Gut microbiota composition in Himalayan and Andean populations and its relationship with diet, lifestyle and adaptation to the high-altitude environment

Andrea Quagliariello[1#], Monica Di Paola[2#], Sara De Fanti[3], Guido Alberto Gnecchi-Ruscone[3±], Lucia Martinez-Priego[4], David Pérez-Villaroya[4], Mingma G. Sherpa[5], Phurba T. Sherpa[5], Giorgio Marinelli[6], Luca Natali[6], Marco Di Marcello[6], Davide Peluzzi[6], Patrizia Di Cosimo[7], Giuseppe D'Auria[4,8], Davide Pettener[3], Marco Sazzini[3§], Donata Luiselli [9*§] & Carlotta De Filippo[10*§]

1) Human Microbiome Unit, Bambino Gesù Children's Hospital and Research Institute, IRCCS, Rome, Italy

2) Dept. of Biology, University of Florence, Sesto Fiorentino (Florence), Italy

3) Laboratory of Molecular Anthropology & Centre for Genome Biology, Dept. of Biological, Geological and Environmental Sciences, University of Bologna, Italy
e-mail: marco.sazzini2@unibo.it

4) Sequencing & Bioinformatics Service, Fundación para el Fomento de la Investigación Sanitaria y Biomédica de la Comunidad Valenciana (FISABIO), Valencia, Spain

5) Mount Everest Summitter's Club, Rolwaling, Dolakha, Nepal

6) Explora Nunaat International, Montorio al Vomano, Teramo, Italy

7) Takesi Project, University of Bologna, Italy

8) Centro de Investigación Biomédica en Red en Epidemiología y Salud Pública (CIBERESP), Valencia, Spain

9) Department of Cultural Heritage, University of Bologna, Italy
e-mail: donata.luiselli@unibo.it

10) Institute of Biology and Agrarian Biotechnology (IBBA), National Research Council (CNR), Pisa, Italy
e-mail: carlotta.defilippo@ibba.cnr.it

Summary - *Human populations living at high altitude evolved a number of biological adjustments to cope with a challenging environment characterised especially by reduced oxygen availability and limited nutritional resources. This condition may also affect their gut microbiota composition. Here, we explored the impact of exposure to such selective pressures on human gut microbiota by considering different ethnic groups living at variable degrees of altitude: the high-altitude Sherpa and low-altitude Tamang populations from Nepal, the high-altitude Aymara population from Bolivia, as well as a low-altitude cohort of European ancestry, used as control. We thus observed microbial profiles common to the Sherpa and Aymara, but absent in the low-altitude cohorts, which may contribute to the achievement of adaptation to high-altitude lifestyle and nutritional conditions. The collected evidences suggest that microbial signatures associated to these rural populations may enhance metabolic functions able to supply essential compounds useful for the host to cope with high altitude-related physiological changes and energy demand. Therefore, these results add another valuable piece of the puzzle to the understanding of the beneficial effects of symbiosis between microbes and their human host even from an evolutionary perspective.*

Keywords - *Human adaptive evolution, Host-microbe coevolution, High-altitude adaptation, Gut microbiota, Dietary habits.*

[§] Corresponding authors.
[±] Current address: *Department of Archaeogenetics, Max Planck Institute for the Science of Human History, Jena, Germany*
[#] These authors contributed equally.
[*] These authors share joint senior authorship.

Introduction

Over the last 100,000 years, modern humans have been exposed to environmental conditions different from those experienced by their African ancestors and characterized by a wide range of nutritional and climate backgrounds, which have left appreciable marks on their genome (Fumagalli & Sironi, 2014; Sazzini *et al.*, 2014; Quagliariello *et al.*, 2017). This is confirmed by several studies that found more evidence for the action of natural selection on non-African populations than on African ones (Kayser *et al.*, 2003; Akey *et al.*, 2004; Storz *et al.*, 2004). For instance, some of the most outstanding selective pressures acting on the human genome have been proved to be those imposed by high altitude, and especially by hypobaric hypoxia. In particular, human populations from the Himalayas, such as Tibetans and Sherpa, and to a lesser extent those from the Andean cordillera (e.g. Aymara and Quechua), are known to be biologically adapted to a hypoxic environment because of their respectively long and medium time exposure to high-altitude conditions (Beall, 2007; Gilbert-Kawai *et al.*, 2014). However, the genetic bases of their complex adaptive phenotypes are far from being completely elucidated (Beall *et al.*, 2010; Gnecchi-Ruscone *et al.*, 2018; Simonson *et al.*, 2010; Xu *et al.*, 2011; Yi *et al.*, 2010) and other, non-genetic, factors are supposed to contribute to their successful adaptation to this extreme environment.

The human gut constitutes an important ecological niche for a variety of symbiotic microorganisms, namely the microbiota, which tuned out to influence several host physiological processes. The relation between the microbiota and their respective host is so profound that recent evolutionary theories proposed to consider it as holobionts (Rosenberg & Zilber-Rosenberg, 2018). Interestingly, gut microbial communities provide many biological functions, mainly related to host metabolism (Wu *et al.*, 2015) and immune system regulation (Wikoff *et al.*, 2009), thus having the potential to exert remarkable effects on host evolution and adaptation to new environmental conditions. Indeed, comparison of modern human microbiota ecology with that of ancient populations and African apes highlighted how gut bacterial communities have co-evolved in response to the major changes occurred during human evolution (Adler *et al.*, 2013; Warinner *et al.*, 2014; Moeller *et al.*, 2016).

In particular, the effect of high-altitude exposure on gut microbiota has been investigated so far especially in populations from different geographic locations across the Tibetan Plateau (Li & Zhao, 2015; Lan *et al.*, 2017). Nevertheless, the recently attested East-West gradient of admixture between Tibetan and low-altitude East Asian groups (Gnecchi-Ruscone *et al.*, 2017; Jeong *et al.*, 2017) may account, at least partially, for the differences in microbiota composition observed among the examined populations. Therefore, this has the potential to confound the identification of peculiar microbial profiles that may have played a remarkable role during the evolutionary history of high-altitude populations by facilitating their adaptation to such a harsh, energetically demanding and nutritionally limited environment.

To overcome this issue and to identify putative adaptive microbial signatures useful to cope with high-altitude stresses, thus not only associated to the individuals' ancestry, in the present study, we evaluated the gut microbiota composition of a high-altitude Sherpa population from Nepal, which has been recently proved to do not have received recent gene flow from neither East Asian nor South Asian low-altitude groups (Gnecchi-Ruscone *et al.*, 2017). We then compared their gut microbiota with that of another Nepalese Tibeto-Burman ethnic group (i.e. the Tamangs), who likely share an ancient common origin with the Sherpa, but who have originated from a different branch of the ancestral Tibeto-Burman gene pool and spent most of their evolutionary history at low altitude (Gnecchi-Ruscone *et al.*, 2017). Moreover, to validate the identified microbial signatures that are plausibly involved in high-altitude adaptation, we also examined samples from populations with completely different genomic backgrounds with respect to the

Nepalese ones. Accordingly, we studied the high-altitude Aymara population living in the South Yungas Region/Altiplano of La Paz (Bolivia) and a low-altitude cohort of individuals with European ancestry, as a control group.

Results

Characterization of the studied populations

In order to identify gut microbial signatures specific of Himalayan and Andean populations and to evaluate the impact of prolonged exposure to extreme conditions (i.e. high altitude and the related restriction of nutritional resources) on gut microbiota, we considered the Sherpa and Aymara populations living in two typical high-altitude geographical areas, such as the southern slopes of the Himalayan mountain range (Nepal) and the Bolivian Andes, respectively, and we compared them with low-altitude populations from Nepal (i.e. Tamangs) and Europe (Figs. 1A-C).

All individuals were selected because highly representative of their populations of origins according to their genomic backgrounds (Gnecchi-Ruscone *et al.*, 2017, 2019). In particular, we previously proved that the studied Sherpa community is genetically adapted to high altitude (Gnecchi-Ruscone *et al.*, 2018) and did not have received recent gene flow from neither East Asian nor South Asian low-altitude populations (Gnecchi-Ruscone *et al.*, 2017). Conversely, Tamangs live at low-altitude and share a common Tibeto-Burman genomic ancestry with the Sherpa, but did not evolved high-altitude genetic adaptation (Gnecchi-Ruscone *et al.*, 2017). We then considered the Aymara population living in Bolivia, a geographically distant area with respect to the Nepalese populations, and showing a limited ancestry proportion (~12%) ascribable to recent European admixture (Gnecchi-Ruscone *et al.*, 2019). Aymaras are known to be adapted to the high altitude environment (even if at a lesser extent than Himalayan people).

Regarding lifestyle, economic activities of the Sherpa are based on trade and to a very lesser extent on agriculture (predominantly cultivation of potatoes), where conditions allow farming. At highest altitudes, the Sherpa breed the "yak", a domesticated bovid, which is adapted to hypobaric hypoxia as well (Qiu *et al.*, 2012). Generally, the Sherpa sell or barter wool and milk of Yak and dairy products. During the winter, activities are limited, especially agriculture that starts again at the onset of spring, when people move to summer villages.

The recruited Tamangs live at considerably lower altitudes (i.e. no more than 2,000 m a.s.l.) than the Sherpa, and often they work as porters for trekking expeditions, or are engaged as farmers, breeders and day labour.

Aymara is an ethnic group that lives between 2,000 and 4,200 m of altitude in the valleys and the Altiplano of the Andes. In the last century, Aymaras have been suffered by Western influences (i.e. European), especially in the cities. However, in rural and isolated areas, their lifestyle and diet are still traditional. They farm (i.e. tubers) and raise livestock in extreme environments characterized by low temperatures and high-altitude.

