

Gut Microbiome and Host Defense Interactions during Critical Illness

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Introduction

For many years it has been hypothesized that the gut has an important detrimental role in promoting systemic inflammation and infection in the critically ill. During stress and mucosal hypoxia, the mucosa is damaged and host defenses break down causing translocation of bacteria and bacterial toxins which are thought to contribute to the overwhelming inflammation associated with sepsis and multiorgan failure [1, 2]. New emerging data on the role of the microbiome have forced us to reassess the old 'gut as motor of sepsis' hypothesis. The gut microbiome consists of a diverse and vast population of microbes that has an

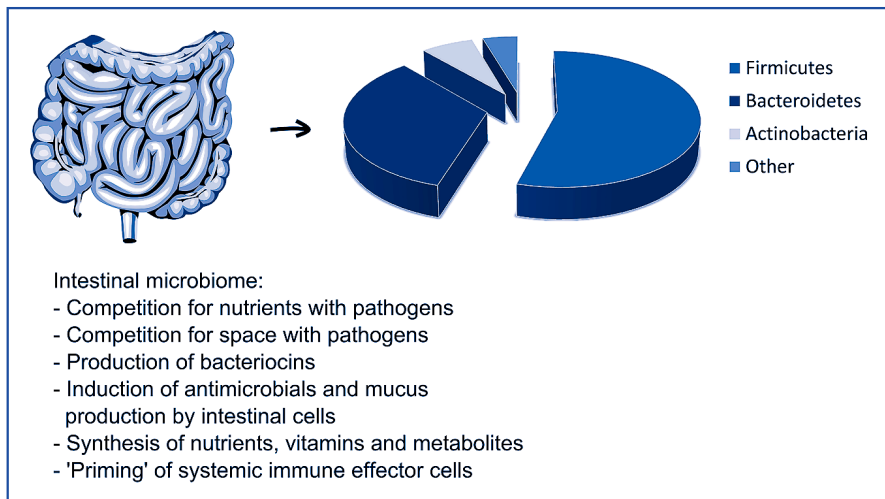


Fig. 1. The intestinal microbiome. The intestinal microbiome is dominated by three phyla: the *Firmicutes* (Gram-positive), *Bacteroides* (Gram-negative) and *Actinobacteria* (Gram-positive). It should be mentioned that significant inter-individual differences in microbiome composition exist. A healthy balanced microbiome is important for the host defense against invading pathogens by preventing pathogenic microorganisms from colonization of the intestine through competition for essential nutrients, space and attachment sites on the epithelium. In addition, members of the intestinal microbiome secrete bacteriocins, defined as toxins produced by bacteria to inhibit the growth of other bacteria, and toxins in order to compete with intestinal pathogens. Other roles of the intestinal microbiome include stimulation of the intestinal immune system, digestion of food, synthesis of essential nutrients for the host and constitutive priming of systemic immune cells.

important protective impact on immune effector functions during both health and disease (Fig. 1). It has become clear that the intestinal microbiome, consisting of more bacteria than the total number of cells in the human body, can be seen as an exteriorized organ that exerts numerous functions in the host response against infections [3, 4]. In addition to the more localized influence of the microbiome on the intestinal immune system, recent data show that the microbiome also plays a key role in systemic activation of the immune system contributing to the effective killing of invading pathogens [5]. The clinical relevance of these new insights is underscored by the notion that antibiotic treatment – which on any given day is received by almost three quarters of all patients on the intensive care unit (ICU) [6] – can largely deplete the microbiome. This review focuses on key aspects of the role of the intestinal microbiome in the immune response against pathogens and the importance of intestinal homeostasis for critically ill patients.

The Intestinal Microbiome during Health

The total bowel surface is $\sim 400 \text{ m}^2$ and provides an important habitat for intestinal microorganisms. In addition to the intestinal microbiome, the intestinal epithelium and the mucosal barrier are regarded as the other major entities of the gut [7].

