Chapter 6

Glucocorticoids and the Intestinal Environment

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1. Introduction

For thousands, perhaps millions of years, human have marveled to understand how the body is formed and function, how it maintains its wholeness and healthy state, and how disordered states occur. In frame of holistic philosophy of ancient eastern cultures, a person had been accepted as physical, emotional, mental, and spiritual ones. In this concept, the balance of the body, mind and spirit were accepted as the healthy state of an individual [1,2]. The ancient Greeks defended the concept of the four humors doctrine; blood, black bile, yellow bile, and phlegm. *Ad modum* this doctrine these four humors in a healthy person were believed to be balanced, and the reason why a person fell ill was due to the fact that these fluids were disturbed [3,4]. With the separation of medicine from superstition, magic and religion by famous Greek physician Hippocrates in around 5th century B.C., more realistic and relevant approaches could be developed to better understanding the life, structures of living creatures and their healthy and diseased conditions. These and the time-dependent innovative developments in technology and science throughout the following centuries led to more concrete observations, mainly on animals. Gathered evidences and additive knowledge base from these observations mankind led mankind to make realistic definitions on the subjects "the structure, integrity and functions" of the animal and human body. In 19th century Louis Pasteur, a French chemist and microbiologist, stated the germ theory of diseases. He believed that micro-organisms (bacteria) infect animals and humans, thus they cause diseases [5]. At the same times, a French Physiologist Claude Bernard tried to understand how living creatures maintain their integrity, and how it is regulated, and defeated. He discriminated the internal environment from the external environment and was in opinion that living creatures maintain their internal milieu relatively constant under continuously changing environmental conditions. Claude Bernard [6] attributed also an important role to nervous system in the maintenance of internal environment in physiological ranges in human and animal organisms and remarked the substantial differences between animals and plants in this respect. Since that time, the interest of the researchers in different fields worldwide is mainly focused on possible regulation

mechanisms of various living species. Thus, it can be said that the philosophical roots of the concept "stress" and "stress physiology" is going back to the early observations that living creatures are exposed continuously to the effects of various environmental challenges against which they have to defend their integrity, and following statement from Claude Bernard that living creatures strive to maintain their internal environments relatively constant *via* various homeokinetic mechanisms even if their environmental conditions are changing, thereby keep their normal physiological functioning and prerequisite for a free, independent live or shortly '*la fixité du miliéu interieur est la condition d'une vie libre et indépendante*' as defined by him "*Il y a pour l'animal deux milieux: un milieu extérieur dans lequel est placé l'organisme, et un milieu intérieur dans lequel vivent les éléments des tissus*". However, Hans Selye, one of the Pioneers and founders of stress from 1930's, was the first who introduced the term "stress" as the real or perceived physical or psychological events which are threaten the homeokinesis in medical terminology. *Ad modum* Selye the stress is somewhat like living, not so easy to define, although there is no doubt about its presence [7]. The environmental challenges affecting a living creature, thus causing stress, can vary from physical, to chemical and bio-psycho-social factors, while all the reactants, their contra- and/or co-players within the living systems are of chemical nature at the last instance; intracellular or intra-bodily signaling elements as either hormones including glucocorticoids and catecholamines or neurotransmitters, cytokines and chemokines, etc., or a group or all of them functioning simultaneously within the body for the same purposes.

Stress and the glucocorticoids are associated or interweaving concepts with each other. Indeed, to physiologists the term "stress" has come to mean any event that elicits increased cortisol secretion [8]. However, as it is well known, glucocorticoids are not only mediators of the stress responses; they take a part in peripheral components of stress responses. The stress response is mediated by the stress system which is composed of two components; central nervous system and the peripheral part. The central, greatly interconnected effectors of this system include the hypothalamic hormones arginine vasopressin, corticotropin-releasing hormone/factor (CRH/CRF) and pro-opiomelanocortin-derived peptides, and the locus ceruleus and autonomic norepinephrine centers in the brainstem. The peripheral components of the stress system include (*a*) the peripheral limbs of the hypothalamic-pituitary-adrenal (HPA) axis; (*b*) the efferent sympathetic-adrenomedullary system, and (*c*) components of the parasympathetic system [9-11]. There are also bidirectional positive feedback regulation between the CRF secreted paraventricular nucleus of hypothalamus and central noradrenergic system [9,11,12].

Stressors activate different physiological processes. The first classical response is the secretion of adrenalin and noradrenalin hormones from the adrenal medulla *via* activation of sympathetic nervous system. This response is called "fight or flight syndrome", because adrenaline and noradrenalin increase the respiratory rate, the heartbeat, the concentration of glucose in circulating blood and the blood flow to the skeletal muscles. This fast response is primarily related to survival. Stressors also activate the HPA axis. The activation of this axis begins with the stimulation of parvocelular neurons of hypothalamus and secretion of CRH. CRH stimulates the secretion of adrenocorticotrophic hormone (ACTH) from the

adenohypophsis and the ACTH acts on cortex of adrenal glands and stimulates the release of glucocorticoid hormones. GC hormones (corticosterone in rodents and cortisol in humans), which are the ultimate product of HPA axis activation, act on multiple bodily systems to maintain homeokinesis. They stimulate protein catabolism, gluconeogenesis and release of glycerol and fatty acids into the blood, maintain the vasoconstructive effect of norepinephrine, inhibit glucose uptake and oxidation by many body cells except the brain (insulin antagonism) and also inhibit inflammation and specific immune response [8-11].

The secretion of ACTH, and therefore of cortisol or corticosterone, is stimulated by several hormones and molecules in addition to hypothalamic CRH. Depending on the stressor, substances such as vasopressin, epinephrine, angiotensin II, various cytokines (tumor necrosis factor (TNF)-α, interleukin (IL)-1, and IL-6), and lipid mediators of inflammation affect the hypothalamic, pituitary, and/or adrenal components of the HPA axis and potentiate its activity. Glucocorticoids play an important role in the regulation of basal activity of the HPA axis, as well as in the termination of the stress response by acting at extra-hypothalamic centers, the hypothalamus, and the pituitary gland. The negative feedback of glucocorticoids on the secretion of CRH and ACTH serves to limit the duration of the total tissue exposure of the organism to glucocorticoids, thus minimizing the catabolic, lipogenic, anti-reproductive, and immunosuppressive effects of these hormones [8,10,11].

The activation of the HPA is a longer-term adjustment by the humans or animals to the changes in their micro- or surrounding environments. Selye [7] called it as the 'general adaptation syndrome' (GAS). The process of adaptation, also known as "allostasis" (literally "maintaining stability, through change"), supports the homeokinesis [13]. In acute stress, the activation of both sympathetic system and HPA axis are essential for survival of individual and they help to re-establish or maintain homeokinesis through adaptation. However, in chronic periods, prolonged or repeated activation of stress systems can result in cumulative biological changes (known as allostatic load) and can alter adaptive mechanism. If the ability of the organisms to maintain their integrity is poor or not strong enough to compensate the effects of environmental challenges, they can also harm the living creatures *via* various mechanisms. Excessive or inappropriate, inadequate adaptive responses to stress may play a causal role in development of certain diseases [10,11,14,15]. Indeed, depending on the stressors and their severity as well as the capability of living creatures to respond them, the reactions of living creatures to the stressors can be systematic, also reflecting itself throughout the whole body, or mainly local or regional, reflecting self at the organel/celluler/organ or system levels [11,14]. Similarly, the application of glucocorticoids for a long time can also cause events with serious consequences [16].

The gastrointestinal tract and the immune system are particularly responsive to different stressors. This system has many cellular targets for the stress mediators such as catecholamines, glucocorticoids and CRF [17-19]. The association between stress and various gastrointestinal diseases, including functional bowel disorders, inflammatory bowel disease (IBD), peptic ulcer disease and gastroeosophageal reflux disease, is being actively

investigated [15,17]. However, there is a parodox that the chronic stress plays a role in inflammatory bowel disease while the glucocorticoid therapy is widely used to cure the same disease.

Gastrointestinal system (GIS) is the biggest surface area (~200 m^2) of the body that is binding the organism to the external environment. This system is continuously exposed to various antigens from consumed foods and resident bacteria and also from potentially harmful pathogens, such as viruses, bacteria, fungi or parasites. Besides digestion and absorption processes, it forms a barrier between the internal and external environments [20]. Intestinal barrier is composed of various components such as tight junction conformation between the intestinal epithelial cells, mucosal immune system, mucin secretion, and intestinal microbiota. The barrier function is quite critical process because of the barrier should confirm the hyporesponsiveness or tolerance towards commensal bacteria while maintaining the ability to fight pathogenic microorganisms. The breakdown of this critical balance usually results in inflammation-associated damages. Gastrointestinal system is rather complex system, which has own nervous and endocrine system. The enteric nervous system is connected bidirectionally to the brain by parasympathetic and sympathetic pathways forming the brain–gut axis. The description of stress-induced alterations in this axis is thought to be important for the solving problems of many stress-related gastrointestinal disorders [17,20]. A number of paracine acting endocrine cells have important functions for the regulation of digestion processes. These hormones exist in both central nervous system and gastrointestinal plexus neurons where they function as neurotransmitters or neuromodulators [8]. Recent findings suggest that glucocorticoid hormones may also be synthesized from extra-adrenal tissues such as brain, skin, vascular endothelium and intestine. These tissues express steroidogenic enzymes and were claimed to be potential extra-adrenal sources of glucocorticoids [21]. It is emphasized that intestinal glucocorticoid synthesis might be of potential importance in regulation of mucosal immune responses and information of inflammatory bowel disease [22].

