Probiotic Approach for Mitigation of Stress Adverse Effects

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“Stress is any threat to the homeostasis of an organism”

Selye H., Nature, 1936
<table>
<thead>
<tr>
<th>Condition</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thermoregulation</td>
<td>Kent et al., 1992</td>
</tr>
<tr>
<td>Neuroendocrine control</td>
<td>Kent et al. 1992</td>
</tr>
<tr>
<td>Sleep</td>
<td>Kent et al. 1992</td>
</tr>
<tr>
<td>Social behavior</td>
<td>Bercik, et al., 2011; Li, 2009</td>
</tr>
<tr>
<td>Cognition</td>
<td>Kent et al. 1992</td>
</tr>
<tr>
<td>Gut neuro-motor function</td>
<td>Verdu et al., 2009</td>
</tr>
<tr>
<td>Muscular activity</td>
<td>Verdu et al, 2006</td>
</tr>
<tr>
<td>Memory</td>
<td>Li, 2009</td>
</tr>
<tr>
<td>Anxiety</td>
<td>Bercik, et al., 2010, 2011a</td>
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</table>
Stress, via signals from the central nervous system, leads to the altered release of and response to neuroendocrine factors (acetylcholine, neurotensin) in the intestinal mucosa. Such factors may act on the epithelium, inducing barrier dysfunction and the uptake of proinflammatory material from the gut lumen. The resultant inflammation causes disability and increases stress, which further amplifies the defect.

Soderholm, Perdue, 2001
Intestinal barrier function - the ability to control uptake across the mucosa and to protect the gut from harmful substances present in the lumen. The intercellular junctions of intestinal epithelial cells are sealed by different protein complexes, including tight junctions (TJs), adherens junctions (AJs), and desmosomes. The TJs, multiple protein complexes, locate at the apical ends of the lateral membranes of intestinal epithelial cells and they act as a primary barrier to the diffusion of solutes through the intercellular space.
Tight Junction Integral Proteins

Claudin
- a family of ≥24 members
- the main structural components of intramembrane strands
- determine ion selectivity of paracellular pathway

Occludin
- regulates paracellular diffusion of small hydrophilic molecules
- has been linked to the formation of the intramembrane diffusion barrier
- regulates the transepithelial migration of neutrophils

Junctional adhesion molecule (JAMs)
- JAM is involved in formation and assembly of TJs in intestinal epithelial cells
The intestinal TJ barrier is dynamically regulated by physiological and pathophysiological factors:

- microorganisms (probiotics and pathogens)
- cytokines
- food factors
Downregulation of Intestinal Tight Junction by Pathogens

- *Vibrio cholerae*
- Enteropathogenic *E. coli*
- *Clostridium perfringens*
Upregulation of Tight Junction Proteins by Probiotics

- *Streptococcus thermophilus*
- *Lactobacillus acidophilus*
- *Escherichia coli Nissle 1917*
- *Saccharomyces boulardii*
Effect of Probiotic Bacteria on Stress-Inhibited Intestine

Healthy intestine (1):
- physical barrier to hinder invasion of pathogens
- immune system development
- activation of immune and inflammatory response

Stress effects (2):
- depression of mucosal barrier function
- immune system depression
- reduction of the bacteria of the normal microflora

Effect of probiotic (3):
- restoration of normal microflora and mucosal barrier function
- activation of immune system
Types of Stressors

- Physical
- Psychological
- Chemical
Experimental Design

Days 0 1 2 3

PBS (groups 1 and 3)

*B. subtilis* probiotic (groups 2 and 4)

Heat stress 45°C (groups 3 and 4)
No stress 25°C (groups 1 and 2)
Protective Effect of *Bacillus subtilis* Probiotic on Gut Epithelial Cells

**Villi height, µm**

<table>
<thead>
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<th>Control</th>
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<tr>
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<td>800</td>
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<tr>
<td>PBS</td>
<td>400</td>
<td>600</td>
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**Total mucosal thickness, µm**

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<th>Control</th>
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<td>800</td>
</tr>
<tr>
<td>PBS</td>
<td>600</td>
<td>600</td>
</tr>
</tbody>
</table>

*P < 0.05*
Histological images of intestinal mucosa
Prevention of Bacterial Translocation by Probiotic

