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Characterization of bacterial heme oxygenase ChuS of probiotic strain *E. coli* Nissle 1917 and its potential therapeutic uses

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ABBREVIATIONS

Amp: Ampicilin

BV: Biliverdin

CFU: Colony forming units

Cm: Cloranphenicol

CO: Carbon Monoxide

CoPP: Cobalt protoporphyrin X

DC: Dendritic cells

DNA: Desoxiribonucleic acid

EcN: *Escherichia coli* Nissle 1917

EHEC: *Escherichia coli* O157:H7

FRT: Flippase recognition target

Gn: Gentamicin

HK: Heat killed

HO: Heme oxygenase

HO-1: Heme oxygenase 1

IFN-γ: Interferon gamma

IL-10: Interleukin 10

IL-12: Interleukin 12

IL-17: Interleukin 17

IL-1β: Interleukin 1 beta

IL-2: Interleukin 2

IL-4: Interleukin 4

IL-6 Interleukin 6

Kan: Kanamycin

LPS: Lipopolysaccharide

MAPK: Mitogen-activated protein kinase

MLN: Mesenteric Lymph node

MOI: Multiplicity of infection

MSDC: Myeloid suppressor derived cells

NFKB: Nuclear factor kappa-light-chain-enhancer of activated B cells

OVA: Ovalbumin

RNA: Ribonucleic acid

SEN: *Salmonella enterica* serovar Enteritidis

SnPP: Tin protoporphyrin

STM: *Salmonella enterica* serovar Typhimurium

TIP dendritic cells: TNF/iNOS - producing dendritic cells

TJ: Tight junction

TNF-α: Tumor necrosis factor alpha

ABSTRACT

Heme-oxygenase (HO) is an enzyme that catalyzes the metabolism of heme group. Products derived from this reaction are biliverdin (Bv), carbon monoxide (CO), and iron (Fe^{++}). Many properties have been described for this enzyme, such as antioxidant, anti-proliferative, anti-apoptotic, and anti-inflammatory. Most of them are related with CO production, which is able to attenuate the immune response by reducing immunogenicity of dendritic cells and modulating the T cells populations. HO was initially associated with mammal cells (HO-1) specially in humans, but some studies have identified a bacterial heme oxygenase. Some *E. coli* strains possess *chuS* gene that encodes bacterial HO ChuS and it is located into an operon that regulates the heme uptake with the subsequent CO production. On the other hand, lately microbiota influence on immune regulation have reached a high importance due to its capability to modulate directly and indirectly the function of immune cells. Indeed, as part of microbiota, probiotics play a key role promoting anti-inflammatory environments, acting against pathogens, modulating barrier function, even ameliorating autoimmune diseases. In that sense, *E. coli* Nissle 1917 (EcN), is a widely used commercial probiotic, originally used to treat bowel inflammation. Interestingly, *chuS* gene is present in its genome. The focus of this project is to assess whether anti-inflammatory properties of probiotic strain *E. coli* Nissle 1917 (EcN) are due to the presence of bacterial heme-oxygenase enzyme (ChuS) produced by this bacterium. For that, we developed a mutant strain which lacks *chuS* gene. The first part of the project was directed to evaluate both strains (WT and mutant) in DC and macrophages *in vitro*

cultures, infected with different MOI of bacteria. The results of the first part evidenced that both strains were able to activate and induce maturation of DC and macrophages, despite the mutation. Interestingly, WT bacteria induced higher levels of PD-L2, a tolerance marker, than mutant strain. Additionally, IL-10 secretion showed the same pattern. Furthermore, results from antigen presenting assay with DC infected with the probiotic strains showed that T cells were activated and that the production of IL-4 was reduced in the mutant probiotic group. The second part of the project was focused on evaluating ChuS role *in vivo*. For this, we treated mice with different doses of both probiotics. The first assay evaluated the higher dose tolerated by mice, which was $1*10^9$ CFU. After probiotic treatment, a group of mice were euthanized, and we found that T cells population in liver decreased in mice treated with the mutant strain. Mice were then infected with *Salmonella enterica* serovar Enteritidis (SEN) to assess whether treatment could affect in some way the outcome of the infection. After 48 h of infection, all mice were euthanized. Interestingly, we found bacterial load in spleen, liver and MLN in mice treated with mutant probiotic and infected with SEN. T cell populations showed differences between groups infected treated with mutant probiotic and WT strain. Also, B cells population in MLN showed to be higher for mice treated with mutant strain. Moreover, 4 additional doses were evaluated for each strain ($1*10^4$, $1*10^5$, $1*10^6$, and $1*10^7$ CFU). This assay allowed us to assess differences between strains in inflammation patterns and cytokine profile. For instance, mice from groups of all doses for mutant strain presented inflammation in colon sections and mucus in feces or soft feces. Also, IL-10 production, detected in blood, was higher for mice treated with WT probiotic that those treated with mutant strain. Besides. IL-12p70 remained low for

lower doses and increased for higher doses, for groups of mice treated with the WT probiotic. In the case of mice treated with mutant strain IL-12p70 levels were always lower than IL-10. Together, results allowed us to suggest that ChuS apparently has ability to modulate EcN immunomodulatory properties *in vitro* and *in vivo*. Which behave similar to HO-1, modulating IL-10 and IL-12p70 profile. Also, effect of EcN and ChuS is not only local but systemic, because we were able to detect differences in spleen, liver, MLN and blood.

RESUMEN

La Hemoxigenasa (HO) es una enzima que cataliza el metabolismo del grupo hemo. Los productos derivados de la reacción son biliverdina (Bv), monóxido de carbono (CO) y hierro (Fe++). Varias propiedades han sido descritas para esta enzima, tales como antioxidante, anti-proliferativa, anti-apoptótica y anti-inflamatoria. La mayoría de ellas están relacionadas con la producción de CO, el cual es capaz de atenuar la respuesta inmune mediante la reducción de la inmunogenicidad de las células dendríticas y la modulación de las poblaciones de células T. Esta enzima se asoció inicialmente con células de mamíferos, especialmente humanos (HO-1), pero algunos estudios han identificado hemoxigenasas bacterianas. Algunas cepas de E. coli tienen el gen chuS que codifica para una HO bacteriana llamada ChuS, que está localizada en un operón que regula la captación y absorción del grupo hemo, con la subsecuente producción de CO. Por otro lado, últimamente la influencia de la microbiota en la regulación del sistema inmune ha alcanzado mucha importancia, dada su capacidad de modular directa e indirectamente la función de las células inmunes. Es más, como parte de la microbiota, los probióticos tienen un rol clave promoviendo ambientes anti-inflamatorios, actuando en contra los patógenos, modulando la función de barrera, incluso mejorando enfermedades autoinmunes. En ese sentido, E. coli Nissle 1917 (EcN) es un probiótico comercial ampliamente utilizado, originalmente enfocado al tratamiento de la inflamación intestinal. De manera interesante, el gen chuS está presente en el genoma de esta bacteria. El propósito de este proyecto es evaluar si las propiedades anti-inflamatorias de la cepa probiótica EcN están dadas por la

presencia de la hemoxigenasa bacteriana (ChuS) producida por esta bacteria. Para esto, desarrollamos una cepa mutante que carece de gen chuS. La primera parte del proyecto está dirigida a evaluar ambas cepas (WT y la mutante) en cultivos in vitro de DC y macrófagos, con diferentes MOI de la bacteria. Los resultados de la primera parte evidenciaron que ambas cepas son capaces de activar e inducir la maduración de DC y macrófagos, a pesar de la mutación de una de las cepas. LA segunda parte del proyecto estuvo enfocada en evaluar el rol de ChuS in vivo. Para esto, tratamos a ratones con ambas cepas probióticas. El primer ensayo evaluó la dosis más alta tolerada por ratones, que fue 1*109 CFU. Después del tratamiento con probióticos, se practicó eutanasia a un grupo de ratones, los resultados mostraron que la población de células T en el hígado disminuyó en los ratones tratados con la cepa mutante. A continuación, los ratones fueron infectados con Salmonella entérica serovar Enteritidis (SEN) para evaluar si el tratamiento pudiese afectar de alguna manera el resultado de la infección. Después de 48 h de infección, se practicó eutanasia a todos los ratones. De manera interesante, encontramos carga bacteriana en bazo, hígado, y nódulos linfáticos mesentéricos (MLN), en los ratones que fueron tratados con la cepa probiótica mutante y luego infectados con SEN. Además, entre los ratones tratados con la cepa mutante y la cepa WT, se observaron diferencias en la población de células T. También, la población de células B en MLN fue mayor en ratones tratados con la cepa mutante. Adicionalmente, 4 diferentes dosis fueron evaluadas para cada cepa (1*104, 1*105, 1*106, y 1*107 CFU). Este ensayo nos permitió evaluar las diferencias ente las cepas en cuanto a los patrones de inflamación y el perfil de citoquinas Es así que los ratones de los grupos tratados con la cepa mutante, sin importar la dosis,

presentaron inflamación en los cortes histológicos de colon, moco en las heces o heces suaves. Además, la producción de IL-10, medida en sangre, fue mucho mayor para los ratones tratados con la cepa probiótica WT, que para los ratones tratados con la cepa mutante. También, la producción de IL-12p70 se mantuvo baja para los grupos tratados con las dosis más bajas y fue incrementando para las dosis mayores, en los ratones tratados con la cepa probiótica WT. En el caso de los ratones tratados con la cepa mutante, los niveles de IL-12p70 fueron siempre menores a los de IL-10. En conjunto, los resultados nos permiten sugerir que ChuS aparentemente tiene la habilidad de modular las propiedades inmunomoduladoras de EcN in vitro e in vivo. Lo cual es similar al comportamiento de HO-1, modulando el perfil de IL-10 e IL-12p70. Además, el efecto de EcN y ChuS no es solamente local, si no también sistémico, porque fue posible detectar diferencias en bazo, hígado, MLN y sangre.

1. INTRODUCTION

1.1. Mammalian heme-oxygenase and its role as immune system modulator

Heme-oxygenase (HO) is an enzyme that was described and characterized 50 years ago. Its principal function is to catalyze metabolic pathways in the conversion from heme to biliverdin and bilirubin, in the presence of biliverdin reductase. This reaction requires three molecules of oxygen and a total of seven electrons. The metabolic products derived from this reaction are biliverdin (BV), carbon monoxide (CO) and iron (Fe) (Liu & de Montellano, 2000). The electrons needed for HO-1 reaction (mammalian HO) are transferred from NADPH and are mediated by cytochrome P450. HO-1 was described as an intracellular enzyme (Tardif *et al.*, 2013) expressed in spleen and participates in degradation of erythrocytes in the liver and bone marrow (Ryter *et al.*, 2006). However, HO-1 is expressed in basal levels in all cells. Additionally, two other

HOs were described in mammals, those are HO-2 and HO-3 (Cruse & Maines, 1988; McCoubrey *et al.*, 1997)

Many properties have been described for this enzyme. For example, an antioxidant, anti-proliferative, anti-apoptotic and anti-inflammatory role has been assigned to HO-1 (Ryter *et al.*, 2006) (Figure 1). In particular, CO molecule is able to attenuate immune response, specifically by reducing immunogenicity of dendritic cells (DC). Also, this molecule can stimulate the tolerance of autoreactive T cells, thus reducing their pathogenic potential (Simon *et al.*, 2013).

The anti-inflammatory capacity of HO-1 may act in many ways. The regulation of heme amount in serum is important because its accumulation is considered as a pathological condition. In the case of Malaria, the homeostasis of tissue is maintained by HO-1 through the degradation of heme and the production of CO, which inhibits hemoglobin oxidation and thus the release of heme group (Dutra & Bozza, 2014).

Additionally, it has been observed that HO-1 may modulate DC by inhibiting its maturation after a treatment with lipopolysaccharide (LPS) and promoting tolerogenic phenotypes (Wong *et al.*, 2016). LPS is a bacterial component located at the outer membrane of Gram-negative bacteria, one of its effects is to trigger DC maturation. Some of the observed effects in stimulated DC are the reduction of pro-inflammatory cytokine secretion, impaired capacity to induce T cell proliferation, and sustained IL-10 production. Furthermore, HO-1 and CO can inhibit the pro-inflammatory cytokine production of DC induced by TLR3 ligand (Rémy *et al.*, 2009). As recently mentioned, IL-10 remains constant despite the inhibition of IL-12p70 and IL-12p40 secretion, and

IL-2 production (Chauveau *et al.*, 2005). However, IL-10 does not mediate the anti-inflammatory effects of HO-1 by itself. The treatment with anti-IL-10 antibody does not

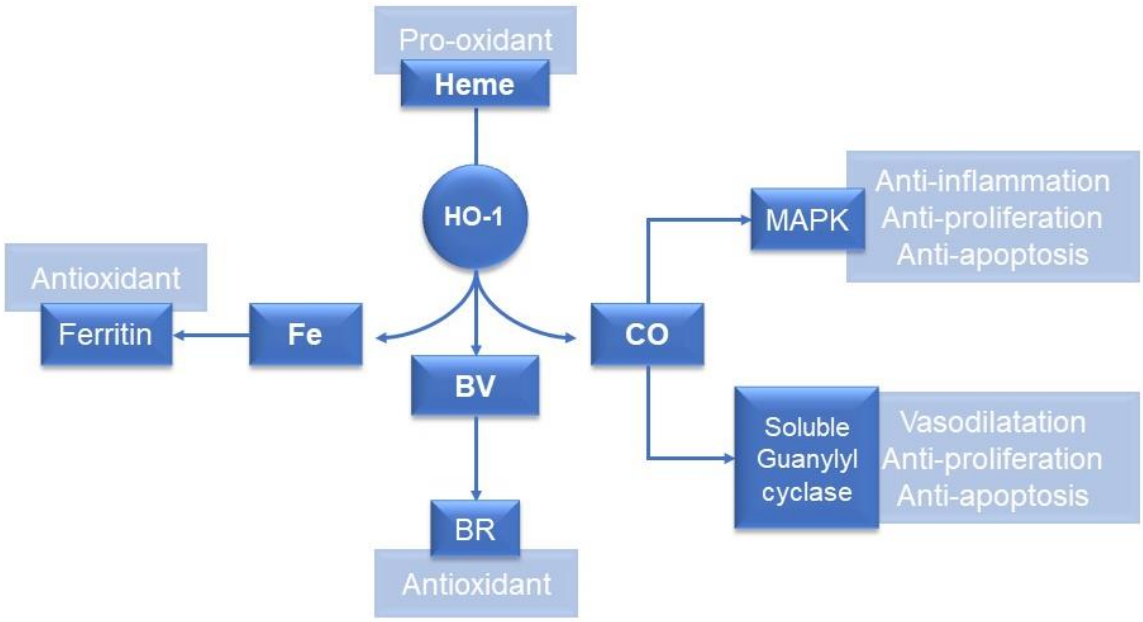

Figure 1. HO-1 products and effects on the organism (Modified from Pat et al., 2008). Enzymatic process starts with heme group that is catalyzed by HO-1 enzyme. Products: Fe (iron), CO (Carbon monoxide), and BV (Biliverdin), are released, each of these products has an its own functions. Fe is the precursor of ferritin, which is an antioxidant. BV is the precursor of bilirubin (BR) that also functions as antioxidant. CO is involved in two signaling pathways, MAPK (Mitogen-activated protein kinase) and Soluble Guanylyl cyclase, promoting some major effects of HO-1, such as anti-inflammation, anti-proliferation, anti-apoptosis, and vasodilatation.

reverse the inhibitory effect of HO-1 activity and CO regarding to secretion of other cytokines, indicating that inhibition of DC by the action of HO-1 and CO is independent of IL-10 (Rémy *et al.*, 2009).

DC previously treated with CO may contribute to autoreactive T cell tolerance. Therefore, pathogenic potential of these T cells would be decreased, and the incidence of autoimmune diseases mediated by them may be ameliorated. In a Diabetes murine model, it was observed that the expression of CD29 receptor (or β1-integrin) significantly diminished on $CD8^+$ T cells after CO treatment. Also, the capacity of these cells to lyse Langerhans islets was decreased (Simon *et al.*, 2013). Additionally, in presence of CO treated DC, $CD8^+$ T cells were not able to accumulate in the pancreas of mice.

Recently, it was described that HO-1 and CO may inhibit antigen presentation of LPS treated DC to naive T cells (Tardif *et al.*, 2013). This process occurs trough less endosome-lysosome fusion mediated by CO, which may be due to a reduction of MHC-II molecules presentation. These studies highlight the importance of immune regulation by HO-1 and CO.

1.2. Heme-oxygenase as a therapeutic target for inflammatory and autoimmune diseases

Some important studies have shown evidence of a protector role of metabolic products of HO-1 in autoimmune and inflammatory diseases. These diseases are characterized by autoreactive immune responses against the body itself. Studies with

pharmacological induction of HO-1, HO-1 over expression or CO administration, have shown promising results (Mackern-Oberti *et al.*, 2014). Some animal models of inflammatory diseases that have been evaluated are lupus, arthritis, multiple sclerosis, diabetes and inflammatory bowel disease (Ryter *et al.*, 2006; Llanos *et al.*, 2013; Simon *et al.*, 2013; Onyiah *et al.*, 2014). In general, the action of this enzyme may modulate inflammation by attenuating autoimmune response. The effects of this modulation are activation of specific cells, proliferation, apoptosis, cytokine response and bacterial clearance (Mackern-Oberti *et al.*, 2014; Chung *et al.*, 2008; Onyiah *et al.*, 2014). A mechanism that has been described for CO produced by mammal HO-1 is the inhibition of DC maturation without altering IL-10 production (Chauveau *et al.*, 2005).

Autoimmune diseases are characterized by an immune auto-reactive response able to cause damage on healthy cells and tissues. Determinant factors responsible for autoimmunity may be environmental and genetic, however other factors remain unknown (Mackern-Oberti *et al.*, 2014; Mackern-Oberti *et al.*, 2015).

For instance, in the case of inflammatory bowel disease (IBD), some treatments have been tested in which HO-1 is induced or CO is administered in animal models, some examples are described below. In murine model IL-10 $^{-/-}$, reduction of tissue damage and inflammatory infiltrate in the gut are observed when HO-1 is induced (Hegazi *et al.*, 2005). Levels of pro-inflammatory cytokines such as TNF-α, IFN-γ, IL-17A also decrease with treatment with CO and this is one of the most potent anti-inflammatory effects of this molecule. Also, in IL-10$^{-/-}$ mice that have been induced by trinitrobenzene

sulfonic acid (TNBS) to produce ulcerative colitis, the administration of CO significantly promotes the return of the histologically integrity of the colon (Fukuda et al, 2014).

Also, in murine model of Experimental Autoimmune Encephalomyelitis (EAE), HO-1 was found in inflammatory tissue during lesion formation and even on the onset of neurologic disease (Schluesener *et al.*, 2000; Liu *et al.*, 2001). This disease will be explained with more detail later.

Therapies for autoimmune and inflammatory diseases currently used are constantly evolving in order to achieve more specific drugs with fewer side effects. An alternative is to focus on treating inflammation caused by the immune system and thus reduce the impact of the disease (Mackern-Oberti et al, 2014; Mackern et al, 2015). Thus, HO-1 modulation would be a potentially effective alternative treatment.

1.3. Bacterial Heme-oxygenase

Even though heme-oxygenase was firstly described in mammals, the identification of bacterial heme-oxygenase has been done. *Corynebacterium diphteriae* has a heme-oxygenase (HmuO) that has 33% sequence identity and 70% of homology with human HO-1 (Wilks & Schmitt, 1998). Most of the known bacterial HO are described for pathogens. Some pathogens that have this protein are *Yersinia pestis* (Hmu), *Neisseriae spp.* (HemO), *Pseudomonas aeruginosa* (PigA), *Mycobacterium tuberculosis* (MhuD), *Staphylococcus aureus* (IsdG, IsdI) and *Escherichia coli* (ChuS), all of them have genes for HO that encode functional proteins (Engel et. al, 1972; Schmitt *et al.*, 1997; Thompson *et al.*, 1999; Zhu *et al.*, 2000; Ratliff *et al.*, 2001; Skaar

et al., 2004; Suits *et al.*, 2005). Initially, the only purpose reported for these bacterial proteins was the iron acquisition from the environment as a source of energy, but no functions have been reported for the CO produced by them (Frankenberg-Dinkel, 2004).

Furthermore, metabolic products of all bacterial HO are not always BV and CO (Wilks & Ikeda-Saito, 2015). Gram-positive bacteria like *M. tuberculosis* and *S. aureus* are able to capture and degrade heme to use it as energy source. However, CO is produced in small amounts (*M. tuberculosis*) or even not produced (*S.aureus*). This is because CO acts as a bactericide for *S. aureus* and activates dormancy genes in *M. tuberculosis*. Metabolic products in these bacteria are formaldehyde, staphylobilin and mycobilin without reported functions in the host (Matsui *et al.*, 2013; Nambu *et al.*, 2013).

On the other hand, Gram-negative bacteria as *P. aeruginosa* and *E. coli* have a HO that produces same metabolic products than human HO-1. In the case of *E. coli* O157:H7, ChuS enzyme is composed of 337 residues, and heme binds specifically to His 193 and Arg-100 from C-terminal half and N-terminal half respectively (Figure 2). Additionally, enteric bacteria as *Enterobacter* and *Shigella* may have similar proteins (Suits *et al.*, 2005).

Especially, the HO enzyme produced by *E. coli* can produce CO efficiently despite having a different structure from HO-1 (Suits *et al.*, 2006; Suits *et al.*, 2005). In laboratory conditions NADPH and cytochrome p450 reductase are needed for ChuS reaction to releases CO (Suits, 2005; Suits 2006). Also, enzyme needs hydrogen

peroxide for the degradation reaction. Products are meso-hydroxyheme, ferric verdoheme and CO. Additionally, at the end of reaction hematinic acid, tripyrrole product, and non heme iron are released. The reaction process and products suggest that this enzyme may be involved in oxidative-stress response of bacteria (Oullet, 2016).

E. coli heme uptake system, composed by a group of proteins with different functions, in addition to ChuS (Figure 3). ChuA is the outer membrane receptor (Mills, 1995) which needs TonB system as energy source for the transport of heme (Torres, 2001). ChuT is a periplasmic binding protein. ChuU is the inner membrane permease. ChuV functions as an ATPase. ChuX has affinity for binding heme but as a transport protein, that can regulate heme processing by storage (Suits, 2009). Finally, in an anaerobic scenario ChuW and ChuY play an important role. ChuW is an aerobilin synthase that catalyzes anaerobic degradation of heme (LaMattina, 2016), and ChuY is an anaerobilin reductase (LaMattina, 2017).

HO properties described before have an influence on the bacteria infection cycle, therefore metabolic products of the reaction of HO are required. Probably, bacterial HO is involved in other mechanisms used by bacteria to colonize and spread, besides allowing iron acquisition (Hagan, 2009; Suits *et al.*, 2008). However, how this enzyme specifically affects the pathogenesis of bacteria has not been studied but it may be a regulation by the metabolic products, such as CO.

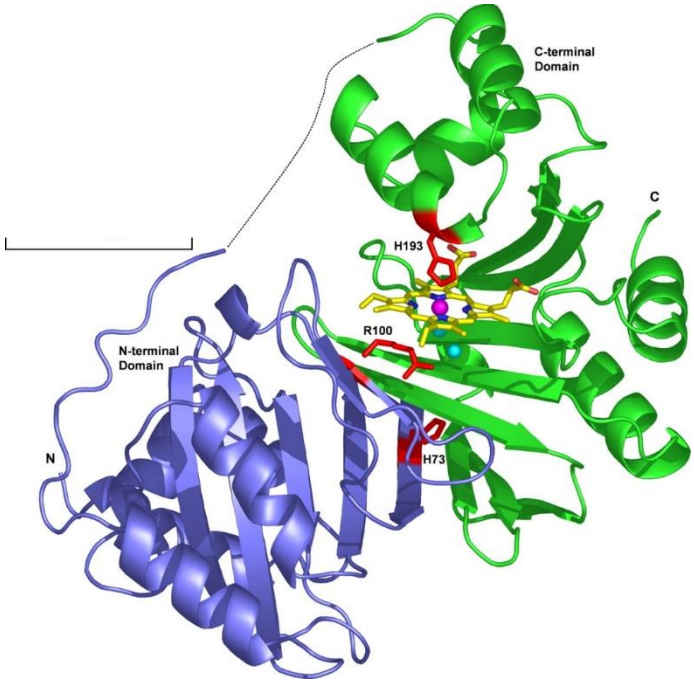

Figure 2. Ribbon diagram of ChuS when bound with heme. ChuS structure consists in two antiparallel β-sheets, one is flanked by N-Terminal (Blue) and the other by C-Terminal (Green). Heme (represented in yellow) binds ChuS between His-193 and Arg-100 in the presence of two molecules of water (represented in light blue). Figure extracted from Suits, 2005.

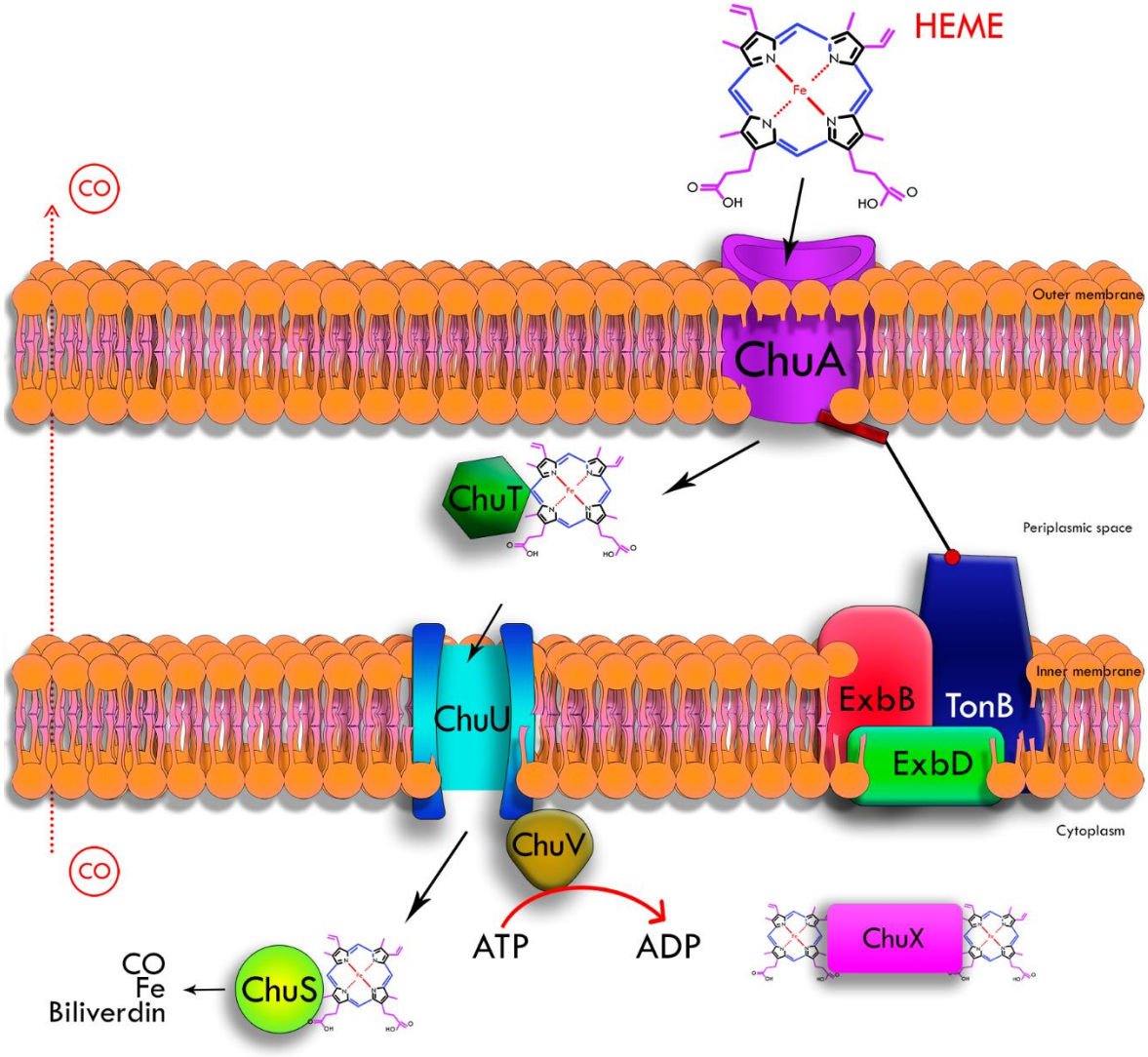

Figure 3. Scheme of heme uptake system in *E. coli* O157:H7. Heme binds ChuA and is transported through membranes to bacteria cytoplasm, where ChuS binds heme and enzyme reaction takes place. As result of enzyme reaction, CO is released to cytoplasm and then to exterior space. ChuA: outer membrane receptor. ChuT: periplasmic binding protein. ChuU: inner membrane permease. ChuV: ATPase. ChuX: transport protein. ChuS: heme-oxygenase. ATP: Adenosine triphosphate. ADP: Adenosine diphosphate. CO: Carbon monoxide. Fe: iron.

Recently, HO activity was found in a gut-isolated *E. coli* strain designated as NC101. *chuS* expression correlated with inflammation in non-associated $IL10^{-/-}$ mice. Additionally, pro-inflammatory cytokine IL-12p40 was measured and levels reduced when *chuS* was highly expressed by means of an expression vector. On the other hand, IL-10 production increased, promoting an anti-inflammatory scenario. These changes in cytokines expression were promoted by a soluble factor released by bacteria that may have similar effects than CO (Maharshak, 2015). These findings show up and strengthen the hypothesis that bacterial HO may have an important effect in host immune response.

1.4. Microbiota

Mammals are strongly associated to microorganisms all over environmental exposed surfaces. Besides, there are more than 10^{14} microorganisms in the gastrointestinal tract, which correspond to 1.000 species (Gill *et al.*, 2006) and live principally in the colon. Most of these bacteria (>90%) belong to two different phyla, Gram negative are Bacteroidetes and Gram positive Firmicutes (Peterson *et al.*, 2008). Microbiota plays an important role in development and differentiation of local and system immune components (Sartor *et al.*, 2008). For example, some specific bacteria determine adaptive immune functions that are upregulated in the intestine and are related with intraepithelial lymphocytes, TCRαβ, Treg cells and Th17 cells (Mazmanian *et al.*, 2008). Furthermore, studies have shown that butyrate is able to promote an immunomodulatory action. This molecule is a secondary metabolite produced by

commensal Clostridial bacteria. The mechanism of action favors differentiation of T naive cells into Treg and their proliferation (Arpaia *et al.*, 2013).

