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ORIGINAL ARTICLE

Study of the cultivable microflora of the large intestine of the rat under varied environmental hyperbaric pressures

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KEYWORDS

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Background/Purpose(s): We conducted an *in vivo* experiment to investigate the effect of hyperbarometric air pressure on the quantity and composition of the cultivable microflora of the large intestine.

Methods: Using selective culture-based methods, we enumerated from the large intestine total aerobes and total anaerobes, and indicator bacteria such as *Escherichia coli*, other *Enterobacteriaceae*, *Bifidobacterium spp.*, *Lactobacillus spp.* and *Clostridium perfringens*, and studied their quantitative variation.

Results: Total aerobes and facultative anaerobes (*E. coli* and other *Enterobacteriaceae*) were increased with an increase in air pressure, whereas the increase caused a drastic reduction in the numbers of total anaerobes and *Clostridium perfringens*. *Bifidobacterium spp.* and *Lactobacillus spp.* were affected slightly by the altered air pressures. Variation in the numbers of these groups of bacteria was correlated to dose and duration of hyperbaric treatment.

Conclusion: We conclude from our results that air pressure is an important exogenous factor that strongly modulates bacterial colonization of the large intestine and the composition of the intestinal microflora, and that the occurrence of gastrointestinal disorders during hyperbarism is a result of alteration in the indigenous microflora.

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Introduction

The gastrointestinal tract of the human and other mammals is colonized by a vast and diverse group of microbes in a complex manner.^{1,2} Bacteria are the predominant inhabitants of the alimentary tract and this indigenous microflora is popularly designated the "gut microflora".³ The mainly symbiotic and dynamic interaction between host and microbes has profoundly advantageous effects on human health and nutrition.^{4–6} The symbionts are metabolically active and often referred to as a "forgotten organ", in view of their collective activity resembling that of the liver.⁷ Benefits to the host of the microbial activity include, among others, the breakdown of undigested food, metabolism of drugs, synthesis of vitamins, prevention of the establishment of pathogens, induction of host immunity, and stimulation of intestinal maturation.^{8–10} Recent research has revealed an intricate relationship between the gut flora and the brain, and hence implications for the overall physiology of the host.^{11–13} Colonization of the gastrointestinal epithelial lining is disturbed by numerous host-induced^{14,15} and exogenous factors, such as antimicrobial agents,¹⁶ other drugs, disorders of peristalsis, inflammatory bowel diseases, cancer, stress, redox potential, temperature, food contaminants, and others.^{17–19} The gut microflora is also highly sensitive to oxygen and air pressure, as was established in our previous study.⁸

Individuals who undertake certain activities, such as deep-sea diving or digging tunnels beneath a river or in a mine, and passengers in submarines, are exposed to a hyperbaric atmosphere (an increase of 0.1 kPa air pressure per 1 cm drop below sea level, sea-level pressure being 101.3 kPa).^{8,20} This can induce several gastric disorders,^{21,22} of which the most common symptoms are indigestion and acid and gas (flatus) formation.²³ Such problems are mostly associated with the ecological disturbances of gastrointestinal microflora^{8,9} but there is still no detailed record that can correlate variations in composition of the indigenous microflora with atmospheric pressure.

In the present study, quantitative variation of some common bacteria of the large intestine, including total aerobes and prominent anaerobes, an indicator strain (*Escherichia coli*), other Enterobacteriaceae, *Bifidobacterium* spp., *Lactobacillus* spp. and *Clostridium perfringens* were studied during exposure of an experimental model animal to different durations of graded hyperbaric atmospheric pressure.

Methods

Animals and diet

We used healthy male albino rats with an average body weight of 115 ± 7 g. They were housed in metal cages ($34 \times 28 \times 19$ cm³). All animals had access to boiled rat feed (containing carbohydrates (74.05%); proteins (10.38%); fibre (2.20%); iron (56 ppm); calcium (400 ppm) and sodium (500 ppm), and water *ad libitum*. The animals were maintained without interrupting their normal activity.

Sample size and experimental set up

A set of 30 healthy rats was subjected to two different simulated hyperbarometric pressures (122 kPa and 170 kPa) for periods of 10, 20 and 30 days, at the rate of 5 h duration daily. Control animals (a set of 15 rats) were maintained alongside the experimental animals, with adequate supplementation of food and water. At the end of each 10-day period, five rats from the control group and five from each pressure-treated group were killed. After scarification, particular sections of large intestine were dissected aseptically. Intestinal segments were suspended in sterilized phosphate-buffered saline (PBS; pH 7.0 and 9 g/l NaCl) and homogenized thoroughly using a glass homogenizer for 5 min. The content was then centrifuged (1000 g for 5 min) and the clear supernatant used for microbial analysis.

