Letter to the Editor

Hypobaric-hypoxia Induces Alteration in Microbes and Microbes-associated Enzyme Profile in Rat Colonic Samples

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Present study deals with the straight impact of hypobaric hypoxia on the quantity and composition of some predominant fecal microflora and its functional aspects. For that, isolated fecal contents of rat were exposed to two different simulated air pressures (70 kPa and 40 kPa) for different time durations (1, 3, and 5 h) and the bacterial community composition was compared with normobaric groups (101.3 kPa). It was found that the total anaerobes, *Escherichia coli, Enterbacters spp., Bifidobacterium spp., Clostridium spp.* were increased whereas total aerobes were decreased at both hypobaric treatments. The increased number of amplicon was detected in the pressure-treated groups than the control that clearly mentioned the disruption of microbiota structure at different simulated hypobaric-hypoxia. The amylase, protease, tannase, β-glucuronidase, and alkaline phosphatase activities were increased at these atmospheric pressures. Thus, the present investigation demonstrates that the hypobaric hypoxia is an important environmental factor which can strongly modulate the composition of intestinal flora as well as microflora-derived functional aspects.

Hypobaric hypoxia is a hallmark environmental state at high altitude area and people are known to develop varieties of physiological complications in such conditions. The integrity of hypobaric stresses is solely dependent on to the elevation of altitude from sea level [an increase of 1 km above sea level drops 10 kilopascal (kPa) air pressure; with a sea level air pressure of 101.3 kPa (at 15 °C and 0% humidity)]. Amongst several patho-physiological disorders, gastrointestinal (GI) turmoil develops and it is collectively called as altitude-related sickness (ARS). Symptom of GI disorders includes indigestion, acid and gas (flatus) formation, headache, anorexia, nausea with or without vomiting, fatigue, etc.[1].

As it is well-known, GI tract is the 'enchanting bed' for vast and diverse type of bacterial community[2]. Functionally, they are very active and have profound impact on human health and diseases[3]. The intricate relationship between gut flora and brain is known to maintain the homeostasis of the whole physiology[4-5]. The colonization of gastrointestinal flora is driven by the numerous host-induced and exogenous factors[5]. It is clear that gut microbiota appears to contribute nearly at every aspect of the host's growth and development. Concurrently, imbalance in composition, numbers, or changing habitat of this microbiota initiates a tremendous array of dysfunctions by an array of altered factor. That is why the altitude related GI problems are believed to arise mainly as the cause of altered microbial ecology. Considering this, the present study has been undertaken to analyze changes in microbial structure under hypobaric hypoxia.

The main purpose of our study was to identify the direct impacts of graded hypobaric hypoxia (70 kPa and 40 kPa which are above ~3 and ~7 km altitude from the sea level, respectively) on the microbial populations in the rat faeces using culture-dependent as well as metagenomic approach. Apart from microbial population analysis, the functional integrity of different microbiota associated enzymes like α-amylase, protease, β-glucuronidase and tannase were also evaluated.

The study was conducted with the ethical approval from the Vidyasagar University Ethics Committee. Animals were reared and fed according to suggested rules of the committee. Healthy male albino rats (*n*=30, 10 at each group including control) with average body weight 115.0±4.0 g were used in the present study. They were housed in metal-made cages, accessed to sterile rat feed and water *ad libitum*. Rat faeces (~1 g) were collected just after dropping onto clean paper underlying the cage. For anaerobic sampling, it was suspended immediately in 10 mL of sterilized phosphate-buffer saline (PBS; pH 7.0 and 9 g L^{-1} NaCl) supplemented with sodium thioglycollate (0.02 g) and sodium formaldehyde sulphoxylate (0.01 g) and homogenized. Another

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solution was prepared only with PBS solution for aerobes. By a pressure simulation chamber, homogenates were exposed at two different hypobaric hypoxic conditions [70 kPa (14.5% O_2 isobaric) and 40 kPa (8.0% O_2 isobaric) with decompressed air content, these are respectively 1.5 and 2.0 times lower than normal atmospheric pressure] for different time periods (1, 3, and 5 h). The suspension was centrifuged (1000×g for 5 min) and the clear supernatant was collected and used for microbial analysis[5]. Microbial load was expressed as log of colony forming units (cfu)/g of wet feces and growth direction index (gdi) was calculated using the formulae previously described by Maity et al.[5].