Recently, associations between microbiota and metabolic processes that occurs in human body have been done (Clemente, 2012; Tremaroli & Bäckhed, 2012). For instance, a study has shown that bacterial metabolites such as short chain fatty acids (SCFA) affect microglia maturation, morphology and function (Erny, 2015). These SCFAs may be able to translocate from the intestine, reaching systemic circulation.

1.5. Immunomodulatory properties of probiotic strains

According to FAO (FAO & WHO, 2006), probiotics are defined as live microorganisms that confer a health benefit on the host when administered in adequate amounts. Humans have used microbial fermented food by centuries, due to their beneficial properties. Modern probiotics are mostly based on ancient fermentation process such as yogurt and cheese (Gogineni, 2013).

Administration of probiotics such as *Lactobacillus* GG may diminish intestine inflammation. Treatment with this strain for 14 days has ameliorated intestinal permeability (Isolauri *et al.*, 1993). Additionally, Bourrel *et al.* (2003) evaluated *Lactobacillus bulgaricus* y *Lactobacillus casei* and *E. coli* probiotic strains and results showed that probiotics interacted with immune cells, diminishing considerably inflammation, by inducing pro-inflammatory cytokines secretion.

In addition, a pilot random study made by Rembacken *et al.* (1999) and finished by Kruis *et al.* (2001) has evaluated 327 patients with ulcerative colitis. All patients were

treated with mesalazine (500 mg) or *E. coli* Nissle 1917 (200 mg) and were monitored for 12 months. Relapse rates were 36.4% for *E. coli* Nissle 1917 and 33.0% for mesalazine. Consequently, *E. coli* Nissle 1917 represents powerful alternative for treating patients with ulcerative colitis, which is been used currently (Sonnenborn, 2009). Recently, this probiotic strain has been studied in an ulcerative colitis murine model induced by Dextran sulfate sodium (DSS). Mice were treated with *E. coli* Nissle 1917 by a period of time and results have shown a decrease in clinical and histopathological symptoms of disease. In addition, inflammation process in these mice was controlled largely by *E.* Nissle 1917, because levels of pro-inflammatory cytokines such as IL-1ß were reduced as well as inflammatory cells. Moreover, Treg cells were increased and permeability of intestinal epithelium was preserved (Souza *et al.*, 2016). EcN showed to ameliorate inflammation, reducing levels of neutrophils and chemokines in a DSS-induced colitis murine model.

Furthermore, probiotics administration has demonstrated to be able of restoring normal gut microbiota and reducing inflammatory response (Al-Salami *et al.*, 2012). An important clarification is that modulation of intestinal epithelium cell responses by different induction or suppression process, is probiotic-specie dependent (Llewellyn, 2017). Therefore, probiotics effects may vary between species.

1.6. Escherichia coli Nissle 1917

Escherichia coli 1917 (EcN) serotype O6:K5:H1 is known as a probiotic bacterium originally isolated from gut and commercially available as Mutaflor® manufactured by Ardeypharm GmBH (Germany). Some of its properties are the conformation of biofilm

on intestinal epithelium, inhibition of invasion of gut epithelial cells, strengthen tight junction (TJ), immunomodulation and anti-inflammation (Sonnenborn, 2016). For instance, study performed by Boudeau (2003), has shown that *E. coli* Nissle 1917 biofilm conformed on intestinal cells was strong enough to prevent pathogenic microorganisms from reaching the cells. Additionally, these probiotic bacteria can induce the activation of human β defensing-2 through their flagellin. These features enable these bacteria to strengthen barrier function (Wehkamp, 2004; Schlee, 2007) and prevent or ameliorate leaky gut. One of the pathways to reach that goal, was recently described by Guo *et al.*, 2019. Their results showed EcN protected the intestinal barrier function in sepsis by ameliorating the altered expression and localization of TJ proteins and inhibiting the NF-κB-mediated activation of the MLCK-P-MLC signaling pathway which might be one of the mechanisms underlying the effect of EcN (Guo *et al.*, 2019).

As mentioned before, EcN has shown the capability of inhibit the invasion of pathogenic bacteria and yeasts in the gut, with some features that even allow it to kill them (*Salmonella*, EIEC, AIEC, *Shigella*, *Yersinia*, *Listeria*, Candida) (Sonnenborn, 2009). Also, a study performed with piglets that were treated with EcN before an infection with human Rotavirus (HRV), showed that virus titers were significantly less when compared to infected piglets without probiotic. In addition, splenic and ileal mononuclear cells (MNCs) from piglets were treated with EcN for 24 h, then cytokine secretion was evaluated. Findings evidenced that levels of IL-6 and IL-10 were higher than control group, in splenic as well as ileal cells (Kandansamy, 2016).

Recently, some reports of EcN capability to ameliorate autoimmune diseases have been done. For instance, a study evaluated EcN immunomodulatory properties in mice with Experimental Autoimmune Encephalomielitis (Secher, 2017). Mice were treated daily with EcN. and exhibited reduced migration of CD4+ T cells from the periphery to the central nervous system (CNS) when treated with EcN. Also, when lymph nodes MOG-specific T cells from EcN-treated mice were extracted and stimulated with MOG_{35-55} for 72 h, IL-10 was found highly secreted as compared to control group. EAE clinical score also showed changes with EcN treatment, total score was lower than EAE mice without probiotic treatment. Moreover, EcN treatment for CACo-2 cells, enhanced and maintained the permeability, by acting against alterations induced by inflammatory bowel syndrome (Barbaro, 2018).

Additionally, sequence analysis of EcN genome showed up many features that have not been studied yet, one of them is the presence of a *chu* heme transport locus, considered as a fitness factor (Grozdanov, 2004).

Despite many studies which have evaluated EcN immunomodulatory properties, not all mechanisms are dilucidated. Most research is focused on structural components of EcN as flagella and capsule proteins. Furthermore, less is known about the specific effect on immune system of compounds produced by these bacteria as secondary metabolites. On the other hand, the presence of *chu* whole heme transport locus in its genome may be conferring some of its anti-inflammatory properties. As described before, CO is one of ChuS products, similar to HO-1 from mammals. Due to known CO

anti-inflammatory properties, it is possible that by releasing CO as a result of ChuS action, EcN may exert part of its immunomodulatory features by this via.

2. HYPOTHESIS

Anti-inflammatory properties of probiotic strain *E. coli* Nissle 1917 (EcN) are due to the presence of bacterial heme-oxygenase enzyme (ChuS) produced by this bacterium.

3. AIMS

3.1. General aim

To evaluate ChuS role from *Escherichia coli* Nissle 1917 strains in an inflammation context.

3.2. Specific aims

3.2.1. To assess the role of ChuS in *E. coli* Nissle 1917 strain *in vitro* with murine cells.

3.2.2. To evaluate ChuS anti-inflammatory capacity of *E. coli* Nissle 1917 strain in mice.

4. MATERIALS

4.1. Equipment

- Agarose electrophoresis chambers, Fermelo.
- Analytical balance, Precisa XB220A.
- Balance Clever.
- PowerPac Basic Power Supply, BioRad.
- Biosafety cabinet Class II LABGARD, Nuaire.
- Centrifuge, Eppendorf model 5702R.
- Centrifuge, Eppendorf model 5804.
- Centrifuge HERAEUS FRESCO 21, Thermo ScientificTM
- Chemical fumehood, BIOBASE FH1000X.

- Gene Pulser Xcell Electroporation Systems, BioRad.
- Homogenizer, ScilogexR.
- Incubator, LabTech LIB 080M.
- Environ-Shaker Orbital Incubator, Lab-Line.
- Microcentrifuge SPROUTTM, LabScientific.
- NanoDrop, Thermo ScientificTM.
- StepOne plus thermocycler, Applied Biosystems.
- Spectrophotometer Spectronic BIOMATE 3, Thermo ScientificTM.
- Maxygene Gradient Thermal Cycler, Axygen.
- Vortex Select Vortexer SBS100-2 Select BioProducts.
- Cell incubator Forma Scientific model 3110
- Biosafety hood Sterile Gard Hood from The Baker Company
- Microcentrifuge Eppendorf 5415R
- Microcentrifuge Refrigerated IEC Micromax RF, Thermo Electron Corporation
- Centrifuge Eppendorf 5702R
- Centrifuge Eppendorf 5804R

- Centrifuge Centra CL3R
- Freezer -80°C REVCO model Ultima III
- BD LSR Fortessa X-20 flow cytometer
- Thermo regulated water bath LP/BX
- Microm HM 325 Rotary Microtome Thermo Scientific
- Enclosed automatic tissue processor Leica ASP300S
- Cold plate for Modular Tissue Embedding System Leica EG 1150 C
- Heated Paraffine embedding Module Leica EG 1150 H
- Histology Water Bath for Paraffin section Leica HI 1210
- Microscope Olympus BX51
- Microscope Olympus CKX4
- Automated Cell counter BIO RAD TC20
- Microscope camera Infinity 2
- Olympus camera adaptor U-TV0.5XC-3
- pH reader Hanna HI 9321PH
- Vortex Labnet International, model VX100

- Analytic balance Adam, model AFA-180LC
- 35 Liters Liquid Nitrogen Tank: Taylor Wharton
- Neubauer camera
- Nano Drop 2000 UV-Vis Spectrophotometer, Thermo Scientific
- Maxygene Thermocycler, Axygen.
- Veriti Thermal Cycler, Applied Biosystems
- Real-Time PCR System Applied Biosystem Step OneTM
- Microplate Reader Multiskan EX, Thermo LabSystems
- Microplate Reader 800 TS Biotek
- Thermo Scientific™ myECL™ Imager.

4.2. Reagents, enzymes, and buffers

- 2-mercaptoethanol Gibco® 21985-023
- Agarose Lafken, cat. Number FER/00A200.
- Ampicilin Calbiochem ® Cat # 171254-5GM
- Chloramphenicol, Calbiochem, cat. Number 220551.
- Chlorophorm, Merck EMPARTAR, CAS 67-66-3, cat. Number 107024.

- Colagenase IV Gibco Cat # 1985941
- Deoxynucleotide triphosphates (dNTPs), InvitrogenTM.
- DNAse Thermo Scientific EN0521
- DNAse I Roche 10104159001
- Ethanol molecular grade, Merck, CAS 64-17-5, cat. Number 108543.
- Ethanol, Merck EMSURER, CAS 64-17-5, cat. Number 100983.
- GeneRuler 1kb Plus DNA Ladder ThermoFisher cat # SM1331
- GeneRuler 100bp DNA Ladder ThermoFisher cat # SM0241
- Accuruler 100bp Plus DNA MaestroGen Cat # 0200250
- 6x loading DNA dye solution cat # R0611
- Glycerol, Winkler, cat. Number BM-0017.
- Isopropanol, Merck EMSURER, CAS 67-63-0, cat. Number 10963.
- iScript cDNA Synthesis Kit Biorad. cat. Number 1708891.
- Kanamycin, Calbiochem cat. Number 420311.
- L-Glutamine 200mM Cat # 25030-081
- Luria Bertani (LB) Agar, MoBio cat. Number 12107-1.

- Lipopolysaccharide (LPS) Sigma
- Lysis buffer (Ethanol 19%: Phenol 1%: SDS 0.1%).
- LB medium: Tryptone 10g, Yeast extract 5g, NaCl 10g Distilled Water complete to 1L; for LB agar add Agar 15g.
- MacConkey (MAC) Agar, Difco, BD, USA. cat. Number 215197.
- Nuclease-Free Water, UltraPure™, InvitrogenTM, cat. Number 10977015.
- Phenol, Merck, CAS 108-95-2, cat. Number 100206.
- Phenol: chloroform: isoamylic alcohol, Winkler 50:48:2, cat. Number BM-0770
- Proteinase K (10mg/mL), Sigma-Aldrich, cat. Number P2308.
- Penicilin/streptomycin Gibco Cat nº: 15140122
- RNAse A (5mg/mL), Sigma-Aldrich, cat. Number R4875.
- SDS, Sigma-Aldrich, CAS 151-21-3, cat. Number L3771.
- Sodium Acetate 3M.
- Sodium Citrate 0.1M, 10% Ethanol.
- Streptomycin, Gibco, cat. Number 11860-038.
- SYBRTM Safe DNA gel stain, InvitrogenTM, cat. Number S33102.

- TAE buffer (40 mM Tris-HCl, 1 mM acetic acid, 1 mM EDTA) for agarose gel preparation and electrophoresis.
- Taq DNA Polimerase, InvitrogenTM, Life Technologies.
- Platinum Pfx DNA Polymerase Invitrogen TM cat #11708039
- TaqManR Fast Advanced Master Mix Applied BiosystemsTM.
- TE buffer (10mM Tris-Cl (pH 8.0) 1mM EDTA (pH 8.0)).
- TRIzolTM reagent, InvitrogenTM, cat. Number 15596026.
- Triton 100-X, Merck, CAS 9036-19-5, cat. Number 108643.
- Wizard Plus SV Miniprep, Promega, cat. Number A1330.
- Wizard SV Gel and PCR clean-up system kit, Promega, cat. Number A9282.
- TRUE BLUE Peroxidase (KPL)
- 3,3', 5,5-tetramethylbenzidine (TMB, BD Pharmigen)
- Twenn-20 (Biorad)
- Bovin Serum albumin (BSA, Winkler)
- Ovalbumin peptide 257-264 (OVA) (InVivoGen Cat # vac-sin)
- Ovalbumin peptide 323-339 (OVA) (InVivoGen Cat # vac-isq)
- Protoporphyrin IX cobalt chloride (CoPP, Frontiers Scientific)

- Tin protoporphyrin IX dichloride (SnPP, Frontiers Scientific)
- Ethylenediaminetetraacetic acid (EDTA, US Biological E 2210)
- Na_2HPO_4 (Merck)
- KH_2PO_4 (Winkler)
- NaCl (Merck)
- KCl (Merck)
- H_2SO_4 (Merck)
- NH_4Cl (Winkler)
- $KHCO_3$ (Winkler)
- $NaHCO_3$ (Winkler)
- $CaCl_2$ (Winkler)
- Ketamine 10% (Ketostop)
- Xylaxine 2% (Alfasan)
- Isoflurane (Baxter)
- Kit ImProm Reverse Transcription System (Promega)
- SyBR Green QPCR Master Mix (Life Technology).

- GoTaq G2 Flexi DNA Polymerase Promega Cat # M7805.
- IL-6 ELISA KIT (OptEIA; BD Pharmingen) Cat # 555240
- IL-4 ELISA KIT (OptEIA; BD Pharmingen) Cat # 555232
- IL-10 ELISA KIT (OptEIA; BD Pharmingen) Cat # 555252
- IFN-γ ELISA KIT (OptEIA; BD Pharmingen) Cat # 555138
- IL12p70 ELISA KIT (OptEIA; BD Pharmingen) Cat # 555256
- IL1-β ELISA KIT (OptEIA; BD Pharmingen) Cat # 559603
- IL-2 ELISA KIT (OptEIA; BD Pharmagien) Cat # 555-148
- FACs buffer: for a liter of buffer, 20 ml FBS, 10% NaN_3, PBS 10x and 870 ml of H_2O.
- Free-serum medium: 20 UI heparoma. 20.1 mM HEPES, 2.5 mM $CaCl_2$, 2 mM $MgCl_2$ and 250UI/ml of Colagenase IV.
- PEB: PBS 1x, 0.5% EDTA/ 2mM EDTA
- Heat Inactivated Fetal bovine serum (Invitrogen Life Technologies, Carlsbad, CA; catalog # 12484-028)
- IMDM Iscove's Modified Dulbecoo's Medium Gibco TM cat # 12440053

- Paraformaldehyde 2% for cell fixation: 2% paraformaldehyde (Merck Millipore Corporation; catalog # 104005) in Phosphate buffered saline (PBS).
- Paraformaldehyde 4% for tissue fixation: 4% paraformaldehyde (Merck Millipore Corporation; catalog # 104005) in PBS.
- PBS: 0.14 M NaCl, 1.47 mM KH_2PO_4, 7.81 mM, Na_2HPO_4, 2.68 mM KCl, pH 7.2-7.4, in distilled water.
- Red blood cell lysis buffer (ACK): 0.17 M ammonium chloride, 10 mM potassium bicarbonate, 0.1 mM EDTA, pH 7.2
- Staining buffer for flow cytometry: PBS with 10% fetal bovine serum.
- Trypan blue 0.4% (Invitrogen Life Technologies, Carlsbad, CA; catalog # 15250061).
- Washing buffer (ELISA): PBS 0.05% Tween-20.
- RPMI 1640 (Gibco, Thermofisher, Massachusetts, USA)
- Granulocytes Monocytes-Colony Stimulating Factor (GM-CSF) Peprotech Cat # 315-03
- Macrophage Colony Stimulating Factor (M-CSF) Peprotech Cat # 315-02
- HEPES Gibco TM Cat # 15630080

4.3. Bacterial strains:

Salmonella enterica serovar Typhimurium 14028s (*S*. Typhimurium) was used in this study for *in vitro* assays, which was donated by Guido Mora Ph.D. from Universidad Andres Bello. *Salmonella enterica* serovar Enteritidis (*S.* Enteritidis) was used for *in vivo* assays (reference strain NCTC 13349, isolate P125109) donated by Carlos Santiviago Ph.D. from Universidad de Chile.

Escherichia coli K12 was commercially obtained from ATCC, *E. coli* SM10λpir and *Escherichia coli* O157:H7 (EHEC) strains used in this study were provided by Roberto Vidal Ph.D. from Universidad de Chile.

Escherichia coli Nissle 1917 (EcN) strain used in this study was kindly provided by Ardeypharm GmbH (Germany).

4.4. Plasmids

- pKD3: Containing *cat* gene. Chloramphenicol resistance.
- pKD4: Containing *aph* gene. Kanamycin resistance.
- pKD46: Containing the Red recombinase genes from Lambda phage. Ampicillin resistance.
- pCP20: Containing FLP recombinase gene, temperature inducible (43°C), ampicillin and chloramphenicol resistance and thermosensitive replication
- pKD3_*chuS*: custom made and synthetized by EPOCH.

4.5. Primers and probes

Primers were designed using Vector NTI for each specific PCR run (Table 1). Probes and primers for RT PCR were designed using Primer Express® Software v3.0.1 License

1.1. Mice for cell primary cultures

For DC and macrophages cultures, 6-8 weeks old C57BL/6 male mice were used. Also, these mice were used to purify T cells for proliferation assays. OT-I and OT-II lymphocytes were obtained from 6-8 weeks old C57BL/6 OT-I and C57BL/6 OT-II male mice were used. These mice were originally purchased from Jackson Laboratories and maintained in an animal facility at Facultad de Ciencias Biológicas of Pontificia Universidad Católica de Chile.

OT-I and OT-II C57BL/6 are transgenic mice, which have and exclusive lymphocyte type, TCD8$^+$ (OT-I) or T CD4$^+$ (OT-II). This type of lymphocyte has a unique Receptor of T Cells (TCR) that can recognize only one MHC class from C57BL/6 mice, MHC–I (OT-I) or MHC-II (OT-II), associated to an Ovalbumin derivate peptide (OVA).

- OT-I C57BL/6 mice: Tg (TcraTcrb)1100Mjb. These mice have an only variant in T CD8$^+$ TCR. This variant is composed by two TCR chains, alpha (Tcra-V2, Tcra-J(alpha26)) and TCR beta (Tcrb-V5, Tcrb-D(beta2), Tcrb-J(beta2.6)). As mentioned before, this receptor only recognizes MHC-I from C57BL/6 mice, which specifically binds to residues 257-264 of ovalbumin peptide (OVA $_{257\text{-}264}$).

Table 1. List of primers and probes used in this work

Name	Fw sequence (5'-3')	Rev sequence (5'-3')
rpoD	Fw_2 TGA GTC TGA AAT CGG TCG TAC G	TTC GCG GGT AAC ATC GAA CT
CHUS	AAA GAG GCC CAG CAA TAA ATA G	GGG GCA TTT ATC TGG AAG GAA G
OPCHU1	CGA CGT AAT GTT GCA TCA GA	TGT CGT ATA ACG TCC CGA AC
OPCHU2	GTT CGG GAC GTT ATA CGA CA	GCG CAG GAT AGG TTT CAT AA
OPCHU3	GCC TCG ATA TCT CTG GGT TC	GGT ACG GTA GTA CGC CCA TT
OPCHU4	AAT GGG CGT ACT ACC GTA CC	AAA ACC CGT GGT AGA CGA AT
OPCHU5	ATG GCT GAC CCA GGA TTA CT	GCA GGT AAC ATT CTC GTT CG
EHEC-ETEC putAP::Cm	(H1+P1)** TGT TAA TAA AAG AAA TCG ATA TGA CAG GGA TTA AAA AAA Tgt gta ggc tgg agc tgc ttc	(H2+P2)** ATT CAC AAT CGA TTT AAC ACA CCA TTT ACA TTA AAT TTT Acc ata tga ata tcc tcc tta
Kan-chu	(H1+P1)** ATA AAC TCA AAC GCA ACG AGG TAA ATT GCG GAC GTG ACA Tgt gta ggc tgg agc tgc ttc	(H2+P2)** TCT GAA TTT TAA ATA AAT TTA TTT TTC GCC AGC CAC CCC Gca tat gaa tat cct cct tag
chuS	(H1+P1) AAG AGG CCC AGC AAC AGC CAG ACC TCC CGT TAA TTA TAC Ggt gta ggc tgg agc tgc ttc	(H2+P2) CCC GAT ATT TCG GGG CAT TTA TCT GGA AGG AAG AGA GAC Aca tat gaa tat cct cct tag
Nissle PutA		(H2+P2)** ATT CAC AAT CGA TTT AAC ACA CCA TTT ACA TTA AAT TTT Ata acg gct gac atg gga att
Nissle putAP	GAG GAA AGG CTG GTG TGG TTC CTC	CGT CAC GAA TGG CCC AAG AC
qchuS Nissle	TGC GCT TGC TCC TGT TCA C	CTG GAA TTG TTT GCC CAT GA
rpoD-RT	ACC CGG GAA GGC GAA A	TGG TTG ATC CCG TCT TCG AT
Probe chuS	TGT ACG TTG ACC ATA AAG	
Probe rpoD	CGA CAT CGC TAA ACG	

**Capital letters correspond to sequence that hybridizes EcN chromosome. Lowercase letters correspond to sequence that hybridizes pKD3, pKD4 and pKD3_*chuS* plasmids.

- OT-II C57BL/6 mice: Tg (TcraTcrb)425Cbn. These mice have an only variant in T $CD4^+$ TCR. This variant is composed by two TCR chains alpha (Tcra-V2) and TCR beta (Tcrb-V5). As mentioned before, this receptor only recognizes MHC-II from C57BL/6 mice, which specifically binds to residues 323-339 of ovalbumin peptide ($OVA_{323-339}$).

1.2. Mice for *in vivo* assays

6 – 8 weeks old C57BL/6 male mice were used. These were maintained at the free-germ animal facility at Facultad de Ciencias Biológicas of Pontificia Universidad Católica de Chile. All animal work was performed according to the Guide for Care and Use of Laboratory Animals (National Institute of Health, Bethesda, MD) and Institutional guidelines.

1.3. Software

- Vector NTI 11
- BLAST ® from NCBI
- G*Power 3.1.9.2
- Flow Jo V_10
- Infinity Analyze Software
- GraphPad Prism 6

- Primer Express® Software v3.0.1
- StepOne Software v2.2

5. METHODS

5.1. pKD3_*chuS* development

Construction was based on pKD3 basic structure and properties. Principal feature is the presence of a copy of functional *chuS* gene from *E. coli* O157:H7. *trc* promoter from pKK233 was chosen because a strong promoter was needed and for its constitutive expression. In addition, restriction enzyme sites NdeI and XhoI were added. Antibiotic resistance *cat* gene, *bla* gene, and FRT sites, were conserved, see Figure 4. The design was made using Vector NTI.

5.1. Mutant strains development

Three mutant strains of *E. coli* Nissle 1917 were prepared. Heme oxygenase gene *chuS* was depleted for the first strain. The second one has depleted gene *chuS* and complemented with the same gene. The third strain has an extra copy of *chuS*.

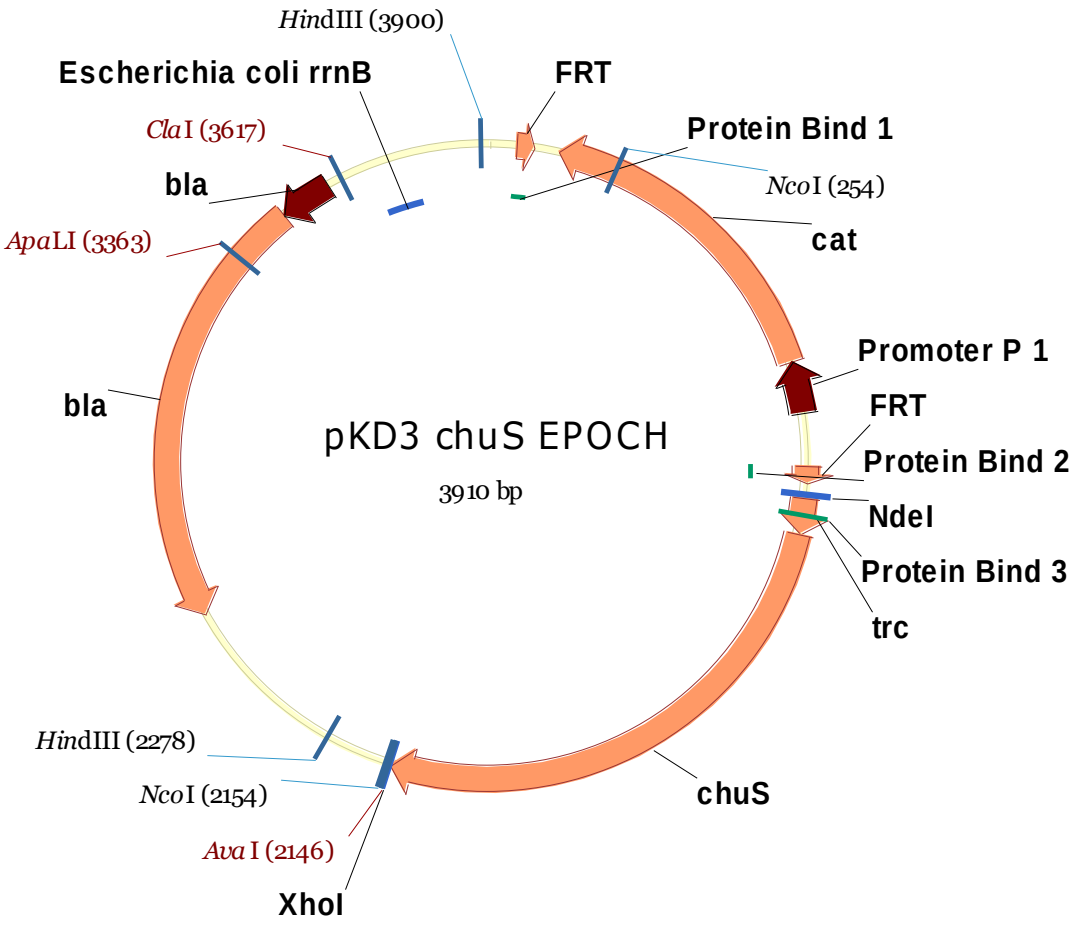

Figure 4 . pKD3_*chuS* plasmid designed with an additional copy of *chuS*.

E. coli Nissle 1917Δ*chuS* was developed following Datsenko & Wanner protocol (2000). First, a PCR product of antibiotic resistance gene *aph* from pKD4 was generated, primers were 60 nucleotides-long that included 40 nucleotides homology extension of loci among *chuS* gene and 20 nucleotides priming pKD4. The respective product was purified and used to transform *E. coli* Nissle 1917 pKD46, which was previously prepared. After transformation, colonies that grew in medium with antibiotic were selected.

The second strain is *E. coli* Nissle 1917Δ*chuS::putA::chuS*. Depletion of *chuS* was done as described previously. Complementation was done in trans. PCR product was amplified from pKD3_*chuS*, which contains *chuS* from *E. coli* O157:H7. Primers were designed with 40 nucleotides homology extension of *putA-putB* region in EcN chromosome and 20 nucleotides priming pKD3_*chuS* (including antibiotic resistance gene *cat, chuS*, trc and FRT sites). Transformants were selected by antibiotic resistance to Kan and Cm. In both strains, confirmation was done by conventional PCR.

The third strain is EcN *putA::chuS*. This strain has an extra copy of *chuS* inserted in the *putA – putP* loci. An additional copy of *chuS* was obtained from pKD3_*chuS*, previously described. Primers that were used to amplify this fragment (including antibiotic resistance gene *cat*, *chuS*, *trc* and FRT sites) were the same used for the EcNΔ*chuS::putA::chuS* strain. Transformants were selected by antibiotic resistance to Cm.

In all these strains, confirmation of the correct deletion and/or insertion was done by conventional PCR. Antibiotic resistance gene was eliminated by pCP20 action, as described by Datsenko & Wanner (2000).

5.2. DNA extraction

DNA extraction was performed by phenol-chloroform method, as described below. Pelleted bacteria were resuspended with 570µl of TE buffer, 5µl of RNAse A, 10µl of proteinase K, 30µl of SDS 10%, and incubated at 37°C for 1 h. Then, 600µl of phenol:chloroform:isoamylic alcohol (25:24:1) were added and solution was mixed vigorously until became milky. Later, the solution was centrifuged at 21,000 x g for 10 min at 4ºC, the organic phase was removed and prior step was repeated twice with 300µl and 200µl of phenol:chloroform:isoamylic alcohol (25:24:1). After the third centrifugation, aqueous phase was recovered and a solution of 0.6 volumes of isopropanol and 0.1 volume of sodium acetate 3 M was added, incubation lasted 1 h at -20°C. Samples were centrifuged at 21,000 x g for 10 min at 4ºC, the supernatant was discarded and 200µl of cold ethanol (molecular biology grade) were added for a final incubation of 5 min at room temperature. The DNA was recovered by centrifugation at 21.000 x g for 10 min at 4ºC, the supernatant was discarded, and the pellet was dried at room temperature for 15 min. Finally, the gDNA was resuspended in 50µl of nuclease-free water, and the final concentration was measured using NanoDrop.

