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REVIEW ARTICLE

Gastrointestinal stress as innate defence against microbial attack

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Summary

The human gastrointestinal (GI) tract has been bestowed with the most difficult task of protecting the underlying biological compartments from the resident commensal flora and the potential pathogens in transit through the GI tract. It has a unique environment in which several defence tactics are at play while maintaining homeostasis and health. The GI tract shows myriad number of environmental extremes, which includes pH variations, anaerobic conditions, nutrient limitations, elevated osmolarity etc., which puts a check to colonization and growth of nonfriendly microbial strains. The GI tract acts as a highly selective barrier/platform for ingested food and is the primary playground for balance between the resident and uninvited organisms. This review focuses on antimicrobial defense mechanisms of different sections of human GI tract. In addition, the protective mechanisms used by microbes to combat the human GI defence systems are also discussed. The ability to survive this innate defence mechanism determines the capability of probiotic or pathogen strains to confer health benefits or induce clinical events respectively.

Introduction

The human gastrointestinal (GI) tract is charged with the most difficult task of rendering protection against uninvited pathogens (Hecht 1999). It is at constant and significant risk of infection and microbe induced inflammation (Dale and Fredericks 2005). Besides having the vital role in human digestion, GI tract offers the primary innate defence against intruding microbial communities, albeit maintaining the beneficial commensal/resident flora. To perform such multi-faceted roles, human GI tract is equipped with an array of features (Hecht 1999).

Food serves as a primary vehicle for providing safe passage to microbial strains through human and animal GI tract. It is believed that bacteria in the food are partially shielded from stomach acids until they remain covered in the food matrix (Ray and Didier 2014). Upon reaching the intestine, they establish themselves inside the gut. Contemporary probiotic research focuses on development of new health promoting foods that enhance the microbial transit tolerance as well as on selecting new beneficial strains having an enhanced ability to survive the stress offered by human GI ecosystem, and to colonize therein (Valerio *et al.* 2006). In terms of dietary intervention, fermented foods are acknowledged as

vehicle of choice for maintaining bacterial supply to gut; as besides offering favourable growth environment and transit protection to resident microbes; they present added advantage of delayed transit time associated with fewer GI symptoms (Labayen *et al.* 2001).

This review focuses on defence mechanisms that are presented by the epithelial cells of oral cavity and GI tract. This review is divided into three major sections, that is, oral cavity, stomach and intestine that represents range of different environmental conditions within human GI tract, which strongly influence their innate and immune defence response.

Gastrointestinal stress (hurdles)

The mucosal lining of GI tract serves as front line of defence against myriad of microbial species. The healthy intestinal mucosal lining is vital for proper gut function such as digestion, nutrient absorption and assimilation, as well as for maintaining immune system. The gut mucosal surface is the largest organ of body in contact with external environment that maintains a physical barrier to the environmental stimuli thereby acting as an interface between external environment and internal organs. Besides mediating the selective intake of essential nutrients, it offers protection against potential pathogens, and maintains adequate number of commensal bacterial population thereby preventing opportunistic infections (Dommett *et al.* 2005). Different defensive mechanism(s) are at play throughout the human GI tract starting from the human oral cavity (Fig. 1). GI tract offers broad spectrum anti-bacterial, anti-fungal, anti-viral, anti-protozoan and antisepsis properties. The antimicrobial defence(s) of human GI tract and the mechanisms have been detailed below in the respective sections and summarized in Table 1.

Oral cavity

The human oral cavity contains a number of physical, chemical and physicochemical agents that offers protection to oral tissues against harmful compounds, in particular those of microbial origin. Among others, human saliva is most important compound, not only because of its continuous flushing effect, ensuring effective removal of exogenous and endogenous microbes and their derivatives into the gut but also due to its role in maintaining nonimmune and immune factors in the oral cavity (Fig. 2a). Human saliva contains several innate (buffering system), nonimmune (lysozyme, salivary peroxidase, hypothiocyanite, lactoferrin, myeloperoxidase) and immune (salivary antibodies) factors, which are responsible for its protective and antimicrobial functions (Tenovuo 1998). The high antimicrobial potency of lysozyme, peroxidase, hypothiocyanite and lactoferrin has been well documented earlier (Scannapieco 1994; Valima *et al.* 2009; Arslan *et al.* 2009; Panwar 2014). Salivary nonimmune and immune factors synergistically prevent infections that can occur in between the transit passage from oral cavity to stomach, provided that their supply is constant to the oral cavity. The salivary components are known to inactivate mutagenic activities of certain carcinogens such as AF-2, MNNG, 4NQO, aflatoxin B1, benzo [α] pyrene, Trp-P-1 etc. (Nishioka *et al.* 1981; Toda *et al.* 2002; Kuboyama *et al.* 2008) and exhibit antagonistic activity against bacteria, fungi and viruses (Tenovuo 1998). Parotid gland releases its secretions to the oral cavity and synthesizes a complex mucous layer consisting of mucins, Trefoil factor family peptides and various carbohydrates. The lamina propria of parotid duct contains granulocytes, T lymphocytes and macrophages. This complex layer confers protection against multiple infectious agents (Kutta *et al.* 2006).

The interaction of oral epithelial lining with microbial cells also lead to induction of cytokines, chemokines and antimicrobial peptide secretions, that contribute to healthy oral environment (Dale and Fredericks 2005). Trefoil factor family peptides are group of such molecules that are secreted in epithelial lining along with mucins, and render protection and helps in its restitution during mucosal injury and inflammation. Additionally, these peptides also possess antimicrobial activity, specifically against *Helicobacter pylori* infection (Aihara *et al.* 2016). Several other antimicrobial factors viz. defensins (α, β), calprotectin, cathelicidin, histatin, adrenomedullin etc. block microbial self-aggregation and adherence to epithelial lining, thereby limiting infection caused by bacteria, fungi and viruses. Defensins are small cysteine rich β-sheet peptides that kill microbial cells by forming micropores in their plasma membrane. α-defensins displays antimicrobial activity against Gram-positive and Gram-negative bacteria, several mycobacterial species, yeast, filamentous fungi and enveloped viruses; while, β-defensins have strong bactericidal effect on Gram-negative bacteria and weak bacteriostatic activity against few Gram-positives, along with strong anti-mycotic potency (Dunsche *et al.* 2001). Human β-defensin 3 displays broad spectrum antimicrobial activity against both Gram-positive and Gram-negative bacteria, besides have antagonistic effect over multi drug-resistant *Staphylococcus aureus* sp. (Dunsche *et al.* 2002). β-defensins also displays anti-viral activity via interacting directly with the virus and/or indirectly with its target cells. Noticeably, in mammals, β-defensins are produced by the oral mucosa and they are active against HIV-1 virus: in particular, hBD1 (human β-defensin 1) is constitutively expressed, whereas the

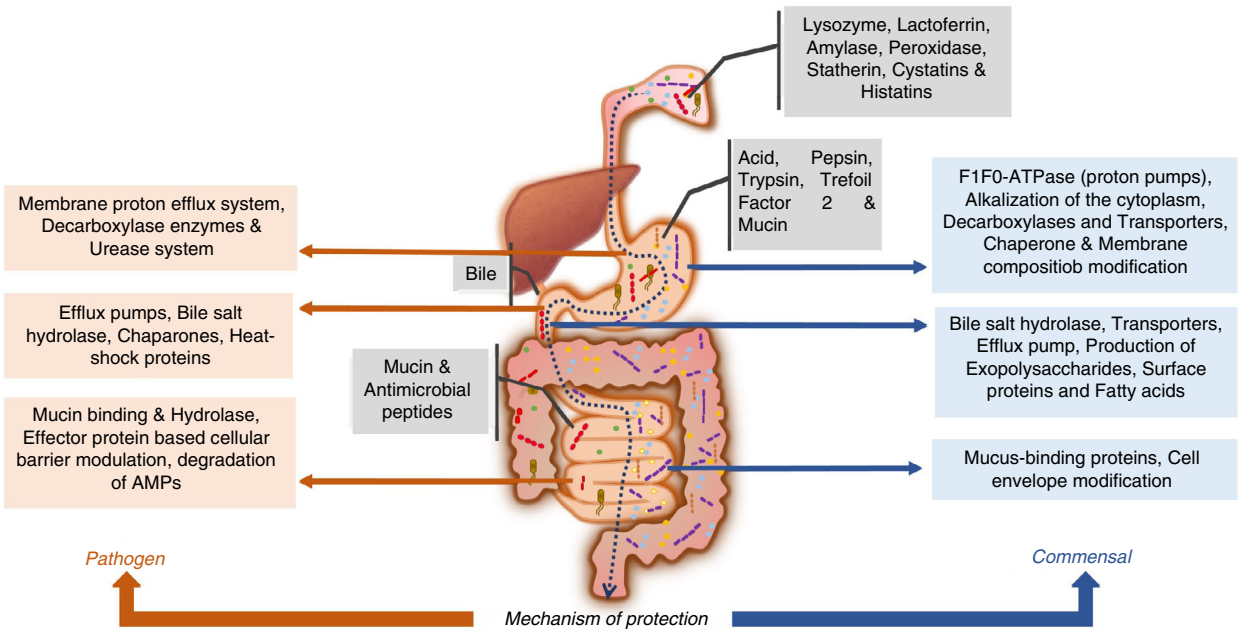

Figure 1 Components of gastrointestinal innate defence and the protection mechanisms of pathogen v/s commensal micro-organisms. Text in grey box represents components of gastrointestinal innate defence mechanism whereas, text in blue and brown boxes represent the protection mechanisms of commensals and pathogens respectively.

presence of a low HIV-1 viral load can stimulate the expression of hBD2 and hBD3 gene products through direct interaction with the virus. More specifically, hBD2 has been shown to down-regulate the HIV transcription of early reverse transcribed DNA products (Sun *et al.* 2005) and hBD2 and hBD3 induced an irreversible effect on virion infectivity through attachment to viral particles. hBD-2 and -3 blocks HIV -1 replication through direct interaction with virus via down regulation of C-X-C chemokine co-receptor 4, which was an important receptor for HIV-1 to infect $CD4^+$ T cells. Inhibition was recorded greater against CXCR4-tropic as compared to CCR5-tropic HIV-1 (C-C chemokine receptor type 5) isolates in both peripheral blood mononuclear cells and T lymphocytic cells (Quiñones-Mateu *et al.* 2003). These mechanisms diminish the chances of infection (Weinberg *et al.* 2006) and along with other salivary gland components, could help explaining the oral mucosal natural resistance to HIV infection. hBD3 also blocks the fusion of influenza virus membrane with the host cell endosome, through cross linking of the viral glycoproteins (Leikina *et al.* 2005).

