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Letters to the Editor

Prerequisite Evaluation of Anaerobic Settings for Gut Microbiome Functional Studies

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Dysbiosis of the gut microbiota has been associated with colorectal cancer (CRC),[1] where microbiome sequencing studies revealed over-representation of pathobionts in CRC patients. Nevertheless, definite determination of tumorigenic properties of these CRC-associated pathobionts require functional studies involving co-culture of these bacteria and colon cells. Colon cells, regardless of the normal or cancerous phenotypes are usually grown in aerobic conditions in the laboratory. On the other hand, the *in vivo* gut surface environment is anaerobic, allowing only the growth of strict and facultative anaerobic bacteria. In this study, we set out to investigate and compare survival and viability of the human colon adenocarcinoma cell line HT-29 in aerobic and anaerobic culture environment.[2] We also determined gene expression changes of the cells when they were cultured in an anaerobic environment.

HT-29 was purchased from American Type Culture Collection (ATCC) (Manassas, Virginia, United States) and maintained using the Roswell Park Memorial Institute (RPMI) 1640 media (Pan Biotech, Germany) supplemented with 12% fetal bovine serum (Tico Europe, Netherlands), 1% of sodium pyruvate (HyClone™, USA), and 1% of penicillin/streptomycin (Nacalai Tesque, Inc., Japan). An anaerobic culture environment was established using a 3.5L AnaeroGen™ sachet (Oxoid, Thermo Scientific, USA) placed inside an AnaeroPack™ 2.5L rectangular jar (Mitsubishi Gas Chemical, Inc., USA), to achieve a culture environment of <0.1% oxygen (O_2) and 7-15% carbon dioxide (CO_2).[3] This was indicated by an anaerobic indicator strip (Oxoid, Thermo Scientific, USA) which was also placed inside the AnaeroPack™ jar. For aerobic culture, plates of cells were cultured in a rectangular jar without the AnaeroGen™ sachet. Both aerobic and anaerobic culture jars were placed inside a 37 °C incubator during culture experiments.

To determine survival, viability and cell count of the HT-29 cells in aerobic and anaerobic culture conditions, 5×10^5 cells/mL were first seeded in a 24-well plate and grown in an aerobic incubator until 80-95% confluency. Subsequently, the cells were then seeded onto two plates: one was incubated in the aerobic jar, while and the other was placed in the anaerobic set-up. The plates were exposed to either aerobic or anaerobic environment for 5 consecutive days. Cells were then dissociated, stained and counted daily using a Countess™ automated cell counter (Invitrogen, USA). Experiments were carried out in triplicates.

To determine if the cells were in higher hypoxic stress in the anaerobic environment compared to aerobic culture, we determined the expression of six hypoxia-associated genes (HIF1α, GLUT1, LDHA, SLC16A1, SLC16A3, and CA9) of the cells in both culture conditions. All markers were categorized as important endogenous hypoxia markers in anaerobic glycolysis. [4,5,6] The Ki-67 marker was measured to determine the proliferation rate of HT-29 for both culture conditions. Total RNA for both aerobic and anaerobic conditioned cells were extracted at the end of day 5 via guanidinium thiocyanate-phenol-chloroform extraction and converted to cDNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). Real-time polymerase chain reaction was carried out with a QuantiNova SYBR® Green PCR Kit (QIAGEN, USA). Statistical analysis was performed using GraphPad PRISM 8.0 (GraphPad Software, USA). Data was analyzed using two-way ANOVA test where $P<0.05$ indicated significant difference in gene expression.

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HT-29 was found to be able to grow in both aerobic and anaerobic culture conditions, with similar epithelial-like cell morphology and formation of adherent cell colonies observed throughout the five days (Figure 1 (a)).
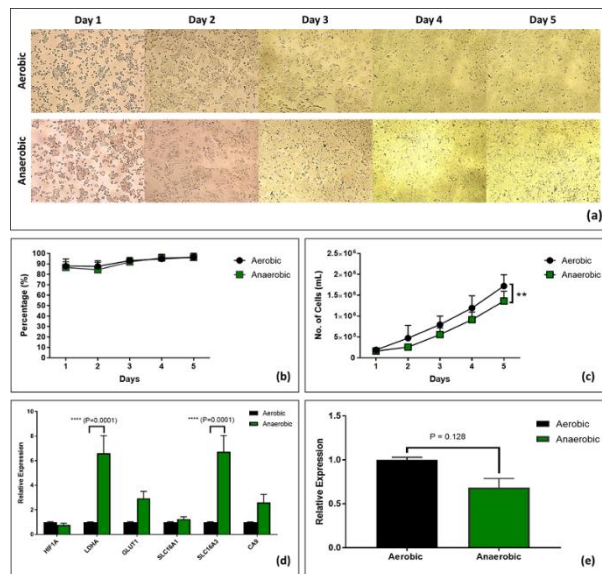

