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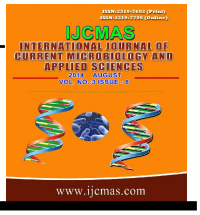

Original Research Article

Impact of Probiotic supplementation on intestinal microflora of rat under environmental hypobaric pressure

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A B S T R A C T

The gastrointestinal tract (GI) is a complex and active network where a sophisticated and mutualistic symbiosis modulates the relationship between the host and the microbiota in order to establish and persuaded gut homeostasis. This study was aimed to clarify the physiological and microbial change in the intestinal microbiota at hypobaric condition and evaluate the improvement of gut microflora including the health condition of rats by probiotic treatment. Twenty four healthy male albino rats randomized in 4 groups; normobaric control, hypobaric control and two types of commercially available probiotic treatment groups (NC, HC, CP1 and CP2 respectively) (n=6). Plasma and faecal sample were used for hematological and microbiological parameter analysis. At hypobaric pressure (429 mm Hg), faecal sample analysis revealed that the count of total aerobes, facultative anaerobes and *Salmonella* spp. increased while those for total anaerobes and lactic acid bacteria (LAB) were decreased at significantly ($p<0.05$). The growth direction index (GDI) and hematological parameter is altered interestingly in probiotic treated group compare to HC group. This result suggests that air pressure is a significant exogenous factor that strongly regulates the composition of the gut microflora and probiotic has potential effect for gut homeostasis at the stress condition and improving health condition by reducing pathogens.

K e y w o r d s

Altitude; GDI; Gut microflora; Hypobaric pressure; Probiotic

Introduction

The gastrointestinal tract of the human and other mammals is populated by a vast and diverse group of microbes in a composite manner (Frank and Pace, 2008; Ito, 2005). Bacteria are the major population of the alimentary tract and this native microflora is commonly designated the "gut microflora" (Ito, 2005). Microbial flora present in the microenvironment of the gastrointestinal tract performs several important and essential activities of the host (Straw, 1989) such as the breakdown of undigested food, metabolism of drugs, enhanced absorption of foodstuffs, synthesis of vitamins, creation of resistance against pathogenic bacteria by colonization resistance, stimulation of host

immunity and induction of intestinal maturation (Mitsuhara *et al*., 2001; Samanta et al., 2004). The gastrointestinal micro ecosystem is always changeable. This condition arises due to the high sensitivity of microflora to frequent host-induced physio-chemical and environmental factors such as antimicrobial agents, disorders of peristalsis, inflammatory bowel diseases, cancer, stress, redox potential, drugs, temperature and nutrients (Heavey and Rowland, 1999; Kleessen et al., 2000). The microflora is also highly sensitive to oxygen tension (Kaye, 1967; Loesche 1969) and correlated with the atmospheric pressure.

Individuals who are exposed to high altitude (HA) are characterized by hypobaric hypoxia environmental conditions that induce several physiological changes like body weight, hematological changes (Paula and Josef, 2012; Winslow et al.,1984), including gastrointestinal disorders in human (Shao and Wan, 2005). Acute mountain sickness (AMS) is a frequent complication for military personnel, veterans, athletes, and travelers at high altitudes. One of the important problems in AMS is the gastrointestinal disorders that consist of indigestion, acid formation, flatulence, vomiting, anorexia, diarrhoea, etc. Irritable bowel syndrome (IBS) in intestine, and high altitude flatus expulsion (HAFE) in rectum are very frequent infection at high altitude (Rook and Brunet, 2005) and others mentioned that these gastric disorders are due to hypobaric hypoxia stress and mediated by local hormones (e.g. leptin and cholecystokinin) and by vagal stimulation. But clear cut or any state forward clue behind these disorders has not yet been explored. Confusion like intestinal flatulence [composed of H_2, carbon dioxide (CO_2) and methane (CH_4)], which is completely a fermentative product of commensally bacteria, not restricted by hormones or neural stimulations (Brock et al., 1982).