5.3. 4.2 RNA purification

Total RNA was obtained from each sample using TRIzol reagent. 1ml of reagent was added to each sample and incubated at room temperature for 5 minutes.

Then, samples were centrifuged at 12,000 x g for 15 min at 4ºC, and aqueous phase transferred to a clean a tube. Next, 200 µl of chloroform per ml of TRIzol applied during the homogenization were added and solution was shaken vigorously for 15 sec and incubated at room temperature for 3 min. After, samples were centrifuged at 12,000 x g for 15 min at 4ºC. The supernatant was transferred to a clean tube and 500µl of isopropanol were added, solution was incubated at -20ºC for at least 30 min and centrifuged at 12,000 x g for 10 min at 4°C. The supernatant was discarded, and the RNA pellet was washed with 500 ul of cold ethanol 75% (prepared in nuclease-free water), homogenized and centrifuged at 7,500 x g for 5 min at 4ºC. Finally, RNA pellet was dried for 10 to 15 min, resuspended in 50µl of nuclease-free water, and stored at -80ºC.

For RNA purification from organs same protocol was used after organs were homogenized.

5.4. cDNA synthesis

Reverse transcription was performed with iScript cDNA Synthesis Kit, and the reaction parameters were stablished according to the manufacturer's instructions. Briefly, 1mg of total RNA, 4µl of 5x iScript Reaction Mix and 1µl of iScript Reverse Transcriptase were mixed in a final volume of 20µl. The reaction was incubated in a thermal cycler

using the following protocol: 5 min at 25ºC (priming), 20 min at 46ºC (reverse transcription), 1 min at 65ºC (RT inactivation) and hold at 4ºC. cDNA samples were stored at -20ºC.

5.5. Standard PCR

PCR amplifications were performed in a MaxiGene Gradient Thermocycler. To prepare the PCR mix, 10 to 100ng of DNA were incubated with 0.5µl of Taq Polymerase (5U/µl), 1.25µl of each primer (10µM), 0.5µl of dNTP (10mM mix), 2.5µl of Buffer 10X, 0.75µL of MgCl2 (50mM) and complete with nuclease-free water to 25µl. The PCR products were resolved by electrophoresis (60 min at 90V) in an agarose gels containing 0.5µg/ml SYBR Safe and visualized under UV light.

For Tiling PCR Platinum *Pfx* polymerase was used including an enhancer in the mix.

PCR cycles were adjusted to specific primers manufacture recommendations, especially for annealing temperature. General conditions are specified on Table 2.

5.1. Quantitative real-time PCR

Quantitative real-time PCR (qPCR) was performed for the quantification of bacterial *chuS* expression, using TaqMan probes and TaqMan TaqMan RNA-to-Ct 1-step kit (following the manufacturer's instructions for 20µl of the reaction mixture). The qPCR mix was prepared using the following reagents: TaqMan Fast Master Mix 2X, Mix of Primers 20X (100µM of each primer and 100µM of the probe) and 2µl of RNA adjusted to the final reaction volume.

Table 2. PCR cycles

	1st Stage x1	**2nd Stage x35**			**3rd Stage x1**	
Step	1st	1st	2nd	3rd	1st	2nd
Temperature °C	95	95	57	72	72	4
Time min.	0:15	0:15	0:15	1:30	7:00	∞

The mRNA gene expression of every sample was compared to *rpoD* expression. After the standardization, the abundance of each target mRNA was determined by the comparative method (2−ΔΔct) of StepOne software.

5.2. Viability Assays with mutant bacteria strains

E. coli Nissle 1917 WT and *E. coli* Nissle 1917Δ*chuS* were cultured in LB medium. When OD_{600} reached 0.3 independent cultures were supplemented with 50µM CoPP (Cobalt protoporphyrin IX, which is a HO-1 inductor), 50µM SnPP (Tin protoporphyrin IX, which is a HO-1 inhibitor), or vehicle (NaOH 1N).

Parameters that were measured are growth rate, CFUs and *chuS* expression by RT-PCR (probes are specified in Table 1).

For growth rate, 100 µl were diluted 1:10 in LB then measured with spectrophotometer OD 600 nm.

For CFUs, 100 µl were used for serial dilutions 1:10 until 10^{-7}. Then bacteria were seeded in LB agar (with antibiotic when necessary) in drops in triplicate. Cultures were incubated for 18 hours and then colonies counted for CFU calculations.

For expression evaluation 1 ml of culture was centrifuged and pelleted, then RNA extraction was done with TRIzol. *chuS* expression was evaluated by Real Time PCR, using *rpoD* as control.

5.3. Bacterial culture conditions for infections

All bacteria strains' stocks were stored at -80º in Cryobank® system. To perform the infection assays, one bead from Cryobank® tube was grown with agitation at 37°C in LB broth overnight and then sub-cultured in LB broth until OD600 equal to 0.6 was reached. Then, the volume of the inoculum with the CFU necessary for the infection was calculated and bacterial culture containing the required CFUs was centrifuged at 6,200 x g for 10 min, using a refrigerated microcentrifuge. For *in vitro* infections, bacterial pellet was resuspended in a volume of 10 µl for each dose. In the case of *in vivo* assays, bacterial pellet was resuspended in 200 µl of sterile PBS and then used to immediately infect mice by intagastric gavage (i.g.). Next, to verify the infective dose, serial dilutions of the bacterial inoculum were performed and seeded by microdrop in LB agar plates (with or without antibiotic, according to the assay).

5.4. DC, macrophages and lymphocytes obtention, isolation and preparation

Cells were purified from C57BL/6 bone marrow. Femur, tibia and humerus were extracted from mice and then perfused with medium, RPMI for DC and IMDM for macrophages (Ying, 2013). Approximately 24 million cells were obtained. For cell differentiation, 1 million of cells were seeded by well in a 24 well plate in the case of DC and 4 million of cells for macrophages (Salazar *et al.*, 2017; Gómez *et al.*, 2017; Tardif *et al.*, 2013).

Lymphocytes were obtained from C57BL/6 OT-I and OT-II mice, which were previously described. Spleen was extracted and cell were purified by Magnetic-activated cell sorting (MACS). Cells were resuspended in RPMI. Approximately 12 million cells were obtained by mice. For assays, 200.000 cells were seeded by well in a 96 well plate.

In the case of lymphocytes, cells were activated with anti-CD3/anti-CD28 (Trickett, 2003) previously to assays. A group of T cells remained without activation for being used as control group.

5.5. Dendritic Cells cultures

- Viability assay for Dendritic Cells:

An initial assay was performed, DC were cultured in presence of EcN WT and EcNΔ*chuS* strains, with a MOI (Multiplicity of Infection) of 12 and 25. A group of infected DC were treated with gentamicin 100 µg/ml (Gibco Cat # 1570064) for 1 hour, then with gentamicin 25 µg/ml for 22 hours. Additionally, a group of DC was treated with LPS 1 µg/ml as control of cells activation. Microscopic observations were periodically made for evaluating cellular viability and extracellular bacterial growth. Photographic record was made of each observation until cellular death. Each group of cells were evaluated using flow cytometry, by observing surface activation markers (CD11c, CD40, CD80, CD86) and viability marker (BV510).

This assay allowed us to determine maxim time for DC viability when exposed to these bacterial strains, without visibly affecting DC.

- Evaluation of DC phenotype against different EcN strains stimulus:

EcN WT and mutant strains were used to stimuli DC. *E. coli* K12 and *S.*Thyphimurium (STM) were used as well to infect cells as a control. MOIs used were 2.5, 12, 25 and 50. The time of infection was 1 hour, after this, DC were treated with gentamicin 100 µg/ml for 1 hour, then cells were treated with gentamicin 25 µg/ml for 22 hours, in order to complete 24 hours since the time of infection. Parameters that were evaluated are cytokine secretion in culture supernatant by ELISA (IL-1ß, IFN- γ, IL-6, IL-12p70, IL-10), cellular viability (BV510), activation markers (CD80, CD86, CD40, CD11c, MHC-II), and tolerance markers (PDL-1, PDL-2) by flow cytometry.

- Evaluation of DC Antigen presenting capacity to T lymphocytes

DC were infected with EcN strains as well as before, MOI of 12. At 24 hours post infection, OVA peptide was added for additional 24 hours. $OVA_{257-264}$ 20µM for cultures that will be cocultured with OT-I lymphocytes and $OVA_{323-339}$ 20µM for cultures that were cocultured with OT-II lymphocytes.

Then coculture with OT-I and OT-II lymphocytes were performed. For this, DC were removed from wells and counted, then were placed 200 000 cells by well in a 96 well plate. Lymphocytes were added to each well in a ratio 1:1. Coculture lasted 24 hours.

Additional wells were used for controls: activated lymphocytes with uninfected DC treated with respective OVA peptide, activated lymphocytes with respective OVA peptide, naïve lymphocytes with respective OVA peptide, activated lymphocytes, and just naïve lymphocytes.

Finally, supernatants were collected, and cytokine secretion by T cells evaluated (IL-2, IFN- γ, IL-4, IL-10) by ELISA. Surface activation markers of lymphocytes were also checked (CD69, CD25, CD71).

5.6. Macrophages cultures

Macrophages assay were performed the same way as described for DC cultures with some differences:

- For flow cytometry the markers used were: F4/80, CD11b, CD80, CD86, CD40.
- Presentation antigen assay was not performed with macrophages.

5.7. Lymphocytes proliferation assay

T cells were obtained from a C57BL/6 mice spleen, as described before. Then cells were stained with CFSE (Thermo Fisher Cat # 34554), adding 1µl per ml of cells, for a final concentration of 5 µM (Lyons, 2013). Cells were incubated at 37ºC for 20 minutes. After, RPMI 10% FBS is added 5 times the original volume and incubated for 5 minutes in ice. Then cells were centrifugated and resuspended in warm medium (37ºC) RPMI 10%FBS /L-glutamine /HEPES / 2-mercaptoethanol. This protocol is optimized for $1*10^6$ cells concentration. 200 000 were placed by well in a volume of 190 µl in a 96 well plate.

Infection was made with three different strains, EcN WT, EcN Δ*chuS*, and STM. MOI was 12. In parallel, Heat Killed (HK) bacteria were prepared, using same MOI, bacteria were exposed to 90ºC for 10 min. Cells were infected with respective bacteria for 3 h

at 37ºC, 5% CO_2, following this, they were centrifugated and washed with sterile PBS 1x. Then lymphocytes were resuspended in medium supplemented with 2% of penicillin-streptomycin, gentamicin 50 µg/ml, and 1 µg/ml of anti-CD28. Subsequently, cells were transferred to a new plate with wells activated a day before with 2.5 µg/ml of anti-CD3. Finally, cells incubation lasted 3 days at 37ºC, 5% CO_2.

At the end of the experiment, supernatants were collected for cytokine secretion evaluation (IL-2) and cells were observed by flow cytometry using surface markers (CD4, CD8).

5.8. *In vitro* infection procedure for probiotic strains (WT and mutant) and control strains (STM and *E. coli* K-12)

Before infection, cells were centrifugated and washed twice with sterile PBS 1x. Then resuspended in respective medium without antibiotic. Bacteria in respective MOI were added in a volume of 10 µl to each well for the time specified in each assay. After infection, cells were centrifuged again and washed twice with sterile PBS 1x. Cells were then resuspended in medium supplemented with antibiotic and incubated untill end of the experiment at 37ºC, 5% CO_2.

5.9. Cells preparation from *in vitro* assays to be analyzed by flow cytometry

Cells were scrapped off each well, with PBS 1x, then stained with respective mix of antibodies. 1.2 µg/ml final concentration of antibody per sample was used. Viability stain BV510 was done with 1µl/ml of antibody in PBS. Washes between staining were

made with PBS 1x – 1% BSA. When necessary, cells were fixed with paraformaldehyde 1% for 15 min. Antibodies that were used are listed in Table 3.

Samples were evaluated with BD LSR Fortessa X-20 flow cytometer, in 96 well plates using BD™ High Throughput Sampler. All data were analyzed using FlowJo V.10

5.10. *In vivo* evaluation of ChuS anti-inflammatory properties

To asses second aim of this project, *in vivo* evaluation of anti-inflammatory properties of ChuS was performed.

- First *in vivo* assay

For the first assay, doses of $1*10^9$ CFU of EcN WT and EcNΔ*chuS* were administered to mice, every 48 h. The final volume of each dose was 200 µl. These probiotic administrations were designed to be from day 0 to 14. In total there were 7 administrations. Control group was treated with 200 µl of PBS 1x.

At 14 days post probiotic administration (dpa), a group of mice of each experimental group was infected with $1*10^6$ CFU of *S.* Enteritidis (SEN).

For all mice, euthanasia was performed 48 h post SEN infection (hpi) by anesthesia overdose ketamine 80mg/Kg – xilazyne 10mg/Kg and posterior cervical dislocation. Samples taken in necropsy and evaluations performed are detailed below:

- Spleen, liver and mesenteric lymph nodes: Bacterial load and cytometry (T cells, B cells, Myeloid cells).

Table 3. Antibodies conjugated to fluorophores used for flow cytometry

Cell Marker	Antibody	Cell used for
Fixable Viability Stain	BV510 BD Pharmingen Cat # 564406	DC, Macrophages and T cells.
MHC-II (I-A/I-E)	BV650 BD Pharmingen Cat # 563415	DC, Macrophages
CD11c	PE-Cy7 BD Pharmingen Cat # 558079	DC
CD40	APC BD Pharmingen Cat # 558695 PE-Cy7 BioLegend Cat # 124621 PE BD Pharmingen Cat # 561846	DC Macrophages
CD80	PE BD Pharmingen Cat # 561955 PerCP-Cy5.5 BD Pharmingen Cat # 560685	DC DC, Macrophages
CD86	APC-Cy 7 BD Pharmingen Cat # 560582	DC, Macrophages
PD-L1 (CD274)	BV605 BD Pharmingen Cat # 745135	DC, Macrophages
PD-L2 (CD273)	PE BD Pharmingen Cat # 557796	DC, Macrophages
CD11b	BB515 BD Pharmingen Cat # 564454	Macrophages
F4/80	PE BD Pharmingen Cat # 565410 APC BD Pharmingen Cat # 566787	Macrophages
TCR-β	PE-Cy7 BD Pharmingen Cat # 560729	T Cells, Proliferation assay
CD3	PerCP-Cy5.5 BD Pharmingen Cat # 560527	OT-I, OT-II
CD4	PE BD Pharmingen Cat # 561832 APC-H7 BD Pharmingen Cat # 560246 APC BD Pharmingen Cat # 561091	OT-II OT-II T cells, Proliferation assay
CD8	APC-Cy7 BD Pharmingen Cat # 561967 BV605 BD Pharmingen Cat # 563152	OT – I T Cells, Proliferation assay
CD25	APC BD Pharmingen Cat # 561048 FITC BD Pharmingen Cat # 561779	OT-I, OT-II OT-II
CD69	FITC BD Pharmingen Cat # 561929 PE BD Pharmingen Cat # 561932	OT-I, OT-II
CD71	PE-Cy7 BioLegend Cat # 113811 FITC BD Pharmingen Cat # 561967	OT-I, OT-II

- Ileum and colon are longitudinally dissected in two equal parts, for histology and RNA extraction (Cytokine expression).

- Caecum: RNA extraction (Cytokine expression).

Cell markers mixes used for flow cytometry evaluation:

- T cells: CD45, TCR-β, CD3, CD4, CD8, CD25.

- Myeloid cells: CD45, CD11b, Ly6G, Ly6C, F4/80, CD11c, CD103.

- B cells: CD45, B220, CD19.

Since day 0 post probiotic administration till the end of the experiment, each 48 h feces samples were taken for evaluation of the presence of respective bacteria, by PCR.

- Second *in vivo* assay: doses curve

In this assay, 4 different doses of each strain (EcN WT and EcNΔ*chuS*) were evaluated. Experimental groups are listed below:

1. Vehicle: 3 mice
2. Treated with EcN WT $1*10^7$ CFU: 3 mice
3. Treated with EcN Δ*chuS* $1*10^7$ CFU: 3 mice
4. Treated with EcN WT $1*10^6$ CFU: 3 mice
5. Treated with EcN Δ*chuS* $1*10^6$ CFU: 3 mice

6. Treated with EcN WT $1*10^5$ CFU: 3 mice

7. Treated with EcN Δ*chuS* $1*10^5$ CFU: 3 mice

8. Treated with EcN WT $1*10^4$ CFU: 3 mice

Both strains were intragastrically administered to mice each 48 h, from day 0 to day 28. In total 14 administrations were made (days: 1, 3, 5, 7, 9, 11,13, 15, 17, 19, 21, 23, 25 and 27). Vehicle groups will receive sterile PBS 1x in the same volume as infection dose. Mice were daily monitored by registering a clinical score and recording feces by photographs. Feces samples were taken each 48 h. On day 28 was the end of the experiment.

For all mice, euthanasia was performed at 28 dpa by anesthesia overdose ketamine 80mg/Kg – xilazyne 10mg/Kg and posterior cervical dislocation. Blood samples were taken for cytokine secretion evaluation by ELISA (IL-10, IL12p70). Organs collected and analyses performed are listed below:

- Spleen, Liver and Mesenteric Lymph nodes: Bacterial load and Flow Cytometry (T cells, Myeloid cells and B cells).

- Ileum, Caecum and colon: Histology

Cell markers used for flow cytometry evaluation were the same described above.

5.11. Infection of mice with probiotic strains (WT and mutant)

For infection assays, mice were anesthetized with isoflurane and infected via intragastrically with appropriate doses of every EcN strain, according to the experimental design described above, in 200µl of PBS, using mouse feeding tube (for gavage). Infectious doses were corroborated by dilution and seeding in LB agar.

Experiments using animals were daily overseen by trained personnel and clinical score (Table 4) was evaluated as previously described. Weight loss of more than 20% of the initial one and/or manifestation of severe signs of pain as depression, lethargy, weakness and uncoordinated movements on cage, were considered humanitarian criteria to end point.

Clinical score is designed with considerations according to punctuation described below:

- 0 to 3: Normal
- From 4 to 7: Careful supervision
- From 8 to 12: Intense suffering. Evaluate the possibility of suppressing the experiment and performing euthanasia.
- Over 12: Euthanasia

Table 4. Specific Clinical Score

Variables	Observations	Score
Physiological Data: Corporal weight loss	Normal	0
	Weight loss less than 10%	1
	Weight loss beween 10 and 20 %	2
	Weight loss superior to 20%.	3
Aspect	Normal (erect, hangs in its cage, lustrous fur).	0
	Mouse avoids moving, hirsute fur, there may be porphyric secretion in the nose and eyes (red).	1
	Bent over, motionless at the bottom of the cage, hollow, hirsute fur, porphyric secretion of nose and eyes (red).	2
	Prostrate, lying on the side, obvious dehydration, sunken eyes.	3
Spontaneous behavior	Normal: mouse país attention to the environment, interact with your peers, groom yourself	0
	Small changes: reduces grooming and displacement, rapid respiratory movements, tends to stay at the bottom of the cage.	1
	Shaky, inactive or retracted displacement at the bottom of the cage, abdominal breathing.	2
	Immobile animal, prostrate on the lateral side, opens its mouth when breathing.	3
Hydration parameters	Return time of the skin fold of the torso, less than 2 seconds; normal position; eyeball without alterations.	0
	Return time of the skin fold of the torso, less than 2-4 seconds; piloerection; weight loss between 10-15%, decreased movements inside the cage.	1
	Return time of the skin fold of the torso of 2-4 seconds, weight loss greater than 10%, piloerection and arched posture, weakness and dry stools.	2
	Time of return of the fold of the skin of the torso greater than 5 seconds, arched posture, obvious sinking of the eyeball, inability to incorporate itself.	3
General evaluation of colitis	Normal: Stools formed, without alterations in the anus.	0
	Altered stool consistency: pasty or firm / dry but formed, presence of mucus.	1
	Soft or absent stools; hematochezia, mild perianal abnormalities such as edema, perianal dermatitis.	2
	Diarrhea or constipation with tenesmus; hematochezia that can reach mane, obvious perianal alterations.	3

5.12. Bacterial Load in organs

Spleen, liver and mesenteric lymph nodes were homogenized and then seeded in LB agar for EcN WT and LB agar with Kanamycin for EcNΔ*chuS*. Cultures were incubated overnight at 37ºC. CFUs were registered.

Each organ recovered after euthanasia were weighted, disrupted in sterile PBS, serially diluted in sterile PBS and seeded on LB agar with antibiotic when needed, using microdrop technique. Briefly, after disruption of organs, 10µl of each sample was serially diluted five times on 90µl of sterile PBS and three 10µl drops were seeded for each dilution. Seeded plates were incubated overnight at 37°C for 18 h. CFUs were counted and adjusted to total CFUs, according to the dilution normalized by initial weight (mg) of the organ or volume (ml).

5.13. Flow cytometry of organs

Organs were recovered at different hours post-infection (hpi), minced with sterile scissors and placed in sterile PBS 1x in ice. Homogenized organs were filtered using a 70-µm cell strainer (Falcon Cat nº: 352350).

- For liver: Cells were centrifuged, and the pellet was placed at 37°C with stirring for 30 min with serum-free medium (5 to 10 ml). Then, maximum volume of 15 ml falcon tubes was completed with PEB with DNase and samples were centrifuged at 30g for 6 min to pellet the hepatocytes. Supernatant is recovered and the pellet is discarded. Finally, supernatant is brought back to maximum

volume and centrifuged at 300g for 10 min to pellet the immune cells. Cells were counted and resuspended for a final volume of 100 µl of cells for well in a 96 well plate.

- For spleen: 0.5 ml of 1x ACK was added for 3 min to remove red blood cells. Then, tissue was passed through cell strainer and 0.5 ml of 1x ACK was added again, this procedure is repeated twice. In between repetitions, cells were centrifuged at 1800 rpm for 5 min each time. Then, 5-10 ml of FACs buffer were added. Next, samples were centrifuged at 1500 rpm for 10 min at 4ºC. Supernatant was discarded. And cells were resuspended with FACs buffer for being counted. Finally, cells were served in a volume of 100 µl per well in a 96 well plate.

- For mesenteric lymph nodes: cells were pelleted and resuspended in 1 ml of PBS 1x - 2% FBS, then were counted. Finally, they were resuspended for a final concentration of $1x10^6$ cells / 100 µl. Cells were served in 96-well plates in a volume of 100 µl.

Staining of cells was done with respective mix of antibodies. 1.2 µg/ml final concentration of antibody per sample was used. Viability stain BV510 was done with 1µl/ml of antibody in PBS. When necessary, samples were fixed with 2% of paraformaldehyde, for 15 minutes at 4ºC. Antibodies used are listed in Table 5. Samples were evaluated with BD LSR Fortessa X-20 flow cytometer, in 96 well plates using BD™ High Throughput Sampler. All data were analyzed using FlowJo V.10

Table 5. Fluorophores conjugated antibodies used for organs flow cytometry

Cell Marker	**Antibody**
Viability stain for all cells	BV510 BD 564496
T cells mix	
CD 45	BV786 564225
TCR-β	PE-Cy7 BD 560729
CD3	PerCP-Cy5.5 560527
CD4	APC – H7 BD 560246 APC BD 561091
CD8	BV605 BD 563152
CD25	BV421 BD 566228
Myeloid cells mix	
CD45	BV786 BD 564225
CD11b	PE BD 561689
Ly6G	A700 BioLegend 127622
Ly6C	BV605 BD 563011
F4/80	APC BD 566787
CD11c	FITC BD 561045
CD103	PerCP-Cy5.5 BD 563637
B cells mix	
CD45	BV786 BD 564225
B220	PE BD 561878
CD19	BB515 564531
CD3	PerCP-Cy5.5 BD 560527
CD4	BV650 563232
CD11b	APC 561690

5.14. Histological analysis

Organs were fixed in formalin for subsequent paraffin embedding and hematoxylin-eosin staining. The tissue was observed by microscopy to determine changes in the structure of the colon.

5.15. Statistical analyses

Finally, sample size for experiment with animals were calculated using G*Power 3.1.9.2. Necessary statistical analyses were performed using PRISM 5 software. Variance analysis and Student's t-test were carried out. One way or 2way ANOVA were performed depending on data type and comparisons wanted. Turkey's test was used for multiple comparisons. Linear and Non-Linear regression were performed to analyze clinical score and weight loss curves. For all analysis, P value = 0.05 was considered as statistically significant.

6. RESULTS

6.1. Determination of the presence of *chu* operon in *E. coli* Nissle 1917

Sequence analysis were performed with EcN genome comparing to EHEC specifically looking for complete *chu* operon presence. Basing on EHEC *chu* operon sequence, primers for Tiling PCR (Table 1) were designed: OPCHU1, OPCHU2, OPCHU3, OPCHU4, OPCHU5 (Figure 5). These primers were used to detect whole *chu* operon in EcN.

Tiling PCR amplicons for EcN showed the presence of complete set of genes for *chu* operon as observed in Figure 6 below. Finally, EcN genome region 4018329 – 4027281 hosts the eight genes of *chu* operon.

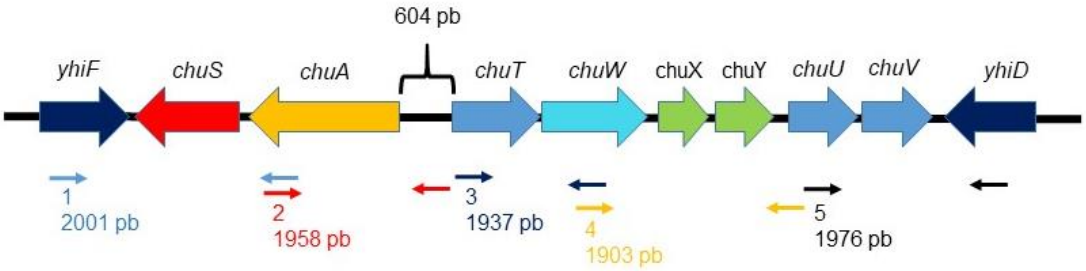

Figure 5. Tiling PCR primers alignment to EHEC chu operon sequence. Primers are placed in pairs by colors (Forward and reverse) and amplicon size are specified below each pair.

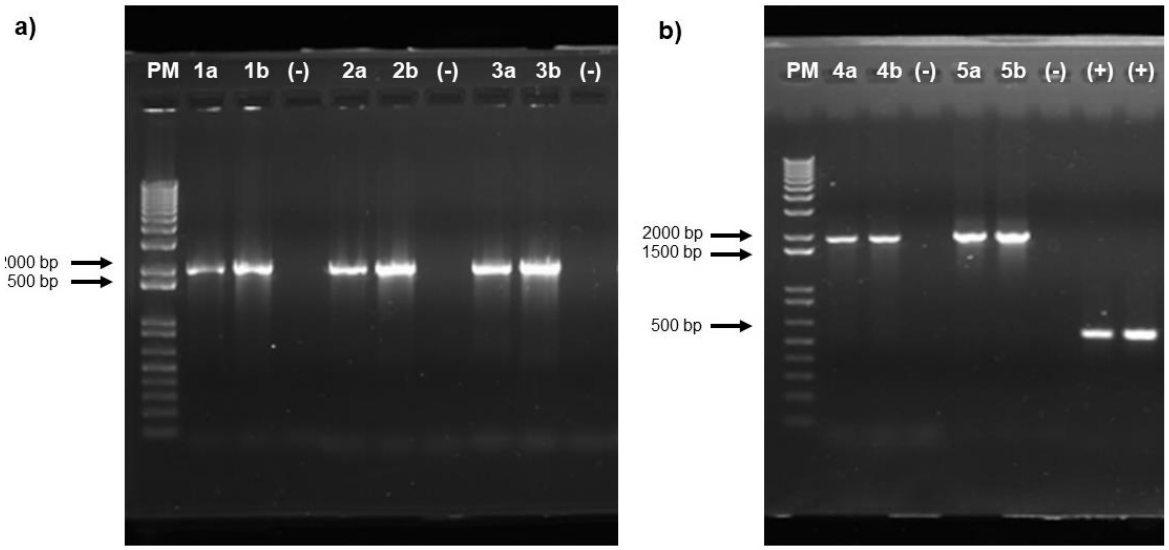

Figure 6. Detection of chu operon presence in EcN WT. PCR was performed in set of primers OPCHU1 (1), OPCHU2 (2), OPCHU3 (3), OPCHU4 (4), and OPCHU5 (5), detailed before. Control (+) corresponds to rpoD amplification. Control (-) corresponds to a sample that does not have DNA. Each amplicon was done in duplicated (e.g. 1a and 1b). a) Agarose gel was exposed to UV light for 300 ms. Amplicons sizes are 1: 2001 bp, 2: 1958 bp, and 3: 1958 bp. b) Agarose gel was exposed to UV light for 300 ms. Amplicons size are: 4:1903 bp, 5: 1903 bp, and control (+): 586bp. PM: Molecular weight marker.

6.2. Mutants generation and viability assays of mutant strains

Mutant strains were developed used method described by Datsenko & Wanner (2000). Once generated, strains were confirmed by PCR. EcNΔ*chuS* strain with kanamycin resistance were correctly generated as seen in Figure 7.

On the other hand, EcN Δ*chuS::putA::chuS* and EcN *putA::chuS* were no viable strains, apparently extra copy of *chuS* impaired in some way bacteria survival since only bacteria that rejected this transformation survived. In PCR confirmation, amplicons in three mixes were necessary to confirm correct modifications in both strains. Despite of presenting expected amplicons in mix 1, there were no amplicons at all in mix 2, but the presence of correct size of amplicons in mix three confirms the presence of bacteria DNA (Figure 7).

However, it is worth to mention that when whole pKD3_*chuS* plasmid was inserted in *E. coli* k -12 and *E. coli* SM10λpir, bacteria survival was not affected.

In viability assays only EcN WT and EcNΔ*chuS* were assessed. Each strain was evaluated in 4 different cultures at the same time: 1. without treatment, 2. treated with CoPP, 3. treated with SnPP, and 4. treated with vehicle. Treatments with CoPP and SnPP did not alter normal growth in comparison with the culture of EcN that did not received any treatment (Figure 8a). CFU counting have shown that neither CoPP nor SnPP are toxic for bacteria, CFU/ml values are similar between groups (Figure 8b).