Bacteria count, CFU/g

Probiotic
PBS

Stress
Control
Protective Effect of Probiotic

- LPS in serum, ng/ml
  - Stress: Probiotic vs. PBS
  - Control: Probiotic vs. PBS

- IL-10 in serum, pg/ml
  - Stress: Probiotic vs. PBS
  - Control: Probiotic vs. PBS

P < 0.05
Beneficial Effect of *Bacillus* Probiotic on Intestinal Tight Junction

Claudin, Arbitrary Units

- **Probiotic**
- **PBS**

**Control**
- Claudin
- Actin

**Stress**
- Claudin
- Actin

* p<0.05
Beneficial Effect of *Bacillus* Probiotic on Intestinal Tight Junction

![Graph showing the effect of Probiotic and PBS on ZO-1 expression.](image)

**Probiotic**
- Control: 0.8
- Stress: 1.2

**PBS**
- Control: 1.6
- Stress: 1.2

**ZO-1, Arbitrary Units**

**Actin**

---

*ZO-1* expression levels were assessed under control and stress conditions with and without the administration of *Bacillus* Probiotic.
Mechanism of Action

Bacillus subtilis

- Normalized intestinal microbiota composition
- Metabolic effects
  - Antibiotics, biosurfactants production
  - Quorum-sensing peptides production
- Protection of intestinal cells against tissue damage and loss of barrier function
- Control of stress-induced adverse effects in the gut
- Colonisation resistance

- Immunomodulation
  - Balanced immune response
  - Antiallergic effects
- Strengthened innate immunity
Conclusion

- *Bacillus subtilis* probiotic prevents heat stress-related complications:
  - changes in morphology of intestinal cells
  - translocation of bacteria into lymph nodes and liver
  - elevation of LPS level in serum
  - changes of serum cytokines composition
  - changes of TJ proteins composition

- Upregulation of TJ proteins with probiotic in rats exposed to heat stress is one of the mechanisms of animal protection against stress-related adverse effects.
Probiotics:

“Live microorganisms which when administered in adequate amounts confer a health benefit on the host”

Joint FAO/WHO Expert Consultation, October 2001

Probiotic Microorganisms

- Bifidobacterium breve
- B. bifidum
- B. adolescentis
- B. infantis
- B. lactis
- B. longum
- B. thermophilum
- Lactobacillus acidophilus
- L. delbrueckii subs. bulgaricus
- L. casei
- L. johnsonii
- L. reuteri
- L. crispatus
- L. fermentum
- L. Gasseri
- L. brevis
- L. plantarum
- L. ramnosus
- L. salivarius
- Lactococcus lactis
- Enterococcus faecium
- Streptococcus salivarius
- Pediococcus acidilactici
- Bacillus cereus
- B. clausii
- B. coagulans
- B. subtilis
- B. licheniformis
- Escherishia coli
- Propionibacterium shermanii
- Saccharomyces cerevisiae
- S. boulardii
Modulation of tight junctions

Upregulation of tight junction proteins (*occludin, claudin, and junctional adhesion protein*) might help to limit the damage that is caused to epithelia by inflammatory processes or pathogens. The probiotic-coated surface retains an intact junction.
Beneficial Effect of Probiotic on Intestinal Tight Junction

Effect of probiotic bacteria *Escherichia coli* Nissle 1917 on changes in ZO-2 mRNA of T84 epithelial cell after infection with enteropathogenic *E. coli* (EPEC)

Zyrek, 2006
Beneficial Effect of Probiotic on Intestinal Tight Junction

ST/LA - *Streptococcus thermophilus* and *Lactobacillus acidophilus*

EIEC - enteroinvasive *Escherichia coli*

Resta-Lenert, 2003
Occludin distribution after infection with enteropathogenic *E. coli* (EPEC)

Occludin Actin                  Merge Occludin Actin                  Merge

<table>
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<tr>
<th>Control</th>
<th>EPEC</th>
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</thead>
<tbody>
<tr>
<td>Occludin</td>
<td>Actin</td>
</tr>
<tr>
<td>Merge</td>
<td>Merge</td>
</tr>
</tbody>
</table>

Ileal (a) and colonic (b) epithelium

Shifflett, 2005
Pathways of epithelial permeability. Transcellular permeability is associated with solute or water movement through intestinal epithelial cells. Paracellular permeability is associated with movement in the intercellular space between epithelial cells and is regulated by TJs localized at the junction of the apical-lateral membranes.