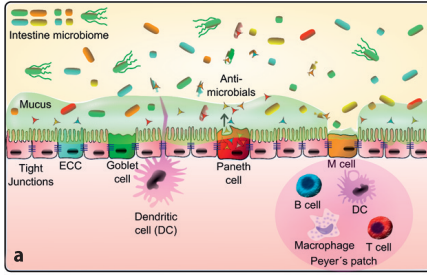
The Intestinal Epithelium and the Mucosal Barrier

The intestinal epithelium is a major hurdle for invading pathogens and is composed of several cell types; key intestinal epithelial cells (IEC) include the enterocytes, Paneth cells, enterochromaffin cells (ECCs), microfold cells and goblet cells which are tightly bound together by tight junctions (Fig. 2). Epithelial tight junctions regulate the permeability of the intestinal barrier and control the passage of large molecules. Paneth cells are found throughout the small intestine primarily in the crypts of Lieberkühn [8]. When exposed to bacteria or bacterial antigens, Paneth cells, characterized by large cytoplasmic granules, secrete innate immune effector molecules such as defensins, but also lysozyme, phospholipase A2 and tumor necrosis factor (TNF)- α , all of which have antimicrobial activity [8]. ECCs are enteroendocrine cells that secrete hormones,

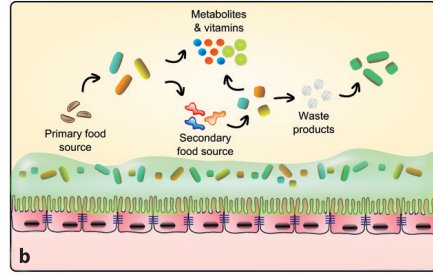
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Fig. 2. a The intestinal epithelium is composed of enterocytes (pink), goblet cells (green), Paneth cells (red), microfold (M) cells (orange) and enterochromaffin (ECC) cells (blue). Goblet cells continuously produce and secrete mucins which form a thick mucus layer (green layer). The mucus layer consists of two layers; only the upper layer is inhabited by members of the intestinal microbiome. Paneth cells contain granules with antimicrobials such as defensins and lectins which are released into the mucus layer. M cells facilitate the transport of microbes to the underlying Peyer's patches which are aggregated lymphoid nodules. To facilitate ingestion of bacteria by M cells these cells are not covered by the thick mucus layer. The intestinal epithelial cells (IECs) are packed tightly together by tight junctions. Strict regulation of the tight junctions makes the epithelial tissue a selectively permeable barrier. ECCs are enteroendocrine cells that secrete various hormones, like serotonin, in response to several stimuli including toxins produced by bacteria. **b** Several bidirectional relationships between the host and the intestinal microbiome and between bacteria within the microbiome take place in the intestine. Intestinal bacteria provide the human body with metabolites such as short-chain fatty acids, but also with vitamins and other nutrients. Strong mutualism exists between bacteria within the microbiome as well.

