Intestinal *in vitro* and *ex vivo* Models to Study Host-Microbiome Interactions and Acute Stressors

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The gut microbiome is extremely important for maintaining homeostasis with host intestinal epithelial, neuronal, and immune cells and this host-microbe interaction is critical during times of stress or disease. Environmental, nutritional, and cognitive stress are just a few factors known to influence the gut microbiota and are thought to induce microbial dysbiosis. Research on this bidirectional relationship as it pertains to health and disease is extensive and rapidly expanding in both *in vivo* and *in vitro/ex vivo* models. However, far less work has been devoted to studying effects of host-microbe interactions on acute stressors and performance, the underlying mechanisms, and the modulatory effects of different stressors on both the host and the microbiome. Additionally, the use of *in vitro/ex vivo* models to study the gut microbiome and human performance has not been researched extensively nor reviewed. Therefore, this review aims to examine current evidence concerning the current status of *in vitro* and *ex vivo* host models, the impact of acute stressors on gut physiology/microbiota as well as potential impacts on human performance and how we can parlay this information for DoD relevance as well as the broader scientific community. Models reviewed include widely utilized intestinal cell models from human and animal models that have been applied in the past for stress or microbiology research as well as *ex vivo* organ/tissue culture models and new innovative models including organ-on-a-chip and co-culture models.

Keywords: physiology, stress, *ex vivo*, *in vitro*, intestine, DoD

INTRODUCTION

The mammalian intestine is critically important for nutrient digestion and absorption, ion and water transport, as well as maintaining a homeostatic relationship with immune cells and the trillions of bacteria that reside there collectively known as the gut microbiome. The gut microbiota consists of not only bacteria, but also viruses, fungus, yeast, and archaea. *Bacteroidetes* and *Firmicutes* phyla make up ∼90% of the human adult gut microbiota but there is a wide range of

species diversity numbering in the hundreds to thousands (Macfarlane and Macfarlane, 2004; Eckburg et al., 2005). Bacterial diversity within the human microbiota demonstrates inter-individual variability, and can be influenced by the environment, genetics, diet, antibiotic use, and geographical location (Zhu et al., 2015). Normally, the gut microbiome significantly contributes toward protection against pathogens by competing for shared nutrients and niches or through enhancing host defense mechanisms (Kinross et al., 2011).

A balanced host-microbe interaction is necessary for maintaining homeostasis. Intestinal epithelial cells can sense and respond to the microbial environment by secreting signaling compounds, such as cytokines or chemokines as well as anti-microbial peptides and hormones, reflecting its notoriety as an endocrine organ (Smirnova et al., 2003; Clarke et al., 2014). The normal intestinal epithelium consists of several cell types including enterocytes, goblet cells, stem cells, enteroendocrine cells, Tuft cells, M cells and Paneth cells, all of which can sense and respond to bacteria by several means including producing and secreting anti-microbial peptides (Paneth cells, enterocytes), producing and secreting mucin (goblet cells), as well as secreting cytokines, and expressing toll-like receptors (TLR's) and nod-like receptors (NLR's). The epithelium of the small intestine consists of a single mucus layer that acts as a protective barrier between cells and bacteria whereas in the colon there is a double mucus layer consisting of a sterile, tightly-adhered mucus layer and a loose layer which provides a niche for bacteria (Johansson et al., 2008). This mucus layer produced by mucin-secreting goblet cells can influence the types of bacteria that reside in the gut (Van den Abbeele et al., 2013).

Aside from epithelial cell interactions, the gut microbiome also interacts with the host immune system largely through gut-associated lymphoid tissue (GALT) system (Doe, 1989; Mowat, 2003; Round et al., 2010; Hooper et al., 2012) and in some ways the immune system is actually "educated" by the gut microbiota to distinguish friend or foe. The gut comprises the largest lymphoid system in the human body. The GALT is separated from the lumen by the epithelial cell layer. Underneath this epithelial layer is an underlying layer called the lamina propria as well as lymphatic circulation that can house several different types of immune cells including macrophages, mast cells, and plasma cells. In the small intestine there are Peyer's patches that consist of isolated lymphoid follicles that house B cells, T cells and dendritic cells (Doe, 1989; Mowat, 2003). The host is equipped with pattern recognition receptors (PRR's) that recognize pathogen-associated molecular patterns (PAMPs); recognition of these PAMPS leads to immune activation and production of anti-microbial peptides and cytokines/chemokines. As briefly mentioned earlier, PRR's include the family of TLRs and NLR's that recognize bacterial and viral ligands. Epithelial cells are capable of producing various anti-microbial peptides (AMPS) including defensins and c-type lectins. These systems are designed to defend against pathogens but in some cases are also thought to be associated with auto-immunity (van Kooyk, 2008; Frasca and Lande, 2012).

The enteric nervous system (ENS) is also important for maintaining homeostasis and is involved in the host-microbiome response. The ENS is a large neural network embedded in the tissue of the GI tract and has been referred to as the body's "second brain." It helps with peristalsis in the gut, hormone secretion, neurotransmitter release, and signaling to the central nervous system (CNS) (Zhu et al., 2017). It is also known that stress and the microbiota can have effects on the ENS (Mayer et al., 2015) affecting both GI function as well as CNS function. Also, the gut microbiome has an important relationship with the endocannabinoid system, which is a complex system involved in energy homeostasis and metabolism (Cani et al., 2014). Enteroendocrine cells are the most numerous endocrine cells in the human body that produce and secrete gastrointestinal hormones/peptides, and are involved in appetite regulation. Although they are the most numerous type of endocrine cells, they represent <1% of all intestinal epithelial cells. Additionally, these cells are known chemosensors that interact with the host immune system and express several functional TLR's (Yoo and Mazmanian, 2017). Although there has been increasing evidence that the microbiome influences the gut-brain axis; little in vitro/ex vivo work has examined this potential link, thus this topic will not be discussed in detail in this review (Carabotti et al., 2015).

The epithelial layer works in conjunction with immune cells, smooth muscle layer, and nerve cells in a coordinated effort to promote homeostasis. The host-microbiome interaction is largely symbiotic and mutualistic in nature. However, perturbations in this homeostasis can lead to dysbiosis, decreased intestinal barrier function, nutrient malabsorption/diarrhea, infection (sepsis), and/or auto-immune disorders (Aleksandrova et al., 2017). Dysbiosis is characterized by a compositional shift from obligate anaerobes to facultative anaerobe classes within the gut microbiome community (Winter and Baumler, 2014). Specifically, the gut microbiome plays a large role in maintaining gut health by metabolizing nutrients to create short chain fatty acids (SCFA) and other beneficial metabolites, such as polyphenols to the host (Russell et al., 2013). In addition, bacteria normally found in the mammalian gut, synthesize vitamins including Vitamin K and Vitamin B12 (LeBlanc et al., 2013). The gut microbiota is also involved in intestinal barrier defense, and priming the immune response (Elson and Alexander, 2015) and interact with the neuroendocrine system (Farzi et al., 2018). Various conditions and environments have been shown to alter microbial composition and cause decreased epithelial barrier function, and inflammation, such as intense exercise/training, hot or cold environments, high altitude, sleep deprivation, caloric deprivation, psychological stress and changes in diet. However, there is evidence to suggest that the resilience of the gut microbiome may positively or negatively impact health (Sommer et al., 2017).

