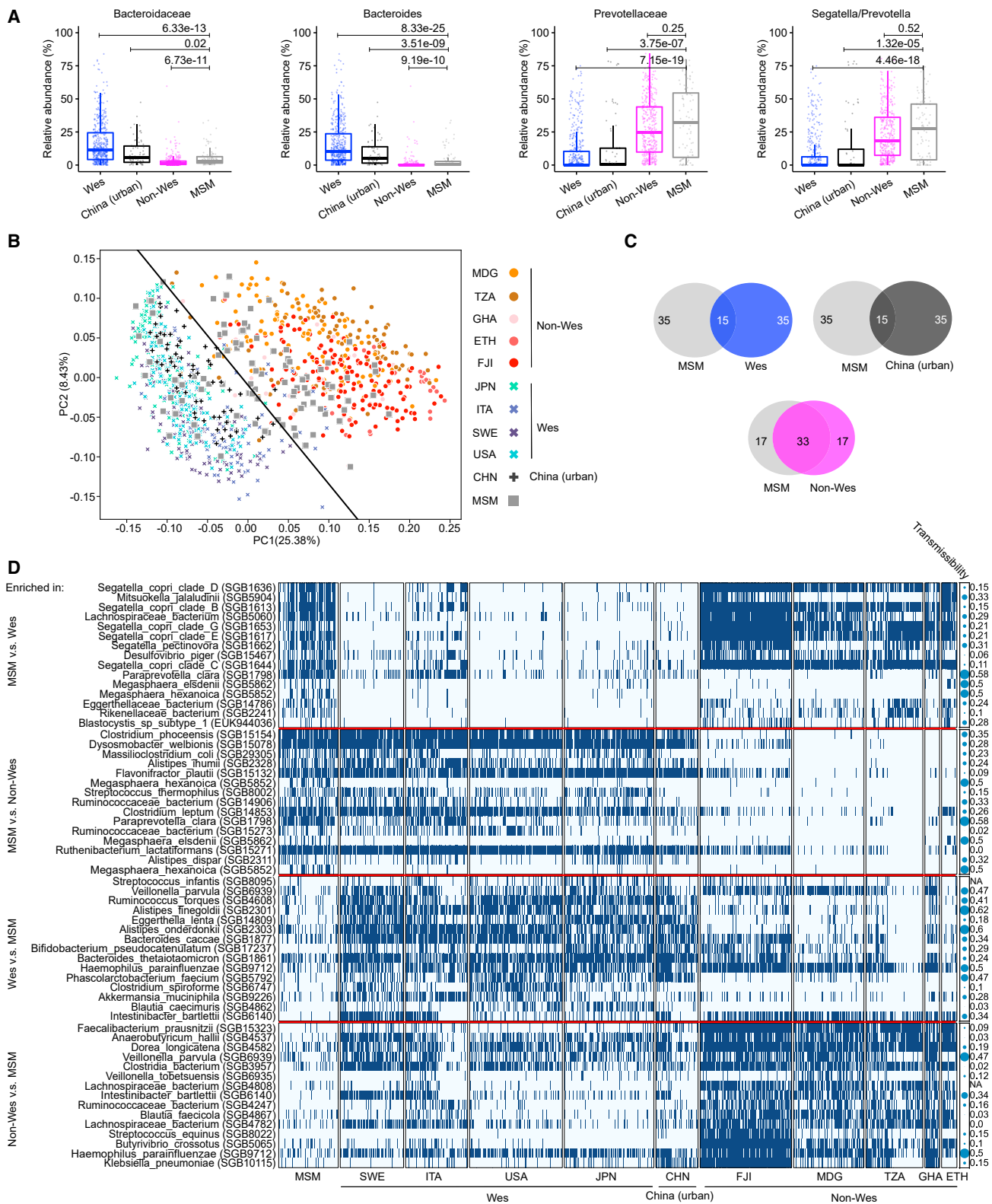
Figure 3. Gut microbiome community of MSM in the global context

(A) Differential abundance of *Bacteroidaceae* and *Prevotellaceae* families and *Bacteroides* and *Segatella/Prevotella* genera between MSM (n = 93), Westernized (n = 481), non-Westernized (n = 389), and urban Chinese (n= 66) samples (Wilcoxon rank-sum test with FDR correction).

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Risky sexual activities such as condomless intercourse and multiple sex partners were thought to increase pathogen exchange between individuals to increase infection incidences; thus, we hypothesize they might contribute to shaping a distinctive gut microbiome in MSM as well. We next sought to identify microbial biomarkers associated with sexual practices using linear discriminant analysis effect size (LEfSe).[58] For comparison, we divided sexual practices into risk-increasing and risk-reducing categories in analogy to their STI risk (see STAR Methods). Of note, we generally observed a higher number of microbial biomarkers associated with the risk-increasing behaviors, e.g., not using condoms during RAI, in comparison to risk-reducing behaviors, e.g., always using condoms during RAI (Figure 4D; Table S4). This suggests that risk-increasing activities introduced more microbial features differentiating gut microbiome structure compared to risk-reducing ones. Along these lines, having >3 sexual partners was linked to a co-existence of multiple *Segatella/Prevotella* members including *S. copri* clades B and C as well as *P. pectinovora,* which were mutually enriched for subjects with >3 partners (Figure 4C). Notably, the majority of identified biomarkers for specific sexual practices were exclusively associated with the specific practice, however, we noted a few biomarkers that were common to multiple practices in both risk-increasing and risk-reducing categories, respectively (Figure 4D). For example, uSGB2230 (*Rikenellaceae* spp.) was an overlapping biomarker across anal intercourse receivers, oral sex practitioners, STI carriers (in the last 24 months), and those not using condoms during RAI (Figure 4D; Table S4). By contrast, kSGB5075 (*Lachnospira pectinoschiza*) was mutually associated with those who always used condoms during RAI and had 0–3 sexual partners (in the last 12 months) (Figure 4D; Table S4). Overall, it appeared that risk-increasing behaviors share a higher number of microbial biomarkers than risk-reducing behaviors (Figures 4D and 4E; Table S4).