Information about dietary habits of these populations were collected by dietary questionnaires and interviews. We calculated daily food intake (Fig. 1D) by estimating average quantities of food ingested per day, food energy (kcal/day), grams of proteins, fats, carbohydrates, including simple sugars, and fibres (Supplementary Tab. 1).

As expected, the dietary habits of Nepalese and Bolivian populations appear to be considerably poor in terms of food variety when compared to that of Europeans (Fig. 1D). In fact, they eat mainly starchy foods (i.e. potatoes for the Sherpa, and other variety of tubers, such as chuño, caya, and camote, for the Aymara) and cereals (i.e. rice and millet for Sherpa and Tamangs; quinoa, rice and amaranth for Aymara), supplemented by spices (Sherpa and Tamangs) or vegetables, such as carrots, chili peppers, tomatoes, and onions (Aymara), and eggs. Meat (i.e. chicken and yak for the Sherpa, chicken and buffalo for the Tamangs, freeze-dried camelid meat, i.e. charque, steer, sheep, chicken for the Aymara) is consumed a few times a week.

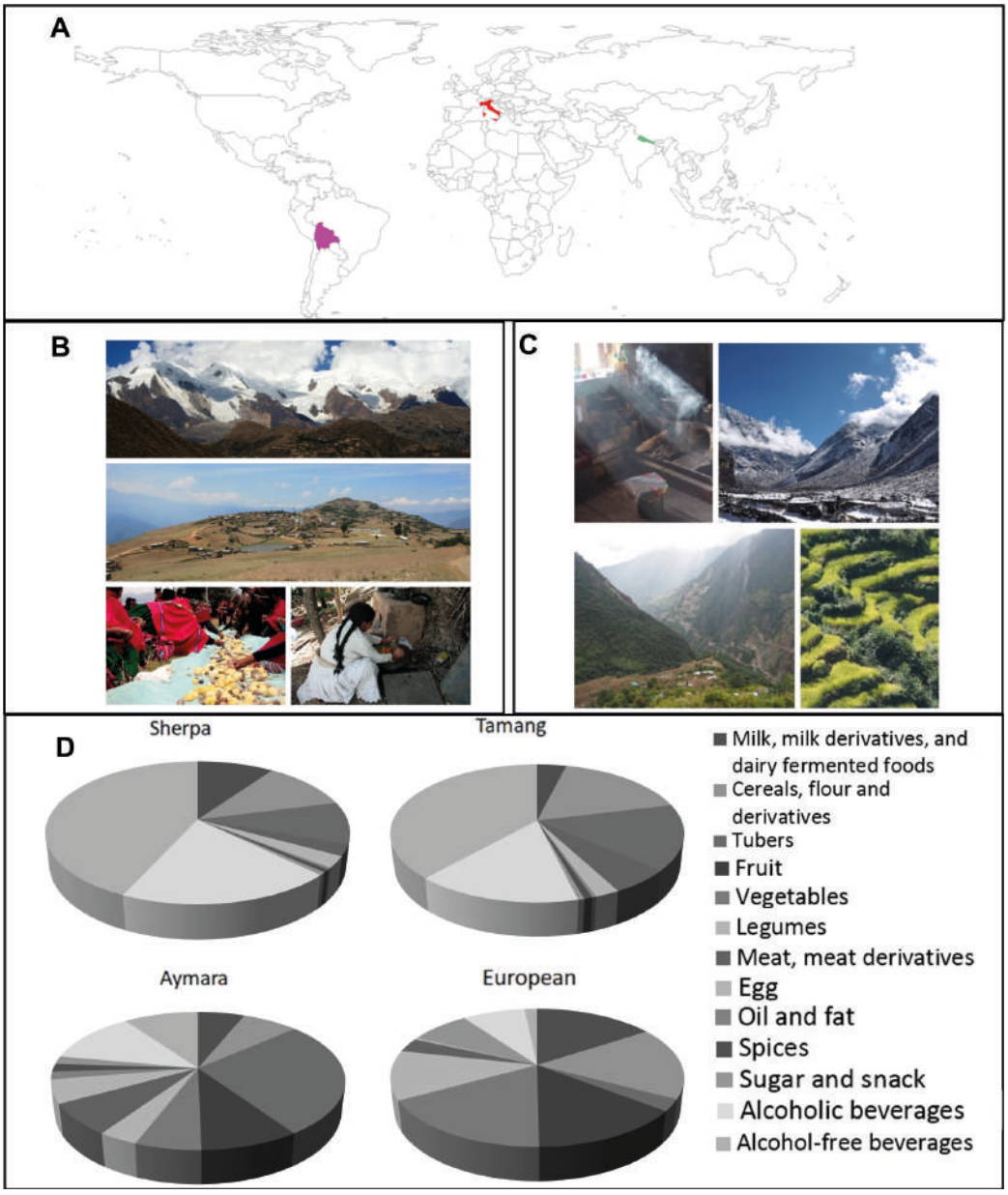

Fig. 1 - A) Geographical distribution of the populations considered in this study. B) Pictures of the Taca's village and of Aymara daylife (photos by Patrizia Di Cosimo). C) Pictures of the Na Sherpa village and of a fireplace in a Sherpa house (top of the plot), as well as of the Simigaon Tamang village and of the surrounding millet crops (photos by Marco Sazzini). D) Results from diet analysis based on food questionnaires. Pie charts represent the food intake for each ethnic group considered. The colour version of this figure is available at the JASs website.

Aymaras usually drink coffee, coca leafs tee, chocolate, industrial beer, and alcoholic (1-3% alcohol) beverages derived from grains, maize, amaranth or fruit.

Interestingly, both Sherpa and Aymara people usually drink fermented beverages, although they are derived from different components (i.e. chang, a typical Sherpa beverage obtained from millet and yak's milk, and chicha a typical Bolivian fermented beverage obtained from maize or amaranth).

Differential microbial richness and biodiversity among the examined populations

When considering their country of origins, the Nepalese, Bolivian and European populations showed a trend of variability in microbial richness (Fig. 2A and Supplementary Tab. 3A), although the observed differences were not statistically significant according to a Mann-Whitney test. Differential microbial richness was observed also between the two Nepalese populations, with the Sherpa showing higher microbial biodiversity than Tamangs (Supplementary Tab. 3A). By means of beta diversity analyses (Figs. 2B,C; Supplementary Tab. 3B), we observed that microbial community of Western subjects clustered together and separately from those of non-Western populations, as expected, and irrespectively from the high-altitude or low-altitude settings of the latter. Among the Nepalese samples, the Sherpa showed a strong dispersion within the group, while Tamang samples clustered together, similarly to Western ones (Figs. 2B,C and Supplementary Fig. 1). Among the considered indexes, Weighted UniFrac showed a significant separation between the two Nepalese populations (Supplementary Fig. 1 and Supplementary Tab. 3B). As regards the distribution of the samples from Bolivian Aymaras, a statistically significant difference (PERMANOVA test; p-value < 0.001) was observed with respect to the Western ones, but not when compared to the samples from Nepalese populations (Supplementary Tab. 3B).

The strong differentiation in terms of microbiota profiles of native Himalayan and Bolivian populations with respect to that of European ancestry was further supported by random forest classifier analysis, which allows to assign and classify each sample according to its microbial profile (Fig. 2C). Samples from Western subjects were assigned to their source population with 100% accuracy (global OOB error = 31.7%, Supplementary Tab. 4). Classification accuracy for Sherpa and Aymara samples also maintained high values (75% and 80%, respectively), while that for the Tamang ones dropped to 50% of assignation.

Microbial signatures shared among populations living at high altitude

To identify the principal taxa driving gut microbiota differentiation across the studied populations, abundance and distribution of bacterial phyla were compared. Accordingly, Spirochaetes and Elusimicrobia phyla were found to be significantly enriched in samples from high-altitude populations, and to a lesser extent in those from Tamangs (Fig. 3A). Although the relative abundances were variable, Proteobacteria were enriched in all traditional populations (10%-18% range of relative abundance vs. <5% in Western samples). We also considered Firmicutes/Bacteroidetes ratio (F/B) (Fig. 3B). Samples from the Sherpa and Western populations were characterized by a high ratio (i.e. predominance of Firmicutes), while those from Aymara and, especially, from Tamangs showed a lower ratio (i.e. predominance of Bacteroidetes). The Mann-Whitney Wilcoxon test indicated a significant difference when comparing Aymara and Tamangs, with respect to the Western population, while both Sherpa and Aymara showed a significant different F/B ratio with respect the Tamang group (Supplementary Tab. 5).