Genomic DNA was extracted from rat faeces using the Bioline genomic DNA isolation Kit (Bioline, India) following user's instructions. The flanked conserved sequences of the variable V3 region of the 16S rDNA were amplified by GC-clamped universal primer and used for DGGE analysis (341F, 5'CCT ACGGGAGGCAGCAGCCG3' and 907R, 5'TCAATTCMTT TGAGTTT3' and GC clamp, CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGG GGGG 3' end of the forward primer). The reactions program was described previously by Muyzer et al.[6]. The cell free supernatant was used for the assay of different enzymes. Amylase, protease, β-glucuronidase, and tannase were assayed using starch, casein, phenolphthalein para-nitrophenyl β-D-glucuronide and tannic acid as substrate respectively. The activity was expressed as unit per gram of wet faeces (U g^{-1}).

It was observed that rat faeces occupy (cfu g^{-1} of wet content) total aerobes of 8.70×10^6, total anaerobes of 3.63×10^{11}, *E. coli* of 1.58×10^5, *Enterobacters spp*. of 2.75×10^4, *Bifidobacterium spp*. of 3.98×10^4, *Clostridium perfringens* of 3.54×10^5 and *Lactobacillus spp*. of 1.58×10^4 (Table 1) at normobaric air pressure. The population density of total aerobes was reduced and the growth direction index was found to descend by the trivial modification (1.00, -1.01, -1.04 at 70 kPa and -1.02, -1.05, -1.07 at 40 kPa after 1, 3, and 5 h). The number of anaerobes were heightened polynomially up to 14.32 folds (5.2×1012 cfu g^{-1}) at 70 kPa and 35.81 folds (1.3×10^{13} cfu g^{-1}) at 40 kPa after 5 h exposure in comparison with the control (P<0.05) (Table 1). Growth direction index also showed their rapid expansion with the values of 1.00, 1.00, 1.09 (at 70 kPa) and 1.00, 1.06, 1.13 (at 40 kPa) after 1, 3, and 5 h (Figure 1) respectively. The results clearly indicated the impact of hypobaric hypoxia which remarkably influenced the stable microbial ecology of rat faeces with an expansion of facultative anaerobes and total anaerobes by dose and duration dependent manner. Hypoxia at reduced atmospheric pressure may implement directly to the cellular metabolism of aerobes present in the feces, besides that hypoxia alters the redox of the surrounding microenvironment, which distorted the electron balance of the functional cellular proteins. In case of anaerobes, the reduced microenvironment possibly induced the metabolic activities and thus their proliferation, but the mechanism lay possibly at transcription level where, rate limiting enzymes are over-expressed.

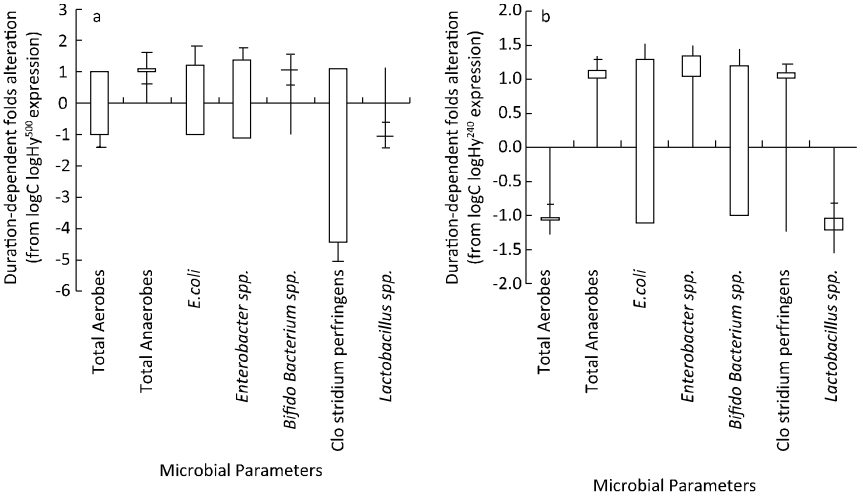

Figure 1. Changes of growth direction index (gdi) of different bacteria in faecal contents during exposure of 70 kPa ($logHy^{500}$) (a) and 40 kPa ($logHy^{240}$) (b) air pressure for different time duration (1, 3, and 5 h). The pattern of expression towards positive site (along with y-axis) indicating growth expansion and towards negative direction indicating growth contraction from the control (logC).