Next, we analyzed whether the biomarkers associated with risk-increasing practices are overall more transmissible or likely to engraft in the recipient's gut than those with risk-reducing practices (see STAR Methods). We did not observe either a significant difference in horizontal transmissibility (Wilcoxon rank-sum test p = 0.1616) or transmission-related properties including being spore forming, anaerobic, motile, or Gram-negative (Fisher's exact test FDR >0.05; Table S4). Counterintuitively (as pathogens tend to display more aggressive colonization strategies[59]), species associated with risk-reducing practices seemed to show a higher engraftment rate than those associated with risk-increasing ones (Wilcoxon rank-sum test p = 0.0001; Figure 4F).

Finally, we assessed links between the sexual practices of MSM living and raised in a Wes society and Westernization or non-Westernization of the gut microbiome. We quantified the similarity of MSM compared to Wes and non-Wes populations (see Figure 3B) based on principal coordinates calculated using Bray-Curtis distances of species-level relative abundances (see STAR Methods). Interestingly, risk-increasing behaviors, e.g., practicing oral sex, having >3 sexual partners, and not using condoms during RAI, were linked to a microbiome composition significantly closer to that of non-Wes subjects (Figure 4G; practicing oral sex p = 0.01, having >3 sexual partners p = 0.02, condomless [during RAI] p = 0.02). This suggests that specific sexual practices were associated with distinctly different microbiota in MSM that shared considerable commonality with the gut microbial composition of non-Wes individuals.

Enrichment of undescribed microbial species in the gut microbiome of MSM

The above reference-based approach allowed us to easily analyze our cohort in the context of numerous previous studies, yet this approach is limited by the existing databases, which are most likely from populations dominated by non-MSM. Thus, to characterize the microbial species that were not yet classified with described species-level taxonomy, we performed *de novo* metagenomic assembly on all samples in this study (see STAR Methods). Based on commonly used quality criteria,[21,60] we recovered medium quality (50% < completeness ≤90% and contamination <5%) and high quality (completeness >90% and contamination <5%) genomes for a total of 6,065 putative genomes representing 765 species (Figure 5A). 183 genomes (=strains) belonged to 72 previously undescribed species, as they lacked a characterized species-level taxonomy assignment by the GTDB-Tk database[61] (Table S5). These previously undescribed species were distributed across nearly all phyla represented by all genomes reconstructed in this study with the exception of *Cyanobacteria*, *Elusimicrobiota*, *Methanobacteriota*, and *Myxococcota* (Figure 5A; Table S5). Their strain genomes were significantly more prevalent in MSM samples than non-MSM samples, with 86% of MSM carrying at least one previously undescribed strain genome compared to 55% in non-MSM (Figure 5A; Table S5; Fisher's exact test p = 3.5e−04). To estimate the read abundance of strains from previously undescribed species in MSM and non-MSM samples, we sought to align the reads of each metagenomic sample against 183 previously undescribed strain sequences. Because some strains were underrepresented in both MSM and non-MSM samples, for stringency, we only considered 130 strain sequences, which attracted, on average, >0.001% of total reads per sample from both MSM

(B) PCoA based on MetaPhlAn 4[25] microbial abundances (log2 transformed) (PERMANOVA, p < 0.001). The hyperplane drawing a boundary between Westernized and non-Westernized individuals was generated using support vector machine (SVM).

(C) Venn diagram of the number of species shared and distinct between MSM and Westernized, non-Westernized, and urban Chinese individuals, respectively. Only the 50 most abundant species were considered based on the 90th percentile in each population.

(D) Presence-absence heatmap (blue, present; white, absent) for differentially enriched species (Fisher's exact test, FDR correction). For each comparison, only 15 species were displayed (a complete list is included in Table S3). Circles illustrate the transmissibility[47] of each enriched species and ranges from 0 (low) to 1 (high) with circle size increasing proportional to the value. Country abbreviations: MDG, Madagascar; TZA, Tanzania; GHA, Ghana; ETH, Ethiopia; FJI, Fiji; JPN, Japan; ITA, Italy; SWE, Sweden; USA, United States; CHN, China.
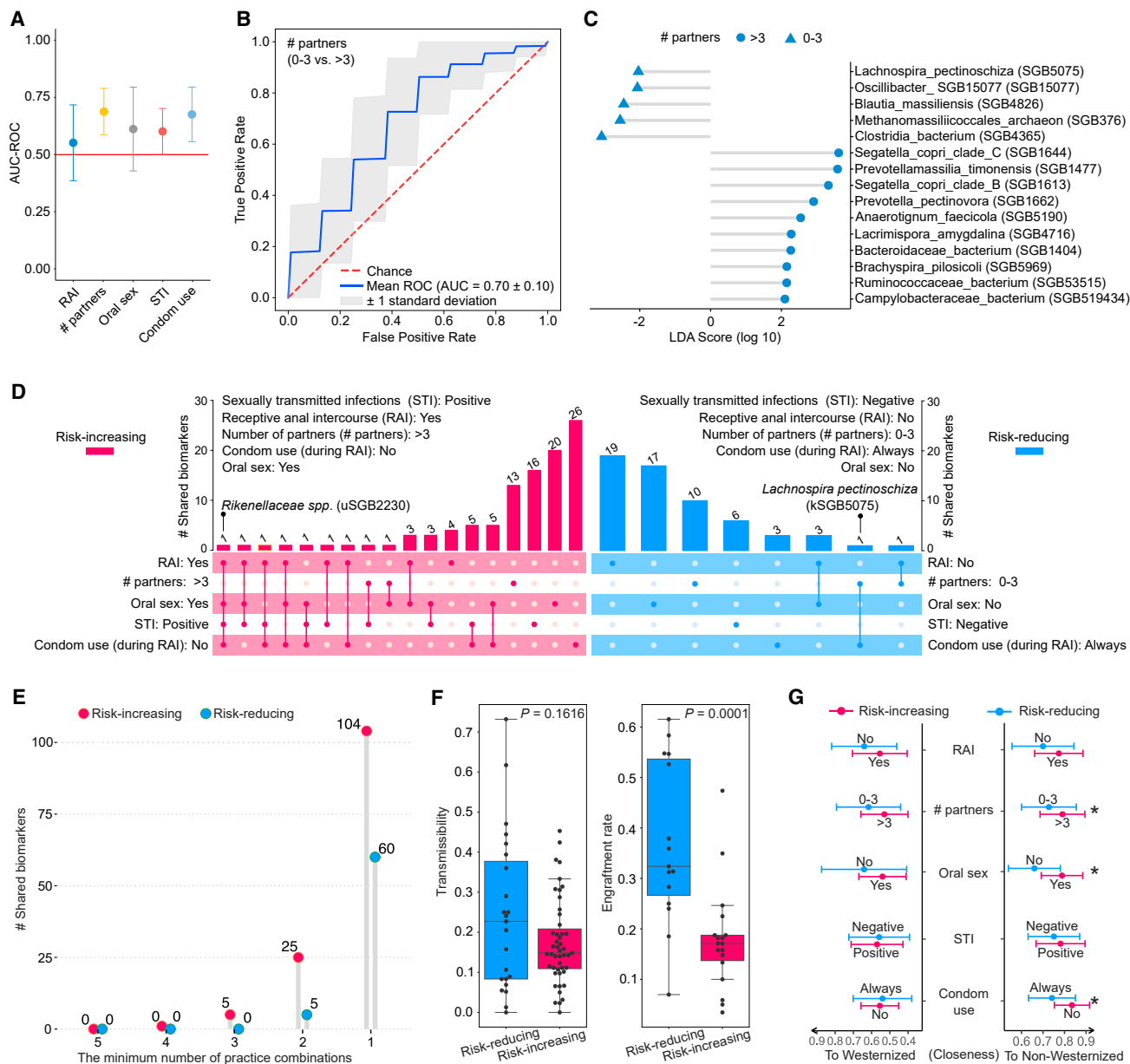See also Table S3.