Within the Firmicutes phylum (Supplementary Fig. 2), a great bacterial biodiversity was observed among the studied populations (Fig. 2B). In particular, *Faecalibacterium* was the most abundant, especially in the microbiota of Western people. Conversely, some genera, such as *Catenibacterium, Lactobacillus, Anaerovibrio* characterized the microbiota of traditional populations. Within the Bacteroidetes phylum (Fig. 3D), the abundance of *Prevotella* characterized the microbiota of traditional rural populations,

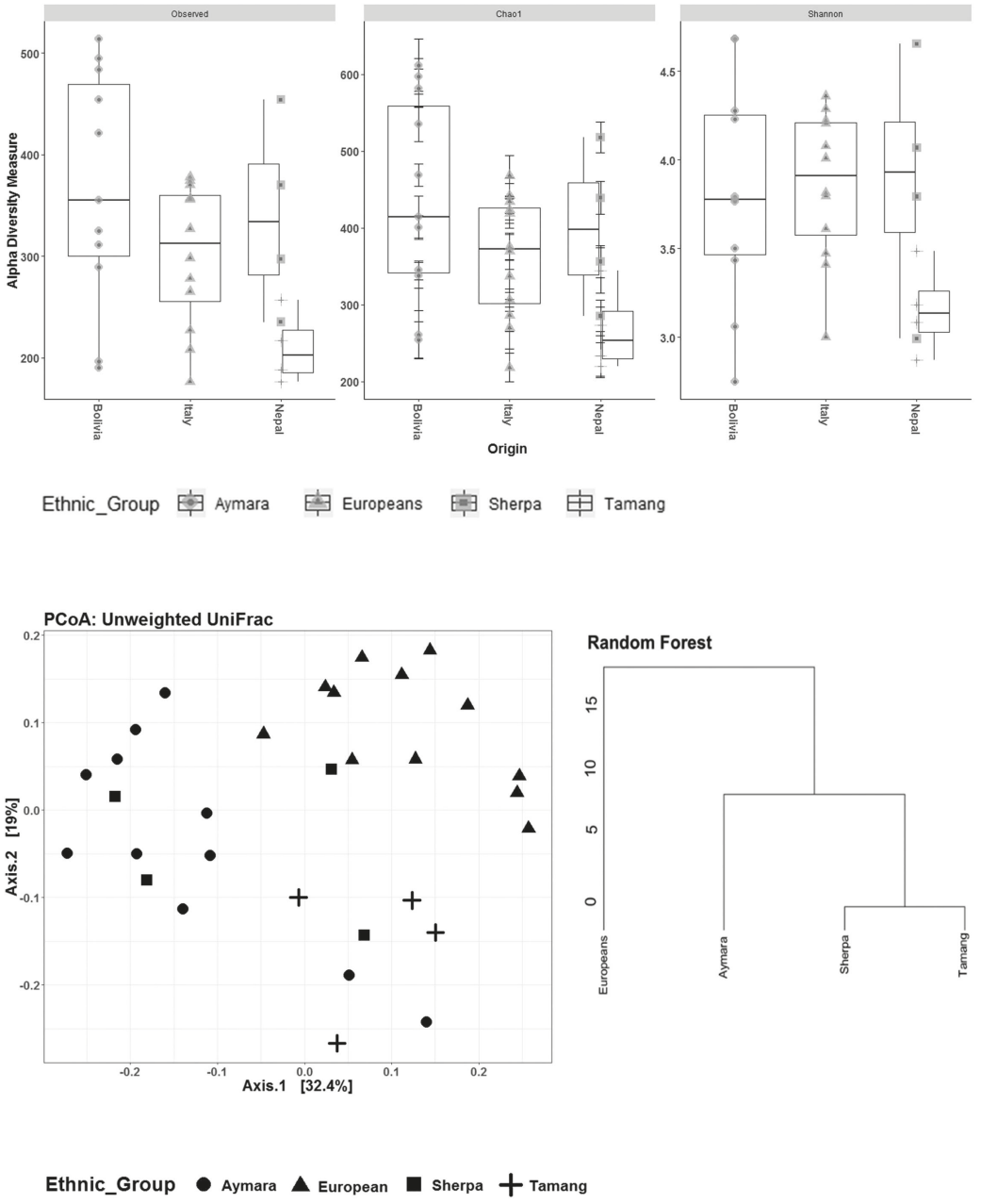

Fig. 2 - A) Alpha diversity indices calculated by considering Observed OTUs, Chao1 index and Shannon entropy. For each country (reported on the x axis), ethnic groups are reported with different shapes. B) Unweighted UniFrac distance across the studied populations. PERMANOVA test reported p < 0.001. C) Hierarchical cluster plot based on Random Forest classifier analysis: 5,000 trees were used to classify samples with an out-of-bag (OOB) error rate of 31%.

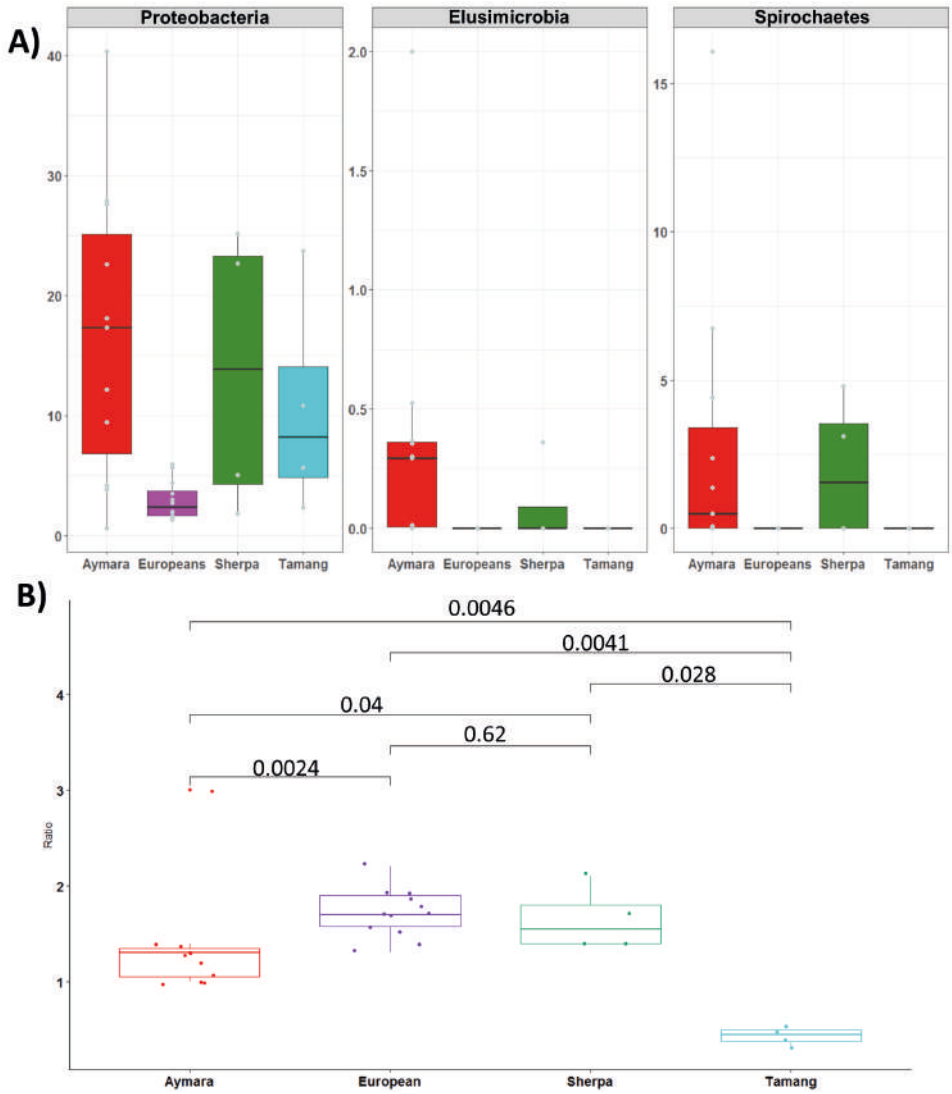

Fig. 3 - Overview of microbial profiles of traditional and European populations. A) Relative abundance distribution of the significant phyla across populations, as resulted from the DESeq2 analysis. B) The boxplots indicate values of Firmicutes/Bacteroidetes ratio: the p-values have been estimates through Mann-Whitney test. (C) and Bacteroidetes (D) phyla. The colour version of this figure is available at the JASs website.

while *Bacteroides* and *Parabacteroides* were more abundant in the Western population when compared to the other groups. Interestingly, the microbiota of Tamangs, which presented predominance of Bacteroidetes with respect to the other populations, was enriched in *Prevotella* (either of *Prevotellaceae* or *Paraprevotellaceae* families; Fig. 3D).

At the genus level, 23 genera were differently distributed across populations (Fig. 4). Among them, some characterized the non-Western populations, such as *Bulleidia*, *Succinivibrio,*