Studying these complex-interactions in vitro and ex vivo is a constantly evolving area. Therefore, this review addresses the current knowledge along with strengths and limitations of intestinal models utilized in the study of host-microbiome interactions. This review will pertain mainly to gut models and mechanisms of bacteria-epithelial interactions, although where appropriate will also include co-culture models that mimic the gut-immune or gut-brain axes.

MILITARY RELEVANCE

Military training and combat is frequently characterized by exposure to various stressors. These stressors are psychological (e.g., fear, anxiety, trauma, cognitive demands), environmental (e.g., heat, cold, high altitude, pathogens), and physical (e.g., strenuous exercise, undernutrition, sleep deprivation) in nature, and are often experienced at extremes and in combination (Weeks et al., 2010; Henning et al., 2011). Associated physiological effects are myriad (Nindl et al., 2013), and affect every organ system in the body including the nervous and immune systems and gastrointestinal tract. For example, it is well-established that hypoxic (i.e., high altitude), heat and cold stress cause changes in cognition, appetite, intestinal function and permeability, and immune function (Saunders et al., 1994; Coskun et al., 1996; Anand et al., 2006; Lambert, 2008; Zhou et al., 2011; Pearce et al., 2014; Dokladny et al., 2016; Khanna et al., 2017). For instance, cold exposure, which is a period of high energy demand, can shift the composition of the gut microbiota that can favor energy extraction and intestinal absorptive surface (Chevalier et al., 2015). In military personnel, these effects are often compounded by sleep deprivation and concomitant increases in physical exertion and inadequate dietary intakes (Friedl et al., 1994; Montain and Young, 2003; Tharion et al., 2005; Margolis et al., 2014, 2016; Karl et al., 2017b) that independently influence nervous system, immune, and gastrointestinal function (Everson and Toth, 2000; Lambert, 2008; Li et al., 2013, 2014; Zuhl et al., 2014; Schmid et al., 2015; Clark and Mach, 2016; Karl et al., 2017a). In deployed settings in particular, traumatic psychological events (Creamer et al., 2011; Bonde et al., 2016) and exposure to enteric pathogens (Porter et al., 2017; Riddle et al., 2017) are additional stressors potentially impacting nervous system, immune, and gastrointestinal function (Lafuse et al., 2017; Mackos et al., 2017). A summary of effects of military-relevant stressors on the gut microbiota was recently published by Karl et al. (2018).

Growing evidence from animal, and, to a far lesser extent, human studies indicates that stressor-induced changes in nervous system, immune, and gastrointestinal function alter the gut microbiome, and the gut microbiome, in turn, alters physiologic and cognitive responses to these stressors (Kleessen et al., 2005; Zhou et al., 2011; Holmes et al., 2012; Galley and Bailey, 2014; Chevalier et al., 2015; David et al., 2015; Clark and Mach, 2016; Thaiss et al., 2016; Voigt et al., 2016; Zietak et al., 2016; Allen et al., 2017; Mackos et al., 2017). This suggests that the gut microbiota could be an underappreciated mediator of stress responses and associated outcomes in military personnel. Indeed, gut microbiota composition and activity has recently been associated with gastrointestinal permeability, inflammation, gastrointestinal symptoms, and psychological metrics during military training (Li et al., 2013, 2014; Phua et al., 2015; Karl et al., 2017a,b). Elucidating mechanisms by which host-gut microbiome interactions impact host stress responses could therefore facilitate development of gut microbiome-targeted interventions for health and performance optimization in military personnel. Of note, potential benefits of such interventions extend beyond the military as military-relevant stressors are not uncommon, alone or in combination, in some

civilian populations, such as athletes (Clark and Mach, 2016) and first responders, such as firefighters (Alexander and Klein, 2009).

Our current understanding of the mechanisms underpinning host-gut microbiome interactions is largely derived from rodent studies, particularly germ-free mice. However, the relevance of these models to human host-gut microbiome interactions has been questioned (Nguyen et al., 2015). Moreover, many of these studies have focused on chronic health and disease rather than shorter-term outcomes. From a military and athletic perspective, elucidating mechanisms of host-gut microbiome interactions and how they can be leveraged to build resiliency, and optimize physical and cognitive performance under transient stress exposure is perhaps of greater interest. *In vitro* and *ex vivo* models have become an integral tool in examining mechanisms underlying host-pathogenic and host-commensal bacterial interactions. As such, although many of the *in vitro* and *ex vivo* models reviewed below have been used for examining mechanisms of disease, it is important to consider how these models can be applied to study the complex interplay between stress exposure, the gut microbiome, and host physiology.

INTESTINAL HOST MODELS FOR MICROBIOME RESEARCH

In vitro

Caco-2

Developed in the 1970's, the Caco-2 cell line has been widely studied in the pharmacological, nutritional, and microbiological fields. This cell line was derived from human colorectal adenocarcinoma and can either function as undifferentiated large intestinal cells, or can spontaneously differentiate to resemble a small intestine-like phenotype with enterocyte-like absorptive properties. A brush border expressing clone of these cells (Caco-2 BBe) has also been produced to act as a small intestinal mimic. Due to the spontaneity of this cell line, it has been difficult to control and reproduce data; however it is desirable for studying transport kinetics and can act as a small or large intestine mimic (Shi et al., 2017). Over 200 papers have examined bacteria and bacterial metabolites using the Caco-2 model to study intestinal barrier function, bacterial adhesion/invasion, and innate immune response. Beneficial and pathogenic bacteria have been used to treat Caco-2 cells and elucidate protective effects of *Lactobacillus rhamnosus GG* and *Lactobacillus casei* against inflammation (Toki et al., 2009). This study found that *L. rhamnosus GG* and *L. casei* do not induce pro-inflammatory cytokine expression and actually suppress certain cytokines induced by *E. coli*, or bacterial ligands. Caco-2 cells have also been shown to express functional toll-like receptors that can respond following bacterial challenge (Furrie et al., 2005). Interestingly, Caco-2 cells have also been used to study prebiotic oligosaccharides. Prebiotic oligosaccharides have been shown to positively modulate the intestinal microbiota and Caco-2 cells treated with oligosaccharides exhibit reduced inflammatory activity through the PPARγ pathway (Zenhom et al., 2011). A comprehensive and eloquent review on human colon cancer cell lines that discusses mechanisms of pathogenesis of human enterovirulent bacteria was written in 2013 that also encompasses

the following two cancer cell lines (Lievin-Le Moal and Servin, 2013).