Analytical measurements

The quantities of prominent cultivable indicator groups of large intestinal bacteria were enumerated on the basis of colony-forming units (cfu). We used selective media following the standard protocol set out in the HiMedia Manual (www.himedialabs.com). Total aerobic and anaerobic bacteria were enumerated by a standard pour-plate technique using single-strength trypticase soya agar (TSA, HiMedia, Mumbai, India) and reduced Wilkins Chalgren agar (WCA, Micromaster, Mumbai, India), respectively. For anaerobic culture we used an anaerobic jar from which oxygen was removed catalytically before filling it with 10% of both CO₂ and H₂ gas (Micromaster). Enumeration of *E. coli* and *Bifidobacterium* spp. was carried out using selective media such as MacConkey and bifidobacterium agar (HiMedia), respectively. Enterobacteriaceae (other than *E. coli*) were differentially enumerated on eosin methylene blue (EMB) agar. For selective cultivation and enumeration of *Lactobacillus* spp. and *C. perfringens*, we used, respectively, Rogosa SL agar and reduced perfringens agar base (Himedia).⁸

Growth direction index (GDI)

Colony-forming units represent the actual number of bacteria present in the 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 a clear picture of the expansion or contraction of bacterial populations in a particular biosystem.

Statistical analysis

Collected data are presented as the arithmetic mean of three replicas (mean \pm standard error). The variations in microbial count were examined by one-way ANOVA (Kruskal–Wallis) and the multiple comparisons of all possible pairs were made using the Tukey *t* test (SPSS version 10; SPSS Inc., Chicago, IL, USA). The alteration

in bacterial quantity at different air pressures (122 kPa and 170 kPa) for each specific time period (10, 20 and 30 days) was tested by Fisher's *t* test. Significant variation was accepted at the level of 5% and 1% (i.e., $p < 0.05$ and $p < 0.001$) and measured using Sigmasat 11.0 (Systat Software Inc., San Jose, CA, USA) statistical software.

Results

In control (normobaric) animals, the large intestine contains (in cfu/g wet weight): total aerobes 7.21×10^4 , total anaerobes 1.58×10^{11} , *Escherichia coli* 1.91×10^5 , *Bifidobacterium* spp. 2.45×10^3 , *Clostridium perfringens* 1.42×10^3 and *Lactobacillus* spp. 4.52×10^4 .