Researchers were investigated that by accumulation of probiotic with feed may stimulate early gut development and progress overall efficiency of the intestinal microflora. Alteration of intestinal flora by supplied probiotics, which are bacteria directly influence development of the gut microflora and have been manipulated to achieve increased feed conversion and pathogen reduction (Patel et al., 2005), can improve the metabolism of host animals, develop gut efficiency by escalating nutrient absorption (Gritsenko et al., 2000) and accelerating gut development (Erickson and Hubbard, 2000). Most of probiotic microorganisms belong to Lactic Acid Bacteria (LAB), such as *Lactobacillus spp.*, *Bifidobacterium sp.* and *Enterococcus sp.* Among lactic acid bacteria, *Lactobacillus* has attracted a lot of attention for their potential probiotic effects in human health (Gilmore MS and Ferretti, 2003) like improve humoral immune responses (Isolauri et al.,1993; Perdigón et al., 1998), altered cell numbers within White Blood Cell (WBC) subsets and enhanced phagocytic capacity in the peripheral granulocyte population. Plasma endotoxin concentrations were decreased during probiotic feeding and Red Blood Cells (RBCs) were decreased susceptibility to osmotic pressure (Zoe et al., 2006).

In the present study, quantitative variation of some common bacteria of the feacal sample likes total aerobes and prominent anaerobes, an indicator strain (*Escherichia coli*), Lactic acid bacteria and a pathogenic strain *Salmonella spp.* were studied during exposure of environmental hypobaric pressure on experimental rat model and to search out whether there is any impact of the hypobaric hypoxic conditions of host on the

gastrointestinal lactic acid bacteria (LAB) by modifying the intestinal flora to refrain generation of toxins by probiotics, which are bacteria administered as food components.

Materials and Methods

Animal study

Selection of animals and care

Twenty four healthy, adult, male albino Wistar strain rats weighing 110±12 g (supplied by Ghosh Animal, Animal Foods and Animal Cages Supplier, Kolkata 54) were used. They were acclimatized to laboratory condition for 2 weeks prior to experimentation. Animals were housed six per cage in a temperature-controlled room (24 ± 2 ºC) with 12-12 h dark-light cycle at 60±10% RH. The principle of laboratory animal care of National Institute of Health USA guideline was followed throughout the duration of experiment.

Grouping of animals and experimental procedure

Animals were randomized and divided into four groups of six animals each. Group NC and HC served as control and hypobaric control (Exposed to hypobarometric pressure) and Group CP1, and CP2 was administered with commercially available probiotic VSL3 and Lactobacillus Plus respectively, with adequate supplementation of food and water.

Exposure was carried out in a decompression chamber (Instrumentation India, India) maintained at 10 ± 2.5◦C temperature and 65 ± 10 per cent relative humidity. Group HC, CP1 and CP2 was exposed to 429 mm Hg (555 mbar) pressure equivalent to 15000 feet for 5 h/day for 7 days (Anjana et al., 2012). NC group was exposed to normal room air (normoxia).

Feeding procedure: They were provided with standard boiled rat feed (carbohydrates, 74.05%; proteins, 10.38%; fiber, 2.20%; iron, 56 ppm; calcium, 400 ppm and sodium, 500 ppm) and water *ad libitum* (Maity et al.,2012). While CP1 and CP2 groups were administetived with probiotics *VSL3 cap* (3 × 10^9 cfu/dose/day) (Shibolet et al.,2002) and *Lactobacillus Plus* (1×10^9 cfu/dose/day) (Arpita et al., 2012). (Table-1)

Sample collection

Rat faecal samples were collected just after dropping onto clean paper underlying the cage and collected prior to feeding at everyday during the total experimental period. Fresh faecal sample was suspended in sterilized phosphate-buffered saline (PBS; pH 7.0 and 9 g l^{-1}NaCl) using a manual glass homogenizer for 5 min. After 7 days, all experimental animals from all groups were sacrificed by chloroform anesthesia. Blood samples were collected by hepatic artery punch under diethyl ether anesthesia, using 21 gauge (21 G) needles mounted on a 5ml syringe (Hindustan syringes and medical devices ltd, Faridabad, India.) into heparin coated sample bottles for analyzed Hematological parameters (Arpita et al., 2012).