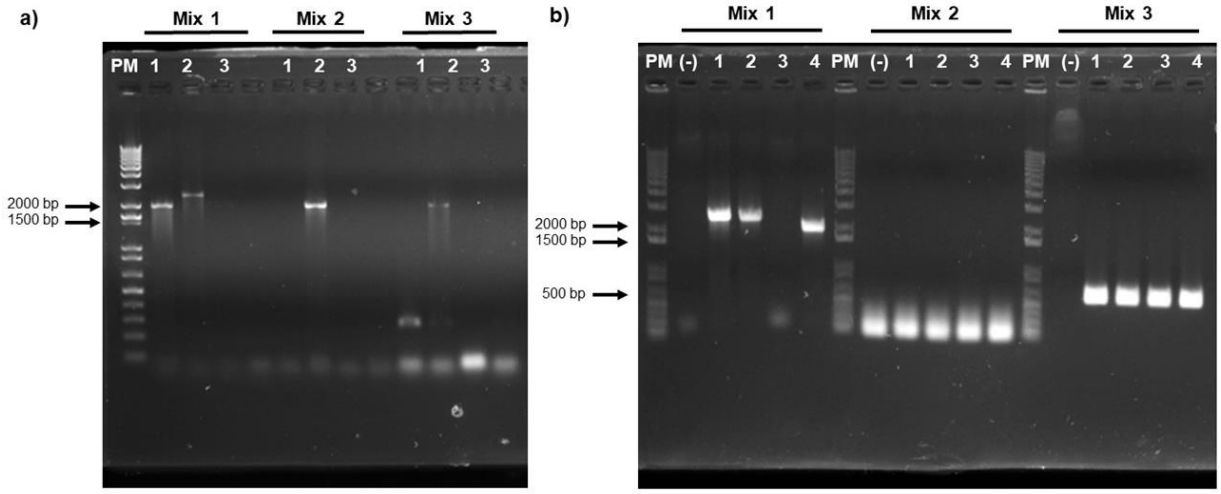

Figure 7. Mutant strains confirmation by PCR. a) Samples correspond to 1: EcN WT, 2: EcNΔ*chuS*, and 3: Control (-) without DNA. Mix 1 corresponds to external primers for *chuS*: OPCHU1 forward and reverse, amplicons sizes are 1: 1997 bp, 2: 2445bp, and 3: no amplicon. Mix 2 corresponds to an external forward primer with an internal reverse one, OPCHU1 Fw and *chuS*(H2+P2). Amplicons sizes are 1 and 3: no amplicon, 2: 2006 bp. Mix 3 corresponds to and internal forward primer and an external reverse one, *chuS*(H1+P1) and OPCHU1 Rev. Amplicons sizes are 1 and 3: no amplicon, 2: 1996 bp. B) Samples correspond to 1: EcNΔ*chuS*, 2: EcNΔ*chuS*/*putA*::*chuS*, 3: EcN *putA*::*chuS*:, 4:EcN WT, and control (-) without DNA. Mix 1 corresponds to OPCHU1 forward and reverse. Expected amplicons sizes are 1 and 2: 1054 bp, 3 and 4: 1997 bp. Mix 2 corresponds to EHEC-ETEC PutA::Cm H1+P1 and Nissle PutA H2+P2. Expected amplicons sizes are 1 and 4: no amplicon, 2 and 3: 2330 bp. Mix 3 corresponds to rpoD Fw_2 and rpoD Rev. Expected amplicon size for all samples is 586 bp. PM: Molecular weight marker.

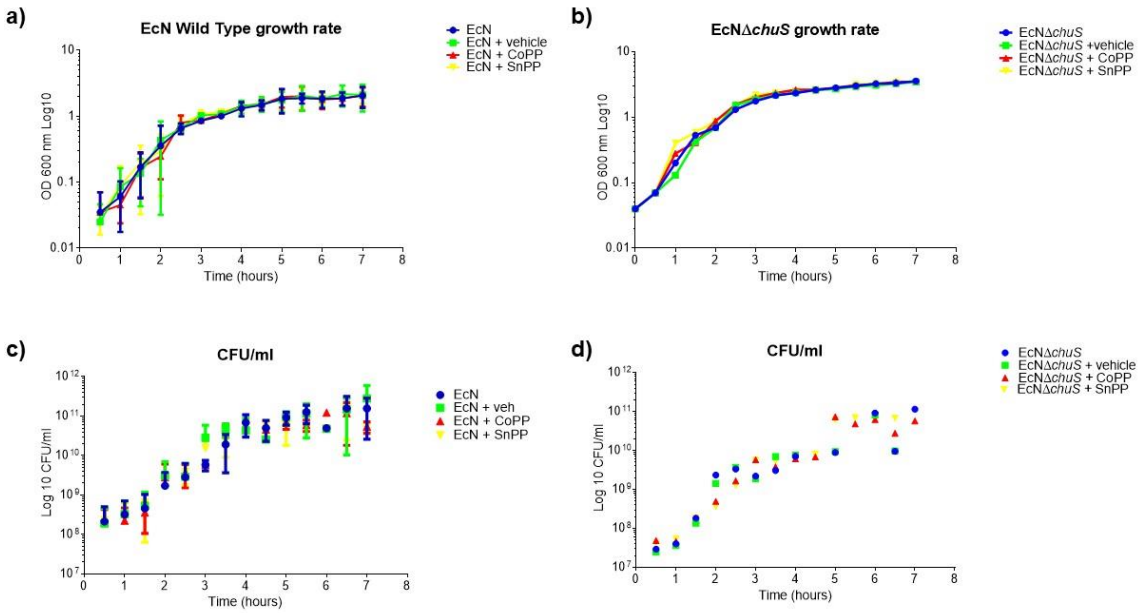

Figure 8. CoPP and SnPP treatment did not alter growth rate and CFU/ml of EcN WT and EcNΔ*chuS*. Growth rate was measured in spectrophotometer, OD 0.6 nm. Measurements were done using 100 µl of culture and diluted 1:10 in LB. CFU values were calculated for 1 ml of culture. a) EcN WT growth rate. b) EcNΔ*chuS* growth rate c) EcN WT CFU/ml. d) EcNΔ*chuS* CFU/ml. For EcN WT data correspond to two independent experiments. For EcNΔ*chuS* data correspond to a unique experiment.

chuS basal expression was evaluated, which was low. But in presence of an inducer *chuS* expression was highly increased in comparison to the inhibition seen with SnPP treatment (Figure 9). This behavior is similar to HO-1 which highlights the hypothesis that both enzymes may have similar mechanism of action.

6.2. Viability of DC and macrophages infected with EcN WT and EcNΔ*chuS*

The major purpose for this assay was to stablish the time of infection with two different MOI (12 and 25) for which DC and macrophages will remain viable. For this, DC and macrophages were infected and a group of each one treated with gentamicin to evaluate whether cells can survive with active infection or not. Pictures were taken periodically. Despite of MOI, at 6 h post infection, DC infected with EcN WT and treated with gentamicin survived, cells shape was not altered, and medium remained clear in comparison to control group (Figure 10 d and f,). On the other hand, DC infected with EcN WT without gentamicin treatment did not survive regardless of MOI (Figure 10 c and e), medium was full of active bacteria and with a murky appearance in comparison to control group. DC infection with EcNΔ*chuS* showed same behavior as described for EcN WT infection (Figure 10 g, h, i, and j).

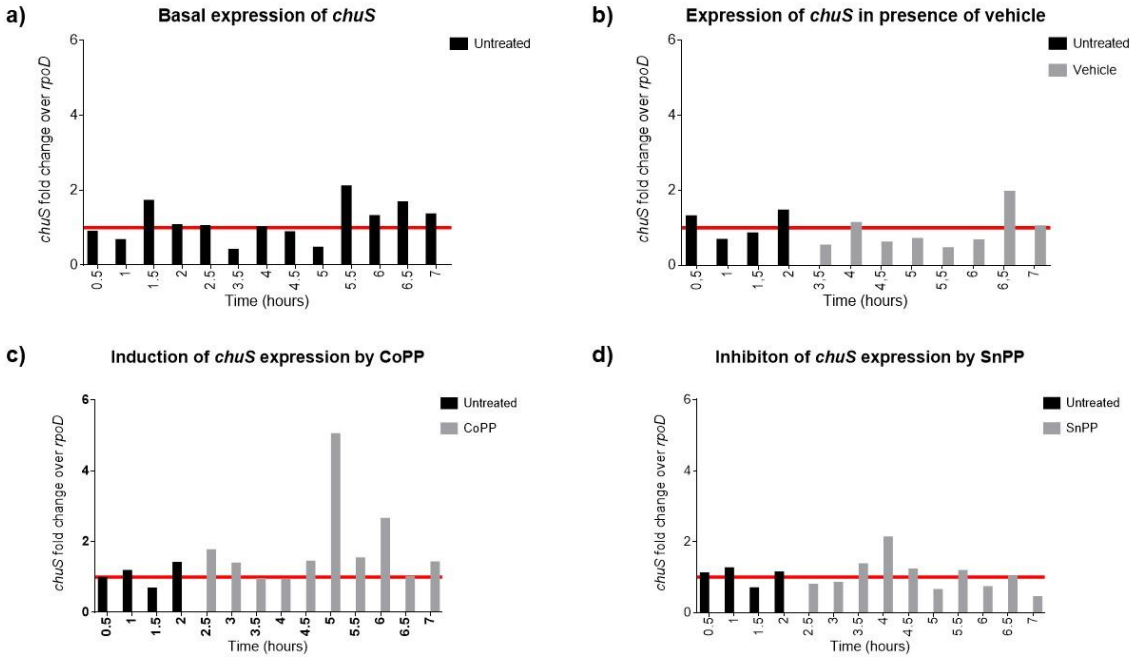

Figure 9. *chuS* expression is modulated by inducer CoPP and inhibitor SnPP during time. a) This group did not receive any treatment during time. b) Vehicle NaOH 1 N used for doses preparation was administered at the point of 2.5 h. c) Doses of CoPP and d) doses of SnPP were administered at the point of 2.5 h. For data analysis each point was compared to *rpoD* expression. Then an average of the first four points of each experimental group which correspond to untreated points of each group were calculated and used to compare each sampling point with it. Data is representative from 2 independent experiments.

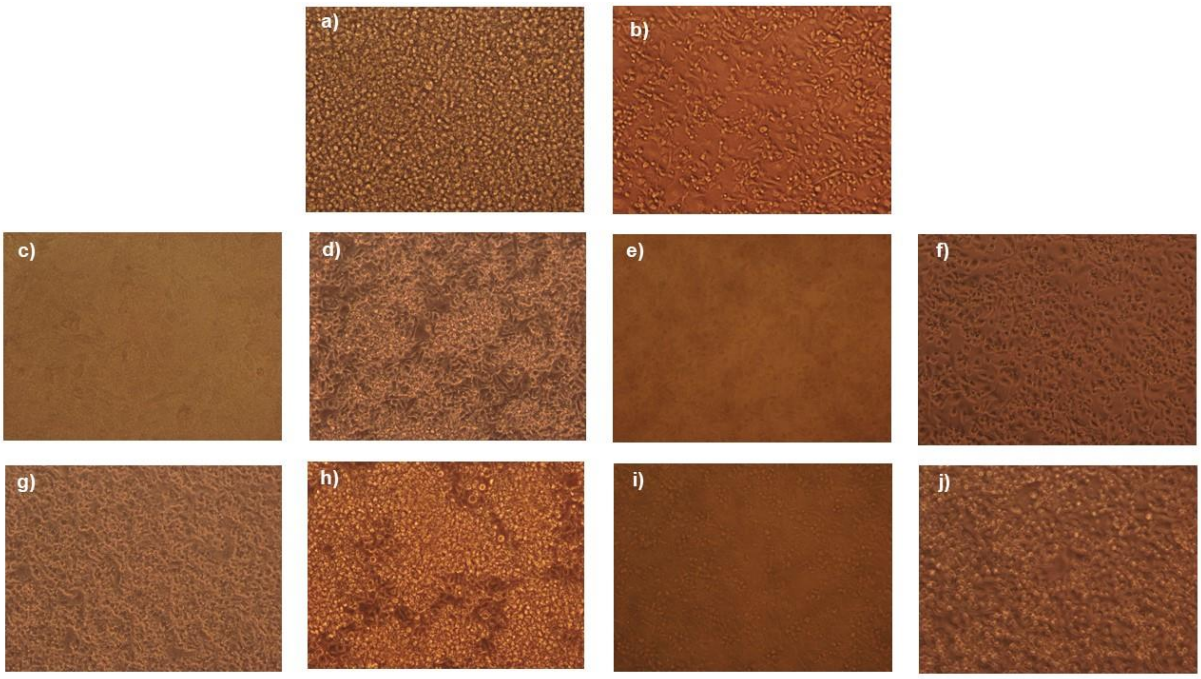

Figure 10. Representative photographs of dendritic cells 6 h post infection with EcN WT and EcNΔ*chuS*. a) control group: not infected cells and without antibiotic treatment. b) DC treated with LPS 1 µg/ml as control of cell activation. c) DC infected with EcN WT MOI 12 without antibiotic treatment. d) DC infected with EcN WT MOI 12 with gentamicin treatment. e) DC infected with EcN WT MOI 25 without antibiotic treatment. f) DC infected with EcN WT MOI 25 with gentamicin treatment. g) DC infected with EcNΔ*chuS* MOI 12 without antibiotic treatment. h) DC infected with EcNΔ*chuS* MOI 12 with gentamicin treatment. i) DC infected with EcNΔ*chuS* MOI 25 without antibiotic treatment. j) DC infected with EcNΔ*chuS* MOI 25 with gentamicin treatment. Microscopical observations for this figure were performed at 20x for all samples. Photographs were taken using an Infinity 2 camera in microscope using Infinity Analyze software. Data set represents a unique experiment.

The next analysis was at 18 h post infection. For both strains and both MOIs, DC that did not receive antibiotic treatment died before 6 hours post infection. Thus, observation at 18 h have shown viability only in DC with gentamicin treatment post infection. There were not evident differences between groups infected with EcN WT and EcNΔ*chuS* (Figure 11). Observations ended at this point because data was enough to determine the capability of DC to survive only when treated with gentamicin after infection, despite of MOI and strain.

For same analysis with macrophages, these cells shown to be more resistant to EcN strains infection. In the first observation at 6 h post infection all groups of macrophages were viable, they did not change morphology, and bacteria multiplied considerably in groups without antibiotic treatment (Figure 12). However, bacteria population was controlled in some way by macrophages themselves.

The next analysis was 18 h post infection. Macrophages treated with the highest MOI (25) off both strains and without antibiotic treatment started to die (Figure 13 e and i). Also, bacteria population continued growing. Besides, experimental groups that were treated with gentamicin remained completely viable, with no changes in morphology (Figure 13 d, f, h and j)).

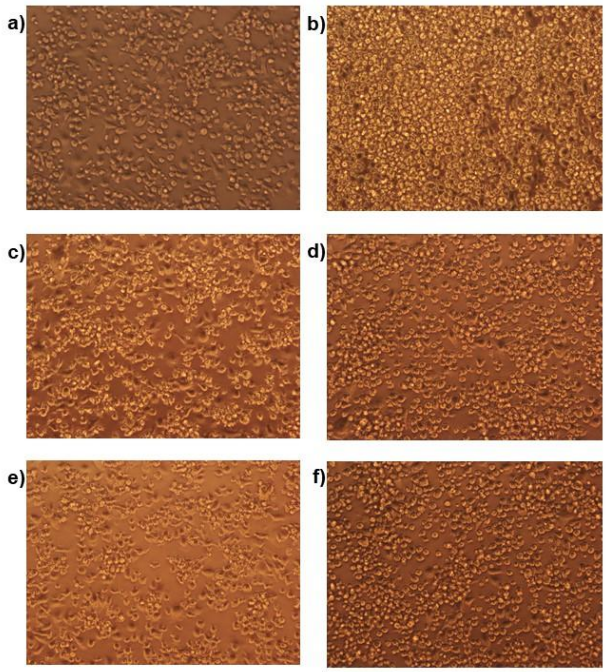

Figure 11. Representative photographs of dendritic cells 18 h post infection with EcN WT and EcNΔ*chuS*. a) control group: not infected cells and without antibiotic treatment. b) DC treated with LPS 1 µg/ml as control of cell activation. c) DC infected with EcN WT MOI 12 with gentamicin treatment. d) DC infected with EcN WT MOI 25 with gentamicin treatment. e) DC infected with EcNΔ*chuS* MOI 12 with gentamicin treatment. d) DC infected with EcNΔ*chuS* MOI 25 with gentamicin treatment. Microscopical observations for this figure were performed at 20x for all samples. Photographs were taken using an Infinity 2 camera in microscope using Infinity Analyze software. Data set represents a unique experiment.

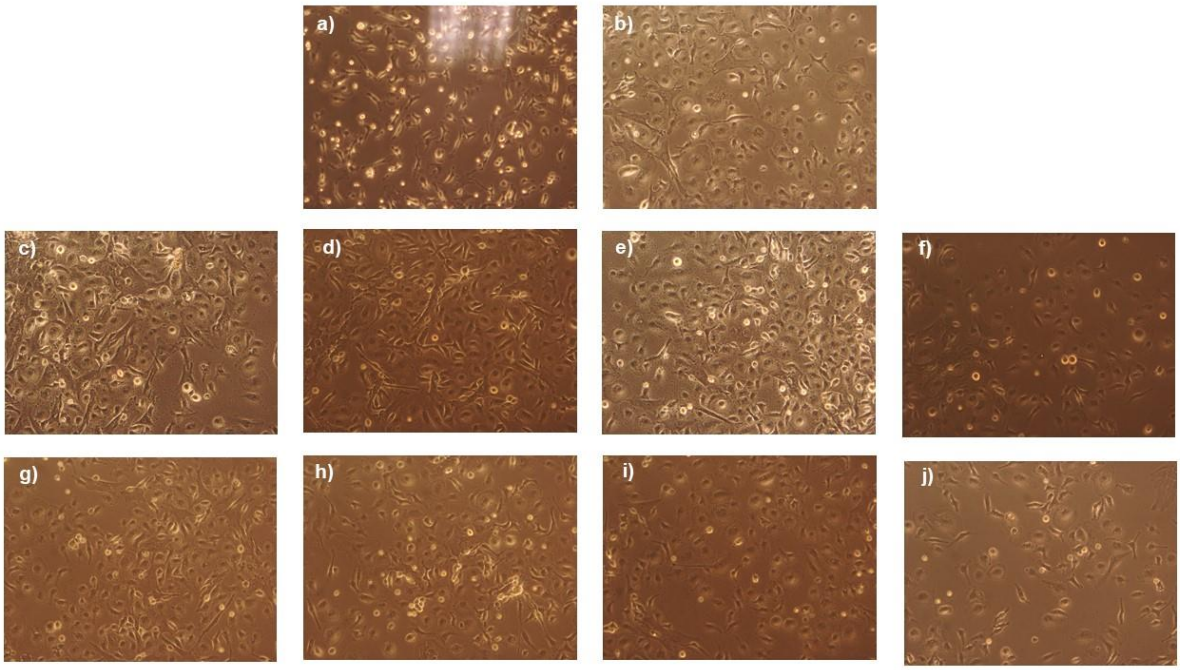

Figure 12. Representative photographs of macrophages 6 h post infection with EcN WT and EcNΔ*chuS*. a) control group: not infected cells and without antibiotic treatment. b) DC treated with LPS 1 µg/ml as control of cell activation. c) Macrophages infected with EcN WT MOI 12 without antibiotic treatment. d) Macrophages infected with EcN WT MOI 12 with gentamicin treatment. e) Macrophages infected with EcN WT MOI 25 without antibiotic treatment. f) Macrophages infected with EcN WT MOI 25 with gentamicin treatment. g) Macrophages infected with EcNΔ*chuS* MOI 12 without antibiotic treatment. h) Macrophages infected with EcNΔ*chuS* MOI 12 with gentamicin treatment. i) Macrophages infected with EcNΔ*chuS* MOI 25 without antibiotic treatment. j) Macrophages infected with EcNΔ*chuS* MOI 25 with gentamicin treatment. Microscopical observations for this figure were performed at 20x for all samples. Photographs were taken using an Infinity 2 camera in microscope using Infinity Analyze software. Data set represents a unique experiment.

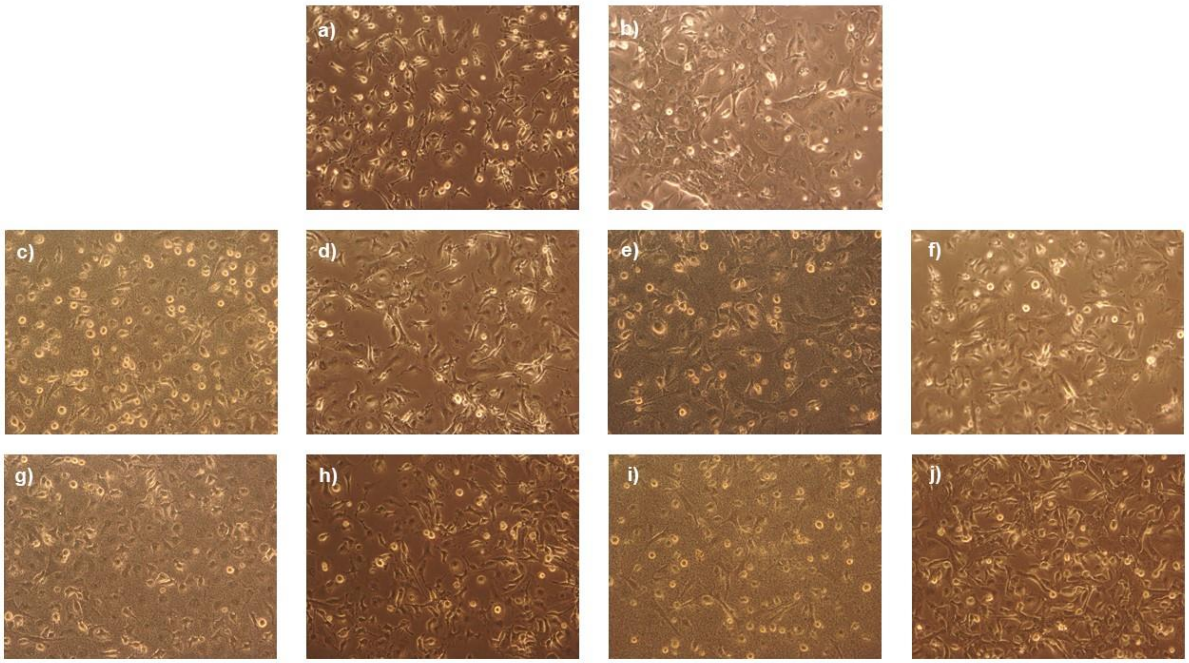

Figure 13. Representative photographs of macrophages 18 h post infection with EcN WT and EcNΔ*chuS*. a) control group: not infected cells and without antibiotic treatment. b) DC treated with LPS 1 µg/ml as control of cell activation. c) Macrophages infected with EcN WT MOI 12 without antibiotic treatment. d) Macrophages infected with EcN WT MOI 12 with gentamicin treatment. e) Macrophages infected with EcN WT MOI 25 without antibiotic treatment. f) Macrophages infected with EcN WT MOI 25 with gentamicin treatment. g) Macrophages infected with EcNΔ*chuS* MOI 12 without antibiotic treatment. h) Macrophages infected with EcNΔ*chuS* MOI 12 with gentamicin treatment. i) Macrophages infected with EcNΔ*chuS* MOI 25 without antibiotic treatment. j) Macrophages infected with EcNΔ*chuS* MOI 25 with gentamicin treatment. Microscopical observations for this figure were performed at 20x for all samples. Photographs were taken using an Infinity 2 camera in microscope using Infinity Analyze software. Data set represents a unique experiment.

The next analysis was at 30 h post infection. At this final point, only groups without antibiotic treatment remained in observation. The other groups were used for activation markers evaluation. Observations showed that despite of strain and MOI, cells died. Bacteria population greatly exceeded macrophages (Figure 14).

DC and macrophages maturation markers CD40, CD80, and CD86, were evaluated at 24 h post infection by flow cytometry. Also, viable cells were counted. Results showed that DC that were not treated with antibiotic were less activated compared to DC treated with gentamicin (Figure 15) There were not significant differences in the expression of maturation markers between DC infected with MOI12 and MOI 25 (Figure 15 b, c, d, e, f and g). Maturation of macrophages was evaluated only in groups that were treated with gentamicin. It's important to mention that both strains were capable to activate DC and macrophages. There were no significant differences between strains.

In conclusion, results have showed that antibiotic treatment is necessary to maintain viability after infection until at least 24 h post infection. Also, probiotic strains were able to activate DC and macrophages in both MOI.

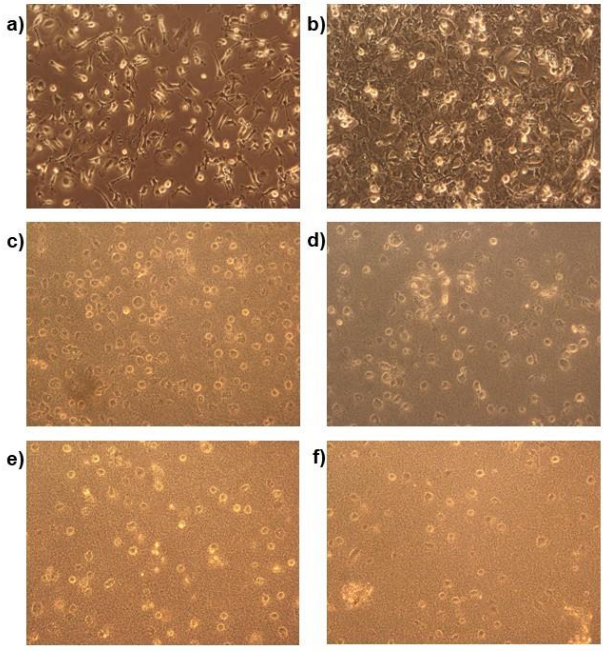

Figure 14. Representative photographs of macrophages 30 h post infection with EcN WT and EcNΔ*chuS*. a) control group: not infected cells and without antibiotic treatment. b) DC treated with LPS 1 µg/ml as control of cell activation. c) Macrophages infected with EcN WT MOI 12 without antibiotic treatment. d) Macrophages infected with EcN WT MOI 25 without antibiotic treatment. e) Macrophages infected with EcNΔ*chuS* MOI 12 without antibiotic treatment. d) Macrophages infected with EcNΔ*chuS* MOI 25 without antibiotic treatment. Microscopical observations for this figure were performed at 20x for all samples. Photographs were taken using an Infinity 2 camera in microscope using Infinity Analyze software. Data set represents a unique experiment.

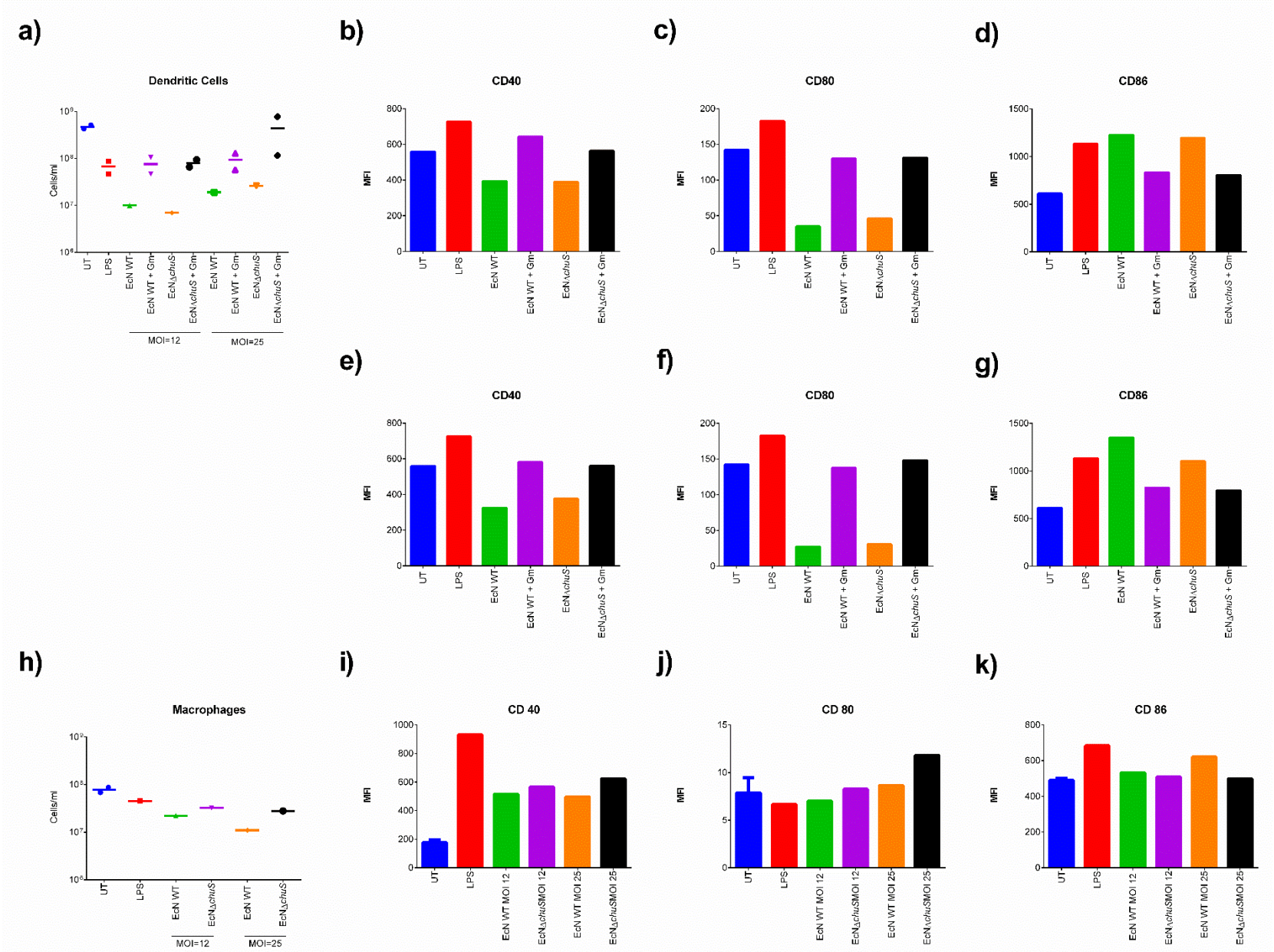
a)
Dendritic Cells
Cells/ml
UT
LPS
EcN WT
EcN WT + Gm
EcNΔchuS
EcNΔchuS + Gm
MOI=12
MOI=25
b)
CD40
MFI
c)
CD80
d)
CD86
e)
CD40
f)
CD80
g)
CD86
h)
Macrophages
i)
CD 40
j)
CD 80
k)
CD 86
EcN WT MOI 12
EcNΔchuSMOI 12
EcN WT MOI 25
EcNΔchuSMOI 25

Figure 15. (Next page) DC and macrophages are activated after being infected with EcN and EcNΔ*chuS*. a) Graph showing the quantity analysis of DC (cells/ml) after they were infected with MOI 12 and 25 and treated or not with gentamicin. b) MFI analysis of CD40 in DC infected with MOI 12 and treated or not with gentamicin. c) MFI analysis of CD80 in DC infected with MOI 12 and treated or not with gentamicin. d) MFI analysis of CD86 in DC infected with MOI 12 and treated or not with gentamicin. e) MFI analysis of CD40 in DC infected with MOI 25 and treated or not with gentamicin. f) MFI analysis of CD80 in DC infected with MOI 25 and treated or not with gentamicin. g) MFI analysis of CD86 in DC infected with MOI 25 and treated or not with gentamicin. h) Graph showing the quantity analysis of macrophages (cells/ml) after they were infected with MOI 12 and 25 and treated with gentamicin. i) MFI analysis of CD40 in macrophages infected with MOI 12 and 25 and treated with gentamicin. j) MFI analysis of CD80 in macrophages infected with MOI 12 and 25 and treated with gentamicin. K) MFI analysis of CD86 in macrophages infected with MOI 12 and 25 and treated with gentamicin. Gn: Gentamicin, UT: Untreated. LPS: group treated with LPS 1 µg/ml (used as positive control of cell activation). Data sets correspond to unique experiment.