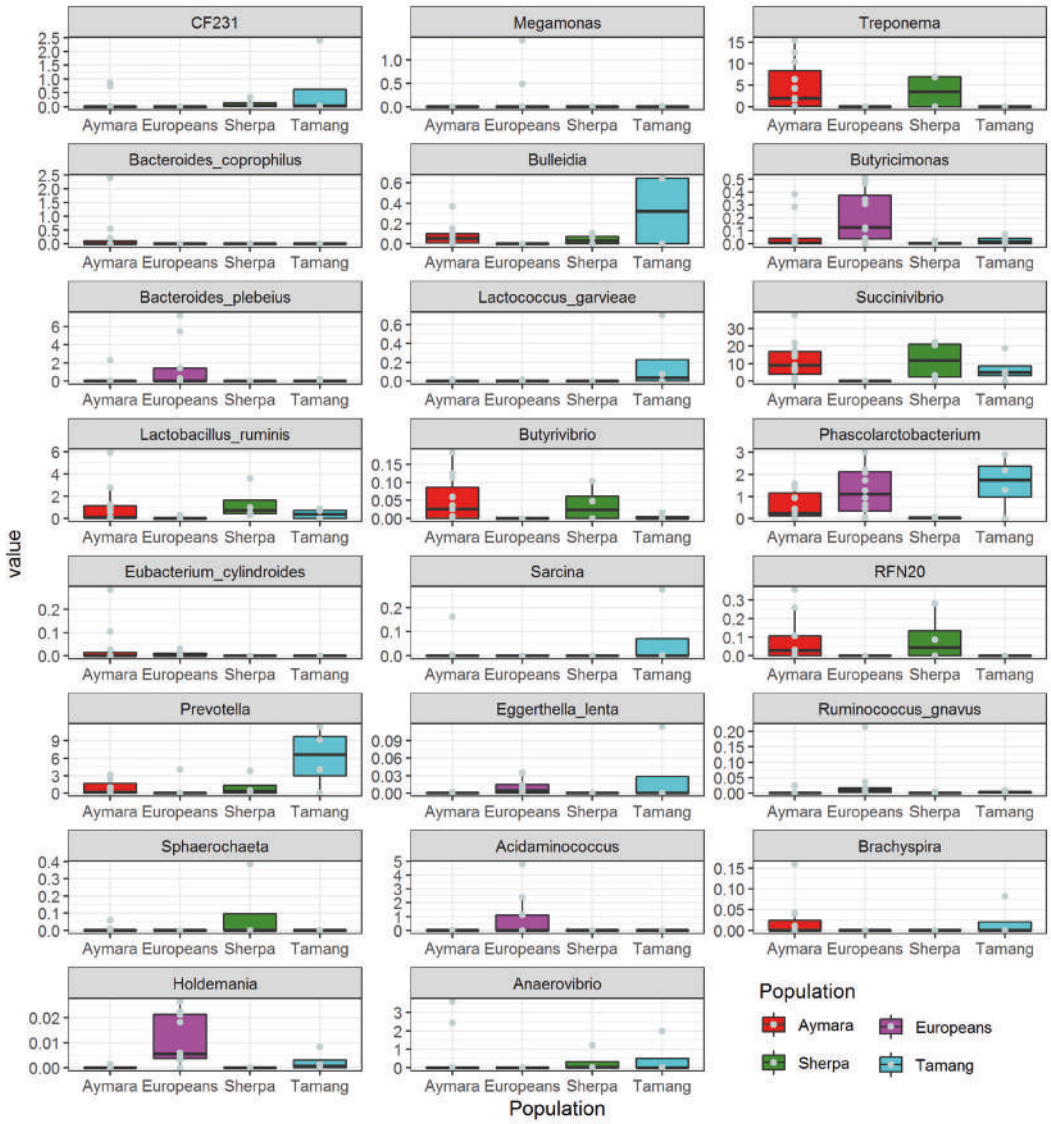

Fig. 4 - Representation of relative abundances of significant taxa, obtained through the DESeq2 analysis performed across the examined ethnic groups. The colour version of this figure is available at the JASs website.

Lactobacillus ruminis and *Prevotella*. Western population samples were instead strongly enriched for bacteria belonging to either the Bacteroidetes phylum (*i.e. Butyricimonas* and *Bacteroides plebeius*) or Firmicutes (*Acidaminococcus*, *Ruminococcus gnavus* and *Holdemania*). Among the non-Western populations, differential microbial profiles were then strongly associated to each ethnic group, independently from their geographical location. For example, *Treponema,*

Butyrivibrio and the unidentified rumen bacterium *RFN20* characterized the microbiota of high-altitude populations (i.e. Sherpa and Aymara). Interestingly, Basic Local Alignment Search Tool (BLAST) alignment of *Treponema* sequences confirmed that samples from both Aymara and Sherpa populations showed a significant sequence identity (99%; E-value 0.0) with *Treponema succinifaciens*, while low abundance of this species was observed for the Tamang group. On the other hand, the microbiota of both the Nepalese populations (i.e. Sherpa and Tamangs) were enriched of *Anaerovibrio* and *CF231* (a member of *Paraprevotellaceae*), when compared to those of the Aymara and Western populations. However, *Prevotella*, *Bulleidia, Lactococcus* and *Sarcina* were more abundant in the Tamang group, while *Sphaerochaeta* was more abundant in the Sherpa (Fig. 4).

To deepen the impact of environment, diet, culture and lifestyle on gut bacterial communities of the two Nepalese populations, a direct comparison between Sherpa and Tamang microbiota was performed. A great difference in Operational Taxonomic Units (OTUs) distribution between the two populations was detected at the phylum level (Supplementary Fig. 2). Notably, samples from Sherpa were enriched in *Bifidobacterium adolescentis*, *Rikenellaceae*, *Treponema* and *Elusimicrobiaceae*. On the other hand, the microbiota of Tamangs was characterized by *Enterobacteriaceae*, *Blautia producta*, *Eggerthella lenta*, *Lactococcus garviae* and *Bacteroides plebeius* (Fig. 4).

Furthermore, network analysis was performed in order to further investigate the influence of single OTUs in the global microbial communities in each population (Supplementary Fig. 3). After filtering the core microbiota for each ethnic group by using 80% threshold of relative abundance, Pearson's correlation was calculated between OTUs. The four distinctive networks showed different microbiota profiles across the populations, not only in terms of species presence, but also according to relationships among bacterial species in the communities. Specific OTUs could be considered as milestone bacteria of the intestinal communities in each population due to their highest node degree and highest betweenness centrality with respect to the whole bacterial community. For instance, in the Sherpa network betweenness centrality of *Prevotella*, *Oscillospira* and *Faecalibacterium* pointed to these genera as important for shaping the microbial community. In the Tamang network, *Faecalibacterium* showed a central role in the intestinal community. The microbiota profile of Aymara instead seems to be more influenced by *Parabacteroides*, *Dorea* and *Prevotella*, while in the Western population network, *Bifidobacterium* and *Phascolobaterium* were the two driving taxa.

Detecting the influence of high-altitude life conditions on the functional potential of gut microbiota

Finally, we evaluated the potential of the gut microbial functions of the two high-altitude populations in comparison with that of the low-altitude Western and non-Western groups through the identification of specific Kyoto Encyclopedia of Genes and Genomes (KEGGs) profiles (functional inference by 16S rRNA dataset).

Interestingly, both Sherpa and Aymara microbiomes showed several common functional pathways that distinguish them from that of the Western population (Supplementary Figs. 46A,B). Among the enriched metabolic functions, in the microbiome of high-altitude populations we observed the synthesis and degradation of ketone bodies, vitamin B6 metabolism, degradation of caprolactam, as well as the biosynthesis of terpenoid backbone and unsaturated fatty acids. Moreover, the Aymara microbiome was enriched even for other predicted KEGGs pathways involved in amino acids metabolism (e.g. lysine, valine, leucine and isoleucine degradation, as well as D-arginine, D-Alanine, D-glutamine and D-ornithine metabolism), energy production (i.e. oxidative phosphorylation) and vitamins metabolism (i.e. Riboflavin). In parallel, the microbiome of Western people were enriched in differential pathways when compared to both Sherpa and Aymara, especially those related to glycan degradation, carbohydrate metabolism, lipid metabolism and vitamins metabolism.

Comparing the two Nepalese populations, the Sherpa microbiome was mainly characterized by functions involved in the metabolism of amino acids, fatty acids and glycerophospholipids (Supplementary Fig. 6C). The microbiome of Tamangs was instead enriched in KEGGs pathways involved in lipopolysaccharide, polyketides and N-glycan biosynthesis.

Discussion

Since their expansion out of Africa approximately 100,000 years ago, modern humans have experienced considerably different environmental conditions that forced them to adopt a wide range of lifestyles and diets, as well as to biologically adapt to challenging ecological settings (Brown, 2012; Sazzini *et al.*, 2014). Comparison of the gut microbiome across several traditional and rural populations geographically isolated with respect to urban populations has showed how these changes could have affected the human microbiota profile during our recent evolutionary history (Adler *et al.*, 2013; Moeller *et al.*, 2016). In particular, the present study aims to evaluate whether gut microbial communities, which are shaped by dietary habits and environmental conditions, in turn may contribute to the adaptation of the host to peculiar ecological and cultural settings. Therefore, we focused on the "high-altitude" case study by characterizing the gut microbiota of two populations with different genetic backgrounds, dietary habits and lifestyles, but exposed to similar and highly challenging high-altitude environments in the Himalayas (the Sherpa) and the Bolivian Andes (the Aymara), respectively. We then compared the observed microbial profiles with those of low-altitude populations, one (the Tamangs) who shares an ancient common genetic ancestry with the Sherpa (Gnecchi-Ruscone *et al.*, 2017), and another being instead representative of Western populations of European ancestry.

By comparing traditional rural populations adapted to high-altitude with Western people, alpha diversity analysis showed a trend of higher microbial richness in Aymara samples and, on average, lower diversity in Nepalese samples, even with respect to Western ones. Recently, Jha and colleagues (Jha *et al.*, 2018) reported a trend of lower values of alpha diversity in traditional Nepalese populations (but significant only for the Tharu ethnic group) when compared to people of European ancestry from the United States. A similar trend is observable according to our data, thus being in contrast with previous results on traditional populations, in which higher levels of bacterial richness are generally found when compared to European populations (Yatsunenko *et al.*, 2012). However, the samples of traditional populations analysed in most of the previous studies were collected from countries located at tropical latitudes (Yatsunenko *et al.*, 2012; Schnorr *et al.*, 2014; Martínez *et al.*, 2015), which are generally characterized by high endemic biodiversity. Our results support the hypothesis of the existence of a relationship between microbiota richness and geographical location of host populations (Jha *et al.*, 2018), and thus that environmental richness could affect microbial biodiversity. Indeed, the ecological context of the Bolivian Yungas, in which the Aymara population lives, presents higher endemic biodiversity than all the other geographic areas considered in this study (Kier *et al.*, 2009). On the other hand, the Himalayan valleys are characterized by a lower endemic biodiversity (Kier *et al.*, 2009) and this may explain the lower bacterial richness observed in Nepalese people than Aymara. Interestingly, a previous observation showed an increased trend of endemic richness in Nepal along an altitude gradient and even this finding is in agreement with the alpha diversity values calculated for the Sherpa and Tamang microbiotas (Vetaas & Grytnes, 2002). Therefore, diversity of the microbial communities examined in the present study seems to reflect the overall ecological diversity experienced by their host populations.

Beta diversity analysis and random forester classifier also showed that the traditional non-Western populations were characterized by a different gut microbial structure when compared to Western people. This result is in agreement with

the general pattern of microbiota differentiation between Western and non-Western human groups, as reported in literature (De Filippo *et al.*, 2010; Yatsunenko *et al.*, 2012; Schnorr *et al.*, 2014).