The microbe mediated reduction in nitrate ion has been identified as another innate antimicrobial defence mechanism. Presence of nitrate in saliva promotes growth of nitrate reducing bacteria, resulting into nitrite, which upon acidification forms several antimicrobial oxides of nitrogen, including nitric oxide (Doel *et al.* 2005). Nitric and other oxides of nitrogen lead to modification of DNA and respiratory complexes. Another highly reactive molecule, peroxynitrite, produced upon reaction between nitric oxide and superoxide further adds to antimicrobial potency of oxides of nitrogen (Fang 2003).

Stomach

Microbes that survive the challenges of oral cavity travel to the stomach through oesophagus. Since the transit time of foods through the oesophagus is considerably short, it is unlikely to contribute nutrients to resident microbes, preventing adhesion and survival (Wilson 2008). The stomach serves as the primary organ for storage and consecutive digestion of dietary components. While, a substantial body of scientific literature exists on the antimicrobial defence of the intestinal tract itself, there is a relative paucity of data on those defences found in the stomach (Kim *et al.* 2000). Structurally, the gastric mucosa comprises of several exocrine and endocrine glands, secreting a mixture of important substances, collectively referred to as gastric juice. Mucus, several digestive enzymes, intrinsic factor and hydrochloric acid forms the major components of gastric juice ensuring proper digestion. Gastric juice has been recognized as an early line of defence against invading foreign particles since

Table 1 Antimicrobial components in GI tract and their role in innate defence against pathogens

Antimicrobial components	Apparent molecular weight	Nature	Source of secretion	Functions	References
Oral mucin					
MUC5B	620 kDa	High molecular weight glycoprotein	Salivary glands, tracheobronchial tissue, Brunner's glands, endocervix, gall bladder, pancreas	Reduces pathogen adhesion and biofilm formation	Nielsen *et al*. (1997); Thornton *et al*. (1999); Derrien *et al*. (2010); Pedersen and Belstrøm (2019)
MUC7	41·5 kDa		Salivary glands	Promotes bacterial agglutination (clearance)	Loomis *et al*. (1987); Derrien *et al*. (2010); Pedersen and Belstrøm (2019)
MUC19	805·3 kDa		Salivary glands, tracheobronchial tissue	Prevent dental carries. Promotes aggregation of pathogenic bacteria to enhance bacterial clearance (*S. mutans*)	Derrien *et al*. (2010); Culp *et al*. (2015); Pedersen and Belstrom (2019)
Epithelial mucin					
MUC1	122 kDa		All epithelial cells	Helps in cell signal transduction and mucosal pellicle formation	Li *et al*. (2003); Derrien *et al*. (2010); Kullaa *et al*. (2014)
MUC4	930 kDa		Virtually all epithelial cells	Helps in cell-cell and cell-extracellular matrix interactions and signalling	Frenkel and Ribbeck (2015); Ukkonen *et al*. (2017); Derrien *et al*. (2010)
Gastric mucin					
MUC5AC	641kDa		Secreted by gastric gland cells	MUC6 and MUC5AC: gastric epithelium protection from HCl	Kawakubo *et al*. (2004); Derrien *et al*. (2010)
MUC6	257 kDa			MUC6: Anti *H. pylori* activity	
Intestine/colon mucin					
MUC2	540 kDa		Secreted by gastric gland cells	Anchors to epithelium reducing bacterial penetration	Derrien *et al*. (2010); Arike and Hansson (2016)
MUC3A/ MUC3B	345 kDa/ 131·4 kDa		Small intestine, colon, gall bladder	Barrier to pathogens	Derrien *et al*. (2010); Putten and Strijbis (2017); Pelaseyed and Hansson (2020)
MUC12	558·2 kDa		Colon	Resistance to harmful conditions and toxic compounds	
MUC13	54·7 kDa		Trachea, small intestine, colon	Causes receptor shielding and dampening of immune response	
MUC15	120 kDa		Spleen, small intestine, colon, prostate, lung	Decoy receptor for invasive pathogens	
MUC17	452 kDa		Pancreas, small intestine, colon	Phosphorylation of cytoplasmic tail	
				Activation of pro- or anti-inflammatory pathways and cytokine secretion	
				Modulation of the NF-κb pathway	
				Inflammasome regulation	
				Regulation of cell proliferation and apoptosis	
				Serve as cancer progression markers	

(Continued)

Table 1 (Continued)

Antimicrobial components	Apparent molecular weight	Nature	Source of secretion	Functions	References
Lysozyme	14·7 kDa	Muramidase	Lysozyme secreted from duodenal mucosa, Paneth cell and epithelial gland of brunner's gland, stomach mucinous granules, epithelial cells of pyloric glands, mucous neck of fundic glands and epithelial surfaces of pyloric and fundic region, Paneth cells of small intestine	Hydrolyse Gram-positive bacterial cell wall. Aggregate oral bacteria promoting clearance and activation of endogeneous bacterial autolysins	Scamnapieco *et al.* (1994); Saito *et al.* (1998)
Lactoferrin	80 kDa	Transferrin family of iron-binding proteins	Secreted by serous acinar cells of salivary glands	Sequester growth essential iron from bacteria Promotes microbial adherence and aggregation Apo-lactoferrin mediates agglutination of multiple *Streptococci P. gingivalis* and *Aggregatibacter actinomycetemcomitans* Broadly effective against many bacteria, fungi and viruses	Arslan *et al.* 2009; Valima *et al.* 2009
Anti-microbial peptides					
Alpha defensins (HD 5 and HD 6)	3·5–4·5 kDa	Small cationic peptides containing 6 disulphide linkage with cystiene	Expressed in intestinal Paneth cells also in some villous epithelial cells in duodenum, jejunum and ileum	Involved in direct killing of pathogens Promote the growth of 'healthy' microbial communities Protect host against pathogenic insult HD5 involved in sequester and neutralize free bacterial LPS in the gut. Implicated in the repair of damaged epithelial tissues Play a mechanistic role in age associated microbial dynamics Suppress gut pathogenic communities	Salzman *et al.* (2003); Eriguchi *et al.* (2012); Jandhyal *et al.* (2015); Wang *et al.* (2016); Walters *et al.* (2017)
Beta defensins	3·5–4·5 kDa		Secreted from leukocytes, epithelial cells and Paneth cells	Antimicrobial, chemotactic and induction of IL-8 Stimulate cytokine secretion Induce MUC2 expression Activate dendritic cells and monocytes	Jager *et al.* (2010); Blyth *et al.* (2020)
Elafin	9·8 kDa	Peptidase inhibitor 3	Epithelial cells and leukocytes	Antiprotease with antimicrobial and chemotactic properties	
Secretory phosphor-lipase A2	14 kDa	Phospho-lipase	Epithelial and inflammatory cells, Paneth cell granules	Involved in eicosanoide metabolism and small intestinal mucosal defence	
Cathe-licidins	18 kDa	Peptide LL-37 with two leucine residues on left side	Present in the phagocytic leukocytes and in various epithelial Cells, including Paneth cells	Antimicrobial, chemotactic Alter chemokine response Increase MUC2 expression	

(Continued)

Table 1 (Continued)

Antimicrobial components	Apparent molecular weight	Nature	Source of secretion	Functions	References
Resistine like molecules (RELM)	12·5 kDa	Secreted proteins having conserved cysteine-rich C-terminus	Expressed in large intestine specifically in colon and cecum	Broad spectrum bactericidal and anti-helminth	Blyth *et al*. (2020)
Regenerating islet derived protiens (REGIII)	~16–17 kDa	C- Type lectins	Mostly expressed in intestines specifically small intestine	Antibacterial activity against both Gram positive and negative bacteria Expression increased during intestinal inflammation	
Salivary amylase	62 kDa	Glycoside hydrolase family	Secreted from serous acinar cells in the and submandibular glands	Modulate adhesion, co-adhesion and colonization of micro-organism. Specifically agglutinate certain streptococci for easy clearance	Marsh (2009); Pedersen and Belstrøm (2019)
Salivary peroxidase system	80 kDa	Heme peroxidases,	Produced by the parotid and submandibular glands	Protects host cellproteins from hydrogen peroxide. Inhibits *S. mutans*, lactobacilli, yeasts, several Gram-negative species including periodontal pathogens and certain viruses	Tenovuo *et al*. (1991); Lumikari *et al*. (1991); Kho *et al*. (2012); Pedersen and Belstrøm (2019)
Salivary proline rich proteins	45–15kDa	Acidic, basic and glycosylated	Produced by the parotid and submandibular glands	Glycosylated PRP's cause agglutination of oral bacteria clearance Basic PRPS exhibit anti-HIV-I activity	Azen *et al*. (1993); Robinovitch *et al*. (2001); Pedersen and Belstrøm (2019)
Statherin	5·38 kDa	Acidic tyrosine-rich phosphor-protein	Produced by the parotid and submandibular glands	Bind to mucins and form protective protein complexes resistive of microbial proteolytic activity Promote microbial aggregation and clearance	Blankenvoorde *et al*. (1998); Bruno *et al*. (2005); Pedersen and Belstrøm (2019)
Salivary cystatins	13·260 kDa	Cysteine-containing phosphor-proteins	Secreted along with submandibular sublingual saliva	Helps in the formation of the salivary pellicle Antagonist effect on *Actinomycetemcomitans* (periodontal pathogen) Strong antifungal potential	Ganeshnarayan *et al*. (2012); Pedersen and Belstrøm (2019)
Salivary Histatins	4·928 kDa	Histidine-rich, cationic peptides	Released by ductal cells of the parotid, sublingual and submandibular salivary glands	Anti *C. albicans activity* Promote microbial aggregation eventually leading to microbial cell death	Xu *et al*. (1991); Li *et al*. (2003a); Li *et al*. (2006); Wiesner and Vilcinskas (2010); Pedersen and Belstrøm (2019)
Pepsin	34·5 kDa	Endo-peptidase	Secreted by the chief cells in the oxyntic glandular area	Causes rapid loss of motility the proteolytic destruction of bacterial cell wall.	Schreiber *et al*. (2006)