Gastrointestinal system hosts also a large number of microorganisms. They all together are called as gastrointestinal microbiota, and form the great part of the system. The microbiota–host interactions play an active role in many physiologic and pathophysiologic processes of the host [19,23,24]. While gastrointestinal microbiota could affect the stress response [25] and intestinal structure and functions of the host [23,24], stress hormones of the host organism such as the catecholamines can also alter directly growth, motility, biofilm formation and/or virulence of pathogens and commensal bacteria [19,26,27] or they may affect their microbiota indirectly by changing the intestinal environment [20].

The effects of stress on important physiological functions of the gut include; motility, secretion and absorption, visceral sensitivity, mucosal integrity, permeability, immune system, blood flow, microbiota and microbiota-host interactions [17,20]. Some stress-related alterations in gut physiology also can be induced by exogenous glucocorticoids [28-30]. However; recent findings using central and peripheral CRF administration showed that CRF and their receptor subtypes (CRF1 and CRF2) are playing important roles in many stress-

related alterations of the gut. The stress-related alterations including intestinal permeability, mast cell activity [20], goblet cell and mucin formation [31], and motility alterations [32] can be mimicked by the CRF agonists and inhibited by CRF receptor antagonists. Therefore; CRF have been suggested as a target to treat stress-induced functional gastrointestinal disorders.

In this chapter, the effects of stress and stress-related hormones, especially glucocorticoids on the components of intestinal environment suc as intestinal epithelium, mucin formation, mucosal immune system, intestinal microbiota and microbiota-host interactions, and also the role of corticosteroids and stress in bacterial colonization and intestinal diseases were reviewed.

2. Stress models for gastrointestinal studies

A great number of animal models of stress (acute/chronic, early life/adult, physical/ psychological or both physical and psychological) have been described for studying the effects of stress on gastrointestinal functions. For this purpose, rather naturally (Wistar-Kyoto rats) or genetically modified stress susceptible animals were also used. As denoted by Soderholm and Perdue [20], stress models that psychological effects are more powerful than the physical effects are preferred for mimicking the effects of the life and environmental stress on several pathologies. However, the kind and duration of stressors and other factors including genetic or perinatal environment, etc. might be a key factor in the evaluation of the stress effects on specific gastrointestinal functions such as stress-induced visceral hyperalgesia. Depending on the characteristics of the stressors and the time-course of their effects, alterations caused by stress can be immediate, delayed, transient or sustained or never be seen [33,34]. For example, the small intestinal transit was significantly inhibited by restraint stress, but not by footshock stress although plasma corticosterone levels were significantly elevated to the same extent by restraint stress and foot-shock stress [33].

Williams et al [35] reported that acute mild restraint (wrap restraint) elevated plasma levels of adrenocorticotropic hormone and beta-endorphin, and caused analgesia. Gastric ulcer did not form, gastric emptying was not affected, however, small intestinal transit was inhibited, and large intestinal transit was stimulated by wrap restraint stress, and there was an associated increase in fecal excretion. Because of neither adrenalectomy nor hypophysectomy have prevented the response of the intestine to stress, they suggested that adrenal or pituitary-derived factors are not responsible for mediating the effects of stress on the gut.

Restraint stress is most widely used as an acute stress model. Restraining can be supplied with restraint devices or by wrap restraint for times varying from 30 min to 4 hours. This model could be modified with a cold environment (cold restraint stress) or water immersion (water immersion restraint stress) for creating both physical and psychological stress [20]. Cold enhanced the changes in rat intestine caused by restraining. However, plasma corticosterone levels increased in both restraint and cold restraint stressed rats in same extent compared with fasted unstressed rats. Fasting corticosterone levels also increased compared with the fed rats [36].

The acute (one time) and chronic (1 hour/day for 10 consecutive days) models of water avoidance stress are also preferred potent psychological models in determination of stress-induced gastrointestinal functions. These models result in elevations of ACTH and corticosterone within 30 min [37], and induce enlargement of the adrenal glands [38]. Recently, Vicario et al [39,40] reported that crowding stress (8 rats/cage) or sham-crowding (2 rats/cage) for up to 15 consecutive days triggers reversible inflammation, mast cell-mediated barrier dysfunctions, persistent epithelial dysfunction, and colonic hyperalgesia. Crowding-stressed rats showed higher plasma corticosterone levels than sham-stressed animals from day 1 and up to day 15. After 15 days of crowding stress, corticosterone levels decreased %38 (slightly adaptation), but HPA reactivity to incoming stressors was preserved. Crowding stress, differently from the stress models induced in laboratory, is actualized in natural environment of animals and may reflect life stress well for humans. Thus, this model has been also suggested as a suitable animal model to unravel the complex pathophysiology underlying to common human intestinal stress-related disorders, such as IBS.

Early life stress models such as maternal deprivation of pups from the dams have also been widely used [31,41,43,44], because stressful events in the early period of life (in the form of abuse, neglect or loss of the primary caregiver) have been shown to modify adult immune and gastrointestinal tract functions [12].

3. Intestine and its microenvironment

The gastrointestinal system is a tubular organ and the part 'intestine' extends downwards from the pyloric sphincter. With its many loops and coils, small and large intestines fill much of the abdominal cavity. Microscopically, both the small and large intestine are composed of four distinct layers; the mucosa covering the internal site of the tube, the submucosa, the muscularis externa and the serosa. The mucosa consists of layer of epithelia, loose connective tissue layer (lamina propia) containing blood vessels, lymphatics, and some lymphoid tissue, and muscularis mucosa. They both have different types of cells as building blocks; however, some of them are especially important in relation to luminal challenges *via* different agents including undigested or partly digested dietary proteins, gut microbiota and its metabolites. Located among the enterocytes are goblet cells secreting mucin which *per se* build a barrier; scattered enteroendocrine cells with paracrine and endocrine actions; Paneth cells characterized by their granules containing lysozyme, tumor necrosis factor-α and defensins have antibacterial roles; M cells with their well-known roles in antigen sampling and transportation; and the intestinal subepithelial myofibroblasts located in proximity to the mucosal epithelium and produce growth factors including hepatocyte growth factor promoting the proliferation of the intestinal epithelial stem cells, thus responsible for regeneration and maintaining of the integrity of the intestinal mucosa; and dendritic cells (DCs) highly specialized for antigen presentation which can capture non-self proteins and recognize microbial products [45-55].

Intestinal epithelial cells continuously contact with two different environments; first, luminal environment, which include intestinal secretions, food antigens, commensal

bacteria, and also noxious or pathogenic materials, and second, the interstitial fluid surrounding the cells at the basolateral side. The single layer intestinal epithelium along the small and large intestine has a number of physiologic functions; it forms a barrier between the external (luminal) and internal environments besides its digestive and absorptive functions, and this epithelial barrier limits the space for bacterial growth. The ability of the epithelium to control uptake of molecules into the body is denoted as the intestinal barrier function [20,56,57]. The characteristics of intestinal epithelium for participation to the barrier function include; tight junction adherence between the epithelial cells, fluid and mucin secretions, secretions of numerous antimicrobial peptides, transepithelial transport of secretory IgA and the antigen presenting cell activity. However, intestinal epithelium is not only component of the intestinal barrier. Mucosal immune system, microbiota and microbiota-host interactions exist in major components of intestinal barrier [23,24]. All these components of intestinal barrier are controlled by the mediators of neuro-endocrine-immune network, and stress and stress mediators have significant impact on these components and regulatory network [20,24].

4. The effects of stress and glucocorticoids on intestinal barrier function

Intestinal mucosal epithelium is very sensitive against different types of stress because of the half-time of mucosal epithelia which may be as short as one and half day in certain parts. In other words, compared to many other tissues in the human and animal body, it is not well differentiated and very fragile [8,57]. The effects of acute or chronic stress on intestinal barrier function or intestinal permeability does not appear very different from each other in animal models of stress. However, the duration and repetition of stressors may influence severity, and the alterations may be temporary or permanent [34,58]. Generally, intestinal ion secretion [40,59], macromolecular permeability [60,61], inflammation [40,59], visceral hypersensivity and colonic motility increased [40], while gastric motility decreased in various animal models of stress [62-64]. These stress responses have been also described in IBD patients and involve dysregulation of HPA axis [12,15,17]. They are mediated by stress-related neuropeptides such as CRF, neural mechanisms and mast cells [18,20,65]. Various stress factors including heat, nutritional alterations, overcrowding, physical restraints and transporting also destroy the microbial balance and their microenvironment in the gut [66,67]. When the stressful events cause a decrease in beneficial bacteria, they generally increase the pathogenic species within the gut microenvironment [67-69]. In following sections, the effects of stress mediators on each component of barrier were discussed in details.

