**CENTRO DE INVESTIGACIÓN Y DE ESTUDIOS**

**AVANZADOS DEL INSTITUTO POLITÉCNICO NACIONAL**

UNIDAD ZACATENCO

DEPARTAMENTO DE BIOMEDICINA MOLECULAR

# “Análisis funcional del soluto compatible homoectoina en la estabilización de la barrera epitelial intestinal durante la inflamación”

T E S I S

Que presenta

**M.V.Z. KARLA FABIOLA CASTRO OCHOA**

Para obtener el grado de

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**Director de la Tesis:** Dr. Michael Schnoor

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# "Functional analysis of the synthetic compatible solute homoectoine in the stabilization of intestinal epithelial barrier during inflammation"

T H E S I S

Presented by

**M.V.Z. KARLA FABIOLA CASTRO OCHOA**

To obtain the degree

**MASTER IN SCIENCES**

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**ABBREVIATIONS**

**AJ.** Adherens Junction

**AJC.** Apical Junction Complex

**ATG16L.** Autophagy Related 16-Like 1

**BR.** Bacteriorhodopsin

**CD.** Crohn's Disease

**CNF-1.** Cytotoxic Necrotizing Factor-1

**CNPs.** Carbon Nanoparticles

**DAI.** Disease Activity Index

**DSS.** Dextran Sulfate Sodium

**HNF4A.** Hepatocyte Nuclear Factor-4-Alpha

**IBD.** Inflammatory Bowel Diseases

**IFN-γ. I**nterferon-γ

**IL-.** Interleukin-

**IRI.** Ischemia Reperfusion Injury

**JAM.** Junctional Adhesion Molecules

**KO.** Knock-out

**LPS.** Lipopolysaccharide

**MAPK.** Mitogen-Activated Protein Kinase

**MDP.** Muramyl-Dipeptide

**MEP1A.** Meprin A Subunit Alpha

**MLCK.** Myosin Light Chain Kinase

**MPO.** Myeloperoxidase

**MUC-.** Mucin-

**NF-kB.** Nuclear Factor kappa-light-chain-enhancer of activated B cells

**NOD2.** Nucleotide-binding Oligomerization Domain containing 2

**NSAID.** Nonsteroidal Anti-Inflammatories drugs

**PGE2.** Prostaglandin E2

**PRR.** Pathogen Recognition Receptors

**RBC.** Red Blood Cells

**SDS.** Sodium Dodecyl Sulfate

**SNP.** Single Nucleotide Polymorphism

**TEER.** Transepithelial Electrical Resistance

**TEWL.** Transepidermal Water Loss

**TJ.** Tight Junction

**TLR.** Toll-Like Receptor

**TNBS.** 2,4,6-Trinitrobenzenesulfonic Acid

**TNF-α.** Tumor Necrosis Factor alpha

**UC.** Ulcerative Colitis

**ZO.** Zonula Occludens

## 1. ABSTRACT

**Background and Aim:** Inflammatory bowel diseases (IBD) are multifactorial disorders that comprise ulcerative colitis (UC) and Crohn's disease (CD) affecting millions of people worldwide with alarmingly increasing incidences every year. Dysfunction of the intestinal epithelial barrier has been associated with the pathogenesis of IBD and therapies include anti-inflammatory drugs that enhance intestinal barrier function. However, these drugs often have adverse side effects. Compatible solutes, such as bacterial ectoines, have been shown to stabilize cell membranes and proteins. Here, we tested if ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidinecarboxylic acid) and homoectoine (4,5,6,7-tetrahydro-2-methyl-1H-(1,3)-diazepine-4-carboxylic acid), a synthetic derivative of ectoine, prevent excessive intestinal epithelial permeability during inflammation.

**Results:** Ectoine and homoectoine treatments alone in C57Bl/6J mice did not affect the disease activity index (DAI) suggesting that these compounds do not affect colon functions in healthy mice. Importantly, treatment with ectoine or homoectoine in colitic mice significantly reduced the DAI score with greater effects on intestinal bleeding and stool consistency compared to weight loss. Histologically, edema formation, leukocyte influx and tissue damage induced by dextran sulfate sodium (DSS) were strongly reduced. Moreover, intestinal epithelial permeability was reversed to control levels in the groups of colitic mice receiving the compatible solutes. Downregulation of junction proteins, commonly observed during DSS colitis, was prevented in mice treated with ectoine or homoectoine. Moreover, the switch between claudin-1 and claudin-2 protein levels during DSS colitis was reversed only by homoectoine.

**Conclusions:** Both ectoine and homoectoine have protective effects on the epithelial barrier during inflammation and may thus serve in the future as diet supplementation in IBD patients to reach or extend phases of remission.

## RESUMEN

**Antecedentes y objetivo:** Las enfermedades inflamatorias intestinales (IBD) son desórdenes multifactoriales que comprenden a la colitis ulcerativa (UC) y a la enfermedad de Crohn (CD), afectando a millones de personas en todo el mundo y cuya incidencia aumenta alarmante cada año. La disfunción de la barrera epitelial intestinal se ha asociado con la patogénesis de la IBD y actualmente drogas anti-inflamatorias son utilizadas como terapia para mejorar la función de la barrera intestinal. Sin embargo, estas drogas comúnmente causan efectos adversos. Se ha demostrado que solutos compatibles, como las ectoinas bacterianas, son capaces de estabilizar membranas celulares y proteínas. Nosotros probamos si ectoina (Ácido 1,4,5,6-tetrahidro-2-metil-4-pirimidinacarboxilico) y homoectoina (Ácido 4,5,6,7-tetrahidro-2-metil-1H-(1,3)-diazepina-4-carboxilico), un derivado sintético de ectoina, previenen la excesiva permeabilidad intestinal epitelial que se presenta durante la inflamación.

**Resultados:** Los tratamientos con ectoina y homoectoina por si mismos no afectaron el índice de actividad de la enfermedad (DAI) de ratones C57Bl/6J, sugiriendo que estos compuestos no afectan las funciones del colon en ratones sanos. De manera importante, el tratamiento de ratones enfermos con ectoina u homoectoina reducen el DAI de manera significativa, observándose mayores efectos en sangrado intestinal y consistencia de las heces comparado con pérdida de peso. Histológicamente, hubo una fuerte disminución de la formación de edema, flujo leucocitario y daño del tejido inducidos por dextran sulfato de sodio (DSS). Además, la permeabilidad intestinal epitelial se revirtió a niveles control en aquellos animales enfermos que recibieron los solutos compatibles. La desregulación de las proteínas de las uniones intercelulares, que comúnmente se observa durante la DSS-colitis, fue prevenida en ratones tratados con ectoina u homoectoina. Además, el intercambio de los niveles proteicos de las claudina-1 y claudina-2 durante la DSS-colitis fue revertido solo por homoectoina.

**Conclusiones:** Tanto ectoina como homoectoina tienen un efecto protector en la barrera epitelial durante la inflamación y, por lo tanto, podrían servir en un futuro como un suplemento alimenticio para pacientes con IBD para alcanzar o extender la fase de remisión.

## 2. INTRODUCTION

### 2.1 Inflammatory Bowel Disease

Inflammatory bowel diseases (IBD) are complex inflammatory disorders comprising ulcerative colitis (UC) and Crohn's disease (CD). They usually develop in the second and third decade of life. UC is more frequent in males while Crohn's disease occurs more often in females (Loftus & Sandborn 2002). IBD is more common in developed countries, with around 10,000 new cases being diagnosed every year suggesting that western diet, smoking, pollution and exposure to industrial chemicals can influence the pathogenesis. There is not much epidemiologic information about IBD in Latin America. Historically, IBD has been considered rare in Mexico; however, it has been significantly increasing in recent years. A study realized in the northeast area of Mexico covering a period of 5 years from 2004 to 2008, showed that UC incidence has been increasing from 2.3 to 4 of 1000 hospital admissions (Bosques-Padilla et al. 2011). Another epidemiologic study realized in a referral hospital in Mexico City showed a 2.6 fold increase in UC incidence over a 20 year period from 1987 to 2006 (Yamamoto-Furusho 2009).

CD causes a transmural inflammation, affects any part of the gastrointestinal tract in a discontinuous pattern, and is associated with complications like granulomas, fistulas and strictures. By contrast, UC is a confined inflammation of the colonic mucosa affecting mainly the rectum and the distal colon in a continuous manner (Zhang 2014; Abraham & Cho 2009).

### 2.2 Pathogenesis

In both forms of IBD, a chronic and recurrent inflammation is caused in part by an uncontrolled immune response to the antigens of the intestinal microflora (Zhang 2014). The specific etiology of the disease is still unknown. However, emerging evidence suggests that genetic, environmental and immunological factors play a role in the development of IBD.

### *2.2.1 Genetic susceptibility*

Genome-wide association studies have helped to discover single nucleotide polymorphisms (SNPs) that are associated to the risk of developing IBD. 163 gene loci associated to IBD have been identified with 30 of them being specific for CD and related to innate immunity, autophagy, and phagocytosis such as autophagy related 16-like 1 (ATG16L) and nucleotide-binding oligomerization domain containing 2 (NOD2). 23 gene loci have been associated to UC such as hepatocyte nuclear factor-4-alpha (HNF4A) and Meprin A Subunit Alpha (MEP1A) (Corridoni et al. 2014; Ibd et al. 2012).

Polymorphisms within the NOD2 gene are frequently associated with CD. NOD2 is an intracellular pattern recognition receptor that recognizes muramyl-dipeptide (MDP), a derivative of peptidoglycan which is present in the cellular wall of both Gram-positive and Gram-negative bacteria. NOD2 activation induces nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) and mitogen-activated protein kinase (MAPK) pathway signaling promoting the production of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1) and interleukin-6 (IL-6) (Atreya et al. 2008). It has been proposed that this association is dose-dependent, with heterozygous individuals of these mutations having incremented risk of 2-4 times of suffering the disease, whereas homozygous individuals have a 20-fold incremented risk (Xavier & Podolsky 2007; Cuthbert et al. 2002).

Past studies have associated mutations in pathogen recognition receptors (PRR) with IBD. Intestinal epithelial cells express PRRs which include the toll-like receptors (TLR) family and the NOD intracellular receptors. These receptors monitor the luminal microflora and respond to a variety of microorganisms. During normal conditions, the response triggers signaling pathways leading to the activation of non-specific killing mechanisms of innate immunity such as synthesis of reactive oxygen species, activation of the complement protein system, and secretion of antimicrobial proteins and cytokines. Mutations in these receptors cause an uncontrolled innate immune response (Goyette et al. 2007).

### *2.2.2 Environmental factors*

Not only genetic factors play an important role in the pathogenesis of IBD, but also environmental factors have a considerable influence. Smoking is one of these factors, but with opposite roles. While it has a protective effect in UC, in CD smoking increases the risk of further triggering the disease (Corridoni et al. 2014).

The consumption of drugs like nonsteroidal anti-inflammatories (NSAID) increases the risk of developing IBD or worsens existing disease since they block the production of protective prostaglandins such as prostaglandin E2 ($PGE_2$). $PGE_2$ is capable of inhibiting the production of lipopolysaccharides (LPS)-induced TNF-α by macrophages (Kunkel et al. 1988). It has also been observed that an appendectomy in individuals younger than 20 years has a protective effect against UC, however, the mechanism responsible for this is still unknown (Danese et al. 2004).

Moreover, diet plays a major role in IBD. High dietary intake of fatty acids and proteins is associated with the development of the disease whereas high fiber intake is associated with a decreased risk (Yamamoto 2013)(Bier 2003). Diet alters the composition of the gut microbiota. Gut microbiota has been clustered into three enterotypes: *Bacteroides*, *Prevotella*, and *Ruminococcus* generae. High levels of *Bacteroides* have been found in individuals with a “Westernized” diet which is rich in fat and protein and are related to higher IBD risk. *Prevotella* is mainly found in individuals with high fiber diets associated with lower IBD risk (Wu et al. 2013).

It has been suggested that aberrant interactions between commensal flora and the host’s immune response are important in the development and progression of IBD. Evidence suggests that the imbalance of the intestinal microbiota can be a predisposing factor for IBD. Several studies have been realized about this topic, but it is still unclear if this dysbiosis is actually a cause or a consequence of IBD (Nguyen 2011).

## 2.3 Intestinal epithelial barrier

The main function of the intestinal epithelial barrier is to control the passage of nutrients, water and electrolytes, meanwhile impeding the passage of pathogens and toxins. It is composed of a layer of mucins which are produced by specialized epithelial cells called goblet cells. The function of mucins is to prevent the contact of large molecules and bacteria with the epithelial layer (Turner 2009). The glycocalyx is located under the mucin layer, it is constituted of glycoproteins and glycolipids attached to the apical side of the epithelial cell membrane and also functions as a physical barrier. The third component is the epithelium itself which is formed by a monolayer of epithelial cells that originate from pluripotent stem cells at the bottom of the intestinal crypts. The space between adjacent epithelial cells must be sealed in order to prevent the passage of particles and microorganisms and this is achieved by the intercellular junctions (Turner 2006).

### *2.3.1 Intercellular junctions*

Epithelial cells are attached to each other by 3 types of junctional complexes: tight junctions (TJ), adherens junctions (AJ) and desmosomes. TJ and AJ form the apical junction complex (AJC). While TJ are mainly in charge of regulating barrier permeability, AJ and desmosomes maintain cell adhesion and polarity (Figure 1).

Tight junctions are located at the most apical end of the lateral membrane surface and have two main functions, the gate (barrier) function and the fence function (Figure 2). Gate function regulates the passage of ions, water, nutrients and electrolytes through the paracellular space. Fence function maintains cell polarity by preventing the intermixing of junction molecules in the apical membrane with those from the lateral membrane. TJ are formed by transmembrane proteins such as claudins, occludin and junctional adhesion molecules (JAM) which are linked to the actin cytoskeleton by interactions with intracellular adaptor proteins such as the Zonula Occludens (ZO) (Sawada et al. 2003).

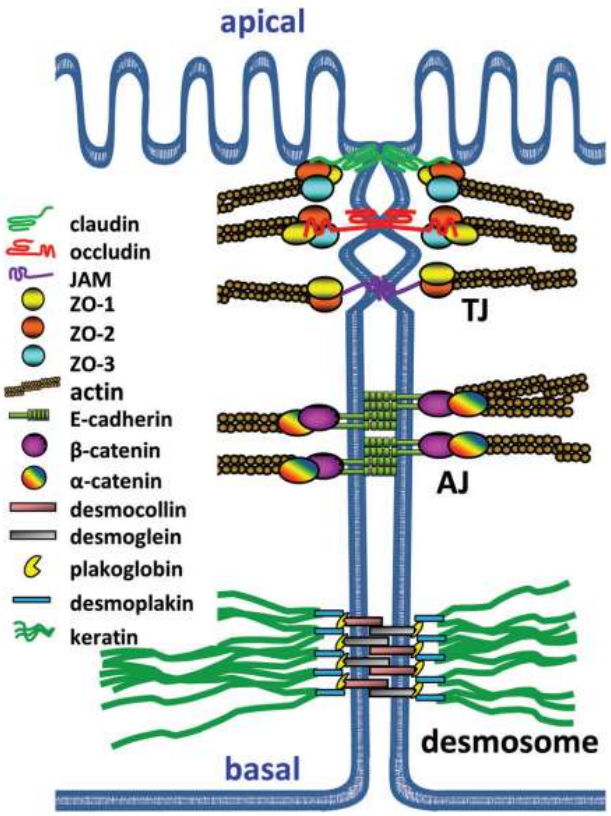



**Fig. 1 Epithelial intercellular junctions.** Intercellular junctions maintain epithelial cell polarity and control permeability. Tight junctions (TJ) are the apical-most junctional complex consisting of claudins, occludin, junctional adhesion molecules (JAM) and zonula occludens (ZO) proteins. Adherens junctions are formed by E-cadherin and catenins. Desmosomes consist of desmogleins, desmocollins, desmoplakin and plakoglobin. (Citalán-Madrid et al. 2013)

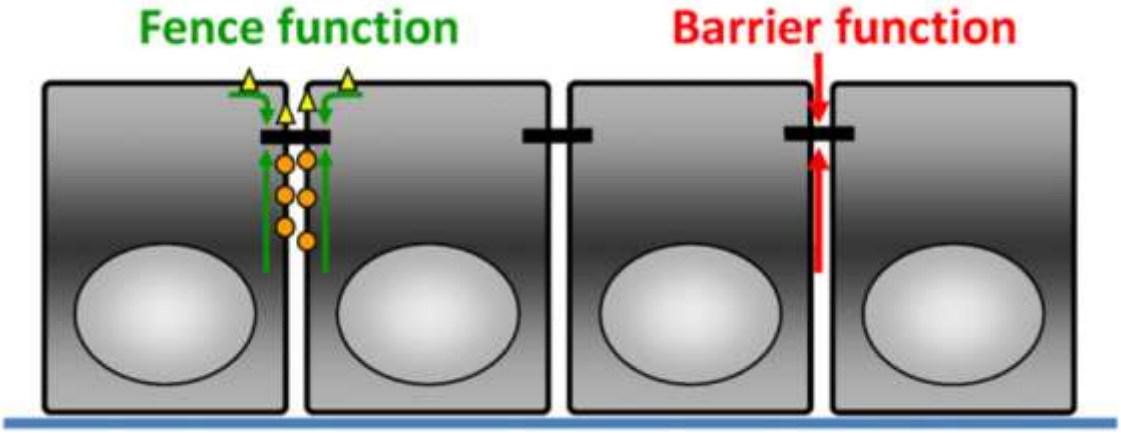



**Fig. 2 The fence and barrier functions of tight junctions.** Fence function prevents intermixing of apical and basolateral junction proteins. Barrier or gate function regulates the transport of molecules through the paracellular space (Sawada 2013).

