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Comparative analysis of the gut microbiota in centenarians and young adults shows a common signature across genotypically non-related populations

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ABSTRACT

Gut microbiota is among the factors that may be involved in healthy aging. Broader and geographically spread studies on gut microbiota of centenarians can help in identifying a common signature of longevity. We identified an endogamous Indian population with high centenarian prevalence. Here, we compared the gut microbiota composition and fecal metabolites of a centenarians group (˜100 years) with young people (25–45 years) of the region with the high centenarian prevalence and the nearby region of low centenarian prevalence to decipher microbial-related longevity signatures. Also, we compared our results with publicly available datasets of similar groups including 125 centenarians from three countries (Italy, Japan, China). Our comparative analysis resulted in higher biodiversity within *Ruminococcaceae* in centenarians, with respect to younger adults, irrespective of their nationality. We observed bacterial signatures that are common among extremely old people of different nationality. Comparative metabolites profiling identified the fecal metabolic signature of extreme aging in the Indian study population. Our analysis of the co-occurrence network and bimodal distribution of several taxa suggested the establishment of a pervasive change in the gut ecology during extreme aging. Our study might pave the way to develop gut microbiota based biomarkers for healthy aging.

1. Introduction

Age-related healthcare is a leading expenditure cause in many countries (de Magalhaes et al., 2017). The average life expectancy has increased by four years during the past decade (Kassebaum et al., 2016), and the modern society is progressively aging with a prediction of 1.2 billion people aged over 60 years by 2025 (Jackson et al., 2016). In such a scenario, facilitating healthy aging and preventing aging-associated chronic disabilities are great challenges and important research priorities. Food is among the most important factors that influence the possibilities of attaining healthy aging and prolonged life expectancy. Elie Metchnikoff published the role of fermented foods in prolonging Bulgarian life about 100 years ago (Cavaillon and Legout, 2016). Nowadays, researchers widely accepted the traditional Mediterranean diets (De Filippis et al., 2016) and Japanese Washoku (Gabriel et al., 2018) as a possible means of improving and maintaining health throughout the whole life. Other than this, genetic factors, lifestyle and environmental factors are also linked with lifespan extension. Gut microbiota composition is among the factors that have been proposed to be involved in longevity (Biagi et al., 2016; Gruber and Kennedy, 2017; Han et al., 2017; Santoro et al., 2017), based on both human and animal models. Many observational studies focused on the sequential changes in gut microbiota over a wide range of years established that the gut microbiota structure in elderly is considerably different from that of the younger adults (Claesson et al., 2012; Gruber and Kennedy, 2017; Park et al., 2015; Wang et al., 2015). Moreover, the age-related decline in butyrate producers (i.e., *Faecalibacterium*) and colonization of opportunistic pathobionts (i.e., *Enterobacteriaceae* members) have been linked with frailty (Biagi et al., 2017). Broader and geographically spread studies on gut microbiota of long-living

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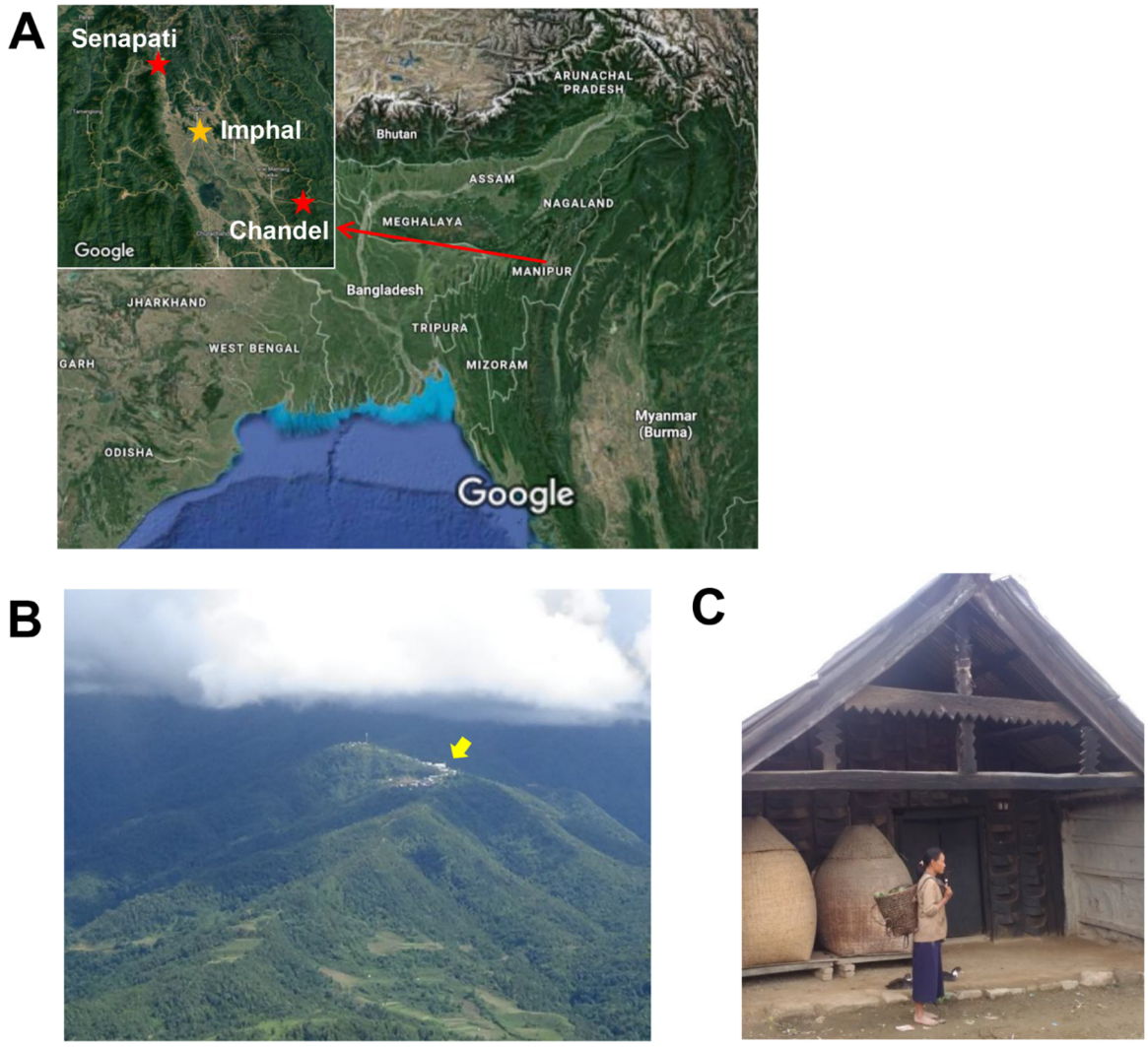


Fig. 1. Geography of the study area (Manipur, India) and lifestyle of the study population. (A) Location of Chandel and Senapati districts with high centenarian prevalence (red), and Imphal district with low centenarian prevalence (yellow). (B) Remoteness of the Maiba village in the hilltop of Senapati district. (C) Traditional wooden house of Poumai tribe of Naga community in Saranamai village of Senapati district. Photos by K.J.

individuals will help in understanding the complex phenomenon of longevity. Indeed, centenarians, having reached the extreme limits of the human lifespan, are the best model for understanding the phenomenon of longevity. Few comparative analyses have identified signatures of longevity in the gut microbiota of Italian (Biagi et al., 2016) and Chinese (Kong et al., 2016) centenarians. Study groups were often imbalanced in sex due to the longer life expectancy of females. However, the availability of these data will help in looking for a common signature of longevity independent of nationality, directing future researches on healthy aging and longevity.