As emerged from the analysis of dietary habits of the Sherpa and Aymara populations, the poor variety and restrict typology of nutritional resources on which they rely are unavoidably conditioned by their settlement in extreme environments. Particularly, high-altitude, arid soils and low temperatures limit farming to a few cereals and tubers cultivations, as well as to livestock breeding of Yak (in the Himalayas) or alpaca (in the Bolivian Andes) that are adapted to hypobaric hypoxia (Qiu *et al.*, 2012; López *et al.*, 2018), together with traditional food preparation, such as the use of fermented foods (i.e. the Sherpa *Chaang* beer and the Aymara *Chicha*) and poor hygienic conditions. These environmental settings and lifestyles are thus supposed to heavily affect the gut microbiota composition of these traditional populations.

Metataxonomic analyses pointed to significant differences in the gut microbiota of these traditional populations with respect to Western people. Concerning the F/B ratio, Tamangs showed a microbial profile enriched in Bacteroidetes, in agreement with previous observations for other traditional populations (De Filippo *et al.*, 2010). Interestingly, the two high-altitude populations showed a high F/B ratio similarly to Western people. However, within the Firmicutes phylum, a great bacterial biodiversity was observed among high-altitude populations and Europeans. In particular, bacterial genera able to degrade fibers (i.e. *Butyrivibrio, Bacterium RFN20*) and associated to dairy products and milk fermentation (i.e. *Lactobacillus*) differentiated the traditional groups from the Western one. This finding plausibly reflects the different dietary habits of the examined populations, in particular in relation to the consumption of cereals and fiber-rich foods, as well as of fermented foods, which are typical in the diets of traditional populations.

Within the Bacteroidetes phylum, differential abundances of *Prevotella* (in general enriched in all traditional populations, but prevalent in Tamangs) and *Bacteroides* (more abundant in Western people) were observed among the studied populations.

Consumption of fiber-rich foods, such as millet or corn, respectively by the Nepalese and Bolivian populations, could again explain the enrichment of *Prevotella, Succinivibrio* and *Treponema* among the examined non-Western populations due to the ability of these bacteria to degrade plant polysaccharides, such as cellulose and xylans (De Filippo *et al.*, 2010, 2017). Previous studies have already disclosed the presence of *Treponema succinifaciens* and *porcinum* across different traditional rural populations, despite they use different subsistence strategies (Schnorr *et al.*, 2014; De Filippo *et al.*, 2017; Jha *et al.*, 2018). It was thus hypothesized that the higher amount of this bacterial genus in non-Western and rural populations is related to their higher consumption of plant fibres with respect to Western groups.

Prevotella, *Treponema* and *Butyrivibrio* and other degrading-fibres bacteria are also known to produce high levels of short-chain-fatty-acids (SCFAs) (Flint *et al.*, 2008), which are important for energy yield and play a key role in the host gastrointestinal homeostasis (Pluznick, 2017). In particular, SCFAs were found to be associated with low blood pressure (Raizada *et al.*, 2017). Thus, SCFAs production potentially represents an important advantage for the fitness of high-altitude living populations, because they may further contribute to reduction of acute mountain sickness susceptibility, as previously suggested by evidence from Tibetan populations (Li & Zhao, 2015). It is noteworthy that in all non-Western populations, network analysis pointed to a central role of the *Prevotella* genus in the bacterial community, together with other SCFAs-producer bacteria (i.e. *Faecalibacterium* for Sherpa and Tamangs, *Dorea* for Aymara). This evidence supports the fact that SCFAs-producing bacteria are fulfilling functions essential for the host physiology in these populations. The high-altitude life conditions, especially due to hypobaric hypoxia and low temperatures, indeed require high energy demands that could

be satisfied by contribution of bacterial metabolism deputed to ferment polysaccharides (i.e. *Prevotella*, *Treponema* and *Butyrivibrio* found to be abundant in the Sherpa and Aymara).

Tubers are a typology of food that can be easily cultivated in poor and extreme environments, such as the high-altitude one, and represent a staple food for Sherpa and Aymara. In line with this evidence, a trend towards the enrichment of *Catenibacterium*, a bacterial genus involved in starch degradation and sugar fermentation (Kageyama & Benno, 2000), was found in both Himalayan and Bolivian high-altitude groups. Further diet-induced bacterial markers were inferable when comparing the microbiota composition of Sherpa and Tamangs. In fact, despite the close genetic and geographical proximity of these populations (Gnecchi-Ruscone *et al.*, 2017), they showed differences in dairy product sources that could be at the base of their differences in *B. adolescentis* and *Lactococcus garvieae* abundances. This finding may be particularly relevant since both of these groups were found to do not present adaptive alleles at the *MCM6/LCT* locus, which are known to confer lactose tolerance in adulthood, when genome-wide genotype data were *ad hoc* investigated (Gnecchi-Ruscone *et al.*, 2017). In detail, the Sherpa consume mainly yak milk-based products, which are rich in polyunsaturated fatty acids (PUFA), conjugated linoleic acid (CLA), antioxidant elements (e.g. vitamin C) and a number of probiotic bacteria (Guo *et al.*, 2014). Moreover, yak k-casein has been demonstrated to increase *Bifidobacterium* spp. overgrowth (Tang *et al.*, 2013). On the other hand, Tamang samples were characterized by the presence of *L. garviae*, which is associated to the consumption of dairy products, usually consumed by this population (Fortina *et al.*, 2007), and whose presence is often associated to traditional populations in close contact with cattle, as is the case of Tamangs.

Results by pathways prediction analysis further supported the hypothesis that a peculiar gut microbiota structure coevolved with Sherpa and Aymara populations adapted to high-altitude and limited nutritional resources. For instance, despite their considerably different genomic and cultural backgrounds, these populations were both found to be enriched in microbial genera involved in the metabolism of vitamin B6, which is essential for glycogen conversion into glucose and for conversion of amino acids into oxaloacetate (Rosenberg *et al.*, 2017). Vitamin B6 plays an important role in energy yield, and its deficiency induce a decreased level of glucose conversion, thus leading to a loss of energy fuel. Moreover, the two forms of vitamin B6 (i.e. pyridoxal 5'-phospate and pyridoxal) influence the oxygen binding affinity of erythrocytes (Reynolds and Natta, 1985), having the potential to contribute to adaptive processes in response to hypobaric hypoxia. It has been observed that vitamin B6, together with folate, can influence the one-carbon pool metabolism, which is another common signature between Sherpa and Aymara microbiome, and it has been recently proved to exert a neuroprotective effect in hypoxic conditions (Yu *et al.*, 2016).

In line with these findings, enrichment of microbial profiles involved in unsaturated fatty acids metabolism was also observed in the microbiome of both high-altitude populations, being potentially associated to their adaptation to hypobaric hypoxia. Indeed, a study has reported that unsaturated fatty acids are mandatory for hypoxia-inducible transcription factor 3α (HIF3A) stability and thus for modulation of HIF processes in response to hypoxia (Fala *et al.*, 2015).

Interestingly, in both the Sherpa and Aymara microbiotas, enrichment of the pathway related to synthesis and degradation of ketone bodies suggests a potential role in conferring an advantage in high-altitude environments. It is known that when humans are exposed to a sustained cellular oxygen reduction due to high altitude, there is an alteration in oxygen use, which drives hypoxia-related genes expression (Semenza, 2007; Wheaton & Chandel, 2011). These genes influence the human metabolism by inducing loss of weight due to a decrease of muscle mass, decrease of energy and oxygen expenditure and catabolism with the production of ketone bodies

through amino acids breakdown (Boyer & Blume, 1984; Westerterp & Kayser, 2006; Sanchez *et al.*, 2012). In particular, the synthesis of ketone bodies from endogenous resources would be a major advantage under hypoxic conditions as they can act as an energy substrate instead of glucose, especially to sustain brain metabolism (Hasselbalch *et al.*, 1995; Veech, 2004). Moreover, the use of ketone bodies, instead of glucose, require a lower oxygen cost for ATP biosynthesis, improves mitochondria efficiency, and help in modulating HIF response to hypoxia (Masuda *et al.*, 2005; Puchowicz *et al.*, 2008; Semenza, 2011). Amino acids conversion to ketone bodies is performed by the liver (Murray & Montgomery, 2014), but very few information are available so far about the activation of this metabolic function by the microbiota under hypoxic conditions. Therefore, the obtained results could suggest the hypothesis that the gut microbiota has the potential to contribute to host physiological functions useful for adaptation to high-altitude and to life conditions characterized by poor nutritional and energy resources.

Furthermore, other signatures associated to vitamins and ketogenic amino acids pathways were found exclusively in the Aymara population. This may be related to an even more relevant role of microbiota-mediated high-altitude adaptation in such a group with respect to the Sherpa, due to their less optimized and genetically fixed adaptive phenotype resulted from a less prolonged evolutionary history at high altitude (Beall, 2007). Among these signatures, Riboflavin has been reported to increase in soldiers working in Bolivia and during Operation Everest II (Rose *et al.*, 1988). Moreover, supplementation of Riboflavin/B6/Folate showed ameliorate hypoxia-induced memory deficits in mice (Yu *et al.*, 2016). In parallel, enrichment of microbial profiles associated to other vitamins and amino acids pathways was observed in the Sherpa, thus giving further support to the hypothesis that environmental conditions (e.g. diet and high altitude) exert a strong influence on the gut microbiota ecology irrespectively of the genetic background of the host population.

In conclusion, although being performed on a limited number of samples *per* group, this preliminary study showed how the high-altitude environment affects nutritional sources, consequently impacting on the gut microbiota structure of populations exposed to such a selective pressure for most of their evolutionary history. Despite considerable genetic, geographic and cultural differences, the two examined high-altitude populations were found to share important aspects of their gut microbial composition and metabolic profiles. Overall, our results suggest that the gut microbiota has the potential to mitigate the effect of nutritional restrictions and environmental pressures induced by high altitude, by providing metabolic functions able to supply compounds, such as vitamins, ketone bodies and amino acids, which are useful for the host to cope with the challenging physiological stresses and energy demand imposed by life in this extreme environment (Fig. 5).