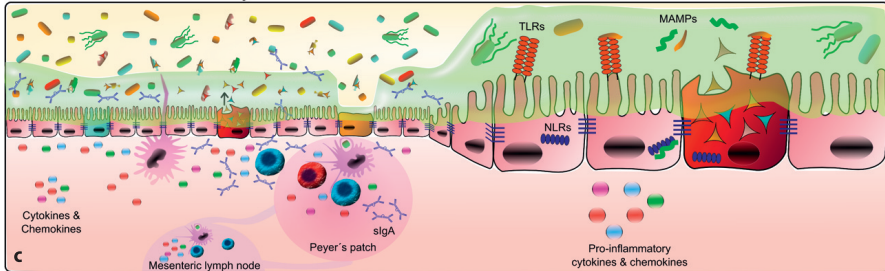
Major entities of the gut



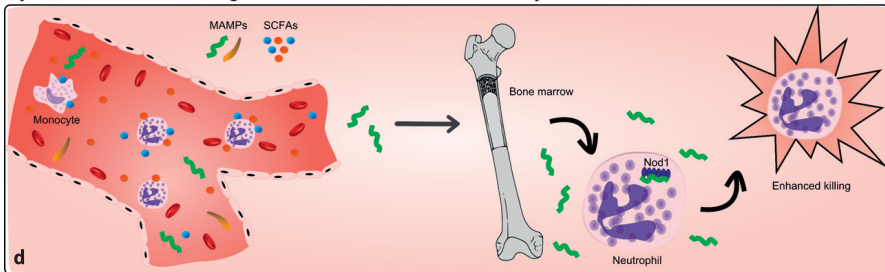
Mutualism



Crosstalk with immune system



Systemic effects of the gut flora on the host's defense system



While some intestinal bacteria can process a primary food source, other bacteria are dependent on metabolic products (secondary food source) produced by these bacteria. Other members of the microbiome play a role in the removal of waste products. **c** Components of the microbiome express conserved molecular structures termed microorganism-associated molecular patterns (MAMPs) which are sensed by pattern recognition receptors (PRRs) – such as Toll-like receptors (TLR) – expressed on IECs, which continuously respond to these compounds in order to orchestrate the immune response. MAMP and metabolite release are a signal for intestinal cells to continuously produce and secrete mucus, antimicrobials and cytokines. Nutrients are also able to activate the immune system directly. M cells are the most important cells in sampling antigens and bacteria from the gut lumen by transferring the material to the underlying Peyer's patches. Dendritic cells (DCs) process and present antigens of T-cells and B-cells in the Peyer's patch or migrate to mesenteric lymph nodes to do this. B-cells that mature become IgA-secreting plasma cells. This dimeric soluble IgA (sIgA) is transported through epithelial cells into the mucosal surface. sIgA is not only important in eradication of pathogens but also in transporting antigens back into the gut lumen in order to maintain hemostasis. Besides M cells, intraepithelial DCs are also involved in direct uptake of antigens and bacteria from the mucosal surface. **d** The impact of the intestinal microbiome does not stop at the gut. Metabolites, such as short-chain fatty acids (SCFA), are partly taken up by IECs while another portion enters the systemic circulation. SCFAs and butyrate have an anti-inflammatory effect on leukocytes. Additionally, components of the intestinal microbiome are translocated into the systemic circulation and continuously prime neutrophils leading to enhanced capability of these cells to kill pathogens.

such as serotonin in response to various stimuli including bacterial toxins. Microfold cells can transport bacteria and secreted parts of bacteria across the intestinal barrier to immune cells located in Peyer's patches. Peyer's patches, which are lymphoid follicles, contain antigen presenting cells such as dendritic cells and macrophages. Peyer's patches are part of the gut-associated lymphoid tissue (GALT) of which the other components are the mesenteric lymph nodes and lamina propria lymphocytes.

Before potential intruders meet the intestinal epithelium, they are first awaited by the mucosal barrier. Epithelial and, in particular, goblet cells produce and secrete mucins, which are major components of a thick mucus layer. This layer coats the epithelial surface to protect against pathogens and other irritants such as enzymes, chemicals and mechanical damage. The mucosal barrier itself consists of an inner and outer mucus layer. The outer mucus layer is inhabited by commensal bacteria whereas the inner layer is sterile under normal conditions [9].

Composition of the Intestinal Microbiome

The human intestinal microbiota is composed of 10^{13} to 10^{14} microorganisms whose collective genome ('microbiome') contains at least 100 times as many genes as our own genome [10]. The gut hosts $\sim 1 \times 10^{14}$ bacteria from 500–1,000 different species of which three bacterial divisions – the *Firmicutes* (Gram-positive), *Bacteroides* (Gram-negative) and *Actinobacteria* (Gram-positive) – dominate (Fig. 1) [11]. Although the majority of the intestinal microbiome is composed of bacteria, other members have also been identified, including viruses (5.8 %), archaea (0.8 %) and eukaryotes (0.5 %) [12]. Significant differences in the composition of the intestinal microbiome exist between individuals, which can be explained by host genetic and environmental factors [13]. Recent metagenomic studies, that combined 22 sequenced fecal metagenomes of individuals from four countries, have stratified the differences in gut microbiome composition into three robust clusters, termed enterotypes, which are not nation or continent specific [12]. This remarkable finding demonstrates the existence of only a very limited number of well-balanced host-microbial symbiotic states [12]. Differences in gut flora composition may not only contribute to variations in normal physiology as seen among individuals but most probably also affect susceptibility to infection and variations seen in the subsequent immune response [10, 11]. It is anticipated that the identification of enterotypes in patients might allow classification of human groups that respond differently to diet or drug intake [12].

Bidirectional Relationship Between the Immune System and the Intestinal Microbiome

Insight into the close and intense relationship between the intestinal microbiome and the cells of the intestinal epithelium is just beginning to emerge. A role of the microbiome in protection against epithelial cell injury, optimization of host immune systems and resistance against colonization by pathogens have all been described [14, 15]. Furthermore, intestinal bacteria are of importance in the development of mucosal immunity. Germ-free mice, which lack the intestinal microbiome, have underdeveloped Peyer's patches, immature germinal centers, reduced epithelial antimicrobial production and diminished antibody responses [16].