Adherens junctions establish a strong adhesive link between epithelial cells in order to maintain their polarity and proximity. They are mainly composed by a family of transmembrane proteins called cadherins with E-cadherin being mainly expressed in the intestinal epithelium. E-cadherin directly interacts with the adaptor proteins p120-catenin and β-catenin. Meanwhile, β-catenin interacts with α-catenin which connects the E-cadherin/catenin complex to the actin cytoskeleton (Turner 2009; Lee 2015; Suzuki 2012).

Desmosomes give mechanical strength to the epithelium by connecting to intracellular intermediate filaments. The transmembrane proteins desmocollins and desmogleins interact with plakoglobin and plakophilin which are bound to the intermediate filaments by desmoplakin (Garrod & Chidgey 2008).

### *2.3.2 Epithelial barrier regulation*

Barrier function is regulated by the AJC. Physiologic and pathological stimuli can induce the endocytosis of AJC components and thus a weakening of the barrier. Endocytosis of AJC proteins can occur via three classical endocytic pathways, i.e. clathrin-mediated endocytosis, micropinocytosis and caveolar-mediated endocytosis. Clathrin-mediated endocytosis participates in normal constitutive internalization of TJ and AJ proteins without affecting its function and integrity (Utech et al. 2010). Micropinocytosis is responsible for the internalization of TJ proteins under inflammatory conditions. In presence of interferon-γ (IFN-γ) this endocytic pathway is upregulated causing increased paracellular permeability, while cell-cell contacts remain intact (Nusrat et al. 2005). Caveolar-mediated endocytosis of occludin has been observed in T84 polarized intestinal epithelial monolayers stimulated with *Escherichia coli* cytotoxic necrotizing factor-1 (CNF-1). Studies suggest that under physiologic conditions, AJC proteins undergo endocytic recycling resulting in a rapid remodeling of the complex without altering barrier function (Morimoto et al. 2005). Furthermore, endocytosis of AJC proteins induced by cytokines or bacterial toxins can result in both a recycling pathway or degradation, with the latter causing barrier dysfunction (Fletcher & Rappoport 2014).

Loss of epithelial barrier integrity allows for the passage of bacteria which are detected by cells of the immune system residing in the lamina propria. These immune cells respond by producing pro-inflammatory cytokines. The exacerbated production of pro-inflammatory cytokines further contributes to the dysfunction of the epithelial barrier (Figure 3). For example, elevated levels of IFN-γ increase actomyosin contraction inducing the internalization of TJ proteins via macropinocytosis. TNF-α is principally produced by macrophages and activated T cells and induces epithelial cell apoptosis and activation of myosin light chain kinase (MLCK) leading to actomyosin contractibility and eventually disruption of the AJC. However, neither IFN-γ nor TNF-α directly affect AJ composition and function. Interleukin 1-β (IL-1 β) increases epithelial permeability by decreasing the expression and distribution of occludin and by increasing the activation of MLCK and thus actomyosin contractility (Utech et al. 2010)(Coskun 2014).

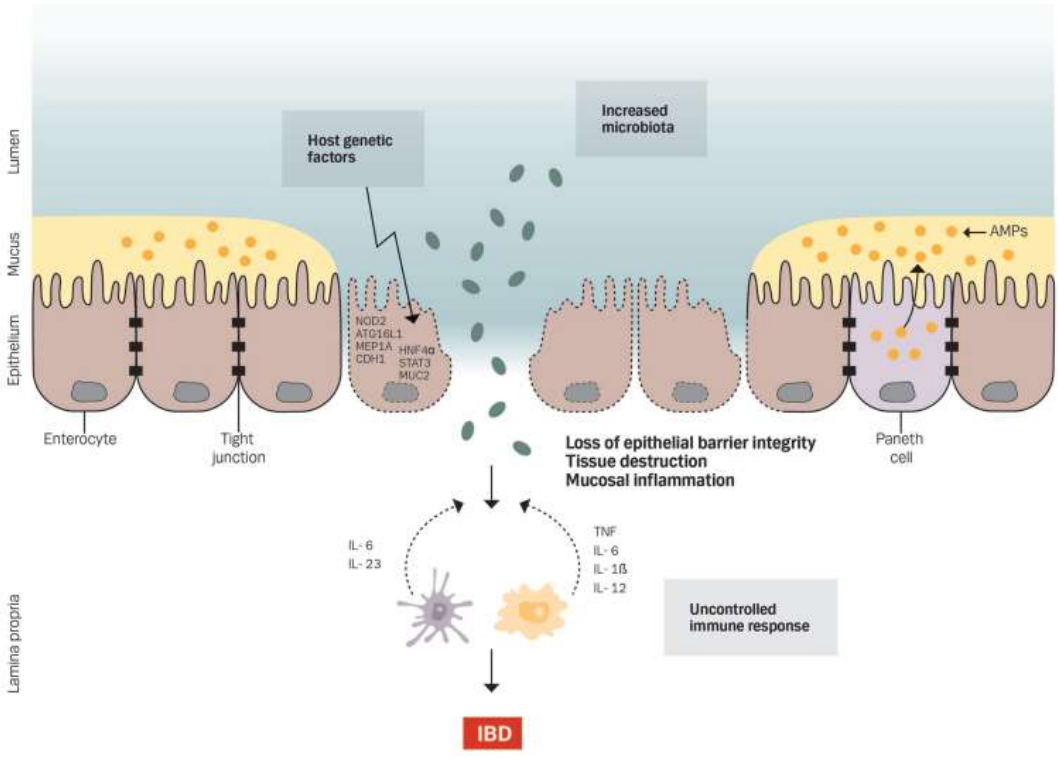



**Fig. 3 Loss of epithelial barrier integrity induces an uncontrolled immune response.** Genetic, environmental and immunological factors cause epithelial barrier dysfunction, affecting its integrity and increasing its permeability thus triggering bacterial translocation and an uncontrolled immune response (Coskun 2014).

Production of macrophage-derived chemokines induce recruitment of neutrophils which upon contact with the antigens will release myeloperoxidase, proteases, reactive oxygen species, antimicrobial peptides and hydrolytic enzymes. If this immune response is not controlled and terminated properly, it can contribute to mucosal tissue damage (Fournier & Parkos 2012).

## 2.4 Compatible Solutes

Compatible solutes are soluble, uncharged or zwitterionic organic molecules of low molecular weight including saccharides, polyols, betaines, amino acids and their derivatives. They are mainly produced by extremophilic bacteria and accumulate in the cytoplasm to protect against environmental stress. Compatible solutes mitigate the negative effects of osmotic stress, heat stress, freezing, drying, oxygen radicals, radiation to maintain the integrity of biopolymers like proteins, nucleic acids, biomembranes and even whole cells. All these functions are performed without interfering with essential cellular processes and the normal metabolism (Pastor et al. 2010).

### *2.4.1 Mechanism of action*

Different theories have been described trying to explain the mechanism by which compatible solutes can exert their protective function, with the most accepted being the preferential exclusion model (Figure 4).

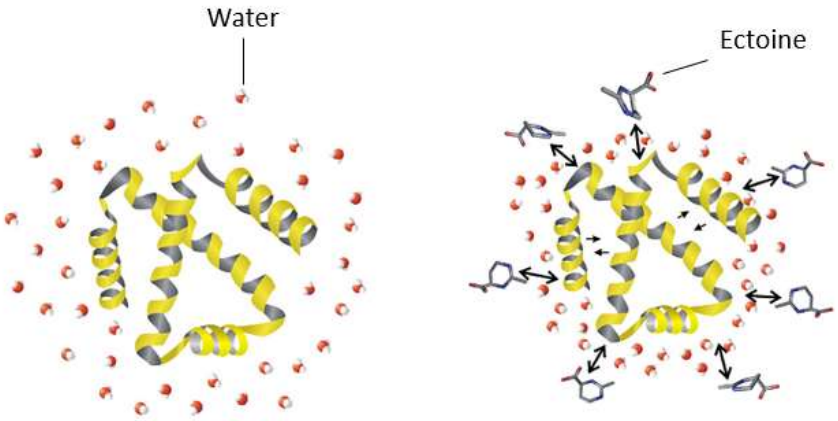



**Fig. 4 The preferential exclusion model.** Ectoine favors the formation of water-water complexes thus hydrating the protein surface and stabilizing its native conformation (Pastor et al. 2010)

This model proposes that solutes act as a co-solvent leading to thermodynamic interactions with the macromolecule that favor the formation of water-water complexes expulsing ectoine from the macromolecule surface thus resulting in a preferential hydration of the macromolecule. In the case of proteins, the recruitment of water to its surface will induce the refolding of the protein into its native conformation or prevent its denaturation (Smiatek et al. 2012).

### *2.4.2 Ectoines*

Ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidinecarboxylic acid) is a heterocyclic amino acid or a hydrogenated pyrimidine derivative and has a molecular weight of 142.16 g/mol (Figure 5a) (Held et al. 2010). It was discovered by Galinski and collaborators in the halophilic bacteria *Halorhodospira halochloris* (previously known as *Ectothiorhodospira halochloris*) while studying the cellular response to sudden changes in salinity (Galinski et al. 1985).

Hydroxyectoine was discovered in *Streptomyces parvulus* which is chemically similar to ectoine with an additional hydroxyl group and has a molecular weight of 158.2 g/mol (Held et al. 2010). It also shows properties as an important heat stress protector in the halophilic bacteria *Chromohalobacter salexigens* (Figure 5b) (García-Estepa et al. 2006). Homoectoine is a synthetic derivative of ectoine with a molecular weight of 156 g/mol and contains an extra $CH_2$ group in its ring making it more hydrophobic (Figure 5c) (Schnoor et al. 2004).

Ectoine and its derivatives have been implicated in the stabilization and protection of lipid membranes (Harishchandra et al. 2010). Ectoine is being used as cell protectant in skin care products and currently studied for a potential therapeutic use in inflammatory diseases (Marini et al. 2014)(Pastor et al. 2010).

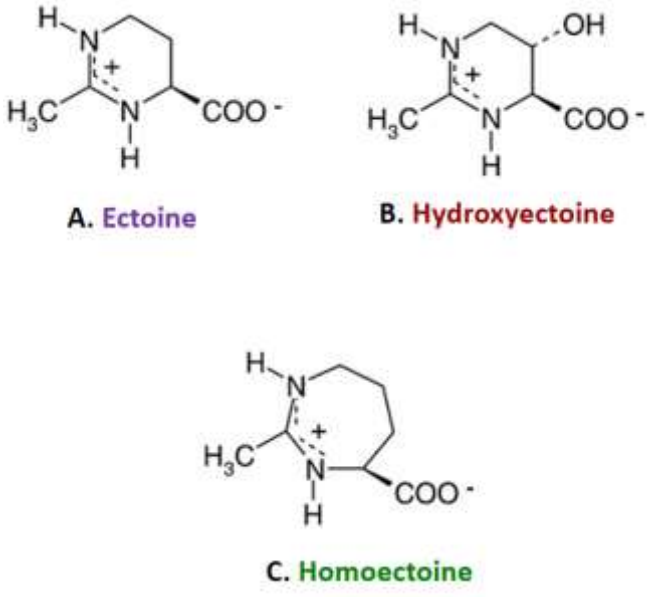



**Fig. 5 Chemical structure of compatible solutes**: A) ectoine, B) hydroxyectoine and C) homoectoine. (Held et al. 2010)

### *2.4.3 Cell membrane and protein stabilization by ectoines*

Utilizing artificial lipid layers as models for cell membranes, Harishchandra and colleagues demonstrated that ectoine and hydroxyectoine are capable of stabilizing lipid monolayers and bilayers by forming ectoine-water complexes, thus increasing the hydration of the surface resulting in increased fluidity (Harishchandra et al. 2010).

Graf and colleagues showed that ectoine has a protective and stabilizing effect on cell membranes by applying the red blood cell (RBC) test. This assay is an *in vitro* test to estimate the denaturing properties of surfactants by a photometric quantification of the hemoglobin released by RBCs with membrane damage (Figure 6). RBCs were incubated with sodium dodecyl sulfate (SDS) for 10 minutes and treated with ectoine at different concentrations for 1 hour. Cells without ectoine suffered maximum damage and represent 0% increase of membrane stability. The protective effect of ectoine increased with higher concentrations.

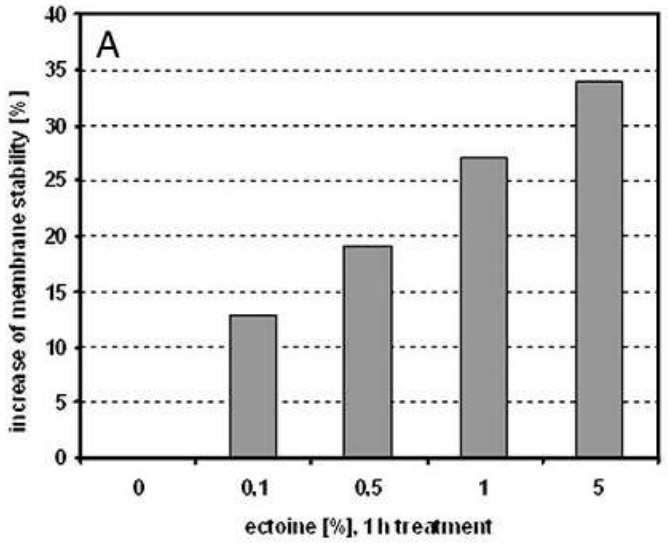



**Fig. 6 Protective effect of ectoine against membrane damage in red blood cells.** Human erythrocytes were treated with 0, 0.1, 0.5, 1 and 5% ectoine during 1 hour and stressed with 0-0.04% SDS for 10 minutes. The number of lysed cells was determined spectroscopically via the content of free hemoglobin and represented as the increase of membrane stability. (Graf et al. 2008)

Furthermore, it has been demonstrated that ectoine has a protective effect against denaturation of transmembrane proteins. The investigators applied mechanical force in order to unfold Bacteriorhodopsin (BR), a 26 kDa transmembrane protein that acts as a light-driven proton pump in *Halobacterium salinarum*. The amount of applied force and the persistence length of the unfolded protein were measured in the presence and absence of ectoine. The persistence length of an unfolded amino acid chain is a measure for its tendency to form a compact coil. A short persistence length indicates a higher tendency to coil up, and a longer persistence length indicates a more extended conformation. Both parameters were obtained from all recorded force curves and displayed as shown in figure 7. In the presence of ectoine the amount of applied force required for denaturation of BR was increased suggesting that there are intra-molecular interactions that stabilize the protein structure. The persistence length of the unfolded protein in the presence of ectoine was significantly decreased indicating that ectoine assists in the refolding of the protein (Roychoudhury et al. 2012).

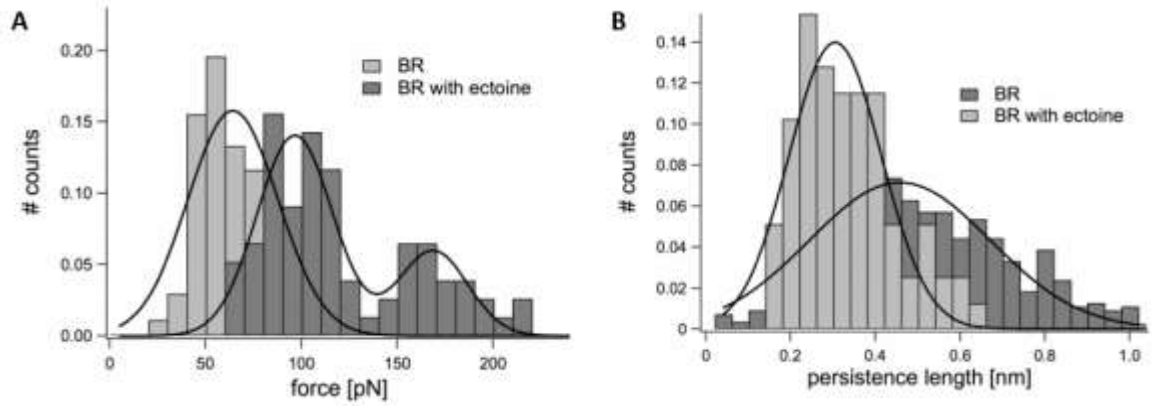



**Fig. 7 Dependency of unfolding forces and persistence length of Bacteriorhodopsin on ectoine.** Histograms of the parameters obtained from all recorded force curves. A) In the presence of ectoine, the force required to unfold Bacteriorhodopsin was higher than in its absence. B) The persistence length of the unfolded protein was reduced in the presence of ectoine (Roychoudhury et al. 2012).

Moreover, the stabilizing effects of ectoine against protease-catalyzed of macromolecular substrates have been studied (Kolp et al. 2006). The zymogens trypsinogen and chymotrypsinogen were incubated with the proteases enteropeptidase and trypsin respectively in the presence of the compatible solute ectoine at different concentrations. Ectoine protected the zymogens against proteolysis in a dose-dependent manner (Figure 8). In presence of ectoine, zymogens adopted a more compact conformation that impaired the interaction between zymogens and proteases.

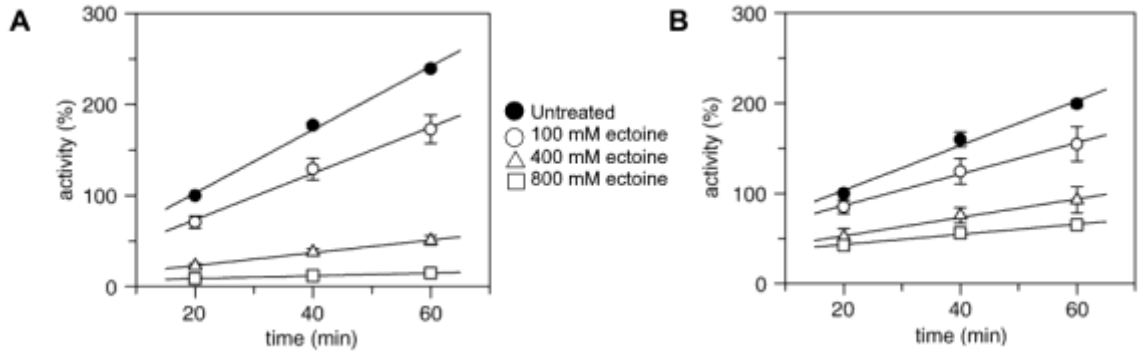



**Fig. 8 Effect of ectoine on zymogen proteolysis.** A) trypsinogen activation by enteropeptidase and B) chymotrypsinogen activation by trypsin in the absence (●) and presence of 100 mM (○), 400 mM (△) and 800 mM (□) of ectoine (Kolp et al. 2006).