6.1. Evaluation of DC and Macrophages phenotype against different EcN strains stimulus. DC and macrophages phenotype is modulated by different EcN strains stimulus.

The assay described before allowed us to determine 24 h post infection as the time of evaluation for the next experiments. Cells were infected with three different MOI: 2.5, 12, 25 of EcN WT and EcNΔ*chuS*. Then, activation of DC and macrophages was evaluated observing CD80 and CD86 surface markers. Results in DC have shown that both strains were equally capable of activating cells (Figure 16). Also, PD-L1 and PD-L2, which were evaluated as tolerance markers, did not shoe either significant differences (Figure 16 c and d). However, in PD-L2 marker a little tendency was observed in which treatment with EcN WT stimulated a higher expression than treatment with mutant strain (Figure 16d). Most remarkably differences were found in MOI 12 in IL-10 (Figure 18a). Indeed, secretion of this cytokine presented differences statistically significant between EcN WT and EcNΔ*chuS*, promoting WT strain a major level of IL-10. Also, a tendency is observed in IL-1β secretion, where levels were lower for treatment with EcN WT and higher for EcNΔ*chuS* (Figure 18a).

In the case of macrophages results were similar to the findings in DC. In almost every MOI there were no differences in activation between cells infected with EcN WT and EcNΔ*chuS* (Figure 17). In cytokine secretion in MOI 12, in both cases IL-1β and IL-10, cells treated with EcN WT had shown higher levels of secretion than EcNΔ*chuS* group. Additionally, IL-6 was evaluated in both cell cultures, but no significant difference between probiotic strains was found (Figure 18b).

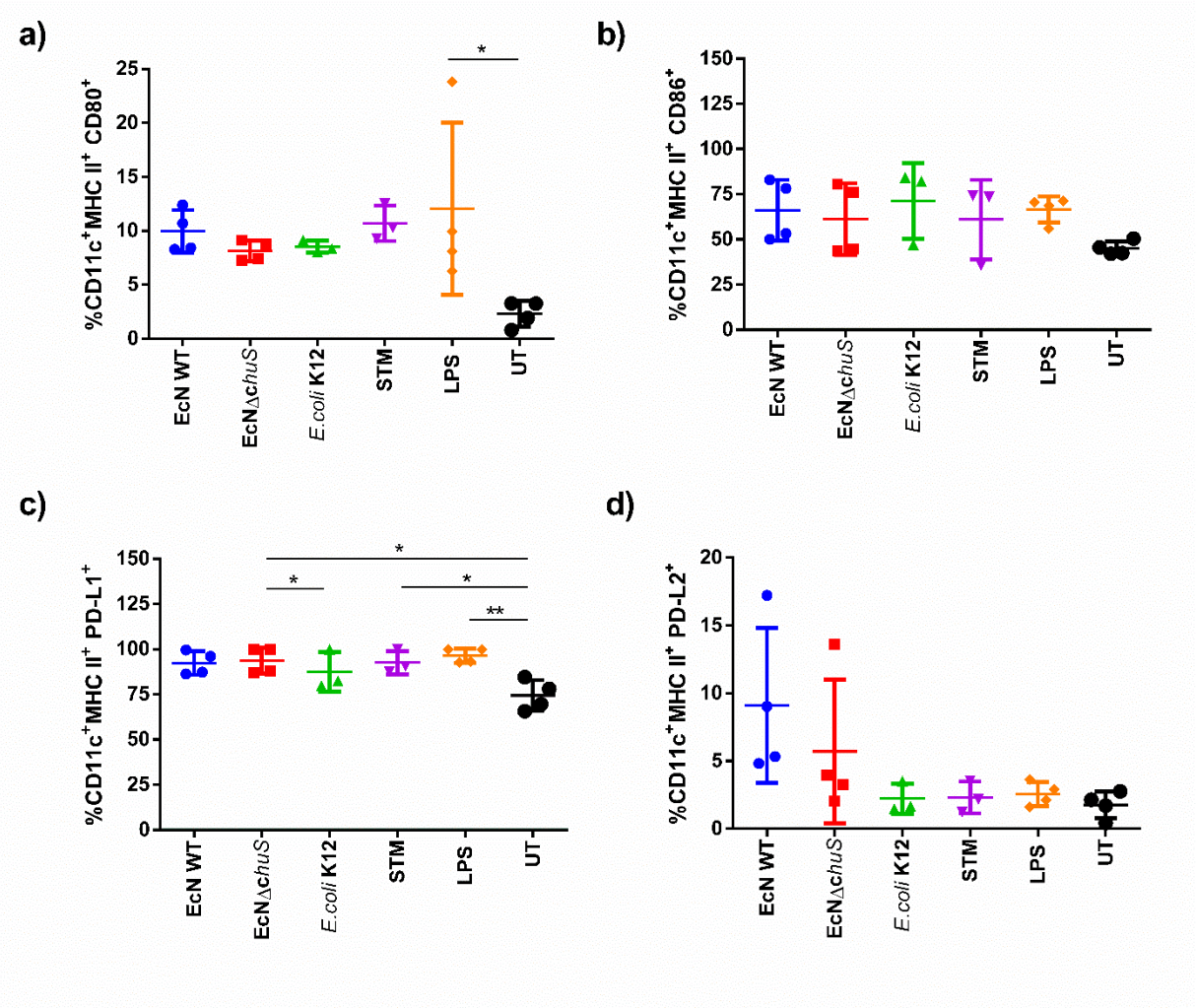

Figure 16. Dendritic cells activation and maturation after a treatment with different EcN strain using a MOI 12. a) Graph showing the analysis of the percentage of DC expressing CD80 by flow cytometry. b) Graph showing the analysis of the percentage of DC expressing CD86 by flow cytometry. c) Graph showing the analysis of the percentage of DC expressing PD-L1 by flow cytometry. d) Graph showing the analysis of the percentage of DC expressing PD-L2 by flow cytometry. Data were evaluated with On-way ANOVA and Turkey's Multiple comparison test, with α=0.05 for both tests. Statistically significant differences were found for a) P value= 0.0293, and c) P value= 0.0088. Data were compilated from 3 independent experiments. EcN WT: group treated with EcN WT. EcNΔ*chuS*: group treated with EcNΔ*chuS*. E. coli K12: group treated with E. coli K12. STM: group treated with *Salmonella enterica* serovar Typhimurium. LPS: group treated with LPS 1 µg/ml. UT: group untreated. STM and *E. coli* K-12 strains were used as control of infection and no pathogenic scenario, respectively. Data set corresponds to the sum of 2 independent experiments.

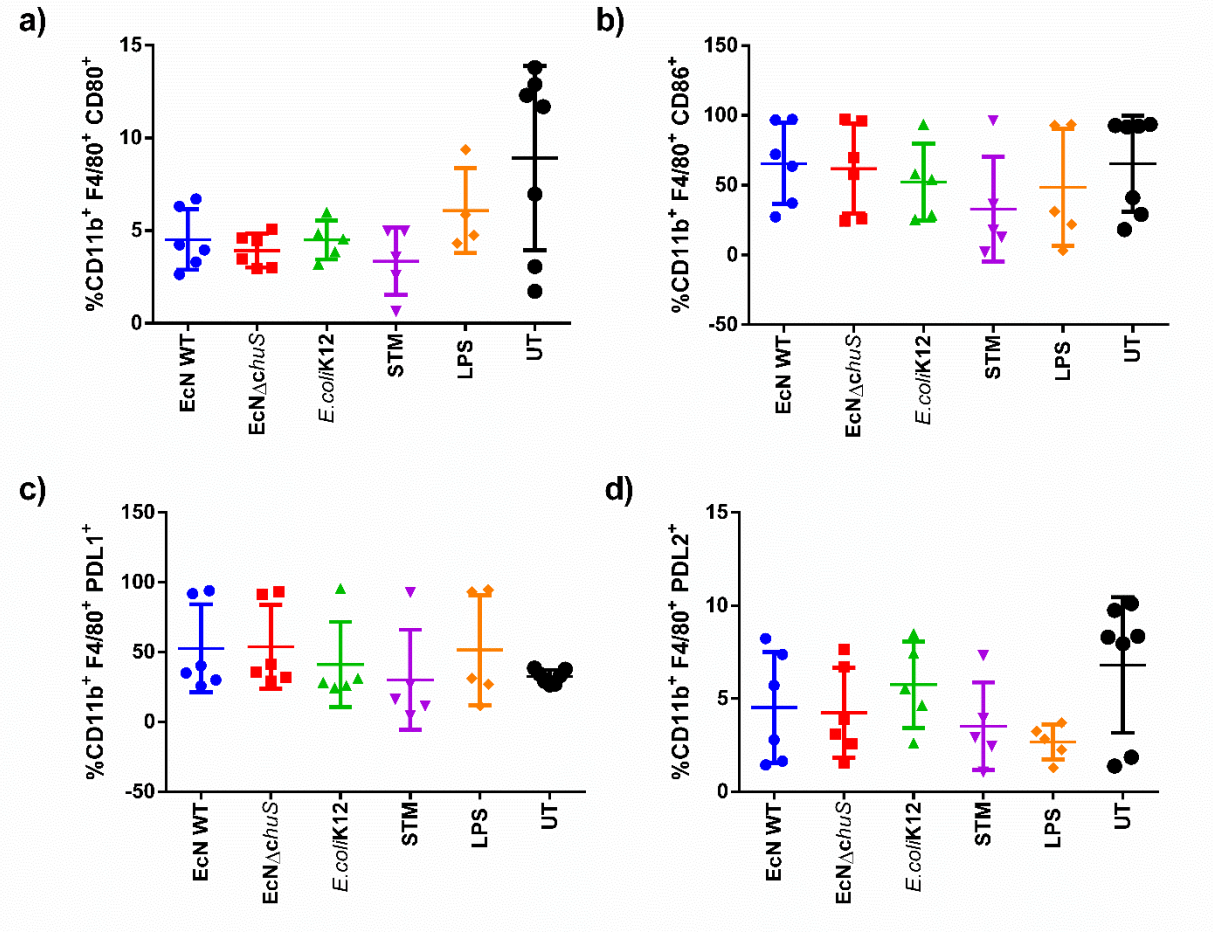

Figure 17. Macrophages activation and maturation after a treatment with different EcN strain using a MOI 12. a) Graph showing the analysis of the percentage of macrophages expressing CD80 by flow cytometry. b) Graph showing the analysis of the percentage of macrophages expressing CD86 by flow cytometry. c) Graph showing the analysis of the percentage of macrophages expressing PD-L1 by flow cytometry. d) Graph showing the analysis of the percentage of macrophages expressing PD-L2 by flow cytometry. Data were evaluated with On-way ANOVA and Turkey's Multiple comparison test, with α=0.05 for both tests. Data were compilated from 3 independent experiments. EcN WT: group treated with EcN WT. EcNΔ*chuS*: group treated with EcNΔ*chuS*. *E. coli* K12: group treated with *E. coli* K12. STM: group treated with *Salmonella enterica* serovar Typhimurium. LPS: group treated with LPS 1 µg/ml. UT: group untreated. STM and *E. coli* K-12 strains were used as control of infection and no pathogenic scenario, respectively. Data set corresponds to the sum of 3 independent experiments.

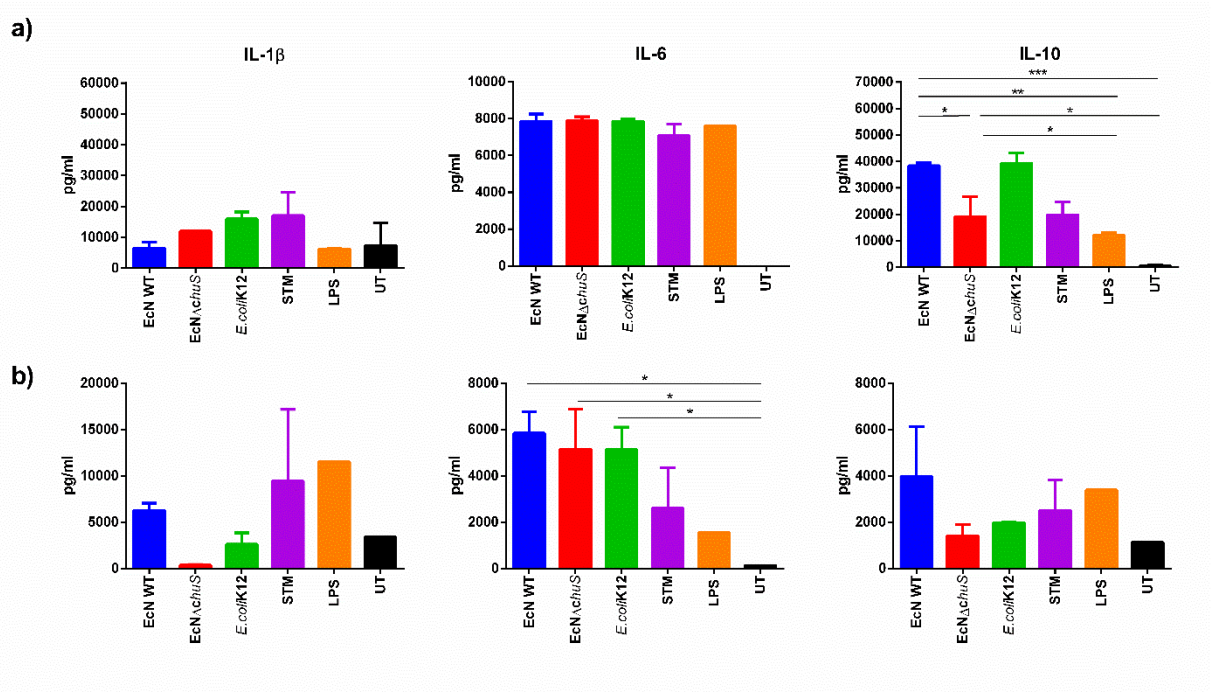

Figure 18. Cytokine secretion after cells were treated with probiotic strains with at MOI 12. a) Cytokine secretion for DC groups, from left to right: IL-1β, IL-6, and IL10. b) Cytokine secretion for macrophages groups, from left to right: IL-1β, IL-6, and IL10. Data were evaluated by On-way ANOVA and Turkey's Multiple comparison test, with α=0.05 for both tests. Statistically significant differences were found for DC IL-6 secretion (P value <0.0001), DC IL-10 secretion (P value=0.0005), and Macrophages IL-6 secretion (P value=0.0116). EcN WT: group treated with EcN WT. EcNΔ*chuS*: group treated with EcNΔ*chuS. E. coli* K12: group treated with *E. coli* K12. STM: group treated with Salmonella enterica serovar Typhimurium. LPS: group treated with LPS 1µg/ml. UT: group untreated. STM and *E. coli* K-12 strains were used as control of infection and no pathogenic scenario, respectively. Data set corresponds to the sum of 3 independent experiments.

Finally, both probiotics strains were capable of promoting maturation of DC and macrophages as well. Also, a modulation of tolerance markers was observed. Importantly, secretion of IL-10 was modulated by probiotic strains, being EcN WT the one that stimulated higher levels of this cytokine than mutant strain. For instance, is important to remark that mutant strain lacks heme oxygenase gene.

6.2. Evaluation of DC Antigen presentation capacity to T lymphocytes

Antigen presentation assay results showed that DC were able to activate lymphocytes. OT-I lymphocytes activated equally for both strains infection. CD25, DC69, and CD71 were evaluated and no differences were found. Only a little tendency of a higher CD71 MFI in group infected with EcN WT than the group with EcNΔ*chuS*. For OT-II lymphocytes, results were similar to those recently described for activation markers (Figure 19).

In the case of OT-I lymphocytes, cytokine secretion showed the tendency of a higher production of IL-4 in group infected with EcN WT compared to group treated with EcNΔ*chuS*. IL-10 and IFN-γ showed slightly the same behavior. On the other side, for OT-II lymphocytes IFN-γ secretion was higher when cells were treated with mutant strain and IL-10 and IL-4 were lower. IL-2 did not showed difference between probiotic strains treatment (Figure 20).

In general, ability of DC and macrophages of antigen presenting was not impaired. Results have highlighted that activated T cell populations percentages were mostly lower for OT-II lymphocytes.

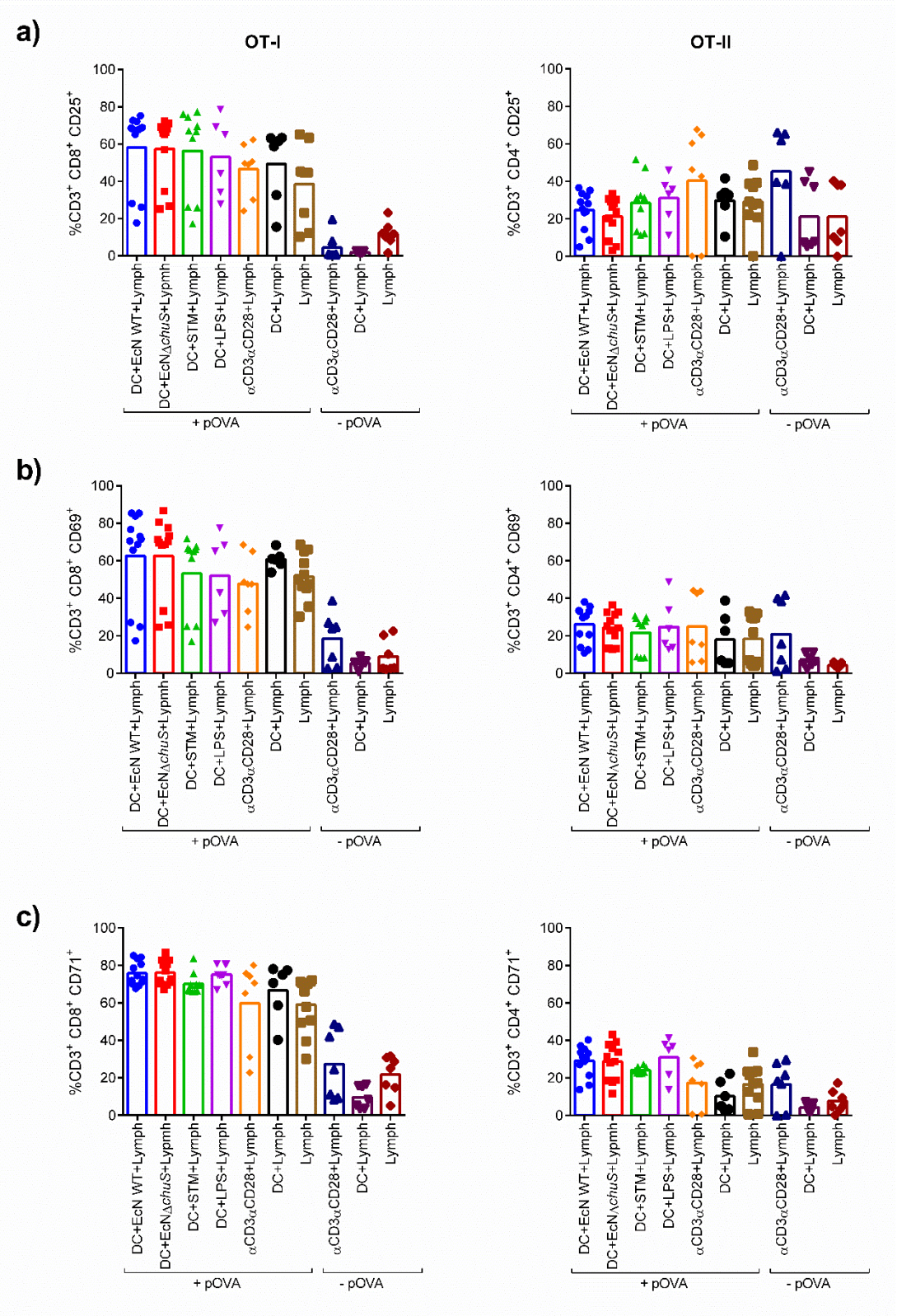
a)
OT-I
OT-II
%CD3+ CD8+ CD25+
%CD3+ CD4+ CD25+
b)
%CD3+ CD8+ CD69+
%CD3+ CD4+ CD69+
c)
%CD3+ CD8+ CD71+
%CD3+ CD4+ CD71+
DC+EcN WT+Lymph
DC+EcNΔchuS+Lypmh
DC+STM+Lymph
DC+LPS+Lymph
αCD3αCD28+Lymph
DC+Lymph
Lymph
+ pOVA
- pOVA

Figure 19. (Previous page) T cells activated populations after co-culture with DC infected with probiotic strains. a) T cells evaluated for activation marker CD25, in the left graph is OT-I population and in the right graph is OT-II population. b) T cells evaluated for activation marker CD69, in the left graph is OT-I population and in the right graph is OT-II population. c) T cells evaluated for activation marker CD25, in the left graph is OT-I population and in the right graph is OT-II population. LPS: Lipopolysaccharide. LPS was used as a positive control of DC activation. STM: *Salmonella* Typhimurium. STM was used as a positive control of Dc infection and activation, and T cell activation. All data were analyzed with One-way ANOVA and Turkey's multiple comparison test, for both cases $\alpha=0.05$. Statistically significant differences among means were obtained for CD25, CD64, CD71 OT-I lymphocytes populations (for all cases P value <0.0001), CD69 OT-II lymphocytes population (P value= 0.0026), and CD71 OT-II lymphocytes population (P value <0.0001). Data set corresponds to the sum of 2 independent experiments.

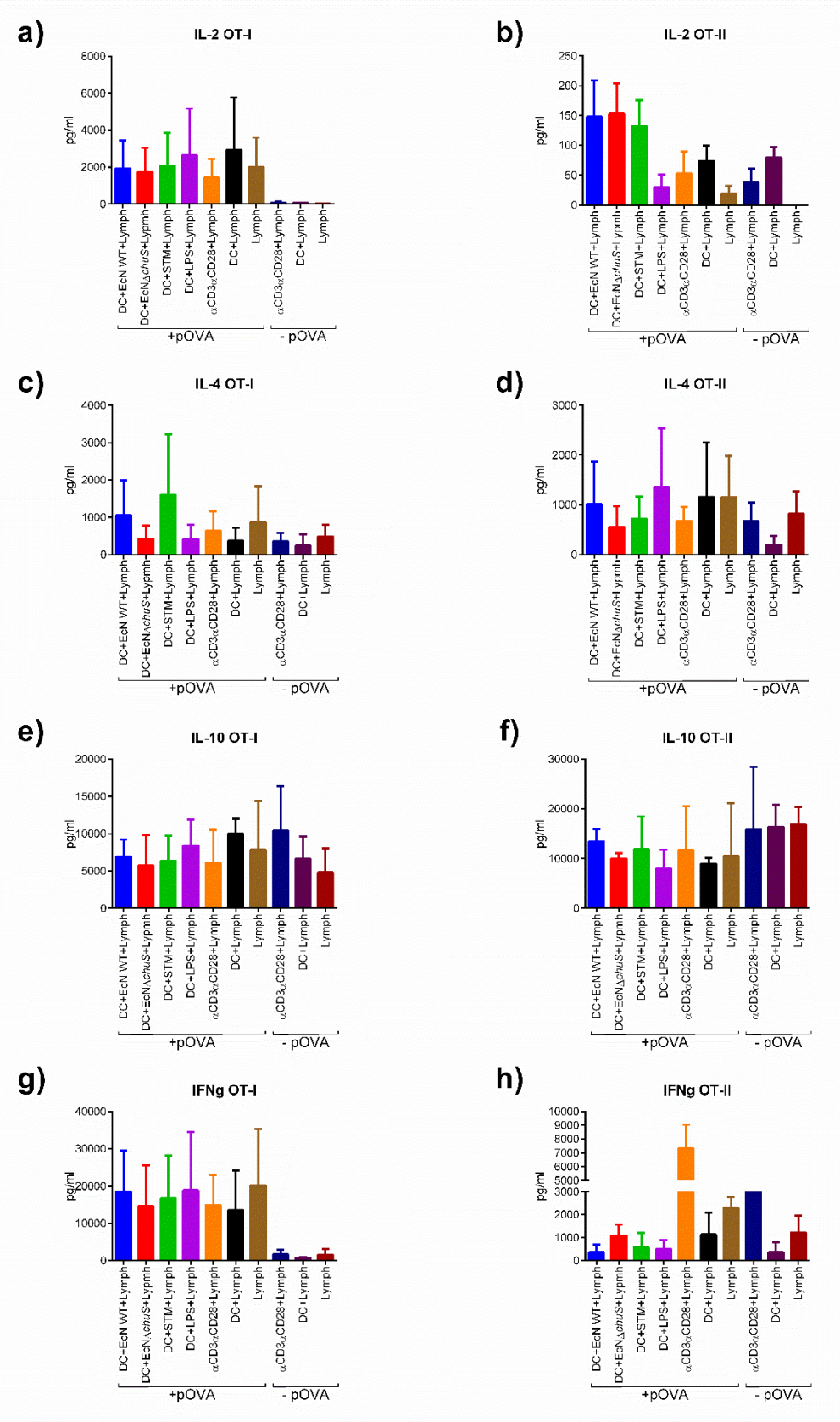
a)
IL-2 OT-I
b)
IL-2 OT-II
c)
IL-4 OT-I
d)
IL-4 OT-II
e)
IL-10 OT-I
f)
IL-10 OT-II
g)
IFNg OT-I
h)
IFNg OT-II
pg/ml
DC+EcN WT+Lymph
DC+EcNΔchuS+Lymph
DC+STM+Lymph
DC+LPS+Lymph
αCD3αCD28+Lymph
DC+Lymph
Lymph
+pOVA
- pOVA

Figure 20. (Previous page) Cytokine secretion after whole treatment with probiotics strain. a) IL-2 secretion in OT-I cells group. b) IL-2 secretion in OT-II cells group. c) IL-4 secretion in OT-I cells group. d) IL-4 secretion in OT-II cells group. e) IL-10 secretion in OT-I cells group. f) IL-10 secretion in OT-II cells group. g) IFN-γ secretion in OT-I cells group. h) IFN-γ secretion in OT-II cells group. LPS was used as a positive control of DC activation. STM: *Salmonella* Typhimurium. STM was used as a positive control of DC infection and activation, and T cell activation. All data were analyzed with One-way ANOVA and Turkey's multiple comparison test. All data were analyzed with One-way ANOVA and Turkey's multiple comparison test, for both cases $\alpha=0.05$. Statistically significant differences among means were obtained for a) P value = 0.0343, b) P value= 0.0091, g) P value <0.0001, and h) P value <0.0001. Data set corresponds to the sum of 3 independent experiments.

6.3. Lymphocytes proliferation assay

This assay was performed with the purpose of identifying whether probiotic strains WT and mutant were able to activate lymphocytes directly. Different groups of T cells were infected with viable bacteria and Heat Killed (HK) inactivated bacteria in parallel. HK bacteria were used to asses if some their structural components affect T cell proliferation. T cells were first separated in $CD4^+$ and $CD8^+$ (gating strategy is in Appendix 1). Then each sample was compared to the control group, which were not activated cells. Proliferation by CFSE method evaluates how diluted is the reagent by measuring flow cytometry signal in the FITC channel. As more diluted is CFSE, more proliferation represents and lesser is the signal read. Therefore, results represent a ratio between each sample read and control group read.

Also, control groups were infected with STM and HK STM. This group allowed us to check the assay. A previous report has shown that STM was able to inhibit proliferation of T cells (van der Velden, 2005).

Interestingly, a major percentage of $CD8^+$ T cells were activated as compared to $CD4^+$ when infected with EcN strains. Inhibition of proliferation was observed in groups infected with alive bacteria. But the most remarkably result is that EcN WT showed inhibition of the proliferation more than EcNΔ*chuS* for both cases $CD4^+$ and $CD8^+$, but with a major effect in $CD4^+$ T cells (Figure 21).