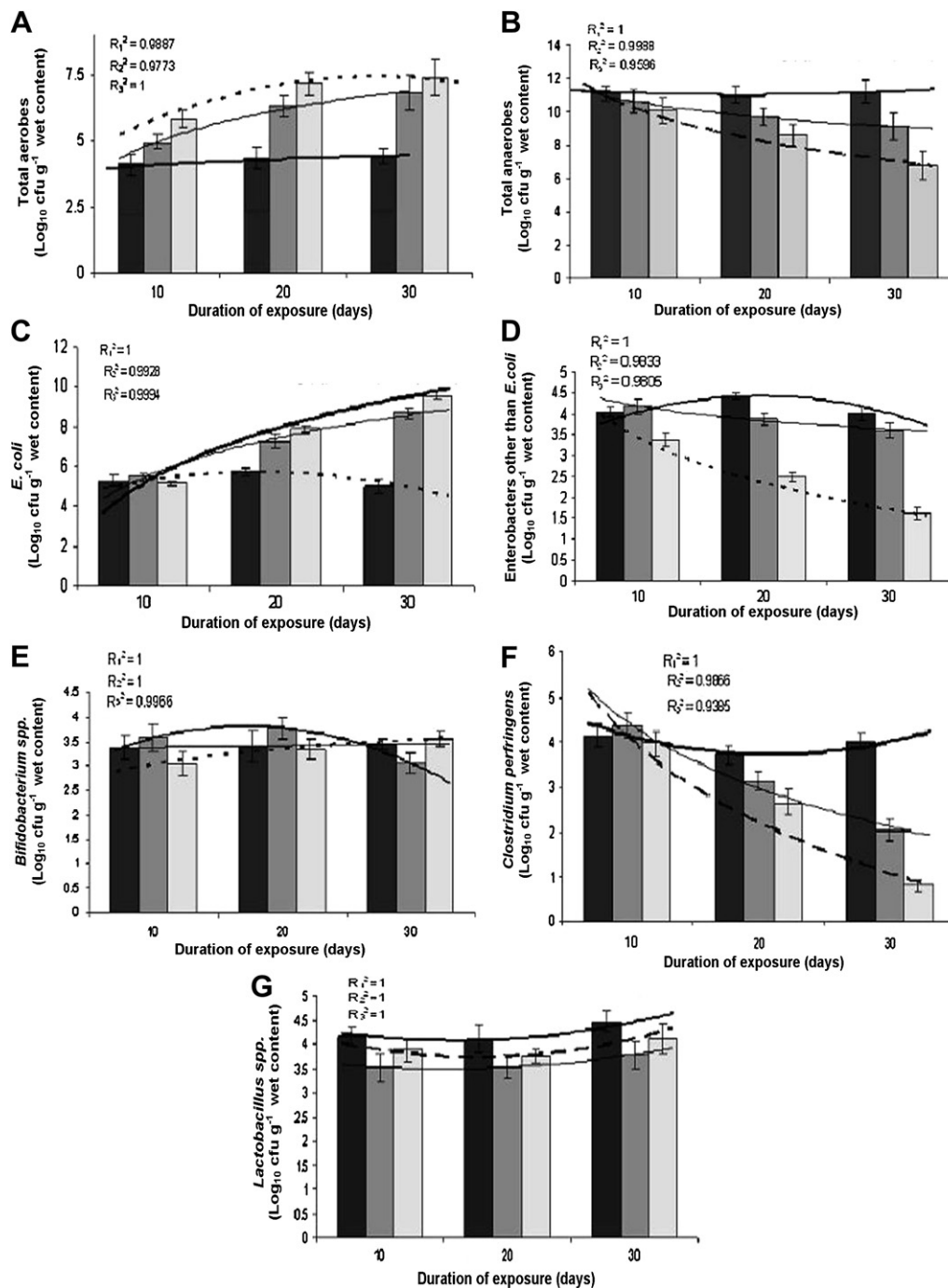


Figure 1. Alteration in the population density of total aerobes. (A) total anaerobes; (B) *E. coli*; (C) Enterobacters other than *E. coli*; (D) *Bifidobacterium* spp.; (E) *Clostridium perfringens*; (F) and *Lactobacillus* spp.; (G) in the large intestine of rat during exposure of 122 and 170 kPa air pressure for different day duration. R_1^2 , regression coefficient for control rat groups; R_2^2 , regression coefficient for hyperbaric pressure (122 kPa) exposed rat groups; R_3^2 , regression coefficient for hyperbaric pressure (170 kPa) exposed rat groups; T, standard error of mean.

After exposure to both hyperbaric pressures (122 kPa and 170 kPa), the counts of total aerobes and of *E. coli* were increased while those for total anaerobes and *Clostridium perfringens* were decreased significantly (Fig. 1A–G) in a duration-dependent manner; other studied microbial populations remained constant. The quantity of total aerobic bacteria increased up to 100-fold [7.21×10^7 at 122 kPa (logarithmically, $R_2^2 = 0.9773$) and 1.01×10^8 at 170 kPa (polynomially, $R_3^2 = 1.000$)] in respect to control (9.62×10^5 with $R_1^2 = 0.9887$). The increment of the total aerobic population was statistically significant, with $p < 0.05$ (Fig. 1A). The growth direction index of selected microbes indicated that they had occupied the large intestinal microecosystem and expanded conservatively at varied pressures (+ 1.12, + 1.07, + 1.17 at 122 kPa and + 1.42, + 1.64, + 1.67 at 170 kPa at Days 10, 20 and 30 respectively) (Figs. 2A and 2B).

Anaerobes are the most populous organisms in the large intestine. After exposure to 122 kPa and 170 kPa for 30 days, the count of prominent anaerobes was reduced about 126-fold (1.25×10^9 cfu g⁻¹) and 2.5×10^4 -fold (6.30×10^6 cfu/g) respectively in respect to the control counts (1.58×10^{11} cfu/g wet weight). Counts of total anaerobes in the control groups were maintained polynomially in a steady-state condition with R_1^2 of 1.000 ($p < 0.05$), but in pressure-stressed rats, this group of bacteria was reduced logarithmically (R_2^2 , 0.9988 at 122 kPa and R_3^2 , 0.9596 at 170 kPa, $p < 0.05$) (Fig. 1B). During graded hyperbaric exposure, diminution of total anaerobic populations was also observed, with GDI moving in a negative direction (logC/logHy at D₁₀ = -1.21; logC/logHy at D₂₀ = -1.38; logC/logHy at D₃₀ = -1.67 at 122 kPa and logC/logHy at D₁₀ = -1.03; logC/logHy at D₂₀ = -1.95; logC/logHy at D₃₀ = -3.85 at 170kPa) (Figs. 2A and 2B).