HT-29

HT-29 is another polarized, but largely undifferentiated, human colorectal adenocarcinoma that was isolated in the 1970's. Since this time, several sub-populations have been created to be more "enterocyte-like." HT-29 cells were originally used to study cancer biology but were transitioned over to other research due to their phenotype (Rousset, 1986). HT-29 cells can now be grown under a non-polarized, undifferentiated state or be cultured to differentiate and obtain a polarized membrane depending on the objective. This cell line also produces cytokines, such as interleukins, TNF-α (De Simone et al., 2015; Khan Mirzaei et al., 2016). For this reason they have been extensively studied in the field of nutrition and host-microbiome interactions. Like Caco-2, using a Transwell® system with apical and basolateral polarity allows for studies of bacterial adhesion to the epithelium, as well as bacterial transport. This model is unique compared to Caco-2 in that HT-29 cells contain mucus-producing goblet cells and for that reason they have also been used as models to study immune function and bacterial-host interactions with greater success (Raja et al., 2012; Altamimi et al., 2016). For example, this cell line has been a useful tool to show that oligosaccharides and a functional mucin layer can reduce bacterial adhesion to the epithelium (Altamimi et al., 2016). This cell line has been well-characterized in regards to host response to bacterial infection including adhesion, migration and internalization of pathogens including *Salmonella*, *E. coli*, and others. HT29-MTX cells, which are HT-29 cells treated with methotrexate, express higher proportions of mucin-secreting goblet cells and have been utilized to study bacterial survival and adhesion (Dahiya et al., 1992). Mucins expressed in this line include both secretory (MUC2, MUC5AC, MUC6) as well as membrane bound (MUC1, MUC3, MUC4) types (Huet et al., 1995). Researchers have co-cultured Caco-2 and HT-29 to make the model more physiologically and functionally relevant, especially for the study of microbiology. HT-29 cells in combination with Caco-2 have been used to study protective effects of probiotics against pathogenic bacteria (Resta-Lenert and Barrett, 2003) by showing that pre-exposure of epithelial cell monolayers to live probiotic *Streptococcus thermophilus* and *Lactobacillus acidophilus* limits invasion and adhesion of enteroinvasive *Escherichia coli* (EIEC). Interestingly, HT-29 cells have become a host model to test *in vitro* fermentation of prebiotics (Maccaferri et al., 2012) and fecal fermentation. One study examined modulation of immune response by two different probiotic *Bifidobacterium* species in a single-stage continuous-culture system combined with epithelial cells (Arboleya et al., 2015). This study showed that HT-29 cells combined with fermentation, can be a tool to screen different bacterial species probiotic potential. It showed that. For these to be an effective model to utilize for host-microbiome interactions, they need to have a viable mucin layer.

T84

Similar to Caco-2 and HT-29, T84 cells were derived from human colon cancer cells. However, unlike the former two lines, T84 cells were isolated from metastasized cells found in the lungs. T84 cells express several brush border properties including brush border enzymes and transporters. These have been extensively utilized as a model for studying epithelial barrier function and electrolyte transport as they attain tightly formed tight junctions and express tight junction proteins including claudins, occludin, and ZO-1, as well as ion transporters (Madara and Dharmsathaphorn, 1985). These cells were initially used to study heat-stable *E. coli* enterotoxin. These toxins induce intestinal secretion but the mechanisms were not previously understood. T84 cells were utilized to study binding of the enterotoxin, activation of guanylate cyclase and cGMP production to provide new information on the mechanism of action (Guarino et al., 1987). T84 cells have also been expanded to study probiotic bacterial supernatants and their protective effects during EIEC invasion. Results determined that probiotics may produce beneficial metabolites that can prevent bacterial invasion (Khodaii et al., 2017). This has been one of the lesser utilized models for microbiome research however researchers have still found value in this model to study bacterial interactions, especially when conducting experiments in parallel with other related cell lines for a more robust outcome. An interesting new study was published using T84 cells to elucidate the effects of a microbial-derived protein by-product and its ability to maintain intestinal barrier integrity. Tryptamine was able to reduce inflammatory cytokine-induced monolayer permeability. In addition, indole-3-propionic acid (IPA) had positive effects on fructose metabolism (Jennis et al., 2018). Additionally, T84 cells have been utilized to study protective effects of Lactoferrin on bacterial-induced barrier dysfunction showing that Lactoferrin maintains tight junction structure during *Yersinia enterocolitica* infection (Hering et al., 2017).

IEC-6 and IEC-18

IEC-6 and IEC-18 are lines derived from the small intestine of rats. IEC-6 is derived from the whole small intestine while IEC-18 is ileum derived. As these lines are small intestinal, they do not accurately reflect colonic morphology and physiology; however, they have become a useful tool to study bacterial adhesion (Cinova et al., 2011). Adhesion of multiple bacterial strains including *E. coli* CBL2 and *Shigella* CBD8 were tested using IEC-6 cells. IEC-6 was initially utilized to study transport of microflora-derived products (Osborne and Seidel, 1989) and attachment of *Giardia intestinalis* (McCabe et al., 1991) due to its similarity to primary cultures and previous lack of a convenient *in vitro* model. These cells, like many of the lines, express cytokines, such as interleukin-6 as well as toll-like receptors (Li et al., 2011). IEC-6 has also been utilized to study small-intestinal microbiota-related bacteria. More recently it has been used to examine potential probiotics including *Bifidobacterium bifidum* (Khailova et al., 2010) and *Lactobacillus reuteri* (Liu et al., 2010). IEC-6 cells have been able to show protective effects against LPS-induced intestinal inflammation using *L. reuteri* (Liu et al., 2010). Additionally, *Escherichia coli Nissle* 1,917 supernatants were shown to be effective in reducing apoptosis, and improving barrier function during simulated epithelial damage (Wang et al., 2014). IEC-18 cell lines have been used to examine protective

effects of *Bifidobacteria spp.* against injury through enhancement of intestinal barrier function and alterations in TLR signaling (Yang et al., 2017). IEC-18 cells have also been shown to have bactericidal capabilities (Deitch et al., 1995).

IPEC-J2 and IPEC-1

The IPEC cell lines are porcine intestinal epithelial cells isolated from neonatal piglet small intestine (Rhoads et al., 1994). IPEC-J2 are jejunum derived while IPEC-1 are from the jejunum and ileum. IPEC-J2 and its usefulness in microbiological investigations was more thoroughly investigated in 2011 (Brosnahan and Brown, 2012). It is unique to most of the other polarized cell lines in that it is non-transformed, and not of cancerous origin. In addition, pig intestine much more closely resembles human intestine compared to mice or rodent models. IPEC cells express tight junction proteins (Schierack et al., 2006), multiple mucins, and are able to express and secrete many types of cytokines/chemokines. They also express inflammatory pathway markers NFKB and MyD88 (Mariani et al., 2009), and toll-like receptors (Arce et al., 2010) that are necessary for assessing host-microbe interactions. They have been utilized to study bacterial infections that affect both swine as well as humans due to physiologic similarities.

The first use of IPEC-J2 cells in microbiological studies involved the pathogenic bacteria *Lawsonia intracellularis* (McOrist et al., 1995). This study showed that the *in vitro* culture model closely resembled that of *in vivo* infection including mechanism release of internalized bacteria into the cytoplasm and release of bacteria from the epithelial monolayer. They have been largely utilized to study innate immune responses to various pathogens including *Salmonella enterica* (Schierack et al., 2006), *S. typhimurium* (Boyen et al., 2009), *E. coli*, as well as Chlamydia (Schierack et al., 2006). In these studies, all infected IPEC-J2 cells encoded mRNA's for several cytokines including IL6, TNF-alpha, and IL1-alpha. *Salmonella* alone was shown to enhance IL-8 expression. Additionally IPEC-J2's have been increasingly used for probiotic research. Adhesion abilities of 11 strains of *Lactobaillus* spp. were analyzed using IPEC-J2 cells, determining that *L. reuteri* and *L. plantarum* have the highest binding capacities (Larsen et al., 2007). *Bacillus licheniformis* was shown to induce IL8 mRNA expression. However, when co-cultured with *Salmonella enterica*, *B. licheniformis* inhibits basolateral IL8 secretion. (Skjolaas et al., 2007). *Bifidobacterium* was shown to have anti-viral activity including inhibition of viral invasion of host cells, and production of anti-viral metabolites (Botic et al., 2007). These cells have also been utilized to study cell proliferation, nutrient and ion transport, viral infection (rotavirus, vesicular stomatitis virus) and fungal infection (toxin from *Fusarium* fungus).