Secretion of Antimicrobials and Competition for Nutrition

Members of the intestinal microbiome secrete antimicrobials and compete for nutrients and space with pathogens. In health, secretion of toxins and antimicrobials such as bacteriocins by bacteria will outcompete pathogens in the intestinal microbiome [17]. For instance, bacteriocins, defined as toxins produced by bacteria to inhibit the growth of similar or closely related bacterial strains, that are produced and secreted by Gram-negative bacteria in the intestine, can inhibit enteropathogenic bacteria such as *Salmonella*, *Shigella*, *Klebsiella*, *Enterobacter*, and *Escherichia* [17]. Competing nutrition is an alternative strategy used by both the 'good' bacteria and the host in order to prevent pathogen invasion [18]. Not surprisingly, diet and nutrition contribute to microbiome composition and immune function [8]. This is the rationale used by those who propagate a prebiotic diet consisting of non-digestible ingredients that can stimulate the health and survival of these 'good' bacteria in order to keep or restore a healthy microbial balance and immune homeostasis. Humans also need certain enzymes from intestinal bacteria that humans themselves do not have; e.g., to digest polysaccharides derived from plant cell walls, enzymes derived from intestinal microbes are essential (Fig. 2). Other much needed metabolites such as the short-chain fatty acids (SCFAs), acetate, propionate and butyrate, are main end-products of bacterial metabolism in the intestine as well. Butyrate is an important energy source for enterocytes [19] and plays an important role in the regulation of enterocyte immune effector functions as discussed below.

Interactions between MAMPs and PRRs

Bacteroides, *Firmicutes*, and *Actinobacteria* and all other components of the microbiome express conserved molecular structures termed microorganism-associated molecular patterns (MAMPs) [20]. Examples of MAMPs include lipopolysaccharide (LPS), peptidoglycan, lipoteichoic acid (LTA) and flagellin. MAMPs are recognized by pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) and nucleotide oligomerization domain-like receptors (NLRs) [21, 22]. Thirteen TLRs have been identified in mammals. TLR4 is regarded as the LPS receptor, whereas TLR2 is seen as the most important receptor for Gram-positive bacteria (e.g., LTA is recognized by TLR2). TLR5 recognizes flagellin. The recognition of MAMPs by PRRs results in the activation of signaling cascades that in turn lead to activation of the nuclear factor-kappa B (NF- κ B) pathway and initiation of the antibacterial immune response [21, 22] (Fig. 2). The intracellular NLRs, Nod1 and Nod2, respond to the peptidoglycan components, meso-diaminopimelic acid (DAP) and muramyl dipeptide (MDP), respectively [20]. Nod1 is expressed in many cell lines, including IECs. Nod2, however, is not expressed in enterocytes, but is expressed by Paneth cells in the small intestine [20, 23]. TLRs are expressed on, among other cells, neutrophils, macrophages and enterocytes [21, 22]. Of note, not only the composition of the microbiome but also the expression of PRRs varies considerably in different parts of the lower gastrointestinal tract [20, 24]. As a consequence, cross-talk between the intestinal microbiome and the immune system will be different in the colon compared to the small intestine [20]. IECs of the human small intestine express TLR1, TLR2, TLR3, TLR4, TLR5 and TLR9 at low levels, which will in general increase during inflammation [24]. In addition, the polarity of enterocytes also implies different TLR