### *2.4.4 Protective effects of ectoine in human skin*

Ectoine has been implicated in the protection of the skin against UVA-induced cell damage preventing premature photoaging (Buenger & Driller 2004). UVA radiation is known to induce photoaging and photodermatosis. UVA induces the release of ceramides that act as second messengers and initiate activation of the transcription factor AP-2 and expression of pro-inflammatory genes. The release of ceramides was measured in human keratinocytes untreated or pretreated for 24 hours with 1mM of ectoine and after exposure to a single dose of 30 J/cm2 UVA radiation (Figure 9). The UVA radiation-induced ceramide release was prevented by ectoine.

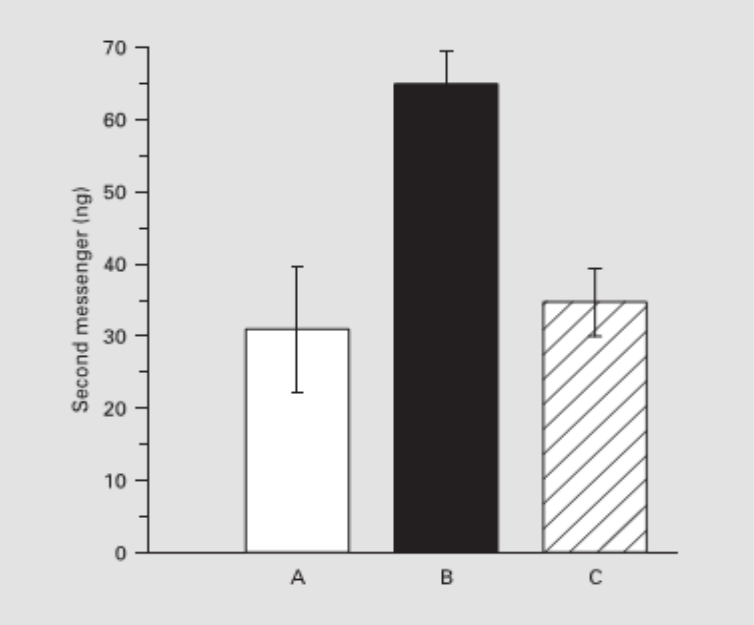



**Fig. 9 Prevention of UVA radiation-induced ceramide release by ectoine.** Human keratinocytes release ceramides upon UVA radiation which act as second messengers that initiate an inflammatory response. A) Untreated and non-irradiated cells. B) Untreated and irradiated cells. C) 1mM ectoine pretreated and irradiated (Buenger & Driller 2004).

Graf and colleagues demonstrated that ectoine is capable of strengthening skin barrier when applied topically (Figure 10). The volar forearm of 5 individuals was

treated with an oil in water emulsion containing different concentrations of ectoine once a day during 1 week. Transepidermal water loss (TEWL) determines the integrity of the skin barrier by measuring the density gradient of water evaporation from the skin. An increase in water loss indicates an affected skin barrier. TEWL was measured on the skin of these individuals before the administration of treatment, 1-week post-treatment and after SDS-induced skin barrier damage. It was observed that the skin of those individuals who received the treatment containing 5% ectoine had a significant reduction of TEWL compared to the untreated group. This suggests that ectoine at that concentration prevented to some extent the damage induced by SDS. (Graf et al. 2008).

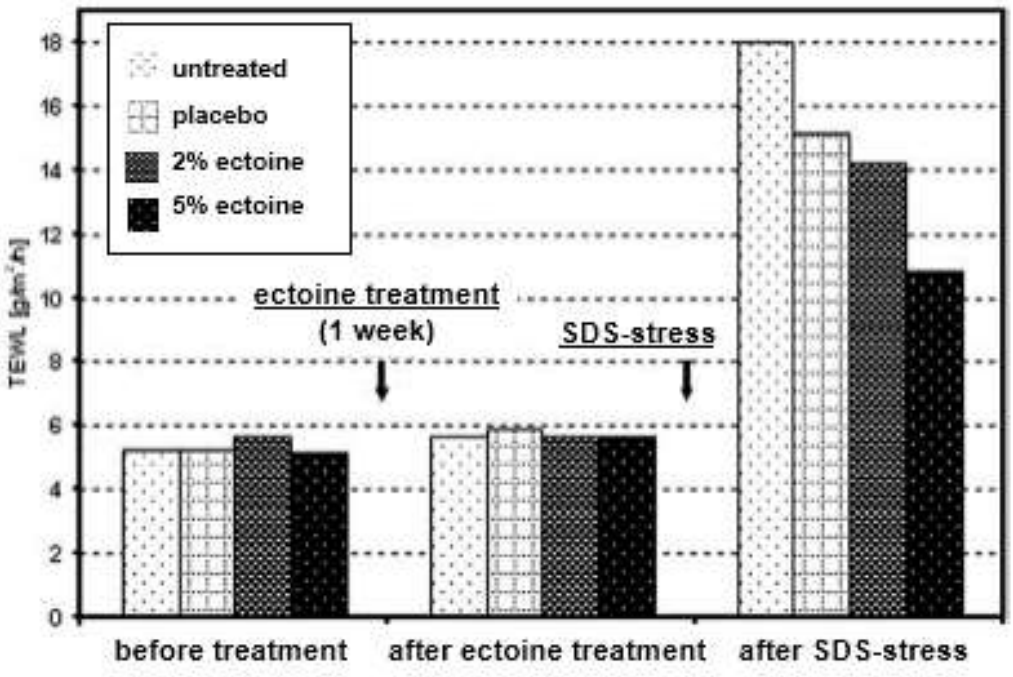



**Fig. 10 Ectoine strengthens skin barrier and diminishes transepidermal water loss.** The volar forearm of 5 individuals were treated with different concentrations of ectoine. Skin barrier damage was induced with SDS and analyzed by TEWL in the different groups: untreated (clear dotted bar), placebo (clear squared bar), 2% ectoine (dark squared bar) and 5% ectoine (dark dotted bar) (Graf et al. 2008).

### *2.4.5 Protective effects of ectoine in lung inflammation*

The anti-inflammatory effect of ectoine has also been assessed *in vivo* in rats. Lung inflammation was induced by carbon nanoparticles (CNPs) in the absence or presence of ectoine at different doses in female Fisher 344 rats (Sydlik et al. 2009). The quantity of neutrophils was measured in the bronchoalveolar lavage and a dose-dependent decrease in neutrophil numbers was observed when ectoine was administered (Figure 11). However, ectoine did not have this effect when the inflammation was induced with LPS. This might be explained by the fact that CNP and LPS use different cell signaling mechanisms to induce an inflammatory response. CNP activate the MAPK-ERK1/2 pathway, whereas LPS activates the TLR4/MyD88 pathway.

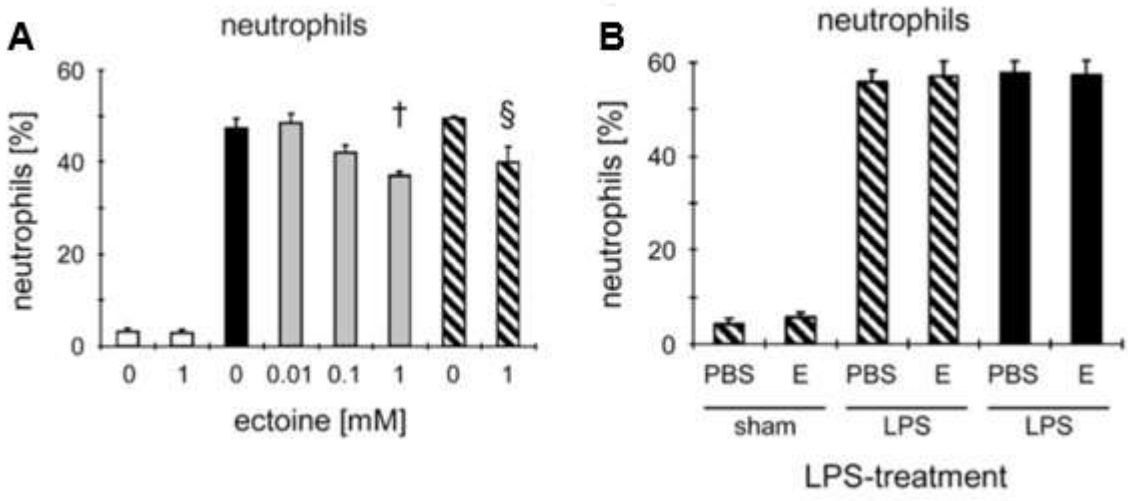
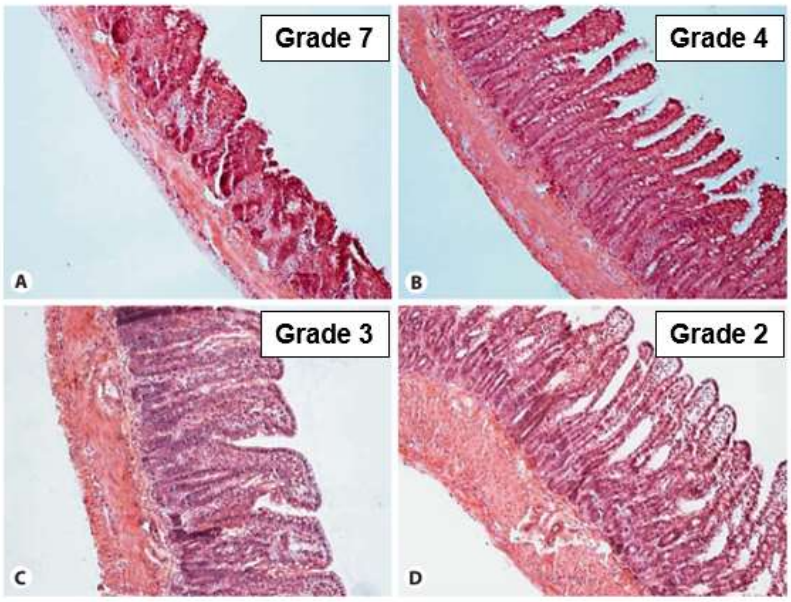



**Fig. 11 Ectoine dose-dependently reduces carbon nanoparticle (CNP) but not lipopolysaccharide (LPS)-induced inflammation *in vivo*.** (A) CNP-induced inflammation in the absence of ectoine (black bar), in the presence of ectoine at different concentrations (grey bars), or ectoine (1mM) as pretreatment (hatched bar 1). Control group (white bars). (B) LPS induced inflammation in the presence or absence of ectoine (1mM) as pretreatment (hatched bars) or treatment (black bars), control group (sham, hatched bars) (Sydlik et al. 2009).

### *2.4.6 Protective effects of ectoine in intestinal inflammation*

Ischemia reperfusion injury (IRI) is the cellular damage that results from a period of ischemia followed by the reestablishment of blood supply (reperfusion) to the tissue. Transplantations are frequently affected by IRI diminishing its success since it induces graft motor dysfunction and inflammatory reactions.

Ectoine has been shown to offer protection against the effects of IRI in intestinal transplantation by reducing tissue damage (Figure 12), infiltration of neutrophils into the intestinal muscularis and mRNA expression of pro-inflammatory cytokines such as IL-6 (Pech et al. 2012).




**Park's score grading system**

| Grade | Description |
|---|---|
| 0 | Normal mucosa |
| 1 | Subepithelial space at villous tip |
| 2 | More extended subepithelial space |
| 3 | Epithelial lifting along villous sides |
| 4 | Denuded villi |
| 5 | Loss of villous tissue |
| 6 | Crypt layer infarction |
| 7 | Transmucosal infarction |
| 8 | Transmural infarction |

**Fig. 12 Ectoine improves intestinal tissue quality evaluated with Park's score grading system.** (A) Animal with vehicle treatment at 3 h after transplantation. (B) Animal with ectoine treatment at 3h after transplantation. (C) Animal with vehicle treatment at 24h after transplantation. (D) Animal with ectoine treatment at 24h after transplantation. (Pech et al. 2012).

Furthermore, Abdel-Aziz and colleagues administered ectoine prophylactically to adult male Wistar rats at different doses via oral gavage once a day during 11 days. 2,4,6-trinitrobenzenesulfonic acid (TNBS) colitis was induced on day 8 of the experiment and on day 12 the rats were euthanized. The protective effects of

ectoine were compared to those obtained with sulfasalazine, an anti-inflammatory drug currently used for the treatment of IBD and other chronic inflammatory disorders such as rheumatoid arthritis and psoriasis. Ectoine protected against weight loss and reduced the area of colonic lesions (Figure 13). Reduction of the levels of some inflammatory mediators such as TNF-α, IL-1β and $PGE_2$ was also significant. The most significant protective effect was observed when ectoine was administered at a dose of 100 mg/kg of body weight. However, further increasing the doses to 200 or 300 mg/kg did not reduced the protective effect observed at lower doses. The reason for this is currently unknown but it indicates that the correct doses will be critical for treating excessive inflammation.

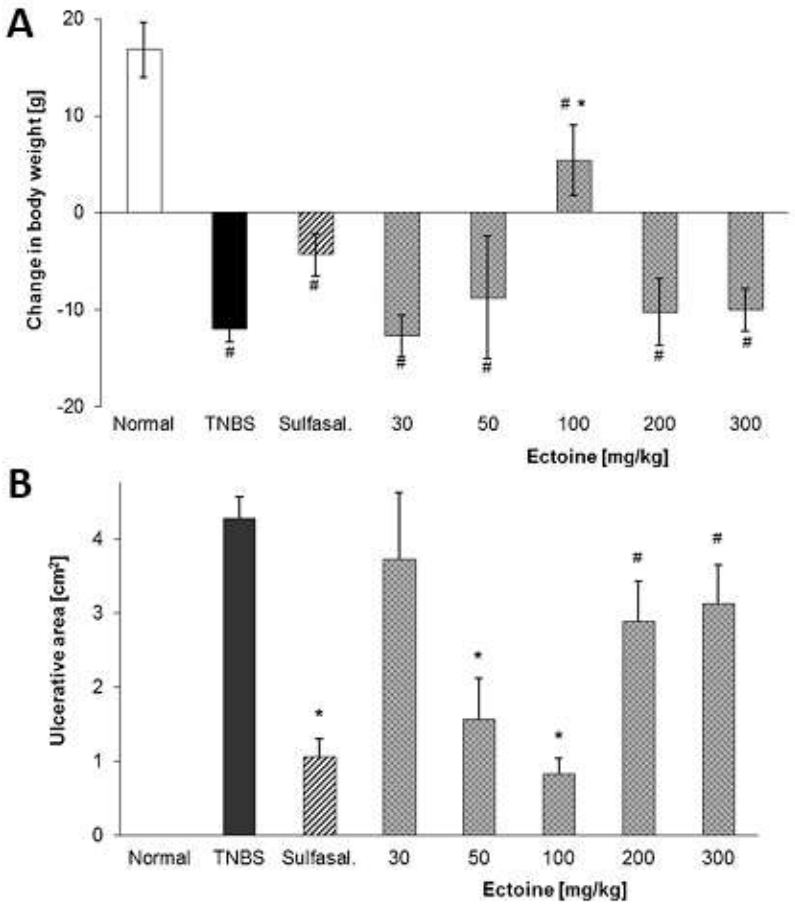



**Fig. 13 Effect of pre-treatment with ectoine on TNBS-induced colitis.** Ectoine and sulfasalazine were administered orally to rats for 11 days, on day 8 TNBS-colitis was induced and rats were sacrificed on day 12. A) Changes in body weight were determined as the difference between baseline weight prior to colitis induction and final weight at the end of the experiment. B) Mucosal damage was assessed by measuring ulcerative area in $cm^2$ (Abdel-Aziz et al. 2013).

Histologically, colon tissues of ectoine pre-treated rats showed reduced lymphocytic infiltration of the submucosa and fewer areas of ulceration compared to the TNBS group and the results were similar to those observed in sulfasalazine-treated rats (Figure 14) (Abdel-Aziz et al. 2013).

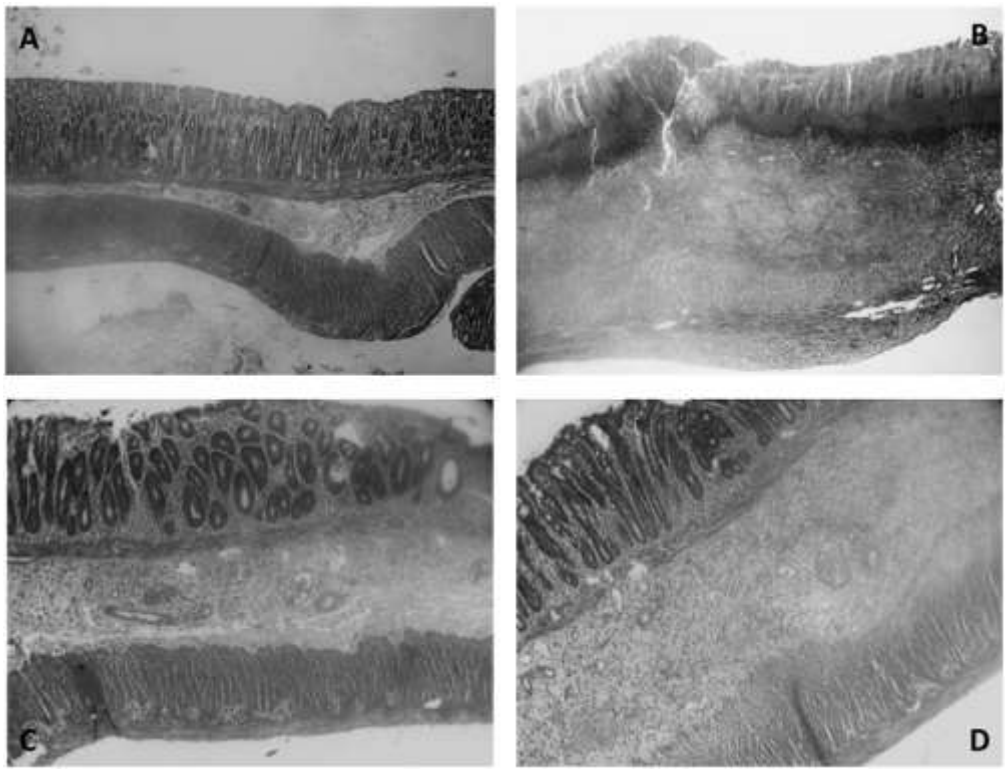



**Fig. 14 TNBS-induced histological damage in the colon is less severe in ectoine pre-treated rats.** A) Normal control group. B) TNBS-colitis group showing necrotic areas and severe infiltration that extends into the muscularis (H & E; magnification, ×60). C) Ectoine pre-treated group showing few areas of ulceration, no necrotic areas, moderate edema and reduction of lymphocytic infiltration. D) Sulfasalazine treated group showing no necrosis, moderate edema and few neutrophilic infiltrations. (H & E; magnification, ×100) (Abdel-Aziz et al. 2013).