(Continued)

Table 1 (Continued)

Antimicrobial components	Apparent molecular weight	Nature	Source of secretion	Functions	References
Trypsin	22·9 kDa	Pancreatic serine protease	Secreted by pancreatic acinar cells	Disrupt cell membrane and causes microbial lysis	Zhang *et al.* (2016)
Human trefoil factor 2	14·284 Da	Secretory peptide lectin	Secreted by surface epithelium of the stomach, gastric glands, pyloric glands, and Brunner's glands, also been detected in the ducts of the pancreas	Protect the gastric epithelium similar to MUC6	Hanisch *et al.* (2014)
Bile	408·6 Da	Aqueous solution of bile acids, cholesterol, phosphor-lipids and the pigment biliverdin	Synthesized in pericentral hepatocytes of liver	Causes emulsification and solubilization of fats, thus damage of cell integrity of pathogen Induce oxidative stress via producing oxygen-free radicals	Begley *et al.* (2005a)

almost a century. Hydrochloric acid secreted from parietal cells, besides having role in digestion, is also the major defence against intruding microbes. It has been known for decades that subjects with impaired gastric acid secretion are more susceptible to bacterial dysenteries (Giannella *et al.* 1972). The human gastric acid pH varies from 1 to 5 and is influenced by several factors viz. age, diet, alcohol consumption etc (Fig. 2b). Food has a strong buffering activity and can significantly shift the gastric pH towards neutral, particularly in the immediate post-prandial state (Wilson 2008).

Analysis of un-fractionated human gastric contents revealed the presence of lactoferricin, a natural antimicrobial component (Kuwata *et al.* 1998). Ingestion of food having bovine lactoferrin results in formation of significant amount of bovine lactoferricin in human stomach. Physiologically functional quantities of lactoferricin could be generated in infant and adult stomach (Kuwata *et al.* 1998) and exerts broad spectrum anti-bacterial activity. High antimicrobial potency of lactoferrin has been reviewed earlier by Panwar (2014). It has also been proposed that the salivary nitrate mediates the formation of a bactericidal compound in the stomach (Benjamin *et al.* 1994). A high concentration of nitric oxide has been observed in expelled stomach air which is further enhanced by dietary nitrate uptake (Lundberg *et al.* 1994; Dykhuizen *et al.* 1996). Nitric oxide in stomach works by improving the gastric mucosal blood flow. The concentrations of nitric oxide in the stomach are several orders of magnitude higher than those required for vasodilation (Lundberg *et al.* 2004). It has earlier been suggested that the acidified nitrite in the stomach augments the antimicrobial potency of gastric acid, displaying more susceptibility of *Candida albicans* and *Escherichia coli* towards the nitric oxide and gastric acid combination together (McKnight *et al.* 1997).

Gastric mucins themselves have also been shown to display natural antimicrobial activity. Mucin produced by deeper human gastric mucosa possess terminal α 1,4-linked N-acetyl-glucosamine residues attached to *O*-glycans, and have been shown to function as a natural antibiotic against *H. pylori*. Glycan chains serves as ligands for cell surface receptors and as modulators of adhesive proteins and receptors (Kawakubo *et al.* 2004). Besides other components, Kim *et al.* (2000) showed the presence of the potent antimicrobial peptide, buforin I in the stomach of Asian toad. Buforin I produced by the action of pepsin isozymes over unacetylated histone H2A, displays antagonistic activity against invading microbes. Presence of buforin has also been documented in human gastric mucosa. Histone 2A is synthesized in excess by human gastric mucosal cells, which accumulates in the cytoplasmic secretory granules. Histone gets processed by

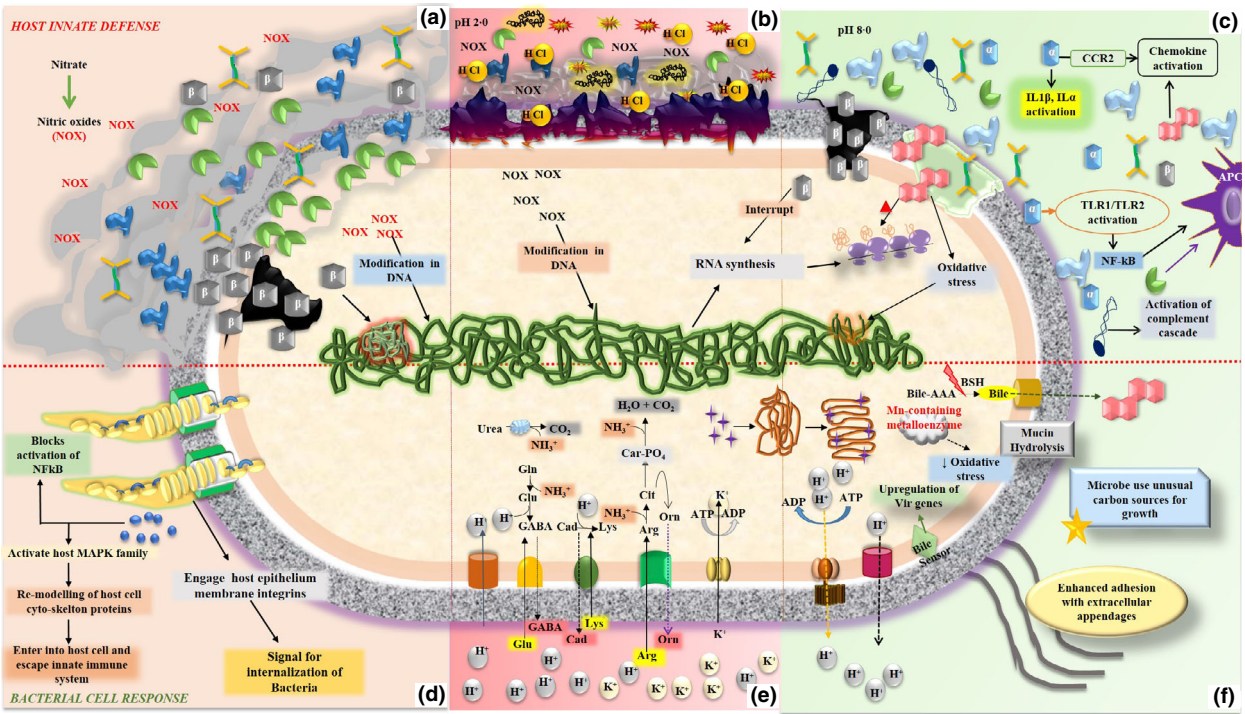

Figure 2 Schematic representation of impact of GI stress on bacterial cell (a–c) and bacterial cell response (d–f) to different defence mechanism (a & d, oral cavity; b & e, stomach; c & f intestine): (a) in oral cavity mucins ( ), β-defensins ( ), secretory antibodies ( ), lysozyme ( ) and nitric oxides (NOX) starts to attack microbial cell surface and destroy its integrity. (b) In stomach, HCl ( ), antimicrobial peptides ( ), proteolytic enzymes ( ), gastric mucin ( ) disrupt cell surface and cause killing of bacterial cell. (c) In intestine bile ( ), secretory antibodies ( ), α and β-defensins ( ), c type lectins ( ), intestinal mucins ( ), lysozyme ( ) attacks genetic material and induce chemokine activation and inflammatory response. (d) Presence of fimbrae type adhesion molecules ( ) and secretory proteins ( ) induce signalling cascade for bacterial internalization, remodelling of host cell cytoskeleton structure, inactivation of NF-kB system thus helps to escape oral innate defence. (e) Proton influx mechanism ( ), arginine deiminase mechanism ( ), cation influx channel ( ), Lysine decarboxylase system ( ), glutamate decarboxylase system ( ), urease enzyme ( ), chaperones proteins ( ) help bacteria to establish in low pH. (f) Bile salt hydrolase enzyme ( ), proton efflux pump ( ), F0F1-ATPase proton pump ( ), Mn containing metalloenzymes ( ), mucin hydrolysis, alternative energy sources, upregulation of *Vir* genes via bile sensor ( ) and extracellular appendages help bacteria to evade intestinal innate defence.

gastric pepsin to buforin II. This peptide remains in close contact to the gastric mucus biofilm coating providing a protective coat to stomach cells (Zasloff 2002). Furthermore, presence of lysozyme (muramidase) has also been detected in mucinous granules of stomach (Saito *et al.* 1988). Lysozyme catalyses the hydrolysis of β 1,4 glycosidic linkage of NAM-NAG subunit of bacterial cell wall peptidoglycan. Its activity is stable at acid and neutral pH but labile to alkaline conditions (Panwar 2014). The human gastric juice contains more than one proteolytic enzyme with optimum activity at about pH 2. Pepsin, one of the most important proteolytic gastric enzymes also exists in different forms (Etherington and Taylor 1969). The gastric proteolytic activity in association with low pH is known to exert potent antimicrobial effect. Zhu *et al.* (2006) reported a marked increase in bacterial susceptibility to low pH (<2·5) in presence of pepsin, suggesting a role of pepsin mediated proteolysis in the killing of bacteria. Pre-incubation of *E. coli* and *H. pylori* in Eagle's growth medium of pH 2·5 increased the survival of pathogen as compared to incubation in PBS at same pH. The pre-incubation in Eagle's growth medium (composed of natural or synthetic nutrients) is indicative of the buffering action of food in the stomach, as pathogen survives via attaching to the food components. After preincubation in growth medium of pH 2·5, pepsin (1 mg ml^{-1}) treatment to pathogens significantly lower the viable count, indicating that hydrochloric acid is not the solitary bactericidal component in the stomach. In actual fact, synergistic action of low pH and different hydrolytic enzymes are effective in effective killing of pathogens in human stomach (Zhu *et al.* 2006).