Analytical measurement

Microbial analysis. The quantities of prominent cultivable microflora were enumerated on the basis of colony-forming units (cfu). The total aerobic and anaerobic faecal bacteria were enumerated by standard pour-plate technique in single-strength trypticase soya agar (TSA, Himedia, India) and reduced Wilkins Chalgren agar (WCA, Micromaster, India), respectively. For anaerobic culture we used an anaerobic jar from which oxygen was removed catalytically before filling it with 10% of

both CO_2 and H_2 gas (Micromaster).Total Lactic acid bacteria were enumerated by a standard spread-plate technique using MRS agar (HiMedia, Mumbai, India). Enumeration of *Escherichia coli.* was carried out using MacConkey agar (HiMedia) (Maity et al.,2009) and Brilliant green agar modified (HiMedia) were used for cultivation *of Salmonella spp.* (Wehr and Frank, 2004).

Growth direction index (GDI). Colony-forming units (cfu) represent the actual number of bacteria present in the faecal sample. These cfu values were converted to their logarithmic value and tallied with the corresponding experimental set of specified conditions. When the log value of control cfu is higher than the log value of test cfu, then GDI is designated as negative and the reverse event is designated as GDI positive. GDI gives the expansion or contraction of bacterial populations in a particular biosystem.

Somatic index and haematological parameter. Body weight of all experimental rats was measured before anaesthesia. Haematological parameters like RBC and WBC count by haemocytometer and hemoglobin (Hb) by standard kit method (Merck, Japan) (Arpita et al., 2012).

Statistical analysis. Collected data are presented as the arithmetic mean of three replicas (mean±SE). The variations in microbial count hematological parameters were examined by one-way ANOVA. The alteration in bacterial quantity was tested by Fisher's t test. Significant variation was accepted at the level of 5%, i.e. $p<0.05$.

Results and Discussion

The effect of hypobaric pressures (429 mm Hg) on the faecal microflora ('0' day, 1st day and 7th day) was evaluated and is presented in Figure 1. In control conditions (normobaric), rat faeces (per gram) contained total aerobes 1.6×10^6, total anaerobes 1.5×10^{11}, total lactic acid bacteria 1.4×10^7, *E. coli* 3.8×10^5 and *Salmonella sp.* 1.3×10^2. When the animals were subjected to lower atmospheric pressure upto 429 mm Hg for seven days (daily 5 h) the count of total aerobes, *E. coli* and *salmonella spp.* were increased while those for total anaerobes and LAB were decreased significantly. The quantity of total aerobes, *E. coli* and *salmonella spp.* was increased up to 1.1×10^2 fold, 0.96×10^2 fold and 10 fold in respect to control group [at 7th day cfu g^{-1} was 1.9×10^8, $3.7 \times 10^7$and 1.3×10^3 respectively (Fig. 1A, 1D, 1E)] and the quantity of total anaerobes and lactic acid bacteria was decreased up to 3.8×10^4 fold and 1.0×10^4 fold in respect to control group [at 7th day cfu g^{-1} was 4.1×10^6 and 1.4×10^3 respectively (Fig. 1B and 1C)]. The changes of the above population were statistically significant, with $p < 0.05$. The growth direction index (GDI) for of total aerobes, E. coli and salmonella spp. moving positive direction (at day 7 $\log HC/\log NC_{(\text{total aerobes})}$=+1.33; $\log HC/\log NC_{(E.\ coli)}$= +1.35; $\log HC/\log NC_{(salmonella\ spp.)}$ = +1.47) and GDI of anaerobes and lactic acid bacteria moving negative direction (at day7 $\log NC/\log HC_{(\text{total anaerobes})}$ = -1.96; $\log NC/\log HC_{(LAB)}$ = -2.26) in respect to control group.