5. The physical barrier of epithelium

Cell-cell and cell–basement membrane interactions of intestinal epithelial cells control the transcellular and paracellular transports of luminal macromolecules and prevent bacteria from translocating into the subepithelial layer. Tight junctions (TJ) are primary physical components of intestinal barrier, located at the most apical part of lateral membranes of

epithelial cells and restrict paracellular passage. The breakdown of tight junctions during bacterial infections results in gut barrier failure, often termed "leaky gut" [20,23]. Proteins that constitute the TJ complex include transmembrane proteins such as occludin, claudin, junction adhesion molecules and intracytoplasmic proteins zonula occludens 1 and 2 (ZO1-ZO2) and members of the membrane-associated guanylate kinase (MAGUK) protein family [70]. Tight junctions are highly dynamic structures, and their permeability is regulated by several physiological and pathophysiological conditions. Signals from intestinal microbiota may promote integrity of the epithelial barrier and have been shown to regulate tight junctions and protect intestinal epithelial cells (IECs) from injury by controlling the rate of IEC proliferation and inducing cytoprotective proteins [23]. Inflammatory cytokines can disrupt tight junctions and impair gut barrier integrity. Treating epithelial monolayers with TNF-α or IL-1b increased the permeability of tight junctions by stimulating transcription and activation of myosin light chain kinase (MLCK) [71-73]. The acute partial wrap restraint stress increased colonic permeability and rectal hypersensitivity *via* epithelial cell cytoskeleton contraction through myosin light chain kinase activation [74]. Acute immobilization stress also induced an increase in TJ permeability in the rat terminal ileum. These changes were mainly due to irregularly distribution of TJ transmembrane protein occludin and of the plaque protein ZO-1 which were seen after 2 hours from the stress induction and returned to a normal pattern within 24 hours [70]. Mazzon and Cuzzocrea [75] also suggested that TNF-α has active roles in the increase of tight junction permeability during acute restraint stress. They demonstrated *in vivo* in a TNF-α R1 knock-out mouse (TNF-α R1KO) model of restraint stress that the inhibition of TNF-α attenuates the development of TJ alteration in the ileum. Restraint stress caused the increase of heat shock protein-70 expression and associated decrease in the expression of type 1 (ZO-1) protein in the colonic epithelium of mice. These stress-induced changes can be inhibited by the glucocorticoid receptor antagonist mifepristone [76]. However, Boivin et al. [77] reported that glucocorticoids enhanced epithelial barrier function by suppressing transcription of myosin light chain kinase. Bacterial pathogens target tight junctions and breach epithelial integrity to promote colonization, obtain nutrients and access the underlying tissues [23]. So, alterations in gastrointestinal microbiota induced by several stressors or exogenous glucocorticoids might be an important threat for the barrier disruption.

6. Fluid and ion secretion

Due to their diluting and flushing effects, fluid and ion secretion of epithelial cells is another protective mechanism contributing to the barrier function [20]. In humans, the jejunal net water and sodium chloride absorption decreased during both psychological (induced by dichotomous listening) [78] and physical stress (induced by cold pain) [79], and also ion absorption is changed toward secretion in psychological form [78]. Both acute and chronic stress inductions increased short circuit current (an *in vitro* technique used for measuring the secretory response of intestines) in several parts of rodent intestines [36,41,59,80]. In addition, the peripheral non-selective CRF antagonists astressin or α-helical CRF9-41

abolished stress- induced alterations [41,80,81]. Intraperitoneal injection of a newly developed selective CRF(1) peptide agonist cortagine also induced an increase in defecation and watery diarrhea in mice and rats [82]. The effects of mineralocorticoids and glucocorticoids on intestinal water and ion movements are well known. Methylprednisolone for 3 days increased Na^+K^+adenosinetriphosphatase activity and Na^+ absorption [83]; it also increased guanylate cyclase activity and Cl^- secretion in the jejunum and ileum 6 h after administration [84,85]. However, there is no direct information about the increase of water and ion secretion related with increased glucocorticoid secretions in different stressful conditions.

7. Intestinal permeability and mast cell functions

An increase in intestinal permeability was reported in animals [61,86] and humans [87] submitted to acute or chronic stress, and in IBD and IBS patients [88,89]. Increase of intestinal permeability or macromolecular permeability also involves mucosal inflammation, mucosal damage, and mast cell hyperactivity. The barrier properties of the intestinal epithelium are usually studied by assessing the permeability to various probe/marker molecules (such as horseradishperoxidase, ^{51}Cr-EDTA, mannitol) *in vivo* or *in vitro* with intestinal segments mounted in Ussing-type chambers [20]. Bagchi et al [90] investigated the effects of acute (90 minute by water immersion) and chronic (15 min/day for 15 consecutive days by water immersion) stress on the production of reactive oxygen species (ROS) and oxidative tissue damage in gastric and intestinal mucosa. Both acute and chronic stress increased ROS production, lipid peroxidation and DNA fragmentation in both gastric and intestinal mucosa, but acute stress produced greater injury when compared to chronic stress. Colonic myeloperoxidase, mucosal mast cell activity and colonic permeability increased (as assessed with macroscopic damage and bacterial translocation to mesenteric lymph nodes, liver and spleen) at 12 weeks-period in maternally deprived rat pups induced by separation from their mothers on 2-14 days period for 3 hours a day [91]. Besides, the responses of maternally deprived rats to the TNBS-induced colitis were more prominent compared with the control rats. Both acute and chronic cold stress could cause oxidative stress of duodenum and a change in iNOS, which was related to the intestinal damage process in broiler chicks [92]. There are also evidences about the bacterial production of iNOS [93]. Boudry et al [58] suggested that both apoptosis in the crypts and an immature epithelium covering the villus surface can be responsible for a barrier defect in rats submitted to WAS (1 h/day) for 5 or 10 days. Morphologic [39,94] and enzymatic alterations [40] in mitochondria of the intestinal epithelium can also participate to promotion of intestinal dysfunctions in stress-induced animals.

Mast cell activity of gastrointestinal mucosa has altered in both acute and chronic stress models. They contain inflammatory and immunmodulating mediators such as prostaglandins, histamine and serotonin that directly alter epithelial transport properties [80] and nerve and muscle functions [95]. So it has a pivotal role in visceral hypersensivity [34], intestinal inflammation, intestinal mucin secretion [96] and epithelial barrier

disruption [20,61]. Castagliuolo et al. [96] found that mast cells are essential for the colonic mucin and prostaglandin secretions in immobilization stressed mice, because of these secretions were absent in mast-cell deficient animals. Even, reconstitution of bone marrow with mast cells reversed that response to normal stress values [96]. Mast cell-deficient rats (Ws/Ws) and their normal mast cell-containing littermates (1/1) were submitted to water avoidance (1 h/day) or sham stress for 5 consecutive days. Stress increased baseline jejunal epithelial ion secretion, ionic permeability, macromolecular permeability [61,94], and the number and proportion of mucosal mast cells [94] in 1/1 rats but not in Ws/Ws rats, compared with non-stressed controls. Morphological, inflammatory and permeability changes were not seen in ileum and colon of mast cell-deficient rats in a chronic stress model [42]. Kim et al [97] reported that acute stress increased mast cell number and mucosal proteinase-activated receptor-2 (PAR2) expression (G-protein coupled receptor which can be activated by mast cell tryptase and modulate gastrointestinal functions) in the rat colon. Because of CRF-antagonist astressin inhibited these alterations, they suggested that CRF can be mediator of these events. Dexamethasone treatment improves PAR-2 agonist-induced visceral hypersensitivity, but does not prevent PAR-2 agonist-induced increase in colonic permeability in rats. This effect is coupled with a reduction of colonic mast cell numbers and RMCP-II contents [98]. Chronic stressful stimulus also caused greatest numbers of degranulated mast cells [99] and mast cell hyperplasia in the intestine of rats [100]. In our unpublished study, we found that while acute cold swimming stress (swimming in 18 ^{0}C water, for 15 min) increased the mucosal mast cell numbers in ileum, dexamethasone decreased their numbers significantly. It has been reported that mast cell numbers and their protease II activity were decreased in different dexamethasone treatments [98,101,102]. Wrap restraining stress in rats for 2 hours increased histamine content in colonic mast cells without degranulation, and this was found to be mediated by interleukin I and CRF [103]. Santos et al [80] reported that CRH, when injected intraperitoneally, mimicked the effects of acute restraint stress on colon epithelium such as increased colonic ion secretion, macromolecular permeability *via* cholinergic and adrenergic nerves and mast cells. Because CRH-induced alterations in colon epithelium inhibited by CRH-antagonist, adrenergic, nicotinic and muscarinic receptor antagonists and mast cell stabilizing agent doxantrazole, but not by aminoglutethimide (mineralcorticoid and glucocorticoid synthesis blocker, they suggested that steroids have no role in CRH-induced colonic pathology. They also denoted that stimulatory effects of CRH on mast cells can be mediated by direct or indirect neural pathways. Also in humans, CRH mediates transcellular uptake of HRP in colonic mucosal biopsy samples *via* CRH receptor subtypes R1 and R2 on subepithelial mast cells [104].