Intestine

Bile

Bile is involved in digestion and absorption of dietary fats and liposoluble vitamins as well as play an important role in biliary lipid secretion, cholesterol solubilization, vitamin and calcium absorption, pancreatic enzyme secretion and cholecytokinnin release (Monte *et al.* 2009). Bile is a yellow-green aqueous solution synthesized hepatically from cholesterol and periodically released into the small intestine in relatively large quantities–as high as a litre per day. It consists of approximately 95% water having a number of endogenous solid (bile salts, bilirubin, phospholipid, cholesterol, amino acids, steroids, enzymes, porphyrins, vitamins and heavy metals) and exogenous drugs, xenobiotics and environmental toxins (Boyer 2002). It is composed of a variety of compounds, with sodium (145 mmol l^{-1}), chloride (90 mmol l^{-1}) and various bile salts (40 mmol l^{-1}) as major components. Bile salts, also referred to as bile acids, is made up of 24 carbon atom steroid nucleus consisting of three six membered rings and one five membered ring. In mammals, bile salts have 5β cis-configuration while in lower vertebrates some bile acid exhibit allo-trans configuration (Monte *et al.* 2009). Bile acids constitute a major part of the bile and are found in conjugated form with either 75% glycine or 25% taurine (Warren *et al.* 2006).

Bile primarily acts as a detergent, which forms the basis of its potent antimicrobial activity via cell membrane damage. Bile acids affect the phospholipids and proteins of bacterial membrane disrupting cellular homeostasis. They may also cause oxidative stress and misfolding or denaturation of proteins. Presence of immunoglobulin A (IgA) and inflammatory cytokines in bile provide protection against enteric infections as well as stimulates intestinal innate immune system (Boyer 2013). It serves as potential excretory pathway for harmful exogenous substances (like drugs, xenobiotics and environmental toxins) as well as some endogenous substances such as high molecular weight bilirubin and bile salts (Boyer 2013). Inagaki *et al.* (2006) reported that conjugated bile acids mediate antimicrobial effects through regulating host gene expressions involved in innate defence against luminal pathogens. Conjugated bile acids activate cellular pathway involving nuclear farnesoid X receptor (FXR) in the distal small intestine. The activation of FXR receptor causes induction of gene expression that results in limiting bacterial overgrowth and enhanced epithelium integrity (Inagaki *et al.* 2006). Bile acids directly influence the genetic make-up of cells by inducing DNA damage and secondary structure formation in RNA. Intracellular dissociation of bile salts in cells, chelate calcium and iron, reducing their intracellular concentrations, hampering several vital processes (Sanyal *et al.* 1991). Combined action of above mentioned mechanisms likely to regulate microbial levels in the small intestine (Hofmann and Eckmann 2006).

An optimum level of bile acids is required for maintaining proper lipid homeostasis. Decreased bile acids may result into lipid malabsorption and supersaturation of bile with cholesterol, leading to kidney stones; however, excessive bile salts may induce oxidative stress, apoptosis and diarrhoea. Bile salts at higher concentration rapidly dissolve membrane lipids and at lower concentration affect the hydrophobicity and potential difference of cell surfaces (Begley *et al.* 2005).

Mucins

The mucus layer is primary defence barrier between GI epithelium cells and microbes. Mucous layer serves as primary framework that provide enriched environment having antimicrobial peptides, secretory immunoglobulin and other important effector molecules that impose physical barrier against nonmotile bacterial pathogens (Johansson *et al.* 2011; Dupont *et al.* 2014; Martens *et al.* 2018).

Mucins are structurally diverse moieties which provide favourable niche for attachment of microbes thus helps in bulk removal of pathogen by peristaltic movement. Hence, mucins impede any direct interaction between the epithelial and microbial cells that may cause injurious/inflammatory/beneficial response depending upon type and composition of microbial inhabitants. Properties of mucus layer, as well as density and diversity of resident bacterial community continuously change over the length of GI tract (Ermund *et al.* 2013).

Other intestinal components

A diverse assortment of intestinal epithelial cells secretes compounds with important roles in the innate immune response, including alpha/beta-defensins, histone proteins, lectins, lysozyme etc (Fig. 2c).

Intestinal colonization with symbiotic/commensal bacteria promotes the epithelial expression of lectins (specifically c-type lectins), antimicrobial molecules that functions as pattern recognition receptors having ability to recognize bacterial targets via various cell surface glycan carbohydrates. Soluble c-type lectins can enhance microbial phagocytosis, differentiate between self and nonself ligands, activate complement cascade, modulate inflammation and causes direct killing of various microorganism (Brown and Janet 2018). Membrane bound lectins (specifically Glucan Receptor Dectin 1) can elicit intracellular signalling to induce various antimicrobial associated cellular and immune responses. Hence, the commensal gut microbiota drives the intestinal cells to

prepare against the potential intruding pathogens. It is postulated that these antimicrobial proteins represent an evolutionarily primitive form of innate immunity (Cash *et al.* 2006).

Microbe mediated inflammatory stimuli or mucosal damage elicits expression and secretion of such intestinal antimicrobial peptides (Ogawa *et al.* 2003; Pull *et al.* 2005). These antimicrobial peptides play an important role in maintaining the mutual beneficial host–microbial associations by limiting contact between the resident microbes and mucosal surfaces (Cash *et al.* 2006).

Paneth cells of small intestine harbour abundant cytoplasmic secretory granules containing antimicrobial proteins, including α-defensins (Cash *et al.* 2006). A large number of defensins (>19) had been identified from mice intestine; however, human intestinal paneth cells are known to secrete only few (HD-5 and 6) of them (Mallow *et al.* 1996; Lisitsyn *et al.* 2012; Walters *et al.* 2017). Presence of bacteria and lipopolysaccharide (LPS) in intestine is known to induce the secretion of defensins from Paneth cells (Qu *et al.* 1996). Both the human intestinal α-defensins (HD-5 and 6) are localized to small intestine, with more expression in adults followed by newborns and foetus (Hecht 1999). In intestine, β-defensin protein-1 (hBD-1) is present in goblet cells, enterocytes and paneth cells of the ileum. Expression of hBD-1 remains unaffected by microbial invasion and pro-inflammatory molecules (Wehkamp *et al.* 2004). In human GI tract, hBD-1 is constitutively expressed; however, hBD -2, -3 and -4 are expressed in response to infection or inflammatory response and are known as host defence proteins. β defensins are cationic peptides which significantly attract the negatively charged microbial group through electrostatic force of attraction. Following initial attraction, the microbial lipid layer is invaded by hydrophobic portion of defensin peptides, and charged arginine side chains binds to polar side to form transmembrane channel. Defensin proteins enter via this channel and cause membrane remodelling (Yang *et al.* 2000). After membrane translocation these peptides causes nutrient leakage, depolarization, impaired cell integrity and eventually cell death (Yeaman and Yount 2003). Some defensins can also damage pathogen via nonmembrane lytic actions, including binding to strong negative charge nucleic acids and direct inhibition of nucleic acid synthesis (Cobo and Chadee 2013). β-defensins function as chemoattractant for immature dendritic cells and memory T-cells to microbial invasion mucosal sites thus regulating adaptive immune response (Yang *et al.* 1999). hBD-2 and hBD-3 binds to CCR2 (C-C chemokine receptor type 2) chemokine and induce chemotaxis in monocytes, dendritic cells and macrophages (Rohrl *et al.* 2010). Likewise, they cause TLR1 and TLR2 mediated recruitment of antigen presenting cells in an NF-κB dependent manner (Funderburg *et al.* 2007). The hBD-2 mRNA expression has been reported to be enhanced in presence of pro-inflammatory cytokines such as IL-1β, IL-1α and tumour necrosis factor (TNF)-α in Caco-2 and HT-29 human colonic epithelial cells through an NF-κB-mediated mechanism (Witthoft *et al.* 2005). Microbial components such as LPS and peptidoglycan induce β-defensins through PAMP (pathogen-associated molecular patterns) receptors in the colonic epithelium (Vora *et al.* 2004).

Lysozyme, another important component of innate immune defence has been found in a variety of cell and tissue-types (Klockars and Reitamo 1975) including the Brunner's glands of the duodenum and the paneth cells of the small intestine (Montero and Erlandsen 1978; Bel *et al.* 2017). Within the small intestine, lysozyme can interact with antigen presenting cells in the gut-associated lymphatic tissue and activate the host immune system (Sava *et al.* 1996). Further in depth studies in this line have elucidated the mechanism behind and have explained that lysozyme could modulate the host immune response by enhancing the expression of anti-inflammatory cytokine, that is, TGF-β in the small intestine (Cooper *et al.* 2011).

Besides Paneth cells, villus cells of human mucosa also possess antimicrobial activity, in form of *histone proteins*. Histone H1 demonstrated antimicrobial protection within the human intestinal tract (Rose *et al.* 1998). Both histone H1 and fragments of histone H1 extracted from human ileal mucosa possess antimicrobial properties, providing a broader level of protection against luminal micro-organisms. Histone H1 is released from apoptotic cells induced with pathogens, further preventing pathogenic organism in itself (Hecht 1999).

Response of invading pathogens to GI tract defence

A wide range of microbial pathogens including bacteria, virus, fungi and protozoans may be acquired from faecal oral route and are capable to dodge the innate and adaptive defence system of GI tract using different mechanisms. These pathogens may survive and confine to the GI tract or spread to other parts of host to evade the hostile environment of intestinal tract. The ability of pathogenic bacteria to survive in the face of host defence systems is intimately linked to virulence. This is exemplified by several gastrointestinal pathogens that must survive exposure to extreme conditions within the stomach and within the intestine (Merrell *et al.* 2002; Finlay and McFadden 2006; McGuire and Arthur 2015). These organisms have evolved complex systems to respond to

acid, bile stress, gut barriers and innate immunity of host and several studies has revealed diverse mechanistic and genetic components involved in survival and virulence of pathogens. Here we will discuss the major mechanisms through which pathogen survive GI tract defence mechanisms.