The count of *E. coli* increased gradually from the normal counts by approximately 10³ times (4.83×10^8 and logarithmically, 0.9928) and 10⁴ times (3.72×10^9 and logarithmically, 0.9994), respectively, at 122 kPa and 170 kPa air pressure ($p < 0.001$) (Fig. 1C). The GDI of *E. coli* was as follows: logHy/logC at D₁₀ = + 1.02; logHy/logC at D₂₀ = + 1.79, logHy/logC at D₃₀ = + 2.92 at 122 kPa (Fig. 2A) and logC/logHy at D₁₀ = - 1.02; logHy/logC at D₂₀ = + 1.19; logHy/logC at D₃₀ = + 1.92 at 170 kPa (Fig. 2B). Enterobacteriaceae other than *E. coli* were reduced significantly (logarithmically, 0.9833 at 122 kPa and logarithmically, 0.9805 at 170 kPa) from their normal count (polynomially,

1.000 and $p < 0.05$) (Fig. 1D). The count of *Bifidobacterium* spp. was not significantly altered throughout the experiment ($p > 0.05$) (Fig. 1E). The count of *Lactobacillus* spp. was increased (Fig. 1G) from their normal count but the increase was statistically insignificant ($p > 0.05$).

Clostridium perfringens, the only pathogenic marker organism of this experiment was decreased logarithmically ($R_2^2 = 0.9866$ at 122 kPa) and ($R_3^2 = 0.9385$ at 170 kPa) after 30 days of hyperbaric exposure (Fig. 1F). The decline of *C. perfringens* was statistically significant at 95% level ($p < 0.05$). Growth direction index of *C. perfringens* at 122 kPa as follows: logC/logHy at D₁₀ = - 1.44; logC/logHy at D₂₀ = - 1.40; logC/logHy at D₃₀ = - 1.48 (Fig. 2A) and at 170 kPa was as follows, logHy/logC at D₁₀ = + 1.09; logC/logHy at D₂₀ = - 1.4; logC/logHy at D₃₀ = -4.45 (Fig. 2B).

Discussion

The microecology of the gastrointestinal system is important to the health of an individual, and imbalances of the microbiota promote illness and contribute to the establishment and persistence of various diseases.^{24–27} A wide variety of host-induced, dietary and environmental factors can modulate the community and colonization of intestinal flora.²⁸ Two hyperbaric pressures, i.e., 122 kPa and 170 kPa (equivalent to the depths of 2.07 m and 6.87 m, respectively, below sea level) were chosen in this study, as humans generally encounter such air pressures during such activities as deep-sea diving, the cleaning of underground municipal drainage systems and wells, working in tunnels, or spending a long time in a submarine. At elevated partial pressure, hyperoxia is generated within the body. The body adapts to this in different ways, depending on the type of exposure.^{21,22} In addition to many physiological disturbances, humans also experience gastrointestinal disorders, particularly severe flatus formation in the large intestine and colon, which are primarily attributable to the intestinal flora. An alteration in the neuroendocrinal axis could modulate the blood circulation as well as the microenvironment of the intestinal tract.

The large intestine is commonly occupied by a huge population of anaerobes and facultative anaerobes; these organisms are nearly 10⁹ times more numerous than the aerobic bacteria occurring in the gut.²⁹ More than 700

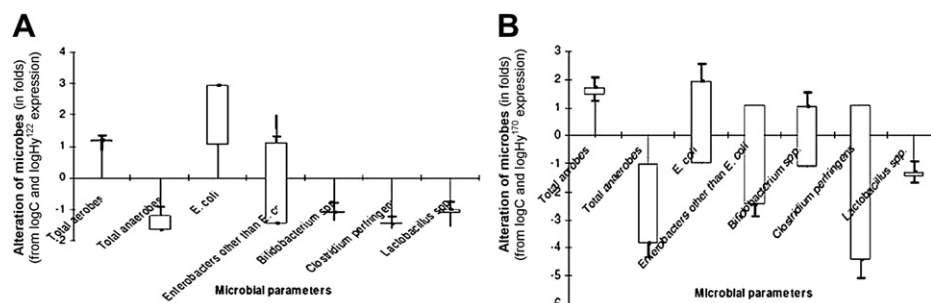


Figure 2. Changes of growth direction index (GDI) of different bacteria in large intestine during exposure of 122 kPa (A) and 170 kPa (B) air pressure for different day duration. The pattern of expression towards positive site (along with y-axis) indicating growth expansion and towards negative direction indicating growth contraction.