The effectiveness of ectoine and hydroxyectoine was also tested by treating an already established TNBS-induced colitis. TNBS colitis was induced in adult male Wistar rats and 48 h post-induction they were treated by oral gavage with ectoine or hydroxyectoine at different doses or sulfasalazine as a reference drug once per day during 1 week.

Ectoine and hydroxyectoine accelerated weight gain and reduced the ulcerative area in the colon thus promoting recovery from colitis (Figure 15). mRNA levels of TNF-α and IL-1β (Figure 16) and reduced levels of myeloperoxidase activity, an important indicator of neutrophilic infiltration of the colon, were observed after treatment with ectoine or hydroxyectoine. (Figure 17) (Abdel-Aziz et al. 2015).

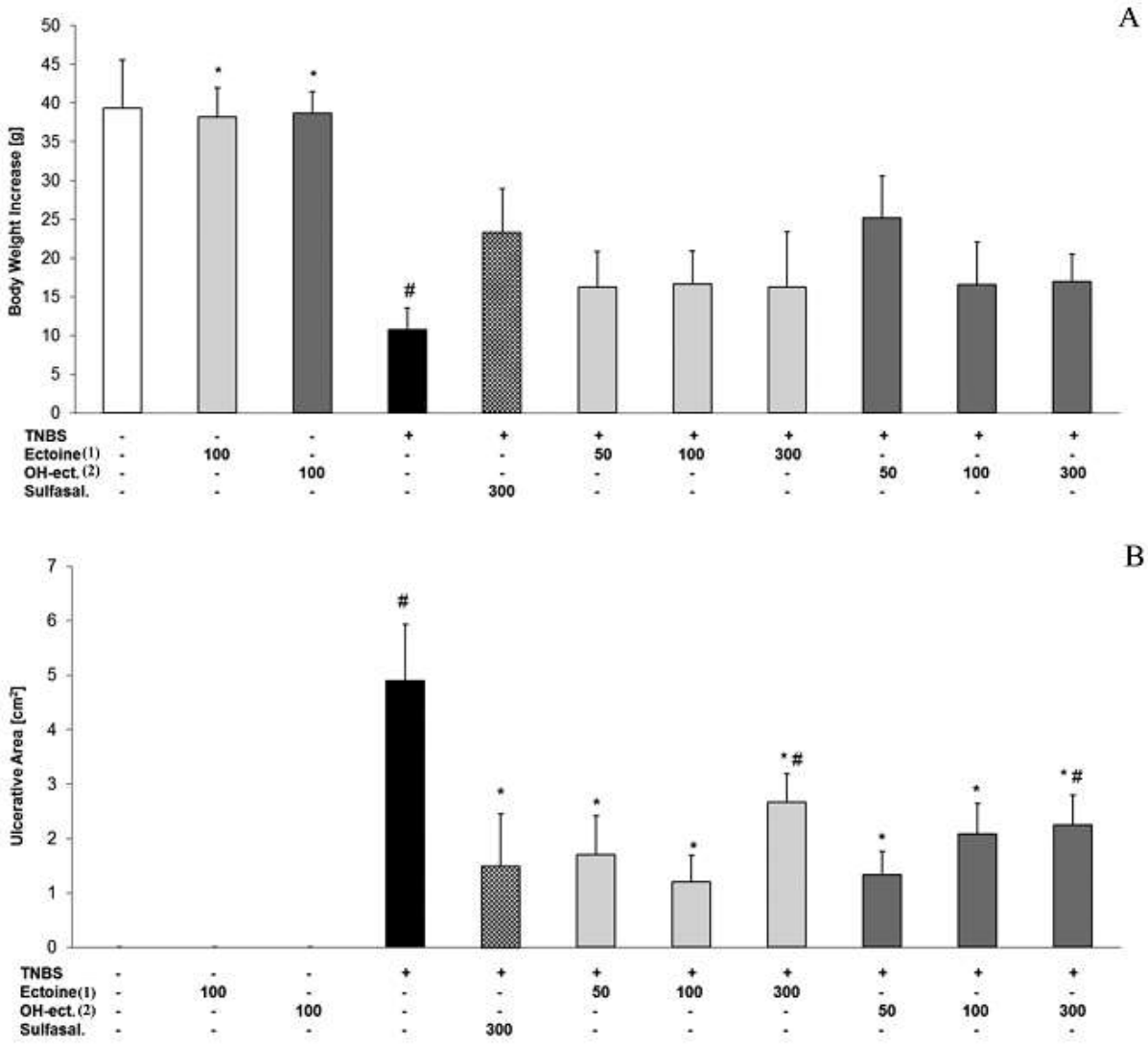



**Fig. 15 Effect of treatment with ectoine or hydroxyectoine on TNBS colitis.** Forty-eight hours after TNBS-colitis induction, rats were treated with ectoine, hydroxyectoine or sulfalazine administered orally once per day for 7 days at the indicated doses [mg/kg]. A) Weight gain and B) Ulcerative area were determined (Abdel-Aziz et al. 2015).

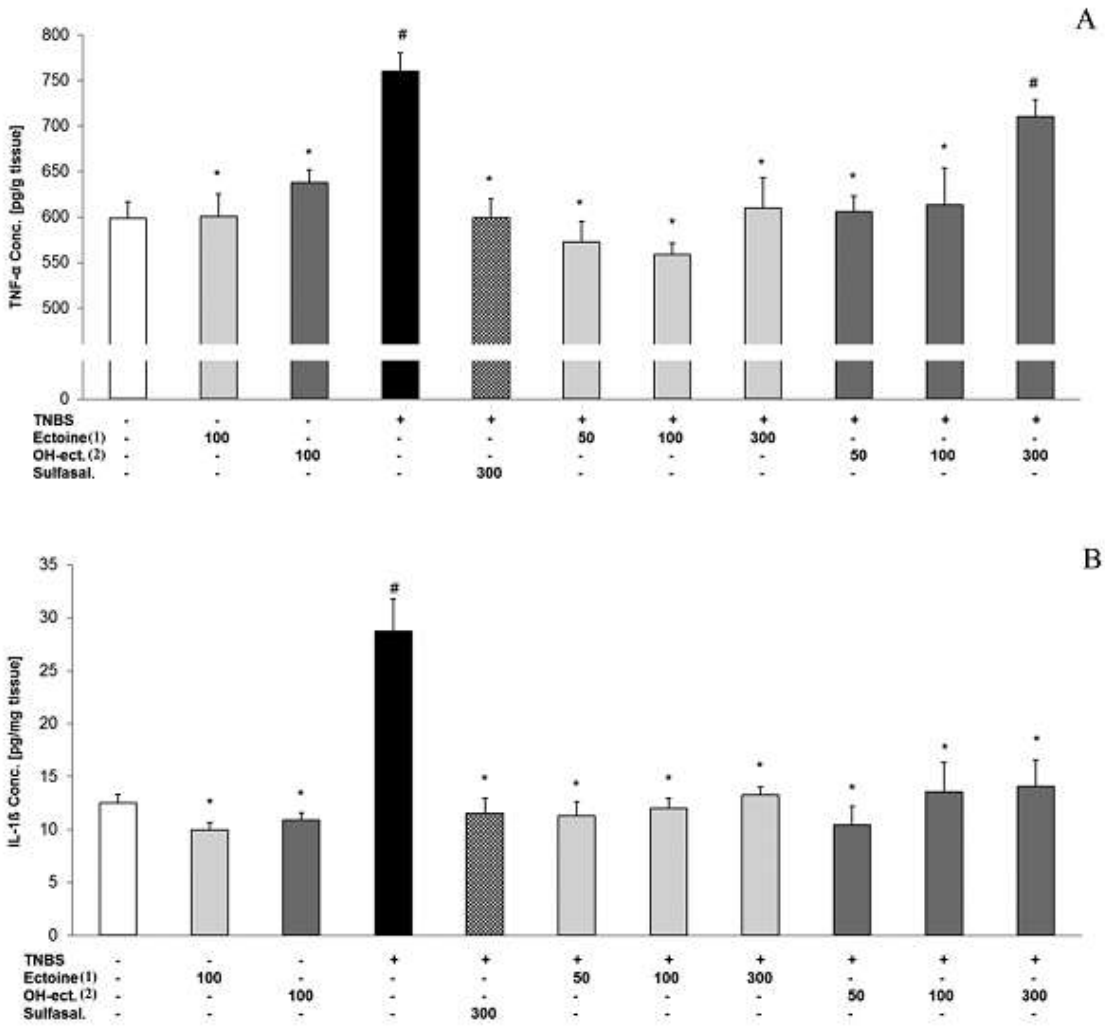



**Fig. 16 Ectoine and hydroxyectoine reduce A) TNF-α and B) IL-1β levels in TNBS-induced colitis.** Forty-eight hours after induction of TNBS-colitis, rats were treated with ectoine, hydroxyectoine or sulfalazine administered orally once daily for 7 days at the indicated doses [mg/kg] (Abdel-Aziz et al. 2015).

Ectoine showed a maximum effect at a dose of 100 mg/kg, as previously reported (Abdel-Aziz et al. 2013), and hydroxyectoine had a maximum effect at a dose of 50 mg/kg and the effects of these compatible solutes were comparable to those obtained with sulfasalazine. Both studies suggest that ectoine and its derivative hydroxyectoine can promote intestinal barrier stabilization; however, the exact mechanisms remain largely unknown.

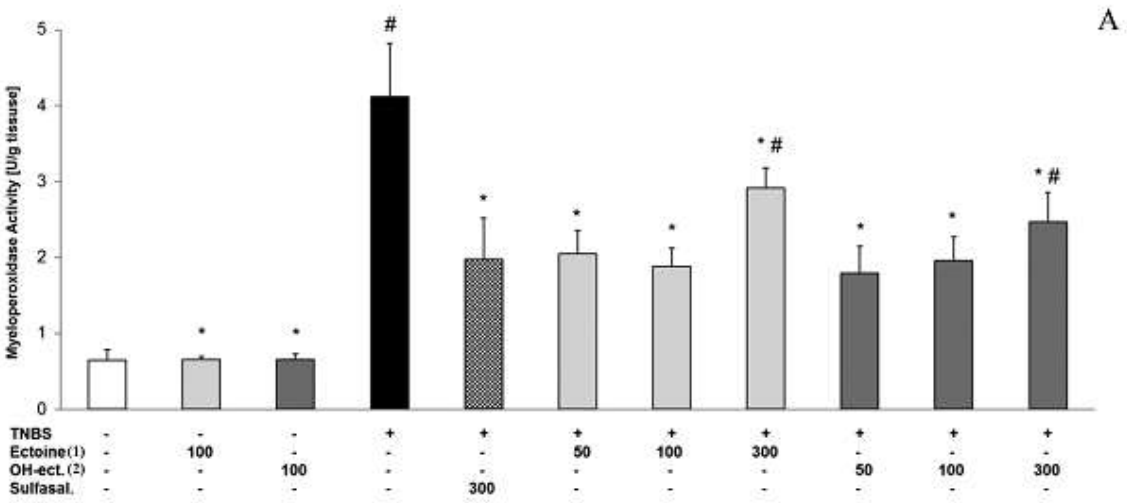



**Fig. 17 Ectoine and hydroxyectoine reduce MPO activity in TNBS-induced colitis.** Forty-eight hours after induction of TNBS-colitis, rats were treated orally with ectoine, hydroxyectoine or sulfalazine once per day for 7 days at the indicated doses [mg/kg] (Abdel-Aziz et al. 2015).

## 2.5 Homoectoine

While protective effects of ectoine and hydroxyectoine have been studied in several models, virtually nothing is known about the synthetic compatible solute homoectoine. Homoectoine is synthetized from ortho-acetic–acid trimethylester and varying diamino carbonic acids, it contains one extra $CH_2$ group in its ring and it can be produced at industrial scale at low cost (Schnoor et al. 2004).

The properties of homoectoine as a PCR enhancer were studied by Schnoor and colleagues. The effects of the compatible solutes betaine, β-hydroxyectoine, L-ectoine and homoectoine on the melting temperatures of GC-poor (SRP14) and GC-rich (COL18A1) templates were compared. They observed that homoectoine lowered melting temperature on both types of templates more efficiently than the other compatible solutes and that salt ions contained in the PCR buffer were not influencing this effect (Table 1).

**Table 1. Effects of compatible solutes on melting temperatures of dsDNA** (Schnoor et al. 2004).

| Compound | 0.25× PCR buffer | | 1× PCR buffer | |
|---|---|---|---|---|
| | SRP14 | COL18A1 | SRP14 | COL18A1 |
| Betaine | −0.75 | −3.6 | −0.65 | −1.45 |
| β-Hydroxyectoine | +0.9 | +0.2 | +0.40 | −1.3 |
| L-Ectoine | −2.4 | −5.05 | −3.15 | −5.4 |
| Homoectoine | −10.1 | −13.4 | −6.75 | −7.05 |

Changes in melting temperature in degrees Celsius per mole

The effects of these compatible solutes were also tested on PCR amplification of GC-rich cDNA. Amplification was more specific and started at lower concentrations of homoectoine compared to the other compatible solutes (Figure 18). These data suggest that homoectoine can stabilizing the interactions between enzymes and their targets more efficiently than ectoine, hydroxyectoine and betaine.

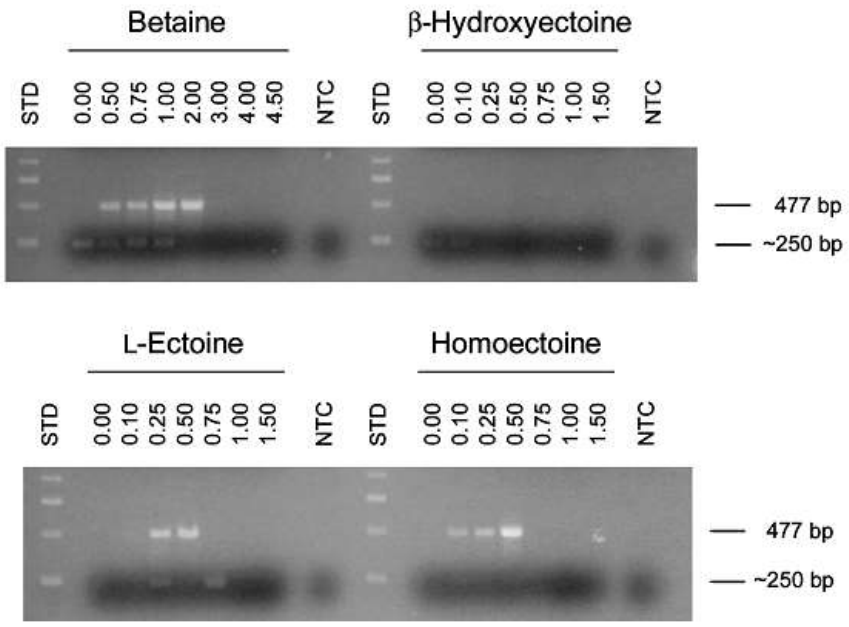



**Fig. 18 Effects of compatible solutes on PCR amplification of GC-rich cDNA.** Betaine, L-ectoine and Homoectoine amplified GC-rich cDNA at different concentrations. However, amplification was more specific with homoectoine and achieved at lower concentrations. However, β-hydroxyectoine was not able to amplify the GC-rich cDNA (Schnoor et al. 2004).

Since ectoine and hydroxyectoine had protective effects during lung inflammation and experimental colitis, we wanted to know whether improved permeability could be a reason for this effect. To this end, we treated TNF-inflamed Caco-2 cell monolayers with different ectoines including homoectoine and measured effects on paracellular permeability. Homoectoine showed a stronger protective effect on barrier function than ectoine and hydroxyectoine *in vitro*. Homoectoine was able to ameliorate the decrease in transepithelial electrical resistance (TEER) induced by the pro-inflammatory cytokines IFN-γ and TNF-α (Figure 19A).