8. Mucin – Physicochemical barrier

Mucin secretion is also a major component of intestinal barrier which protects the mucosa by forming a coating layer over the epithelium against bacterial penetration. In addition to providing a biophysical barrier, mucus forms a matrix that allows the retention of high concentrations of specific and nonspecific antimicrobial molecules, such as secretory IgA and

defensins in close proximity to the epithelial surface [23,105,106]. The secreted mucus forms two layers, a thinner inner layer that is accepted to be sterile and difficult to dislodge and an outer layer that is not sterile and is more easily removed. Mainly MUC2 type mucin is synthesized from goblet cells in small and large intestines. Epithelial cells and Paneth cells secrete antimicrobial peptides that help preventing of bacteria to penetrate the inner mucus layer. Both the physicochemical structure and thickness of mucin coating show differences through the gastrointestinal canal. It was suggested that mucus thickness is increased and the increase was correlated with luminal bacterial concentrations of related parts of gastrointestinal canal. The inner layer is ~15–30 μm and the outer layer is 100–400 μm in small intestine and it is thickest in ileum because there are approximately 10^5–10^7 bacteria per gram of faeces in the lumen. Otherwise, the inner layer of ~100 μm and a thick outer layer of ~700 μm in large intestine where 10^{10}–10^{12} bacteria per gram of luminal content resides [106]. Mucin is not only a barrier against the bacteria but also nutritional source for bacteria. In addition, bacteria capable of colonizing mucus can avoid rapid expulsion *via* peristalsis of the intestine and take an advantage for transmitting their signaling pathway to the host [107]. Microbiota can stimulate mucin secretion *via* bacterial products and increase MUC2 expression *via* activation of TLRs and NOD-like receptors or other signaling pathways at transcriptional level. Mucin secretion is also influenced by hormones, inflammatory mediators, several signaling peptides, growth factors and infectious bacteria [106,107]. Castagliuolo et al. [108] reported that acute stress caused a depletion of goblet cells and an increase of mucin secretion related with decrease of mucin containing goblet cell numbers *via* an increase of CRF secretion [20]. They proposed that although rapid mucin release during acute stress would increase the barrier properties and provide a degree of protection against invasion of a leaky epithelium, goblet cell depletion would be deleterious in a longer time period because of the reduced capacity to respond to ongoing or new threats. A 10-day chronic stress model was resulted in barrier dysfunction in the ileum and colon (increased macromolecular permeability and depletion of mucus) and ultrastructural changes in epithelial cells (enlarged mitochondria and presence of autophagosomes) associated with bacterial adhesion and their penetration into enterocytes [42]. Studies revealed that CRF signaling can activate mucin secretion because goblet cells have CRF1 receptors and stress and peripheral injection of CRF induces mucus depletion in rat distal colon [18]. On the other hand, maternal separation stress increased mucus secretion and thus caused an elevation in the number of mucosal goblet cells in rats [109]. In our unpublished study, both acute cold swimming stress and dexamethasone injection increased the goblet cell counts in the ileum within six hours, but the effects of dexamethasone were more prominent than the swimming stress. Further, while the effect of the dexamethasone on goblet cells maintained in 24 hours period that of swimming stress was disappeared [Ünsal et al., unpublished data]. Finnie et al. [110] reported that exogenous prednisolone and hydrocortisone also increased the mucin secretion significantly in left slightly and in right uninvolved colonic biopsies of patients with ulcerative colitis. They suggested that therapeutic effects of corticosteroids in ulcerative colitis may be related partly with their stimulatory effects on mucin synthesis.

As partly mentioned above, most stress-induced gastrointestinal (GI) dysfunctions can be induced by peripheral CRF agonists and prevented by CRF receptor antagonists [18]. In a

review article Larauche et al [18] reported about "CRF signaling" and emphasized that peripheral injection of CRF or urocortin stimulates colonic transit, motility, Fos expression in myenteric neurons, and defecation through activation of CRF1 receptors, whereas it decreases ileal contractility *via* CRF2 receptors. Additionally, intraperitoneal administration of CRF induces colonic mast cells degranulation *via* both CRF1 and CRF2 receptors and increases ion secretion and mucosal permeability to macromolecules, which can in turn promote intestinal inflammation and alters visceral sensitivity. Furthermore, CRF peptides can reproduce secretomotor and mucosal alterations *in vitro*. Although there are a lot of events that CRF is primary mediator in stress-induced alterations of GIS in animals and humans [17,18,32,80-82,111], similar reports for the glucocorticoids are limited [28-30]. Meddings and Swain [28] reported that stress-induced increases in gastrointestinal epithelial permeability seemed to be mediated by adrenal corticosteroids and disappeared after adrenalectomy or pharmacologic blockade of glucocorticoid receptors. Besides, dexamethasone treatment of control animals increased gastrointestinal permeability and mimicked the effects of stress. Spitz et al. [29,30] evaluated the effects of dexamethasone on intestinal barrier functions in various conditions such as starvation and after bacterial contamination. They found that starvation significantly impairs secretory IgA, promotes bacterial adherence to the mucosa and increases intestinal permeability to f-MLP in rats given 0.8 mg/kg dexamethasone intraperitoneally. These effects are significantly attenuated by the feeding of rat chow [30]. In other study, they also found out that dexamethasone administration increased intestinal permeability and bacterial adherence to the mucosa [29]. However, antibiotic decontamination of the intestine completely abrogated the intestinal permeability defects observed in this model. Basing these findings they concluded that bacterial-mucosal cell interactions may be responsible for alterations in intestinal permeability after dexamethasone administration.

9. Intestinal immune system

Intestinal homeokinesis depends on complex interactions between the microbiota, the intestinal epithelium and the host immune system. Innate and acquired immune cells of the intestine have critical roles in barrier function because of they should tolerate the antigens belonging to the food and commensal microbiota as well as they should protect the body against pathogen microorganisms [20,57]. Systemic nonresponsiveness to antigens that are introduced orally is a phenomenon known as "oral tolerance". Oral tolerance is typically characterized by the suppression of the systemic T helper 1 (Thl) response to antigens and elevated levels of IL-I0, TGF-β and antigen-specific sIgA at the mucosal surface. The T helper 2 (Th2) response also promotes the induction of tolerance in the gut. Production of IL-4 and IL- 5 during Th2 response acts synergistically to enhance IgA production. These cytokines also act further to inhibit the Thl response [57]. The cells of the innate immunity discriminate potentially pathogenic microbes from harmless antigens through pattern recognition receptors (PRR). Toll-like receptors (TLRs) are a family of pathogen-recognition receptors of the innate immune system. TLRs are present on a variety of cell types such as intestinal epithelium, monocytes, and dendritic cells. They recognize conserved molecular motifs on microorganisms called pathogen associated molecular patterns (PAMPS). TLRs

are activated by various components of microorganisms, e.g. TLR4 binds lipopolysaccharide (LPS) in gram-negative bacteria. Besides, TLR5 binds to flagellin, TLR2/6 binds to fungal zymosan and TLR7 binds single stranded RNA (ssRNA) from viruses. Activation of the TLRs by either pathogenic ligands or host factors results in downstream activation or inhibition of pathways involved in inflammation. Toll-like receptor activation by commensal bacteria plays an essential role in maintaining colonic homeostasis and controlling tolerance in the gut [57,112,113,114]. However, inappropriate activation of their signaling pathways may lead to deleterious inflammation and tissue injury. TLRs have been implicated in the pathogenesis of many GI disorders [57,114]. Although intestinal immune system is thought to have a critical role in stress-induced alterations of GIS functions, the studies about this situation are limited as this also the case for other functions of GIS such as motility, secretion and permeability [18,115,116]. McKernan et al. [116] investigated for the first time the regulation of TLR expression in the colonic mucosa in two distinct chronic stress models; Wistar-Kyoto (WKY) rats and maternally separated rats where Sprague Dawley rats were used as controls. Significant increases are seen in the mRNA levels of TLR3, 4 and 5 in both the distal and proximal colonic mucosa of MS rats compared with controls. No significant differences were noted for TLR 2, 7, 9 and 10 while TLR 6 could not be detected in any samples in both rat strains. The WKY strain showed increased levels of mRNA expression of TLR3, 4, 5, 7, 8, 9 and 10 both in the distal and proximal colonic mucosa compared to the control animals of Sprague-Dawley strain. No significant differences in expression were found for TLR2 in all samples of both strains. These authors suggested that the up-regulation of TLR 4 and 5 may indicate increases in cytokine production in response to the increases in sensitivity to gut bacteria. In addition, they suggested that the observed differences in TLR expression activity between MS and WKY rats might be related with their different neuroendocrine responses or microbiota. In spleens isolated from mice subjected to chronic 12-hour daily physical restraint for two days, TLR-4 expression significantly increased, T helper 1 (Th1) cytokine IFN-γ and IL-2 levels were found to be decreased, but Th2 cytokine and IL-4 increased. They suggested that stress modulates the immune system through a TLR4-dependent mechanism, because TLR4-deficient mice are resistant to stress-induced lymphocyte reduction and the restraint stress significantly inhibits changes of Th1 and Th2 cytokines in TLR4-deficient mice compared with the wild type mice [117]. Repeated social defeat stress (SDR) has been shown to increase the expression of TLR2 and TLR4 [118] and can activate dendritic cells for enhanced cytokine secretion in response to TLR specific stimuli. Besides, glucocorticoid resistance was determined in CD11+ dendritic cells isolated from spleens of SDR mice, whereas under baseline conditions DCs are highly sensitive to glucocorticoids [119]. Glucocorticoids and catecholamines appear to be able to regulate the expression of certain TLRs [116]. Toll-like receptor agonist-induced cytokine (IL1b, IL6, IL8 and TNF-α) release was markedly enhanced in stimulated whole blood samples from IBS patients compared with healthy controls. Plasma levels of cortisol, IL-6 and IL-8 were also significantly increased in IBS patients [120].