ADVANTAGES AND DISADVANTAGES OF CURRENT *IN VITRO* MODELS

There is an ongoing discussion amongst researchers on when to utilize primary cell models and when to utilize established cell lines. Transformed cell lines have long been considered the most cost-effective and enduring tool in basic research as they can be passaged indefinitely and are indispensable for preliminary screening and mechanistic studies. Primary cell lines are often considered to be more biologically and physiologically similar to *in vivo* models. Caco-2 cells grown as confluent monolayers are extremely useful to study absorptive and transport kinetics, especially in the drug transport field, under basal and bacteria exposed conditions. The main drawbacks of this cell line are its cancer cell origins, its homogeneity, and the fact that Caco-2 cells do not produce significant amounts of mucins under normal growth conditions (Pan et al., 2015). HT-29 cells contain a mucus layer that is useful for microbiome research, especially when treated with methotrexate. They have similar drawbacks to Caco-2 however, in that they are transformed cells from colon cancer and they are not normally utilized to study barrier function due to their inability to form proper tight junctions. T84 has very similar advantages and disadvantages to Caco-2 and HT-29 due to its ability to be grown as monolayers and its cancer origins but conversely to HT-29, T84 has been an excellent model to examine effects of microbes and stressors on epithelial barrier function due to its high TER properties. Overall, to utilize these cancer cell lines one must take into account several factors including culture conditions (media formulation, differentiated vs. undifferentiated), passage number, potential HeLa cell contamination, and whether they express the desired genes/proteins before determining whether these will be suitable for microbiological investigations.

IEC-6 cells are non-transformed and recapitulate normal small intestinal physiology. However, disadvantages are that it may not accurately reflect a human response in metabolism and absorption or colon physiology which is needed to study microbiology. Additionally, it is more useful to study small intestinal physiology and translatability from rodent to human is more difficult. IPEC-J2 cells are of porcine origin, and most similar to humans compared to other animal cell lines. These cells are non-transformed and mimic normal intestinal physiology and function. IPEC's are able to be polarized and contains multiple cell types, including mucus producing cells. However, its small intestinal origins make it difficult to translate to colon research. Also, like the other lines, media formulation and culture conditions can change the phenotype of the cell line.

In conclusion, *in vitro* models to date have not readily provided a non-cancerous colon model that accurately recapitulates a healthy large intestine but these models do have relevance depending on the application. Overall drawbacks of the above cell types are the lack of cellular diversity in a single cell type system. The normal intestinal epithelium consists of several cell types including enterocytes, goblet cells, stem cells, enteroendocrine cells, Tuft cells, M cells and Paneth cells that are not accurately represented. Additionally, translatability is also a concern in terms of species differences as well as cancerous vs. non-cancerous. They also lack the ability to culture bacterial community dynamics and are only able to study single bacterium-host interaction.

Ex-vivo

InTESTine™ System by TNO

Ex vivo models are models cultured outside of an organism, but contain functional live tissues with complex cellular environments found *in vivo*. TNO, who makes the TIM-1, TIM-2, and TinyTim models of digestion and absorption, has recently developed a new *in vitro* system called the InTESTine™ that utilizes fresh healthy porcine intestinal tissue from multiple segments of the GI tract in parallel. This model was originally intended for drug discovery research but is meant to work in the presence of absence of microbiota. It is a more sophisticated model from the TIM systems as it incorporates actual intestinal tissue as opposed to mimicking conditions of the intestinal tract. This model contains a mucus layer that enables it to be more successfully utilized in conjunction with single or mixed communities of bacteria (TNO). Thus, far it has not been used in published microbiological investigations, but may provide a new way to study host-microbe interactions.

Ussing Chamber

The Ussing chamber was initially developed by Hans Ussing to study transport across a variety of epithelial tissues, and has become a powerful *ex-vivo* tool for studying transport across different segments of the intestine which is a major advantage. It consists of two halves separated by polarized epithelia (tissue or cell monolayers) and the chamber is set up to isolate apical and basolateral sides. It works using electrodes that can measure voltage and short-circuit current to determine permeability/transport. This system has been utilized to study bacterial-host interactions largely via bacterial toxins, but also intact bacteria. The first such study examined the effects of *Shigella* enterotoxin on intestinal ion transport and showed that *Shigella* increases fluid and electrolyte accumulation, as well as net sodium secretion (Donowitz et al., 1975). Additionally, this technique was utilized to show that pathogenic *E. coli* increased Cl^- secretion by the intestinal epithelium (Hecht et al., 1999). Colonic mucosa has been mounted on Ussing chambers to study the effects of *Enterococcus faecalis* to look at bacterial invasion (Isenmann et al., 2000). This study determined that an aggregation substance (a bacterial adhesion product of *E. faecalis*) promotes bacterial translocation into colonic mucosa. *Clostridium difficile* was also tested in a Ussing chamber to test the hypothesis that anaerobic *C. difficile* interactions with host epithelial cells involve bacterial and toxin-mediated cellular events (Jafari et al., 2016). *Campylobacter jejuni* infection has been examined in this system using human colonic monolayers. *C. jejuni* was able to translocate across monolayers and cause an increase permeability by disruption of tight junctions. Other effects included increased release of lactate dehydrogenase, IL8, and prostaglandin E_2 (Beltinger et al., 2008).

Potentially beneficial bacteria have also been studied in the Ussing chamber system including commercially available probiotic blend *Bifco* (Shi et al., 2014) and *Lactobacillus plantarum* (Chen et al., 2010). Bifico was able to improve epithelial barrier function, enhance resistance to EIEC infection, and reduce proinflammatory cytokine secretion. L. plantarum was also demonstrated to improve colonic epithelial barrier dysfunction in IL-10 knockout mice, by modulating epithelial junctions and PepT1-mediated transepithelial transport. The Ussing chamber model has been useful in demonstrating the ability of probiotics to promote intestinal barrier function.