expression at the apical and basolateral surface of these cells. TLR9 activation through apical and basolateral surface domains of IECs has distinct transcriptional responses: Basolateral TLR9 signals $\text{I}\kappa\text{B}\alpha$ degradation and activation of the NF- κB pathway, while apical TLR9 stimulation invokes a unique response in which ubiquitinated $\text{I}\kappa\text{B}\alpha$ accumulates in the cytoplasm preventing NF- κB activation [25]. Furthermore, apical TLR9 stimulation confers intracellular tolerance to subsequent TLR challenges, which implies that polarized IECs are in the unique position to maintain colonic homeostasis and regulate tolerance and inflammation [25]. The differentiated expression of TLR5 on IECs is another example of the polarized function of IECs. TLR5 is expressed only on the basolateral surface of enterocytes, where it can trigger the production of cytokines and chemokines, such as interleukin (IL)-8 and CC-chemokine ligand (CCL)-20 in response to flagellin [24, 25]. Of note, TLR5-deficient mice develop spontaneous colitis and show a large increase in intestinal bacteria density [26]. Just recently it was found that TLR5 deficiency induced changes in gut microbiome composition in mice leading to a full blown metabolic syndrome, including hyperlipidemia, hypertension, insulin resistance, and increased adiposity [27]. Excitingly, transfer of the gut microbiota from TLR5-deficient mice to wild-type germ-free mice conferred many features of metabolic syndrome to the recipients [27]. Lastly, MAMPs also trigger mucin release in order to increase the thickness of the mucosal barrier [9]. In line, PRR mediated responses towards MAMPs will lead to the release of antimicrobials, such as defensins, lectins and lysozymes, into the mucus layer [9].

Microbiome-derived Antigen-induced Production of Antibodies

In humans, the majority of activated B cells reside in the gastrointestinal tract making the gut the largest antibody-producing organ in the human body (reviewed in [20]). Bacteria and their antigenic wall fragments can cross the intestinal barrier primarily through microfold cells that transfer these antigens to immune cells located in Peyer's patches. Dendritic cells in the Peyer's patches present antigens to T cells and B cells either directly in the Peyer's patch or they migrate to the mesenteric lymph nodes to do so. B cells that are located in the lamina propria constantly produce and secrete IgA and IgG after which enterocytes transport and secrete the antibodies into and through the mucus layer [28]. sIgA recognize and bind several pathogens and are, therefore, often named 'natural IgA', whereas infection with a particular pathogen results in production and secretion of specific high-affinity sIgA antibodies [28].

Distant Effects of the Gut Flora on the Host Defense

Perhaps not surprisingly, the impact of the intestinal microflora on the innate immune system does not stop at the gut. Recently it was very elegantly shown that peptidoglycan from the intestinal microbiota constitutively translocates to the circulation and remotely primes bone marrow neutrophils via the NLR, Nod1 [5] (Fig. 2). These constitutively primed neutrophils then show an increased capacity to kill microorganisms [5]. In other words, MAMPs – such as peptidoglycan – are released into the circulation from gut bacteria, are sensed by PRRs and subsequently help host defense mechanisms by positively priming distant immune effector cells in order to be prepared in the case of an invasion by pathogens. In addition to MAMPs, metabolites produced by intestinal bacteria, such as

SCFAs, vitamins and ATP, are absorbed from the gut and released systemically (Fig. 2). These compounds are known to directly affect several parts of both the innate and adaptive immune system. As an example, butyrate has a broad influence on various immune cells and pathways both in the intestine and systemically [19, 29]. Depending on the cell type and concentration of butyrate, SCFA exert anti-inflammatory activities by inhibiting histone deacetylase (HDAC) and thus NF- κ B signaling [29].

The Intestinal Microbiome during Critical Illness

The importance of the gut microbiome in maintaining homeostasis during health has led to a reappraisal of the function – and potential dysfunction – of the gut microbiome during severe illness and infection.

Disturbed Intestinal Homeostasis in the Critically Ill

Both the composition and performance of the gut microbiome, the intestinal epithelium, and the mucosal barrier are all severely affected during critical illness. Studies performed by Shimuzu et al. have shown that the composition of the intestinal microbiome is significantly changed in patients with severe systemic inflammatory response syndrome (SIRS) and that these changes are associated with septic complications and mortality [30, 31]. Using simple Gram-stains to classify the fecal flora into three patterns (diverse, single, depleted), it was shown that mortality due to multiple organ dysfunction syndrome (MODS) for the single pattern (52 %) and the depleted pattern (64 %) was significantly higher than that for the diverse pattern (6 %) [32]. Depletion of the normal gut flora can give