Chronic stress (induced by water avoidance stress for 1 hour/day for 10 consecutive days) induced the infiltration of neutrophils and mononuclear cells, and increased

myeloperoxidase (MPO) activity in ileum and colon mucosa, but these changes were not shown in mast cell deficient rats [42]. Similary, jejunal inflammatory cells such as neutrophils, eosinophils and mononuclear cells and expression of IL-4 (TH2 type cytokine) increased, while interferon–γ (IFN- γ) (TH1 type cytokine) decreased in same chronic stress model of rats. Treatment of stressed rats with an antagonist to CRH eliminated the manifestations of intestinal hypersensitivity [121].

Velin et al [122] evaluated the M cell-containing follicle associated epithelium (specialized in antigen uptake) in acute and chronic stress conditions. Acute stress increased horseradish peroxidase (HRP) flux in villus as well as in follicle-associated epithelium (FAE), and chronic stress increased *E. coli* passage in follicle-associated epithelium whereas there was no significant increase in villus epithelium. In patients with Crohn's disease (CD), transmucosal uptake of non-pathogenic *E. coli* across the FAE increased in ileum, despite unchanged macromolecular permeability, but these changes were not observed in patients with ulcerative colitis [123]. Recently, Keita [124] showed that application of CRF agonist increased HRP and *E. coli* passage, stress-induced increases in uptake across FAE of HRP, and *E. coli* were reduced by CRF antagonist, mast cell stabilizer and atropine. Chronic restraint stress increases eosinophils expressing CRF in the jejunum, which participate to the recruitment of mast cells and epithelial barrier dysfunction [125]. The influence of CRF signaling on intestinal mast cell activity is detailed above. However, the information about their effects on other immune components of intestine (such as intestinal epithelial cells, TLR expression or intraepithelial lymphocytes) is limited. Larauche et al [82] reported that treatment of mice with CRF-1 agonist cortagine exhibits a dose-related interferon-γ (IFNγ) response indicating T cell and/or natural killer (NK) cell activation, which is followed by tight junction deregulation and dose dependent apoptotic loss of different cell populations in ileum.

IECs participate in initiating adaptive immune responses in the gut by transporting luminal antigens to underlying immune cells for presentation by professional antigen presenting cells or can present antigen themselves [57]. intraepithelial lymphocyte (IEL) and paneth cells (specialized IEC located at the base of intestinal crypts in small intestine) do also synthesis the antimicrobial peptides such as lysozymes, alpha defensins, cathelicidins, lipocalins, and C-type lectins such as RegIIIγ [126]. Production of RegIIIg [127] and alpha-defensins [128] as well as that of secretory IgA [129] are induced by commensal bacteria. Nutritional and infection stress affected the secretory activity of Paneth cells in human [130]. In women, acute cold stress induced the release of α-defensin in the jejunum [131]. Evidences suggested that dysfunction of Paneth cells and impaired defensin secretion may contribute to IBD susceptibility [57,105].

Corticosteroids and catecholamines are well recognized and accepted powerful regulators and players of the body in its response to environmental challenges including biological factors of stress. Stress or corticosteroid applications are known to have also profound effects on intestinal wall structure and functioning. Intestinal submucosa is the place where lymphocytes, eosinophils and mast cells reside in men and animals under normal conditions. Jarillo-Luna et al. [115] investigated the effects of chronic restraint stress in mice

submitted to different procedures (adrenalectomy, chemical sympathectomy, and treatment with a glucocorticoid antagonist (RU486), dexamethasone, and epinephrine) on intraepithelial lymphocyte (IEL) numbers. They found that chronic restraint-stress reduced the IEL population in the small intestine and adrenal catecholamines and glucocorticoids are essential in preserving IEL population because adrenalectomy, treatment with RU-486 and chemical sympathectomy decreased the number of γδ, CD4+ and CD8+ T cells in non-stressed groups. They also found that adrenalectomy did not buffered the stress-induced reduction in CD8 lympocytes, but glucocorticoid receptor antagonist RU-486 buffered stress-induced decrease in γδ and CD8+, but not in CD4+ T cells. Besides, low and high doses of dexamethasone (5 and 50 mg/kg BW) significantly reduced the number of γδ and CD8+ T cells, and epinefrin (0.1-0.5 mg/kg) reduced the number of γδ, CD4+ and CD8+ T cells in intact mice. Also many other studies reported that both stress-related endogen rises of glucocorticoids [115,132-134] and exogenous glucocorticoid administrations [135,136] induce decreases in intraepithelial lymphocytes and/or those in ileal Peyer patches. Pretreatment with glucocorticoid receptor antagonist mifepristone significantly reduced apoptosis in both T- and B-cell populations in intraepithelial lymphocytes after the burn injury [133]. Experimental studies also suggested that single or repeated parenteral applications of cortisone cause a decrease in eosinophil concentration in all parts of examined gastrointestinal wall from stomach up to colon [137,138]. Immunosuppressive effects of glucocorticoids are explained mainly by an increase of apoptosis and a decrease of cytokine production. Corticosterone impaired the maturation of DCs and cytokine production and reduced the ability of DCs to prime naive CD8+ T cells *in vivo*; there was no reduction in surface TLR4 expression in CORT-treated DCs [139]. However, McEwen et al. [140] proposed that although glucocorticoids are mainly known with and used widely for their immunsuppressive aspects, adrenal steroids play also different roles as important modulators of the immune system. Pharmacological as well as physiological changes in glucocorticoids result in a decrease in lymphocyte, monocyte and eosinophil numbers and an increase in neutrophil numbers in the blood of men and rodents. These changes are not related with the glucocorticoid-induced leukocyte deaths. The nonspecific stress and glucocorticoid administration cause redistribution of leukocytes from peripheral blood into various tissues and organs, such as bone marrow, spleen and lymph nodes [140-142].

10. Intestinal microbiota

One of the important groups of the environmental biological stressors belongs to the microbiota, and it is associated with every multicellular organism on earth. It is estimated that in humans and many animals reside at least 10^{14} microorganisms, making approximately 1.5 kg biomass, in various parts of the body, most abundant of them residing the distal part of the gut in humans and animals with exception of ruminants [143-146]. These parts of the body lined by the skin or mucous membranes and all are in direct contact with the environment. Although the microorganisms are preferable grouped as pathogenic, while they harm the multicellular organisms, and saprophytic, while they seemingly do not harm their hosts instead they build symbiotic relationships, but possibly this depends only

on the balance or imbalance among numerous groups of microorganisms constituting the microbiota in a definite part of the body and between the microbiota and its host, in general.

The first days, weeks, months or years, the time spent in mothers' womb or in egg-shall of the metazoan life is the only time which they are free of microbes. The delivery into the outside exposes them to an enormous range of microbes from diverse environments. This is the first encounters with life forms with different morphology and functions. The studies have shown that within a short time following delivery, the microrganisms are present on the skin and mucosal surfaces of the body. With time, a dense, complex gastrointestinal microbiota develops [147,148]. Due to the unique properties of microorganisms including their small size, metabolic versatility and genetic plasticity, microorganisms can tolerate and easily adapt to unfavorable and immense variety of continuously changing environmental conditions [149]. However, although a wide variety of microorganisms were exposed to representative individuals from start up throughout the life periods, only a limited numbers of species are able to colonize permanently in available body surfaces of man and animals. The microorganisms display a tissue tropism; e.g., they colonize predominantly only certain body sites. Consequently, each side is inhabited by only certain species of microorganisms. The microorganisms found at a particular body site constitute what is known as the indigenous or normal microbiota of this site, wherein the term 'indigenous' include all of viruses, protozoa, archae, and fungi [150,151]. The GIT is inhabited with 10^{13}–10^{14} microorganisms, approximately 500 to over 1000 different species and more than 7000 strains. Their counts exceed ten times the numbers of somatic cells of their hosts [144,151-154].

The very complex and diverse gastro-intestinal microbiota differs from species to species, in dependence of nutritional habits with some geographic motives. Besides, it varies from one segment to another and varies over time in the same individual, because the environment of the GIT varies considerable along its length and with the lifetime of an individual [144]. Thus, the composition and intensity of the microbiota of a newborn is quite different from those of an adult which are in turn quite different from that of an elderly individual [155,156]. Colonization begins at birth with facultative bacteria and the colonization of anaerobic bacteria which are composed of more than %90 of GIS microbiota develops later. In humans, the microbiota has a stable adult-like signature by 1 year of age [113,154]. Similarly, the composition and intensity of the microbiota varies along the gastrointestinal tract where they are attached to the mucosa or are present in the contents. In stomach and duodenum of humans, microorganism numbers is 10^3CFU/ml and include more lactobacilli, streptococci and yeasts species. In jejunum (10^4CFU/ml) and ileum ($10^{7\text{-}8}$ CFU/ml) *Lactobacillus, Bacteroides*, *Enterobacteriaceae, S*treptococci, *Bifidobacterium and Fusobacteria species* are more existent. The highest numbers of bacteria ($10^{11\text{-}12}$ CFU/ml) displaying enormous diversity are found in colon, predominant species being *Bacteroides, Fusobacterium, Eubacterium,Peptococcus, Peptostreptococcus, Veillonella, Bifidobacterium, Escherichia, Clostridium, Lactobacillus* and others [147,154]. Although only 40-45% part of GI microbiota could be growth with classical microbial culture tecniques, recently developed moleculer techniques allow the definition of non-culturable members of microbiota. The vast majority of

microorganisms belong to the phyla of *Firmicutes, Bacteroidetes, Actinobacteria,* and *Proteobacteria* while *Fusobacteria, Verrucomicrobia and TM7* are represent in relatively lower numbers [144,145,157,158]. There are also some fungi and *Archaea* present in the gastrointestinal tract [144,159,160], but they comprise less than 1% of total inhabitants. The majority of the intestinal bacteria are composed of gram-negative anaerobes [143].