Intestinal Enteroids and Organoids

Organ culture of intestinal tissue was first described in 1969 by Browning and Trier (1969). This method utilized biopsy tissue and a traditional culture-dish system but was limited in the amount of time tissue could be cultured. For decades the limiting factor in culturing intestinal cells was their life span. In the last decade, Dr. Hans Clever's lab developed a method for isolating intestinal crypts from the small or large intestine of mice and propagating LGR5$^+$ stem cells from mice that can be continuously passaged and propagated (Sato et al., 2009), termed intestinal organoids. This scientific breakthrough has led to an exponential increase in publications using these models and opened an entire new world not previously and readily available. Additionally, in 2014 Yin et al., published a reproducible method to direct the differentiation of LGR5$^+$ stem cells to become a specific cell type (i.e., enterocytes, goblet cells, stem cells, enteroendocrine cells; Yin et al., 2014). Directed differentiation methods have allowed researchers to examine cell-type specific responses and properties including barrier function and would be useful tools to examine host-microbe interactions (Pearce et al., 2018). Organoids can now be isolated from several species and methods for culturing stem cells from human biopsy tissue, termed human intestinal enteroids have been greatly refined. As organoids/enteroids have a variety of functional enteroendocrine cells, they are now thought to be a useful model to study the gut-brain axis (Hampton, 2017) which has potential implications for host-microbe interaction research. On the microbial side, human enteroids have been utilized for host-pathogen studies including *Enterohemorrhagic E. coli* (In et al., 2016), *Enterotoxogenic E. Coli* (Rajan et al., 2018), *Enteroaggregative E. Coli* (Noel et al., 2017), as well as Cholera toxin (Zomer-van Ommen et al., 2016). Additionally, host-commensal bacterial interactions including *Lactobacillus rhamnosus GG* (Aoki-Yoshida et al., 2016), *L. acidophilus and Bacteroides thetaiotaomicron* have been examined. A full list of organisms, bacterial ligands, and bacterial metabolites utilizing, organoids and enteroids is listed in **Table 1** along with main findings of each study. Examples of organoid morphology and structure of human and mouse samples are shown in **Figure 1**.

Another source of primary human intestinal epithelium are derived from inducible pluripotent stem cells (iPSC), also known as human intestinal organoids (HIO). These organoids are generated from iPSC's by a multistep process first involving development of an endoderm, and later development of epithelial and organoid structures (Workman et al., 2018). These iPS organoids have been utilized to study how *clostridium difficile* infection affects ion transport (Engevik et al., 2015) as well as intestinal barrier function

TABLE 1 | Bacterial organisms and components that have been utilized for intestinal organoid research and main findings.

	Organoid	Main Findings	References
PATHOGENIC			
Clostridium difficile	iPS organoid	*C. difficile* inhibits Na^+/H^+ transport to create more favorable environment for growth. *C. difficile* decreases paracellular barrier function.	Engevik et al., 2015; Leslie et al., 2015
Salmonella enterica	iPS organoid, mouse ileal enteroid	*Salmonella* decreases stem cell markers and disrupts barrier function. *Salmonella* invades the epithelial barrier using vacuoles.	Zhang et al., 2014; Forbester et al., 2015
Enterohemorrhagic *E. coli*	Human colonoid, ileal/rectal enteroid	EHEC reduces mucus and affects the brush border cytoskeleton.	In et al., 2016
E. coli ECOR2	iPS organoid, duodenal enteroid	Colonization of organoids with non-pathogenic bacteria induces functional maturation of the intestinal barrier.	Hill et al., 2017
Enteroaggretative *E. coli*	Human duodenal/ileal enteroid, colonoid	EAEC adheres to small intestinal segments differently and is host specific.	Rajan et al., 2018
Enterotoxigenic *E. coli*	Human enteroid	Immune and epithelial cells coordinate to combat infection by improving barrier function and cytokine response.	Noel et al., 2017
E. coli O157:H7	iPS organoid	*E. coli* 0157:H7 causes loss of actin and epithelial integrity and increases ROS production and inflammatory cytokines.	Karve et al., 2017
Helicobacter pylori	iPS/human gastric organoid	Organoids are a good model to study *H. pylori* pathogenesis.	Pompaiah and Bartfeld, 2017
Listeria monocytogenes	Murine small intestinal enteroid	*L. monocytogenes* was used in enteroid protocol development for host-pathogen interactions.	Rothschild et al., 2016
Cholera toxin	Human jejunal/rectal enteroid	Enteroids were utilized to test preclinical drug inhibitors of Cholera toxin.	Zomer-van Ommen et al., 2016
COMMENSAL			
Akkermansia muciniphila	Murine ileal enteroid	*A. Muciniphila* modulates expression of important regulators of transcription factor regulation, cell cycle control, lipolysis, and satiety.	Lukovac et al., 2014
Faecalibacterium prausnitzii	Murine ileal enteroid	*F. prausnitzii* weakly promotes host regulation of cellular processes, neurological system processes, and cell differentiation.	Lukovac et al., 2014
Lactobacillus acidophilus	Chicken enteroid	*L. acidophilus* promotes organoid growth and increases production of beneficial prostaglandins.	Pierzchalska et al., 2017
Lactobacillus rhamnosus GG	Murine small intestine enteroid	*L. rhamnosus* GG increases toll-like receptor 3 expression.	Aoki-Yoshida et al., 2016
Bacteroides thetaiotaomicron	Murine ileal enteroid	*B. thetaiotaomicron* induces host epithelial changes via increased fucosylation	Engevik et al., 2013
BACTERIAL LIGANDS			
Lipopolysaccharide	Murine colonoid	LPS is the dominant bacterial agonist that affects cell proliferation in a toll-like receptor 4 dependent manner.	Naito et al., 2017
Flagellin	Murine small intestinal enteroid	Flagellin was used in protocol development to study host-microbe interactions.	Rothschild et al., 2016
Poly(I:C)	Murine small intestine enteroid	Poly(I:C) combined with *L. rhamnosus* GG increases expression of interferon-α and chemokine CXCL1	Aoki-Yoshida et al., 2016
Muramyl-dipeptide	Murine small intestinal enteroid	MDP induces higher yield of intestinal organoids, and induces stem cell protection via NOD2.	Nigro et al., 2014
BACTERIAL METABOLITES			
Short-chain fatty acids	Murine small intestinal crypts	Propionate and butyrate but not acetate regulation expression of HDAC activity, and expression of adipose related genes Fiaf, PPARγ and Gpr3. SCFA's promote organoid development. Butyrate stimulates epithelial production of retinoic acid via HDAC inhibition.	Lukovac et al., 2014; Park et al., 2016; Schilderink et al., 2016
Indoleacrylic Acid	Human colonoid	IA promotes intestinal epithelial barrier function and reduces inflammation.	Wlodarska et al., 2017

(Leslie et al., 2015). Additionally they have been used to study bacterial colonization of *E. coli* ECOR2 effects on the host (Hill et al., 2017), host response to infection of Shiga toxin producing *E. coli*, (Karve et al., 2017) and *Helicobacter pylori*

pathogenesis in gastric organoids (Pompaiah and Bartfeld, 2017).