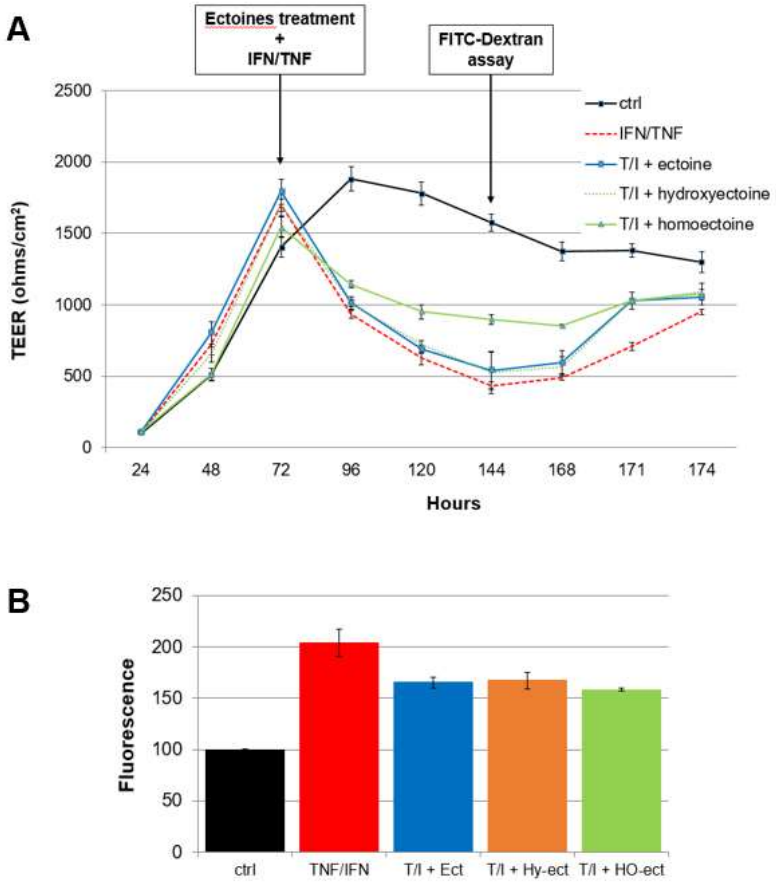



**Fig. 19 Effects of ectoines on ion and macromolecule flux in Caco-2 cells.** Caco-2 cells were grown to confluence on transwell filters. A) Transepithelial electrical resistance (TEER), an indicator of ion flux, was measured every 24 hours. Once cultures reached confluence they received an inflammatory stimulus with 100U/ml IFN-γ and 5nM TNF-α. In parallel, they were treated with 1mM ectoine, 1mM hydroxyectoine, 1mM homoectoine or left untreated. B) Once TEER was established, 0.25 mg/ml 4kDa FITC-Dextran was placed in the upper chamber for 24 h and 100 µl of the lower chamber were measured fluorometrically at 488 nm.

Additionally, paracellular permeability of macromolecules was measured using 0.25 mg/ml 4 kDa FITC-labeled dextran. Reduction of paracellular flux induced by IFN-γ and TNF-α was observed with all ectoines to a similar extent (Figure 19B), suggesting that homoectoine affects both claudin-mediated ion flux and occludin-mediated paracellular flux of macromolecules and that the other ectoines promote occludin but not claudin functions.

## 3. PROBLEM STATEMENT AND JUSTIFICATION

IBD incidence is alarmingly increasing every year worldwide and therapies that enhance intestinal barrier function with anti-inflammatory properties without the adverse effects of current therapies are yet to be developed. Ectoine and hydroxyectoine have shown protective effects in the intestinal, lung and skin epithelium during inflammatory conditions. However, the molecular mechanisms remain largely unknown, but an involvement of junction stabilization seems likely since ectoines can stabilize both transmembrane proteins and lipid bilayers. Given the anti-inflammatory and barrier stabilizing effects of the compatible solutes ectoine and hydroxyectoine and that homoectoine has shown a stronger barrier-stabilizing effect in Caco-2 cells, we speculate that homoectoine may also have stronger anti-inflammatory and barrier stabilizing effects in experimental colitis.

## 4. HYPOTHESIS

Homoectoine and ectoine stabilize tight and adherens junctions to prevent excessive intestinal epithelial permeability during inflammation.

## 5. GENERAL OBJECTIVE

Investigating the effects of homoectoine and ectoine on intestinal epithelial permeability and AJ/TJ architecture under inflammatory conditions *in vivo*.

## 6. PARTICULAR OBJETIVES

1. To unravel whether homoectoine and ectoine reduce disease activity index (DAI) during DSS-induced experimental colitis *in vivo*.
2. To analyze whether homoectoine and ectoine improve mucosal damage and epithelial permeability during DSS-induced experimental colitis *in vivo*.
3. To investigate whether homoectoine and ectoine affect the architecture of TJ and AJ *in vivo*.

# 7. MATERIALS AND METHODS

## 7.1 Materials

### *7.1.2 Chemical reagents*

| **Chemical** | **Company** |
|---|---|
| Dextran Sulfate Sodium (M.W. 40,000-50,000) | Affymetrix |
| Homoectoine and ectoine | Kindly provided by Dr. Galinski University of Bonn, Germany. |
| ColoScreen | Helena Laboratories |
| Ketamine | PiSA Agropecuaria |
| Xylazine | PiSA Agropecuaria |
| Evans Blue | Sigma-Aldrich |
| N-acetylcysteine | Sigma-Aldrich |
| N,N dimethylformamide | J.T. Baker |
| Histosette (Embedding cassette) | Simport |
| 10% formaldehyde | J.T. Baker |
| Tissue-tek. (O.C.T.) | Sakura Finetek |
| Glass Slides | Corning |
| Absolute ethanol | J.T. Baker |
| Tween 20 | Sigma-Aldrich |
| Bovine Serum Albumin | Sigma-Aldrich |
| VECTASHIELD medium | Vector Laboratories |
| DC Protein Assay | BioRad |
| Nitrocellulose membrane, pore 0.45 µm | BioRad |
| SuperSignal® West Pico | ThermoFisher Scientific |
| SuperSignal® West Femto | ThermoFisher Scientific |
| Trizol | ThermoFisher Scientific |
| Chloroform | Sigma-Aldrich |
| Isopropyl alcohol | Sigma-Aldrich |
| 8M Lithium Chloride | Sigma-Aldrich |

| | |
|---|---|
| 3M sodium acetate | Sigma-Aldrich |
| DNase I | Invitrogen |
| SuperScript II Reverse Transcriptase | Invitrogen |
| Taq DNA Polymerase | ThermoFisher Scientific |
| Absolute xylene | J.T. Baker |
| Eosin-Y | J.T. Baker |
| Harris hematoxylin | Sigma-Aldrich |
| Liquid paraffin | Paraplast |
| Lithium carbonate | Sigma-Aldrich |
| Synthetic resin | Poly Mont |
| Hydrochloric acid 1 M | J.T. Baker |
| Plastic cubes | Electron Microscopy |

### *7.1.3 Buffers*

Buffers were prepared in deionized water purified using a Mili-Q-system (Millipore)

| | |
|---|---|
| **PBS** | 138 mM NaCl<br>3 mM KCl<br>8.1 mM Na2HPO4<br>1.5 mM KH2PO4 |
| **PBS-T** | 100ml 10x PBS<br>0.05% Tween20 |
| **IF Blocking buffer** | PBS-T<br>2% BSA |
| **RIPA** | 50 mM TrisHCl pH7.4<br>150 mM NaCl<br>2 mM EDTA<br>1% NP-40<br>0.1% SDS |
| **5X SDS Loading Buffer** | 250 mM Tris-HCl pH6.8<br>10%SDS |

| | |
|---|---|
| | 30% glycerol<br>5% β-mercaptoethanol<br>0.02% bromophenol blue |
| **SDS-page Buffer** | 25 mM Tris<br>192 mM glycine<br>0.1% SDS<br>pH 8.3 |
| **Transfer Buffer** | 20% methanol<br>25 mM Tris<br>192 mM glycine<br>pH 8.3 |
| **TBS** | 150 mM NaCl<br>10 mM Tris<br>pH 8.0 |
| **TBS-T** | 100ml 10X TBS<br>0.1% Tween20 |
| **Blocking Buffer** | TBS-T<br>5% Skim milk |

### *7.1.4 Antibodies*

Primary antibodies:

| Antigen | Source | Company | Catalog number |
|---|---|---|---|
| ZO-1 | Rabbit | Invitrogen | #40-2200 |
| Occludin | Rabbit | Thermo Fisher Scientific | #PA5-20755 |
| Claudin 1 | Rabbit | Thermo Fisher Scientific | #71-7800 |
| Claudin 2 | Rabbit | Thermo Fisher Scientific | # PA5-13334 |
| E-cadherin | Rabbit | Santa Cruz | #sc-7870 |
| β-catenin | Rabbit | Santa Cruz | #sc-7199 |
| γ-Tubulin | Mouse | Sigma-Aldrich | #T6557 |

Secondary antibodies:

| Antigen | Source | Company | Catalog number |
|---|---|---|---|
| anti-mouse IgG-HRP | Goat | Santa Cruz | sc-2005 |
| anti-rabbit IgG-HRP | Goat | Santa Cruz | Sc-2004 |
| Alexa Fluor 488 anti-rabbit IgG (H+L) | Goat | Invitrogen | A11008 |

### *7.1.5 Equipment*

| Equipment | Company |
|---|---|
| Spectrophotometer | Beckman Instruments |
| Nikon Eclipse 80i microscope | Nikon |
| Cryostat | Leica |
| FV-300 | Olympus, Miami, FL |
| Polytron homogenizer | OMNI International Tissue Master 125 |
| ChemiDoc MP System | BioRad |

### *7.1.6 Animals*

Adult male C57BL/6 mice, weighing 20-25g were obtained from the animal facility at CINVESTAV. They were provided with a standard pellet diet and water *ad libitum*. All animal experiments have been approved by the institutional animal care and use committee of Cinvestav.

## 7.2 Methods

### *7.2.1 Induction of colitis*

Sex- and age-matched C57Bl/6 mice were used to induce acute colitis by administration of 3.5% w/v DSS in drinking water *ad libitum* for 7 days.

### *7.2.2 Homoectoine or ectoine treatment*

Homoectoine or ectoine solutions in water (1M stock solutions) were administered by oral gavage once daily at a dose of 100mg/kg in parallel to the induction of DSS colitis (7 days).

### *7.2.3 Assessment of disease severity*

The clinical course of colitis was monitored by a daily disease activity index consisting of the three parameters: weight loss, stool consistency and intestinal bleeding (Mennigen et al. 2009). For each parameter a score of 0-4 was given, so that the maximum disease index would be 12 (Table 2).

Weight Loss percentage

Baseline weight was recorded before starting the experiment. Body weight was measured daily and assigned a score of 0 to 4 according to weight loss percentage which is calculated as the difference in percent between baseline weight and actual weight.

Stool consistency

Stool consistency was determined by observing a fresh stool sample according to the criteria in table 2. A score between 0 to 4 was assigned.

Detection of intestinal bleeding

Presence of fecal occult blood was detected by the guaiac slide test. A fresh stool sample was smeared on one of the test's windows, two drops of developing solution were applied and the result was read after 30 s. Any trace of blue color is positive for fecal occult blood. A score of 0 to 2.5 was assigned depending on signal strength. Visible blood in stool was assigned a score of 3 to 4 depending on the amount of blood present.

**Table 2. Disease activity index score system.** (Mennigen et al. 2009)

| SCORE | WEIGHT LOSS % | STOOL CONSISTENCY | INTESTINAL BLEEDING |
|---|---|---|---|
| **0** | None | Normal | None |
| **1** | 1 – 5 | | |
| **2** | 5 – 10 | Pasty stools | Occult bleeding |
| **3** | 10 – 20 | | |
| **4** | >20 | Diarrhea | Gross Bleeding |

### *7.2.4 In vivo permeability to Evans blue*

Colonic permeability to Evans blue was measured *in vivo*. Animals were anaesthetized by intraperitoneal injection of ketamine/xylazine (100 mg/kg and 13 mg/kg of body weight, respectively) and depth of anesthesia was monitored by the pinch-withdrawal reflex. They were placed in a supine position to perform a laparotomy, the colon was exposed and a small polyethylene tube (G22) was inserted into the proximal segment of the colon ascendens (immediately adjacent to the cecum) and secured by a ligature. PBS was carefully flushed trough the tube to rinse out the feces from the colon. Then Evans blue solution (1.5% w/v in PBS) was instilled into the colon until it reached the anus and was left in place for 15 min. Subsequently, the colon was rinsed with PBS until the washout was clear.

Animals were euthanized by cervical dislocation, the colon was excised, rinsed with abundant PBS, followed by 1ml of 6 mM N-acetylcysteine in PBS to eliminate dye sticking to the colonic mucus. The colon was cut longitudinally and rinsed once more with PBS, and its length and weight were recorded. The whole colon was placed in 2ml N,N-dimethylformamide overnight to extract the Evans blue dye. The dye concentration in the supernatant was determined spectrophotometrically at 610nm and given as extinction per gram colonic tissue.

### *7.2.5 Tissue collection*

Animals were euthanized by cervical dislocation. A laparotomy was performed to expose abdominal cavity, the entire colon was dissected and its weight and length were recorded. Then the colon was carefully flushed with chilled PBS to remove feces and prepared for subsequent experiments as follow:

For histopathological analysis 0.5-1 cm of colon sample was placed inside an embedding cassette and submerged in 5 ml of 10% formaldehyde for 48 h at room temperature for fixation.

For immunofluorescence staining 0.5-1 cm of colon sample was placed inside an aluminum cup (~1.5 $cm^3$) filled with optimal cutting temperature compound (OTC) and frozen at -80°C until further use.

For protein isolation ~100mg of colon sample was obtained, placed inside a tube and snap-frozen in liquid nitrogen and then stored at -80°C until further use.

### *7.2.6 Histopathological analysis*

Colon sections were collected and prepared as described in point 7.2.5, embedded in paraffin and cut in 5 µm sections that were mounted onto glass slides. Tissues were deparaffinized in an incubator at 60°C for 18 h. Then slides were immersed in absolute xylene (1 min), 96% ethanol (1 min), Harris hematoxylin (7 min), acidic alcohol (7 s), lithium carbonate (7 s), 80% ethanol (1 min), Eosin-Y (15 s), 96% ethanol (1 min), absolute ethanol (1 min), absolute ethanol/xylene mix (1 min) and absolute xylene (1 min). Between each step, the slides were washed under tap water. Synthetic resin was added to the samples, covered with coverslips and then dried for 24 h. All steps were carried out at room temperature. Images were taken using a bright-field microscope with 40x magnification to determine tissue damage.

Histological inflammation score was determined by degree of inflammation, extent of inflammation and crypt damage in relation to the percentage of epithelium involved in each slide (Table 3) (Mennigen et al. 2009). The score of the three parameters were added, the total score was multiplied by the percentage involvement factor.

**Table 3. Calculation of histological inflammation score.** (Mennigen et al. 2009)

| SCORE | INFLAMMATION | EXTENT | CRYPT DAMAGE |
|---|---|---|---|
| **0** | None | None | None |
| **1** | Slight | Mucosa | Basal 1/3 lost |
| **2** | Moderate | Mucosa and submucosa | Basal 2/3 lost |
| **3** | Severe | Transmural | Only surface epithelium intact |
| **4** | - | - | Entire crypt and epithelium lost |

| PERCENTAGE INVOLVEMENT % | Factor |
|---|---|
| **0-25** | 1 |
| **26-50** | 2 |
| **51-75** | 3 |
| **76-100** | 4 |

### *7.2.7 Immunofluorescence (IF)*

Colon sections prepared as described in point 7.2.5 were cut in 8 µm thick cryosections at -20°C using a cryostat and mounted on glass slides. Sections were fixed and permeabilized with 96% ethanol for 30 min at -20°C, rinsed with PBS, and blocked for 2 h at room temperature in 1X PBS containing 0.01% Tween and 2% BSA. Colon sections were incubated overnight at 4°C in primary antibodies diluted in PBS. Slides were washed and then incubated for 2 h with Alexa Fluor 488 labeled species-specific secondary antibodies with gentle agitation. Cover slips were mounted in Vecta-Shield medium and analyzed on a confocal laser microscope. Pixel density was determined using ImageJ software.

### *7.2.8 Tissue lysate preparation for western blot*

For ~100mg of tissue 500 µl of ice cold RIPA were added to the tube and homogenized with an electric homogenizer. Then, samples were centrifuged at 4°C for 30 min at 15,000 rpm and supernatants were transferred to fresh tubes. Protein concentration was determined by DC™ Protein Assay. 1X SDS Loading Buffer was added to the sample and the mixture was boiled at 100°C for 5 min.

### *7.2.9 Western blot*

For detection of ZO-1, E-cadherin, β-catenin and occludin, tissue lysates were separated in 8% SDS-PAGE for 120 min at 120 V and transferred to a nitrocellulose membrane (pore size 0.45 µm) during 100 min at 200 mA. The membranes were blocked with TBST containing 5% skim milk for 1 hour at room temperature and incubated overnight in primary antibodies at 4°C with gentle agitation. Membranes were washed with TBS-T 3 times for 10 min each and incubated with species-specific secondary antibodies conjugated to horseradish-peroxidase (HRP) for 1 h at room temperature with gentle agitation. Membranes were washed 3 times for 10 min each, placed between acetate foil and the signals were developed with SuperSignal West Pico or SuperSignal West Femto substrates and imaged with ChemiDoc device. For Claudin-1 and Claudin-2 cell lysates were separated in 15% SDS-PAGE for 120 min at 120 V and subsequently handled as described above. Pixel density was determined using ImageJ software.

### *7.2.10 Statistical Analysis*

Data are represented as the means ± standard error of the mean (SEM) or standard deviation (SD). The significance between groups was assessed by Student's t-test or ANOVA. Analysis was performed using GraphPad Prism software v5.0. Values of probability (P) < 0.05 were considered statistically significant.

## 8. RESULTS

### 8.1. Homoectoine and ectoine attenuated DSS-induced colitis.

Mice suffering from acute colitis present three characteristic clinical signs: severe diarrhea, intestinal bleeding and significant weight loss. To test if homoectoine and ectoine had an effect on clinical progression of colitis, the disease activity index was determined by measuring these parameters as disease activity index (DAI) during the course of DSS-induced colitis (Figure 20). The administration of ectoine and homoectoine alone did not affect DAI, the score remained at 0 during the entire course of the experiment comparable to the untreated control group. By contrast, mice receiving DSS had a progressive DAI increase reaching a maximum score of 11±0.61 by day seven. Both homoectoine and ectoine significantly reduced DAI beginning 5 days after DSS treatment.