With the acquisition and establishment in the intestinal lumina with various microbial populations bidirectional interactions between microbiota and host organism starts [161,162]. There are symbiotic relationships between the host and its GI microbiota in steady-state conditions. The gastrointestinal tract serves a natural habitat for a dynamic microbial community of different origins while the microbiota is very essential for both gastrointestinal integrity [163] and general health of host organisms [113]. Comparison between the conventional and germ-free animals has allowed obtaining information about the effects of microbiota on morphologic, functional and metabolic characteristics of host organisms [23,113,154,160]. Germ free animals have enlarged cecum [164], they have thinner gut wall, smaller total surface area and more cubic epithelium than the columnar, increased enterochromaffin cell area and smaller Peyer's patches, and intestinal epithelium has slower turnover rate compared with the conventional animals [160,165]. The materiality of the gut microbiota also for many gut functions such as motility, immune and barrier functions are reviewed by several authors [23,113].

Complex association between the host and its microbiota collectively extends the processing indigestible parts of food to the benefits of the host organisms *via* metabolic capacities which are not coded in host genomes like mammalian and avian species [166-168]. They digest the unusable parts of the diets; metabolites reach to the intestinal lumina within various secretions and desquamated cells of their hosts for growth and proliferation. At the same time, they also supply the host organisms with a considerable amount of nutrients, which makes this ecosystem an invaluable, essential metabolic organ, which contributes significantly to the homeokinesis of the host organisms [23,113,152,169]. They produce substances including vitamins, volatile or short-chain fatty acids (SCFAs) and polyamines which are absorbed throughout the intestinal wall and used directly by intestinal epithelial cells or other cells of the body [170,171]. SCFA profoundly influence gut barrier functions, host immunity, epithelial proliferation and bacterial pathogenesis [172]. Zheng et al. [171] showed extensive gut microbiota modulation of host systemic metabolism involving short-chain fatty acids, tryptophan and tyrosine metabolism, and possibly a compensatory mechanism of indole-melatonin production. All these metabolites have also many regulatory functions in host organisms. Tryptophan and tyrosine are precursors of neurotransmitters acting directly at the central nervous system level [113]. Thus, the gut microbiota enhances the host's metabolic capacity for processing nutrients and modulates the activities of multiple pathways in a variety of organ systems, including the brain.

Microbiota acts as a luminal barrier against incoming pathogens; this phenomenon has been described as colonization resistance [23,154]. Beneficial and pathogen microorganism compete with each other for the attachment sites and for nutrients, so microbiota and their

products can prevent pathogen colonization directly. On the other hand, microbiota acts on barrier functions also indirectly by stimulating mucosal immune system [172]. The abundant antigenic stimulus supplied by microbiota and their products are essential for the stimulation of immune system cells locally and systemically [173-175]. In germ-free animals, besides the poor developed Peyer's patches, altered compositions of CD4+ T cells and IgA-producing B cells in the lamina propria [165], TH 17 cells, which is a subset of the T cells and contribute to resistance against colonization by pathogens were virtually absent in germ free animals [176,177]. Chow and Mazmanian [177] denoted that although Th17 cells are essential for immunity, they have also been implicated in the pathogenesis of many autoimmune diseases, including IBD, arthritis, psoriasis, and experimental autoimmune encephalomyelitis (EAE).

The gut microbiota has recently been identified as the main source of highest biological variability confined in an individual [178]. Because the metazoan life-forms and the inhabitation of their gastrointestinal tract with microorganisms evolved together, there are close links between any host or its epi-genome and its very complex diverse gastrointestinal microbiota with their multitude genomes. It is estimated that this microbial community has 70–140 times more total genes than the human host. These functional inter-relationships between host and microbiota or two different genomes determine the health or disease state of the metazoan hosts and the balance among different microorganism populations [113,172,179]. It is well accepted that the intestinal microbiota involves in metabolome of the host, thus promotes actively fat accumulation and weight gain and sustains indirectly a low-grade systemic inflammation especially when imbalanced, and consequently, enhances the risk for complex, multifactorial diseases such as insulin resistance, diabetes, obesity and cardiovascular diseases. The search of global obesity epidemic led to the growing evidences about the possible roles of intestinal microbiota in these respects [180,181,182]. Claus et al. [170] has noted that the colonization of the gut microbiota was associated with a rapid increase in body weights of animals up to 4% within 5 days of colonization. Findings of various studies also revealed that gut microbiota profile of obese and diabetic individuals differ by phylum level both in its quantity and quality from that of lean and nonobese individuals [182,183]. Recent evidences exhibit that the composition of the gut microbiome may influence body weight of the host by various mechanisms including enhancing the ability of intestines to extract energy from food [184], regulating fat storage in tissues [185] and affecting satiety by modulating the levels of local hormones that regulate satiety and by direct effects in central nervous system [186].

There is a growing appreciation of the critical roles played by the commensal microbiota, both in general well-being of the hosts and in the specific functioning of the brain–gut axis [113]. Bidirectional communications of brain–gut–enteric microbiota axis simply actualized by through signals from the brain can influence the motor, sensory, and secretory modalities of the gastrointestinal tract and conversely, visceral messages from the gastrointestinal tract can influence brain functions [187]. Sudo and colleagues [25] showed that gut microbiota effect the stress responses of host organisms. They compared the response of the HPA axis to stress in GF, specific pathogen free (SPF) and gnotobiotic mice that were mono-associated

with a single bacterium. Restraint stress caused an exaggerated ACTH and corticosterone elevation in GF rather than SPF mice. This hyper-response of the HPA axis was reversed by mono-association with *Bifidobacterium infantis*. They also showed in following experiments that the levels of brain-derived nerve factor (BDNF), norepinephrine and 5- 5-hydroxytryptamine (5-HT) in the cortex and hippocampus were significantly lower in GF mice than in SPF mice [188]. Improvements of stress-related symptoms by probiotic administration also support the possible regulatory effects of microbiota on HPA axis and brain functions [44,189]. Gareau et al [44] reported that probiotic treatments improved colonic dysfunction and corrected the higher corticosterone levels in stressed rats induced by maternal seperation. L. rhamnosus (JB-1) reduced stress-induced corticosterone and anxiety- and depression-related behavior in mice. In different region of brain, this therapy also altered GABA receptor expression implicated in the pathogenesis of anxiety and depression, which are highly comorbid with functional bowel disorders. Due to the neurochemical and behavioral effects were not found in vagotomized mice, they suggested that vagus could be a major modulatory constitutive communication pathway between the bacteria exposed to the gut and the brain [189]. The roles of probiotics and gut microbiota in modulation of visceral and even somatic pain perception and their possible roles in alterations of mood and behavior were reviewed by Forsythe et al. [190] and Grenham et al. [113].

As mentioned above, intestinal dysbiosis can adversely influence gut physiology both by direct effects to the surrounding gut wall and by leading to inappropriate brain–gut axis signaling and associated consequences for CNS functions and disease states. Stress at the level of the CNS can also impact on gut functions and lead to perturbations of the microbiota [113].

11. Stress and intestinal microbiota

Intestinal microbiota have once been seen as potential treat for the host organisms, but recently accepted as an integral part of metazoan life and even as an organ with a huge variety of building blocks which mainly cooperate with each other and with the host for maintaining the health and survival [113,144]. However, various stress factors such as heat, cold, nutritional alterations, overcrowding, physical restraints and transporting or fouled or contaminated foods can destroy the microbial balance in the gastrointestinal system [66,67,191-195] and alter their relationships with each other and with their hosts. Stressful stimuli can affect gastrointestinal microbiota directly, for example *via* limited availability of food ingredients or direct actions of stress mediators such as adrenaline or noradrenaline on microbiota [27,196], and indirectly *via* altering the intestinal environment of bacteria such as intestinal secretion, motility, permeability and immune functions as reviewed above.