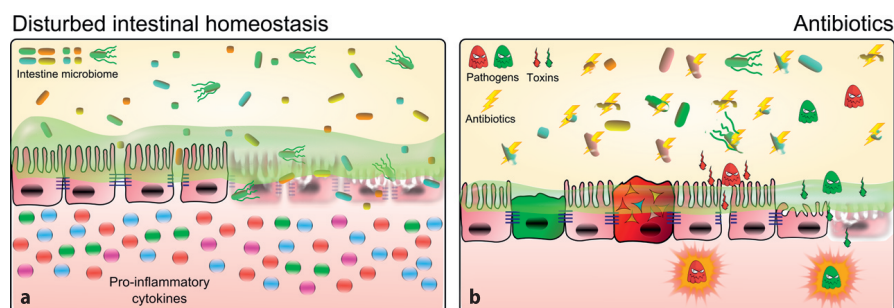


Fig. 3. Altered intestinal homeostasis and the effect of antibiotics on the gut microbiome during critical illness. **a** Pro-inflammatory mediators increase the mucosal permeability by altering the integrity of tight-junctions between enterocytes. Increased apoptosis of intestinal epithelial cells is correlated with sepsis-induced mortality. Loss of the intestinal epithelial barrier function potentially results in translocation of the intestinal microbiome into the circulation. **b** Use of antibiotics can kill members of the intestinal microbiome either directly or indirectly. Indirectly, if these bacteria are dependent on a food source derived from bacteria that are directly affected by antibiotics. Antibiotic treatment decreases the number of intestinal bacteria resulting in diminished release of MAMPs and other metabolites. As a result, fewer antimicrobial molecules, such as defensins and lectins, are produced by Paneth cells, less mucus is produced by goblet cells etc., which makes the host more susceptible to invasion by intestinal pathogens.

I rise to pathogenic bacteria. Pro-inflammatory mediators released during critical illness are able to increase epithelial permeability by influencing the regulation of tight junctions between enterocytes [7] (Fig. 3). Disruption of tight junction barriers results in increased intestinal permeability, infiltration of luminal antigens and the induction of intestinal inflammation [7]. Of note, pathogenic bacteria, such as enteropathogenic *Escherichia coli*, are also known to be capable of increasing tight junction permeability [7]. Increased apoptosis of gut epithelial cells is another characteristic element observed during critical illness. Autopsy studies among patients who died from sepsis have shown a marked increased in intestinal epithelial apoptosis [33]. Inhibition of intestinal epithelial apoptosis in mice showed a survival advantage, although the mechanism underlying this survival advantage is still poorly understood [7, 34]. Finally, it should be emphasized that many treatment modalities that are standard of care for the treatment of critically ill patients on the ICU are also known to affect the composition of the intestinal microflora: Vasopressors can cause splanchnic ischemia and thus local hypoxia and a lower pH; acid suppressive therapies also alter intraluminal pH; highly processed enteral nutrition or parental nutrition can cause a nutrient deficient milieu in the bowel; long term opiate use is associated with bacterial overgrowth and, lastly, antibiotic use causes depletion of the gut flora and potential selection of resistant organisms [2, 35].

The Effect of Antibiotics on the Gut Flora

Several studies have shown that antibiotic therapy has long lasting alterations and implications for the microbial community structure of the intestine [36, 37]. A study among 14,414 patients in 1,265 ICUs in 75 countries, estimated that on any given day 71 % of patients on the ICU receive antibiotic treatment mostly to cover infections considered to originate in the respiratory tract [6]. Antibiotic treatment – which is standard of care for all patients with sepsis – depletes the gut microbiome and is associated with diminished cytokine production in the gut [37, 38] (Fig. 3). Eradication of a specific group of bacteria by antibiotics can indirectly kill other members of the intestinal microbiome that are dependent on food sources provided by these bacteria [39] (Fig. 3). Other members of the intestinal microbiome remove toxic waste products of bacterial fermentation [39]. Of clinical importance, alterations of the intestinal microbiome are associated with nosocomial infections, such as vancomycin-resistant *Enterococcus* and *Clostridium difficile* [15, 40]. Targeted depletion of the gut flora can also be used to preemptively eradicate potential pathogens such as *Enterobacteriaceae*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* in patients on the ICU. Indeed, several studies have shown that selective decontamination of the digestive tract (SDD), in which broad spectrum non-absorbable antibiotics that target yeasts, Gram-positive and Gram-negative pathogens are used to decontaminate the gastrointestinal tract and/or the oropharynx, can decrease ICU and hospital mortality in critically ill patients [41,42]. Worldwide implementation of SDD, however, is hampered by fears of an increase in drug-resistant bacterial strains [43].

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