Figure 4. Associations between gut microbiome alteration and sexual practices in MSM

(A) Association analysis between the whole microbiome composition and sexual practices assessed by machine learning prediction. The approach was based on a 20-repeated 3-fold-stratified cross-validation using random forest approach, given species-level relative abundances as input.

(B) ROC (receiver operating characteristic) curve of the learning model based on the species-level relative abundances of individuals corresponding to having 0–3 and >3 partners (in the last 12 months) using a random-forest-based approach.

(C) Species-level taxonomic biomarkers identified using LEfSe[58] to associate with behaviors having >3 (and 0–3) sexual partners. Only taxonomically known species are displayed (refer to Table S4 for a complete list).

(D) UpSet plot showing overlaps of taxonomic biomarkers identified using LEfSe for sexual practices classified as risk increasing and reducing. Numbers above bars indicate the number of biomarkers, solid dots denote sexual practices, biomarkers shared by multiple practices are denoted by connected dots, and biomarkers exclusively associated with a single sexual practice are denoted by single dots.

(E) The number of taxonomic biomarkers shared by the minimum number of practice combinations in risk-increasing and -reducing categories.

(F) Boxplots showing the transmissibility (left) and engraftment rate (right) of taxonomic biomarkers associated with risk-increasing and -reducing practices.

(G) Closeness of the gut microbiome to Westernized and non-Westernized populations with respect to sexual practices and STIs. Circles show the AUC-ROC mean and error bars represent the standard deviation. Asterisk denotes the statistical significance (*p < 0.05) by Wilcoxon rank-sum test.
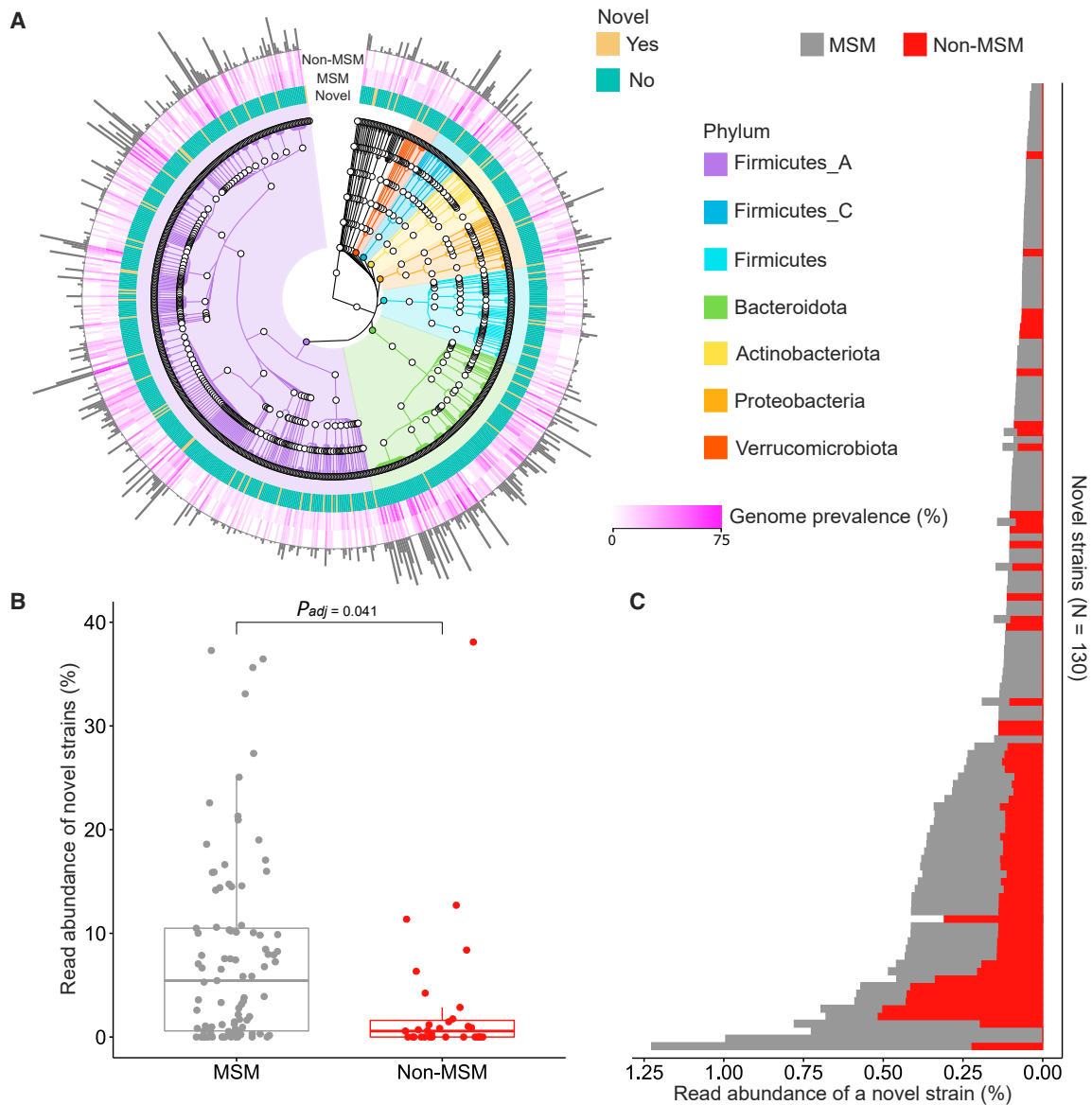
See also Figure S4 and Table S4.