In the present study, we identified areas in the Manipur state of India with a high centenarian prevalence. The rural endogamous population of Naga community (Hodson, 1911) of Maring and Lamkang tribes in Chandel district and Mao and Poumai tribes in Senapati district have high centenarian prevalence. The Nagas live in geographically isolated hill villages in Northeast India with limited contact with other social units (Fig. 1). Traditionally, they hunt in and gathers from surrounding forests (Stirn et al., 2003). At present, they maintain animal husbandry to support the meat requirement. Also, practice Jhum (shifting) cultivation for their agricultural subsistence (Shimray, 1985). They typically follow two meals per day with rice as a staple food and consume heavy meat (pork and beef products). Nagas do not use milk products, consuming instead plentiful rice-based fermented beverages (rice wine). On the contrary, the population with low centenarian prevalence identified for the comparison in the present study is from the sub-urban endogamous Meitei community, living in the valley region of Manipur, Imphal West district, mostly composed of fish eating vegetarian. By comparing the gut microbiota structure of people at extreme aging with young adults, a signature taxa or bacterial community pattern specific to long living people can be established. Here, we analyzed the gut microbiota and faecal metabolites composition of centenarians (˜100 years) in comparison to young adults (25–45 years) from the region with high centenarian prevalence (> 6 centenarians/ 10,000 citizen), as well as to young adults from the nearby region with low centenarian prevalence (< 1 centenarians/ 10,000 citizens). Gut microbiota composition was studied by 16S rRNA gene sequencing, and fecal metabolites were analyzed by LC-HRMS. Also, we compared the results with similar datasets retrieved from the research conducted in three other countries (Italy, Japan, and China) (Biagi et al., 2016; Kong et al., 2016; Odamaki et al., 2016) in the attempt of identifying a common signature of longevity.

2. Results

Thirty centenarians and 30 young adults (internal control) were recruited from the Chandel and Senapati districts of Manipur state,

Table 1
Source and details of subjects in each categorized groups analyzed in this study.

Country	Groups	Number of subjects	Age group	Age mean ± SD range	Sex Female	Source
Indian	Centenarian	30	97-110	99.9 ± 3.55	15 (50%)	This study
	Young Internal Control	30	28-47	35.8 ± 6.34	18 (60%)	
	Young External Control	30	22-50	34.8 ± 7.85	15 (50%)	
Italian	Centenarian	33	99-109	103.9 ± 3.21	27 (81.8%)	Biagi et al., 2016
	Young Control	15	22-48	30.5 ± 7.85	8 (53.3%)	
Chinese	Centenarian	18	96-102	97.7 ± 1.84	14 (77.7%)	Kong et al., 2016
	Young Control	22	24-50	39.7 ± 7.43	15 (68.1%)	
Japanese	Centenarian	17	94-104	97.8 ± 3.19	15 (88.23%)	Odamaki et al., 2016
	Young Control	20	28-41	34 ± 3.26	9 (45%)	

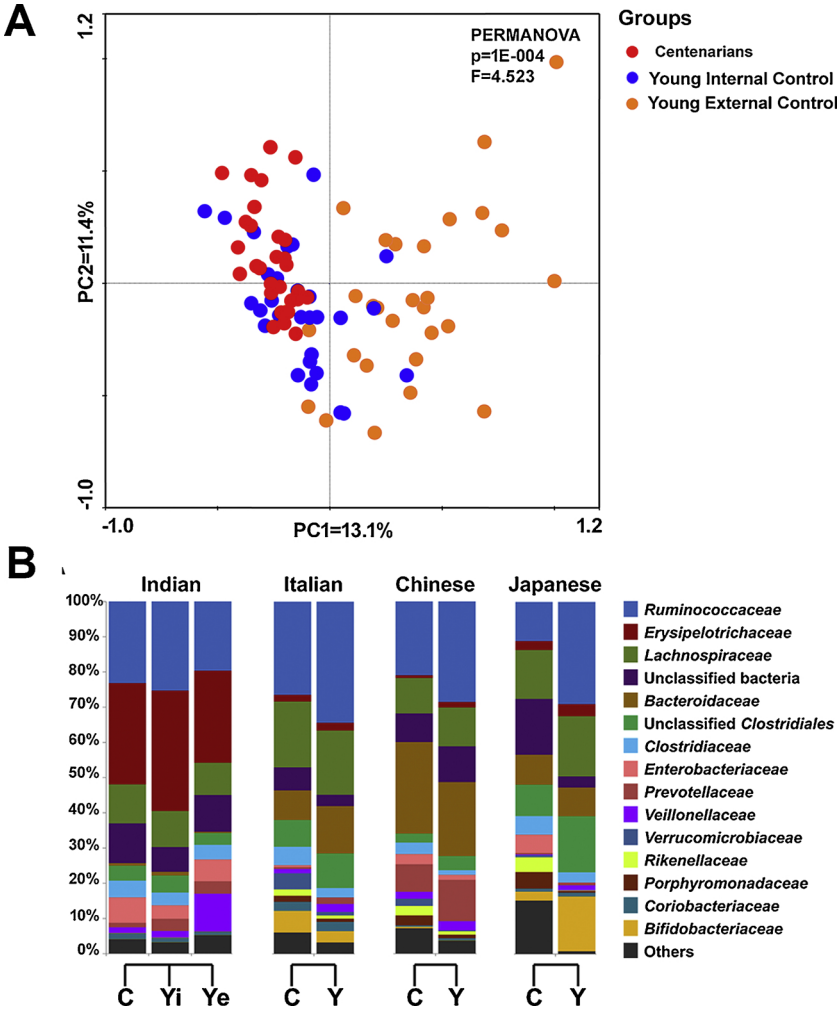


Fig. 2. Comparison of the gut microbiota structure of centenarians and young adults. (A) PCA biplot generated by using the relative abundance profile at the species level. PERMANOVA with 10,000 replicates using Bray-Curtis distances was performed and p-value is indicated in the plot. (B) Bar chart of the relative abundance (%) gut microbiota profiles at the family level of Indian, Italian, Japanese and Chinese centenarians (C) and young adults (Y). For Indian population young adults were enrolled from the same "longevity area" in which centenarians were enrolled (Yi, Young Internal control) and from a separated district with lower centenarians count (Ye, Young External control).

which showed high centenarians prevalence (7.0 and 4.9 centenarians/10,000 citizens, respectively) (Suppl. Table S1). Also, 30 young adults from the Imphal West district, with a lower centenarians prevalence (0.9 centenarian/10,000 citizens) were enrolled as an external control group (Table 1). Fecal microbiota was profiled using Illumina MiSeq sequencing targeting the V4-V5 region of bacterial 16S rRNA gene. Sequencing generated 9.5 million high-quality sequence reads with an average of 106,019 ± 62,005 sequences per subject and grouped them into 1213 OTUs at 97% identity. The gut microbiota structure (at different taxa levels) of centenarians and young adults, both from the longevity area (internal young control, Yi) and from the other Manipur district with low centenarian prevalence (external young control, Ye), was compared. According to the principal component analysis (PCA) using Bray-Curtis distance metrics based on the species level relative abundance profiles (Fig. 2A), the gut microbiota structure of centenarians clustered separately from both the young control groups (PERMANOVA, $p < 0.001$).

In the attempt of identifying a common signature of longevity across geography, gut microbiota data of 125 subjects, both centenarians and young adults, from 3 countries (Italy, Japan, and China) were retrieved from open databases (Table 1). Even if the comparison of gut microbiota structure from the data generated by different studies is generally biased by differences in DNA extraction methods, sequencing platforms, and reference database, primer coverage comparison (Suppl. Table S2) support the reliability of data compared in this study, with the exception of the families *Verrucomicrobiaceae* and *Bifidobacteriaceae*, which resulted not sufficiently covered by the primers used in our and Chinese studies.

We observed an unprecedented predominance of *Erysipelotrichaceae* in the gut microbiota of the Indian study population (> 3, log2 fold