In 3D culture, microbiological research of organoids and enteroids is highly difficult. The 3D architecture of these cell
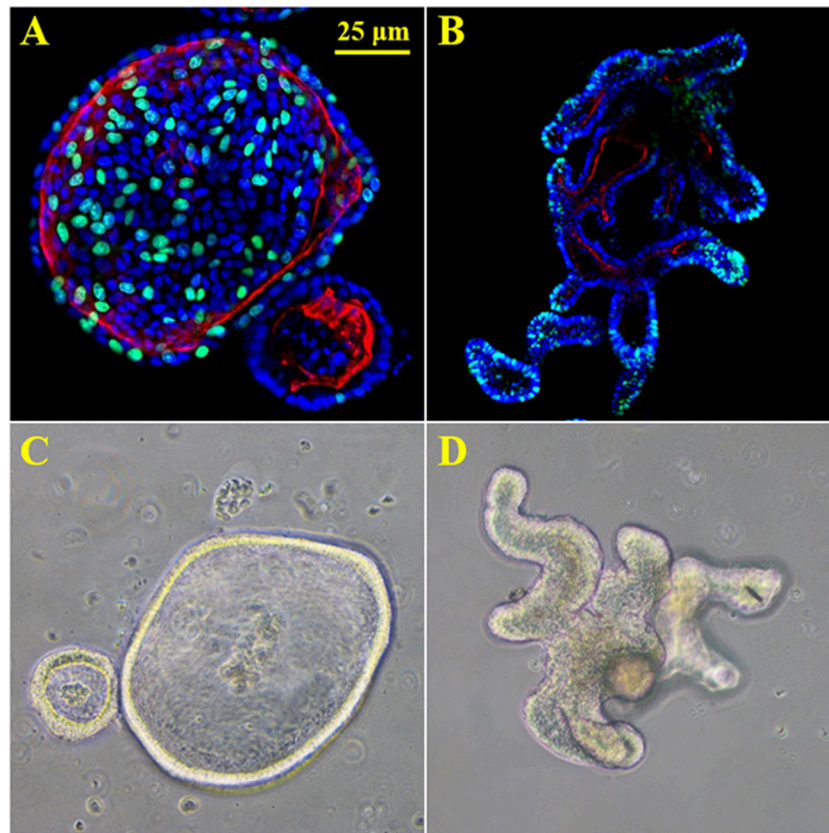
FIGURE 1 | 3D enteroids from human and mouse biopsy samples. **(A)** proliferation stain Edu (green), nuclei (blue) and f-actin (red) in human duodenal organoids. **(B)** proliferation stain Edu (green), nuclei (blue) and f-actin (red) in murine duodenal organoids. **(C)** 20X brightfield image of human duodenal organoids. **(D)** 20X brightfield image of murine duodenal organoids.

systems causes their polarity to be "inside-out" where the lumen is contained in the center of the spherical structure. Accessing the lumen inside the organoid, where bacteria normally reside, is a significant challenge that has been overcome by various laboratories (Hill et al., 2017). Several researchers have found ways to by-pass this via microinjection or trituration (Karve et al., 2017); however, this is a very difficult and time consuming process. In the last few years, there have been large steps forward in organoid technology including the ability to polarize these cells on a 2D monolayer as well as the ability to co-culture with immune cells, neurons and other cell types to make a more physiologically relevant GI model system.

Organs-on-a-Chip and Microfluidic Devices

Organs on a chip utilizing microfluidics is the most recent bioengineering advancement as it pertains to intestinal cell models and may show the greatest potential to mimic complex multi-organ or multi-layer systems found *in vivo*. A recent comprehensive review on organ-on-a-chip models was written by Bein et al. (Kasendra et al., 2018). Several variations of these models now exist and the field is relatively new but expanding at a very rapid rate. One example of the applications for this type of platform utilizes Caco-2 cells cultured in the presence of planar stretch and luminal flow that mimics digestive

shear forces and presumably promotes a more physiologically-relevant state of the cells. Researchers have used this model to screen several different applications including evaluating anti-inflammatory probiotics and analyzing bacterial overgrowth often observed with IBD (Kim et al., 2016b). Additionally, gut-on-a-chip microfluidic devices allow for co-culturing of living microbiome and engineered human intestines (Kim et al., 2016a). Recent advancements in gut-on-chip technology has allowed for electrodes to be embedded into the chips in order to analyze transepithelial electrical resistance (TER) a common functional measure of intestinal barrier integrity (Henry et al., 2017). More recently, a small intestine-on-a-chip has been created using human biopsy derived organoids cultured alone or in conjunction with intestinal microvascular endothelial cells to better mimic the complexity of the *in vivo* intestinal epithelium tissue (Kasendra et al., 2018). A recent more comprehensive review on these models was recently published (Kasendra et al., 2018).

ADVANTAGES AND DISADVANTAGES OF *EX VIVO* MODELS

Condensed advantages/disadvantages of both *in vitro* and *ex vivo* models are shown in **Table 2**. Overall, *ex vivo* systems contain

added complexity and functional cross-talk between many different cell types that are not generally found in *in vitro systems* (Roeselers et al., 2013). The IntesTINE system has potential to be a very useful model to study bacterial-host interactions in live tissue of a physiologically relevant animal model. However, it has not been fully validated for use and therefore may provide some challenges. Ussing chambers can utilize live mammalian tissue or cultured cells on snap-well dishes. Tissues or cells can be treated before or during Ussing chamber runs and more than one analysis can be conducted including epithelial resistance, FITC-LPS, or FITC-Dextran transport, as well as measures of glucose or amino acid transport. The main disadvantage to this system is that the tissue is only viable for a period of hours and it cannot be used for longer term studies (>5 h). Intestinal organoids, especially from human biopsy samples provide a near-ideal model to utilize for host-microbe interactions. Cell types found in organoids recapitulate the normal epithelium and translatability if using human organoids is not an issue. For bacterial research, the main drawback is the 3D organoid system that has the lumen internalized and provides difficulty in treating with bacteria; however, recent host-pathogen studies have emerged that utilize intestinal organoids grown as confluent monolayers. Organs-on-a-chip are technically challenging and working with small volumes and cell numbers can limit the methods that can be used for downstream analysis. Many times end-point studies alone are used because monitoring changes overtime in a chip is challenging and many methods of detecting cellular changes involve terminating the cells. However, the industry involvement in organ-on-a-chip technology (ALine, Draper) has improved reproducibility and manufacture of chips built to a researchers specifications. Another major disadvantage to the current *ex vivo* systems is the inability of these systems to recapitulate the anaerobic environment of the intestinal lumen as well as the oxygen gradient established at the mucosal layer. With the growing field, increased interest, available organ types and advances in real-time monitoring, it may be possible to replace rodent or other animal models in a variety of scientific applications in the near future. Condensed advantages/disadvantages are shown in **Table 2**.

OTHER POTENTIALLY USEFUL MODELS

Co-culture

With the advancements in technology, including organs-on-a-chip, have come a new wave of more complex, integrated cell systems including the gut-brain axis and immune systems. Advances have been made using Transwell culture plates, where cells and bacteria can be co-cultured and separated by a semi-permeable membrane that allow for bacteria-host interactions to be investigated in a complex culture. For example, Caco-2 cells were grown in a Transwell® system where epithelial cells are grown in the apical chamber, while human dendritic cells are cultured in the basolateral chamber, followed by apical exposure to live probiotic *Lactobacillus paracasei*. This co-culture model showed that probiotics in the presence of epithelial and immune cells exert a different response than observed using single cell cultures (Bermudez-Brito et al., 2015). Additionally, flow and

variation in the environment between a bacteria and host layers can be applied, with the added advantage of the larger size then organ-on-a-chip models. Human non-transformed neonatal small intestinal cells (H4-1) have been used to study bacterial interactions when co-cultured with macrophages and researchers observed a significant difference in bacterial translocation in epithelial cells cultured alone vs. those co-cultured with immune cells (Trapecar et al., 2014). Additionally, a recent model was developed that contains macrophages co-cultured with human organoids (Noel et al., 2017). Another microfluidics system is the HuMiX which incorporates human cells co-cultured with bacterial cells (Shah et al., 2016). Advantages here include more complex systems that may more accurately recapitulate *in vivo* physiology but disadvantages include commercial availability, cost, and culturing conditions.