E. coli and *Enterobacters spp*. have been shown to survive surprisingly throughout the hypoxic exposure as they played some pivotal and adaptive role in these conditions. The population density of *E.coli* was increased 10.00 and 34.74 folds after 5 h of exposure at 70 and 40 kPa respectively. This increment was achieved after a brief shock at 1 h exposure (Table 1). The growth direction index was of -1.04, 1.01, 1.19 and -1.11, 1.11, 1.29 after 1, 3, and 5 h of exposure at 70 and 40 kPa air pressure (Figure 1) respectively. *Enterobacters spp*. was also increased upto 34.18 and 35.63 folds after 5 h of exposure at both the applied pressure (Table 1) than their normal count (P<0.05). *Enterobacters spp*. showed gdi as -1.18, 1.10, 1.34 and 1.03, 1.30, 1.34 after 1, 3, and 5 h of exposure at 70 and 40 kPa air pressure (Figure 1) respectively. A mechanism related to the expression of hypoxia induced factor may be regulated at the transcriptional level, which underlie acclimation/acclimatization and potentially enhanced hypoxic survival. Specially, *E. coli* have very complex adaptation character in broad and extreme environment. The variation of microgravity induces the expression of over 50 genes in *E. coli*, most of which are regulated the expression of sigma factor RpoS (s) and some controlled the expression of universal stress resistant protein (UspA, RpoS, etc.)[7]. Pokorny et al.[8] previously reported the alteration of growth, cytoplasmic membrane polarization and total cellular fatty acid composition of *E. coli* JM109 (Gram-negative) and *Bacillus subtilis* (Gram-positive) under hypobaric conditions.

Bifidobacterium spp. and lactic acid bacteria (LAB) are important pharmaco-biotic organism and predominant at early stage of microbiome development in the intestinal tract. *Bifidobacterium spp.* increased polynomially at graded hypoxic air pressures in duration-depended manner (P<0.05) (Table 1). The gdi showed the expansion in their population by the gdi of -1.00, 1.07, 1.17 at 70 kPa and -1.00, 1.13, 1.21 at 40 kPa (Figure 1A and 1B) respectively after, 1, 3, and 5 h. Whereas, population of *Lactobacillus spp*. was remained constant at 70 kPa but gradually decreased at 40 kPa (Table 1). The growth pattern was followed by a complex gdi (-1.09, 1.12, -1.04 and 1.05, -1.20, -1.41 at 70 and 40 kPa respectively (Figure 1). *Clostridium perfringens* was increased polynomially (P<0.05) after an initial reduction (Table 1). The gdi was as 1.01, -1.05, 1.06 at 70 kPa and 1.00, -1.02, 1.12 at 40 kPa (Figure 1) respectively at 1, 3 and 5 h exposure to hypoxic air pressure. Kawasaki et al.[9] was pointed about the multiplication of *Bifidobacterium spp*. by an increased level of anaerobiosis. But, *Lactobacillus spp*. was shown to be unresponsive and the exact mechanism behind this is unclear. Clostridium belongs to strict anaerobe and highly dependent on the reduced redox potential for their multiplication. At Hypobaric hypoxia, increase in *C. perfringens* has pathological impact in the intestine particularly to initiate the intestinal inflammation from mild to chronic stage.

Table 1. Changes in Microbial Load in Faecal Samples of Rat after *in vitro* Treatments of Graded Hypobaric (70 and 40 kPa) Conditions for Different Time Durations (1, 3, & 5 h), Data Represented as Mean±SD