Figure 5. Quantification of previously undescribed microbial species in the gut microbiome of MSM and non-MSM

(A) GTDB-Tk taxonomy structure of 765 species represented by 6,065 putative genomes reconstructed by *de novo* metagenomic assembly (only top seven phyla are highlighted in colors). The binary color of mustard and teal denote whether a species has been previously described or not, respectively. The gradient of magenta indicates the prevalence of species-assigned genomes in MSM and non-MSM (the color gradient was rescaled by arcsine-square root transformation for enhancing readability). Heights of the outmost histograms are in proportion to the number of genomes corresponding to the assigned species. Genome prevalence is defined as the number of species-assigned genomes divided by the number of individuals of MSM and non-MSM in percentage, respectively.

(B) The distribution of read abundance of previously undescribed strains in the metagenomic samples of MSM and non-MSM (adjusted by fixed-effect linear models,[31] mean difference p_{adj} = 0.041).

(C) Read abundance stratified by strains, averaged over 93 MSM and 31 non-MSM, respectively. The read abundance of previously undescribed strains is defined as the percentage of reads mapped to previously undescribed strains to total reads in a metagenomic sample.

See also Table S5.

and non-MSM samples (see STAR Methods). This resulted in a considerably larger proportion of previously undescribed strain-specific reads contributed by MSM samples relative to non-MSM samples (Figure 5B; p_{adj} = 0.041; adjusted by fixed-effect linear models for confounding effects reported as above). Focusing on single, previously undescribed strains, 76%

(99/130) of strain genomes appeared to attract more reads from MSM samples compared to non-MSM samples (Figure 5C). The *de novo* genome reconstruction complements the reference-based analysis, revealing that the MSM gut microbiome contains more frequently than the non-MSM gut microbiota not-yet-characterized bacterial species.

DISCUSSION

Here, we describe on the species level a distinct gut microbiota with an elevated alpha diversity in MSM compared to their non-MSM counterparts regardless of anthropometric (e.g., BMI) and clinical factors (e.g., HIV infection) (Figures 1 and S1), which corroborates previous *16S rRNA* gene sequencing studies performed using lower taxonomic resolution.[3,4,7] Enabled by deeply sequenced metagenomes, we also identified more not-yet-characterized microbial species in the gut microbiome of MSM relative to non-MSM (Figure 5). Specifically, we uncovered thriving *Prevotellaceae* communities in the MSM gut microbiome including co-existing *S. copri* complex clades and unknown species members (Figure 2). Distinctive species features identified in this study, as well as community-level alteration patterns in MSM, are consistent with previous reports[3,4,7] and implicate that identifying as MSM is a relevant factor for explaining inter-individual variability in the human gut microbiome. Notably, in another study, we recently observed a strong association of *S. copri* species complex members with the male gender in a large cross-sectional analysis of more than 4,000 healthy Wes individuals from 24 studies.[22] Importantly, as sexual orientation is not recorded in these and many other microbiota studies, it may be an underlying confounder that is currently largely overlooked, as MSM are estimated to constitute up to 6% of the male population.[62]

Despite the reported association of *Prevotellaceae* enrichment with fiber-rich diets in Wes individuals,[14,35] the increase in both prevalence and relative abundance of *S. copri* complex members and *Prevotellaceae* in MSM appears independent of diet, as only two were on a high-fiber diet based on our dietary survey in 52 participants (Table S1). Two other studies in MSM also failed to link dietary patterns and *Prevotella* abundance in MSM.[3,4] The *Prevotellaceae* enrichment in MSM has been hypothesized to be related to rectal mucosal injury, as functionalities involved in injury repair were indirectly predicted based on a small group of HIV-negative MSM engaging in condomless RAI.[57] Nonetheless, mechanisms underlying the *Prevotellaceae* expansion in MSM should be further explored regarding functional potentials, particularly in the context of rectal immune environment and mucosal inflammation.

Analyzing the gut microbiome of MSM combining publicly available metagenomic datasets covering many regions of the world (Table S3) led to the observation that the MSM microbiomes frequently bear remarkable resemblance to non-Wes microbiomes (Figure 3). This observation is in line with a similar observation by Lozupone and colleagues[6]: that the gut microbiome of HIV-infected persons in the US resembled that of healthy agrarians in Malawi and Venezuela, largely based on the *Prevotella* enrichment characteristics shared between two populations. Nonetheless, consistent with other studies,[3,4,7,57] our results revealed an increased abundance and diversity of *Segatella/Prevotella* in MSM compared to non-MSM, independently of HIV infection (Figure 2). Moreover, the reminiscent pattern of MSM and non-Wes microbiomes was featured by numerous abundant and prevalent species beyond *Segatella/Prevotella* members. Because the HIV-infected subjects in many enrollment settings are predominantly MSM, comparison

of the gut microbiota of HIV-infected populations with other cohorts without taking into account their MSM status might have been confounded in prior studies. Although a clear effect of Westernization on MSM gut microbiome alteration was apparent (Figure 3), owing to our approach such as selecting samples from healthy adults of different cohorts (Table S3), common confounding factors like batch effects should be better technically controlled in future studies when integrating large public datasets.