When prebiotic VSL 3 were supplied, the GDI of total aerobes, E. coli and salmonella spp. moving negative direction (at day 7 $\log HC/\log CP1_{(\text{total aerobes})}$ = -1.28; $\log HC/\log CP1_{(E.\ coli)}$ = -1.36;$\log HC/\log CP1_{(salmonella\ spp.)}$ = -4.05) and GDI of anaerobes and lactic acid bacteria moving positive direction (at day 7 $\log CP1/\log HC_{(\text{total anaerobes})}$ = +1.53; $\log CP1/\log HC_{(LAB)}$ = +2.37) with respect to

HC group. GDI of CP2 group of total aerobes, total anaerobes, total LAB, *E.coli.* and *Salmonella spp.* is -1.35, +1.35,+1.97,-1.14 and -4.27 in respect to HC group at 7th day of experiment.

Body weight increased at the end of experiment in NC, CP1 and CP2 groups compared to their initial body weight whereas the body weight of HC group was decreased significantly (7.62%) with respect to initial body weight (Table-2). After administration of the probiotic in group CP1 and CP2, the percentage of body weight was increased (5.99% and 6.41% respectively) although it was significantly lower with compared to NC group (17.14%).

Hemoglobin level, total RBC and total WBC count were significantly increased in HC, CP1 and CP2 groups animals (the pressure treated groups), compared to group NC. But expansion in HC group is much higher with respect to group CP1 and CP2 (Table-3).

Microbial flora present in the microenvironment of the gastrointestinal tract performs several important and essential activities of the host. Changes of host and external environment can cause significant change of intestinal flora (Rao et al., 1998). In the present study we have selected two commercially available probiotics to evaluate the effect on the intestinal microbes present in faecal matter at hypobaric pressure.

The populations of total aerobes were increased after the 7th day of exposure at hypobaric chamber, was nearly 118- fold greater than its normobaric population but when probiotic CP1 and CP2 were supplied, the GDI decreased with respect to hypobaric control which nearly 1.72-fold and 7.65 fold greater than normobaric population and does not differ significantly. The GDI for *E. coli* and *Salmonella spp.* moving positive direction and increased 110-fold, 10-fold whereas GDI of total anaerobes and LAB moving negative direction in respect to NC group. Total aerobes, facultative anaerobes (*E.coli)* and total anaerobes resided in a ratio of $4.36:1:4.03\times10^5$, but this may vary within species and even between individuals in the same species (Atanu et al., 2013).

The lower level of oxygen of gastro intestinal epithelium supported the proliferation of *E. coli* as it possessed elaborate genetic regulatory networks for sensing oxygen (Holý and Chmelař, 2012). Researcher revealed that 6-h immobilization stress initiates the increase of the concentration of *E. coli* in the proximal sections (the duodenum and the jejunum) of the digestive tract.

This rapid expansion of *E. coli* population may encourage the growth of other strict (*Bacteroidetes sp*. and *Lactobacillus sp.)* and pathogen (*Salmonella spp.)* in anaerobic respiration (Gombošov et al., 2011). But it was not clear why the growth of lactic acid bacteria was lower than other anaerobes. When probiotics were supplied the GDI of total aerobes, *E. coli* and *salmonella spp.* moving negative direction and GDI of anaerobes and lactic acid bacteria moving positive direction which was nearby the ratio of control microbial population. It was noted that *Salmonella spp.* was decreased 100-fold with respect to control group because probiotics promote gut health by influencing enterocyte turnover, competing with pathogenic bacteria for nutrients and binding sites, and producing bacteriostatic compounds that limit the growth of pathogenic bacteria (Farthing, 2004; Manning and Gibson, 2004).

Loss of body weight at hypobaric hypoxic condition had been described in several studies (Benso et al., 2007; Wall et al., 2009).

Table.1 Dosages used in the studies of different groups:

Groups	Microbial Additive	No. of Rats	Cfu/ day for 7 days
Normal Control (NC)	No microbe(s)	6	-
Hypobaric Control (HC)	No microbe(s)	6	-
Hypobaric + Commercial Probiotic (CP1)	**VSL-3 cap:** *Streptococcus thermophilus , Bifidobacterium breve , Bifidobacterium longum , Bifidobacterium infantis , Lactobacillus acidophilus , Lactobacillus plantarum , Lactobacillus paracasei , Lactobacillus delbrueckii spp bulgaricus*. Manufacturer: SUN	6	3×10^9
Hypobaric + Commercial Probiotic (CP2)	**Lactobacillus Plus** *Lactobacillus acidophilus, Lactobacillus rhamnosus, Lactobacillus longum, Bifidobacterium bifidum, Saccharomyces boulardii,* Fructo- oligo-saccharide, Manufacturer:Infra	6	1×10^9