Microbial Parameters	Control (log cfu g^{-1})	Pressures (kPa)	Population Load (log cfu g^{-1}) at Different Durations (h)		
			1 h	3 h	5 h
Total aerobes (M)	6.95^{a}±0.07	70	6.95^{a}±0.07	6.85^{b}±0.07	6.67^{d}±0.07
		40	6.80^{c}±0.05	6.61^{d}±0.05	6.32^{e}±0.04
Total anaerobes (N)	11.57^{d}±0.26	70	11.58^{d}±0.26	11.57^{d}±0.26	12.71^{b}±0.28
		40	11.57^{d}±0.12	12.37^{c}±0.13	13.11^{a}±0.14
E. coli (O)	5.20^{e}±0.13	70	4.96^{f}±0.13	5.26^{d}±0.14	6.20^{b}±0.16
		40	4.68^{g}±0.15	5.80^{c}±0.18	6.74^{a}±0.21
Enterobacters spp. (P)	4.45^{e}±0.13	70	3.76^{f}±0.09	4.91^{c}±0.12	5.97^{a}±0.15
		40	4.59^{d}±0.18	5.81^{b}±0.23	5.99^{a}±0.24
Bifidobacterium spp.(Q)	4.60^{e}±0.14	70	4.58^{e}±0.14	4.95^{d}±0.15	5.40^{b}±0.17
		40	4.57^{e}±0.15	5.23^{c}±0.18	5.59^{a}±0.19
Clostridium perfringens(R)	5.56^{c}±0.19	70	5.61^{c}±0.19	5.28^{e}±0.18	5.92^{b}±0.20
		40	5.58^{c}±0.19	5.43^{d}±0.19	6.23^{a}±0.22
Lactobacillus spp.(S)	4.20^{c}±0.18	70	3.83^{e}±0.16	4.72^{a}±0.20	4.04^{d}±0.17
		40	4.43^{b}±0.18	3.49^{f}±0.14	2.97^{g}±0.12

Note. Values within a row followed by different superscripts are significantly different according to ANOVA [P<0.05 (M), Duncan's method; P<0.05 (N), Student-Newman-Keuls method; P<0.05 (O), Student-Newman-Keuls method; P<0.050 (P), Student-Newman-Keuls method; P<0.05 (Q), Student-Newman-Keuls method; P<0.05 (R), Student-Newman-Keuls method; P<0.05 (S), Student-Newman-Keuls method].

A unique gut microbiota composition in the fecal digesta of healthy rats was resolved by containing bands of 42 V3 amplicon. Dice cluster analysis reflected an increase in the number of amplicon which was observed in the pressure treated groups (49 and 51 bands in 70 and 40 kPa air pressure respectively after 5 h exposure) than their control group (Figure 2). The microbial profile of fecal digesta at normobaric pressure (1 h) shared a common pattern among 3 h and 5 h treatment groups (with similarity index of 68 and 58% respectively). A less divergence in band similarity was observed in the faeces of control group whereas others showed a likely divergence among them. The remarkable similarity (≥70%) was observed among 70 kPa-1h to normobaric-5h, 70 kPa-3h to 70 kPa-1h, 70 kPa-5h to 70 kPa-3h, 40 kPa-3h to 40 kPa-1h, and 40 kPa-5h to 70 kPa-3h. On the contrary, the least similarity (≤45%) was observed in the fecal digesta among different time treatments at 40 kPa (at all three durations) (Figure 2). The increase in amplicon number clearly depicts and can be correlated with the increase in total anaerobes population, beyond its boundary of culturing protocol.

It was observed that amylase, protease, β-glucuronidase activity were increased at two different simulated hypobaric air pressures. In normobaric condition, the activity of amylase, protease, β-glucuronidase was 123.56, 66.34, 81.54 U g^{-1} respectively. But at graded hypoxic condition, these were changed to 1.01:0.99:1.19 and 1.07:1.33:1.82; 1.01:1.06:1.19 and 1.06:1.27:1.46; 1.01:1.15:1.46 and 0.99:1.14:1.41 (at 1, 3, and 5 h) at 70 kPa and 40 kPa respectively in response to their control group. Tannase and alkaline phosphatase activity were decreased at hypobaric air pressures. Normally, the activity was 0.741 and 18.23 respectively. In graded pressure, the activity of tannase and alkaline phosphatase was changed to 0.99:0.89:0.81 and 0.96:0.82:0.43; 0.94:0.60:0.17 and 0.82:0.27:0.12 (at 1, 3, and 5 h) at 70 kPa and 40 kPa respectively (Table 2). The amylase activity of the faeces was directly correlated with the hypoxic stress (Table 2) and this indicated that overgrowth of