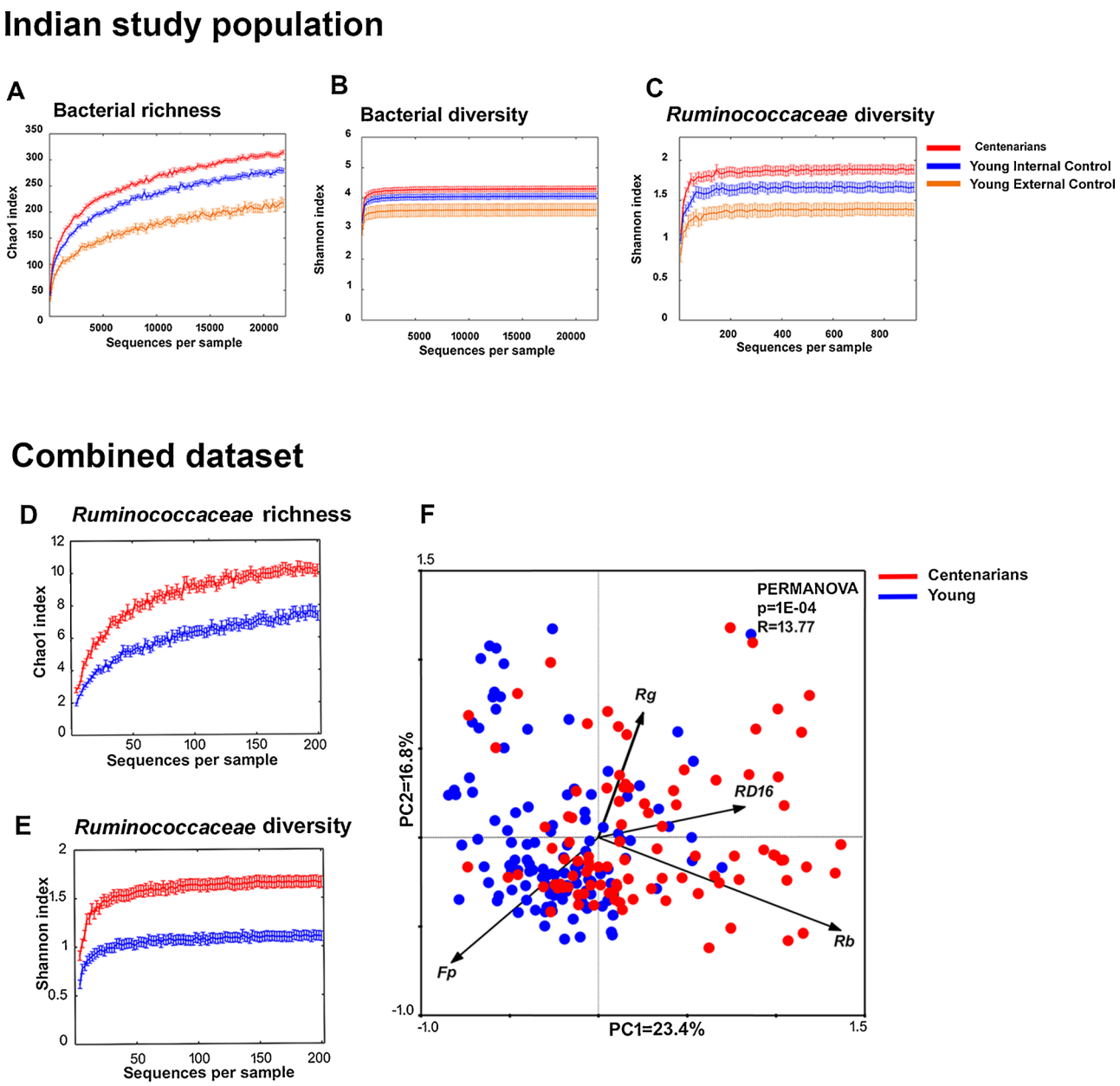


Fig. 3. Higher biodiversity of *Ruminococcaceae* in centenarians. Alpha diversity rarefaction curve for centenarians and young control groups in the Indian study population, showing bacterial species richness expressed as Chao1 index (A), bacterial diversity expressed as Shannon index (B), and *Ruminococcaceae* diversity expressed as Shannon index (C). Alpha diversity rarefaction curve showing *Ruminococcaceae* richness expressed as Chao1 index (D) and diversity expressed as Shannon index (E) in the centenarians and young adults using a combined dataset obtained from Indian, Italian, Japanese and Chines populations. PCA plotting of the species level OTUs belonging to *Ruminococcaceae* family showing the separation between centenarians and young adults irrespective of the country (F). The arrow indicates species contribution to the sample separation in the PCA space (Fp, *Faecalibacterium prausnitzii*; RD16, Unclassified *Ruminococcaceae* bacterium D16; Rb, *Ruminococcus bromii*; and Rg, *Ruminococcus gnavus)*.

difference; $p < 1 \times 10^{-14}$, BH corrected) in comparison to the gut microbiota of three other countries (Italy, Japan, and China) (Fig. 2B, Suppl. Table S3). Also, the gut microbiota of the Indian study population was enriched with *Enterobacteriaceae* and lactic acid bacteria (*Lactobacillaceae* and *Leuconostocaceae*) in comparison to Italian, Japanese and Chinese samples. On the contrary, members of the phylum Bacteroidetes, i.e., *Bacteroidaceae* and *Rikenellaceae,* were relatively lower in the Indian population. Interestingly, we could not see any significant difference in the relative abundance of *Ruminococcaceae* and *Eubacteriaceae* among the different countries study population. In term of species richness, Indian and Italian populations were higher than the Chinese and Japanese populations (Suppl. Fig. S1). However, we noticed an exceptional high bacterial diversity in the Italian population. The comparative differences in the structure of gut microbiota between the countries observed during this study are shown in the Suppl. Table S3.

Focusing on the differences between extremely old people and young adults, we found a higher species richness (Chao1 index) in Indian centenarians ($p < 0.001$, BH corrected) with respect to both the internal and external young control groups (Fig. 3A). We could observe a similar trend in Italian and Japanese datasets (Suppl. Fig. S1A). However, the significant variation in species richness was not reflected in the bacterial diversity (Shannon index) (Fig. 3B, Suppl. Fig. S1B). Moreover, an extremely high variation in bacterial richness and diversity among the centenarians across the four countries study groups was observed (Suppl. Fig. S2). At the family level, *Ruminococcaceae*

showed a mean relative abundance of 25% with a consistent non-significant variation across the four countries (Suppl. Table S3). A significantly higher *Ruminococcaceae* diversity (Shannon index calculated using the OTUs assigned to the family *Ruminococcaceae*) was detected in centenarians of all four countries when analyzed individually (Fig. 3C, Suppl. Fig. S1C). In the combined data set also reflected with significantly higher *Ruminococcaceae* richness (Chao1) (Fig. 3D) and *Ruminococcaceae* diversity (Fig. 3E) ($p < 0.005$, Wilcoxon test, BH corrected) in centenarians. To substantiate this observation, we performed an unsupervised PCA plotting based on Bray-Curtis distance metrics using the species level OTUs belonging to *Ruminococcaceae* family, which showed a separation between centenarians and young adults (PERMANOVA, $R = 13.77$, $p < 0.0001$), irrespective of the country (Fig. 3F). The biplot indicated enrichment in unclassified *Ruminococcaceae* D16 and *Ruminococcus bromii* in centenarians, while *Faecalibacterium prausnitzii* was more abundant in young adults. We detected similar trends in all the four datasets when analyzed separately (Suppl. Fig. S3).

To obtain a more detailed prediction of the key signature taxa that best differentiated between centenarians and young adults, we performed Random Forest analysis on genus level abundance profiles. The top 20 differentiating genera, arranged based on their mean decrease in accuracy (MDA) score, that best discriminates in the Indian study population and combined datasets of four countries together are shown in Fig. 4A and B, respectively. We identified three taxa (*Alistipes* within the family *Rikenellaceae*, *Akkermansia* within the family *Verrucomicrobiaceae,* and unclassified *Ruminococcoaceae* D16) as a key signature of longevity across different populations (top 3 discriminating taxa in the combined datasets) (Fig. 4B). These three signature taxa were present in the top 20 discriminating taxa in at least 3 out of 4 countries when analyzed separately (Suppl. Fig. S4), including India. Among the other differentiating taxa, we detected unclassified *Lachnospiraceae*, *Odoribacter*, *Holdemania*, *Shigella*, and *Yersinia* across the countries (Suppl. Fig. S4). Above results supported our aim of identifying common gut microbiota signatures of extreme aging. Statistically significant differences ($p < 0.01$, BH corrected) in the key differentiating taxa between centenarians and young adults are shown in Table 2 (combined datasets) and Suppl. Table S4 (Indian population) and Suppl. Table S5 (combined datasets, at species level). Among the differentiating taxa within the phylum Bacteroidetes, *Rikenellaceae* and *Porphyromonadaceae* were abundant in centenarians ($p = 0.0001$, BH corrected) (Table 2). However, we noticed a low presence of *Prevotellaceae* in the Indian centenarian ($p = 0.017$, BH corrected) (Suppl. Table S4). We further confirmed the low *Prevotellaceae* load in Indian centenarians by qPCR assay ($P < 0.01$, Students t-test, two-tailed) (Fig. 4C). Within the phylum Firmicutes, *Faecalibacterium* showed a significantly lower presence in centenarians, whereas the unclassified members of *Lachnospiraceae* and *Ruminococcaceae* had a higher abundance while considering the combined datasets (Table 2). In the Indian population, the members of *Peptostreptococcaceae* were in high abundance (Suppl. Table S4). Among the other phyla, *Pyramidobacter* (Synergistetes) were more abundant in centenarians ($p < 0.0001$, BH corrected) (Table 2). The qPCR assay allowed us to point out that both centenarians and young adults from the longevity area showed a significantly higher total bacterial load ($P < 0.001$, Students t-test, two-tailed) (Fig. 4D) than the young group from a non-longevity area, also showing low species richness and bacterial diversity.