*cfu = Colony-forming units

Table.2 Effect of hypobaric pressure on body weight in the four groups

Groups	Initial Body Weight(g)	Final Body Weight (g)	Increases or Decreases in Body Weight (g %)
NC	103.11 ± 2	120.82 ± 3.20	17.14^a ↑
HC	104.01 ± 3.70	96.1 ± 3.77	7.62^b ↓
CP1	107 ± 2.66	113.42 ± 1.86	5.99^c ↑
CP2	102.31 ± 2.69	108.88 ± 1.35	6.41^c ↑

Data are expressed as Mean ± SE (n=6). ANOVA followed by multiple two-tail t-test and data with different superscripts (a, b, c) in a specific vertical column differ from each other significantly ($P< 0.05$).

'↑' indicate increase of body weight and '↓' indicate decrease of body weight.

Table.3 Hematological parameter changes at different condition

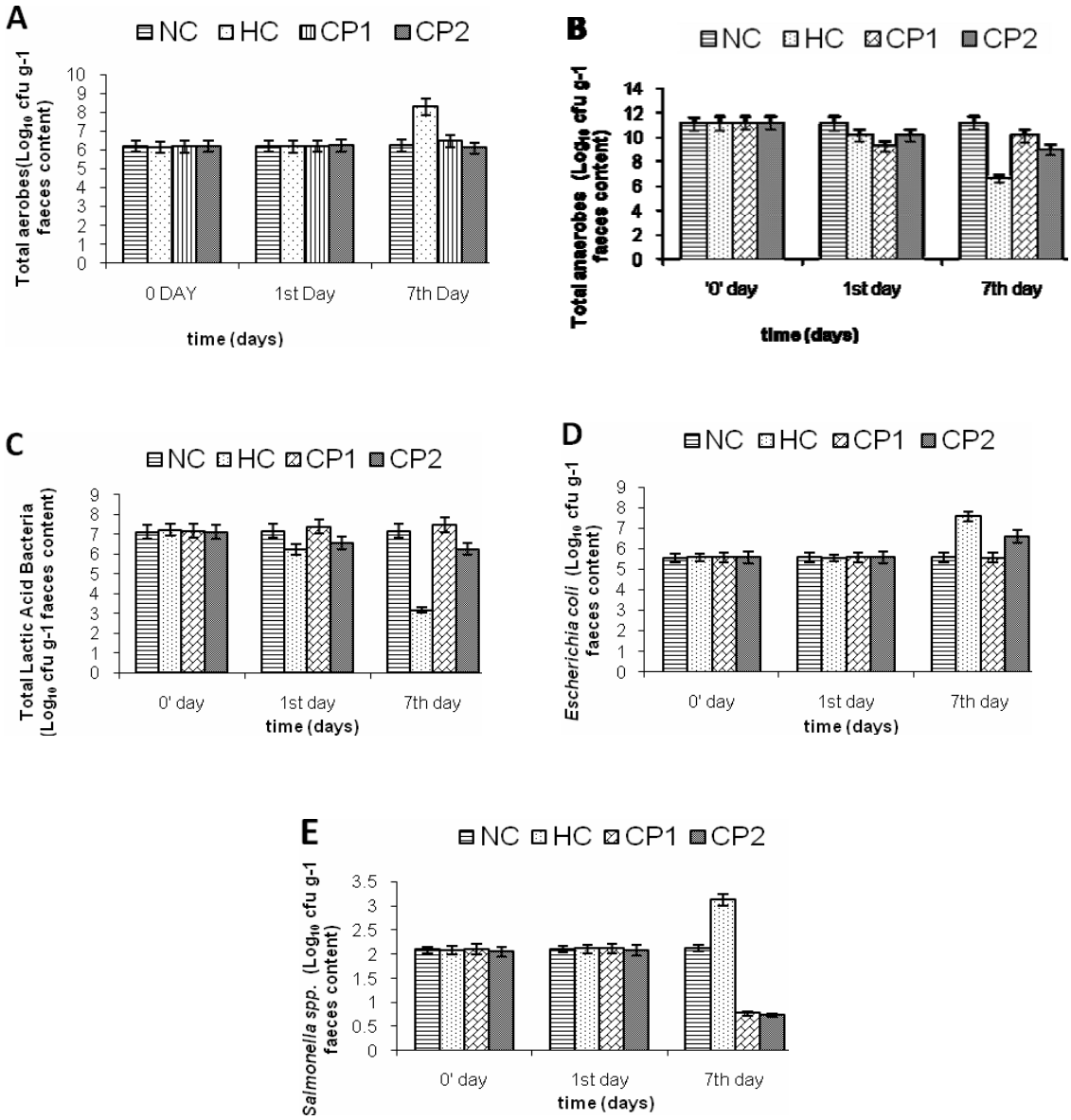
Groups	RBC /cumm×10^6	Hb gm%	WBC /cumm×10^3
NC	6.56 ± 0.72^a	8 ± 0.52^a	8.01 ± 0.57^a
HC	10.17 ± 0.45^b	14.15 ± 0.48^b	13.53 ± 0.32^b
CP1	8.05 ± 0.46^c	10.58 ± 0.37^c	11.06 ± 0.80^c
CP2	7.85 ± 0.31^c	10.93 ± 0.53^c	13.19 ± 0.85^b