Material and Methods

Populations studied and samples collection.

A total of 19 faecal samples were collected from subjects (n = 10 males and n = 9 females, age range 20-41 years old; Supplementary Tab. 2) belonging to three populations with diverse ancestries and living at different altitudes in Asia (Nepal) and South America (Bolivia). Sampling and logistic conditions were not always easy to deal in such remote geographical areas. Several factors have to be considered during the sampling collection, in particular the environmental and hygienic conditions to assure the adequate level of sterility, the transportation and storage of samples in order to preserve the integrity of DNA (e.g., to avoid temperature fluctuation, frozen-thaw cycles, and shorten the transportation time) (Wu *et al.*, 2019). Moreover, during the bacterial genomic DNA extraction, two samples belonging to the Nepalese populations have been discarded due to the poor quality of the DNA that would not have allowed the microbiota sequencing. All these variables constituted a strong limitation to

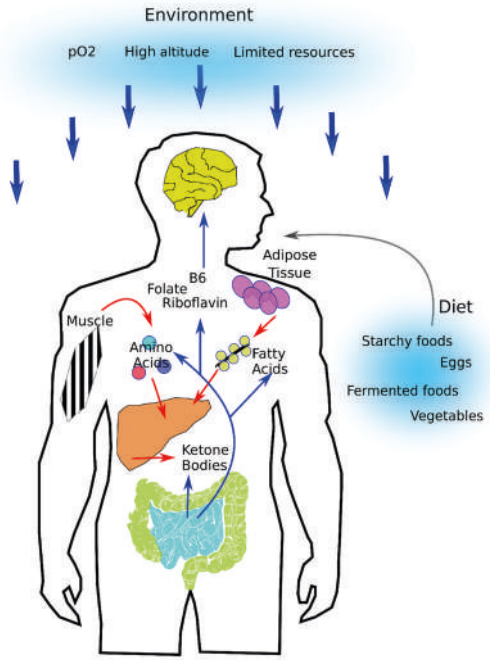

Fig. 5 - Scheme of the effects of the high-altitude environment on the host and role of the gut microbiota in adaptation to such a selective pressure. Murray & Montgomery (2014) reported that exposure to hypobaric hypoxia leads to cachexia (red lines). Fatty acids are released by adipose tissue, while amino acids are released from muscle breakdown. These are converted into ketone bodies by the liver and act as metabolic substrates. According to the scheme of Murray & Montgomery, results obtained for the considered high-altitude populations (i.e. Aymara and Sherpa) have shown how environmental conditions (i.e. high altitude) and diet (i.e. usual consumption of starchy and fermented foods) may influence the gut bacterial ecology and functions (blue lines). It is also plausible to hypothesize that the microbiota of these populations provides an important support to the catabolic response to altitude by metabolism of fatty acids, ketones bodies, ketogenic amino acids and vitamins. The colour version of this figure is available at the JASs website.

the collection of numerous faecal samples, thus only a limited number of samples has been collected, but strictly checking for the biological ancestry of the recruited people.

Indeed, individuals belonging to the Sherpa ethnic group (n = 4), which did not present genomic signatures ascribable to recent gene flow from neither East Asian nor South Asian low-altitude populations (Gnecchi-Ruscone *et al.*, 2017), were recruited at the village of Na (4,180 m a.s.l.) in the Rolwaling Himal (Gaurishankar Conservation Area, Dolakha District, Nepal). Tamang samples (n = 4) were instead collected in the same region (Gaurishankar Conservation Area) at Simigaon (2,000 m a.s.l.). In particular, we have recently proved that Tamangs belong to an ethnic group that shares Tibeto-Burman genomic ancestry with the Sherpa, but who undergone a completely different adaptive evolution (i.e. they are not genetically adapted to high-altitude) (Gnecchi-Ruscone *et al.*, 2017).

People from the Aymara ethnic group (n = 10) were sampled from the Taca and Santiago de Taca Communities (Irupana Municipality, South Yungas Region of La Paz, 2,800 m a.s.l.) on the Bolivian Andes. This Aymara population often moves to the Altiplano of La Paz (4,000 m a.s.l.) for working, studying or for residing with family members for short time during the year.

All the sampled individuals were selected because highly representative of their population of origins according to their genomic backgrounds, which were evaluated by characterization of ~720,000 genome-wide SNPs and by the implementation of ADMIXTURE analysis, haplotype-based estimate of ancestry proportions, linkage disequilibrium decay-based methods, and local ancestry inference within the framework of two previous population genomics studies focusing on the Sherpa, Tamang and Aymara ethnic groups (Gnecchi-Ruscone *et al.*, 2017; Gnecchi-Ruscone *et al.*, 2019). As representatives of a low-altitude control population of European ancestry, Italian subjects (n = 11, n = 4 males and n = 7 females) from the Emilia-Romagna region were also recruited. Moreover, in order to avoid possible bias in the observed microbiota composition due to the health conditions of individuals, subjects were selected basing on the absence of any

chronic, gastrointestinal or extra-intestinal disease and according to the absence of any infection. Furthermore, no antibiotics or probiotics intake was allowed in the four weeks before the enrollment.

During the collection, all faecal samples were preserved in RNAlater kits (Qiagen, Hilden, Germany) and then maintained in RNAlater and stored at −80 °C until the extraction of genomic DNA. Dataset information are reported in Supplementary Table 2.

For all subjects, data on lifestyle and dietary habits were obtained by means of an interview and the completion of a dietary questionnaire to gather information about their usual food consumption. Nutritional values were obtained by quantifying the amount of food consumed, by calculating the daily portion on average and intakes of Energy (Kcal), Carbohydrates (including fibres), Fats, Proteins, Sugar, and Minerals (Supplementary Tab. 1). For this purpose, the food composition tables for Nepal and Bolivia, as reported by the International Network of Food Data Systems (INFOODS; http://www.fao.org/infoods/infoods/tablas-y-bases-de-datos/bolivia/es/; http://www.fao.org/fileadmin/templates/food_composition/documents/regional/Nepal_Food_Composition_table_2012.pdf) were consulted.

This study was designed and conducted in accordance with relevant guidelines and regulations according to the ethical principles for medical research involving human subjects stated by the WMA Declaration of Helsinki. All participants were informed about the research, and informed consent was obtained by anthropologists during the conducted expeditions in accordance with the sampling protocols within the framework of the University of Bologna HUMAN project (for Nepalese and Italian populations), and UNIGEN (Unidad de Identificación Genética de la Universidad Mayor de San Andrés-UMSA) authorization (Dott. Sergio Quispe, Facultad de Ciencias Farmacéuticas y Bioquímicas, UMSA, for the Bolivian population), approved by the Ethical Committee of the University of Bologna (Italy).

DNA extraction and targeted massive parallel sequencing experiments.

Genomic bacterial DNA was extracted from faecal samples by using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany), following manufacturer's instructions. DNA quality was assessed by gel electrophoresis and spectrophotometry, by measuring OD 260/280. The bacterial 16S ribosomal RNA (rRNA) gene was amplified following the 16S rDNA gene Metagenomic Sequencing Library Preparation Illumina protocol (Cod. 15044223 Rev. A). Gene-specific primers (PCR1_f: 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3'; PCR1_r: 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3') targeting V3-V4 region according to Klindworth *et al.* (2013) and the obtained PCR amplicons were purified, quantified and pooled together in a final amplicon library that was sequenced on an Illumina MiSeq platform at the facilities of the FISABIO Sequencing and Bioinformatics Service (Valencia, Spain). All Illumina sequencing raw reads and associated metadata are available at NCBI: Bioprojects: PRJNA488981.

Illumina MiSeq sequencing and data analyses.

The generated sequence reads were analyzed for quality, length and chimera presence by using the *qiime* v1.8 pipeline (Caporaso *et al.*, 2010b). Then, sequences were organized into OTUs with a pairwise identity clustering threshold of 97%. Each representative sequence was classified using the VSEARCH-based consensus classifier (Rognes *et al.*, 2016) and multiple sequence alignment (MSA) was performed against the Greengenes 13_08 database using the PyNAST v.0.1. program (Caporaso *et al.*, 2010a) and a 97% of similarity for bacterial sequences. The MSA was used as well to build a phylogenetic tree (DeSantis *et al.*, 2006).

Alpha diversity was estimated in terms of Observed OTUs, Chao1 index and Shannon entropy. Beta diversity (i.e. Bray-Curtis, Weighted and Unweighted UniFrac) was calculated to evaluate differences in bacterial

communities between samples by use of the R *phyloseq* package (McMurdie & Holmes, 2013).

Further statistical analyses were computed to identify differences in relative abundance of OTUs across samples by using the following R packages: *phyloseq*, *vegan*, *ggplot2* and *microbiome* (Lahti *et al.*, 2014; McMurdie & Holmes, 2013; Wickham, 2016; Oksanen *et al.*, 2018). In order to prevent bias in the obtained results due to OTUs with small mean, taxa present no more than three times in at least 20% of the examined samples were removed. To compare alpha diversity indexes across populations, the Mann-Whitney-Wilcoxon test was performed for each metric, while the PERMANOVA test was computed on beta diversity values. Metagenomic data were also used to construct a one hundred random forest classifiers (RFC) using a range of 50-5,000 trees from the *randomForest* package (Liaw & Wiener, 2002). Differences in taxa abundance across the studied populations were assessed by using the *DESeq2* package, with the Wald test and by applying multiple testing correction via the computation of false discovery rates (FDRs) with the Benjamini-Hochenberg method (Anders & Huber, 2010). Only OTUs with adjusted p-values < 0.05 were considered as significant. Network analysis was performed on the core microbiota at the genus level for each population (by identifying genera present at 80% in each population with the *core* function of the *microbiome* package). Edges were then calculated on Pearsons' correlation and plotted through the *igraph* package. Sequences identified as belonging to the *Treponema* genera were specifically aligned using the BLAST.