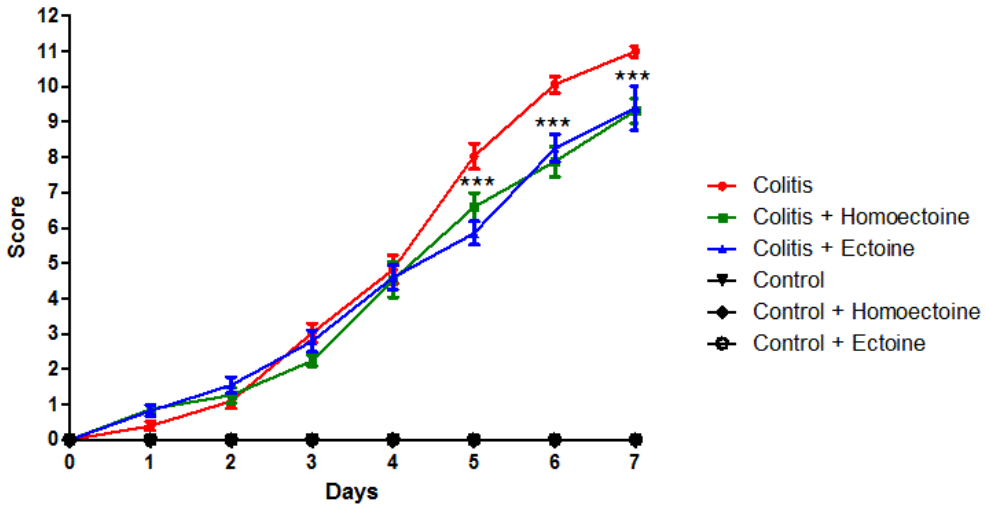



**Fig. 20 Homoectoine and ectoine ameliorated severe colitis.** Disease activity index (DAI) consists of the combination of three parameters: weight loss, stool consistency and intestinal bleeding which were scored daily. Control groups receiving water (n=14), homoectoine (n=4) or ectoine (n=4) maintained a score of 0 during the course of the experiment. DAI of colitic mice co-treated with homoectoine (n=17) or ectoine (n=11) were significantly lower compared to mice treated with DSS alone (n=17). Values given as mean±standard deviation of the mean. ***p<0.001.

### 8.2 Intestinal epithelial permeability was restored to control levels in colitic mice treated with homoectoine and ectoine.

To determine whether homoectoine and ectoine prevent excessive intestinal epithelial permeability during colitis, an Evans blue assay was performed *in vivo* (Figure 21). As expected, the permeability significantly increased in DSS-treated mice in comparison with healthy controls. Importantly, Evans blue uptake in DSS-treated mice that received either homoectoine or ectoine was completely prevented.

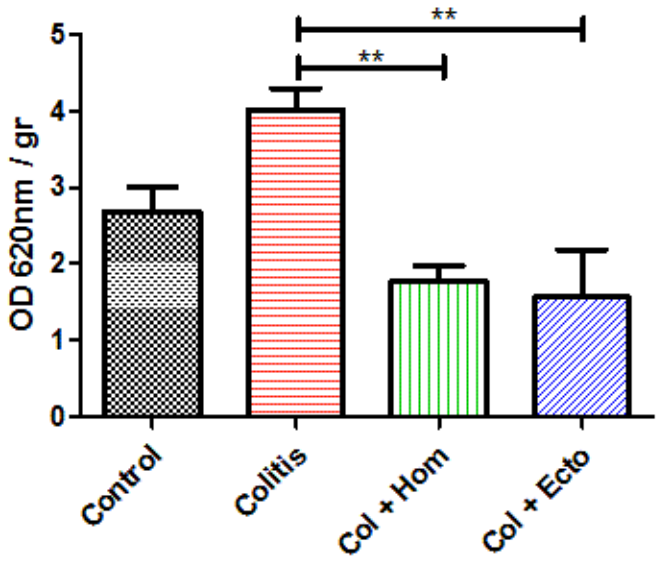



**Fig. 21 The increase of intestinal epithelial permeability during colitis was prevented by homoectoine and ectoine.** An Evans blue-based assay was used to determine intestinal epithelial permeability in the experimental groups: control, colitis, colitis + homoectoine (col+Hom) and colitis + ectoine (col+Ecto). n=4 per group. Values given as mean±standard deviation of the mean. **p<0.01.

### 8.3 Homoectoine and ectoine reduced edema formation, leukocyte influx and tissue damage induced by DSS colitis in C57BL/6 mice.

To determine how homoectoine and ectoine protect against colitis progression, we analyzed tissue morphology by hematoxylin and eosin staining of colon cross sections. Homoectoine and ectoine treatment alone did not significantly affect tissue morphology (not shown). DSS-colitis induced severe edema formation, inflammatory cell infiltration into the mucosa and submucosa, apical erosion and loss of intestinal crypts. Moderate tissue damage was observed in homoectoine and ectoine treated mice, but the surface epithelium remained largely intact with few ulcerative areas (Figure 22). Intestinal crypt depletion was significantly reduced and edema formation was completely prevented. However, some areas with leukocyte infiltration could still be observed.

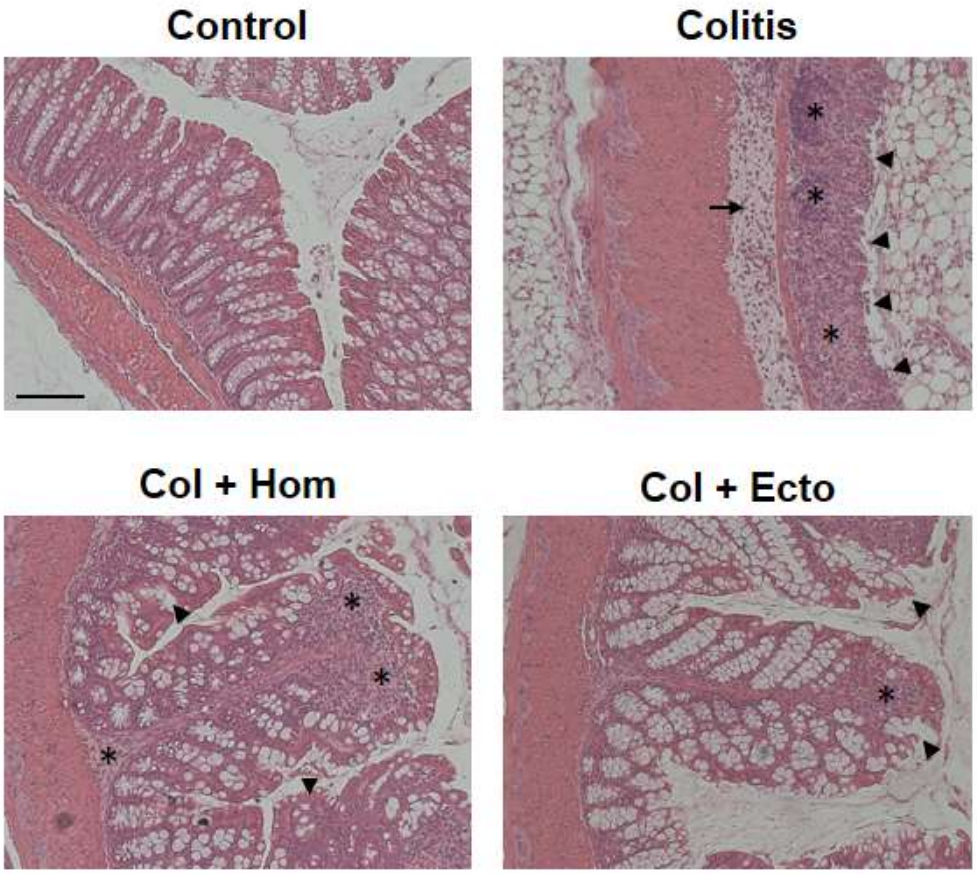



**Fig. 22 DSS-induced tissue damage was less severe in animals co-treated with homoectoine or ectoine.** DSS-colitis was induced in C57Bl/6 mice during 7 days. Homoectoine and ectoine

treatments were administered daily by oral gavage in parallel to colitis induction. Arrows indicate areas of edema; arrowheads indicate apical erosions and asterisks indicate inflammatory cells infiltration. Representative images of 3 independent experimental groups. (H & E; magnification, x40; bar=50µm).

A histological inflammation score was obtained by determining the degree of inflammation, inflammation extent and crypt damage as previously reported (Mennigen et al. 2009). Both compatible solutes significantly reduced the histological inflammation score (Table 4). Interestingly, we observed less severe tissue damage in colitic mice treated with homoectoine compared to ectoine. Homoectoine reduced inflammation and in large part reduced the extent of inflammation and crypt damage, whereas ectoine slightly ameliorated the three features. Furthermore, a high proportion of goblet cells were observed in the colon of mice co-treated with homoectoine and ectoine, despite the induction of acute colitis which usually reduces the number of goblet cells (Gersemann et al. 2009).

**Table 4. Histological inflammation scores.** Hematoxylin and eosin staining of 3 independent tissue preparations were analyzed for the indicated parameters. Scores of control, colitis, colitis + homoectoine, and colitis + ectoine groups are shown. Values are means ± SE. ***p<0.001.

| | CONTROL | COLITIS | COL+HOM | COL+ ECT | *p* VALUE COL vs COL + HOM | *p* VALUE COL vs COL + ECT |
|---|---|---|---|---|---|---|
| **INFLAMMATION** | 0.40 ± 0.13 | 8.71 ± 0.85 | 4.10 ± 0.82 | 5.05 ± 0.85 | *** | *** |
| **EXTENT** | 0.40 ± 0.13 | 11.62 ± 0.26 | 1.65 ± 0.16 | 7.60 ± 0.64 | *** | *** |
| **CRYPT DAMAGE** | 0.35 ± 0.10 | 14.10 ± 0.52 | 1.85 ± 0.22 | 8.80 ± 1.03 | *** | *** |
| **TOTAL SCORE** | 1.15 ± 0.12 | 34.43 ± 0.54 | 7.6 ± 0.40 | 21.45 ± 0.84 | *** | *** |

## 8.4 Colon shortening induced by colitis was prevented by ectoine and homoectoine.

Colon shortening is a characteristic feature of colitis caused by submucosal edema and muscularis mucosa hypertrophy. Given the observed beneficial effects on colon morphology, we wondered whether colon shortening is affected by compatible solutes. Compared with the healthy group (60 mm ±2.04), colons of untreated mice exposed to DSS (43.25 mm ± 1.18) were significantly shortened by 27.92% (Figure 23). Of note, colons of colitic mice treated with homoectoine (54.18 mm ± 1.89) or with ectoine (52.54 ± 0.92) were only shortened by 9.7% and 12.43%, respectively. These results confirm the significant protective effect on tissue morphology and edema formation.

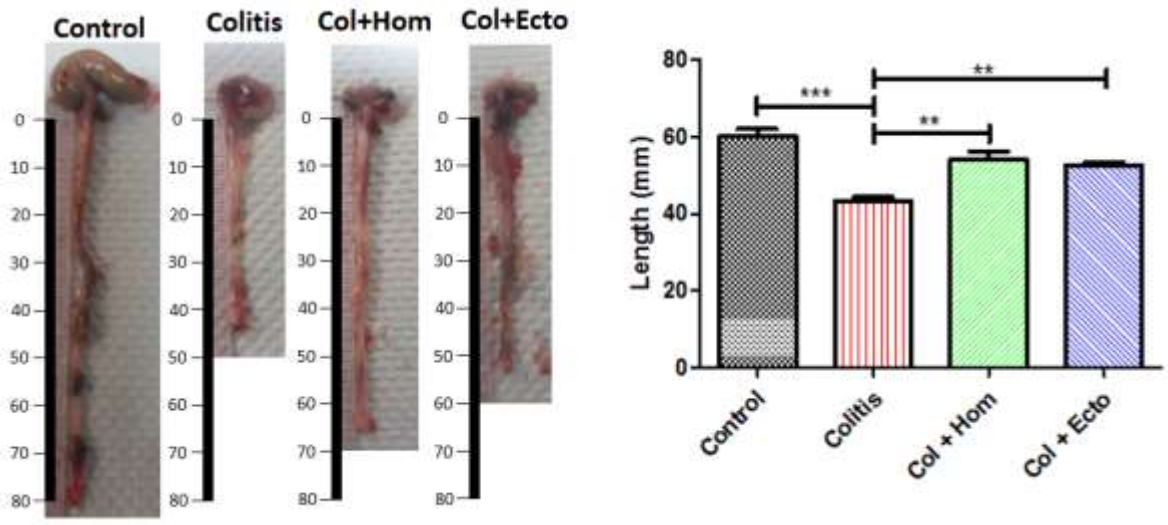



**Fig. 23 Homoectoine and ectoine prevented colon shortening during DSS-induced colitis.** Gross appearance and morphology of colons of healthy mice (60 mm ±2.04) were compared to colons of colitic mice (43.25 mm ± 1.18) and colitic mice treated with homoectoine (54.18 mm ± 1.89) and ectoine (52.54 ± 0.92). n=4 per group. Values given as mean±standard deviation of the mean. **p<0.01, ***p<0.001.

## 8.5 Homoectoine and ectoine prevented changes in expression and distribution of tight and adherens junction proteins during DSS-induced colitis.

Given the observed effect on permeability that is in large part regulated by intercellular junctions, we investigated junction architecture. The effect of homoectoine and ectoine on the expression and distribution of tight and adherens junction proteins during DSS-induced colitis was determined by immunofluorescence staining and western blotting. The TJ proteins ZO-1 and occludin were significantly downregulated during DSS-induced colitis, as expected. Importantly, homoectoine and ectoine prevented this downregulation of ZO-1 and occludin (Figure 24).

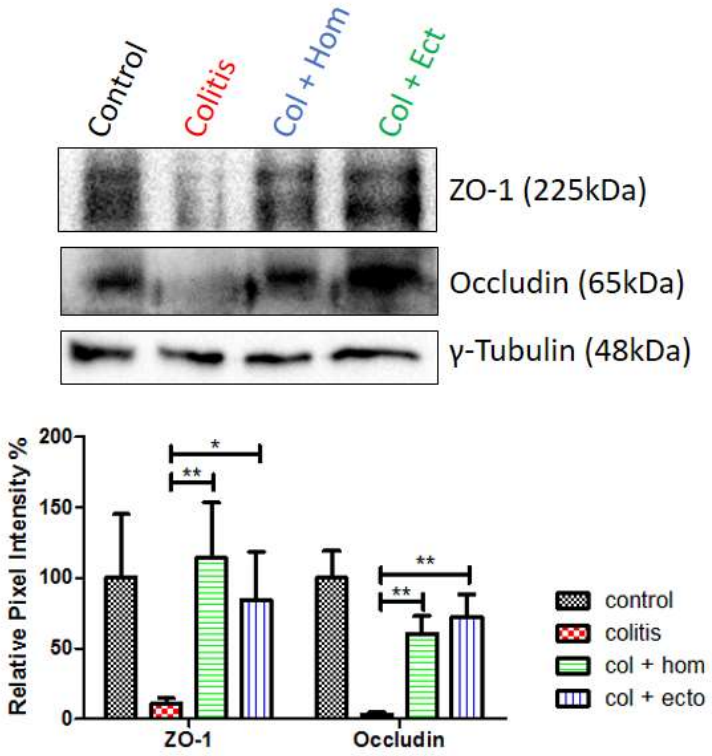



**Fig. 24 ZO-1 and occludin downregulation during DSS-colitis was prevented by homoectoine and ectoine.** ZO-1 and occludin blots from colons of healthy mice, colitic mice and colitic mice treated with homoectoine or ectoine. ZO-1 and occludin were downregulated during DSS-colitis and treatments with both homoectoine and ectoine prevented this downregulation. Images are representative of three independent blots. Values given as mean±standard deviation of the mean. *p<0.05, **p<0.01.

Immunofluorescence analysis confirmed these results. Both ZO-1 and occludin were reduced at cell contacts during colitis but in colitic mice treated with homoectoine localization of both ZO-1 and occludin was maintained at cell contacts suggesting that a better preservation of junction architecture leads to improved intestinal permeability in these mice (Figure 25).

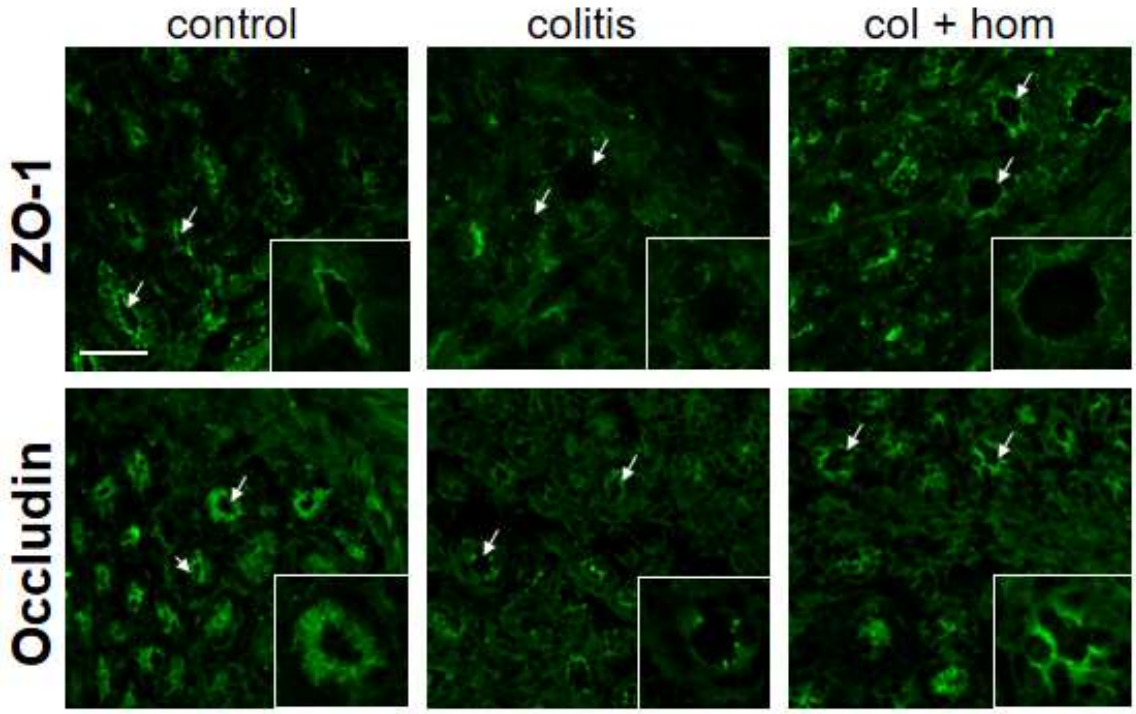



**Fig. 25 ZO-1 and occludin staining decrease during DSS-colitis is prevented by homoectoine.** Images of colon cross sections stained for ZO-1 or occludin of healthy mice, colitic mice and colitic mice treated with homoectoine. Staining intensity is significantly reduced during DSS-colitis, but homoectoine treated colitic mice show a staining intensity similar to the control group. n=3 per group. Arrows indicate apical crypt stainings (Magnifications; x60, and x100, respectively; Bar=50µm).