The effects of several stressors and stress mediators on intestinal microbiota were given in Table 1. Bailey et al [197] induced social disruption stress (SDR) in mice to determine whether the microbiome contributes to stressor-induced immune enhancements. They analyzed bacterial populations in the cecum with using bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) and found that microbiota significantly changed immediately

after stressor exposure as summarized in Table 1, and stress also increased circulating levels of IL-6 and MCP-1, which were significantly correlated with stressor-induced changes to three bacterial genera (i.e., *Coprococcus*, *Pseudobutyrivibrio*, and *Dorea spp*). However in antibiotic treated mice, exposure to SDR failed to increase IL-6 and MCP-1. Bailey et al [191] reported that restraint stress also significantly change the composition of the intestinal microbiota in mice and disruption of the microbiota increased susceptibility to murine enteric pathogen *Citrobacter rodentium* which can be associated with reduced competitive exclusion of commensal bacteria and increased tumor necrosis factor alpha (TNF-α) gene expression in colonic tissue. Reduction in bifidobacteria and lactobacilli numbers in fecal samples of infant monkeys whose mother had been exposed to stress in either early or late pregnancy period showed that maternal pregnancy conditions affect infant health and can enhance susceptibility to infection [68]. Similarly, early life stress induced by maternal separation altered fecal microbiota with concomitant increases in corticosterone and also visceral hypersensitivity and systemic immune response to *in vitro* lipopolysaccharide challenges in rats [198]. Knowles et al [199] reported that non-extreme 'every day' stress events such as exam stress can affect the integrity of the indigenous gastrointestinal microflora of humans but these changes are not supported by cortisol responses. Maternal separation of rhesus macaques also caused to decrease of lactobacilli at 3th days post-separation but significant differences in the cortisol responses did not predict the magnitude of the reduction of lactobacilli numbers. These authors suggested that more than one neuro-hormone can modulate microbiota changes.

Because of their immune-suppressive or stress-mediating effects, some studies have been focused on the effects of glucocorticoids on intestinal microbiota. These studies showed that exogenous glucocorticoid applications to the host organisms are also able to cause changes in gastrointestinal microbiota. We [200,Ünsal et al., unpublished study] and others [29,30,201] demonstrated that exogenous glucocorticoid administrations can also affect gut microbiota by enhancing total aerobe and gram negative enteric bacteria and their translocation to extraintestinal tissues [201]. We compared the effects of different doses of dexamethasone on ileal microbiota and found, in contrast to well-known stress effects, that 5mg/kg dexamethasone injection also increased the numbers of total anaerobe and lactobacilli in ileal content of rats [200]. However, their number did not change in lower doses of dexamethasone. Also in our unpublished study acute cold swimming stress decreased the numbers of lactobacilli, while dexamethasone in dose of 5 mg/kg increased total aerobe, gram-negative enteric bacteria and lactobacilli. Thus, the evidences available suggest that several stressors reduce the number of lactobacilli, while on the contrary, they increases growth, epithelial adherence and mucosal uptake of Gram-negative pathogens. Lactobacilli may possible be defined as stress indicator bacteria of the gut microbiota which is sensible to the effects of various stressors, in general. Although it is known that exogenous glucocorticoids increase the counts of gram negative enteric bacteria [29,30,201], no other information about their effects on the lactobacilli in the gut could be found.

The roles of stress and stress-related hormones in the pathogenesis of infectious diseases are beyond any argument. Microbial endocrinology is a new research area which appeared

from the demand how stress influence the bacterial infections, how neuro-endocrine-immune secretions of host organisms influence their harboring microbiota and how infectious microbes can actively use the neurohormonal products of the stress to their own advantages [202,203]. Recently, several *in vitro* studies have focused on direct effects of stress hormones on bacterial growth and their virulence in an effort to explore and understand the interactions of so-called stress hormones and infections. These studies gathered the evidence that catecholamines stimulate the growth of a wide variety of gram-negative bacterial species, including those of medical importance [27,196,204-209]. Furthermore, catecholamines were also found to be able to induce *E.coli* to produce a heat-stable autoinducer of growth [19,27,204,210] as well as for adhesion required K99 pilus and shigella-like toxins I and II, which may have important roles in its pathogenic activity [210].

The effects of norepinephrine and its receptor antagonists on mucosal bacterial adherence were also determined in sheets obtained from different parts of the gut, mounted in Ussing chamber. Norepinephrine increased the adherence of enterohemorrhagic *Escherichia coli* O157:H7 (EHEC) to colonic epithelium through interactions with α-2 adrenergic receptors [211-213] and it increased the internalization of enterohemorrhagic E. coli O157:H7 (EHEC) and S. enterica serovar Choleraesuis in jejunal mucosa containing Peyer's patch follicles [214,215]. Parallel studies related to the direct actions of other stress mediators such as cortisol or CRF on bacterial growth could not be found. However, Kakuno et al. [216] suggested that hydrocortisone enhance intracellular colonizations of E. coli and Schreiber and Brown [217] reported that ACTH increased EHEC adherence to the porcine colonic mucosa.

Many factors participate in the pathology of inflammatory bowel disease (IBD) such as genetic and immune status of host organism, the gut microbiota, and environmental triggers [218]. Some scientific events support that the enteric microbiota is involved in abnormal inflammatory responses observed in diverse animal models for inflammatory bowel disease [219,220]. These events reviewed by the authors [219,220] are arranged as the reduction or absence of intestinal inflammation in animal models of colitis in antibiotic-treated or germ-free animals; colitis formation with some bacterial inoculations to germ free rats or not to formation with other bacterial species; and beneficial effects of probiotics and prebiotics in IBD patients or animal models of IBD. However, although microbiota has been thought to play an active role in etiopathogenesis of inflammatory bowel disease, it is not clear whether they are cause or outcome. In other words, whether alterations in intestinal microenvironment of microbiota such as mucin secretion, immune modifications and epithelial dysfunctions cause to changes in commensal microbiota or microbial alterations cause to inflammatory responses of intestinal mucosa in stressful conditions is not clear yet [221].

Although the reports in microbiome composition of IBD-patients or models show differences, changes in microbiota composition are characterized more likely by decreases in lactobacilli and bifidobacteria, and increases or unchange in aerobe and facultative anaerobic bacteria [222]. Molecular analysis of the microbiota of IBD patients have shown that temporal stability and diversity of the gut microbiota composition in IBD patients

decreased compared to non-IBD controls [223], and commensal bacteria, particularly members of the phyla *Firmicutes* and *Bacteroidetes* decreased and *Proteobacteria* and *Actinobacteria* increased [224]. These changes look alike to those that seen following effects of stress on intestinal microbiota.

Stress and/or Stress Mediators	Microbiota	Method	References
Social disruption stress (SDR) in mice (total of 6, two-hour cycles of SDR)	Decrease in *Bacteroides* spp., tended to decrease in *Lactobacillus* spp., tended to increase in *Clostridium* spp. and changes in *Coprococcus*, *Pseudobutyrivibrio*, and *Dorea spp. in cecal content*	(bTEFAP)	197
Restraint stress for 7 days in mice (between 18.00- 08.00 h)	Overgrowth of facultatively anaerobic microbiota, reduction in family *Porphyromonadaceae,and genus Tanneralla,*reducing microbial richness and diversity in the ceca	both culture technique and bTEFAP	191
Prenatal stress induced by acoustical startle paradigm for 6 weeks in early or late pregnancy in Rhesus Monkey	prenatal stress reduced the overall numbers of bifidobacteria and lactobacilli in fecal cultures of infants	culture technique	68
Maternal separation in rhesus macaques *(Macaca mulatta)*	Decrease of lactobacilli in fecal samples at 3th days postseparation, not correlated with cortisol responses	culture technique	26
Academic stress in undergraduate students	Reduction in fecal lactic acid bacteria, unsignificantly cortisol enhancement	culture technique	199
Food, water and bedding deprivation in mice	Decrease in lactobacilli in stomach, increase of coliforms in jejunum, ileum and cecum, reduction in fusiform-shaped bacteria associated with mucosal epithelium of cecum and colon	culture technique	192
Dexemethasone (0.8 mg/kg) Dexamethosone (0.8 mg/kg)+ starvation for 48 h in Fischer rat	impairs secretory IgA, promotes bacterial adherence to the mucosa, increase of intestinal permeability		29,30,225

Stress and/or Stress Mediators	Microbiota	Method	References
Dexamethasone 5 mg/kg in rats	Increase of total aerobe bacteria and lactobacilli in ileum	culture technique	200, Ünsal et al., unpublished study
Methylprednisolone (3 mg/kg) in rats subjected to temporary liver inflow occlusion	Increase of intestinal *Klebsiella spp* and *Proteus spp* and of translocation to multiple organs.	culture technique	201
Pregnant rats were treated with either cortisone acetate or normal saline on days 18-21 of gestation	Total bacteria and gram-negatives found in association with the mucosa were significantly lower in pups prenatally treated with steroids.	culture technique	226
Norepinephrine action on porcine or murine cecum/ colon/jejunum explants	Increases cecal-colonic adherence of E. coli O157:H7; changes Salmonella and E. coli uptake into Peyer's patches		211-215
ACTH action on explants of porcine distal colonic mucosa	Increases adherence of E. coli O157:H7 to colonic mucosa		217

Table 1. The effects of some different type stressors and/or stress mediators on intestinal microbiota

12. Nutritional stress

Nutrition and nutrients of metazoans play very important multifunctional key roles both for metazoan host and for its gastrointestinal microbiota. They are essential not only in colonization, growth and survival of the microbiota in intestinal system but also in maintaining the balance among different species and their localization within its lumina [67,192,227,228]. Nutrition plays an important role as stressor for host organisms and their gastrointestinal tract *via* three mechanisms. Firstly, foods supply nutrients for intestinal microbiota and also serve as carriers of various microorganisms into gastrointestinal tract which may under circumstances lead to imbalances among different species; secondly, nutritional deficiencies or imbalances are perceived from the organisms as stressors setting them to the state of well-known alarm reaction of stress; and thirdly, certain food ingredients in their undigested forms as foreign substances accepted as non-self from organisms and stimulate a stress situation. In cases of carrying the microorganisms into gastrointestinal tract or acting as antigens, the foreign treats come into direct contact with the wall of gastrointestinal tract. A great part of microorganisms stay in gastrointestinal canal for a short time period, while others colonize its lumina permanently and their genera can be life-long present there, mostly in a symbiotic relationship with the host organisms.