Furthermore, to gain more insights into the biological processes in which the identified bacterial groups are involved, inferences on their functions were drawn from the 16S rDNA dataset by means of the PICRUSt v.1.1.0 tool (Langille *et al.*, 2013). Functional predictions were then analyzed thought the HUMAnN v0.99 program to identify pathways already annotated in the KEGG database (Abubucker *et al.*, 2012). Finally, to identify KEGGs biomarkers, the linear discriminant effect size (LEfSe) was computed (Segata *et al.*, 2011) with α value equal to 0.05 and a logarithmic LDA score threshold of 2.0.

Acknowledgements

We would like to thank the Sherpa and Tamang communities of the Gaurishankar Conservation Area (Nepal) and the Aymara people of La Paz (Bolivia) for having made this study possible. We also thank the members of the Mount Everest Summitter's Club and of the Explora Nunaat International team who took part to the Extreme Malangur 2015 expedition, and Agnese Gori for the support in elaboration of nutritional analysis. This study was supported by the Italian Ministry for University and Research (MIUR) RFO 2015-2016 grant to M.S. G.A.G.R. and S.D.F. were supported by the ERC-2011-AdG295733 to D.PTT.

Author Contributions

AQ: metagenomic data analysis
MDP, AQ, GDA: performed experiments
MS and GAGR: evaluation of population genetic background
AQ, MDP, MS, GDA, CDF and DL: wrote manuscript
DL, CDF, MS: study conception and design
MS, PTS, MGS, GM, LN, MDM, DPel: Nepalese samples collection
PDC: Bolivian samples collection
LMP, DPV and GDA: data acquisition
LMP, DPV, PDC, MS, GAGR, GDA, SDF and DPTT: revision of the manuscript
All authors read and approved the final version of the manuscript.

References

Abubucker S., Segata N., Goll J. *et al.* 2012. Metabolic Reconstruction for Metagenomic Data and Its Application to the Human Microbiome. *PLoS Comput. Biol.,* 8: e1002358. doi:10.1371/journal.pcbi.1002358.

Adler C. J., Dobney K., Weyrich L. S. *et al.* 2013. Sequencing ancient calcified dental plaque shows changes in oral microbiota with dietary shifts of the Neolithic and Industrial

revolutions. *Nat. Genet.*, 45: 450–455 455e1. doi:10.1038/ng.2536.

Akey J. M., Eberle M. A., Rieder M. J. *et al.* 2004. Population history and natural selection shape patterns of genetic variation in 132 genes. *PLoS Biol.*, 2: e286. doi:10.1371/journal.pbio.0020286.

Anders S. & Huber W. 2010. Differential expression analysis for sequence count data. *Genome Biol.,* 11: R106. doi:10.1186/gb-2010-11-10-r106.

Beall C. M. 2007. Two routes to functional adaptation: Tibetan and Andean high-altitude natives. *Proc. Natl. Acad. Sci.USA*, 104: 8655–8660. doi:10.1073/pnas.0701985104.

Beall C. M., Cavalleri G. L,. Deng L. *et al.* 2010. Natural selection on EPAS1 HIF2alpha associated with low hemoglobin concentration in Tibetan highlanders. *Proc. Natl. Acad. Sci. USA*, 107: 11459–11464. doi:10.1073/pnas.1002443107.

Boyer S. J. & Blume F. D. 1984. Weight loss and changes in body composition at high altitude. *J. Appl. Physiol.*, 57: 1580–1585. doi:10.1152/jappl.1984.57.5.1580.

Brown E. A. 2012. Genetic explorations of recent human metabolic adaptations: hypotheses and evidence. *Biol. Rev. Camb. Philos. Soc.*, 87: 838–855. doi:10.1111/j.1469-185X.2012.00227.x.

Caporaso J. G., Bittinger K., Bushman F. D. *et al.* 2010a. PyNAST: a flexible tool for aligning sequences to a template alignment. *Bioinformatics*, 26: 266–267. doi:10.1093/bioinformatics/btp636.

Caporaso J. G., Kuczynski J., Stombaugh J. *et al.* 2010b. QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods*, 7: 335–336. doi:10.1038/nmeth.f.303.

De Filippo C., Cavalieri D., Di Paola M. *et al.* 2010. Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. *Proc. Natl. Acad. Sci. USA*, 107: 14691–14696. doi:10.1073/pnas.1005963107.

De Filippo C., Di Paola M., Ramazzotti M. *et al.* 2017. Diet Environments and Gut Microbiota. A Preliminary Investigation in Children Living in Rural and Urban Burkina Faso and Italy. *Front. Microbiol.*, 8. doi:10.3389/fmicb.2017.01979.

DeSantis T. Z., Hugenholtz P,. Keller K. *et al.* 2006. NAST: a multiple sequence alignment server for comparative analysis of 16S rRNA genes. *Nucleic Acids Res.*, 34: W394-399. doi:10.1093/nar/gkl244.

Fala A. M., Oliveira J. F,. Adamoski D. *et al.* 2015. Unsaturated fatty acids as high-affinity ligands of the C-terminal Per-ARNT-Sim domain from the Hypoxia-inducible factor 3α. *Sci. Rep.*, 5: 12698. doi:10.1038/srep12698.

Flint H. J., Bayer E. A., Rincon M. T. *et al.* 2008. Polysaccharide utilization by gut bacteria: potential for new insights from genomic analysis. *Nat. Rev. Microbiol.*, 6: 121–131. doi:10.1038/nrmicro1817.

Fortina M. G., Ricci G., Foschino R. *et al.* 2007. Phenotypic typing technological properties and safety aspects of Lactococcus garvieae strains from dairy environments. *J. Appl. Microbiol.*, 103: 445–453. doi:10.1111/j.1365-2672.2006.03265.x.

Fumagalli M. & Sironi M. 2014. Human genome variability natural selection and infectious diseases. *Curr. Opin. Immunol.* 30 9–16. doi:10.1016/j.coi.2014.05.001.

Gilbert-Kawai E. T., Milledge J. S., Grocott M. P. W. & Martin D. S. 2014. King of the mountains: Tibetan and Sherpa physiological adaptations for life at high altitude. *Physiol. Bethesda Md*, 29: 388–402. doi:10.1152/physiol.00018.2014.

Gnecchi-Ruscone G. A., Abondio P., De Fanti S. *et al.* 2018. Evidence of Polygenic Adaptation to High Altitude from Tibetan and Sherpa Genomes. *Genome Biol. Evol.*, 10: 2919–2930. doi:10.1093/gbe/evy233.

Gnecchi-Ruscone G. A., Jeong C., De Fanti S. *et al.* 2017. The genomic landscape of Nepalese Tibeto-Burmans reveals new insights into the recent peopling of Southern Himalayas. *Sci. Rep.*, 7: 15512. doi:10.1038/s41598-017-15862-z.

Gnecchi-Ruscone G. A., Sarno S., De Fanti S. *et al.* 2019. Dissecting the Pre-Columbian genomic ancestry of Native Americans along the Andes-Amazonia divide. *Mol. Biol. Evol.* under review.

Guo X., Long R., Kreuzer M. *et al.* 2014. Importance of functional ingredients in yak milk-derived food on health of Tibetan nomads living under high-altitude stress: a review. *Crit. Rev. Food Sci. Nutr.*, 54: 292–302. doi:10.1080/10408398.2011.584134.

Hasselbalch S. G., Knudsen G. M., Jakobsen J. *et al.* 1995. Blood-brain barrier permeability of glucose and ketone bodies during short-term starvation in humans. *Am. J. Physiol.*, 268: E1161-1166. doi:10.1152/ajpendo.1995.268.6.E1161.

Jari Oksanen F., Guillaume B., Michael F. *et al.* 2018. vegan: Community Ecology Package. R package version 2.5-2. *HttpsCRANR-Proj.*

Jeong C., Peter B. M., Basnyat B. *et al.* 2017. A longitudinal cline characterizes the genetic structure of human populations in the Tibetan plateau. *PloS One*, 12: e0175885. doi:10.1371/journal.pone.0175885.

Jha A. R., Davenport E. R., Gautam Y. *et al.* 2018. Gut microbiome transition across a lifestyle gradient in Himalaya. doi:10.1101/253450.

Kageyama A. & Benno Y. 2000. Catenibacterium mitsuokai gen. nov. sp. nov. a Gram-positive anaerobic bacterium isolated from human faeces. *Int. J. Syst. Evol. Microbiol.*, 50: 1595–1599. doi:10.1099/00207713-50-4-1595.

Kayser M., Brauer S. & Stoneking M. 2003. A genome scan to detect candidate regions influenced by local natural selection in human populations. *Mol. Biol. Evol.*, 20: 893–900. doi:10.1093/molbev/msg092.

Kier G., Kreft H., Lee T. M. *et al.* 2009. A global assessment of endemism and species richness across island and mainland regions. *Proc. Natl. Acad. Sci. USA*, 106: 9322–9327. doi:10.1073/pnas.0810306106.

Klindworth A., Pruesse E,. Schweer T. *et al.* 2013. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Res.*, 41: e1. doi:10.1093/nar/gks808.

Lahti L., Salojärvi J., Salonen A. *et al.* 2014. Tipping elements in the human intestinal ecosystem. *Nat. Commun.*, 5: 4344. doi:10.1038/ncomms5344.

Lan D., Ji W. Lin B., Chen Y. *et al.* 2017. Correlations between gut microbiota community structures of Tibetans and geography. *Sci. Rep.*, 7. doi:10.1038/s41598-017-17194-4.