Tissue Engineering

A comprehensive review of tissue engineering of the gut was written by Bitar and Raghavan in 2012 (Bitar et al., 2014). In brief, tissue engineering involves generation, or in many cases regeneration of complex tissue systems. For the intestine, this includes tubular tissue constructs that contain absorptive villi and crypts, along with associated smooth muscle and enteric nerves to provide a fully integrated system. Cell sources for this can be difficult but strides have been made utilizing stem cells to direct differentiation to a desired cell type.

Host-Microbial Metabolite Models

Dietary tryptophan that is metabolized by both host and bacteria to indole derivatives have been shown to reduce intestinal permeability in T84 cells (Jennis et al., 2018). Intestinal organoids have proven to be a good model to examine mechanisms of host-bacterial interactions in a multi-cellular system (Rothschild et al., 2016; Blutt et al., 2018). Polyphenol metabolites which are biotransformed by the gut microbiota, have also been examined *in vitro* models (mainly Caco-2) including urolithin A (Gonzalez-Sarrias et al., 2015) and tert-butyl hydroperoxide (Deiana et al., 2010) and 3,4-dihydroxyphenyl-ethanol (Manna et al., 1997). Metabolic products produced by bacteria, especially butyrate, have been implicated in intestinal health and Caco-2 cells have been utilized in several studies to elucidate the mechanisms by which SCFA's modulate colonic function (Nepelska et al., 2012).

IN VITRO AND *EX VIVO* MODELS FOR MILITARY RELEVANT STRESSOR RESEARCH

As mentioned earlier, there are several environmental and physical stressors that are experienced by human beings, and in the military these stressors are often exacerbated. Several of these stressor types can be mimicked in the aforementioned *in vivo* and *ex vivo* models to provide a more mechanistic approach that can be utilized for future clinical research. Currently no data is available pertaining to model systems for military specific research in this area.

TABLE 2 | Advantages and Disadvantages of intestinal host models.

	Advantages	Disadvantages
IN VITRO		
Caco-2	Can be polarized, cost effective, ease of use, extensive literature available, commercially available	Cancerous origin, difficult to control differentiation
HT-29	Can be polarized, commercially available, mucus producing cells	Cancerous origin
T84	Can be polarized, commercially available, cost effective, ease of use	Cancerous origin
IEC-6	Can be polarized, commercially available, non-cancerous origin, good for studying small intestine	Not good mimic for colon
IPEC-J2	Can be polarized, contain multiple cell types, commercially available, good for studying small intestine, non-cancerous origin, physiologic relevance to humans	Not good mimic for colon
EX VIVO		
Ussing Chamber	Tissue is polarized, can obtain barrier function and transport data, contain all epithelial cell types	Short Term (up to 5 h)
Organoids	Can be grown 2 or 3D, can be polarized, contain all epithelial cell types, contain transporters and pattern recognition receptors	Human samples difficult to obtain, cost
Gut-on-Chip	More complex system, more physiologically relevant	Cost, not commercially available
OTHER		
Co-culture	More complex system, cell type interactions, more physiologically relevant	Difficult to control conditions for multiple cell types
Tissue Engineering	Translatability	Cost, not fully validated

Heat Stress/Cold Stress

Heat stress (HS) has been studied *in vitro* and *ex vivo* in a number of models covered in this review. Generally, in *in vitro* models, heat stress/hyperthermia is applied using a temperature adjustment on a standard cell culture incubator while in many *ex vivo* models the stressor is applied to the animal prior to tissue being excised, such as in Ussing Chambers. Caco-2 cells have been used to study heat stress effects on the gut (Swank et al., 1998; Hershko et al., 2003; Dokladny et al., 2006; Xiao et al., 2013) to show changes in intestinal permeability, heat shock response, and immune response that mimic human *in vivo* studies. Similarly, IEC-6 cells have been utilized to study heat stress mechanisms and cell death (Xu et al., 1996; Yu et al., 2013). IPEC-J2 cells have been utilized to show that HS affects tight junctions, as well as selenoproteins involved in the oxidative response. In *ex vivo* models, heat stress has been studied extensively using post-stress measurement of barrier function in Ussing chambers (Pearce et al., 2013, 2014). Cold stress has not been studied *in vitro,* and to date there are no *in vitro* models of environmental cold stress.

High Altitude/Hypoxia

Hypoxia, mimicking high altitude has been studied extensively in *in vitro* models, including Caco-2 (Unno et al., 1996; Xu et al., 1999; Lee et al., 2002; Lei et al., 2014; Jin and Blikslager, 2016), IEC-6 (Xu et al., 1999; Li et al., 2003; Miki et al., 2004; Chen et al., 2010) as well as IPEC-J2. This can be a chemically induced hypoxia using a hypoxia-mimetic agent Cobalt Chloride, or environmentally induced utilizing *in vitro* hypoxia chambers that can control oxygen and carbon dioxide concentrations. These studies have mechanistically shown the involvement of myosin light chain kinase in hypoxia-induced barrier dysfunction (Jin and Blikslager, 2016). Interestingly, one study in Caco-2 cells shows that exposing host cells

to hypoxia prior to bacteria treatment decreased bacterial internalization of *Yersinia enterocolitica* (Zeitouni et al., 2016). Hypoxia has also been studied in colonic biopsies in Ussing chambers where hypoxia is induced in one or both sides of the chamber (Carra et al., 2013). Additionally, hypoxia combined with *E. coli* exacerbates intestinal inflammation and cytokine secretion in a Ussing chamber hypoxic model (Ding et al., 2001). Additionally, hypobaric hypoxia, which more accurately mimics environmental hypoxia, such as is seen at high altitudes, has been shown to alter intestinal barrier function in an *ex vivo* rodent model (Saravi et al., 2002).

Nutritional Stress

Nutrient deprivation, including amino acid deprivation (Roussel et al., 2017), folate deprivation (Townsend et al., 2004), fasting (Le Bacquer et al., 2003) have been examined in Caco-2 cells, while glucose deprivation has been studied in depth utilizing HT-29 cells (Hwang et al., 2008; Li et al., 2009). Intestinal organoids are starting to be utilized for more nutritional related research and effects of essential amino acid deprivation have been studied in intestinal stem cells (Saito et al., 2017).

"OMICS" AND BIOINFORMATICS METHODS FOR *IN VITRO* AND *EX VIVO* MODELS

To determine effects of microbes or stressors on the host, or bacteria there are a number of functional, quantitative, and qualitative measurements that have been standard practice. However, in recent years, "Omics" techniques that have been applied to human and animal models have come to the forefront of analyses. Valuable information can obtained through the use of "omics" approaches in host-microbe or

host-metabolite models as they integrate mammalian cells and microbial cells or microbial by-products. Experimental information about the function via RNA (transcriptomics), protein analysis (proteomics), proteins that are externally secreted by cells (secretomes) (Mukherjee and Mani, 2013) and the ability to identify the presence of metabolites (metabolomics) are potential targets. Also, the microbial diversity of a specific community and its genes could be extensively analyzed from any of these models with the current Next-generation sequencing techniques using specific markers, such as the 16s rRNA gene (metagenomics). To date, with advances in bioinformatics, researchers have access to a variety of computational methods to analyze "omics" data (Segata et al., 2013) and the combination of different approaches like metagenomics and metaproteomics

which not only reveal the taxonomy, but also functional activity.