Claudins play a particular role in barrier function. Claudin-1 has a sealing function, whereas claudin-2 forms paracellular channels promoting permeability of small ions (Hering et al. 2012). To determine changes in claudin regulation, we analyzed claudin-1 and claudin-2 expression by western blot. During DSS-induced colitis,

claudin-1 was downregulated which was clearly prevented in colitic mice treated with homoectoine. However, in mice treated with ectoine, claudin-1 levels were comparable to colitic mice. Claudin-2 was basally expressed at low levels in control groups and its expression was significantly upregulated during DSS-colitis. However, when colitic mice were treated with homoectoine or ectoine claudin-2 was undetectable (Figure 26).

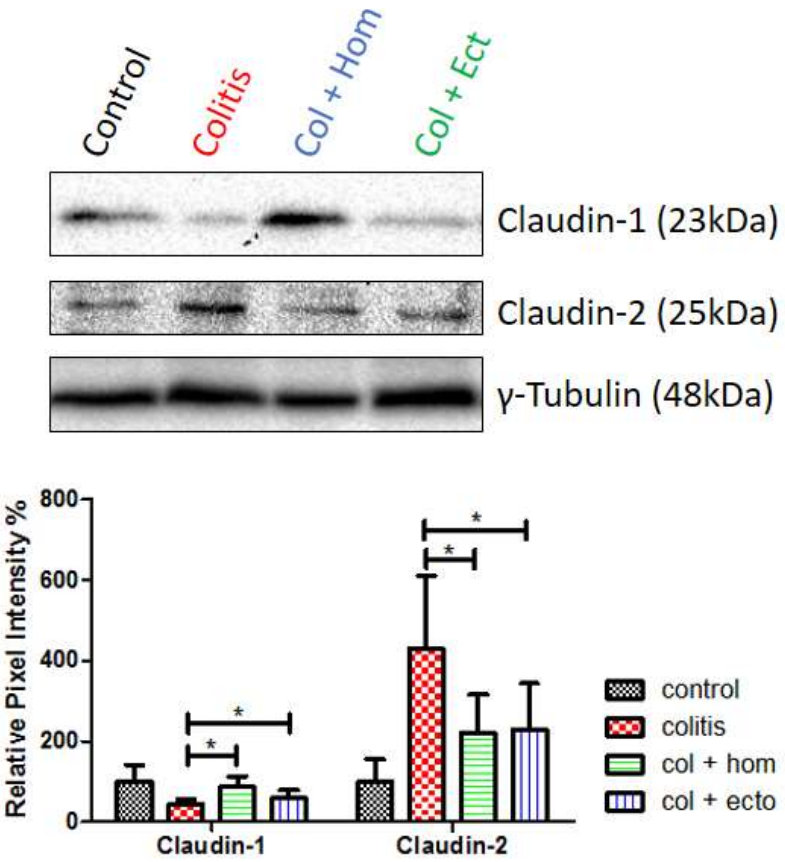



**Fig. 26 The switch of claudin-1 to claudin-2 induced by DSS-colitis was prevented by homoectoine and ectoine treatments.** Claudin-1 and claudin-2 blots from colons of healthy mice, untreated colitic mice and colitic mice treated with homoectoine or ectoine. During DSS-colitis, claudin-1 is downregulated whereas claudin-2 is upregulated. Homoectoine treatment prevented the switch between claudin-1 and claudin-2 completely, whereas ectoin only prevented claudin-2 upregulation. Images are representative of three independent blots. Values are given as mean±standard deviation of the mean. *p<0.05.

To test the effects of homoectoine and ectoine on the architecture of AJ, we analyzed the expression of E-cadherin and β-catenin in colon tissues of the 4 experimental groups. Interestingly, no significant changes were observed in the expression of these proteins by western blot (Figure 27). However, immunofluorescence stainings suggest a redistribution of E-cadherin and β-catenin away from the apical junctions to the cytosol during DSS-colitis. In colon tissues of colitic mice treated with homoectoine this relocalization was prevented (Figure 28).

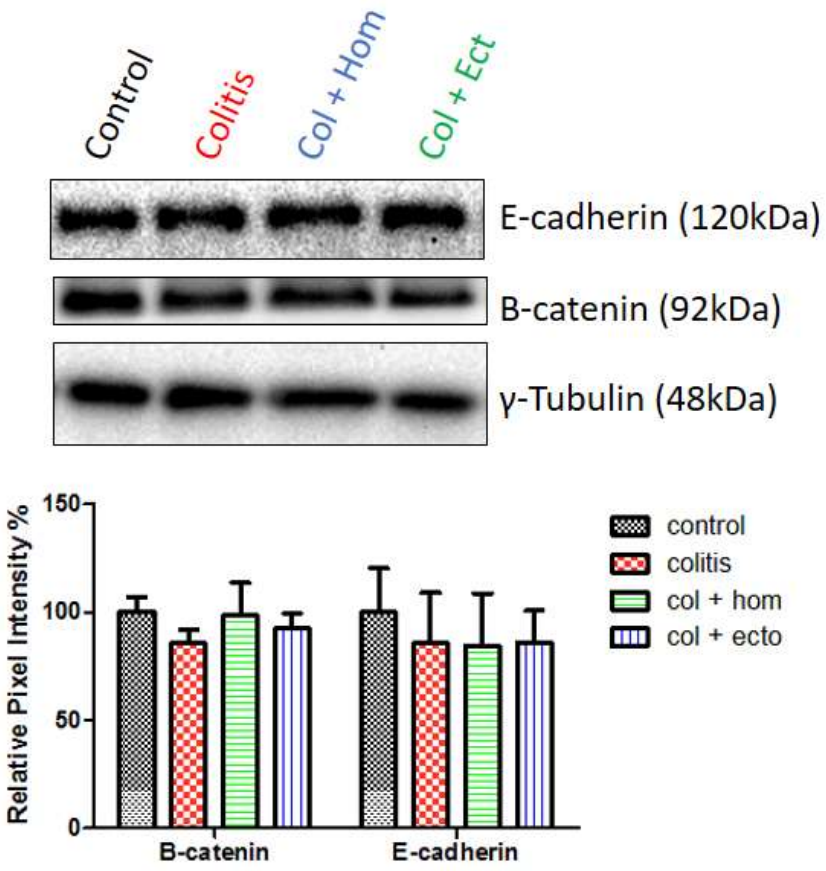



**Fig. 27 Protein levels of the AJ proteins E-cadherin and β-catenin were not significantly affected during DSS-colitis.** E-cadherin and β-catenin blots from colons and of healthy mice, colitic mice and colitic mice treated with homoectoine or ectoine. No significant difference was observed for E-cadherin and β-catenin in the different conditions. Images are representative of three independent blots. Values are given as mean±standard deviation of the mean.

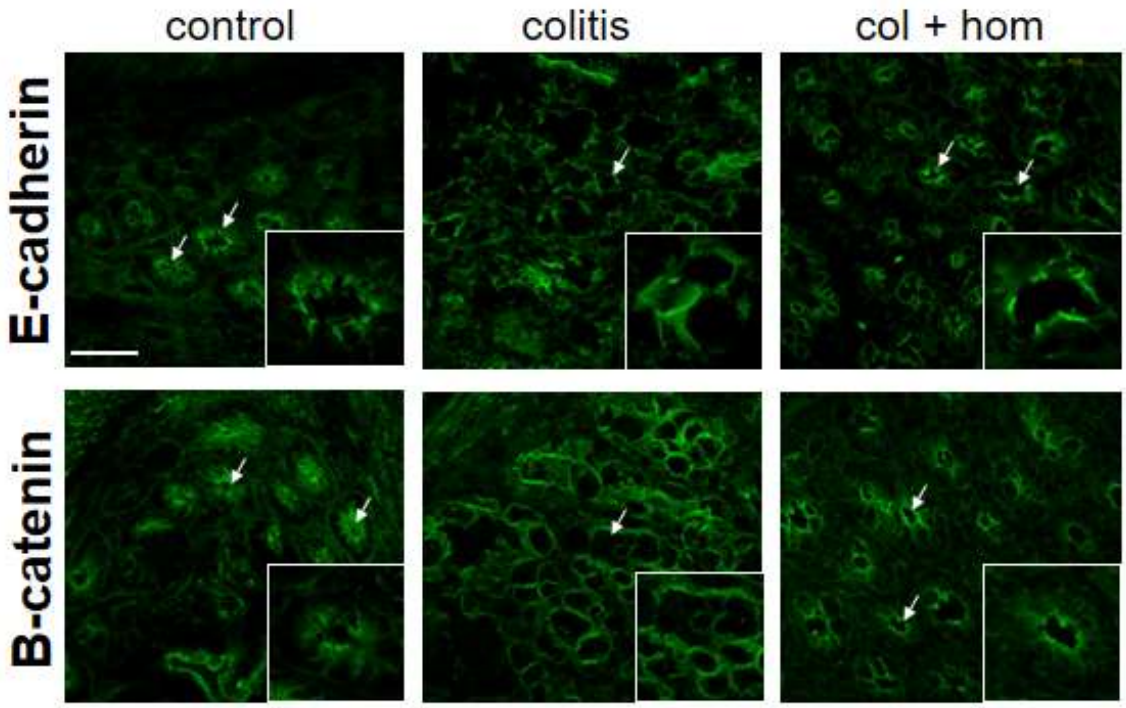



**Fig. 28 E-cadherin and β-catenin internalization during DSS-colitis was prevented by homoectoine.** Images of colon cross sections stained for E-cadherin or β-catenin of healthy mice, colitic mice and colitic mice treated with homoectoine. Arrows indicate epithelial cell contacts within crypts (Magnification, x60, close-up x100; Bar=50µm).

## 9. DISCUSSION

In this thesis, I analyzed the potential beneficial effects of the compatible solutes homoectoine and ectoine during DSS-induced colitis. In particular, I found that these compounds ameliorate DSS-induced tissue damage by reverting excessive intestinal barrier permeability, reducing edema formation, decreasing leukocyte infiltration and stabilizing cell-cell junction architecture.

Ectoine is a compatible solute produced by halophilic microorganisms in response to stressful stimuli that has been shown to have anti-inflammatory properties in different model systems (Pastor et al. 2010). A recent study investigated the effects of ectoine during TNBS-colitis in rats. Administration of this compound previous to the induction of colitis resulted in protection against weight loss, reduction of ulcerative areas in the colon, decrease in pro-inflammatory cytokine levels, reduced MPO activity and less tissue damage. However, it is unknown whether this is also the case in other species using different colitis-inducing agents. DSS colitis is a well-established experimental model often used in mice that resembles histopathological and clinical features observed in human IBD (Perše et al. 2012).

My findings show for the first time that ectoine treatment during DSS colitis diminishes intestinal bleeding and improves stool consistency (Figure 20), however, no effect on weight loss was observed. This could be due to the fact that ectoine was administered in parallel with the induction of acute colitis as compared to a pretreatment used in rats with TNBS-colitis (Abdel-Aziz et al. 2013). This notion is supported by observations of the same group that ectoine did not protect against weight loss when administered as treatment of an already established TNBS-colitis (Abdel-Aziz et al. 2015). Furthermore, ectoine was shown to ameliorate histopathological features of TNBS-colitis: necrosis of the mucosa, severe edema and transmural infiltration, and this effect was comparable to what was observed with sulfasalazine, a drug currently used for IBD treatment. In our model, ectoine treatment led to reduction of edema formation, leukocyte infiltration, inflammation extent and crypt damage. Nevertheless, it will be important to study

whether the administration of ectoine as pretreatment would have even stronger protective effects against DSS-colitis.

By contrast, nothing is known about potential effects of homoectoine during inflammation in general and colitis in particular. Using Caco-2 cells as *in vitro* model of intestinal epithelial barrier, homoectoine showed a stronger protective effect during inflammatory conditions induced by TNF-α and IFN-γ compared to other ectoines. Homoectoine was more efficient in preventing the decreases in resistance and the increase in paracellular flux of 4 kDa FITC dextran. Thus, we also expected a stronger protective effect of homoectoine against DSS-colitis. However, we observed similar results with homoectoine and ectoine in the protection against colitis progression and excessive epithelial permeability. By contrast, homoectoine was more efficient in preventing overall tissue damage than ectoine. Nevertheless, we cannot yet rule out that the different compatible solutes might work more efficiently at different doses. In this work, we only used a dose of 100 mg/kg body weight in our experiments since it has been reported as the optimal dose of ectoine in a different chemical-induced colitis model (Abdel-Aziz et al. 2013). Thus, it will be important to test other concentrations for both ectoine and homoectoine to see whether this could improve the protective effects in the DSS colitis model in mice.

The mechanisms by which ectoine and homoectoine protect against colitis are still largely unknown. In our study, I consistently observed an increase of goblets cells in colitic mice treated with these compatible solutes. Goblet cells are specialized secretory epithelial cells that participate in the maintenance of intestinal epithelial barrier integrity by secreting mucins. Mucins form a protective layer which functions as a physical barrier between the epithelium and the luminal contents (McCauley & Guasch 2015). During ulcerative colitis, the number of goblets cells is reduced and the mucus layer is thinner or completely depleted (Gersemann et al. 2009). Mucin 2 (MUC2) is the principal secretory mucin found in the small and large intestines and it is thought to be essential for epithelial protection. It is stored in goblet cells and is an important goblet cell morphology-determining factor

(Birchenough et al. 2015). The characterization of MUC2 KO mice showed that young animals developed less efficiently due to malnutrition, developed spontaneous colitis as early as 5 weeks of age and were much more susceptible to DSS-induced colitis. Although goblet cells were present in the intestines, their morphology was aberrant (Van der Sluis et al. 2006). By contrast, mucin 4 (MUC4) is a transmembrane mucin which forms part of the glycocalyx on the surface of intestinal epithelial cells and its expression is increased in DSS-treated mice. Interestingly, MUC4 KO mice were more resistant to DSS-induced colitis and MUC2 was significantly upregulated compared to WT mice (Gersemann et al. 2009). Therefore, it will be important to elucidate the expression levels of MUC2 and MUC4 in homoectoine- and ectoine-treated colitic mice. It is tempting to speculate that these compatible solutes could downregulate MUC4 expression, and, as a compensatory mechanism, upregulate MUC2 secretion, thus forming a stronger mucus layer that would protect against translocation of luminal antigens through the epithelial barrier.

Defects in epithelial barrier function have been observed during inflammatory disorders such as IBD (Salim & Söderholm 2011). Excessive intestinal permeability alone is not an onset factor, because it has been observed that asymptomatic first-degree relatives of IBD patients presented increased intestinal permeability without developing the disease (Halme et al. 2006). However, it greatly contributes to barrier dysfunction during inflammation. My results indicate that ectoine and homoectoine protect against permeability increases during DSS-colitis making them interesting therapeutic factors for IBD patients. The epithelium forms a semipermeable barrier which is regulated by intercellular junctions consisting of tight junctions, adherens junctions and desmosomes which require dynamic interactions between their inter- and intra-cellular proteins. These interactions can be affected by many factors such as proinflammatory cytokines. For example, IFN-γ induces internalization of epithelial TJ proteins (Nusrat et al. 2005); immune cells such as dendritic cells open TJ in order to sample bacteria in the lumen (Rescigno et al. 2001); pathogens induce the production of proinflammatory cytokines and some bacteria release proteases that cleave

junction proteins (Nava et al. 2013); drugs like aspirin decrease the expression of claudin-7 (Groschwitz & Hogan 2009). Disruption of junctions leads to the dysregulation of ion and macromolecule fluxes and an increased passage of antigens of the lumen microbiota, thus triggering the inflammatory response. ZO-1, occludin and the family of claudins are key components of the TJ that regulate barrier integrity. ZO-1 is an intercellular protein which binds to the transmembrane proteins occludin and claudins linking them to the actin cytoskeleton. Occludin is mainly involved in the regulation of paracellular flux of macromolecules, whereas claudins permit the selective flux of ions across the barrier (Steed et al. 2010). During DSS-colitis, occludin and ZO-1 levels are significantly reduced until their complete depletion (Li et al. 2014) (Poritz et al. 2007). However, contradictory results have been reported about claudin-1 expression during DSS-colitis. There are reports of claudin-1 increases during inflammatory conditions and it was speculated that this is a compensatory mechanism for the loss of ZO-1. However, other studies report loss of claudin-1 and occludin during DSS-colitis (Mennigen et al. 2009)(Iraha et al. 2013). My results confirm the loss of ZO-1, occludin and claudin-1 during DSS-colitis and I could show that both homoectoine and ectoine prevented the downregulation of these proteins. However, they do it differentially. Ectoine showed a stronger protection against occludin downregulation compared to homoectoine. By contrast, claudin-1 upregulation was only observed with homoectoine. Claudin-2 forms selective pores for small ions and it is normally expressed in leaky epithelia (Amasheh et al. 2002). During inflammatory conditions, claudin-2 expression is upregulated (Ahmad et al. 2014). I confirmed that claudin-2 is basally expressed under normal conditions in the colon and is upregulated during DSS-induced colitis. Of note, both ectoine and homoectoine significantly downregulated the expression of claudin-2 during colitis. These data could explain why both compatible solutes equally reduced intestinal epithelial permeability. These findings may also provide an explanation for our results showing that homoectoine had a stronger stabilizing effect on ion flux permeability (controlled by claudins) than the other ectoines *in vitro*; and that all ectoines similarly reduced paracellular flux of macromolecules (controlled by occludin).