Langille M. G. I., Zaneveld J., Caporaso J. G. *et al.* 2013. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nat. Biotechnol.*, 31: 814–821. doi:10.1038/nbt.2676.

Li L. & Zhao X. 2015. Comparative analyses of fecal microbiota in Tibetan and Chinese Han living at low or high altitude by barcoded 454 pyrosequencing. *Sci. Rep.*, 5: 14682. doi:10.1038/srep14682.

Liaw A. & Wiener M. 2002. Classification and Regression by random Forest. *R News*, 23: 18–22.

López V., Moraga F. A., Llanos A. J. *et al.* 2018. Plasmatic Concentrations of ADMA and Homocystein in Llama Lama glama and Regulation of Arginase Type II: An Animal Resistent to the Development of Pulmonary Hypertension Induced by Hypoxia. *Front. Physiol.*, 9: 606. doi:10.3389/fphys.2018.00606.

Martínez I., Stegen J. C., Maldonado-Gómez M. X. *et al.* 2015. The Gut Microbiota of Rural Papua New Guineans: Composition Diversity Patterns and Ecological Processes. *Cell Rep.*, 11: 527–538. doi:10.1016/j.celrep.2015.03.049.

Masuda R., Monahan J. W. & Kashiwaya Y. 2005. D-beta-hydroxybutyrate is neuroprotective against hypoxia in serum-free hippocampal primary cultures. *J. Neurosci. Res.*, 80: 501–509. doi:10.1002/jnr.20464.

McMurdie P. J. & Holmes S. 2013. phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. *PLoS One*, 8: e61217. doi:10.1371/journal.pone.0061217.

Moeller A. H., Caro-Quintero A. Mjungu D., *et al.* 2016. Cospeciation of gut microbiota with hominids. *Science*, 353: 380–382. doi:10.1126/science.aaf3951.

Murray A. J. & Montgomery H. E. 2014. How wasting is saving: weight loss at altitude might result from an evolutionary adaptation. *BioEssays News Rev. Mol. Cell. Dev. Biol.*, 36: 721–729. doi:10.1002/bies.201400042.

Pluznick J. L. 2017. Microbial Short-Chain Fatty Acids and Blood Pressure Regulation. *Curr. Hypertens. Rep.*, 19. doi:10.1007/s11906-017-0722-5.

Puchowicz M. A., Zechel J. L., Valerio J. *et al.* 2008. Neuroprotection in diet-induced ketotic rat brain after focal ischemia. *J. Cereb. Blood Flow Metab. Off. J. Int. Soc. Cereb. Blood Flow Metab.*, 28: 1907–1916. doi:10.1038/jcbfm.2008.79.

Qiu Q., Zhang G., Ma T. *et al.* 2012. The yak genome and adaptation to life at high altitude. *Nat. Genet.*, 44: 946–949. doi:10.1038/ng.2343.

Quagliariello A., De Fanti S., Giuliani C. *et al.* 2017. Multiple selective events at the PRDM16 functional pathway shaped adaptation of western European populations to different climate conditions. *J. Anthropol. Sci.*, 95: 235–247. doi:10.4436/JASS.95011.

Raizada M. K., Joe B., Bryan N. S. *et al.* 2017. Report of the National Heart Lung and Blood Institute Working Group on the Role of Microbiota in Blood Pressure Regulation: Current Status and Future Directions. *Hypertension*, 70: 479–485. doi:10.1161/HYPERTENSIONAHA.117.09699.

Reynolds R. D. & Natta C. L. 1985. Depressed plasma pyridoxal phosphate concentrations in adult asthmatics. *Am. J. Clin. Nutr.*, 41: 684–688. doi:10.1093/ajcn/41.4.684.

Rognes T., Flouri T., Nichols B. *et al.*. 2016. VSEARCH: a versatile open source tool for metagenomics. *PeerJ*, 4: e2584. doi:10.7717/peerj.2584.

Rose M. S., Houston C. S., Fulco C. S. *et al.* 1988. Operation Everest. II: Nutrition and body composition. *J. Appl. Physiol. Bethesda Md 1985*, 65: 2545–2551. doi:10.1152/jappl.1988.65.6.2545.

Rosenberg E. & Zilber-Rosenberg I. 2018. The hologenome concept of evolution after 10 years. *Microbiome*, 6: 78. doi:10.1186/s40168-018-0457-9.

Rosenberg J., Ischebeck T. & Commichau F. M. 2017. Vitamin B6 metabolism in microbes and approaches for fermentative production. *Biotechnol. Adv.*, 35: 31–40. doi:10.1016/j.biotechadv.2016.11.004.

Sanchez A. M. J., Csibi A., Raibon A. *et al.* 2012. AMPK promotes skeletal muscle autophagy through activation of forkhead FoxO3a and interaction with Ulk1. *J. Cell. Biochem.*, 113: 695–710. doi:10.1002/jcb.23399.

Sazzini M., Schiavo G., De Fanti S. *et al.* 2014. Searching for signatures of cold adaptations in modern and archaic humans: hints from the brown adipose tissue genes. *Heredity*, 113: 259–267. doi:10.1038/hdy.2014.24.

Schnorr S. L., Candela M., Rampelli S. *et al.* 2014. Gut microbiome of the Hadza hunter-gatherers. *Nat. Commun.*, 5: 3654. doi:10.1038/ncomms4654.

Segata N., Izard J., Waldron L. *et al.* 2011. Metagenomic biomarker discovery and explanation. *Genome Biol.*, 12: R60. doi:10.1186/gb-2011-12-6-r60.

Semenza G. L. 2007. Hypoxia-inducible factor 1 HIF-1 pathway. *Sci. STKE Signal Transduct. Knowl. Environ.* 2007 cm8. doi:10.1126/stke.4072007cm8.

Semenza G. L. 2011. Hypoxia-inducible factor 1: regulator of mitochondrial metabolism and mediator of ischemic preconditioning. *Biochim. Biophys. Acta*, 1813: 1263–1268. doi:10.1016/j.bbamcr.2010.08.006.

Simonson T. S., Yang Y., Huff C. D. *et al.* 2010. Genetic evidence for high-altitude adaptation in Tibet. *Science*, 329: 72–75. doi:10.1126/science.1189406.

Storz J. F., Payseur B. A. & Nachman M. W. 2004. Genome scans of DNA variability in humans reveal evidence for selective sweeps outside of Africa. *Mol. Biol. Evol.*, 21: 1800–1811. doi:10.1093/molbev/msh192.

Tang X., Tian Q., Cheng X. *et al.* 2013. Bifidobacterial growth-promoting effect of yak milk κ-casein hydrolysates produced with different proteases. *Int. J. Food Sci. Technol.*, 48: 1682–1687. doi:10.1111/ijfs.12138.

Veech R. L. 2004. The therapeutic implications of ketone bodies: the effects of ketone bodies in pathological conditions: ketosis ketogenic diet redox states insulin resistance and

mitochondrial metabolism. *Prostaglandins Leukot. Essent. Fatty Acids*, 70: 309–319. doi:10.1016/j.plefa.2003.09.007.

Vetaas O. R. & Grytnes J.-A. 2002. Distribution of vascular plant species richness and endemic richness along the Himalayan elevation gradient in Nepal: Diversity along the Himalayan elevation gradient. *Glob. Ecol. Biogeogr.*, 11: 291–301. doi:10.1046/j.1466-822X.2002.00297.x.

Warinner C., Rodrigues J. F. M., Vyas R. *et al.* 2014. Pathogens and host immunity in the ancient human oral cavity. *Nat. Genet.*, 46: 336–344. doi:10.1038/ng.2906.

Westerterp K. R. & Kayser B. 2006. Body mass regulation at altitude. *Eur. J. Gastroenterol. Hepatol.*, 18: 1–3.

Wheaton W. W. & Chandel N. S. 2011. Hypoxia. 2. Hypoxia regulates cellular metabolism. *Am. J. Physiol. Cell Physiol.*, 300: C385-393. doi:10.1152/ajpcell.00485.2010.

Wickham H. 2016. *ggplot2: Elegant Graphics for Data Analysis*. Springer-Verlag New York.

Wikoff W. R., Anfora A. T., Liu J. *et al.* 2009. Metabolomics analysis reveals large effects of gut microflora on mammalian blood metabolites. *Proc. Natl. Acad. Sci. USA*, 106: 3698–3703. doi:10.1073/pnas.0812874106.

Wu H., Tremaroli V. & Bäckhed F. 2015. Linking Microbiota to Human Diseases: A Systems Biology Perspective. *Trends Endocrinol. Metab. TEM*, 26: 758–770. doi:10.1016/j.tem.2015.09.011.

Wu W., K. Chen C., C. Panyod S. *et al.* 2019. Optimization of fecal sample processing for microbiome study - The journey from bathroom to bench. *J. Formos. Med. Assoc. Taiwan Yi Zhi*, 118: 545–555. doi:10.1016/j.jfma.2018.02.005.

Xu S., Li S., Yang Y. *et al.* 2011. A genome-wide search for signals of high-altitude adaptation in Tibetans. *Mol. Biol. Evol.*, 28: 1003–1011. doi:10.1093/molbev/msq277.

Yatsunenko T., Rey F. E., Manary M. J. *et al.* 2012. Human gut microbiome viewed across age and geography. *Nature*, 486: 222–227. doi:10.1038/nature11053.

Yi X., Liang Y., Huerta-Sanchez E. *et al.* 2010. Sequencing of 50 human exomes reveals adaptation to high altitude. *Science*, 329: 75–78. doi:10.1126/science.1190371.

Yu Y., Zhou Y., Cheng T. *et al.* 2016. Hypoxia enhances tenocyte differentiation of adipose-derived mesenchymal stem cells by inducing hypoxia-inducible factor-1α in a co-culture system. *Cell Prolif.*, 49: 173–184. doi:10.1111/cpr.12250.

Editor, Giovanni Destro Bisol