Groschwitz and Hogan, 2009
Overview of intestinal epithelial junctional complexes. The intestinal epithelium consists of a single layer of polarized epithelial cells. Adjacent cells are connected by 3 main junctional complexes: desmosomes, AJs, and TJs. Desmosomes are localized dense plaques that are connected to keratin filaments. AJs and TJs both consist of transcellular proteins connected intracellularly through adaptor proteins to the actin cytoskeleton. The collection of proteins in the junctional complexes forms cytoplasmic plaques.

Groschwitz and Hogan, 2009
TJs are localized to the apical-lateral membrane junction. They consist of integral transmembrane proteins (occludin, claudins, and JAMs) that interact in the paracellular space with proteins on adjacent cells. Interactions can be homophilic (eg, claudin-1/claudin-1) or heterophilic (eg, claudin-1/claudin-3). The intracellular domains of transmembrane proteins interact with PDZ domain–containing adaptor proteins that mechanically link the TJ complex to the actin cytoskeleton. TJ proteins are regulated by means of phosphorylation by kinases, phosphatases, and other signaling molecules.
http://www.dbriers.com/tutorials/2012/12/junctions-between-cells-simplified/
Schematic diagram of interactions of ZO-1 (zonula occludens-1) with transmembrane, cytosolic and cytoskeletal proteins. JAM, junctional adhesion molecule; PDZ, Post synaptic density 95, Disc large and ZO-1 domain; SH3, Src homology domain; GUK, guanylate kinase domain.

Kosinska, 2013
Cai, 2010
Acknowledgement

- Dr. Vitaly Vodyanoy
- Dr. Benson Akingbemi
- Mrs. Ludmila Globa
- Mr. Oleg Pustovyy
Santos, 2008

Genetics, environment, nutrition, enteric flora, gender, age, novelty, learning, memory, emotion endocrine biorhythms (daylight/darkness, asleep/slept, fertility/menopause-andropause)...

STRESSORS

acutec
chronic
intermittent
recurrent

physical
psychological
metabolic
mixed

0-20  20-40  40-60  60-80  years

coping abilities

stress response

homeostasis
dishomeostasis

allostatic load

mucosal homeostasis
mucosal inflammation
transmural inflammation
Live probiotics protect intestinal epithelial cells from the effects of infection with enteroinvasive Escherichia coli (EIEC)

- Infection with EIEC alters phosphorylation of the tight junction proteins occludin and ZO-1.
- *Streptococcus thermophilus* and *Lactobacillus acidophilus* (ST/LA) living and antibiotic killed (a) were tested

Resta-Lenert, 2003
Zonulin is regarded as a physiological modulator of intercellular tight junctions and a surrogate marker of impaired gut barrier.

Increased zonulin concentrations are related to changes in tight junction competency and increased GI permeability.

Stool concentrations of zonulin in trained men before and after 14 weeks of treatment. Pro with probiotics supplemented group, Plac placebo group, Tx treatment, wk week; n = 11 (probiotic supplementation), n = 12 (placebo). Values are means ± SD. There was a significant difference between groups after 14 wk of treatment: PTx < 0.05.

Lamprecht, 2012
An overview of mechanisms involved in probiotic-induced enhancement of epithelial barrier function. These include direct modulation of epithelial cell signaling pathways and tight junctions, as well as effects on microbial ecology and innate and adaptive immune function.