To better highlight differences in the gut microbiota interactions between centenarians and young adults, we performed a co-occurrence network analysis using Spearman correlation coefficients ($r = 0.5$, $p = 0.001$) (Fig. 5). The network plot of gut microbiota at the genus level in the combined datasets generated three main co-occurrence modularity groups (CMG-I, CMG-II, and CMG-III). In centenarians, we observed a negative interaction network between *Alistipes* of CMG-I and CMG-II, and the co-occurrence of unclassified *Ruminococcaceae* D16 and Actinobacteria (*Bifidobacterium* and *Eggerthella*) within CMG-III (Fig. 5A). These features were not present in younger adults (Fig. 5B).

At the family level, we witnessed negative correlations ($r = -0.5$, $p = 0.001$) between common, potentially health promoting, intestinal symbionts (*Bifidobacteriaceae, Verrucomicrobiaceae, Rikenellaceae, Lachnospiraceae, Bacteroidaceae*, and *Porphyromonadaceae*) and potential pathobionts (*Enterobacteriaceae*, *Fusobacteriaceae*, *Erysipelotrichaceae*, and *Prevotellaceae*) (Suppl. Fig. S5). The negative interaction between the clustered taxa supported a bimodal distribution of gut bacteria observed in this study (Suppl. Fig. S6). We showed a shift in the bimodal distribution of some taxa between centenarians and young adults (Fig. 5C).

Finally, in order to look at the longevity phenomenon from the metabolic point of view, and in the attempt of identifying the fecal metabolic signature of longevity, we compared the metabolites present in the fecal extract obtained from the Indian population by LC-HRMS analysis. Out of 871 metabolites detected by LC−MS analysis, 109 compounds (12.5%) differed significantly between centenarians and young adults ($p < 0.05$, BH corrected). PCA analysis based on Bray-Curtis distance metrics using the fecal metabolic profiles separated ($p = 0.0001$, $F = 6.549$, PERMANOVA) the centenarian group from the two young control groups (Fig. 6A). The identity of the compounds that significantly differed was further assigned by comparing acquired MS/MS spectra with those in the mzCloud library (Suppl. Tables S6 and S7). Ten metabolites that significantly differed between the study groups (> 2 log2 fold change, $p < 0.0001$, BH corrected) are shown in a hierarchically clustered heatmap (Fig. 6B). Centenarians clustered together with a metabolite signature including higher levels of DL-3-Aminoisobutyric acid, *N*-Ethylglycine, gamma-Aminobutyric acid (GABA), Imidazoleacetic acid, Niridazole, and four unidentified compounds. On the contrary, internal young controls showed a significantly higher level of cyclohexanecarboxylic acid (> 4 log2 fold change, $p < 0.0001$, BH corrected). Among the unsaturated fatty acids, subjects from longevity villages had higher levels of Erucic acid, and lower levels of 13-cis,16-cis-Docosadienoic acid in feces (> 3 log2 fold difference, $p < 0.0001$, BH corrected) (Suppl. Table S6). Random Forest analysis showed that high β-Alanine in addition to GABA and DL-3-Aminoisobutyric acid were the most differentiating metabolites in Indian centenarians (> 18 mean decrease accuracy). Also, we observed an unidentified compound C1207 with a molecular weight of 294.9995 Da as a key differentiating metabolite (> 3 log2 fold change, $p < 0.0001$, BH corrected) in centenarians than both the control groups (Suppl. Table S6). Other metabolites that significantly differed between the study groups (> 2 log2 fold change, $p < 0.0001$, BH corrected) are listed in Suppl. Tables S6 and S7.

3. Discussion

The study presented here add a significant contribution to the field of aging research, by analyzing the microbiome of a peculiar population of long-living Indian subjects. We observed a common pattern in the gut microbiota composition among extremely old people in comparison to young adults from populations with a different genetic background and lifestyle habits. Progressive loss of biodiversity along with age has been regarded as a common feature across different population (Biagi et al., 2017; Wang et al., 2015). However, our analysis surprisingly allowed us to point out a higher species richness and biodiversity within the family *Ruminococcaceae* in centenarians, regardless of the nationality of the subjects. *Ruminococcaceae* are well known human gut symbionts, commonly reported among the dominant families in a healthy ecosystem, together with *Lachnospiraceae*, and include both cellulolytic and non-cellulolytic species; the latter evolved to degrade a large array of polysaccharides, such as glucans, mannans, resistant starches and simpler saccharides (La Reau and Suen, 2018). Therefore, an increase in *Ruminococcaceae* diversity might be related to higher metabolic plasticity and versatility of the gut microbiome of long-living individuals,

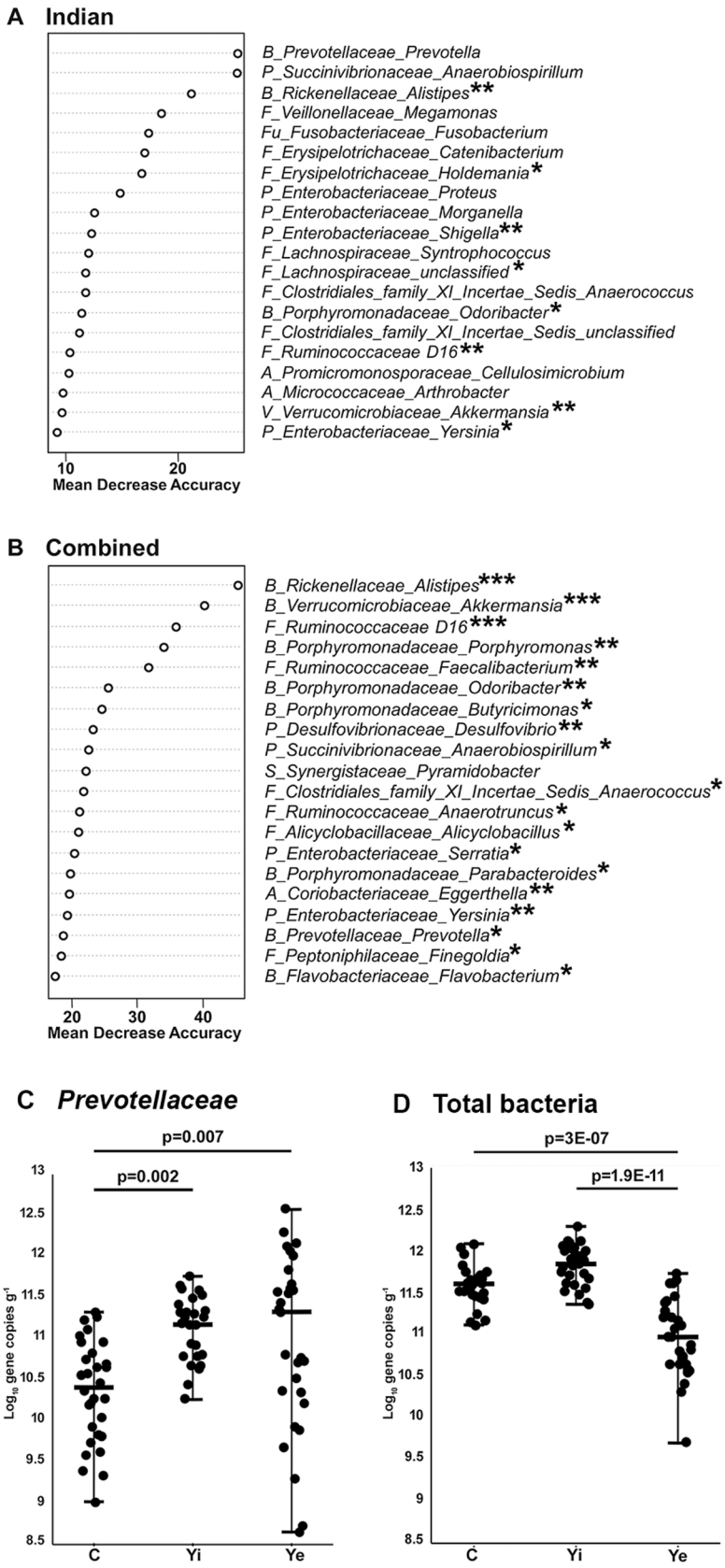


Fig. 4. Gut microbiota signature taxa of longevity. Top 20 signature taxa of longevity at genus level obtained by Random Forest analysis using the Indian study population only (A) and the combine datasets of four study populations (B). The number of the asterisk (*) in each taxon indicates a prediction of its presence in the top 20 discriminating taxa by Random Forest analysis in the study groups from four different countries when analyzed separately. (F: Firmicutes, B: Bacteroidetes, P: Proteobacteria, A: Actinobacteria, Fu: Fusobacteria, and V: Verrucomicrobia.). Absolute quantification of (C) *Prevotellaceae* and (D) total bacteria by qPCR assay in centenarians (C, n = 30), Young Internal controls (Yi, n = 30), and Young external controls (Ye, n = 30). Students t-test, two-tailed, was used to calculate the statistical significance (indicated in the plot).