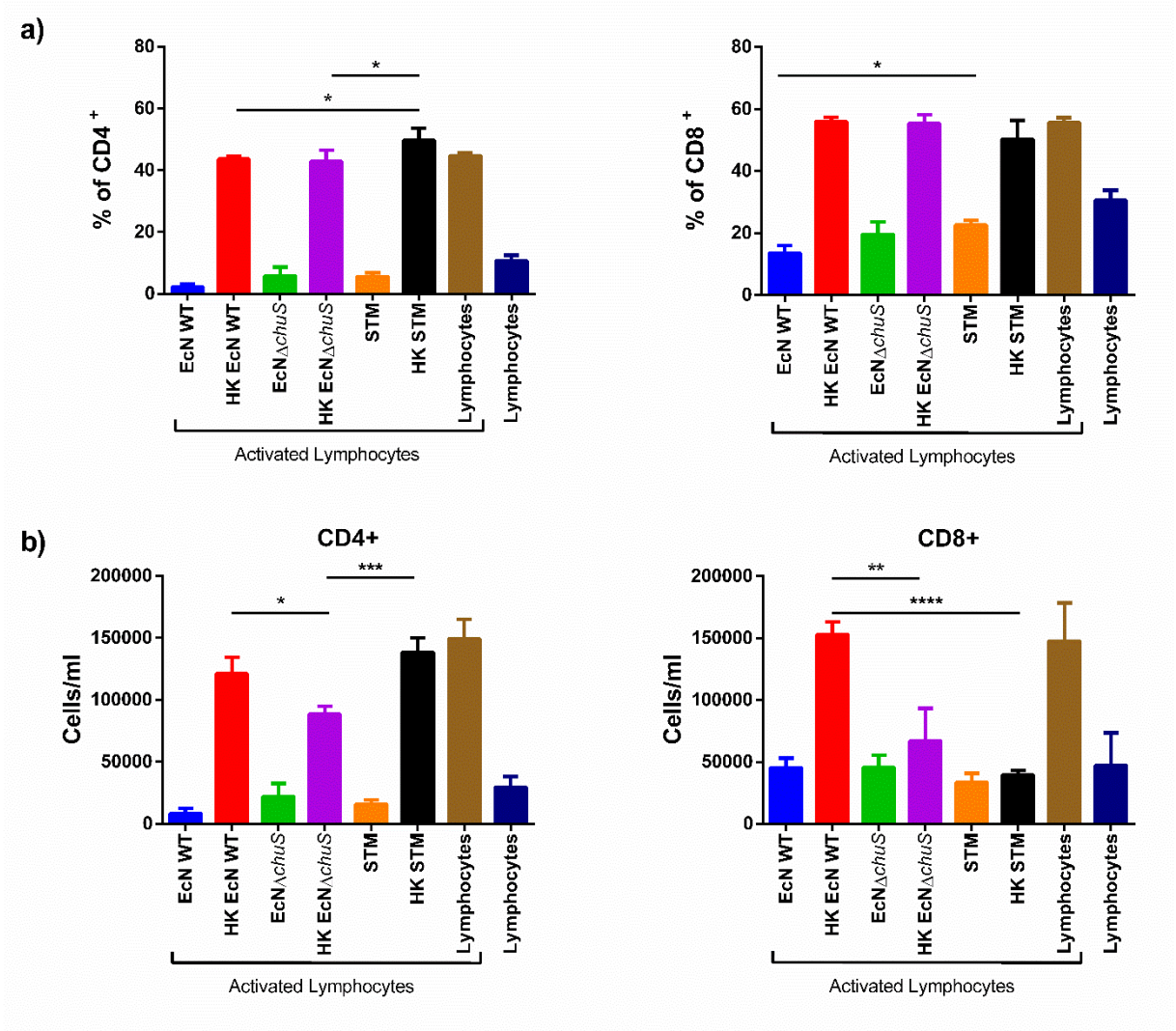

Figure 21. T cells proliferation is altered by probiotic treatment. Three days after infection with probiotic strains, samples were collected and evaluated by flow cytometry to measure cell proliferation by CFSE dilution. a) Graph showing the percentage of cells that proliferated, in the left $CD4^+$ T cells and in the right $CD8^+$ T cells. This percentage of cells considers the $CD4^+$ and $CD8^+$ populations that proliferated with respect to the complete sample. b) Graph showing the quantity analysis (cells/ml) of the cells that proliferated. $CD4^+$ T cells in the left graph and $CD8^+$ T cells in the right. STM: *Salmonella* Typhimurium. STM was used as positive control of the experiment. Data were analyzed with ANOVA-One-way $\alpha=0.05$. Multiple comparison Turkey's test $\alpha=0.05$. P value <0.0001. Data sets correspond to a unique experiment.

6.4. First *in vivo* assay: Probiotic strains are able to modulate differently systemic immune response and play a role in the infection with *S.* Enteritidis.

Experiment was dived in two parts. Initially, mice received probiotic treatment (Figure 22b). After, some mice were infected with SEN (Figure 22d). The first part of the experiment is described below. Mice were treated with EcN WT and EcNΔ*chuS*, in parallel control group received PBS. Weight and physiological score were registered daily, including colitis parameters. In the first 4 days, there were no big changes on weight. But, since day 5 of treatment, groups treated with probiotic started to lose weight (Figure 22b), even dying. Subsequently, those who survived regained weight from day 11 post administration. Furthermore, weight loss was high, some mice reached 20% of loss as compared to initial weight. Group treated with EcN WT had two mice with this characteristic, who required euthanasia. Also, one mice of group treated with EcNΔ*chuS* in the same scenario. In both cases, mice also had a high clinical score (10-12). However, clinical score for the rest of the mice was low. An important observation is that during experiment, 7 mice from EcN WT group and 2 mice from EcNΔ*chuS* were found dead, without any previous physiological signal. Survival curve is observed in Figure 22a. In general, 80 % of mice did not show clinical signs though clinical score is not high (Figure 22c). At day 9 post administration, 6 mice survived in group treated with EcN WT, 12 mice in group treated with EcNΔ*chuS* and 4 mice in control group. At this point, probiotic administrations were suspended due to the high mortality, and mice rested until day 14.

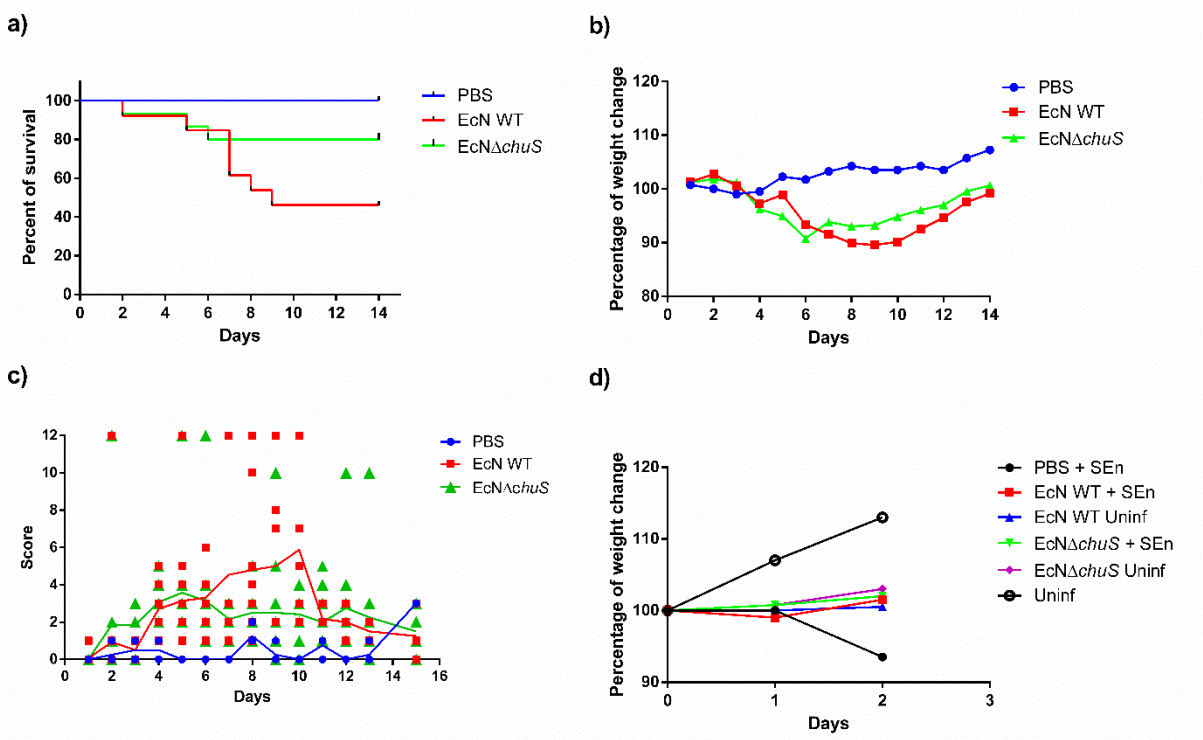

Figure 22. Daily monitoring of survival, weight and clinical score throughout the assay. a) Survival curve during probiotic administration. Survival was checked twice a day and registered. b) Weight loss percentage of mice during probiotic administration. c) Clinical score during probiotic administration (Days 1-14) and after infection with SEN (Day 15). Mice who died are represented with the highest score (12). d) Weight changes during the second part of the experiment showing only days during infection with SEN. Data of a) was analyzed using Kaplan Meier estimator, with Mantel-Cox long-rank test, α=0.05, no significant differences were found. Data of b), c), and d) were analyzed with 2way ANOVA α=0.05. For b) and c) differences in column factor (Experimental groups) and row factor (Days) were statistically significant, P values were <0.0005 and <0.0001 respectively. For d) only column factor (Days) was significant, P value= 0.0077. Data sets correspond to a unique experiment.

At 14 days post administration, a group of mice of each group were euthanized to evaluate immune cell populations. T cells ($CD3^+$ $CD8^+$, $CD3^+$ $CD4^+$), B cells ($B220^+$ $CD19^+$), Neutrophils ($Ly6G^+$ $Ly6C^-$) Monocytes ($Ly6G^-$ $Ly6C^+$)Myeloid cells ($Ly6G^+$ $Ly6C^+$), DC ($CD11c^+$ $CD103^+$), macrophages ($CD11b^+$ $F4/80^+$) and TIP dendritic cells (TNF/iNOS- producing dendritic cells) ($CD11b^+$ $CD11c^+$). Most remarkably finding is the significant differences between T cells activated in liver in groups with the different probiotic strains (Figure 23). In neutrophils, monocytes and MSDC no differences were found between groups. In macrophages a little tendency was observed, when group was treated with EcN WT had a higher percentage of cells in spleen and liver than group treated with mutant strain. On the other hand, in MLN this tendency was the opposite the same as DC populations in MLN. Additionally, TIP DC were analyzed due to their capability of mediating innate immune defense against pathogen infection. Percentages of this cell population are very low but still allowed to observe a bit higher percentage in EcN WT group in spleen and MLN (Figure 24). In the case of B cells, liver and MLN presented a pattern of higher percentage of cells in EcN WT group than EcNΔ*chuS* group. Besides, in spleen this pattern was inverted, higher percentage of B cells for EcNΔ*chuS* group (Figure 25).

At the same point, another group of mice of each treatment, was infected with $1*10^6$ CFU of SEN. Weight loss (Figure 22d) and clinical score (Figure 21c, day 14 and 15) were monitored until 48 hours post infection were reached. Mice who were treated with probiotic did not lost weight during infection.

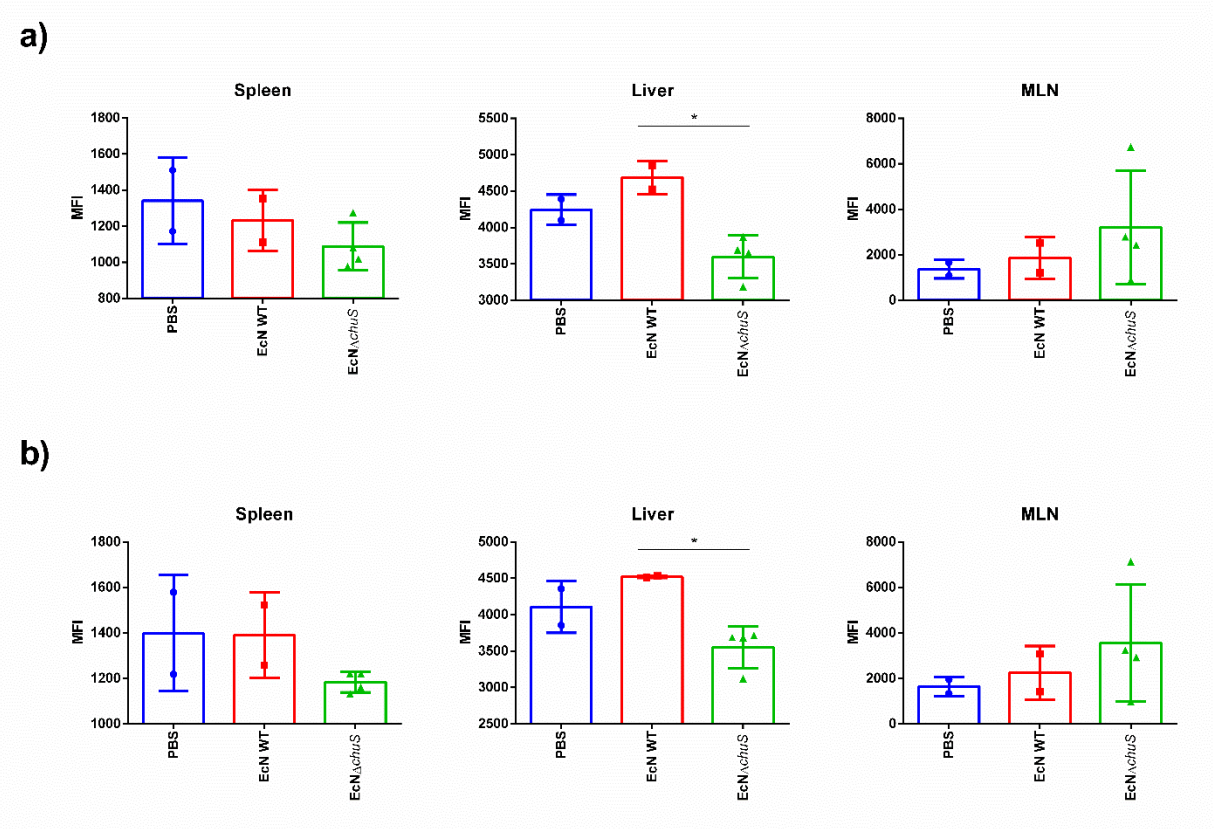

Figure 23. Activation of T cells after treatment with probiotic strains.a) T $CD4^+$ cells of spleen, liver, and MLN. b) $CD8^+$ of spleen, liver, and MLN. Statistical analysis performed are One-Way ANOVA and Turkey's Multiple comparison test, for both tests α=0.05. Only in liver data differences were found statistically significant, P value= 0.0121 for T $CD4^+$ and 0.0227 for T $CD8^+$. Data set corresponds to a unique experiment.

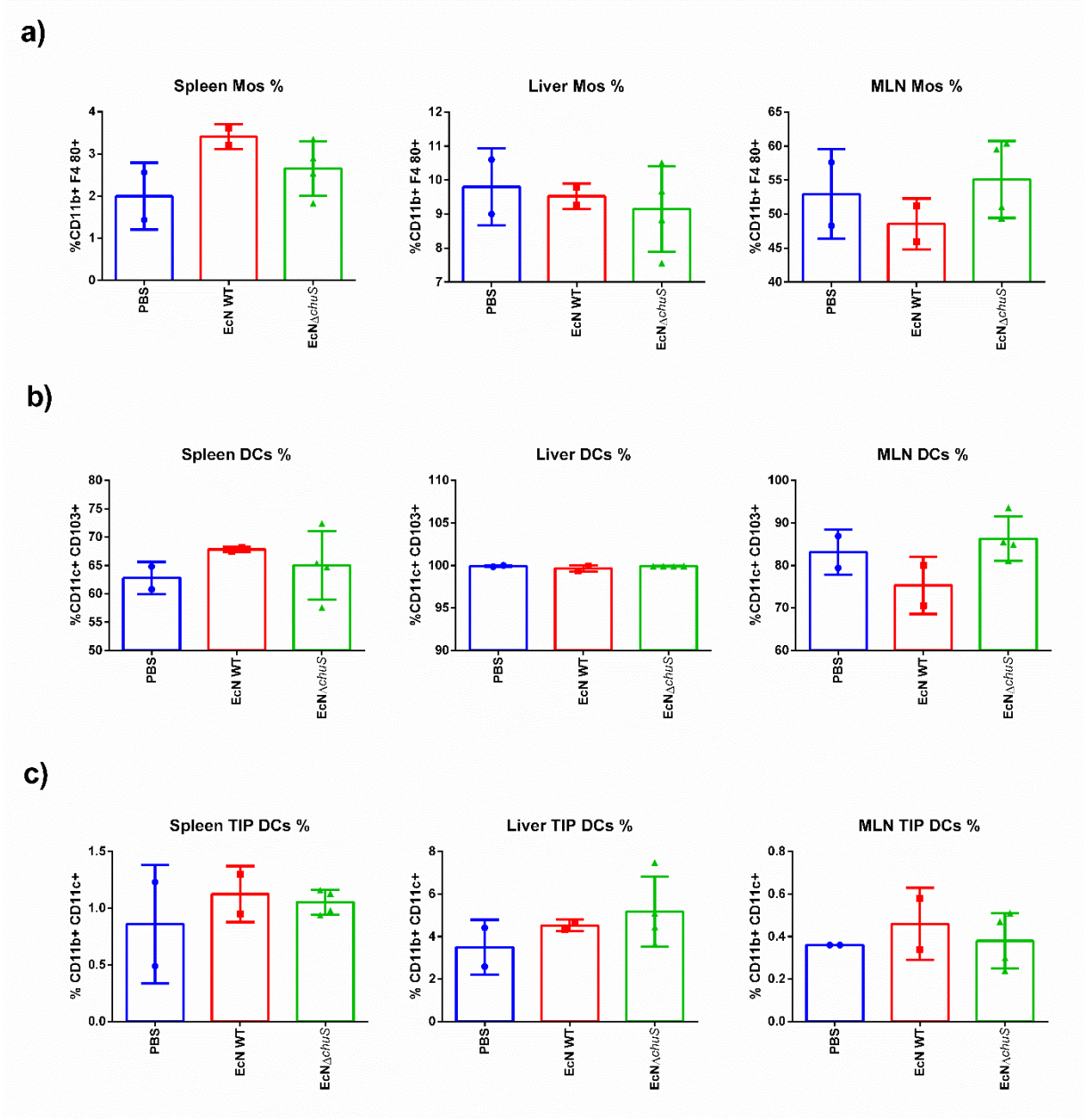

Figure 24. Modulation of myeloid cells population after treatment with probiotic strains. a) Macrophages of spleen, liver and MLN. b) DC of spleen, liver and MLN. c) TIP DC of spleen, liver and MLN. Data were analyzed with One-Way ANOVA and Turkey's Multiple comparison test, for both tests α=0.05. Data set corresponds to a unique experiment.

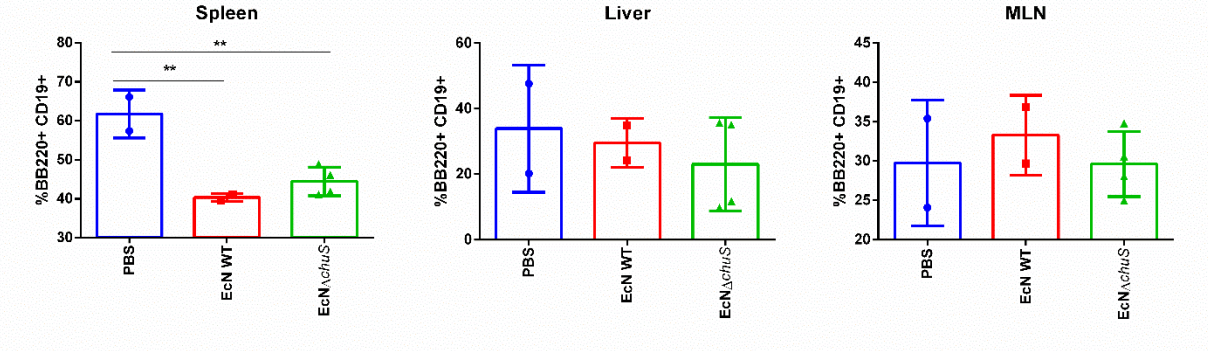

Figure 25. Modulation of B cells population after treatment with probiotic strains. B cells of spleen, liver, and MLN were evaluated. Data were analyzed with One-Way ANOVA and Turkey's Multiple comparison test, for both tests α=0.05. In spleen data, significant differences were found, P value=0.0058. Data set corresponds to a unique experiment.

Necropsy was performed at 48 hours post infection and organs were recovered. Bacterial load in spleen, liver, and mesenteric lymph nodes did not present any bacterial growth for the group of mice treated with EcN WT. In the case of groups treated with EcNΔ*chuS*, there was found SEN growth in one mouse's spleen liver and MLN (Figure 26).

In addition, immune cells populations were evaluated in organs. Percentage of activated T lymphocytes is around 20% of CD4+ in spleen and liver and 40% in MLN. Activation of CD4+ T cells was different between experimental groups treated with probiotics and infected with SEN or uninfected, especially in spleen. In addition, CD8+ T cells showed same behavior. Interestingly, in lymph nodes despite of having high percentages of T cells, CD25 MFI was not as higher (Figure 27). Other cell populations monocytes, neutrophils, MSDC, macrophages and DC did not present any pattern. Only TIP DC and B cells showed significant differences in MLN (Figure 28 and 29).

Results allowed to confirm a systemic effect of probiotic administration, according to physiological score and weight changes, mutant strain may promote inflammation in some way. Interestingly, when mice were infected with SEN, only groups treated with mutant strain presented bacterial load, which highlights one of the benefits of EcN WT against pathogen. Apparently, deletion of *chuS* gene may be impairing this capability *in vivo*.

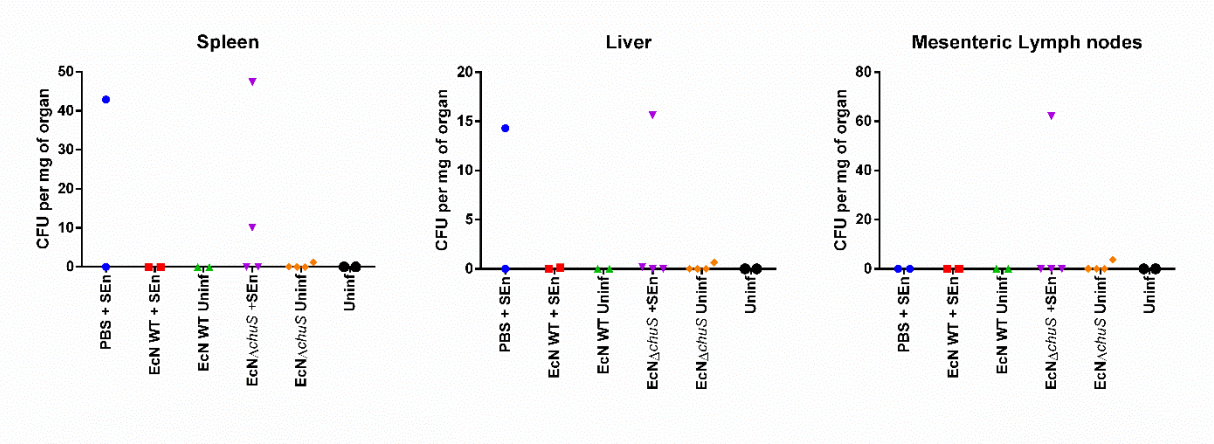

Figure 26. Bacterial load in organs 48 hours post infection with SEN. An aliquot of each homogenized organ was diluted 1:10 and seeded, then counted after 18 hours of incubation. CFUs were calculated according organ weight. Data set corresponds to a unique experiment.

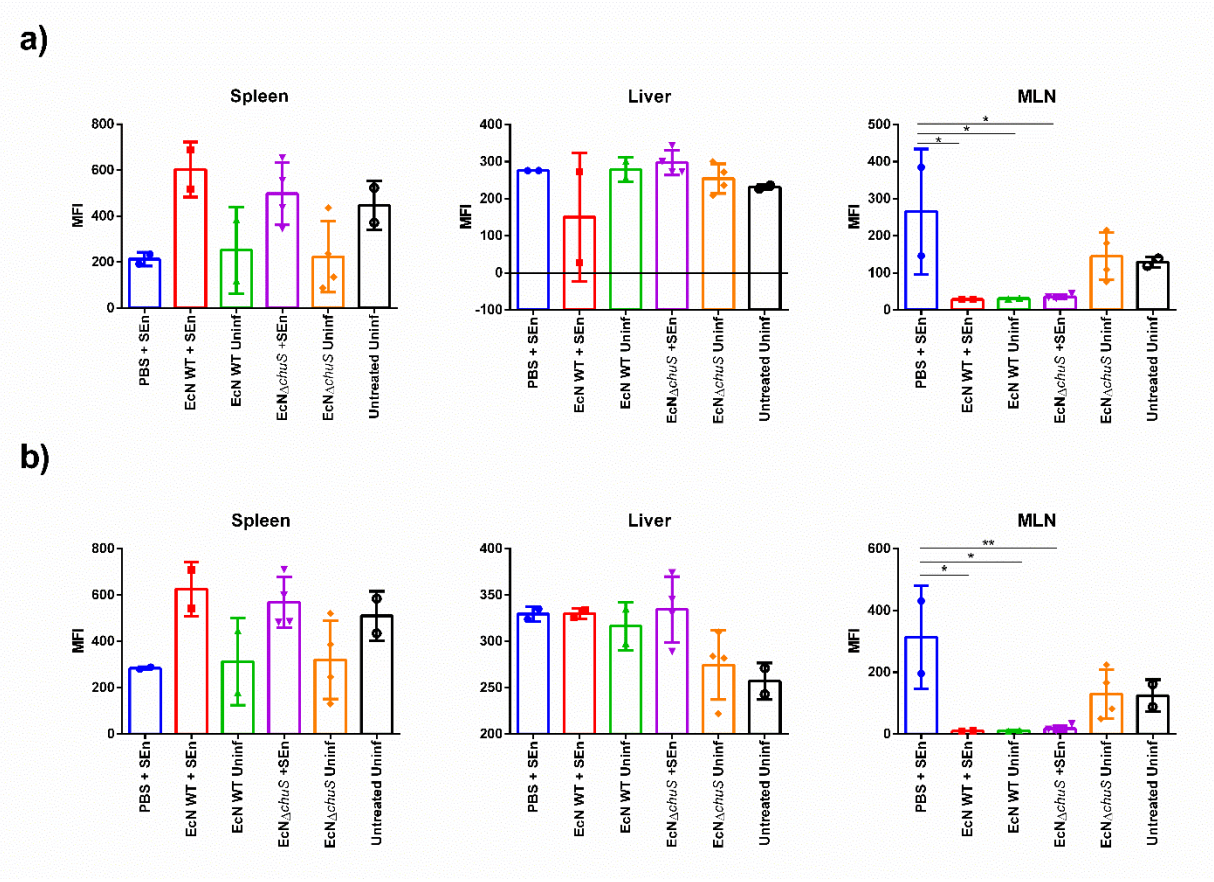

Figure 27. Activation of T cells 48 hours post infection with SEN. a) T CD4$^+$ cells of spleen, liver, and MLN. b) CD8$^+$ of spleen, liver, and MLN. Statistical analysis performed are One-Way ANOVA and Turkey's Multiple comparison test, for both tests α=0.05. Only in MLN data differences were found statistically significant, P value= 0.0158 for T CD4$^+$ and 0.0071 for T CD8$^+$. Data set corresponds to a unique experiment.

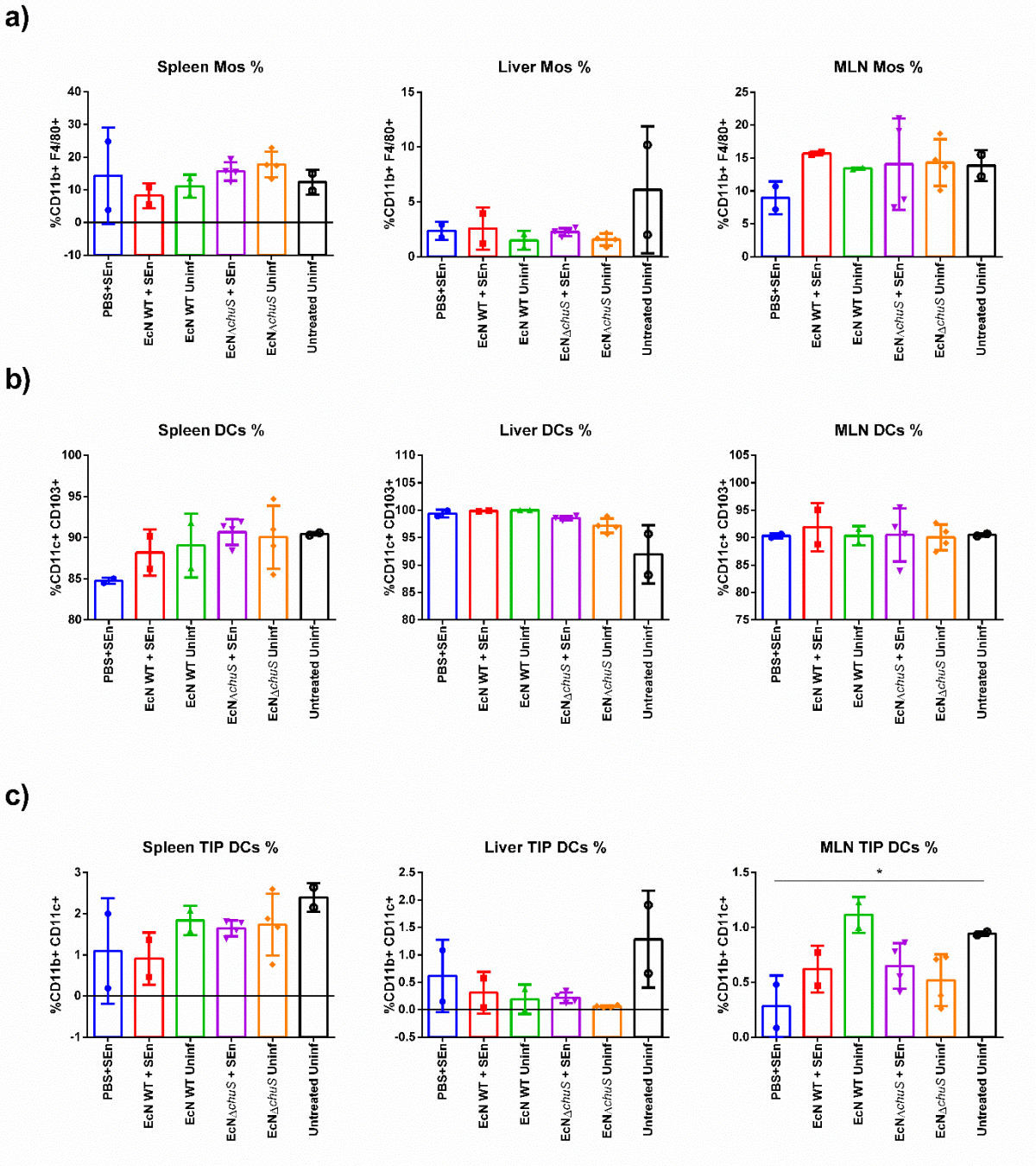

Figure 28. Modulation of Myeloid cells population 48 hours post infection with SEN. a) Macrophages of spleen, liver and MLN. b) DC of spleen, liver and MLN. c) TIP DC of spleen, liver and MLN. Data were analyzed with One-Way ANOVA and Turkey's Multiple comparison test, for both tests α=0.05. Only MLN data of TIP DC were statistically significant. P value=0.025. Data set corresponds to a unique experiment.