Madsen, 2012
Mechanisms of paracellular permeability. (a and b) Epithelial cells are joined to each other by junctional complexes consisting of tight junctions (TJ), adherens junctions (AJ), desmosomes (DE), and gap junctions (GJ). (c) Four transmembrane families of proteins (occludin, claudins, junctional adhesion molecule (JAM) and tricellulin) contribute to TJ formation. The extracellular domains of the transmembrane proteins form a selective barrier through homophilic and heterophilic interactions with the adjacent cells. The intracellular domains of these transmembrane proteins interact with cytosolic scaffolding proteins like zonula occludens (ZO) proteins, which in turn anchor the transmembrane proteins to the perijunctional actinomyosin ring. The interaction of TJ proteins with the actin cytoskeleton is vital to the maintenance of TJ structure and function. The interaction of the TJ with the actinomyosin ring permits the cytoskeletal regulation of TJ barrier integrity. The circumferential contraction of the actinomyosin ring is regulated by the myosin light chain kinase (MLCK) which phosphorylates the myosin light chain.
Putative mechanisms by which enteric bacteria could disrupt the epithelial barrier function. The enteropathogenic bacteria are able to adhere at the surface of the enterocytes and M-cells. (1) When pathogenic bacteria encounter favorable conditions for their adherence and proliferation, they induce a local inflammation. At this place, paracellular and transcellular permeabilities are increased in relation with the recruitment and expansion of immune cells producing inflammatory cytokines like IL-1β, TNF-α and/or IFN-γ. As it has been extensively described these cytokines are able to alter the structure of the TJ by inducing the expression and activity of the MLCK (IL-1β, TNF-α and IFN-γ) and/or triggering the endocytosis (TNF-α and IFN-γ) of TJ proteins. (2) The recirculation of the activated immune cells contributes to propagate the barrier defect at distance of the infected area.

Barreau, 2014
Gastrointestinal selective permeable barrier is achieved by intercellular tight junction (TJ) structures.

Disruption of the intestinal TJ barrier, followed by permeation of luminal noxious molecules, induces a perturbation of the mucosal immune system and inflammation, and can act as a trigger for the development of intestinal and systemic diseases.

Suzuki, 2013
Tight Junction Proteins

Figure 2 The tight junction barrier is composed of tetraspanning membrane proteins claudins and occludin, and the regulatory proteins ZO-1, ZO-2 and ZO-3.

Zuhl, 2014
Structure disruption/protection

gene expression alteration
Intestinal TJ regulation by CYTOKINES
Intestinal TJ regulation by cytokines

The roles of cytokines in intestinal TJ regulation under pathophysiological conditions have been well investigated using cell cultures and animal models.

The cytokine mediated dysfunction of the TJ barrier, resulting in immune activation and tissue inflammation, is thought to be important in the initiation and/or development of several intestinal and systemic diseases. In contrast, some growth factors play roles in the protection and maintenance of TJ integrity.
Cytokines which **increase** intestinal TJ permeability

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>Cell lines</th>
<th>Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIGHT /IFN-γ</td>
<td>Caco-2</td>
<td>MLCK ↑, pMLC ↑, Caveolar endocytosis (occludin, ZO-1 and claudin-1) (Schwarz BT, 2007)</td>
</tr>
</tbody>
</table>
Cytokines which *increase* intestinal TJ permeability

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<tr>
<th>Cytokines</th>
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<th>Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>Caco-2</td>
<td>Occludin ↓ (Al-Sadi RM, Ma TY, 2007)</td>
</tr>
<tr>
<td></td>
<td>Caco-2</td>
<td>MLCK ↑, pMLC ↑ (Al-Sadi R, 2008)</td>
</tr>
<tr>
<td>IL-4</td>
<td>T84</td>
<td>Claudin-2 ↑ (Wisner DM, 2008)</td>
</tr>
<tr>
<td>IL-6</td>
<td>Caco-2, T84</td>
<td>Claudin-2 ↑ (Kusugami K, 1995)</td>
</tr>
<tr>
<td>IL-13</td>
<td>T84</td>
<td>Claudin-2 ↑ (Weber CR, 2010)</td>
</tr>
<tr>
<td></td>
<td>HT29/B6</td>
<td>Claudin-2 ↑ (Prasad S, 2005)</td>
</tr>
<tr>
<td></td>
<td>Caco-2</td>
<td>Potentiate oxidant (Rao R, 1999)</td>
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</table>
Cytokines which **decrease** intestinal TJ permeability

<table>
<thead>
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<th>Cytokines</th>
<th>Cell lines</th>
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<td>IL-10</td>
<td>T84</td>
<td>Decrease Neutralize IFN-c （Madsen KL, 1997）</td>
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<td>IL-17</td>
<td>T84</td>
<td>Claudin-1 ↑, Claudin-2 ↑ (Kinugasa T, et al., 2000)</td>
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<td>TGF-α antibody</td>
<td>Caco-2</td>
<td>Neutralize hydrogen peroxide (Forsyth CB, 2007)</td>
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<td>TGF-β</td>
<td>T84</td>
<td>Claudin-1 ↑ (Howe KL, et al, 2005)</td>
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<td></td>
<td>HT29 / B6</td>
<td>Claudin-4 ↑ (Hering NA, et al., 2011)</td>
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<tr>
<td></td>
<td>T84</td>
<td>Neutralize EHEC, restoration of occludin, claudin-2 and ZO-1 expression (Howe KL, et al, 2005)</td>
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</table>
Cytokines which **decrease** intestinal TJ permeability