Figure 1. Cell morphology, viability, growth rate, and gene expression of HT-29 under aerobic and anaerobic culture conditions. **a)** representative images of HT-29 cell morphology for ; **b)** viability of HT-29 cells ; and **c)** HT-29 cell count; **d)** expression of HIF1α, GLUT1, LDHA, SLC16A1, SLC16A3, and CA9 in HT-29; **e)** expression of Ki-67 in HT-29.s

Interestingly, cell viability was similar between the two experiment setups. HT-29 viability remained stable for 5 consecutive days (aerobic, 91.93%; anaerobic, 90.91%) ((Figure 1 (b)). Nevertheless, we observed consistent slower growth rate for cells cultured in the anaerobic condition, with significant difference in HT-29 cell count throughout the 5 days' exposure (Figure 1 (c)). At day 5, HT-29 cell count for the aerobic setup was $1.72 \times 10^6 \pm 9.04 \times 10^4$ cells/mL, compared to $1.36 \times 10^6 \pm 7.76 \times 10^4$ cells/mL of the anaerobic setup.

To evaluate hypoxic condition, only LDHA and SLC16A3 were found to have significant higher expression in anaerobic HT-29 cells (P=0.0001). These genes were reported to be important for glycolysis,[4] an important source of energy during anaerobic respiration. GLUT1, SLC16A1, and CA9, also important for glycolysis, were also found to be have higher expression in anaerobic HT-29 cells; nevertheless, the difference was not significant compared to aerobic HT-29 (Figure 1 (d)). The increasing patterns of these markers possibly due to their role in the intracellular molecules transport mechanism. These markers are associated with the transportation of available glucose inside the cells to initiate the anaerobic glycolysis, via the transfer of H^+ ion to achieve pH stability, and slight intake of available lactate by neighboring cells, respectively. For LDHA, it catalyzes the conversion of pyruvate to lactate during anaerobic glycolysis. The high level of lactate produced through the pyruvate conversion inside the cells was transported out by SLC16A3.[7] Though the glycolysis process is associated with metabolic pathway re-wiring in cancer progression,[8] the severity is minimal considering only two (LDHA and SLC16A3) of six

common hypoxic markers exhibited enhanced expression levels. Furthermore, this observation supports the hypothesis of the study that anaerobic stress does not affect the tumorigenesis in HT-29 cells.

Expression of the Ki-67 marker showed that cultivation in an anaerobic culture system did not produce any significant effect (P=0.128) on HT-29 cell proliferation activity (Figure 1(e)). Taken together, two of six hypoxic markers showed differential significance of expression, indicating hypoxic stress was not interfering with the proliferative rate in this anaerobic HT-29 setup.

This study provided results on some baseline parameters of an anaerobic colon cell culture system. We observed that HT-29 cells did not differ significantly in viability and were not under severe hypoxic stress when they were cultured in an anaerobic condition, compared to their usual maintenance in an aerobic setup. The cells were able to utilize alternative metabolic pathways such as glycolysis and adapt to an anaerobic environment.[9] Nevertheless, re-programming of the metabolic pathways such as glycolysis could possibly be observed by assessing glucose and lactate uptake in the culture media. Having said that, the small changes in the hypoxic marker expressions in this observation setup may indicate minimal stress was induced thus suggested the stability of this anaerobic system for *in vitro* gut microbiome studies. Additionally, HT-29 appeared to grow slower in anaerobic culture, however, cell viability was not affected. This again, affirmatively supporting our hypothesis and the compatibility of the anaerobic setup. Taking it all together, an anaerobic colon cell culture system can be used to investigate tumorigenic properties of anaerobic bacteria on colon cells. This includes experiments to determine the effects of bacterial oxidative stress and cytotoxicity on host cells. In addition, host cell response towards infection, such as DNA damage mechanisms, enhancement of host cell proliferation, gene over-expression as well as understanding re-wiring of metabolic pathways in the tumor microenvironment (TME) can be studied with this system. Reviewing the tumorigenic properties later contribute towards a more precise treatment and a better understanding of gut microbiota-associated CRC tumorigenesis.

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