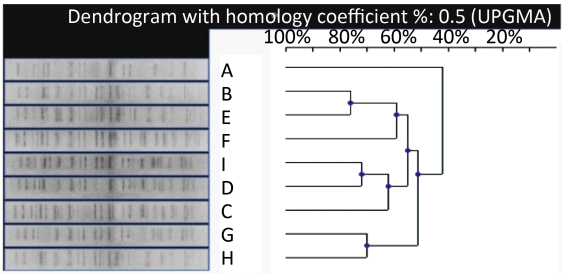

Figure 2. PCR-DGGE profiles of V3 amplicons of the microflora in the rat faecal digesta after the *in vitro* treatment at different hypobaric hypoxic pressures (70 & 40 kPa) for 1, 3, & 5 h exposures. A group of 5 samples from each treatment group were pooled into a single sample. Lane A, B, & C = digesta at normobaric pressure (101.3 kPa) 1, 3, 5 h; Lane D, E, & F = digesta of 70 kPa hypobaric pressure after 1, 3 & 5 h; Lane G, H, & I = digesta of 40 kPa hypobaric pressure after 1, 3, & 5 h.

Table 2. Alteration in Digestive Enzyme Profile in Response to Two Different Hypobaric Pressure Composition (70 and 40 kPa) and Time Duration (1, 3, and 5 h)

Item	70 kPa			40 kPa		
	1 h	3 h	5 h	1 h	3 h	5 h
Amylase	124.49^{e}±3.32	123.1^{f}±1.71	148.17^{c}±1.89	132.24^{d}±1.84	165.13^{b}±1.25	225.29^{a}±1.68
Protease	66.74^{e}±1.85	70.45^{d}±1.58	79.06^{c}±0.944	70.95^{d}±1.45	84.35^{b}±1.82	97.34^{a}±2.80
Tannase	0.74^{a}±0.01	0.66^{c}±0.02	0.60^{d}±0.00	0.71^{b}±0.00	0.60^{d}±0.01	0.32^{e}±0.00
β-glucuronidase	82.55^{d}±0.65	93.84^{c}±2.95	119.52^{a}±6.58	81.11^{d}±0.88	93.25^{c}±5.13	115.14^{b}±4.06
Alkaline phosphatase	17.24^{a}±0.88	11.10^{c}±1.81	3.14^{e}±0.17	15.07^{b}±0.66	5.03^{d}±0.23	2.29^{f}±0.53

Note. Values are the mean of five replicates (±SD) at graded hypobaric hypoxic pressures (70 & 40 kPa) at different time durations (1, 3, and 5 h). The activity of amylase ($F=6069.577$, df=5, 29, $P<0.05$), protease ($F=818.984$, df=5, 29, $P<0.05$), tannase ($F=2084.424$, df=5, 29, $P<0.05$), β-glucuronidase ($F=206.656$, df=5, 29, $P<0.05$) and alkaline phosphatase ($F=1186.503$, df=5, 29, $P<0.05$) was tested by ANOVA (Duncan's multiple range test). Values within a row followed by different lower case letters are significantly different accordingly.

the intestinal anaerobes enhanced the utilization of undigested starch that might increase the acid gas formation ability in the intestine[10]. In altered environmental condition, the inflamed population of microbes tries to harvest energy from alternative carbon sources. The protease and tannase activity were intensified with the increase of hypobaric exposure and this reflected that more energy was harvested from protein and unabsorbed dietary polyphenols. In addition, enhancement of β-glucuronidase activity during graded hypoxic hypobaric conditions may increase the enterohepatic recirculation of toxins, hormones, drugs, carcinogens and the accumulation of local toxic product in the gut lumen. But, the polyphenol transformed into glucose and gallic acid by gut microbial tannase (polyphenoal hydrolase) in which former could serve as a primary carbon source whereas later may contribute to the reduction of the proinflammatory responses.

This *in vitro* study reveals that atmospheric pressure below the ambient pressure drastically alters the composition of gastrointestinal flora at both qualitative and quantitative fashion. Although the selected groups of bacteria are very limited members of the overall microbial population in the gastrointestinal tract, the results from this study indicate that atmospheric pressure has a significant impact on the colonization and ecology of such a group of microflora. Besides this, the functional aspects related to the microbes, i.e., digestive enzyme profile are also changed significantly by that extrinsic factor.

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Biographical note of the first author: Chiranjit MAITY, Ph.D, did his thesis on the impact of atmospheric pressures on gut microbial ecology and studied on the developed patho-physiological conditions.

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