Kelly and colleagues reported for a small group of HIV-negative MSM[57] that sexual practices such as condomless RAI and enema use are linked to an aberrant rectal mucosal environment concerning both immune context and microbiome composition. Mechanical damage of the mucosa and nutrients in seminal fluid might contribute to shifts of the microbiota composition toward microbes that are better adapted to the altered rectal environment. However, we found that sexual practices more than RAI exerted profound effects in differentiating the gut microbiota of MSM and driving the similarity to non-Wes microbiomes (Figure 4). Our observation of distinctive microbial features for different sexual activities suggests that alteration is an additive process driven by combinations of specific sexual practices (Figure 4). While potential confounding effects (e.g., age and BMI) were not included in the machine learning and LEfSe analysis for sexual activities due to the technique limitation to include multiple parameters, association analysis of each sexual activity and confounders revealed that age and BMI showed insignificant confounding effects (Table S4). However, HIV status and antibiotics treatment might still be linked with STI and oral sex, respectively, for which confounding effects can be better controlled with larger recruitment with improved match for covariables in the future.

As high-risk behaviors for acquiring STIs are associated with more diverse and non-Wes gut microbiota, we hypothesized that those behaviors and sexual practices also facilitate the transmission of gut bacteria. Therefore, we attempted to establish the transmission-based mechanism to explain the alteration in the MSM gut microbiome, utilizing species-resolved transmissibility scores and engraftment rates from two recent large-scale studies.[47,50] While the MSM gut microbiome is rich in species with high transmissibility, e.g., *S. copri*, we observed only a slightly increased mean transmissibility and insignificantly different engraftment rate for species that were differentially abundant in MSM (Figure S3; Table S3). This may be partially due to limited transmission data available for MSM-associated species, with a large proportion currently lacking quantitative transmissibility scores or engraftment rates (Table S3). Moreover, the transmission measures were estimated in a global approach independent of potential transfer routes and therefore may not accurately reflect the transmission probabilities in the MSM population. For instance, specific sexual practices and higher numbers of sexual partners probably overcome transmission barriers measured in other settings, such as mother-infant transmission. For transmission quantification per se, the approach was based on species-level marker genes instead of whole-genome sequences and genetic identity without directionality inference, which might be potential caveats in the case of limited strain genetic variation, although it allowed

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inferring transmissibility and engraftment metrics for a much larger number of species. Since the transmission of microbes is a highly complex dynamic affected vastly by factors beyond a species' intrinsic biology, e.g., endogenous colonization resistance,[47,63] clearly more effort is required to determine biological features involved in the gut microbial transmission, to establish transmission dynamics in different scenarios, and to provide insight on the transmission routes and mechanisms.

Along these lines, our cross-sectional cohort identified a strong influence of sexual practices, especially higher numbers of sexual partners, on gut microbiota composition, yet larger cohorts comprising samples from multiple body sites (e.g., oral cavity, penis, and gut) collected at different time points from participants sharing sexual interaction networks are required to reconstruct a more detailed alteration trajectory and transmission landscape between MSM. While our study focused on MSM, it is intriguing to speculate to what degree sexual practices and numbers of sexual partners affect microbiota structure and diversity in the general population and in women who have sex with women.

Limitations of the study

We acknowledge that the self-report questionnaire might introduce biases if participants fail to reliably respond to questions. Although common anthropometric and clinical effects were taken into account in analyzing samples generated in this study, batch effects should be considered when including published samples that were generated from other studies using different methods. In addition, an increasing effort in expanding the dataset would be appreciated, particularly for enhancing the robustness of machine learning and transmissibility analysis.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xcrm.2024.101426.

AUTHOR CONTRIBUTIONS

Conceptualization, K.D.H., T.S., and J.K.; resources, K.D.H., S.E., L.A., A.B., N.S., T.S., and J.K.; data curation, K.D.H., L.A., and A.B.; methodology, K.D.H., T.S., and J.K.; investigation, K.D.H., L.A., E.J.C.G., T.-R.L., R.d.O., A.B.-M., M.V.-C., I.R., E.P., J.B., N.S., S.E., T.S., and J.K.; writing – original draft, K.D.H., T.S., and J.K.; writing – review & editing, K.D.H., L.A., E.J.C.G., M.V.-C., T.S., and J.K.; formal analysis, K.D.H., L.A., T.-R.L., A.B.-M., I.R., and E.P.; visualization, K.D.H. and E.P.; supervision, K.D.H., T.S., and J.K.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Fecal samples	This study	N/A
Critical commercial assays		
Stratec (now Invitek) Stool Collection Tubes	Stratec (now Invitek), Berlin, Germany	1038111200
Stool DNA Stabilizer PSP Spin Stool DNA	Stratec (now Invitek), Berlin, Germany	1038120200
NEBNext Ultra DNA Library Prep Kit for Illumina	New England Biolabs, Massachusetts, USA	E7645L
Deposited data		
Raw sequencing data	This study	PRJNA947377
Metagenome-assembled genomes	This study	PRJNA947377
Software and algorithms		
BBMap	sourceforge.net/projects/bbmap/	https://jgi.doe.gov/data-and-tools/software-tools/bbtools/bb-tools-user-guide/bbmap-guide/
MetaPhlAn 4.0	Blanco-Miguez et al.[25]	https://github.com/biobakery/MetaPhlAn
GTDB-Tk v1.5.0	Chaumeil et al.[61]	https://github.com/ecogenomics/gtdbtk
metaWRAP v1.3.2	Uritskiy et al.[64]	https://github.com/bxlab/metaWRAP
VAMB v3.0.2	Nissen et al.[65]	https://github.com/RasmussenLab/vamb
DAS Tool v1.1.1	Sieber et al.[66]	https://github.com/cmks/DAS_Tool
CheckM v1.0.7	Parks et al.[67]	https://ecogenomics.github.io/CheckM/
Bowtie2 v2.3.5.1	Langmead and Salzberg[68]	https://bowtie-bio.sourceforge.net/bowtie2/index.shtml
CMSeq v1.0.0	Pasolli et al.[21]	https://github.com/SegataLab/cmseq
GraPhlAn	Asnicar et al.[69]	https://github.com/biobakery/graphlan
PhyloPhlAn 3.0	Asnicar et al.[70]	https://github.com/biobakery/phylophlan
iTOL v6.0	Letunic and Bork[71]	https://itol.embl.de/
python package hclust2	N/A	https://github.com/SegataLab/hclust2
python package scikit-bio v0.5.7	N/A	http://scikit-bio.org/
python package scikit-learn v1.1.2	Pedregosa et al.[72]	https://scikit-learn.org/
python package matplotlib v3.5.0	Hunter[73]	https://matplotlib.org/
python package scipy v1.7.3	Virtanen et al.[74]	https://scipy.org/
python package seaborn v0.11.2	Waskom[75]	https://seaborn.pydata.org/
python package pandas v1.3.5	McKinney[76]	https://pandas.pydata.org/
python package statsmodels v0.12.1	Seabold and Perktold[77]	https://www.statsmodels.org/
python package numpy v1.21.2	Harris et al.[78]	https://numpy.org/
LEfSe v1.1.2	Segata et al.[58]	https://huttenhower.sph.harvard.edu/lefse/
R package ggplot2 v3.3.6	Wickham[79]	https://ggplot2.tidyverse.org/
R package ggpubr v0.4.0	N/A	https://rpkgs.datanovia.com/ggpubr
R package ComplexHeatmap v2.8.0	Gu[80]	https://bioconductor.org/packages/ComplexHeatmap/
R package mia v1.0.8	N/A	https://github.com/microbiome/mia
R package lfe v2.8.8	Gaure[31]	https://github.com/sgaure/lfe
R package vegan v2.6.2	Oksanen et al.[81]	https://github.com/vegandevs/vegan