Acid stress tolerance

In addition to elementary acid tolerant mechanism, gastrointestinal pathogens have many acid inducible adaptive mechanisms to survive through hostile environment of stomach. The elementary acid tolerant mechanism includes the rapid response like alteration in ionic flux of the cell membrane and the buffering capacity of intrinsic cellular components. If the first line of defence fails to compensate the drop in cytoplasmic pH, the transcriptional response gets induced to activate several physiological mechanisms (Lund *et al.* 2014). Mainly, to cope up with extreme acidic environment, pathogens rely on pH homeostatic or membrane proton efflux system. In response to acid stress, various decarboxylases which consumes proton during amino acid (primarily lysine, arginine or glutamate) decarboxylation get activated (Begley and Hill 2015) (Fig. 2e). For example, an inducible gene *cadA*, which encodes lysine decarboxylase in *Vibrio cholerae* is expressed during infection and plays role in acid tolerance (Merrell and Camilli 1999). *Listeria monocytogenes* and *E. coli* utilizes an inducible glutamate decarboxylase (GAD) system encoded by *gadB* gene for the same mechanism (Castanie-Cornet and Foster 2001; Cotter *et al.* 2001). Some pathogens express general stress response proteins to protect functional and structural molecules during acid stress. Lowering of cellular pH cause physiological changes to the cytoplasmic and periplasmic components, that is, low pH induced unfolding of enzymes and other vital proteins, DNA damage and membrane destruction. Stress response proteins play an important role in protecting the periplasmic, membrane bound and cytoplasmic proteins against such acid induced damage (Lund *et al.* 2014). In *B. cereus* and *H. pylori*, expression of chaperone DnaK, GroEL and SodA and other DNA binding proteins have been reported to be significantly upregulated during acidic treatments (Jobin *et al.* 2002; Wen *et al.* 2003). *Helicobacter pylori* are an interesting example of acid resistance in pathogens. Despite of its neutrophilic nature, it survives the gastric low pH and also invades the mucus layer of stomach to reside there. This organism has evolved the mechanism to withstand surrounding acidic fluctuations mainly using urease system. Hydrolysis of urea leads to formation of ammonia and CO_2 which further get converted to HCO_3^- by the periplasmic carbonic anhydrase. Both contribute to buffering the cytoplasmic and periplasmic pH near neutrality when the bacterium is exposed to acidic milieu (Pflock *et al.* 2006). It has been reported that *H. pylori* do not survive below pH 4 in the absence of urea and behave like a neutrophilic organism. However, in the presence of urea, *H. pylori* can survive at pH values as low as those found in the healthy human stomach (Stingl *et al.* 2002).

Bile stress tolerance

As noted above in section 2.3.1, bile is a complex mix of compounds that can disrupt the cell envelope of microorganisms. Composition and structural changes in the organism's cell membrane such as charge, hydrophobicity and lipid fluidity can contribute to bile salt tolerance of cells (Urdaneta and Casadesús 2017). In several pathogens, the mechanism of bile tolerance is reported to be mediated by efflux pumps, transcriptional regulators, DNA repair proteins, proteins that maintain membrane integrity and bile salt hydrolase enzymes (Begley and Hill 2015). Generally, Gram-negative bacteria are more bile tolerant due to the presence of some inherent factors, that is, LPS, efflux pumps and bile salt hydrolase enzymes (Fig. 2f). Among Gram positive pathogens, *Listeria monocytogenes* has been well studied for the mechanism of bile resistance. The molecular mechanism reveals that three genes viz. *bsh* (bile salt hydrolase), *pva* (penicillin V amidase) and *btlB* (bile tolerance locus B) plays an important role in resisting the acute toxicity of bile and bile salts (Begley *et al.* 2005). The *bsh* gene can be regulated both by PrfA and σ^B transcription factors, which may be triggered by anaerobic conditions within the host GI tract (Begley *et al.* 2005).

Conversely, some studies have shown that bile can have a stimulatory effect on the pathogenicity of enteropathogens. Letchumanan *et al.* (2017) showed that pathogens such as *Vibrio parahaemolyticus* can sense bile and use its presence as an environmental stimulus to upregulate virulence genes during infection. Similarly, bile salts are also reported to increase expression of LDA, an afimbrial adhesin in atypical enteropathogenic *Escherichia coli* (aEPEC) strain (Torres *et al.* 2007).

Mucin binding and invasion

Mucins secreted by goblet cells are highly glycosylated (>80%) proteins which could expand >1000 fold in volume after secretion and unfolding into the lumen (Johansson *et al.* 2013). The basic function of this layer is to make a hydrated layer over epithelium and to protect intestine by preventing direct interaction between host cells and pathogens. The fundamental difference in their

structure divides them into, secretory and transmembrane mucins. The membrane bound mucins are anchored in the cell membrane by a single membrane spanning domain. The heavily glycosylated N terminal of transmembrane mucins is exposed to the extracellular region; whereas, C terminal makes the intracellular cytoplasmic tail. While secretory type mucins lack membrane anchoring domains and make a loose gel like layer over the epithelium (Dhanisha *et al.* 2018). Peristaltic movements of intestine frequently wash off the outermost layer of secretory mucin to get rid of the overgrowth. However, the layer is constantly targeted by the hydrolytic enzymes of the infusing microbial population. For example, *V. cholerae* secretes a mucinolytic haemagglutinin protease (Hap), *Entamoeba histolytica* secretes cysteine and an M60-like protease, *E. coli* secretes a zinc- metalloproteinase SslE, which cleave the mucin and increase fluidity of mucus gel (Martens *et al.* 2018). Some pathogens like *H. pylori*, binds to specific mucin (MUC5AC and MUC1) to attach with the host (Ansari and Yamaoka 2017). Furthermore, pathogens like *Salmonella* could also modulate the expression of secretory mucin (MUC2) of intestine in order to cause infection by breaching the barrier function of epithelial layer (Rokana *et al.* 2016). Furthermore, another *in vitro* study by Vieira *et al.* (2010) stated that strains of aEPEC increased the production of secretory (MUC5AC) as well as membrane bound (MUC4) mucins in HT29-MTX cells. EPEC cause characteristic cellular lesion to the epithelium after attaching to the enterocyte brush border membrane. The study reported that increased mucin production favoured the growth of adhering bacteria at the apical cell surface. On the same line, an earlier study by Erdem *et al.* (2007), could explain the possible mechanism supporting the attachment and movement of pathogen in mucin layer (mechanism discussed in next section).

Competing commensal microflora

After passing through the extremely acidic environments of the stomach and the upper small intestine (the duodenum), pathogens enter the jejunum of the small bowel to make foundation of infection. However, establishment of pathogens in the intestine where a heavy population of commensal microflora compete with them for space, nutrition and favourable environment is a challenging task. Pathogens, thereby, have to equip themselves to breach the commensal mediated resistance by developing new pathways for carbohydrate metabolism, potential attachment appendages and evolving suppressive mechanism for competitive micro-organisms. Pathogens such as *E. coli* O157:H7, *Campylobacter jejuni* and *V. cholerae* have the ability to catabolize unusual carbon sources (i.e. maltose, free amino, keto acids, succinate, glycine, and chitin etc.) which help them to compete with commensals (Keeney and Finlay 2011). The extracellular appendages of pathogens also offer additional advantage for their colonization and establishment into intestine. For instance, H7 and H6 flagella of two strains of enteropathogenic *E. coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC) poses the ability to bind mucins and extracellular matrix proteins of the host (Erdem *et al.* 2007). This mechanism helps them to get in closer proximity of epithelial layer through the thick layered mucin. Moreover some extracellular components of pathogens also facilitates the access and binding to the host cells. A recent study has recognized the new mechanism of host selectivity and attachment in *Shigella flexneri*. The bacterium interacts with the enteric α-defensin 5 via several membrane proteins, and establishes a close interaction with the epithelium to invade the host cells (Xu *et al.* 2018).

Another important strategy to over compete the commensal microbes is to induce inflammation in the intestine. Some pathogens can take advantage of growth over commensals during the inflammatory conditions. For example, an interesting study by Winter *et al.* (2010) demonstrated that during inflammation, *S. enterica* serovar Typhimurium use a unique mechanism of cellular respiration to survive and overgrow other microbes. The colonic microflora generates a significant quantity of hydrogen sulphide (H_2S) as a metabolic byproduct. This toxic component is eventually converted to nontoxic thiosulfate ($S_2O_3^{-2}$) into the lumen. Winter and coworkers reasoned that during inflammation, neutrophils produce large amount of nitric oxide radicals (NO) and reactive oxygen species which oxidize surrounding thiosulphate into tetrathionate. *S. enterica* serovar Typhimurium reportedly have specific ability to use tetrathionate as a terminal e^- acceptor. Thereby, in such conditions, the pathogen shifts its mechanism of cellular respiration and thrives well in the inflamed intestine. However, the same research group further explored that nitrate generated as a by-product of the inflammatory response also supports the growth of commensal bacterium *Escherichia coli* through nitrate respiration mechanism in similar conditions (Winter *et al.* 2013).

Interestingly, pathogens utilize one of the antimicrobial systems of inflammatory mechanism to outcompete their close competitors. Some siderophores are secreted as antimicrobial agents during inflammation to limit the availability of essential metal ions to inhibit microbial growth. However, pathogens are reported to possess high affinity ion transporter mechanism to overcome such conditions (Costa *et al.* 2016; Diaz-Ochoa *et al.* 2016). For example, *Salmonella* can subvert the calprotectin

mediated chelation of zinc and manganese using high afinity ZnuABC, MntH and SitABCD transporters (Diaz-Ochoa *et al.* 2016). Moreover, the pathogen also utilize Mn-containing metelloenzymes, that is, catalase and superoxide dismutase to survive in oxidative stress condition generated by inflammation (Diaz-Ochoa *et al.* 2016). In addition to the specific microbial physiological responses, induction of diarrhoea in the host can have a generalized effect of reducing the number of total bacteria in the lumen or shift the composition of microflora which supports the establishment of pathogens (Ojetti *et al.* 2009; Stecher *et al.* 2010).

Evading innate immune system

Components of innate immune system continuously remain in contact with internal milieu of the intestine. Intestinal epithelium has some check points for the evaluation of luminal contents by immune system of our body. These check points i.e. Peyer's patches serves as the contact sites and supply the samples of luminal components of intestinal lumen to the immune cells present in the underlying lamina propria. Additionally, the long appendages of dendritic cells also get access to the intestinal lumen by travelling through intercellular space of epithelial layer. The communication between such sampling cells from the epithelium and immune-regulatory cells of lamina propria further develops the immune response accordingly (Gerbe and Jay 2016; Allaire *et al.* 2018).