bacterial species live in the large intestine, predominant residents being *Bacteroides* spp., *Bifidobacterium* spp., *E. coli*, enterococci, clostridia, and anaerobic lactobacilli.²⁹ They perform a variety of functions, generally associated with the production of vitamins, especially vitamin K and biotin.^{8,9,30} They are also involved in the production of cross-reactive antibodies. Other bacterial products include gas (flatus), which is a mixture of nitrogen and carbon dioxide with small amounts of hydrogen, methane and hydrogen sulfide. Alteration of the large intestinal microbial ecology generally induces symptoms such as eructation, aerophagia, bloating and flatulence.^{31,32}

Considering the roles of the microflora in large intestinal homeostasis, the present investigation aimed to evaluate any ecological variation that occurred in different groups of microbial residents during variation of atmospheric pressure, specifically under hyperbaric conditions.

In the large intestine, total aerobes, facultative anaerobes and total anaerobes reside in the ratio of approximately $1.00 : 0.29 \times 10^5 : 1.04 \times 10^7$, although this ratio is variable between species, and between individuals of the same species.^{33–36} It has been found that the quantity of *E. coli*, a dominant species among the facultative bacteria occurring in the large intestine, is greater (by 10^5 times) than the total aerobic population. This is quite different from our earlier finding⁸ where the quantity of *E. coli* in rat feces was approximately one-third of the total aerobic microflora. $R^2 = 1.00$ indicates a linear relationship between two variables. In this particular experiment, we amplified two different graded hyperbaric pressures (122 kPa and 170 kPa), which showed a marked increment in total aerobic flora in comparison to that present at normal air pressure (101.3 kPa). The large intestine is a nutritionally enriched, anaerobic microenvironment, where conditions are such that facultative anaerobes are able to multiply more readily than total aerobes.⁸ Our *in vivo* study of the effects of different durations of hyperbaric pressure revealed significant alterations in the prominence of different types of aerobe, anaerobe and facultative anaerobe in the large intestine, and these were changes analyzed by culture-based methods. During exposure of the experimental animals to an elevated air pressure (122 kPa or 170 kPa) for 30 days, we observed an increase in aerobes, facultative anaerobes, and *E. coli*, and a decrease in anaerobes, including *Clostridium perfringens*, in comparison to the microbial populations in control rats kept at normal pressure (101 kPa).

The composition of the large intestinal flora and its regulation by environmental factors is a complex subject. Changes in the gut composition are likely to have an indirect effect, via the animal's physiology. But in response to a slight pressure increase, as applied in this study, a significant alteration of the microbial community was observed, showing trends that depended on the duration of exposure to the increased pressure. It seems therefore that elevated air pressure can directly modulate bacterial physiology. There is considerable evidence that bacterial behavior alters in response to the environmental oxygen level. Normally bacteria are very sensitive to atmospheric oxygen, and the limit of oxygen tolerance varies from species to species.^{37,38} In our experiment, it is not clear why we saw an increase in the proportion of aerobes and facultative

aerobes at high atmospheric pressure; however, the cause may have been accelerated oxidation of cellular metabolites and over-activation of different oxygen-sensitive rate-limiting enzymes of growth-related metabolism in the presence of high levels of molecular oxygen.^{23,28,39} The quantity of the anaerobic groups of organism was drastically reduced at graded simulated air pressure and this may be because of the production of reactive oxygen species (ROS). Normally, anaerobic bacteria create an anaerobiosis by reducing the action potential of their surroundings.⁴⁰ In hyperbaric conditions, alteration of the electrochemical potential creates an unfavorable environment for their survival. As the counts of *Bifidobacteria* and *Lactobacillus* were not affected by the hyperbaric stresses, it should be possible to design an effective probiotic composition for individuals subject to a hyperbaric conditions that will benefit their health and protect them from hyperbarism-induced gastrointestinal disorders.

In conclusion, this *in vivo* study reveals that atmospheric pressure above the ambient level alters the composition of the gastrointestinal flora of the rat both qualitatively and quantitatively. Although the human large intestine is much longer than that of the rat, thus creating a greater diffusional barrier, we have demonstrated that atmospheric pressure has a significant impact on the bacterial colonization of the gut and on the ecology of the gut microflora, which can be correlated with hyperbaric gastrointestinal disorders.

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