There are several RNA-Bioinformatics tools comprised in different packages, like RNA workbench (Gruning et al., 2017), for the analysis of RNA structures, RNA alignments, RNA-RNA interactions, RNA-protein interactions, RNA sequencing, ribosome profiling, and genome annotation. An important resource since 1995 is the Kyoto Encyclopedia of Genes and Genomes (KEGG) integrated database that can bring information about metabolism and cellular processes from high-throughput genome sequence data (Kanehisa et al., 2014). Another tool for the study of metabolic pathways and enzymes is the MetaCyc reference database (Caspi et al., 2008).
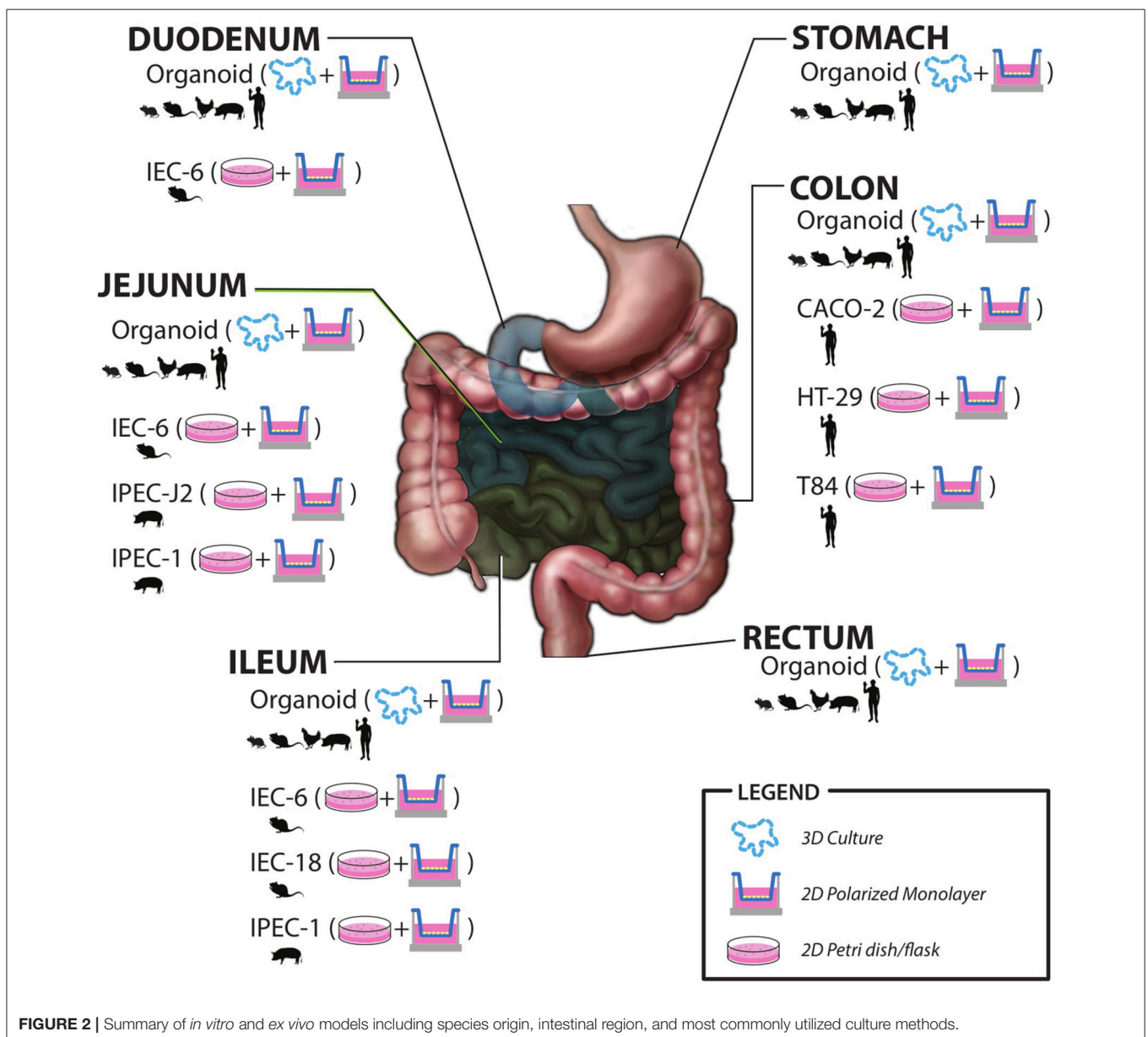

FIGURE 2 | Summary of *in vitro* and *ex vivo* models including species origin, intestinal region, and most commonly utilized culture methods.

Information garnered from "omics" techniques may set a basis to employ a helpful approach called machine learning. It consists of a series of algorithms that after "trained," can predict outcomes and future states in specific areas of the research field, such as shifts in the microbiome structure and function as a result of certain factors (e.g., health vs. disease status; Yazdani et al., 2016). One common machine learning technique is the random forest regression [reviewed on (Knights et al., 2011; Knight et al., 2018)] that generates a large number of trees to select for the best one to carry out taxonomy classification, referring specially to microbiome studies (Breiman, 2001). The integration of these methods could provide information about patterns and understanding on microorganism's abundance as a consequence of a stressor.

CONCLUSIONS, FUTURE PLANS AND IMPLICATIONS FOR MILITARY RESEARCH

As the microbiome field advances it is becoming clearer that humans rely heavily on their microbial counterparts to maintain intestinal homeostasis. The gut microbiota can respond, in parallel with the host to changes in environment, diet, and other common types of stress and this can lead to dysbiosis. This is often compounded in members of the military who often experience stressors in combination during training or on the battlefield. Although there are emerging studies of these stressors in human models, there is a need to further characterize the effects of these stressors via human studies using the military cohort. There is also a need to understand the mechanisms of action via basic and early applied research in physiologically relevant *in vitro* and *ex vivo* models. There have been *in vitro* host models utilized to study host-microbe interactions for the better part of 50 years. New advances in cell biology have allowed for host models much more similar to a healthy human host. The ability to study physiologically relevant models, including the ability to examine specific cell types as well as differentiated vs. undifferentiated allows for in depth examination of cell-specific responses. This is especially true for crypt-residing stem and Paneth cells that will experience microbial and microbial metabolite exposure during times of injury, stress, or dysbiosis. Future research to study host-microbiome interactions using primary cell culture models, such as intestinal organoids will provide new insights into the host-microbe cross-talk. Human gut-on-a-chip platforms also provide a great deal of innovation and opportunity to study the interactions on a more physiologically relevant level. These models can be individualized and used to study military-relevant stressor effects between the gut microbiota and human host and provide input to more applied clinical research studies. There is also potential research areas using new *ex vivo* models to examine the effects of bacterial metabolites on host function. A graphic summary of models discussed in this review is shown in **Figure 2**.

Additionally, clinical studies can help inform the *in vitro/ex vivo* work to explore potential mechanisms. One example might be that a military-relevant stressor, such as high altitude or nutrient deprivation has been identified. This can be modeled in host models to determine effects on the host, as well as in *in vitro* fermentation models to examine effects on bacterial function and metabolites. Thereafter, a potential mitigation strategy can be introduced into both systems and depending on the outcomes, can be applied to clinical research. Ultimately, the goal is to provide potential mitigation strategies to improve the health and performance of US warfighters.

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SP designed, wrote, and edited the paper. KR helped design and edit the paper. HC, JK, IP-F and NZ helped write and edit the paper.

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