These cellular mechanisms are evolved to sense any infection or pathogenesis being caused by harmful microbes into the intestine. The soluble molecules, antigens generated by virulence activity are transported to immune cells through M cells of pear's patches. Whereas, dendritic cell recognize the pathogens by directly sensing them through their long appendages exposed into the intestine. With the help of specific receptors, immune cell could differentiate the harmful organisms. These specific receptors recognize the PAMPs, that is, pathogen associated cell surface molecules, secretory and cytoplasmic components (Abreu 2010). Pathogen recognition is followed by activation of defence mechanism to protect the integrity of mucosal and epithelial lining. The immediate immune response induce localized inflammation and stimulate the expression of various protecting components, that is, regulatory cytokines (to attract more immune cells towards infection site), antimicrobial substances (from immune cells and specific epithelial cells) and mucin etc. (Kim *et al.* 2011; Gallo and Hooper 2012; Benjamin *et al.* 2013). Pathogens attempt to subvert the defence barriers using specific adaptations. During initial phase of establishment of an infection, majority of Gram-negative pathogens modify the functioning of immune components with the help of various effector molecules. The effector proteins are capable of directly modifying host cell signalling pathways tied in membrane transport, cell signalling, cell metabolism, cytoskeletal dynamics and cell death (Pinaud *et al.* 2018).

Even escape from PAMPs associated recognition by host immune cells is seen mediated by some secretory effector proteins. In few strains of *E. coli,* EspF and EspJ effectors inhibit the direct and antibody mediated phagocytosis of cells by macrophages (Wong *et al.* 2011). Some invading bacterial enteropathogens can also manipulate cell death cycle to allow their intracellular replication by suppressing the cell death and then promote it for their dissemination to other host cells. A number of effectors from different pathogens are reported to target mitochondrial pro-death, NF-kB dependent pro-survival and inflammasome-dependent host cell death pathways for manipulation of cell death (Ashida *et al.* 2011).

Several pathogens also intercept the transcription factor NF-κB mediated activation of inflammatory response into immune cells. Pathogens accomplish this task with the help of specific secretory effector proteins. Different type of effector proteins interacts with receptors or functional components in cytoplasm to alter the signalling pathways in immune cells. The altered signalling pathways are associated with regulation of specific transcription factors which direct the expression of innate defence mechanism related genes. Specifically, effector molecules from different invasive or noninvasive type pathogens evidently target toll like receptors (TLR) mediated downstream signalling pathways. Further exploration of molecular mechanism explained that effectors block MAPK and NF-κB signalling by inhibiting the binding of adapter protein to the cytoplasmic domain of TLR (called as Toll and IL-1 receptor: TIR). This inhibition subsequently hinders the degradation of NF-κB associated subunit (i.e. IκBα). Without removal of IκBα subunit, NF-κB cannot be released and translocate to the nucleus, which eventually suppress the inflammatory response of the host cell (McGuire and Arthur 2015).

In response to inflammation, host cells also secrete some α-helical or cyclic antimicrobial peptides to directly destroy the pathogens. However, both Gram-negative and Gram-positive pathogens evolve moderate to high resistance to these peptides using mechanisms such as cell envelope modification, proteolytic degradation/ sequestration/ expulsion of peptides and capsule formation (Abdi *et al.* 2019). Besides, pathogens like *Salmonella* spp. has also been reported to down regulate the expression of AMPs in epithelial cells using TLR mediated pathways (Rokana *et al.* 2016). Pathogens also modulate intestinal microenvironment according to their need. A study by

Lopez *et al.* (2016) revealed that type 3 secretion system of *Citrobacter rodentium* induced an excessive expansion of undifferentiated epithelium. This expansion increased oxygenation of the mucosal surface which supported the growth of aerobic *C. rodentium* in the colon.

Interestingly, reports have shown that pathogen may also manipulate the gene expression of host cell using epigenetic mechanism. A comprehensive review on this topic by De Monerri and Kim (2014) explains that pathogens could manipulate the gene expression of host cell during early stage of infection by altering the DNA methylation. Although the modification are done to favour the pathogenesis during initial phase but, there may be long-term consequences with few infections. For example, oncogenesis caused by *H. pylori* reported to be due to inactivation of fork head transcription factor (FOXD3) promoter by hypermethylation (Cheng *et al.* 2013). FOXD3 transcription factor regulates the expression of proapoptotic factors. It is quite evident that pathogens influence the physiology of host cells using epigenetic manipulation, however, the channel of mechanism still remains to be established.

Commensal/beneficial microbes response to GI tract defence

The resistance to various adverse conditions in the GI tract is an important characteristic of commensals. Commensals along with probiotic micro-organisms have to overcome several hurdles to establish and impart a beneficial effect. Human oral cavity offers first resistance to beneficial microbes administered through functional foods or as pharmacological supplements. Unique as well as the diverse microbial community in the human oral cavity makes it a complex habitat. It is the second highly populated area colonized by microbes, after the GI tract. Microbiome studies and next-generation sequencing techniques revealed that the microbiome is diverse but individual and oral niche specific. Recently, Verma and coworkers presented greater insight into the expanding oral microbiome. As per the Human Oral Microbiome Database (eHOMD), there are 772 prokaryotic species in oral cavity; out of which majority (70%) are cultivable. The six predominant Phyla (Core Phyla) constituting 96% of total oral bacteria includes *Firmicutes, Proteobacteria*, *Actinobacteria, Fusobacteria, Bacteroidetes* and *Spirochaetes* (Verma *et al.* 2018). The development of innate immune system of oral epithelia is also influenced by the commensals. It has been reported that the commensal bacteria can induce hBD-2 in oral epithelia and enhance the innate immune system (Dale and Fredericks 2005).

The predominant breast milk residents like lactobacilli and bifidobacteria are the major microbes that initially colonize in infant's oral cavity (Abrahamsson *et al.* 2009; Gueimonde *et al.* 2013). Some *Bifidobacterium* species were found to be highly resistant to lactoferricin present in mouth (Bellamy *et al.* 1992).

There is a homeostatic coexistence between the commensals, beneficial microbes and pathogens in the oral cavity. This homeostasis is maintained by several mechanisms illustrated in Fig. 3.

The major mechanism is by prevention and inhibition of colonization of pathobionts as well as immune-modulation. It has been reported that commensals produce a wide range of antimicrobial substances such as organic acids, fatty acids, hydrogen peroxide and bacteriocins, as well as antimicrobial peptides like histatins, defensins and cathelicidin LL-37, to limit the growth of pathogens. The regular interaction between the oral commensals and immune cells play a vital role in maintaining oral health (Sultan *et al.* 2018). As these mechanisms of commensal can be extrapolated to probiotic bacteria, probiotic therapy could be applied to restore any dysbiosis in oral homeostasis (Baker and Edlund 2019). The mutualistic and interspecies interactions help to retain homeostasis in the oral cavity.

The extracellular signalling molecules/factors such as adhesins help in social interworking by indigenous oral bacteria, for example, adhesins produced by *Fusobacterium nucleatum* recognizes streptococci, and lectin recognizes *Porphyromonas gingivalis* (Jenkinson and Lamont 2005). Several synergistic commensals and co-dependent metabolic pathways co-exist in the oral cavity. Streptococci produce lactic acid, which in turn is used as a carbon source by Veillonellae (Avila *et al.* 2009) and *Candida albicans* (Jenkinson and Lamont 2005). Moreover Streptococci provide *C. albicans* adhesion sites to survive within the oral cavity (Jenkinson and Lamont 2005). The utilization of lactic acid as a carbon source reduces the oxygen potential, which favours the growth of other commensals (Krom *et al.* 2014). The pH homeostasis is maintained by *S. mutans* and other oral bacteria mainly by two mechanisms: proton-translocating ATPase (H+/ATPase) (Bender 1986; Hamilton and Buckley 1991) mechanism and by acid efflux pumps (Dashper and Reynolds 1996).

Microbes are continuously removed from the oral cavity by processes such as salivary clearance, mastication and oral hygiene. Bacteria reside there either as dynamic oral biofilm or in the planktonic state. In the oral cavity, the ingested strains are first exposed to saliva having bactericidal, bacteriostatic and inhibitory proteins. The probiotic viability, cell surface morphology, the adhesion potential and metabolic activity is influenced by salivary proteins (lysozyme, lactoferrin, histatin, salivary peroxidase, cystatins and secretory IgA) (Stamatova and

Meurman 2009). *Fusobacterium nucleatum*, a commensal strain found in the oral cavity is relatively resistant to the endogenous antimicrobial peptides (Zasloff 2002). One of the common mechanisms exhibited by commensals for survival and prevention of rapid exclusion from the mouth is adhesion and formation of biofilms (dental plaque) on teeth (Bowden and Hamilton 1998). The steady salivary flow can cause the dissolution of some of the microbes from the biofilm surfaces; thereby modulating microbial colonization (Jain and Sharma 2012). The microbial diversity and selective colonization in the mucosal cavity are by specific adhesion-mediated interaction (Ofek and Doyle 1994), aggregation and co-adhesion by the commensals (Bowden and Hamilton 1998).

Adhesion promotes health effect of beneficial strains (Stamatova and Meurman 2009). The specific cell surface component (either carbohydrate or proteinaceous), surface charges as well as the degree of hydrophobicity are responsible for the adhesion of lactobacilli (Jain and Sharma 2012). The cell surface hydrophobicity is found to be more in auto aggregating strains thereby the greater adhesion and colonization potential. The establishment of probiotics in the mouth is also influenced by saliva-mediated aggregation. The co-aggregating microbes have a greater advantage than non-co-aggregating organisms for getting established in the mouth. Pham *et al.* (2009) demonstrated that co-culturing of *L. salivarius* W2431 with other commensal oral micro-organisms enhanced its establishment as compared to monoculture, irrespective of pH conditions in a microplate model (Pham *et al.* 2009).