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources should be requested from the Lead Contact, Prof. Dr. Till Strowig (Till.Strowig@helmholtz-hzi.de).

Materials availability
This study did not generate new unique reagents.

Data and code availability
- Shotgun metagenomic reads, detailed descriptions for each sample and metagenome-assembled genomes in this study are available under NCBI BioProject: PRJNA947377
- Codes and corresponding tutorials for reproducing results in this study are avaialble at: https://github.com/KunDHuang/KunDH-2024-CRM-MSM_metagenomics
- Any additional information required to reanalyze the data reported in this work is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

This study recruited patients from two cohorts treated in the Clinic of Dermatology, Department of Venerology of the University Hospital Essen, Germany. Patients under 18 years of age were excluded for both cohorts. One cohort comprised 57 patients living with HIV of the HIV Heart study group, including 41 MSM and 16 non-MSM. The HIV-heart study is a prospective study assessing the incidence, prevalence and clinical course of cardiovascular diseases in HIV infected patients and is registered under Clinical Trials.gov (NCT01119729). The cohort was initiated for assessing differences in the gut microbiome between people living with HIV linked to coronary heart disease. All patients were treated with antiretroviral therapy. Both groups, with and without coronary heart disease were matched for gender, age, diabetes mellitus and chronic inflammatory bowel disease. The entire characteristics of the cohort is described elsewhere.[82] A second cohort included 67 patients (48 HIV positive and 19 HIV negative individuals, comprising 52 MSM and 15 non-MSM), who additionally completed a questionnaire assessing information about diet, BMI, practice of RAI, use of condoms when practicing RAI, number of sexual partners within last 12 months and STIs of the gut or other gut inflammatory diseases within last 24 months to gain insights into risk factors linked to *Prevotellaceae*-richness of the gut microbiome (Table S1). Human sample and data collections have been performed in agreement with the guidelines of the Ethics Committee of the Medical Faculty of the University of Duisburg-Essen (permit No. 14-5874-BO and 18-8409-BO) and the European Data Protection Laws (Europäische Datenschutz-Grundverordnung DSGVO). All subjects enrolled have signed a letter of informed consent in accordance with the World Medical Association Declaration of Helsinki (Version 2013).

METHOD DETAILS

DNA extraction, library preparation and sequencing
DNA was extracted using the PSP Spin Stool DNA Extraction Kit (Stratec, now Invitek, Berlin, Germany) according to the manufacturer's protocol ''stool samples from difficult to lyse bacteria''. Libraries were constructed using the NEBNext Ultra DNA Library Prep Kit and sequenced on NovaSeq S4 PE150 platform with a target depth of 35,000,000 reads/sample.

Profiling species-level microbial community
For all samples in this study the metagenomic reads were first quality-checked and processed using BBMap (sourceforge.net/projects/bbmap/) with the Ensembl masked human genome GRCh38 and phiX. Afterward, we performed profiling of the species-level microbial community on the pre-processed reads by running MetaPhlAn 4[25] using default settings to identify microbial species and estimate the corresponding relative abundances in the metagenomic samples. To compare the MSM microbiome architecture with that of the global population, we downloaded 936 publicly available shotgun metagenomes (Table S3) spanning ten countries and different lifestyles, and subsequently profiled the species-level microbial community as described above. In each profile, we only considered species with a minimum relative abundance of 0.0001 because the precision of taxonomy identification might be compromised in the case of low-abundance members. All external metagenomes were from adult individuals including males and females who were healthy defined as free of self-reported diseases and medical intervention in order to represent general populations. Each of them was characterized by whether they were from a Westernized or non-Westernized or urban Chinese population (Table S3). The term ''non-Westernized'' describes a population practicing a traditional lifestyle relating to factors such as diet, hygiene, and with limited access to modern medical healthcare and pharmaceuticals (e.g., antibiotics) as previously described.[18,20,21,83] Briefly, Westernization is a complex process with incremental urbanization that has been undergoing for centuries. During the process, lifestyles have changed profoundly, including but not limited to, transition from autarchic means of producing food to controlled food production chain, increased hygiene and accessibility to modern medicals, introduction of food sterilization, increased exposure to pollutants, and switch from high-fiber simple diets to high-fat high-protein processed foods. All these

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factors are thought to play a pivotal part in shaping the human gut microbiome structure. The concept of "Westernization" and "non-Westernization" was adopted here to demarcate populations by combining at least the majority of the factors as discussed above. Although this defines very heterogeneous populations, significance of MSM status to the gut microbiome change could be depicted in a much broader context encompassing many factors. Noteworthily, to complement the binary demarcation, we also included two Chinese cities - GuangDong and TangShan - which are located in Southern China and have been experiencing rapid urbanization in the last decade. They were used to represent populations from non-Western but relatively urbanized communities.