regardless of the dietary habits of the single populations. Interestingly, among the *Ruminococcaceae*, the unclassified species *Ruminococcaceae* D16 emerged as a common longevity signature. This specific unclassified member was reported as the major butyrate producer via *but* gene among herbivorous and omnivorous animals (Vital et al., 2015). Butyrate is a fundamental metabolite for gut health as well as for the homeostasis of the immune system (Koh et al., 2016). Thus, the maintenance of high level of this metabolite in the gut of aging people

Table 2
Gut microbiota that significantly differed between the centenarian and young groups of the combined datasets.

Taxa	Mean Relative abundance (%)		Log2 fold change[a]	p-value[b] (BH corrected)
	Young	Centenarian		
Actinobacteria				
Coriobacteriaceae_Eggerthella	0.06	0.21	1.77	0.0082
Bacteroidetes				
Rikenellaceae	0.44	1.89	2.10	5.7E-07
Rikenellaceae_Alistipes	0.44	1.89	2.10	1.5E-06
Porphyromonadaceae	0.55	1.98	1.85	0.0005
Porphyromonadaceae_Parabacteroides	0.45	1.38	1.61	0.0047
Porphyromonadaceae_Porphyromonas	0.00	0.23	8.03	1.6 E-05
Porphyromonadaceae_Odoribacter	0.02	0.13	3.05	6.4E-05
Porphyromonadaceae_Butyricimonas	0.01	0.09	3.35	0.0082
Firmicutes				
Alicyclobacillaceae	0.00	0.30	8.44	0.0012
Alicyclobacillaceae_Alicyclobacillus	0.00	0.30	8.44	0.0027
Clostridiales_Family_XI_Incertae_Sedis	0.033	0.33	3.32	3.7E-05
Clostridiaceae_Finegoldia	0.00	0.03	2.75	0.0014
Ruminococcaceae	26.56	20.12	−0.40	0.0012
Ruminococcaceae_Faecalibacterium	16.84	10.20	−0.72	5.2E-05
*Ruminococcaceae_*unclassified D16	0.22	0.78	1.80	4.1E-05
Ruminococcaceae_Anaerotruncus	0.03	0.13	1.94	0.0002
Proteobacteria				
Enterobacteriaceae	1.71	3.85	1.17	0.0010
Desulfovibrionaceae	0.01	0.09	3.42	0.0005
Desulfovibrionaceae_Desulfovibrio	0.01	0.09	3.42	0.0009
Synergistetes				
Synergistaceae	0.00	0.08	11.56	0.0003
Synergistaceae_Pyramidobacter	0.00	0.08	11.56	0.0004
Verrumicrobio				
Verrucomicrobiaceae	0.25	2.13	3.11	2.4E-08
Verrucomicrobiaceae_Akkermansia	0.25	2.13	3.11	6.4E-08

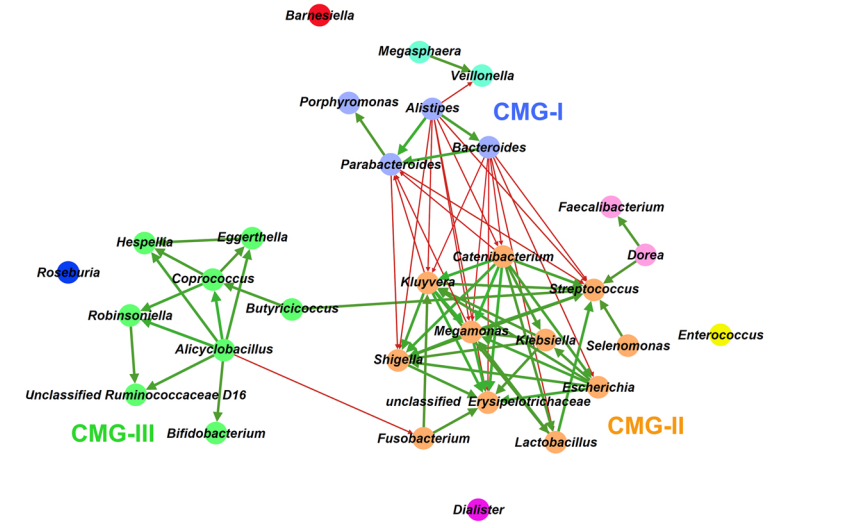
[a] Fold change in the relative abundance of taxa in centenarian in comparison to the young group.
[b] Significance of difference ($p < 0.01$) in the fold change expressed as Benjamini-Hochberg corrected p-value.

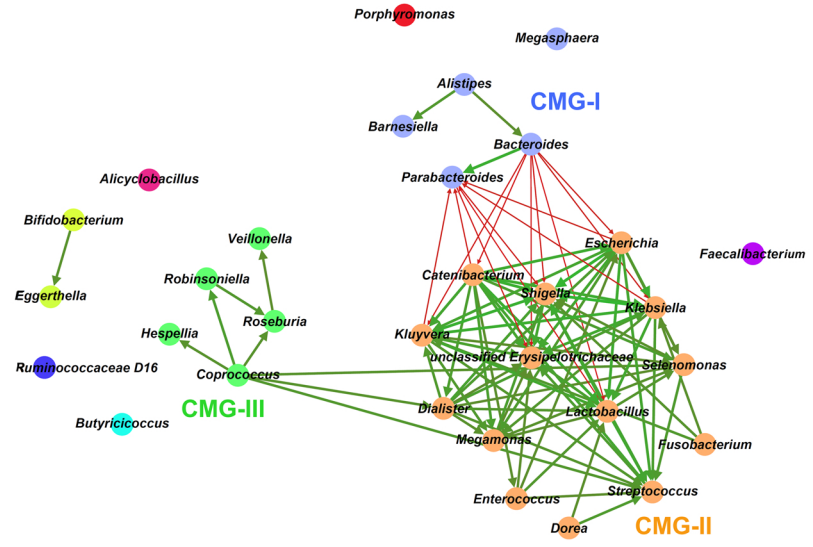
might contribute in preventing inflammation and immunosenescence. We could not provide direct evidence for this hypothesis, but we reported a higher level of butyrate derivatives (DL-3-amino isobutyric acid and GABA) in the centenarian fecal samples, the role of which is worth exploring in future studies.

Among the other putative butyrate producing members of Firmicutes phylum, we were able to confirm the already reported age-related decline in the abundance of *Faecalibacterium* (Biagi et al., 2013; Mueller et al., 2006; Odamaki et al., 2016; Rajilić-Stojanović and de Vos, 2014; Wang et al., 2015). With our study confirming such a considerable amount of literature, the high abundance of *Faecalibacterium* in young adults could be indicated as a microbiota signature of youthfulness. The reduction of *Faecalibacterium* has been linked to inflammatory disorders (Biagi et al., 2013, 2016; Claesson et al., 2012) and, together with an increase in *Enterobacteriaceae*, has been associated with frailty in elderly (Claesson et al., 2012; Ottaviani et al., 2011). As a peculiarity of the Indian population considered in the present study, we reported higher abundance of carnivores-associated butyrate producers, namely *Peptostreptococcacaee* (via butyrate kinase) and *Fusobacterium* (via amino acid fermentation) (Vital et al., 2015) in centenarians, a feature that might be linked to the traditional dietary habits of Naga community, including different type of heavy meat, as well as frogs, dogs and insect larvae, the consumption of which is still in practice in the rural study population (Suppl. Results). Moreover, the Naga community is well known for the fierce headhunting (Thong, 2012).