Data are expressed as Mean ± SE (n=6). ANOVA followed by multiple two-tail t-test and data with different superscripts (a, b, c) in a specific horizontal column differ from each other significantly ($P< 0.05$).

Group NC: Normobaric control; Group HC: Hypobaric control; Group CP1: Hypobaric + commercial probiotic VSL-3 treated; Group CP2: Hypobaric + commercial probiotic *Lactobacillus* Plus treated.

RBC : Red Blood Cell; Hb: Hemoglobin; WBC : White Blood Cell.

Figure.1 Alteration of population density of total aerobes (A); total Anaerobes (B); total lactic acid bacteria (C); *E. coli* (D); *Salmonella spp.* (E); in the feacal sample of rat of control group (NC) and during exposure of 429 mm Hg air pressure for seven day duration for different group (HC, CP1, CP2). ┬, standard error of mean.



In the present study final body weight of HC groups animals were decreased significantly ($P< 0.05$) with compared to initial body weight (table 2) which may due to higher metabolic rate, different energy output, loss of body water and several endocrine factors. When prebiotics were supplied to CP1 and CP2 groups the final body weight increased (5.99% and 6.41% respectively) though it was not similar to CP group (17.14%). The actual cause for increase of body weight is

unknown. The reason for the improvement in body weight observed in pressure treated animals may be beneficial effect of probiotic bacteria which influence the development of gut microflora (Ravi and Patricia, 2010) and had been manipulated to achieve increased feed conversion and pathogen reduction (Shannon et al., 2002), improved the metabolism of host animals and developed gut efficiency by rising nutrient absorption (Gritsenko et al., 2000).

At hypobaric hypoxic condition partial pressure of oxygen (PO_2) was decreased which cause the excessive secretion of erythropoietin to carry out cellular function by increasing blood RBC and Hb (Mizuno et al., 2008; Steven et al., 2000). It was a great capacity for physiological adjustments to compensate for this reduced pressure gradient. In our study blood RBC, Hb and WBC was increased (table 3) which supported the previous work. It was note that RBC, Hb and WBC of prebiotics feed groups were also increased significantly ($P< 0.05$) with compared to control group but lower than HC group. It may be due to beneficial immunomodulatory effects of probiotic (Zoe et al., 2006).

In conclusion It is clear from the study results that hypobaric hypoxic condition altered the faecal flora and showed that probiotic treated animals were significantly reduced the alteration of gastrointestinal microbes when compared with control group at same hypobaric condition. Although the selected groups of bacteria are very limited members of the overall microbial population in the gastrointestinal tract.

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