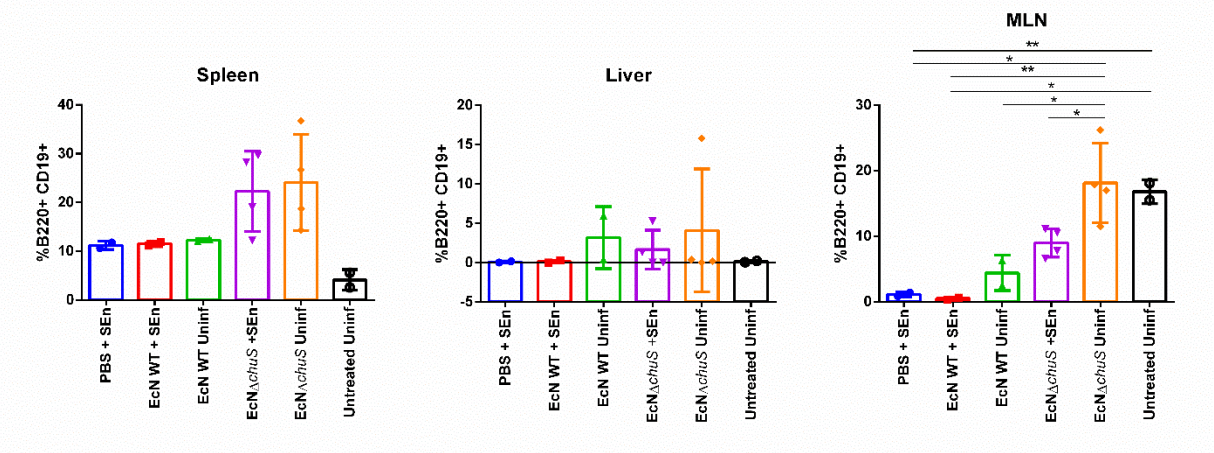

Figure 29. Modulation of B cells population 48 hours post infection. B cells of spleen, liver, and MLN were evaluated. Data were analyzed with One-Way ANOVA and Turkey's Multiple comparison test, for both tests α=0.05. In MLN data, significant differences were found, P value=0.0007. Data set corresponds to a unique experiment.

6.5. Second *in vivo* assay: Different modulation of immune response by different doses of both probiotic strains EcN WT and EcN∆*chuS*

For this assay, 4 different doses were tested of each probiotic strain: $1*10^4$ CFU, $1*10^5$ CFU, $1*10^6$ CFU, and $1*10^7$ CFU. Weight changes and clinical score were monitored daily. Additionally, colitis parameters were included, and feces characteristics were observed as well. An important observation is that weight changes are different in each group so may be a dose dependent effect. Notably at day 2, all mice in the group treated with EcNΔ*chuS* $1 * 10^7$ CFU, lost weight more than 10%. The greatest weight loss registered was for experimental group treated with EcNΔ*chuS* $1*10^6$ CFU, compared to the other groups (Figure 30a).

Weight loss was too high for some mice, which reached even 20% less than initial weight. However, during experiment course all mice recover their original weight even getting to gain more than 100%. Both groups treated with $1*10^6$ CFU doses (EcN WT and EcN∆*chuS*) were the exception to this phenomenon, since mice failed to recover 100% of their initial weight. Also, clinical score was monitored (Figure 30a). In general, 80% of mice did not present any clinical signal, therefore score values were not high.

Experiment end point was at day 28, all mice were euthanized, and organs were recovered by necropsy, spleen, liver, MLN and blood. Results evidenced that there was no bacterial load in any organs nor blood. Immune cell populations were evaluated, due to a technical problem, only B cells behavior was checked (Figures 31, 32 and 33).

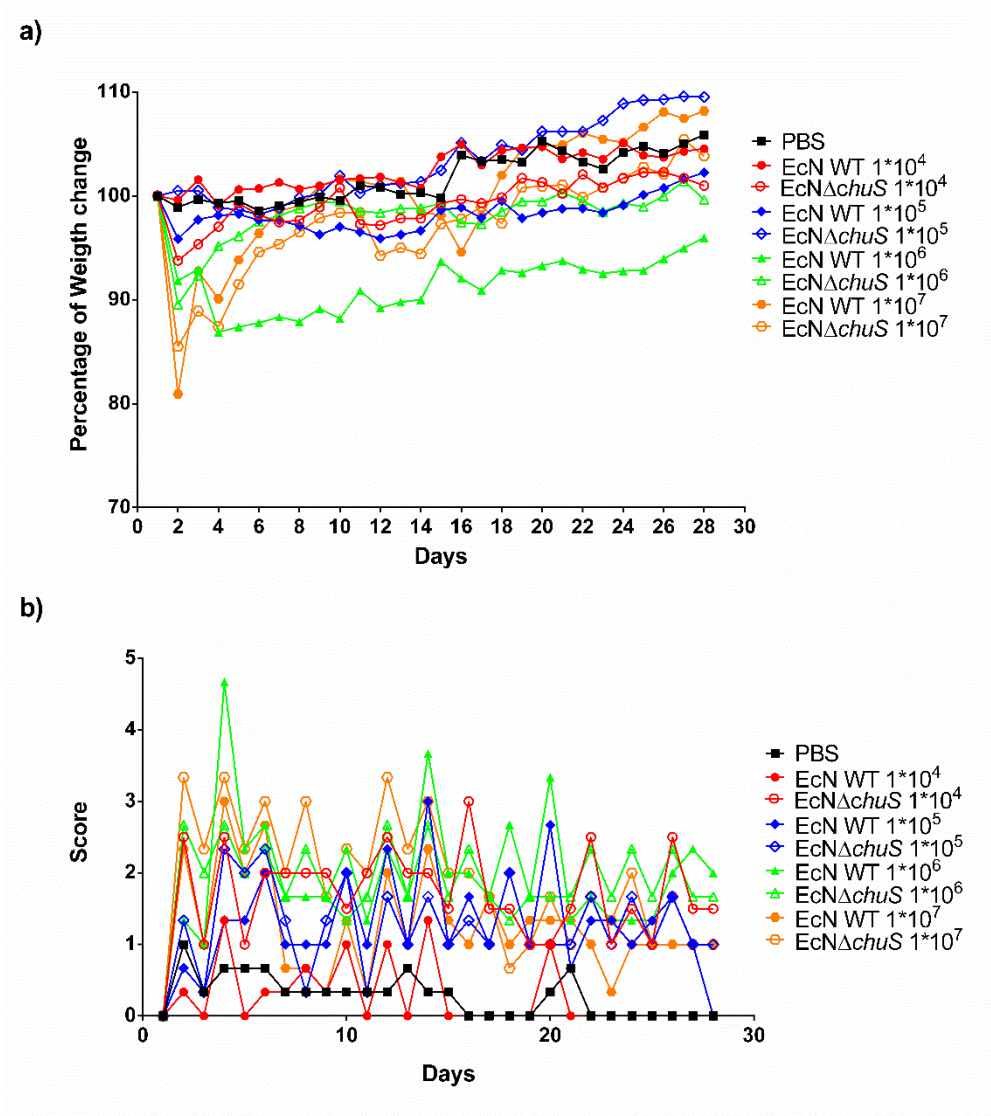

Figure 30. Daily monitoring of weight and clinical score throughout the assay. a) Weight changes during the experiment. A Linear regression was performed, differences between slopes were found to be extremely significant, P value <0.0001. Additionally, a 2way ANOVA (α=0.05) was used to evaluate column (Doses groups) and row (Days) factor, P value <0.0001. b) Clinical score during probiotic administration. A Non-linear Regression was performed, P value <0.0001. Also, data were analyzed by 2way ANOVA α=0.05, P value for column (Doses groups) and row (Days) factors was <0.0001. Data sets correspond to a unique experiment.

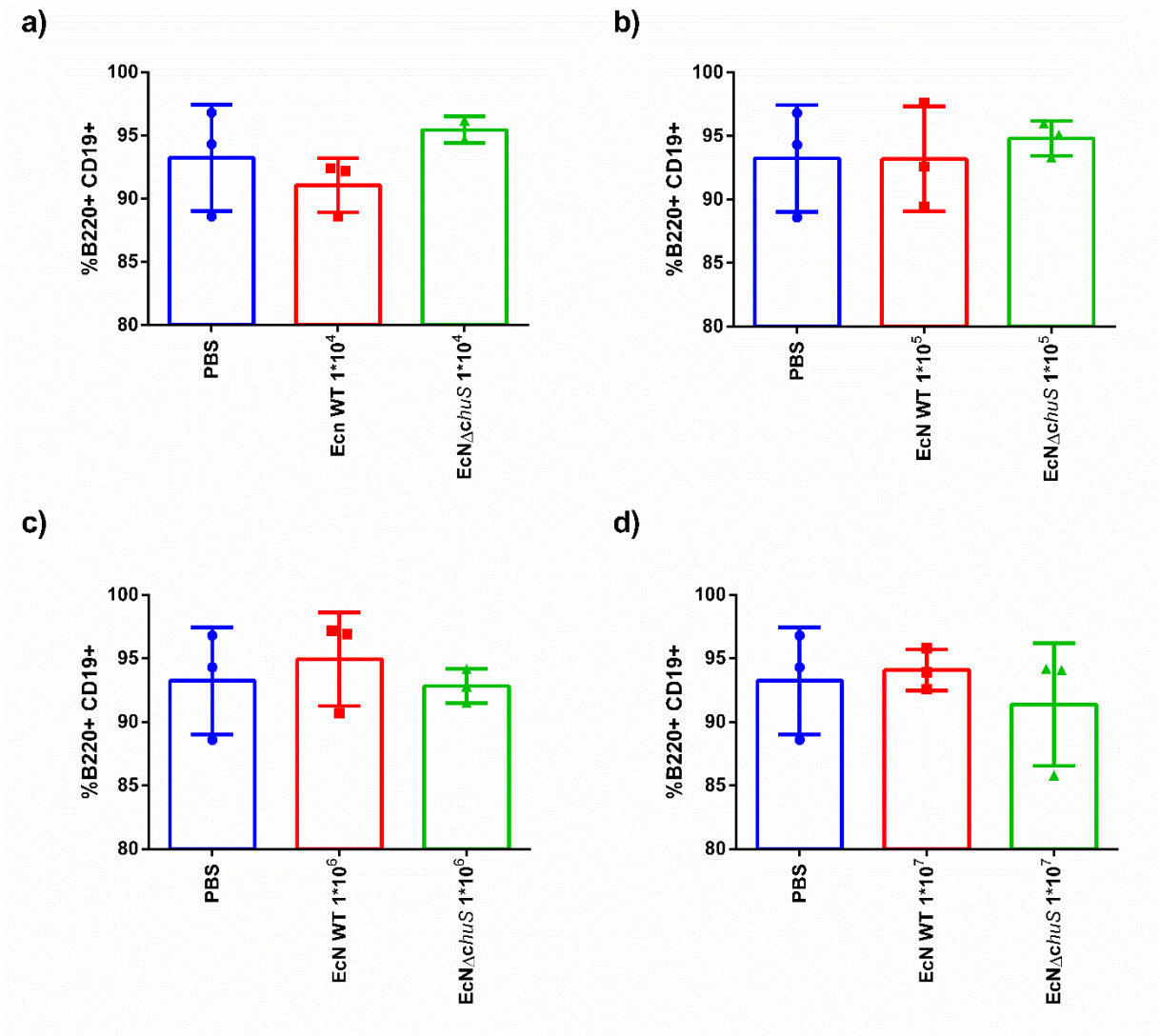

Figure 31. Spleen B cells population after treatment with probiotic strains. a) Groups treated with a dose of $1*10^4$ CFU of respective probiotic strain. b) Groups treated with a dose of $1*10^5$ CFU of respective probiotic strain. c) Groups treated with a dose of $1*10^6$ CFU of respective probiotic strain. d) Groups treated with a dose of $1*10^7$ CFU of respective probiotic strain. Data analyzed with One-way ANOVA α=0.05 and Turkey's multiple comparison test α=0.05. Data set corresponds to a unique experiment.

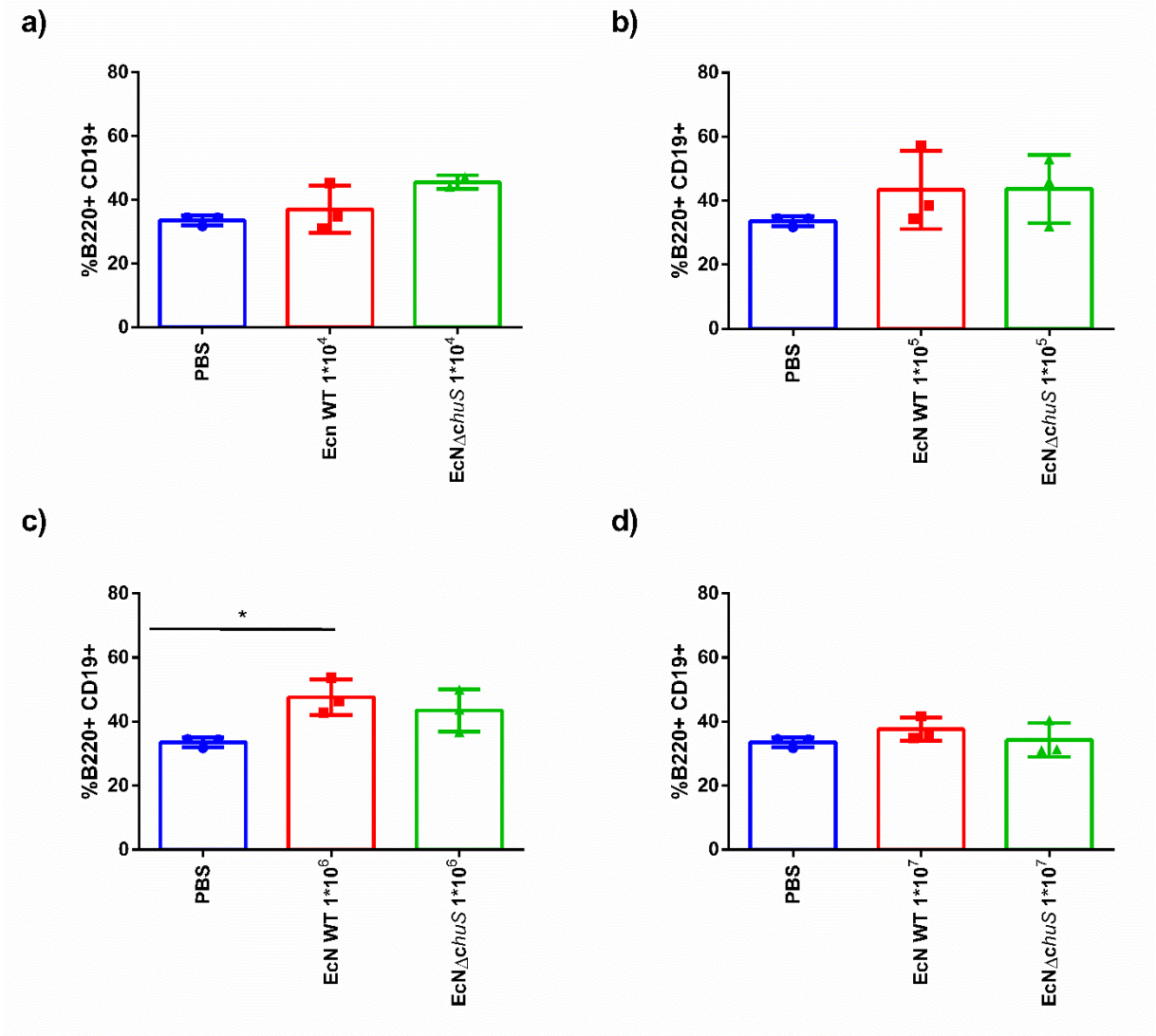

Figure 32. Liver B cells population after treatment with probiotic strains. a) Groups treated with a dose of $1*10^4$ CFU of respective probiotic strain. b) Groups treated with a dose of $1*10^5$ CFU of respective probiotic strain. c) Groups treated with a dose of $1*10^6$ CFU of respective probiotic strain. d) Groups treated with a dose of $1*10^7$ CFU of respective probiotic strain. Data analyzed with One-way ANOVA α=0.05 and Turkey's multiple comparison test α=0.05. For c) P value was 0.0369. Data set corresponds to a unique experiment.

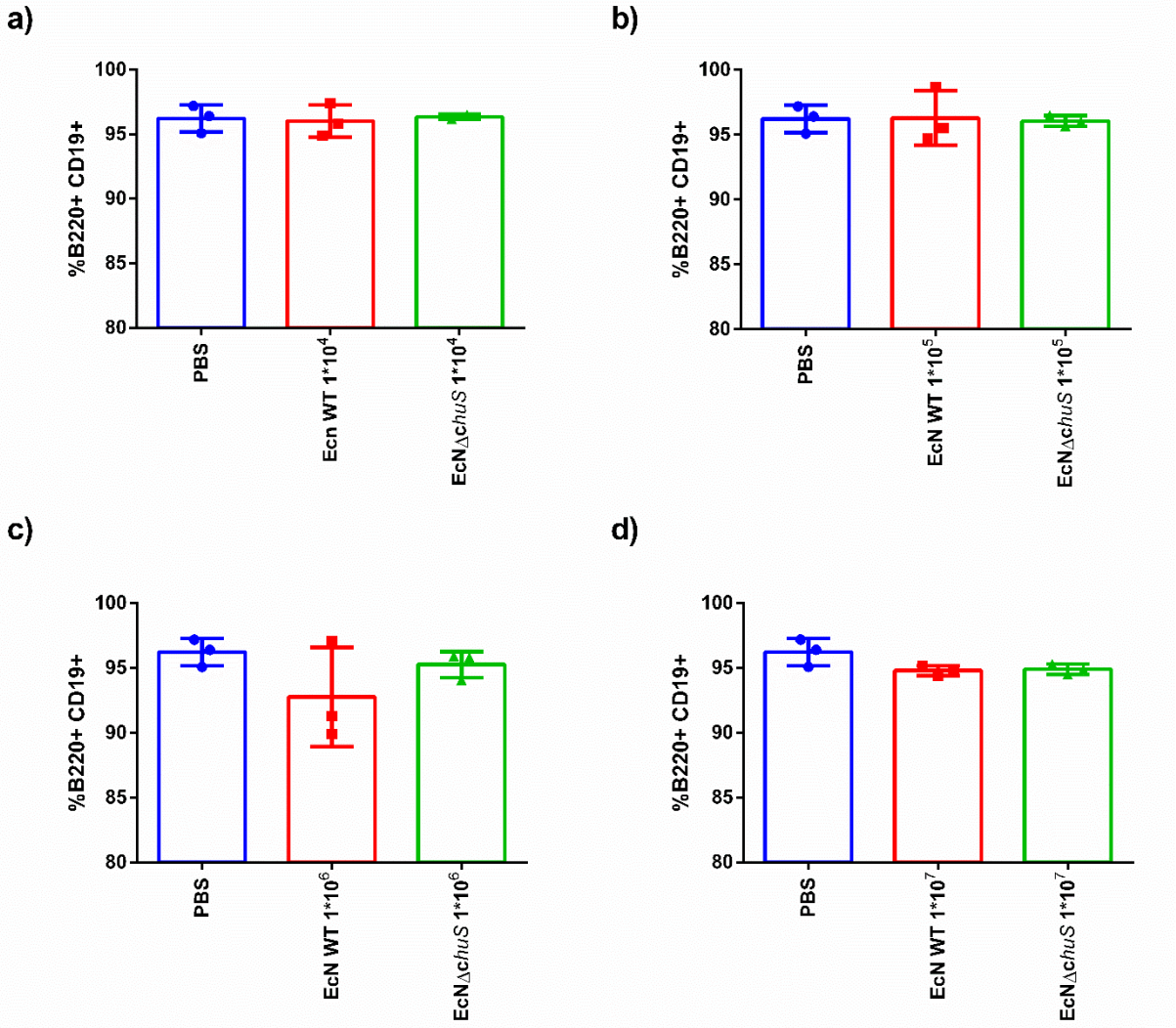

Figure 33. Mesenteric Lymph Nodes B cells population after treatment with probiotic strains. a) Groups treated with a dose of $1*10^4$ CFU of respective probiotic strain. b) Groups treated with a dose of $1*10^5$ CFU of respective probiotic strain. c) Groups treated with a dose of $1*10^6$ CFU of respective probiotic strain. d) Groups treated with a dose of $1*10^7$ CFU of respective probiotic strain. Data analyzed with One-way ANOVA α=0.05 and Turkey's multiple comparison test α=0.05. Data set corresponds to a unique experiment.

Also, blood was used to evaluate cytokine secretion in serum, IL-10 and IL-12p70. In general, secretion of this cytokines was dose dependent. When IL-10 and IL-12p70 production was observed with respect to the treatment mice received (Figure 34a and b) a difference was noticed in dose $1*10^5$ CFU of the respective strain. In the groups with the lower dose of treatment no differences were found in IL-12p70 secretion between EcN WT and EcNΔ*chuS*. Besides, in groups treated with the higher dose ($1*10^7$ CFU), the difference between probiotic strains of cytokine production was inverted for IL-12p70. For IL-10 secretion, both treatments showed the same behavior. But EcN WT groups displayed always higher values than EcNΔ*chuS*.

When both cytokines were analyzed within the probiotic strain groups (EcN WT and EcNΔ*chuS*) a particular behavior is noted for each one. In the case of groups treated with EcN WT (Figure 34c), the pattern has showed that as higher IL-10 secretion is, lower IL-12p70 secretion, when doses are low. But for higher doses this relation is inverted.

On the other hand, cytokine secretion within EcNΔ*chuS* treatment groups has behaved in a particular way (Figure 33d) Indeed, secretion of both cytokines was elevated in the same group and diminished in the higher doses. Relation was as higher IL-10 production is, higher in IL-12p70 as well. However, a remarkably result was that regardless the dose, IL-10 always presented a higher secretion.

In addition, colon was extracted from mice and processed for histological analyses. Apparently, EcNΔ*chuS* treatment in all doses promoted an inflammation in colon tissue compared to control group (Figure 35 and 36).

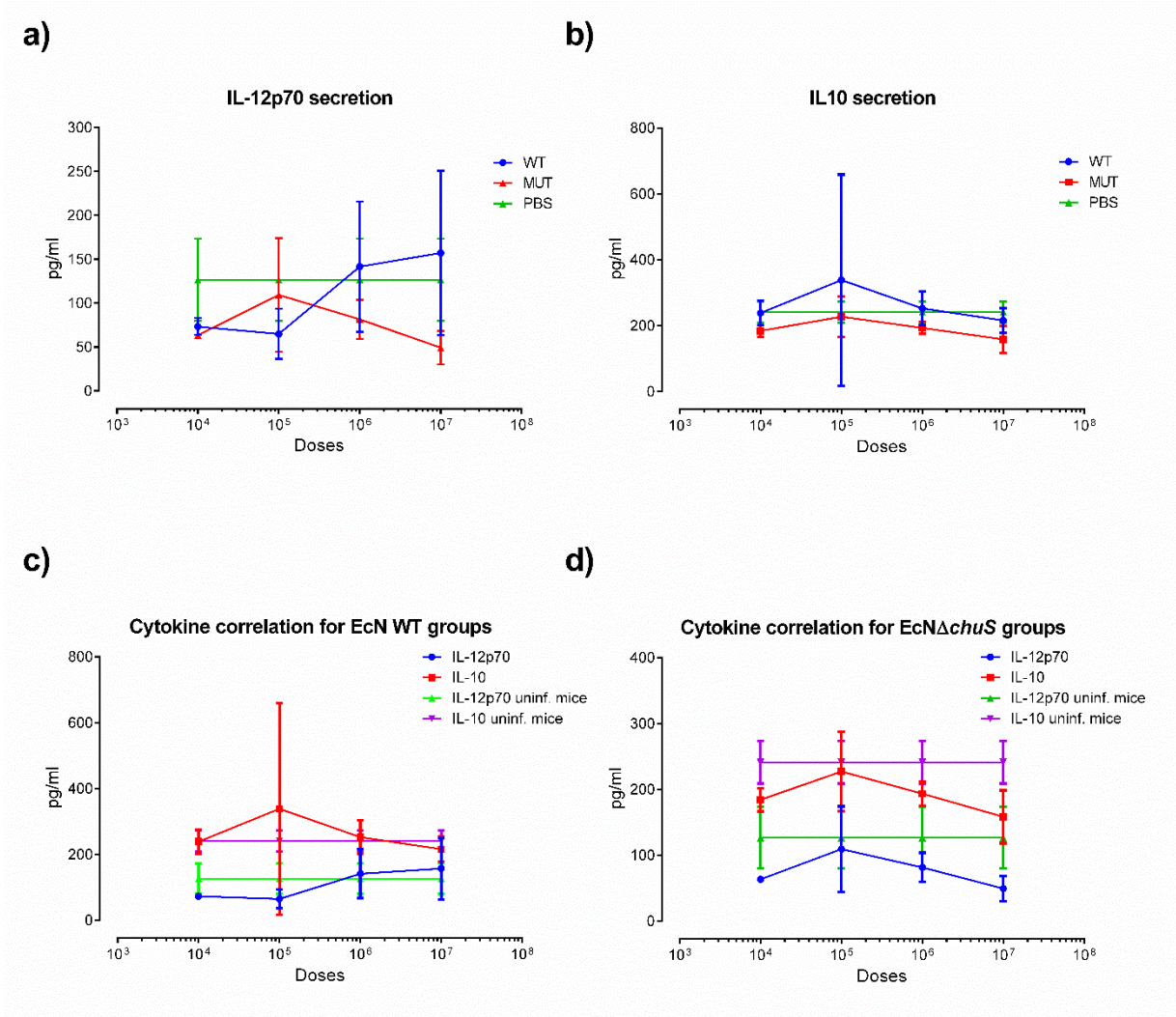

Figure 34. IL-10 and IL-12p70 production in serum after treatment with probiotic strains. a) Correlation between production of IL-12p70 and the treatment with different doses of EcN WT (WT) and EcNΔ*chuS* (MUT). b) Correlation between IL-10 production and the treatment with different doses of EcN WT (WT) and EcNΔ*chuS* (MUT). c) Correlation between treatment of different doses of probiotic EcN WT with the IL-10 and IL-12p70 production. d) Correlation between treatment of different doses of probiotic EcNΔ*chuS* with the IL-10 and IL-12p70 production. Data sets of control group treated with PBS were added to all graphs to show basal production of both cytokines. Data were analyzed with One-way ANOVA, Turkey's multiple comparison, and Pearson correlation coefficient, using $\alpha=0.05$ for all. Data set corresponds to a unique experiment.

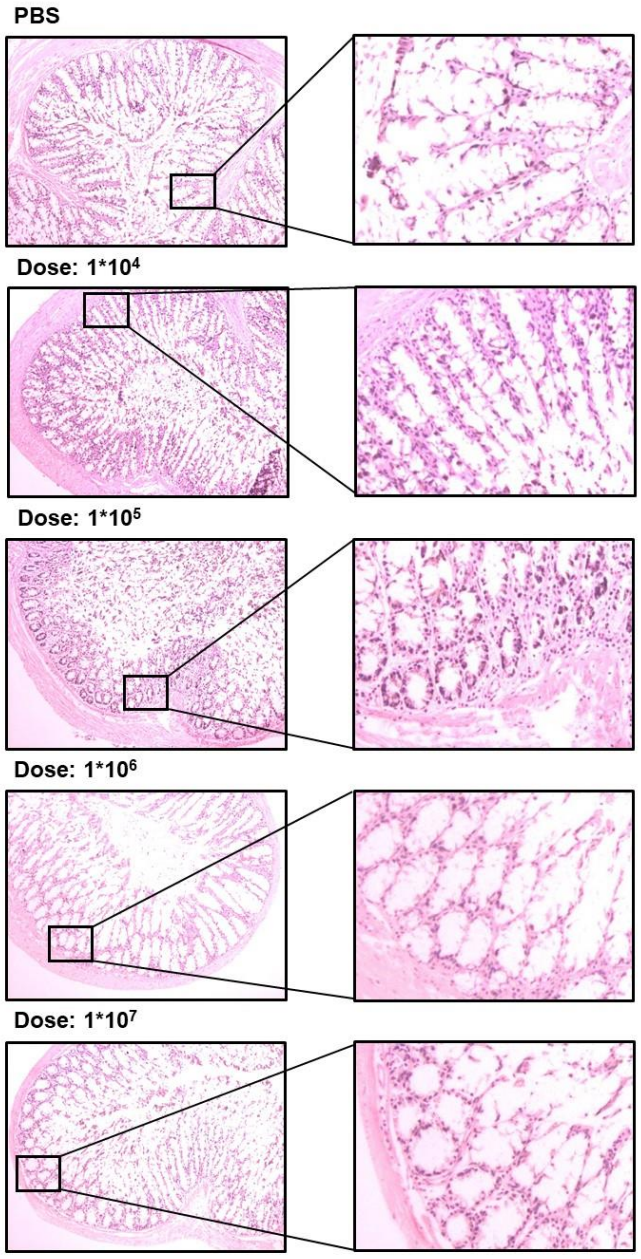

Figure 35. Colon sections after treatment with respective dose of EcN WT. Samples were stained with hematoxylin-eosin method. Sections were observed in microscope, 10X. Pictures were taken with Infinity 2 microscope camera. Pictures are representative from each group. Data set corresponds to a unique experiment.