Similarly, within the Bacteroidetes phylum, *Rikenellaceae* (*Alistipes*) and *Porphyromonaceae* (*Parabacteroides*, *Odoribacter*, *Porphyromonas*), known butyrate producers via amino acid fermentation (Vital et al., 2015), were also reported as increasing in centenarians, regardless of nationality, sustaining a model in which the gut microbiota of long-living people might be enriched in the variety of metabolic pathways for butyrate production. This might increase the possibility of the longevity-associated microbiome to produce the necessary amount of anti-inflammatory, immune modulating butyrate, even throughout the lifestyle and dietary changes that commonly occur during aging, because of the loss of teeth or the altered threshold for taste and smell (Biagi et al., 2012). We observed a decrease in *Prevotella* species richness in Indian centenarians. Our results are in contrast to previous observation of higher abundance of *Prevotella* in a longevity village in Korea (Park et al., 2015). The established anti-correlation of *Prevotella* with the other members of the phylum Bacteroidetes (Ley, 2016) was noticeable in our study. Among the members of the phylum Bacteroidetes, *Prevotella* shows the lowest genetic potential of producing short chain fatty acids (SCFA) and carbohydrate active enzymes (CAZymes) (El Kaoutari et al., 2013). For this reason, this genus might be among the least useful in a model in which versatility in polysaccharide degradation and plasticity in SCFA production are regarded as winning traits. Moreover, *Prevotella* abundance in the gut is mostly linked with chronic inflammation (Dillon et al., 2016; Ley, 2016; Scher et al., 2013), and the countries in which a *Prevotella*-dominated gut microbiota is often retrieved, i.e. African countries, Venezuela, India, Indonesia, and Thailand of the tropical region (Bhute et al., 2016; Dehingia et al., 2015; Nakayama et al., 2015; Yatsunenko et al., 2012), are reported as having a low healthy life expectancy (Vos et al., 2016). This sustains the hypothesis that the negative association of *Prevotella* with longevity, and the negative co-occurrence of *Prevotella* with other longevity signatures (*Alistipes*, *Parabacteroides*, *Odoribacter,* and *Porphyromonas*) may enhance the chances of healthy aging, at least within the Indian population.

Although high abundances of *Prevotella* are known to be common in the Indian microbiota (Bhute et al., 2016; Dehingia et al., 2015), a peculiar trait of the geographically isolated endogamous Indian population considered in this study had *Erysipelotrichaceae* dominance.





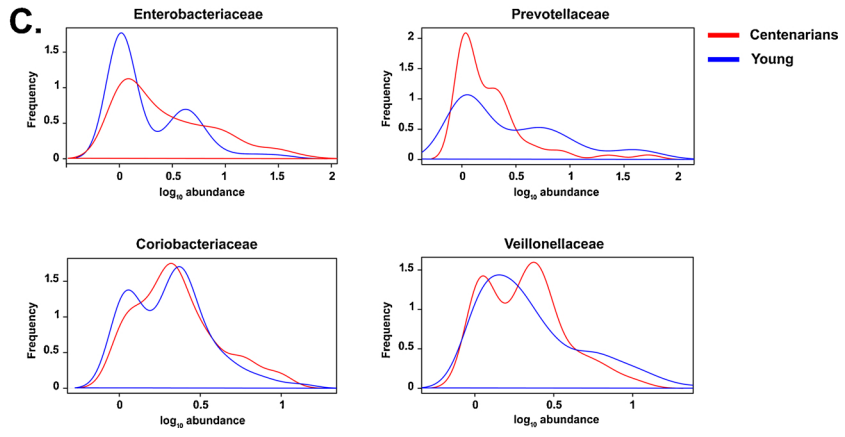


Fig. 5. Differences in the co-occurrence network and a shift in the bimodal distribution of gut microbiota. Co-occurrence network based on Spearman correlation coefficients ($r \geq 0.5$, $p \leq 0.001$) showing the difference in the interaction of gut microbiota at genus level in centenarians (A) and young adults (B) of the combined datasets. Green lines indicate positive co-occurrence, red line indicates negative interaction, and arrows indicate the direction of each interaction. The three Co-occurrence Modularity Groups (CMG-I, CMG-II, and CMG-III) of positively co-occurring taxa are positioned in the network using a color code (light blue, orange and light green respectively). (C) Shifts in the bimodal distribution of *Enterobacteriaceae*, *Prevotellaceae*, *Coriobacteriaceae,* and *Veillonellaceae* in centenarians (n = 98, red) in comparison to the young groups (n = 87, blue) of the combined datasets.

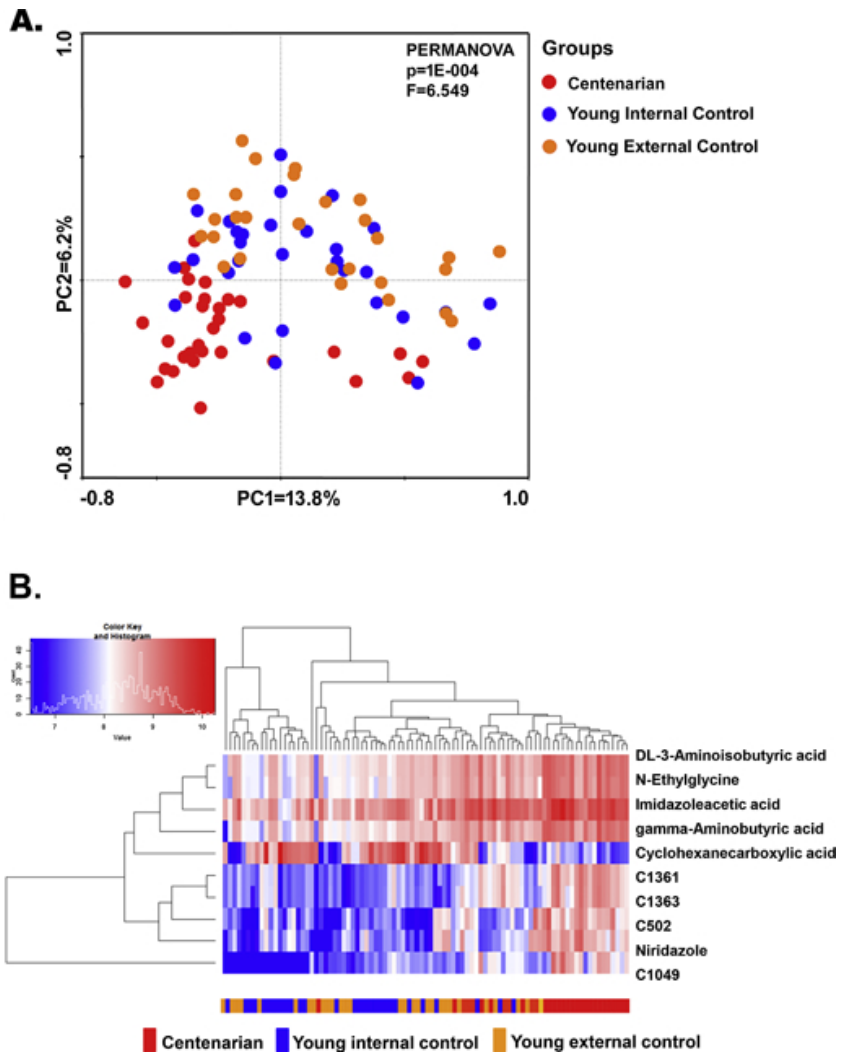


Fig. 6. Comparison of the fecal metabolite profiles of centenarians and young adults.
(A) PCA plot generated by using LC–MS data of fecal metabolites of centenarians (red) and young adults (blue and gold). PERMANOVA with 10,000 replicates using Bray-Curtis distances was calculated and the p-value is indicated in the plot. (B) Hierarchically clustered heat map showing the clustering of centenarians based on ten differentiating fecal metabolites with more than two-fold change (log2 fold change) in the composition with a significance of $p < 0.0001$ (BH corrected). The levels of metabolites are shown with red and blue color gradient.