There are many mechanisms through which biofilms protect commensals against antimicrobials. The modification of the extracellular matrix (glycocalyx) limits the accessibility of the antimicrobials, thereby reducing its effect on cells. The biofilm cells are protected by modulation in physiology and genetic expression of adherent cells (Gilbert *et al.* 1993; Bowden and Hamilton 1998).

Adherence and colonization by oral bacteria is also influenced by the specific serum and salivary antibodies. The commensal micro-organisms escape from the host adaptive immune response by inducing tolerance mechanism. Specifically, the stimulation of $CD4^{+}CD25^{+}$ regulatory T cells (Treg) contributes in maintenance of immune tolerance mechanism (Wu *et al.* 2014). Several commensal micro-organisms have been reported to induce production of TGF-β from host cells which promote the Treg cell mediated tolergenic response (Tourneur and Chassin 2013).

At least 45 identifiable antimicrobial gene products were secreted by the oral epithelial cell, salivary and neutrophils in the oral cavity (Khurshid *et al.* 2016). The antimicrobial peptides like α-defensins (HNP-1, 2, 3 and 4), β-defensins (hBD-1, 2 and 3), Cathelicidins (LL-37), histatin (1, 3 and 5), adrenomedulin etc. plays a key role in maintaining the homeostasis and act as a guardian against pathogens in the oral cavity. hBD-1 is constitutively expressed in oral cavity whereas the other *β*-defensins are expressed upon stimulation by microbes or proinflammatory cytokines. The inducible hBD-2 expression in oral epithelium varies with the presence of commensal and pathogens providing us with a new insight about the innate immunity (Khurshid *et al.* 2016). The expression of hBD-2 and hBD-3 in epithelial cells are modulated by IL-1*β*, TNF-α, and IL-17, which confirms their role in innate immunity. The structural similarities of β-defensins with chemokines make it as a chemoattractant for immune cells. hBD-1 activates immature dendritic cells and memory T cells, hBD-2 can recruit mast cells and neutrophils, and hBD-3 is chemotactic for neutrophils, dendritic cells, mast cells and monocytes. hBD-2

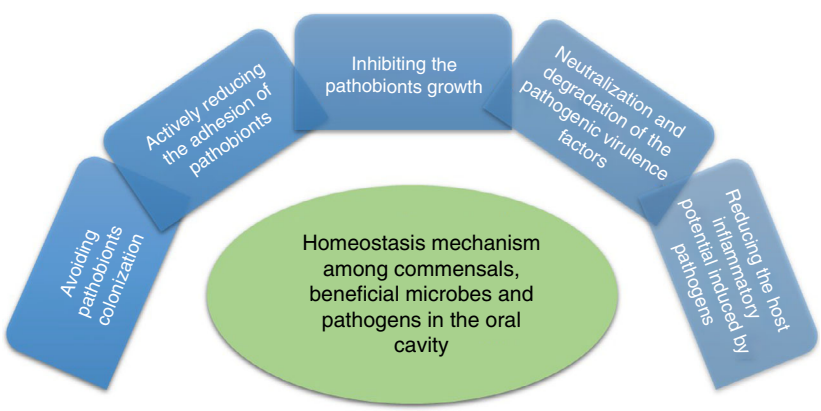

Figure 3 Homeostasis *mechanism among commensals, beneficial microbes and pathogens in the oral cavity.*

and hBD-3 also induce degranulation of mast cells (Hans and Hans 2014).

Human β-defensins in the oral cavity induce the pro-inflammatory pathways including NF-κB and AP-1 as well as MAPKs (Stamatova and Meurman 2009) thereby strengthening the intestinal barrier functions. However, it has been reported that hBD-2 in oral epithelial cells can be induced by commensals and thereby empowering the overall innate immune readiness (Dale and Fredericks 2005). It also has a broad-spectrum antimicrobial activity (Hancock and Diamond 2000) and checks the overgrowth of commensals and prevents the colonization of pathogens. Probiotics help to maintain the health, integrity and homeostasis in the oral cavity. The immune-modulation of pro-inflammatory pathways by probiotic bacteria in the oral cavity can be by altering the toll-like receptor (TLR) signalling, through inhibition of NF-$\kappa\beta$ pathway, and via the release of interleukin-10 cytokine (Hans and Hans 2014). So the anti-inflammatory property and resistance to antimicrobial peptides are the critical factors of consideration during the selection of probiotics for the oral cavity (Bosch *et al.* 2003; Gröschl 2009).

The major probiotic genus which colonizes in the oral cavity is lactobacilli and plays a critical role in sustaining the micro-ecological balance in the oral cavity (Caufield *et al.* 2015; Tester and Al-Ghazzewi 2018). Lactic acid bacteria produce lactic acid by the fermentation of carbohydrates which in turn lowers the oral pH (Tester and Al-Ghazzewi 2018). The protonated acid may enter into the cell and get dissociated increasing intracellular acidity. The maintenance of the intracellular pH homeostasis is the inevitable survival strategy by the probiotics/commensals. The inherent acid tolerance mechanism (proton translocating ATPase) in the LAB helps them to survive the acidic conditions. The basic mechanism includes boosting of the H+'ATPase activity (Bender 1986; Hamilton and Buckley 1991) thereby improving in the potential to manage the transmembrane pH variation (Hamilton and Buckley 1991; Dashper and Reynolds 1992). The cell membrane modification by altering lipid composition, modulating the size of membrane channels is another strategy for restricting entry of protons into the cells. The micro-organisms try to maintain a stable and neutral intracellular pH and a constant pH gradient with the expenditure of a lot of energy which is met by enhancing the glycolytic activity (Hamilton 1987). The switching to predominantly homofermentative metabolism, that is, Glycolytic pathway is induced when pH drops (Hamilton and Buckley 1991). Alkalization is another mechanism of low pH survival adopted by *S. salivarius*, an oral bacterium that converts urea into ammonia (Sissons and Hancock 1993). This can be substantiated by the increased urease gene expression at a lower pH (Li *et al.* 2000). Hence urease enzyme synthesis is significantly enhanced at low pH. Similarly, there is another arginine deiminase (ADI) system which produces CO_2 and NH_3 during the conversion of arginine to ornithine, thereby increasing the pH. Some other reported generic responses include the increased expression of protein repair chaperones and DnaK in *S. mutans* during acid stress (Jayaraman *et al.* 1997) called as 'acid tolerance response' (ATR) (Guan and Liu 2020).

Cell adhesion is a complex process that triggers host immune response which is another classical selection criterion for probiotics. Bacteria adhere to intestinal mucosa through mucus-binding proteins, pili, surface proteins like fibronectin-binding proteins (FBPs) and surface layer proteins; the surface protiens such as fibronectin present in intestinal matrix which can enhance the adherence of probiotic in the intestine. Some probiotics such as *L. rhamnosus* GG have polymeric pilli SpaCBA culter which contain a mucus binding adhesion protein at tip which facilitate the adherence to intestinal epithelial cells (Fig. 2d). The transient colonization of probiotic favours the production of metabolites such as short chain fatty acids as well as release immunomodulatory components which binds to epithelial receptors of host thus inducing host immune signalling pathways (Monteagudo-Mera *et al.* 2019). In addition, probiotics can interact with pattern recognition receptors (PRRs) like TLRs through microbe associated molecular patterns (MAMPs) which triggers the immune responses permitting the specific recognition of both pathogens and commensals by epithelial cells (Kagnoff and Eckmann 1997). The surface bound components and secreted molecules of probiotics remains in close contact with the host immune cells which triggers the host immunomodulation signalling cascade. In some bacteria MAMP are pilli structures which help in adherence as well as function in TLR-2 signalling (Lebeer *et al.* 2012; Turroni *et al.* 2013). Moreover probiotics can also modulate the release of cytokines by inhibiting the pro-inflammatory cytokines and/or stimulating the anti-inflammatory cytokines (Henderson and Wilson 1996). Besides, the protective role of probiotics can be by production of antimicrobial substances, reduction in pathogen adhesion and competition for host cell binding cells. Colonization in intestine not only includes host- microbe adhesion but also the microbe-microbe adhesion ability (co/auto-aggregation). Co/auto-aggregation can be the beneficial strategy that can promote longer colonization in intestine, besides the enteropathogens colonization. Thus, the distribution and stability of microbiota in oral biofilms is influenced by the inter-species cross-talk for binding sites. Some of the probiotic bacteria can persist in the oral cavity after consumption

of products enriched with probiotic bacteria (Caglar *et al.* 2009). After a week of daily consumption of yoghurt containing *L. rhamnosus* GG, lactobacilli were reported to be present in the saliva for up to 2 weeks post discontinuation of the consumption of yoghurt (Näse *et al.* 2001). Few recent studies have reported the transient colonization of probiotics in the oral cavity (Rungsri *et al.* 2017). In a recent study by Dassi *et al.* (2018), an interesting observation was noticed that the probiotic intake minimally influence the overall taxonomic and abundance distribution of bacterial genera (Dassi *et al.* 2018). The permanent colonization of probiotics in the oral cavity is still controversial, as it can affect the stability of oral communities (Monteagudo-Mera *et al.* 2019).

Acid stress in the stomach

Upon entering the stomach, the first adverse condition is the stomach acid (pH 1·5–5) followed by the presence of bile acids along with the digestive enzymes. The exposure to high acid results in the accumulation of protons inside the bacterial cells which adversely affects the transmembrane proton motive force. The acidic condition can cause damage to proteins and DNA (Mills *et al.* 2011). The ATR by lactobacilli and bifidobacteria enables them to survive under acidic conditions (De Dea Lindner *et al.* 2007). Tolerance to stomach and bile acids are the intrinsic characteristic feature of probiotics and has a wide array of defence mechanisms to survive the hostile environment. There are several specific adaptive and protective mechanisms exhibited by commensals as well as probiotics which include proton translocation by F1F0-ATPase (proton pumps), alkalization of the cytoplasm, decarboxylases and transporters, modification of chaperone and proteases, maintaining osmolarity by modulating transport systems, modifications of cell membrane compositions etc. (Ruiz *et al.* 2011).