Alpha and beta diversity calculations

Alpha diversities were calculated using Shannon index estimated by R package *mia* (https://github.com/microbiome/mia) and beta diversities were calculated using Bray-Curtis dissimilarity based on species-level relative abundances, followed by a principal coordinates analysis (PCoA) for the visualization (Python package scikit-bio v0.5.7; http://scikit-bio.org/). To rule out confounding effects, the significance of differences in alpha diversity was additionally measured using fixed effects linear models[31] by accounting for co-variables, including age, ethnicity, antibiotics use, BMI and HIV status. Likewise, the R package *vegan* v2.6.2[81] (https://github.com/vegandevs/vegan) was used to perform covariate-controlled PERMANOVA tests for beta diversities.

Prevotellaceae community comparison in MSM and non-MSM individuals

To compare the *Prevotellaceae* community structure in MSM and non-MSM gut microbiomes, we extracted *Prevotellaceae* members' abundances from MetaPhlAn 4 outputs and removed samples with no *Prevotellaceae* members being identified. Afterward, *Prevotellaceae* community's richness was calculated for the kept samples in this study, and the beta diversity of *Prevotellaceae* community between MSM and non-MSM individuals was estimated using extracted *Prevotellaceae* members' log2-transformed abundances. Besides analyzing the whole *Prevotellaceae* community, we also analyzed *Segatella copri* complex clades due to their likely association with MSM behavior.[19] In particular, we quantified the co-existing pattern of multiple *S. copri* complex clades in the MSM and non-MSM subjects using the method as described elsewhere.[18] The *Prevotellaceae* members' log2-transformed abundances were visualized in heatmap using ComplexHeatmap[80] for only those with a minimum abundance of 0.1 at 90th percentile in all kept samples. To present the phylogenetic structure of all *Prevotellaceae* members identified in this study, we considered one representative genome for each of the 47 *Prevotellaceae* SGBs. The phylogenetic tree was built using PhyloPhlAn (v. 3.0)[70] and by considering the 400 universal marker genes available in PhyloPhlAn. iTOL tool[71] (v. 6.0) was then used to integrate the information about a member's prevalence in MSM/non-MSM and if it belongs to kSGB or uSGB in the tree.[71]

Analyzing MSM microbiome architecture in a global context

To contextualize the MSM microbiome structure in the global population, we used MetaPhlAn 4 outputs to generate a matrix including microbial abundances from 93 MSM individuals of this study and 936 downloaded metagenomic samples which include typical Westernized, non-Westernized societies, and urban Chinese communities whose lifestyles are transitioning into more modern ones[18,21,33–41] (Table S3). Individual abundance profiles were merged into one matrix using merge_metaphlan_tables.py, a utility script in MetaPhlAn 4. Afterward, beta diversity based on log2-transformed relative abundances was calculated on the merged matrix as described above in order to understand the overall difference between MSM microbial community and that of global populations. Afterward, a hyperlane was created using Support Vector Machine (SVM) to draw a boundary between Westernized and non-Westernized individuals (a python package scikit-learn v1.1.2; https://scikit-learn.org/). For identifying the top 50 abundant species in the population of MSM, Westernized and non-Westernized and urban Chinese individuals respectively, we used a python package hclust2 (https://github.com/SegataLab/hclust2) with parameter setting of –fperc 90 –ftop 50 –f_dist_f correlation –s_dist_f braycurtis -s –slinkage complete. To further understand the difference at the single species level, we used the merged matrix for identifying single species which are significantly enriched in MSM individuals, Westernized or non-Westernized (MSM vs. Westernized, MSM vs. Non-Westernized, Westernized vs. MSM and Non-Westernized vs. MSM) using Bonferroni-corrected Fisher's exact test as described elsewhere.[18,60]

Assessing engraftment rate, horizontal transmissibility and relevant biological properties

To examine the horizontal transmissibility, engraftment rate and transmission-related properties including spore formation, anaerobic growth, motility and Gram-negative staining, we retrieved the per-species basis measures from two integrative multi-cohort studies by Valles-Colomer et al., and Ianiro et al.,[47,50] respectively. Briefly, Valles-Colomer and colleagues first aggregated a multi-cohort dataset comprising 9,715 human metagenomic samples with curated metadata on subject identifiers, time points, participant's age, gender, delivery mode, family identifiers, family relationships, two zygosity and age at which twins moved apart, village, and country. Species-level profiling was performed for all samples using MetaPhlAn 4[25] based on the SGB database same as for the 124 samples from this study. Strain-level profiling was subsequently performed for detected SGBs (complying with SGB selection criteria reported elsewhere[47]) using StrainPhlAn4,[25,84] which estimates phylogenetic distances between strains from different samples for each SGB. For inferring horizontal transmissibility, Valles-Colomer and colleagues used StrainPhlAn profiles of 3,192 samples whose subjects shared a household, and calculated the species horizontal transmissibility following *strain-sharing-inference* pipeline (https://github.com/biobakery/MetaPhlAn/wiki/Strain-Sharing-Inference). In the original study, transmission-related properties were predicted for all SGBs using Traitar (v 1.1.12)[85] on the 50% core genes. On the other hand, Ianiro and

colleagues quantified engraftment rate using 1,371 fecal metagenomic samples specific to FMT settings, which mainly reflects the extent of vertical microbiome transmission between donors and recipients. Microbiome species profiles and strain profiles were generated in the same way as described above, which were subsequently used to quantify FMT engraftment rate following the strain-sharing-pipeline (https://github.com/SegataLab/Strain-sharing-pipeline).

As microbial species identified in this study were based on the same SGB database as for those from two large studies, we next annotated the differentially enriched species in this study for their corresponding horizontal transmissibility, engraftment rate and transmission-related properties using the quantifications from Valles-Colomer et al., and Ianiro et al., . For biological properties involved in microbial transmission, we used Fisher's exact test to examine if any of the properties was differentially present in species sets that were enriched in MSM relative to non-MSM, Westernized and non-Westernized individuals, respectively. Afterward, Benjamini-Hochberg methods were used for correcting multiple hypotheses comparison.