<table>
<thead>
<tr>
<th>Cytokines</th>
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<tr>
<td>TGF-β</td>
<td>T84</td>
<td>Neutralize IFN-γ</td>
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<tr>
<td></td>
<td>T84</td>
<td>Neutralize cryptosporidium parvum (Roche JK, et al., 2000)</td>
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<tr>
<td>EGF</td>
<td>Caco-2</td>
<td>Neutralize hydrogen peroxide, restoration of occludin and ZO-1 distribution (Basuroy S, et al, 2006)</td>
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<tr>
<td></td>
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<td>Neutralize ethanol, restoration of microtubule assembly and oxidation/nitration of tubulin (Banan A, et al, 2007)</td>
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<td>Neutralize acetaldehyde, restoration of occludin and ZO-1 distribution (Suzuki T, et al., 2008; Samak G, et al. 2011)</td>
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</table>
• Intestinal TJ regulation by food factors
Nutrients and food factors **decrease** and restore intestinal TJ permeability

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<td>Gln</td>
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<td>claudin-1← →</td>
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<tr>
<td>Gln</td>
<td>Caco-2</td>
<td>Neutralize acetaldehyde, restoration of occludin and ZO-1 distribution</td>
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<td>Trp</td>
<td>Caco-2</td>
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Nutrients and food factors **decrease** and restore intestinal TJ permeability

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<td>EPA, DHA, arachidonic acid, di-homo- γ -LA</td>
<td>T84</td>
<td>Neutralize IL-4</td>
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<td>Acetic acid</td>
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<td>Propionic acid</td>
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<tr>
<td>Butyric acid</td>
<td>Caco-2</td>
<td>Promotion of occludin and ZO-1 assembly in Ca-induced TJ reassembly</td>
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</table>
Nutrients and food factors **decrease** and restore intestinal TJ permeability

<table>
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<tr>
<td>Vitamin A</td>
<td>Caco2</td>
<td>Neutralize Clostridium difficile toxin A (Maciel AA, 2007)</td>
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<tr>
<td>Vitamin D</td>
<td>SW480, not determined permeability</td>
<td>ZO1 ↑, claudin-1 ↑, claudin-2 ↑, E-cadherin ↑ (Kong J, et al, 2008)</td>
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<tr>
<td></td>
<td>Caco-2</td>
<td>Neutralize DSS (Kong J, et al, 2008)</td>
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# Nutrients and food factors decrease and restore intestinal TJ permeability

<table>
<thead>
<tr>
<th>Polyphenol</th>
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<tr>
<td>Genistein</td>
<td>Caco2</td>
<td>Neutralize hydrogen peroxide, occludin ← →, ZO-1 ← → (Rao RK, 2002)</td>
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<tr>
<td></td>
<td>Caco2</td>
<td>Neutralize acetaldehyde, occludin ← →, ZO-1 ← → (Atkinson KJ, 2001)</td>
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<tr>
<td>Curcumin</td>
<td>Caco-2</td>
<td>Neutralize TNF-a (Ye D, 2006)</td>
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<td></td>
<td>Caco-2</td>
<td>Neutralize IL-1b (Al-Sadi RM, 2007)</td>
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<tr>
<td>EGCG</td>
<td>T84</td>
<td>Neutralize IFN-c (Watson JL, 2004)</td>
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<tr>
<td>Quercetin</td>
<td>Caco-2</td>
<td>Claudin-4 ↑, ZO-2 ← →, claudin-1 ← →, occludin ← → (Suzuki T, Hara H, 2009)</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>Caco-2</td>
<td>ZO-2 ↑, claudin-4 ↑ occludin ← →, claudin-1 ← →, claudin-3 ← → (Suzuki T, et al 2011)</td>
</tr>
<tr>
<td>Myricetin</td>
<td>Caco-2</td>
<td>Unknown (Suzuki T, Hara H, 2009)</td>
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