One of the most common mechanisms is the increased activity of proton efflux pump. In proton translocation by F0F1 ATPase, the protons are expelled from the interior of cells and thereby pH homeostasis is maintained. In the ADI (arginine deiminase) pathway, ornithine, ammonia and carbon dioxide are produced from arginine, thereby increasing the alkalinity. The resulting alkalization of cytoplasm and the generated ATP is used for the expulsion of cytoplasmic protons (Corcoran *et al.* 2008; Wu *et al.* 2014). In GAD (glutamic acid decarboxylase) pathway, GABA is produced by the decarboxylation of glutamate. Expulsion by glutamate/GABA antiporter and consumption of intracellular protons increases the internal pH of the cells (Sanders *et al.* 1998; Teixeira *et al.* 2014). Amino acid decarboxylase mechanism has been reported in commensals as well as probiotic lactobacilli (Molenaar *et al.* 1993). Exposure to acidic conditions causes an alteration in the lipid composition of cell membranes in lactobacilli and bifidobacteria (Taranto *et al.* 2003; Ruiz *et al.* 2007). The membranes of acid adapted *L. casei* ATCC 334 showed an increased ratio of saturated to unsaturated fatty acid and cyclopropane fatty acid content which helps in the modulation of fluidity, hydrophobicity and proton permeability of the membrane (Broadbent *et al.* 2010). These mechanisms help the organisms to survive the acidic conditions in the stomach (Fig. 2e). The acid stress results in upregulation of stress proteins and chaperones—GroEL/GroES, DnaK and Clp as a protective mechanism in the LAB (Fiocco *et al.* 2019). During acid stress, the LuxS mediated quorum sensing system significantly produces the universal signalling molecule called autoinducer-2 (AI-2 *L. acidophilus* NCFM and *L. rhamnosus* GG). It is also noticed that the gene encoding the universal stress protein Usp1 was also upregulated during acid stress response of *L. plantarum*.

Bile stress in the intestine

As mentioned above in 2·3.1, bile tolerance is an important property for the survival of beneficial microbes in the small intestine. Bile stress response includes a plethora of phenomenon, that include active efflux of bile salts (Bustos *et al.* 2011; Ruiz *et al.* 2012a, 2012b), hydrolysis of bile salts (Lambert *et al.* 2008), remodelling of the cell membrane and cell wall (Ruiz *et al.* 2007) etc. Bile acid resistance is an added advantage for the microbes to get colonized in the human GIT (Yokota and Moraes 2012; Horackova *et al.* 2018). The probiotic bacteria equip themselves with a plethora of defence mechanisms against bile (Fig. 2f) which include enzymatic hydrolysis of bile, special transport mechanisms, bile efflux pump, the synthesis of various types of surface proteins and fatty acids, production of exopolysaccharides etc. (Sanchez *et al.* 2010; Ruiz *et al.* 2011).

In several probiotic bacteria as well as commensals, bile salt hydrolase—choloylglycine hydrolase (EC 3·5.1·24), a constitutive intracellular enzyme is present which breaks the amide bond between glycine or taurine and the steroid nucleus of bile acids. It aids in the detoxification of bile as well as the incorporation of cholesterol into the cell wall. Bile salt hydrolases de-conjugates bile acids rendering them free to be taken up by the liver for re-conjugation (Monte *et al.* 2009). Only a small portion of the bile acids escapes to the large intestine, where bacterial mediated de-hydroxylation of bile acid is carried out (Ridlon *et al.* 2006).

The accumulation of unconjugated bile acids such as cholic acid inside the cell membranes results in decline in

the intracellular pH leading to cell death. To overcome the situation, commensals exclude bile acids by efflux pumps. This mechanism is adopted as bile stress protection in both lactobacilli and bifidobacteria (Ferreira *et al.* 2013; An *et al.* 2014; Fiocco *et al.* 2019). The induction of several other stresses like acidity, osmolarity and stationary growth phase etc. causes an alteration in cell membrane composition, resulting in increased bile resistance (Begley *et al.* 2005). One of the survival mechanisms of bifidobacteria includes alteration in cell membrane composition by raising the cyclopropane fatty acids and limiting transmembrane proteins thereby reducing the cell membrane permeability (Ruiz *et al.* 2007). It has been reported that during bile stress and a change in environmental conditions like temperature (37–25°C) results in alteration of fatty acid composition by increasing polyunsaturated (C18:2) and saturated (C16:0) fatty acids, thereby maintaining the membrane stability (Fernandez *et al.* 1999).

In lactobacilli, the presence of bile salt causes significant overexpression of phosphofructokinase, phosphoglycerate mutase or elongation factor Tu (a key enzyme of energy metabolism) (Wu *et al.* 2010). Another protective mechanism by the commensals/probiotic is increased production of exopolysaccharide resulting in decreased diffusion rate into the cytoplasm. The protein misfolding during bile stress is tackled by the increased production of chaperones and proteases (Ruiz *et al.* 2011) as well as by the induction of protective proteins like AhpC (alkyl hyroperoxide reductase C) and PNDR (pyridine nucleotide disulphide oxidoreductase). DNA and RNA is protected by the synthesis of proteins like Dpr (dipeptide repeat proteins), NrdA (nuclear pre-mRNA downregulation protein), MutT1 (methyltransferases) and enolase in aerobic conditions (Xiao *et al.* 2011).

Several studies reported that probiotic lactobacilli form biofilm in the presence of bile salts and high osmolarity (Zaidi *et al.* 2011; Aoudia *et al.* 2015). Interestingly, gut commensals including probiotic species can establish biofilms or biofilm like structures in the host. This can be linked to the luxS, a gene involved in the quorum sensing mechanism and biofilm formation which helps in the survival of microbes during acid and bile stress (Lebeer *et al.* 2008; Jia *et al.* 2018).

Oxidative stress in the intestine

Oxidative stress can be overcome by enzyme-dependent and -independent mechanisms. The main mechanism to overcome the oxidative stress is by the synthesis of enzymes such as catalase, superoxide dismutase, NADH oxidase and NADH peroxidase. The Oxygen stress tackled by many lactobacilli is either by the accumulation of manganese or by nonenzymatic superoxide neutralization (Archibald and Fridovich 1981; Barnesea *et al.* 2012; Papadimitriou *et al.* 2016). It has been demonstrated that in the case of *L. lactis*, the metal ions such as $Zn^{2+,}$ protects against oxidative stress induced changes in proteins (Scott *et al.* 2000).

Several lactobacilli and bifidobacteria lack catalase and superoxide dismutase. In oxygenic conditions, the hydrogen peroxide synthesized by a pyruvate oxidase enzyme was removed by NADH peroxidase in *L. plantarum* (Goffin *et al.* 2006). While in the case of bifidobacteria, the NADH oxidase and NADH peroxidase scavenge free radicals (Talwalkar and Kailasapathy 2004). Recently specific mechanisms viz. exopolysaccharide biosynthesis and cellular aggregation to protect itself from ROS have been reported in *L. mesenteroides* (Yan *et al.* 2016). Another survival strategy during oxidative stress by probiotic lactobacilli and bifidobacteria is modifying the membrane lipid composition (Guerzoni *et al.* 2001; Oberg *et al.* 2013).

In case of oxidative DNA damage, the intestinal SOS mechanism is activated. Such as physical protection of DNA by shielding it at large Dps protein DNA complexes (Chiancone 2008). Oxidative stress also induces the production of Dpr proteins (Dps-like peroxide resistance) in the presence of hydrogen peroxide, which in turn produce ferritin like iron scavengers that scavenge the radicals and reduce the damage caused by oxidation (Smith 2004).

Osmotic stress

During osmotic stress, molecular chaperones play a critical role. There are several similar shock proteins produced during different stresses. For example, GroEL and DnaK, which are heat shock proteins, are also induced under osmotic stress (Prasad *et al.* 2003). Synthesis and intracellular accumulation of compatible solutes (osmotic balancers), such as betaine and trehalose is another mechanism to increase the osmotic tolerance by probiotic microbes. The compatible solutes help to maintain the turgor pressure of the cells, as well as prevent denaturation of soluble proteins.

Competition of indigenous microflora

In the large intestine, probiotics have to compete with trillions of microbes primarily the already established commensals. They utilize many mechanisms to help them establishment in this niche. Probiotic organisms have higher competitive adhesion potential due to the numerous surface adhesions such as mucus binding proteins (Dhanani and Bagchi 2013; Singh *et al.* 2018), FBPs (Bisht

et al. 2018) or adhesive pili (Lebeer *et al.* 2012). Probiotics have competitive nutrition strategies, that is, these can break down and metabolite the mucus, a carbon sources generally present in the gut (Becerra *et al.* 2015). Host cells and commensal microbes produce several substances detrimental to the microbes. Probiotics have capability to safeguard themselves against such substances (Lebeer *et al.* 2011; Sims *et al.* 2011). These are some mechanisms which help probiotics in association with commensal to establish the microbial homeostasis in the gut.

The action of probiotics during dysbiosis is entirely different in comparison with normal gut microflora. It is well known that the early colonization in the infant GI tract by commensals determines the establishment of the gut microbiome in later life. The basic rationale behind the use of probiotics is to restore the homeostasis in the gut by replenishing the microbial diversity; however, the mechanisms involved are not yet completely elucidated. The better understanding of gut microbiome composition during normal and diseased conditions is essential to understand the benefits of probiotics in maintaining the microbial biodiversity. Moreover microbe-microbe and host-microbe interactions also help to throw light to understand the mechanisms of probiotic action by beneficial microbes. Based on available knowledge, it can be stated that probiotics can alter the composition and function of the microbiome.

Conclusion

It is evident that the human GIT represents a plethora of challenges to invading microbial communities and also differentiates between the beneficial/ commensal and nonself-microbes. As discussed in this review, several pathogenic strains have evolved to resist its actions and learned to use them even for their advantage. Transfer of the ability to withstand GIT stress from beneficial strains to potential pathogens of clinical relevance can be a worrisome problem in future. Hence, future genetic analysis should focus on mechanistic studies targeted at understanding the molecular mechanisms governing GIT defence. Undoubtedly, genomic insights can help in better understanding the defence mechanisms and exploring them for preventive therapies under clinical scenarios. Furthermore, deeper knowledge of the elaborated defence mechanisms can help to target potential pathogens and would assist in screening for better probiotic strains.

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