Analysis of associations between sexual practices and microbiome composition
To analyze links between sexual practices and gut microbiome characteristics in MSM, we generated six datasets merging MetaPhlAn 4 species-level relative abundances, respectively, each represented by RAI (Yes and No), sexually transmitted infections (Positive and Negative), oral sex (Yes and No), number of partners (0–3 and >3) and condom use during RAI (Always and No). These datasets were then used for assessing the association between sexual practices and microbiome composition based on machine learning, for identifying practice-associated taxonomic biomarker and for estimating microbiome closeness for sexual practices to Westernization and non-Westernization as elaborated below.

Sexual practice-microbiome association analysis using random forest-based machine learning approach
All learning experiments depended on random forest implemented by a python package scikit-learn v1.1.2[12,86] as it has been shown to reach overall a better performance for microbiome data compared to other approaches.[87,88] We created a learning model using 1,000 estimator trees and Shannon entropy to evaluate the quality for each node splitting of a tree. In the hyperparameter setting, we assigned at least one sample per leaf and 30% of features for a tree as suggested elsewhere.[89,90] The prediction capability for each dataset was evaluated by stratified 3-fold cross-validation to ensure that each partition has enough and balanced binary cases. The procedure of dataset folding and model evaluation was repeated 20 times with a result of an average over 60 validation folds per practice.

Identifying taxonomic biomarkers
On a per-dataset basis, linear discriminant analysis effect size (LEfSe) with default parameter settings[58] was used to identify microbial features that were statistically different between sexual practices (e.g., RAI: Yes vs. RAI: No) and to estimate their effect size represented by logarithmic LDA score. Features with a logarithmic LDA score over 2.0 were considered significantly discriminant.

Grouping sexual practices based on risk analogous to STI risk
Risk-increasing category was represented by receiving anal intercourse (RAI: Yes), having >3 sexual partners (#partners: >3), practicing oral sex (Oral sex: Yes), diagnosed with STI in the last 24 months (STI: Positive) or not using condom during RAI (Condom use (during RAI): No). By contrast, risk-reducing category included not receiving anal intercourse (RAI: No), having 0–3 sexual partners (#partners: 0–3), no oral sex (Oral sex: No), free of STI in the last 24 months (STI: Negative) or always using condom during RAI (Condom use (during RAI): Always).

Estimating microbiome similarity to Westernized and non-Westernized microbiomes
We measured the closeness of each sexual practice in MSM to the Westernized and non-Westernized based on the MSM gut microbiome community composition in a global context (Figure 3B). Firstly, species-level relative abundances of 870 publicly available samples (Westernized $N = 481$; non-Westernized $N = 389$) were merged with the dataset of each sexual practice, resulting in six expanded datasets additionally containing global samples characterized by Westernization and non-Westernization. Secondly, Bray-Curtis distances on a per-dataset basis were measured, based on which principal coordinates were estimated for each sample, with only the first two coordinates (PC1 and PC2) being selected to determine a sample's placement. We finally calculated the coordinates distance between each practice-characterized sample and the centroid of the Westernized and non-Westernized samples respectively. The closeness was defined as the complement of the distance (e.g., closeness = 1 - distance).

Metagenomic assembly and estimation of previously undescribed microbial strains
In order to complement the reference-based analysis, metagenomic assembly was performed in order to reconstruct putative strain genomes following the metaWRAP pipeline[64] (version 1.3.2). Briefly, for each of 124 metagenomic samples from this study, reads were first assembled into contigs by a *de novo* metagenome assembler metaSpades[91] whose performance has been reported to outstand among other similar approaches.[21,92,93] Afterward, contigs were binned using five different binning tools including meta-bat1,[94] metabat2[95] and maxbin2[96] which have been implemented in metaWRAP pipeline already and two external methods (VAMB[65] and DAStool[66]). The genome bins generated by five approaches were further consolidated using *bin_refinement* module in the metaWRAP pipeline for producing putative genomes. The putative genomes were assessed for completeness, contamination

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and strain heterogeneity using CheckM[67] (version 1.0.7; lineage specific workflow). We retained only medium-quality (MQ) genomes (50% < completeness ≤90% and contamination ≤5%) and high-quality genomes (completeness >90% and contamination ≤5%) for the analysis to follow as suggested in the previous studies.[21,60] Finally, GTDB-Tk[61] (v.1.5.0; *classify_wf* module; default settings) was used to assign species-level taxonomy to qualified genomes; genomes lacking known species-level taxonomy were identified as previously undescribed microbial strains. The taxonomy structure of genomes was visualized using GraPhlAn.[69]

To estimate the read abundance of previously undescribed microbial strains in the metagenomic samples, we aligned metagenomic reads for each sample in this study against genome sequences of previously undescribed strains using bowtie2[68] (v2.3.5.1; –end-to-end –no-unal -U -S) to generate read alignments in bam files. The resulting bam files, based on each previously undescribed strain sequence, were further cleaned using a python package CMSeq (https://github.com/SegataLab/cmseq) with following criteria: (1) reads alignment quality ≥ 30, (2) reads coverage depth ≥ 5-folds, (3) minimum identity of reads ≥ 96%, (4) aligned read length ≥ 90 nt, (5) minimum dominant allele frequency ≥ 50%, (6) previously undescribed strain sequence coverage breadth ≥ 80%. The read abundance of previously undescribed strains for each sample was then calculated by dividing the filtered aligned reads by the total number of reads of a metagenomic sample. This approach has been validated by other studies for retaining strain-specific reads.[18,93,97] Strains were not considered in reads alignment estimation if the percentage of aligned reads account for, on average, <0.001% of total reads per sample.

QUANTIFICATION AND STATISTICAL ANALYSIS

All statistical significance for mean difference between categories was assessed using the two-tailed Wilcoxon rank-sum test. Statistical significance for multivariate analysis was performed using PERMANOVA test controlled for confounding factors including ethnicity, antibiotics use, BMI, and HIV infection. Statistical significance for enrichment analysis was performed using Fisher's exact test corrected with the Bonferroni method for multiple hypothesis testing. Associations with transmission-related properties were tested using Fisher's exact test followed by Benjamini-Hochberg correction. Tests were considered as significant if *p* values <0.05 ($P_{adj} < 0.05$ in the case of adjusting for confounding effects or FDR <0.05 in the case of multiple hypothesis correction).