Erysipelotrichaceae are known to increase in high-fat diets and to be involved in host lipid metabolism (Kaakoush, 2015). The association of *Erysipelotrichaceae* with inflammation-related disorders and colorectal cancer is an alarm for the study population because of the high incidence of colorectal cancer in this region (Phukan et al., 2006).

Our study population did not show a significant variation in the expected health-promoting, longevity-associated bacteria *Christensenellaceae* (Biagi et al., 2016) and *Bifidobacterium* (Drago et al., 2012). Instead, *Desulfovibrio* (bacteria able to utilize sulfur-containing molecules) (Rabus et al., 2015) was observed as an extreme aging trait in the enrolled Indian subjects. Promotion of *Akkermansia* and *Alistipes* by the sulfur compounds (Wang et al., 2015) and production of sulfonolipids (the bioactive molecules that regulate cell growth, apoptosis, adhesion, cell migration and intracellular trafficking in diverse eukaryotes) by *Alistipes* and *Odoribacter* (Walker et al., 2017) suggest to investigate the role of sulfur compounds in longevity.

We observed changes in the co-occurrence network of some intestinal taxa. For instance, a shift in the interaction among members of *Veillonellaceae* (the predominant member in the small intestine) could indicate a change in the small intestine microbiota of centenarians (El Aidy et al., 2015; Zoetendal et al., 2012), a body environment difficult to explore in healthy people but worth of interest in ageing research because of the reduced intestinal motility commonly showed by elderly (Biagi et al., 2012). The shift in the bistable distribution (bimodality) of *Enterobacteriaceae* and *Veillonellaceae* reflects a change in the negative interaction between the co-occurrence network groups. This shift in the co-occurrence network and bimodal distribution might pave the way for the identification of targets for gut microbiota modulation for promoting healthy aging through dietary intervention.

A final remark concerns our attempt at identifying a fecal metabolic signature of longevity, since this is, to our knowledge, the first study reporting such evidences. A higher level of compounds with neuro-pharmacological properties like GABA (Mittal et al., 2017) and Imidazole 4-acetic acid (Tunnicliff, 1998), as well as azole compounds with antifungal and amebicidal activity (Lamb et al., 2015), were found in Indian centenarians. Also, centenarians showed lower levels of cyclohexanecarboxylic acid in the fecal extract, suggesting that features present in centenarians gut bacteria might degrade this environmental contaminant but not in younger people. It is tempting to hypothesize that these features, which are surely worth exploring further in future studies, might have supported health maintenance and life prolongation. Also, this is an indication that the gut microbiome of long-living individuals, both humans and animals, might be a good place to look at for finding new biological functions useful for health maintenance during aging.

4. Conclusions

We identified common longevity signatures of gut bacteria across four different countries (India, Italy, Japan and China). Also, the first study reporting the fecal metabolite signatures of longevity from the endogamous Indian study population. Data provided by our study might pave the way to develop biomarkers for healthy aging. Our findings related to the high biodiversity of *Ruminococcocaceae* in the rural centenarian sustain the hypothesis of the biodiversity loss due to urbanization, modern foods, and lifestyle habits, which are known to impact human health in westernized countries. Moreover, a pervasive change observed in the gut microbial ecology during extreme aging will allow us to identify targets for gut microbiota modulation for promoting healthy aging through dietary intervention, which has been among the aim of several international research programs to date.

5. Materials and methods

5.1. Sample source

A list of elector data was obtained from the Election Commission Office (ECO) of Manipur, India and subjects above the age of 95 years were segregated. During the process, geographical regions with higher prevalence of centenarian and > 95 years old subjects were identified, i.e. Chandel and Senapati districts, Manipur, India (Suppl. Table S1). Aiming at validating the data and collecting more details, a survey was conducted within the population before samples collection. A questionnaire was administered to obtain information about age, dietary habits, physical condition and health status from the identified subjects. The questionnaire recalled the marital status of the centenarians and their children age during world war-II (1944) to support the age. Also, cross verified the age of family members during the sampling. The subjects who took medical care or received antibiotic treatment within the last six months before the fecal sample collection were excluded from the study. The fecal samples from 30 healthy centenarians and 30 young subjects were collected from the identified longevity regions. Also, we collected fecal samples from 30 young subjects from a population in which the centenarians prevalence is lower than within the longevity region (Imphal West, Manipur, India) and enrolled them as an external young group for comparison (Table 1). Subjects were asked to self-collect fecal samples and provide them to the personnel. Samples were frozen immediately and transported to the laboratory within 12 h of collection, then kept at −80 °C until further analysis. In order to identify a common signature of longevity, we also retrieved the gut microbiota data of centenarians and young subjects from researches conducted in three other countries (Italy, Japan, and China); publicly available sequences were downloaded from MG-RAST (project ID 17761 (Biagi et al., 2016)) and NCBI SRA (accession numbers SRP076167 (Kong et al., 2016) and DRA004160 (Odamaki et al., 2016)).

5.2. Total bacterial DNA extraction

The bacterial DNA from the fecal samples was extracted sequentially by repeated bead beating (RBB) and enzymatic lysis. Briefly, 0.1 mm zirconia beads and QIAmp DNA kit were used for the RBB method, as described by Salonen et al. (2010). Additionally, after RBB extraction, the residual pellet was washed twice with 0.5 M NaCl and deionized MilliQ water, further vortexed with 1 mL petroleum ether: hexane (1:1) at room temperature. After centrifugation, the pellet was subjected to an enzymatic DNA extraction using the method-II described by Keisam et al.(Keisam et al., 2016) without the addition of lyticase. The DNA extracted by both methods were pooled together and quantified fluorometrically using Qubit 2.0 and Qubit® dsDNA BR Assay Kit (Invitrogen). DNA was stored at −20 °C for further use. The laboratory prepared reagents and DNA extraction kit were tested for any DNA contaminant by checking on the blank water sample.

5.3. Barcoded illumina MiSeq amplicon sequencing

The eubacterial V4-V5 region of the 16S rRNA gene was targeted for the paired-end amplicon sequencing by using primers F563–577 (5′-AYTGGGYDTAAAGNG-3′) and R924–907 (5′-CCGTCAATTCMTTTRAGT-3′) (Claesson et al., 2010; Romi et al., 2015). The preparation of metagenomic DNA templates and strategy for sample multiplexing was followed as described earlier (Romi et al., 2015). The amplified products were purified using QIAquick gel extraction kit (QIAGEN) as per the manufacturer's instructions and then quantified fluorometrically. The purified amplicons were finally pooled in equimolar proportion, multiplexed and subjected to Illumina MiSeq sequencing reaction (Xcelris Genomics, Ahmedabad, India). The generated sequence data were analyzed by using MG-RAST (Meyer et al., 2008) and QIIME v1.8.0 bioinformatics pipelines (Caporaso et al., 2010) and taxonomically assigned using Silva database (release version SILVA 119). Chimera checking and removal of chimeric sequences were performed using the ChimeraSlayer algorithm. The quality-filtered sequences were assigned to operational taxonomic units (OTUs) at 97% sequence similarity and clustered into four different taxonomic levels (phylum, family, genus, and species). The unclassified and sequences of Eukaryota origin were removed from the OTU table before performing the statistical analysis. The quality-filtered sequences corresponding to the Indian study population was uploaded to MG-RAST (http://metagenomics.anl.gov/linkin.cgi?project=16687).

5.4. qPCR analysis

The total bacterial load in the fecal samples was quantified by analyzing the extracted DNA by SYBR based qPCR in triplicates using a ABI 7500 instrument (Life Technologies, USA). The primers targeting the V3 region of the bacterial 16S rRNA gene (338f: 5′-ACTCCTACGG GAGGCAGCAG-3′) and 518r: 5′-ATTACCGCGGCTGCTGG-3′) was used. A final reaction volume of 20 µl contained 10 µl of 2X SYBR mix (EXPRESS SYBR® GreenER™ qPCR Supermix, Invitrogen) was used for the qPCR assay. The primer concentration was maintained at 0.2 µM, and the reaction was subjected to the following thermal conditions: 50 °C for 2 min, 95 °C for 5 min, 40 cycles of 95 °C for 15 s, 62 °C for the 30 s and 68 °C for 45 s. A standard curve was generated by using 10^1 -10^8 copies of 16S rRNA gene of *Lactobacillus plantarum* ATCC 8014. Similarly, qPCR for the quantification of *Prevotellaceae* was performed as described above using the primers Prev F: 5′- GGTTCTGAGAGGAA GGTCCCC-3′ and Prev F: 5′- GAGTTTGATCCTGGCTCAG-3′ (Bekele et al., 2010). The primers had a coverage of 78% of *Prevotellaceae* and less than 1% coverage of other members of the phylum Bacteroidetes in ARB-SILVA database. The reaction was carried out in the following condition: 50 °C for 2 min, 95 °C for 5 min, 40 cycles of 95 °C for 10 s, 58.5 °C for the 20 s and 68 °C for 15 s. A standard curve was generated by using 10^1 -10^8 copies of 16S rRNA from *Prevotella copri* DSM-18205 for quantification of *Prevotellaceae*. In all reactions, a melt curve analysis was performed to detect non-specific amplification.