AJ mediate cell proliferation and cell-cell adhesion. They are mainly composed of E-cadherin and members of the catenin family. Loss of E-cadherin in ulcerated mucosa from IBD patients was observed (Mehta et al. 2015). Furthermore, IFN-γ treatment of T84 cells reduced surface expression of E-cadherin and increased its internalization (Smyth et al. 2012). Loss of E-cadherin exacerbated DSS-colitis (Grill et al. 2015) suggesting a role in intestinal epithelial barrier regulation during colitis. During chronic inflammatory conditions, e.g. caused by recurrent administration of DSS, β-catenin translocates to the cytoplasm and/or nucleus (Mehta et al. 2015). However, we observed that in our model of acute DSS-induced colitis, E-cadherin and β-catenin protein levels were unchanged. By contrast, DSS-induced internalization of these proteins was ameliorated by homoectoine. These data demonstrate that compatible solutes of the ectoine family indeed protect epithelial junction architecture thus contributing to the maintenance of barrier integrity during colitis.

Taken together, our data suggest that homoectoine and ectoine ameliorate tissue damage induced by DSS-colitis by stabilizing intestinal epithelial junctions. Combined with previous results showing that ectoine attenuates the expression of pro-inflammatory cytokines (Abdel-Aziz et al. 2013), our data provide a molecular mechanism how ectoines exert their protective effects during colitis. Ectoine is currently used for the treatment of allergic rhinitis (Werkhäuser et al. 2014), conjunctivitis and atopic dermatitis (Marini et al. 2014). At present, no adverse effects have been observed. Our data and those of others clearly demonstrate the beneficial effects of homoectoine and ectoine during acute colitis. Moreover, our data showed that homoectoine and ectoine did not cause negative effects in healthy mice. Taken together, homoectoine and ectoine have the potential to serve as diet supplements for IBD patients to reach or extend phases of remission.

## 10. CONCLUSION

The protective effects of ectoine have been demonstrated in different inflammation models but until now nothing was known about homoectoine. We are the first group to report the beneficial effects of homoectoine and ectoine against DSS-induced colitis. In this study, we observed that both compatible solutes ameliorated intestinal bleeding and diarrhea. Excessive intestinal epithelial permeability was improved equally by both solutes. However, we observed a stronger protective effect on overall tissue damage by homoectoine. Ectoine was more efficient at preventing downregulation of occludin, whereas homoectoine prevented downregulation of claudin-1. Thus, our data proves that both compatible solutes stabilize tight and adherens junctions and in this way prevent excessive intestinal epithelial barrier during inflammation. Ectoines may therefore be interesting candidates for clinical studies to analyze their potential as therapeutic options for IBD patients.

## 11. PERSPECTIVES

- To determine the anti-inflammatory properties of homoectoine and ectoine by evaluating the production of proinflammatory cytokines and chemokines.
- To analyze their effects on other characteristics of colitis such as excessive neutrophil recruitment and oxidative stress.
- To unravel the optimal therapeutic doses of homoectoine and whether it has stronger beneficial effects when given as pre-treatment.
- Given the ability of these compatibles solutes to form hydration shells around biomolecules, it will be interesting to determine if they could be protecting the entire epithelial cell layers like this to reduce DSS uptake.

## 12. REFERENCES


Abdel-Aziz, H. et al., 2015. Bacteria-Derived Compatible Solutes Ectoine and 5α-Hydroxyectoine Act as Intestinal Barrier Stabilizers to Ameliorate Experimental Inflammatory Bowel Disease. *Journal of Natural Products*, p.150528142502005. Available at: http://pubs.acs.org/doi/abs/10.1021/acs.jnatprod.5b00115.

Abdel-Aziz, H. et al., 2013. Novel effects of ectoine, a bacteria-derived natural tetrahydropyrimidine, in experimental colitis. *Phytomedicine*, 20(7), pp.585–591. Available at: http://dx.doi.org/10.1016/j.phymed.2013.01.009.

Abraham, C. & Cho, J.H., 2009. Inflammatory bowel disease. *The New England journal of medicine*, 361(21), pp.2066–2078.

Ahmad, R. et al., 2014. Targeted colonic claudin-2 expression renders resistance to epithelial injury, induces immune suppression, and protects from colitis. *Mucosal Immunology*, 7(6), pp.1340–1353.

Amasheh, S. et al., 2002. Claudin-2 expression induces cation-selective channels in tight junctions of epithelial cells. *Journal of cell science*, 115(Pt 24), pp.4969–4976.

Atreya, I., Atreya, R. & Neurath, M.F., 2008. NF-??B in inflammatory bowel disease. *Journal of Internal Medicine*, 263(6), pp.591–596.

Bier, D.M., 2003. Amino acid pharmacokinetics and safety assessment. *The Journal of nutrition*, 133(6 Suppl 1), p.2034S–2039S.

Birchenough, G.M.H. et al., 2015. New developments in goblet cell mucus secretion and function. *Mucosal immunology*, 8(4), pp.712–9.

Bosques-Padilla, F.J. et al., 2011. Epidemiología y características clínicas de la colitis ulcerosa crónica idiopática en el noreste de México. *Revista de Gastroenterologia de Mexico*, 76(1), pp.34–38.

Buenger, J. & Driller, H., 2004. Ectoin: An effective natural substance to prevent UVA-induced premature photoaging. *Skin Pharmacology and Physiology*, 17(5), pp.232–237.

Citalán-Madrid, A.F. et al., 2013. Small GTPases of the Ras superfamily regulate intestinal epithelial homeostasis and barrier function via common and unique mechanisms. *Tissue barriers*, 1(5), p.e26938. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3942330&tool=pmcentrez

&rendertype=abstract.

Corridoni, D., Arseneau, K.O. & Cominelli, F., 2014. Inflammatory bowel disease. *Immunology Letters*, 161(2), pp.231–235. Available at: http://dx.doi.org/10.1016/j.imlet.2014.04.004.

Coskun, M., 2014. Intestinal Epithelium in Inflammatory Bowel Disease. *Frontiers in Medicine*, 1(August), pp.1–5. Available at: http://journal.frontiersin.org/Journal/10.3389/fmed.2014.00024/full.

Cuthbert, A.P. et al., 2002. The contribution of NOD2 gene mutations to the risk and site of disease in inflammatory bowel disease. *Gastroenterology*, 122(4), pp.867–874.

Danese, S., Sans, M. & Fiocchi, C., 2004. Inflammatory bowel disease: The role of environmental factors. *Autoimmunity Reviews*, 3(5), pp.394–400.

Fletcher, S.J. & Rappoport, J.Z., 2014. Tight junction regulation through vesicle trafficking: bringing cells together. *Biochemical Society transactions*, 42(1), pp.195–200.

Fournier, B.M. & Parkos, C. a, 2012. The role of neutrophils during intestinal inflammation. *Mucosal Immunology*, 5(4), pp.354–366.

Galinski, E. a, Pfeiffer, H.P. & Trüper, H.G., 1985. 1,4,5,6-Tetrahydro-2-methyl-4-pyrimidinecarboxylic acid. A novel cyclic amino acid from halophilic phototrophic bacteria of the genus Ectothiorhodospira. *European journal of biochemistry / FEBS*, 149(1), pp.135–139.

García-Estepa, R. et al., 2006. The ectD gene, which is involved in the synthesis of the compatible solute hydroxyectoine, is essential for thermoprotection of the halophilic bacterium Chromohalobacter salexigens. *Journal of Bacteriology*, 188(11), pp.3774–3784.

Garrod, D. & Chidgey, M., 2008. Desmosome structure, composition and function. *Biochimica et Biophysica Acta - Biomembranes*, 1778(3), pp.572–587.

Gersemann, M. et al., 2009. Differences in goblet cell differentiation between Crohn's disease and ulcerative colitis. *Differentiation*, 77(1), pp.84–94.

Goyette, P. et al., 2007. Molecular pathogenesis of inflammatory bowel disease: genotypes, phenotypes and personalized medicine. *Annals of medicine*, 39(3), pp.177–199.

Graf, R. et al., 2008. The multifunctional role of ectoine as a natural cell protectant. *Clinics*

*in Dermatology*, 26(4), pp.326–333.

Grill, J.I. et al., 2015. Intestinal E-cadherin Deficiency Aggravates Dextran Sodium Sulfate-Induced Colitis. *Digestive diseases and sciences*, 60(4), pp.895–902.

Groschwitz, K.R. & Hogan, S.P., 2009. Intestinal barrier function: Molecular regulation and disease pathogenesis. *Journal of Allergy and Clinical Immunology*, 124(1), pp.3–20.

Halme, L. et al., 2006. Family and twin studies in inflammatory bowel disease. *World Journal of Gastroenterology*, 12(23), pp.3668–3672.

Harishchandra, R.K. et al., 2010. The effect of compatible solute ectoines on the structural organization of lipid monolayer and bilayer membranes. *Biophysical Chemistry*, 150(1-3), pp.37–46. Available at: http://dx.doi.org/10.1016/j.bpc.2010.02.007.

Held, C., Neuhaus, T. & Sadowski, G., 2010. Compatible solutes: Thermodynamic properties and biological impact of ectoines and prolines. *Biophysical Chemistry*, 152(1-3), pp.28–39. Available at: http://dx.doi.org/10.1016/j.bpc.2010.07.003.

Hering, N. a, Fromm, M. & Schulzke, J.-D., 2012. Determinants of colonic barrier function in inflammatory bowel disease and potential therapeutics. *The Journal of Physiology*, 590(5), pp.1035–1044. Available at: http://jp.physoc.org/content/590/5/1035.abstract\nhttp://jp.physoc.org/content/590/5/1035.full.pdf.

Ibd, M. et al., 2012. Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. *Nature*, 490(7422), pp.119–124. Available at: http://dx.doi.org/10.1038/nature11582.

Iraha, A. et al., 2013. Fucoidan enhances intestinal barrier function by upregulating the expression of claudin-1. *World Journal of Gastroenterology*, 19(33), pp.5500–5507.

Kolp, S. et al., 2006. Compatible solutes as protectants for zymogens against proteolysis. *Biochimica et Biophysica Acta - Proteins and Proteomics*, 1764(7), pp.1234–1242.

Kunkel, S.L. et al., 1988. Prostaglandin E2 regulates macrophage-derived tumor necrosis factor gene expression. *The Journal of biological chemistry*, 263(11), pp.5380–5384.

Lee, S.H., 2015. Intestinal Permeability Regulation by Tight Junction: Implication on Inflammatory Bowel Diseases. *Intestinal Research*, 13(1), p.11. Available at: http://synapse.koreamed.org/DOIx.php?id=10.5217/ir.2015.13.1.11.

Li, X.X. et al., 2014. Somatostatin regulates tight junction proteins expression in colitis

mice. *International Journal of Clinical and Experimental Pathology*, 7(5), pp.2153–2162.

Loftus, E. V. & Sandborn, W.J., 2002. Epidemiology of inflammatory bowel disease. *Gastroenterology Clinics of North America*, 31(1), pp.1–20.

Marini, a. et al., 2014. Ectoine-containing cream in the treatment of mild to moderate atopic dermatitis: A randomised, comparator-controlled, intra-individual double-blind, multi-center trial. *Skin Pharmacology and Physiology*, 27(2), pp.57–65.

McCauley, H. a. & Guasch, G., 2015. Three cheers for the goblet cell: maintaining homeostasis in mucosal epithelia. *Trends in Molecular Medicine*, 21(8), pp.1–12.

Mehta, S. et al., 2015. Defects in the adherens junction complex (E-cadherin/ β-catenin) in inflammatory bowel disease. *Cell and Tissue Research*, 360(3), pp.749–760.

Mennigen, R. et al., 2009. Probiotic mixture VSL#3 protects the epithelial barrier by maintaining tight junction protein expression and preventing apoptosis in a murine model of colitis. *American journal of physiology. Gastrointestinal and liver physiology*, 296(5), pp.G1140–G1149.

Morimoto, S. et al., 2005. Rab13 mediates the continuous endocytic recycling of occludin to the cell surface. *Journal of Biological Chemistry*, 280(3), pp.2220–2228.

Nava, P., Kamekura, R. & Nusrat, A., 2013. Cleavage of transmembrane junction proteins and their role in regulating epithelial homeostasis. *Tissue barriers*, 1(2), p.e24783.

Nguyen, G.C., 2011. Editorial: bugs and drugs: insights into the pathogenesis of inflammatory bowel disease. *The American journal of gastroenterology*, 106(12), pp.2143–5. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22138941.

Nusrat, A. et al., 2005. Interferon-gamma induces internalization of epithelial tight junction proteins via a macropinocytosis-like process. *The FASEB Journal*, 19(8), pp.923–933.

Pastor, J.M. et al., 2010. Ectoines in cell stress protection: Uses and biotechnological production. *Biotechnology Advances*, 28(6), pp.782–801. Available at: http://dx.doi.org/10.1016/j.biotechadv.2010.06.005.

Pech, T. et al., 2012. A natural tetrahydropyrimidine, ectoine, ameliorates ischemia reperfusion injury after intestinal transplantation in rats. *Pathobiology*, 80(2), pp.102–110.

Perše, M. et al., 2012. Dextran sodium sulphate colitis mouse model: Traps and tricks. *Journal of Biomedicine and Biotechnology*, 2012.

Poritz, L.S. et al., 2007. Loss of the Tight Junction Protein ZO-1 in Dextran Sulfate Sodium Induced Colitis. *Journal of Surgical Research*, 140(1), pp.12–19.

Rescigno, M. et al., 2001. Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. *Nature immunology*, 2(4), pp.361–367.

Roychoudhury, A., Haussinger, D. & Oesterhelt, F., 2012. Effect of the Compatible Solute Ectoine on the Stability of the Membrane Proteins. *Protein and Peptide Letters*, 19(8), pp.791–794.

Salim, S.Y. & Söderholm, J.D., 2011. Importance of disrupted intestinal barrier in inflammatory bowel diseases. *Inflammatory Bowel Diseases*, 17(1), pp.362–381.

Sawada, N., 2013. Tight junction-related human diseases. *Pathology International*, 63(1), pp.1–12.

Sawada, N. et al., 2003. Tight junctions and human diseases. *Medical Electron Microscopy*, 36(3), pp.147–156.

Schnoor, M. et al., 2004. Characterization of the synthetic compatible solute homoectoine as a potent PCR enhancer. *Biochemical and Biophysical Research Communications*, 322(3), pp.867–872.

Van der Sluis, M. et al., 2006. Muc2-Deficient Mice Spontaneously Develop Colitis, Indicating That MUC2 Is Critical for Colonic Protection. *Gastroenterology*, 131(1), pp.117–129.

Smiatek, J. et al., 2012. Properties of compatible solutes in aqueous solution. *Biophysical Chemistry*, 160(1), pp.62–68.

Smyth, D. et al., 2012. Reduced surface expression of epithelial e-cadherin evoked by interferon-gamma is fyn kinase-dependent. *PLoS ONE*, 7(6).

Steed, E., Balda, M.S. & Matter, K., 2010. Dynamics and functions of tight junctions. *Trends in Cell Biology*, 20(3), pp.142–149. Available at: http://dx.doi.org/10.1016/j.tcb.2009.12.002.

Suzuki, T., 2012. Regulation of intestinal epithelial permeability by tight junctions. *Cellular and Molecular Life Sciences*, pp.631–659.

Sydlik, U. et al., 2009. The compatible solute ectoine protects against nanoparticle-

induced neutrophilic lung inflammation. *American Journal of Respiratory and Critical Care Medicine*, 180(1), pp.29–35.

Turner, J.R., 2009. Intestinal mucosal barrier function in health and disease. *Nature reviews. Immunology*, 9(11), pp.799–809. Available at: http://dx.doi.org/10.1038/nri2653.

Turner, J.R., 2006. Molecular basis of epithelial barrier regulation: from basic mechanisms to clinical application. *The American journal of pathology*, 169(6), pp.1901–1909.

Utech, M., Mennigen, R. & Bruewer, M., 2010. Endocytosis and recycling of tight junction proteins in inflammation. *Journal of Biomedicine and Biotechnology*, 2010.

Werkhäuser, N., Bilstein, A. & Sonnemann, U., 2014. Treatment of Allergic Rhinitis with Ectoine Containing Nasal Spray and Eye Drops in Comparison with Azelastine Containing Nasal Spray and Eye Drops or with Cromoglycic Acid Containing Nasal Spray. , 2014.

Wu, G.D., Bushmanc, F.D. & Lewis, J.D., 2013. Diet, the human gut microbiota, and IBD. *Anaerobe*, 24, pp.117–120.

Xavier, R.J. & Podolsky, D.K., 2007. Unravelling the pathogenesis of inflammatory bowel disease. *Nature*, 448(7152), pp.427–434.

Yamamoto, T., 2013. Nutrition and diet in inflammatory bowel disease. *Current Opinion in Gastroenterology*, 29(2), pp.216–221.

Yamamoto-Furusho, J.K., 2009. Clinical epidemiology of ulcerative colitis in Mexico: a single hospital-based study in a 20-year period (1987-2006). *Journal of clinical gastroenterology*, 43(3), pp.221–224.

Zhang, Y.-Z., 2014. Inflammatory bowel disease: Pathogenesis. *World Journal of Gastroenterology*, 20(1), p.91. Available at: http://www.wjgnet.com/1007-9327/full/v20/i1/91.htm.