Dietary ingredients, which are not digestible for the host organisms or which are digestible but escape from the intestinal digestion can be utilized as substrate for growth from microbiota colonized in the following sections of the gut [229,230]. All microorganisms residing in gastrointestinal tract needs the nutrients that necessary for their growth and proliferation are continuously supplied *via* foods of their hosts and from host organisms in secretions of digestive organs including saliva, gastric juice, pancreatic, hepatic (bile) and intestinal wall secretions and desquamated epithelial structures. The composition and amount of the food, even the compositions of these secrets may undergo substantial changes. This is why the hosts' balanced nutrition and physiological state exerts a strong controlling effect on its microbiota [160,181,192,231-237]. Thus, any nutritional deficiency or imbalance of their hosts serve the most important challenge for the growth, survival and balance of different microbial species within the intestinal lumina.

Malnutritions in different nature or nutritional imbalances had always been and are still very widespread health problems of men and animals worldwide and frequently seen in infants and elderly, and those subjects having malignancies, getting chemotherapy and/or radiotherapy or infected with human immunodeficiency virus. Very common forms of malnutrition are protein, calorie and protein calorie deficiencies which almost always are complicated with deficiencies of other nutrients, especially that of minerals and vitamins [238]. As mentioned above, deficiencies of calories, proteins, minerals or vitamins in hosts' food can influence the indigenous microbiota both directly *via* the restricted availability of the metabolites and their indigestible parts for hosts and indirectly by inducing a stress response within the host organism and affecting the compositions of gastrointestinal morphology and secretions as well as by impairing the general and local immune responses and neuro-endocrine-immune network leading to an imbalance between the host organism and its microbiota, in general. These changed milieus offer possibilities to certain new species of the microbiota for adaptation in gastrointestinal tract or cause their dispersion from their localization areas to others. In protein calorie malnutrition, colonic type microbiota known to spread to and proliferate in the upper small intestine which may cause a variety of metabolic disturbances including steatorrhea, vitamin deficiencies, nutrient malabsorbtions, and consequently water leakage into lumina and diarrhea [160,239-241]. Generally, pathogenic microorganisms including Enterobacteriaceae, Pseudomonas, Klebsiella and Candida were increased [240,241]. In such clinical cases of complicated protein and/or calorie malnutrition, pathogenic microorganisms often cause endotoxemia and infection in addition to intestinal disruptions including diarrhea and metabolic diseases [242,243]. Thus, they all affect the composition of the intestinal digesta and its passage time, which in turn may influence the composition of the indigenous intestinal microbiota and their relationships with the intestinal wall [244-246].

Generally, experimental studies use deficiencies or excesses of a definite dietary component or several components, and animals are held under more hygienic, defined conditions throughout the rearing and experimental periods than their counterparts, whereas clinical cases develop spontaneously in man and animals. Thus, they give the possibility to detect the possible effects of a certain dietary component on the behavior of the gastrointestinal

microbiota. However, such experimental evidences from studies using deficiencies or imbalances of definite nutrients in this respect are very sparse. So, an almost protein-free diet disrupted the cecal microbiota, and made mice more susceptible to bacterial translocation than those mice nourished adequately [227,236,247]. The counts of cecal total aerobic bacteria and Gram-negative enteric bacilli were found to be increased time-dependently when CD-1 mice were fed an almost N-free diet for 21 days [236]. The results of another study on adult female Crl:CD-1[CR]BR mice also showed that both the feeding an almost protein-free and 20% fat containing diet for 14 days and starvation for 3 days resulted in an increase in counts of Gram-negative enteric bacilli and a decrease in counts of lactobacilli and strict anaerobes [227]. In certain studies the effects of dietary manipulations combined and/or compared with those of endotoxemia were investigated. So, Deitch et al. [227] studied the effects of starvation and malnutrition alone or in combination with endotoxemia and found that the spread of bacteria from the gut could not be controlled nor translocated bacteria be cleared in protein malnourished mice as effectively as in the controls. However, no association between protein malnutrition and bacterial translocation could be found by Katayama et al. [247]. Instead, these authors determined that the total numbers of Gram-negative enteric bacilli adherent to the mucosa of ileum and cecum were less in protein malnourished rats than in their adequately nourished controls. Further, there was also a significant negative correlation between the duration of protein malnutrition and bacterial adherence to the intestinal mucosa. Only, *E. coli* binding to insoluble ileal mucus was increased in the rats receiving endotoxin. Tannock and Savage [192] reported that the deprivation of food and water and bedding for 48 hours increased the counts of coliform bacteria while they decreased the counts of cecal lactobacilli of CD-1 and C57BL mice strainsIn a preliminary study, we found that feeding an almost protein-free diet to male Wistar rats for 35 days affected especially the total aerobe microorganisms and lactobacilli while total anaerobe and *Enterobacteriaceae* remained relatively unaffected. Compared to controls with balanced nutrition, both dietary qualitative and quantitative protein malnutrition decreased mean lactobacilli counts. Also, the quality and quantity of the dietary protein made a difference in their effects on intestinal microbiota; compared to gelatin-fed animals, lower aerobe and higher lactobacilli counts could be observed in cecal samples of rats given an almost protein-free diet. Furthermore, it could also be shown that the actual immune status (e.g. suppression of neutrophils) of the host can modify the effects of the qualitative and quantitative protein malnutrition on the intestinal microbiota [193].

Human cultural characteristics may also have implications on the compositions of the gastrointestinal microbiota. Living on a high carbohydrate diet caused also the presence of fewer bacteriodes and more enterococci in feces of the people than those living on a Western diet with more fat and animal proteins [231].

After all, the mechanisms *via* which different type of diets or dietary manipulations affect the host and its guest organ 'gut microbiota' are still not exactly cleared. The effects of dietary qualitative and/or quantitative protein malnutrition on regulatory systems in men and animals are well characterized and are the topic of numerous texts. Earlier studies with definite protein malnutrition were summarized by Aschkenasy [248] and suggest that protein

malnutrition of different types or amino acid imbalances generally result in increases of adrenalin and glucocorticoid concentrations in man and animals. Torún and Viteri [249] also noted that in protein and/or protein-calorie malnutrition, the concentrations of adrenalin and glucocorticoids are either increased or showed no important change while many other hormones with exceptions of aldosterone and growth hormone decreased significantly. However, such studies have very important drawbacks as they look only one aspect of the regulators such as their concentrations in blood. Generally, the concomitant expression status of the enzymes which interconvert active and inactive forms of a given hormone and their receptors in target tissues or cells are ignored or not evaluated concurrently. A study conducted by Marroqui et al. [250] on mice demonstrated that during a protein malnutrition plasma glucagon concentration increased, but the ability of exogenous glucagon to raise plasma glucose levels were lower in mice given a low protein diet.

13. Conclusion

Since their introduction in the terminology of scientific medicine, the terms environment, stress and microorganisms were probably never been so important in mind of mankind for the development, health and welfare of men and animals. Although the roles of stress and stress-dependent disruptions of the intestinal microbiota both in developments and in promotion of the symptoms of various diseases and disorders including those of gastrointestinal system in men and animals are well accepted, there is still a lack of information about many aspects including which strains play really a role in the etiopathogenesis of a given condition, and which mechanisms are effective in such cases [24,113]. The stress-dependent dysfunctions of HPA axis can manifest itself in different ways. In many cases it may be related to high or low cortisol concentrations in blood whereas in other situations no detectable change of cortisol occurs. Further, the response given by hypothalamus and pituitary gland to the cortisol can be increased or decreased depending on the receptor numbers [10,11,14,15]. While in classic stress response sympathetic nervous system and glucocorticoids thought to be responsible for stress-dependent processes, studies within last two decades suggest that many disordered situations of the gastrointestinal system mediated by CRF. Both CRF-related peptides and CRF receptors are also expressed within the intestine, where they may activate directly the enteric, endocrine, and immune cells and may be involved in intestinal manifestations such as mucosal permeability, secretion, mast cell function, motility, mucin formation, immune function and many disorders of the gut. In other words, the peripheral changes produced by stress can be mimicked by CRF-injection and prevented by CRF-receptor antagonists [18,20,31,32,82]. Therefore, CRF have been suggested as a new target in treating stress induced functional gastrointestinal disorders.

Recently, intestinal microbiota imbalances gain growing interests both as the subject and cause of stress and stress-related diseases which are connected with not only the gastrointestinal system but also all other systems or organs of the metazoan hosts including the adipose tissue [24,113, 179, 184,185]. Basing on experimental and clinical studies, certain

phyla and species are currently related with a given specific condition [180,181]. However, all these studies are looking mainly on one side of the iceberg, like for example changes in a specific member of the microbiota in respect to stress stimuli and a specific neurotransmitter in brain, as it also the case in search of the mediating regulatory pathways. Understanding the roles of stress and stress-related microbiotal changes and their mechanisms in the role of various physiopathological conditions would be helpful in improvements of the relationships of the metazoan hosts with their microenvironments including its microbiota and thus, would contribute greatly to the health state of men and animals.

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