5.5. Metabolic profiling by LC-HRMS analysis

500 mg of feces were homogenised in 8 mL of ice-cold extraction solvent (1:1 v/v acetonitrile/water), vortexed for 5 min, and centrifuged at 18,000 *g* for 10 min at 4 °C. The supernatant was stored overnight at -80 °C and centrifuged again at 18,000 *g* for 10 min at 4 °C. The carefully collected supernatant was passed through a 0.2 µm syringe filter (PTFE) and subjected to LC–MS analysis (Ramakrishnan et al., 2016). The LC–MS data was acquired with a Dionex Ultimate 3000 ultrahigh performance Liquid chromatography (UHPLC) coupled to a Q-Exactive Orbitrap (Thermo Fisher Scientific) for untargeted metabolite profiling of fecal samples (C-CAMP MS Facility, Bengaluru). The chromatography was performed on a hydrophilic interaction liquid chromatography column (HILIC, 5µ, 150 mm x 4.6 mm, Phenomenex

Luna) maintained at 40 °C with a flow rate of 0.4 mL/min. Mobile phase of 5 mM ammonium acetate in water (phase A) and 5 mM ammonium acetate in water with acetonitrile in a ratio of 1:9 (phase B) were used to run a gradient as follows: 0–2 min:100% B, 2–15 min:100–90% B, 15–25 min: 90–80% B, 25–30 min: 80–75% B, 30–35 min: 75–20% B, 35–40 min: 20–0% B, 40–45 min: 0% B, 45–45.1 m in. 0–100% B, 45.1–55 min:100% B was formed. The instrument was set up for data acquisition in the full scan/data-dependent scan (FS/DDS) mode in a mass range of 70 to 750 *m/z*, alternating between MS and MS/MS scans. The resolution for the full scan was set from 70,000 to 140,000. MS operating system was run in the negative ionization mode with a spray voltage of 2500 V, a vaporizer temperature of 320 °C, the sheath gas flow rate of 40 arbitrary units, and auxiliary gas flow rate of 10 arbitrary units. Pooled samples were used as quality checks (QCs) for verifying the robustness of the method. Also, spiked taurocholate was used as an internal standard and later used for intensity normalization of the data. For data analysis, raw data files were imported into SIEVE 2.2 for component extraction and generation of peak list, and the Human Metabolome Data Base/Kyoto Encyclopedia of Genes and Genomes (HMDB/KEGG) were used for possible identification of the compounds. The identity of the compounds was further fixed by analyzing the spectral data from acquired MS/MS scans against entries in the spectral database mzCloud.

5.6. Bioinformatics and statistical analysis

The relative abundance of the filtered OTUs at four different taxonomic levels (phylum, family, genus, and species) were normalized by log transformation (log $x_{i\ +}$ 1) before performing statistical analysis. The difference in overall gut microbial community structure between centenarians and young groups was analyzed by an unsupervised principal component analysis (PCA) clustering of species-level OTUs performed using Canoco software v4.52 (Wageningen University, The Netherlands). The relative abundance data of species-level OTUs was plotted using Bray-Curtis distance metrics (Fig. 2A). PERMANOVA test was conducted to calculate the significance of the difference between the groups with 10,000 permutations using Bray-Curtis distances in PAST v3.08. The average relative abundance of taxa at family level assigned to the study groups (centenarian and young adults) were extracted for the four countries and the abundance were made into bar chart (Fig. 2B). Wilcoxon test was conducted on the log transformed data using "svDialogs" in R package (v3.1.3) and the p-value was corrected with "Benjamini-Hochberg" (BH) to detect the significance of the difference in the abundance of taxa between centenarians and young groups. To calculate the alpha diversity and generation of rarefaction curves, the species OTU table was rarefied at a depth range of 50-20,000 sequences. The alpha diversity metrics (Chao1 and Shannon index) were computed for centenarian and young adults for each country separately and for the combined data set (QIIME: alphadiversity.py). Any significant difference in the alpha diversity indices between groups were calculated using compare_alpha_diversity.py script in QIIME (Figs. 3A–C and S1 and S2). The OTUs belonging to *Ruminococcaceae* were segregated by Perl based script for calculating the *Ruminococcaceae* richness and diversity (Fig. 3D and E), used Bray-Curtis distance metrics for plotting the PCA for individual country separately and combined dataset, and the PERMANOVA test was conducted to calculate the significance of the difference between the groups with 10,000 permutations using Bray-Curtis distances (Figs. 3F and S3). Similarly, the quality filtered, normalized fecal metabolite data generated by LC—MS analysis was log-transformed (log $x_{i\ +}$ 1) and used for plotting the PCA using Bray-Curtis distance metrics. PERMANOVA was used to visualize any separation forming among groups with 10,000 permutations using Bray-Curtis distances, and the significance of the difference in the metabolite level was analysed by Wilcoxon test and expressed as BH corrected p-values (Fig. 6A). A hierarchically clustered heat map of log2 fold change in the data was visualized using R library "gplots" using custom made R scripts with a color key representing the intensity of the value (Fig. 6B). The top taxa at genus level and fecal metabolites that best differentiates between the centenarians and the young group were visualized with Random Forest (RF) analysis. The data were log transformed before being used as features for RF model. The RF plot was generated by using "random forest" package with 10,000 trees in R. Top 20 discrimiating taxa or metabolites between the study groups based on the mean decrease of accuracy was plotted (Figs. 4A, B and S4). The qPCR data on absolute quantification of total bacterial and *Prevotellaceae* load were log transformed (log10 gene copies per g of fecal sample) and plotted using Dell Statistica software v13 (Fig. 4C). The statistical significance of difference between centenarian and young adults was obtained by Student's t-test, two tailed. For microbial interaction network, the statistical correlation was calculated from the relative abundance at family and genus level using the Spearman's ranked correlation coefficient in R ("i-graph" package). To get a better visualization of the interaction, we maintained an average relative abundance cut-off of 0.1% at all taxa level. The Spearman's ranked correlation coefficient was computed at a cut-off of r = 0.5 and a p-value of 0.001. Taxa with at least 0.1% relative abundance in at least 30% of the samples in each group were plotted. The network visualization was performed in "Gephi" from the "graphml" file generated by using R scripts. Three co-occurrence modularity groups (CMG-I to CMG-III) with strongest positive correlation were identified in genus level co-occurrence network, and the differences in the network between the centenarian and young adults were compared in the combined dataset (Figs. 5A, B and S5). The bimodal distribution was also drawn using R script based analysis. The relative abundance data at family level was log 10 transformed and the Kernel density plots was generated to visualise the difference in bimodal distribution between the centenarian and young adults in the combined data set (Figs. 5C, S6).

Ethics approval and consent to participate

The study protocol was approved by the Institutional Ethical Committee (IEC) of Institute of Bioresources and Sustainable Development, Imphal, Manipur with ethical approval number IBSD/IEC/2018/002. We followed the standard operating procedures of the Indian Council of Medical Research (ICMR) guidelines (www.icmr.nic.in/ethics_SOP.pdf). Informed consent was obtained from all the participants.

Competing interests

The authors declare that they have no competing interests.

Availability of data and material

The sequence datasets supporting the conclusions of this article are available in MG-RAST (http://metagenomics.anl.gov/linkin.cgi?project=16687).

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Authors' contributions

KJ, NT, and GA conceptualized and designed the study. NT recruited the subject and performed the experiments. RKL supported for data acquisition and R scripts. Imrat supported for qPCR analysis; PR fixed the identity of the fecal metabolites by MS/MS spectra analysis; MCA

supported the anthropological data; KJ, NT, and SK performed the statistical data analyses. KJ and EB critically interpreted the data. KJ, EB, and NT wrote the manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.mad.2019.02.001.

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