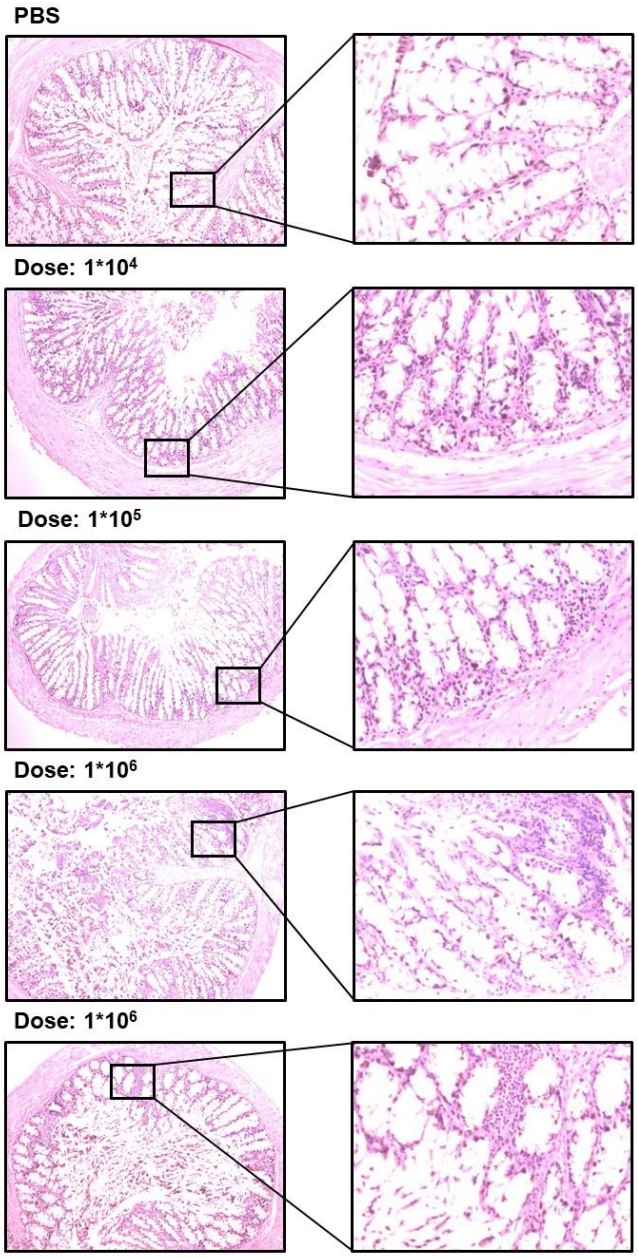

Figure 36. Colon sections after treatment with respective dose of EcNΔ*chuS*. Samples were stained with hematoxylin-eosin method. Sections were observed in microscope, 10X. Pictures were taken with Infinity 2 microscope camera. Pictures are representative from each group. Data set corresponds to a unique experiment.

Furthermore, feces of each group were monitored by taking photographs. In general, feces of mice treated with EcNΔ*chuS* (regardless of dose) showed evident signs of inflammation as the presence of mucus (Figure 37) contrary to feces from mice treated with EcN WT.

Finally, DNA was extracted from feces to detect the presence of probiotic, from EcN WT treated groups. Results have showed that EcN WT was present in all mice of all groups, and that probiotic was stablished in the intestine (Figure 38).

Together, results have shown the property of probiotic strain of modulating immune response at local and systemic level. Also, differences between EcN WT and EcNΔ*chuS* were evidenced specially in inflammation parameters of clinical score (weight, physiological score, behavior, feces) and cytokine secretion in serum. Modulation of IL-10 and IL-12p70 highlighted similitudes with effect of mammal heme oxygenase.

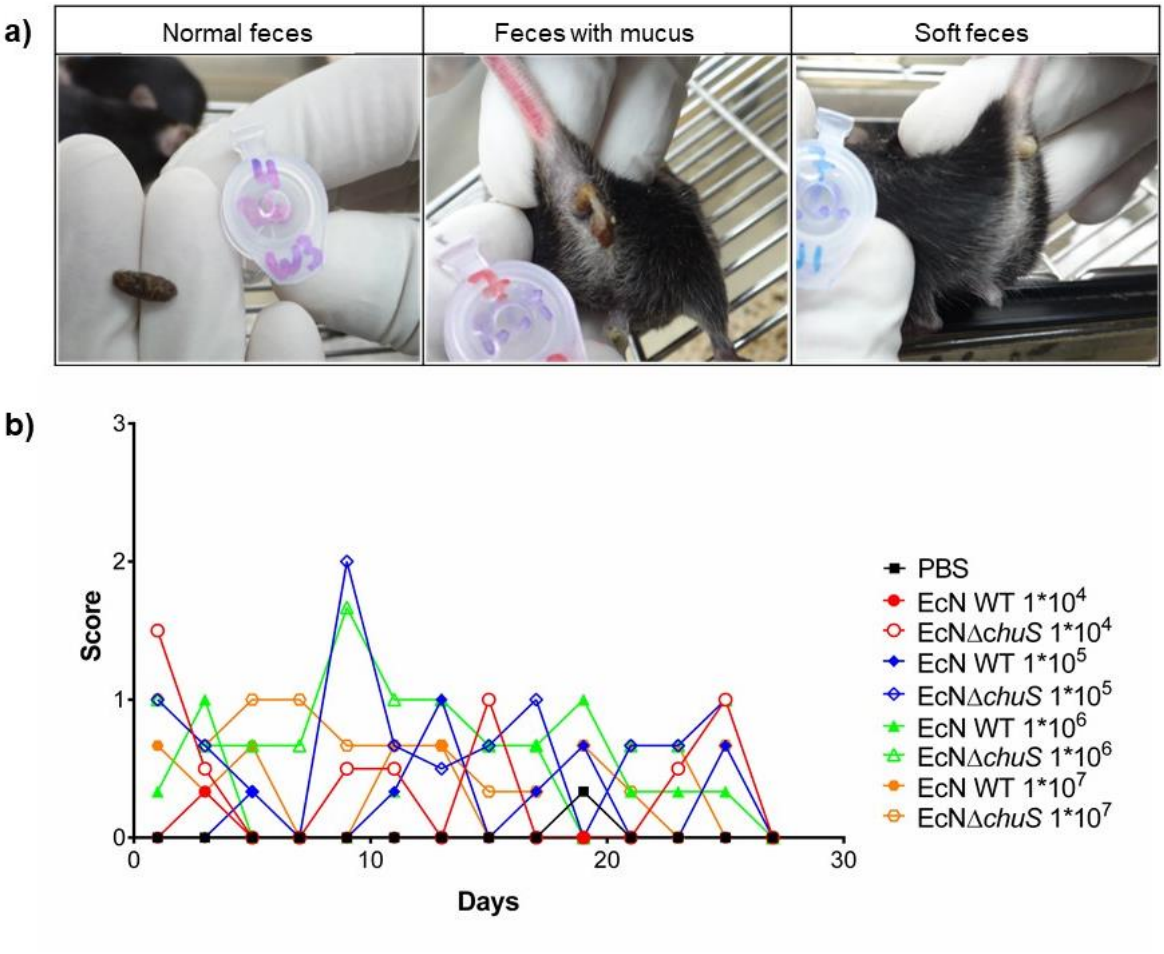

Figure 37. Feces evaluation of mice treated with different doses of probiotic strains (WT and mutant). Observation of feces was done daily and registered in colitis section of clinical score. a) Representative photographs of feces according score used for colitis evaluation. b) Colitis score during probiotic administration. A Linear Regression was performed, P value >0.05 for all slopes. Also, data were analyzed by 2way ANOVA α=0.05, P value for column (Doses groups) and row (Days) factors was <0.0001. Data set corresponds to a unique experiment.

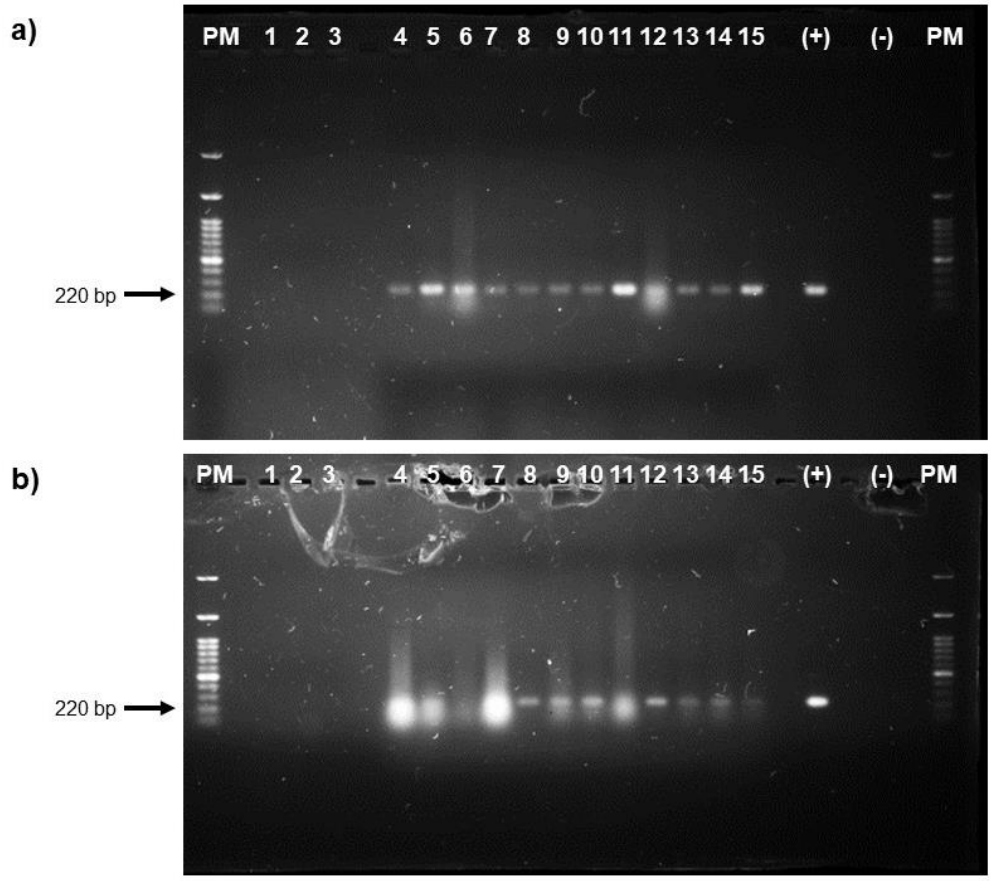

Figure 38. Detection of *chuS* gene from EcN WT in mice treated with EcN WT. a) One day post administration of probiotic. b) Day 27 of probiotic treatment. 1: Mice 1 of PBS group, 2: Mice 2 of PBS group, 3: Mice 3 of PBS group, 4: Mice 1 of EcN WT $1*10^4$ group, 5: Mice 2 of EcN WT $1*10^4$ group, 6: Mice 3 of EcN WT $1*10^4$ group, 7: Mice 1 of EcN WT $1*10^5$ group, 8: Mice 1 of EcN WT $1*10^5$ group, 9: Mice 1 of EcN WT $1*10^5$ group, 10: Mice 1 of EcN WT $1*10^6$ group, 11: Mice 2 of EcN WT $1*10^6$ group, 12: Mice 3 of EcN WT $1*10^6$ group, 13: Mice 1 of EcN WT $1*10^7$ group, 14: Mice 2 of EcN WT $1*10^7$ group, 15: Mice 3 of EcN WT $1*10^7$ group, (+) positive control with DNA from a pure culture of EcN, (-) negative control, PM: Molecular weight marker. Primers used were qchuS forward and reverse, expected amplicon size for treated mice and (+) was 220 bp. Agarose gels were exposed to UV light for 400 ms. Data set corresponds to a unique experiment.

6. DISCUSSION

In general, the last decade probiotics have reached a high level of importance in alternative and “natural” treatments for diseases related with gastrointestinal tract. Additionally, recent studies have proposed probiotics as modulators of immune system in autoimmune diseases (Opazo, 2018). On the other hand, microbiota have shown to have a key role in the immune system at systemic levels, even reaching brain modulation (Petra, 2015; Grigg, 2018).

Beneficial effects of EcN are known, but not all mechanisms by which it promotes them are not clear yet. Such is the case of the presence of a heme oxygenase (*chuS*) in its genome, which had not been previously studied in the context of probiotic function of this strain. Bacterial HOs were only related to pathogens, since functions associated with fitness and competition for nutrients (in this case Fe) were attributed (Sonnenborn,2006; Scaldaferri, 2016). In this project, results allowed to confirm a HO present in EcN and know its potential as immune modulator.

Probiotics may act in two categories in immune regulation according to the mechanism that use. One is Immunostimulatory, in which IL-12 is induced by probiotic, to act against infection and cancer, modulating NK cells and developing Th1 cells. The other one is immunoregulatory, in which IL-10 and Treg are produced, this category works against inflammatory scenarios (Azad, 2018;). Probiotics also, interact directly with gut epithelium, DC, monocytes, macrophages and lymphocytes.

7.1. Determination of the presence of chu operon in *E. coli* Nissle 1917 and development of mutant strains

Bacterial heme oxygenase ChuS exerts its role in conjunction with a set of proteins. Reports for other bacteria like *Yersinia*, *Neisseria* and *Shigella* (Frankenberg-Dinkel, 2004), have shown the necessity of other molecules that work together with heme oxygenase. Furthermore, we detected and confirmed whole *chu* operon with eight genes in EcN.

Mutant strain EcNΔ*chuS* was successfully generated. But, EcN Δ*chuS::putA::chuS* and EcN *putA::chuS* have shown that they are not viable. Apparently, an extra copy of chuS promoted in some way an unsustainable scenario. Construct designed to complement *chuS* in *putA-putP* region, had a strong constitutive promotor *trc*. It is possible that many ChuS were synthesized, and the cell could not stand and died. The other possibility is that having numerous ChuS is useless, without additional equipment to enter and assist in processing the cell heme.

Furthermore, we evaluated ChuS response to an inductor (CoPP) and inhibitor (SnPP) and found that CoPP induces *chuS* expression and SnPP inhibits it, although not completely. This behavior is similar to HO-1 (Shan, 2000; Abraham, 2008). As well as mammal HO, this enzyme expression is heme availability dependent.

7.2. Viability, activation, and maturation of DC and macrophages infected with EcN WT and EcNΔ*chuS*

In general, probiotics should be tolerated by human body, this is one of its most important characteristics. This involves a direct and indirect interaction with immune cells and the intestine. We decided to test our strains behavior *in vitro* in order to assess the way in which they interact with DC and macrophages. Our results have shown that DC and macrophages infected with MOI 12 and 25 of probiotic strains were activated by them. In the periodic observations, we determined that cells must receive an antibiotic treatment after infection, otherwise they died. However, macrophages showed to be more resistant to infection, but after 18 h cells started to die because were exceeded by bacteria. In general, DC are viable for almost 48 hours and macrophages may live for more than a week *in vitro* (Vremec, 2015; Chitu, 2011). Bacteria viability is an important characteristic for probiotic strains, it means that bacteria is metabolically active, study performed by Galdeano (2004) suggested that integrity of bacterial cells is important, because bacteria produces metabolites which are the ones that interact with immune cells. This fact allows the maintenance of epithelial cells. If viability is lost, bacteria will not be able to produce metabolites and then contact immune cells.

Furthermore, in all doses, maturation of cells was observed for both strains treatment. But, tolerance marker PD-L2 was only higher for cells treated with EcN WT MOI 12. This ligand is responsible together with PD-L1 and PD1, to modulate tolerogenic immune response (Wang, 2018). A study suggested the possibility of enhancing the antitumor efficiency of this ligands by modulating the gut microbiota (Sivan, 2015). In that sense, probiotic action may be important since EcN WT has shown the capacity to upregulate PD-L2.

Additionally, secretion of IL-10 was upregulated for cells treated with WT probiotic. Maharshak *et al.* (2015) evaluated the ability to modulate cytokine profile of macrophages treated with a *E. coli* strain isolated from mice gut, which had *chuS* and was modified to overexpress it. Their finds correlate with ours, IL-10 secretion was increased, and also IL-12p40 was downregulated. Many probiotics have the ability of triggering IL-10 (Azad, 2018). This signal promotes a whole signal cascade in an *in vivo* scenario.

7.3. Induction of T cells activation and inhibition of proliferation by probiotics strain infection

Antigen presenting capability of DC were not impaired with probiotic strain. T cells were successfully activated. Probiotic strains must have properties which may be due to its capability of modulating T cells response.

Furthermore, T cells proliferation were inhibited by both probiotic strains, this highlights that bacteria produce signals and molecules to inhibit this process in some way, despite

of activating these cells. Some probiotic bacteria induce cytokine secretion that have as principal objective to decrease T cell populations (Sturm, 2005). In the case of other probiotic strains as *Lactobacillus reuteri*, DC treated with it and then cocultured with splenic T cells from OT transgenic mice, showed that IL-10 induction promoted a lower production of IL-2 and increased TGF-β production (Livingston et al, 2010).

7.4. Modulation of immune system after probiotic treatment *in vivo*

We first evaluated $1*10^9$ CFU as dose for EcN WT and EcNΔ*chuS*, which is reported as probiotic dose for *in vivo* assays (Pavan, 2003). Our results showed that mice started to lose weight and dye in the first days of treatment and even die in some cases. Way of administration was discarded as the cause of deaths, because control group did not show any alteration. Despite of this inconvenient, first mice processing showed differences in immune cell populations in organs. In particular, T cells populations in liver showed to be higher when mice were treated with EcN WT than those treated with mutant strains, for both cases $CD4^+$ and $CD8^+$.

Sturm et al, (2005) showed that cell cycle progression of T cells was inhibited when cultured with EcN, via TLR2 pathway. Suggesting this is a shared characteristic for different E. coli strains. Tol like receptors (TLR) are important for recognition bacterial molecules. *In vitro*, T cells increase TLR2 and TLR4 expression when treated with EcN, suggesting its capability of regulate innate and adaptative immune response by controlling these receptors. EcN was not able to ameliorate colitis in TLR2 and TLR4 knockout (KO) mice DSS induced colitis (Grabig 2006).

Furthermore, we did not evaluate T cells population in the intestine, but antecedents made us suggest that principal T cell modulation differences between probiotic strains (EcN WT and EcNΔc*huS*) would be evident. Intestinal epithelial lymphocytes work in: Immune exclusion, epithelial repair, controlled activation and expression of inhibitory receptor, all of this to promote a regulation and protection scenario. In the case of EcNΔ*chuS* various of these functions would be impaired. Mucosal T cells play an important role in maintaining barrier function and controlling balance between immune activation and immune tolerance (van Wijk, 2009). Additionally, our results showed B cells in spleen were downregulated with probiotic treatment.

Moreover, we evaluated if lack of ChuS had an impact on infection. In this sense, a group of mice treated with both strains of probiotic, were then infected with SEN. Our results suggested that *chuS* depletion impaired in some way antimicrobial capability of EcN. Some previous studies have shown that EcN may diminish and neutralize pathogens infection by many mechanisms as competition for space, food or by the action of antimicrobial molecules (Sonnenborn, 2016; Sonnenborn 2009). However, there are no reports attributing these processes to ChuS action.

In the second *in vivo* assay, we evaluated 4 different lower doses of both probiotics. Results have shown differences in inflammation, evidenced by feces observation, regardless dose, all mice from EcNΔ*chuS* presented mucus in feces. There are few cases in which mucus is observed after a probiotic treatment, but this phenomenon is associated to a previous inflammation or infection (Hong, 2019). So, our results suggest that mutant strain may have lost some antiinflamamtory properties due to ChuS lack.

Constant treatment with probiotic, promotes a particular scenario, according to our results, antiinflamamtory signaling in upregulated. A strategy used by probiotics for promoting tolerization is that pathogen and probiotics may express similar molecules, for example PAMPs/MAMPs, that may overlap. All of which can traduce in inflammatory responses through NFKB and MAPK pathways. Additionally, a second and then constant exposure to probiotics MAMPs induces a suppressive/tolerogenic response by induction of endogenous negative regulators of TLR, to inhibit NFKB and MAPK pathway (Llewellyn, 2017).

In addition, IL-10 secretion in mice treated with EcN WT were higher than EcNΔ*chuS* treated mice for all doses. In the case of IL-12p70 secretion, as higher was de dose of EcN WT, higher was the secretion. On the other hand, EcNΔ*chuS* mice presented the higher secretion in 1*105 CFU dose, then cytokine levels were lower for higher doses. An *in vivo* study performed by Maharshak *et al.* (2015) evaluated an *E. coli* strain isolated from mice gut, they assessed IL-10 and IL-12 in serum. Findings indicated that levels of IL-12 were lower when mice were treated with a *chuS*-overexpressing strain and more CO was found in liver. These outcomes correlate with results that we obtained.

Therefore, the expected result is the correlation of cytokines secretion found in mice treated with lower doses of EcN WT. Where IL-10 levels were high and IL-12p70 levels were low, indicating an interaction between them.

Finally, histological sections of colon, showed inflammation in mice treated with EcNΔ*chuS*, contrary to what is expected for a treatment with probiotics. This result also

supports the thesis that chuS depletion affected immunomodulator properties of EcN. Because a study performed by Souza *et al.* (2016) demonstrated a reduction of inflammation signs of colitis in intestine section, preservation of intestinal permeability, control of the inflammatory process by reducing levels of inflammatory cells and cytokine production (IL-1β) and increasing Treg cells ($CD4^+CD25^+FOXP3^+$) in a DSS-induced colitis model. They highlighted the importance of microbiota interaction with probiotics as resident bacteria.

Together, these findings will allow us to propose a mechanism model of EcN immunomodulatory properties via ChuS action (Figure 38). Remarkably, most of this process is altered when EcN lacks *chuS*.

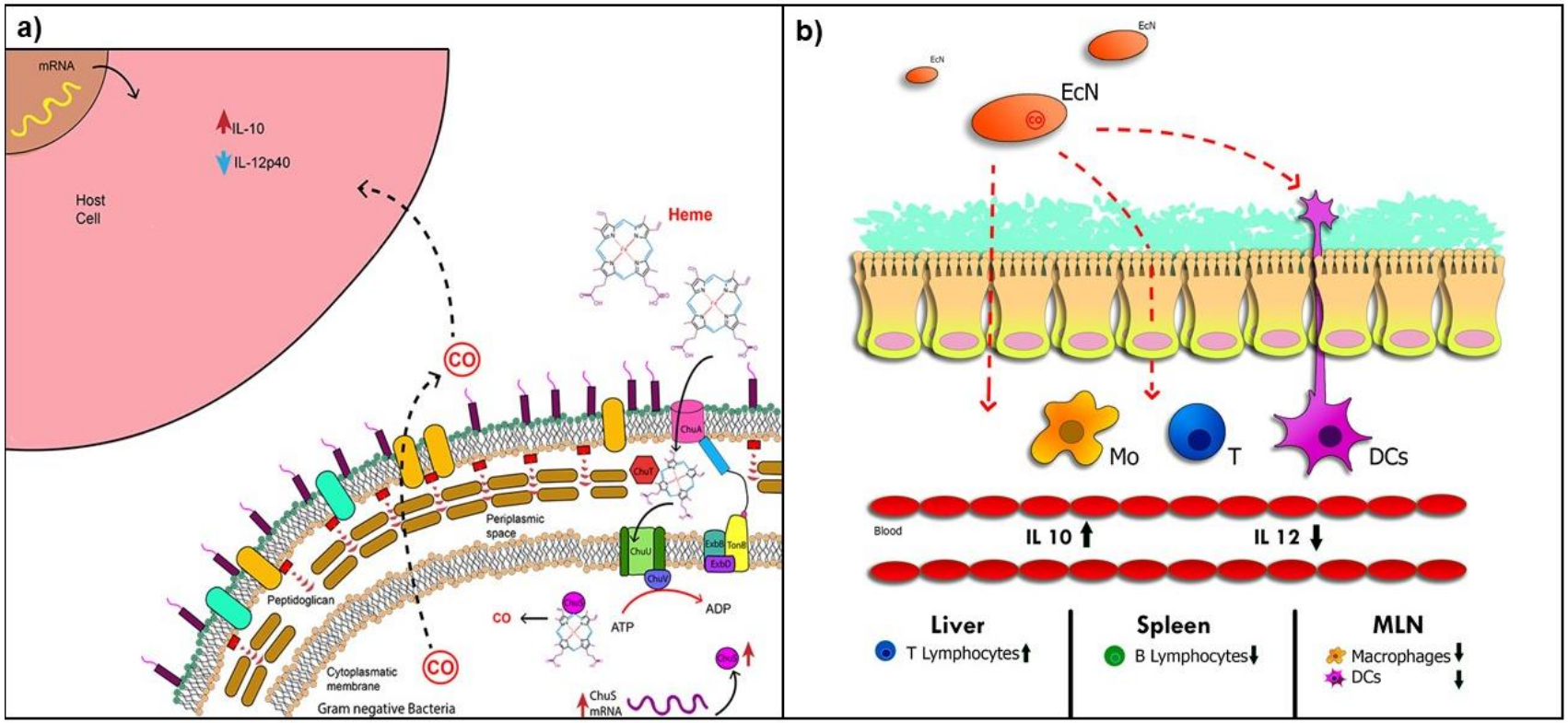

Figure 39. Suggested model of ChuS role in EcN immunomodulatory properties (Legend in next page).

Figure 39. (Previous page) Suggested model of ChuS role in EcN immunomodulatory properties. a) Heme is caught by ChuA and transported to the cytoplasm, where ChuS catalyzes it and produces CO. CO is released to exterior, where may act directly with immune cells or with epithelium. *In vitro* analysis showed that immune cells signaling turns anti-inflammatory secreting IL-10, these levels were measured in supernatant from cell cultures with EcN WT (Figure extracted from Sebastian et al., 2018). b) CO secreted by EcN WT is released to intestinal environment. *In vivo*, CO may interact directly with epithelium and direct or indirectly with immune cells (Mo: Macrophages, T: T Lymphocytes and DC: Dendritic cells) modulating cytokine production. Increased IL-10 and decreased IL-12 secretion measured in serum indicates that immune signals are reaching systemic level, after a treatment with EcN WT strain. Immune cell population measured in organs are shown after treatment with probiotic. In Liver T lymphocytes population of EcN WT group increases compared to mice treated with PBS and EcN∆*chuS*, with a dose of $1*10^9$CFU. In Spleen B Lymphocytes population of EcN WT and EcN∆*chuS* groups decrease compared to mice treated with PBS and EcN∆*chuS*, with a dose of $1*10^9$ CFU. In MLN macrophages and DC populations of EcN WT group decrease compared to mice treated with PBS and EcN∆*chuS*, with a dose of $1*10^9$ CFU.

7. CONCLUSIONS

In conclusion, EcN WT and EcNΔ*chuS* were able to activate and induce maturation of DC and macrophages equally. Differences were evident in IL-10 production, were EcN WT induced more cytokine secretion than mutant strain. Also, a modulation of tolerance marker was observed, giving first insights for EcN modulation of PD-L2. Additionally, DC treated with probiotic strains were capable activating T lymphocytes.

In vivo results allowed to confirm a systemic effect of both probiotics administration. This effect was differentiated for WT and mutant strain, results suggest that mutant strain promotes inflammation. Also, when mice were infected with SEN, only groups treated with mutant strain presented bacterial load, which highlights one of the benefits

of EcN WT against pathogen. Interestingly, deletion of *chuS* gene impairs this capability *in vivo*.

Together, results have shown the property of probiotic strain of modulating immune response at local and systemic level. Also, differences between EcN WT and EcNΔ*chuS* were evidenced specially in inflammation parameters of clinical score (weight, physiological score, behavior, feces) and cytokine secretion in serum. Modulation of IL-10 and IL-12p70 highlighted similitudes with effect of mammal heme oxygenase.

Finally, this project allowed us to elucidate one possible mechanism of action of EcN. Where ChuS plays a key role in modulation of gut environment and immune response in whole organism.

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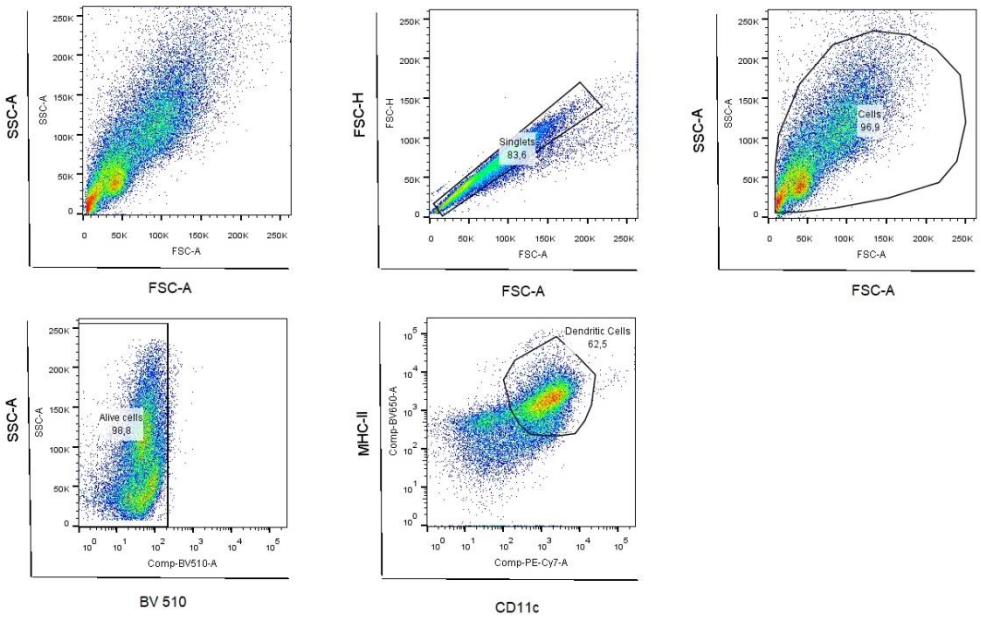
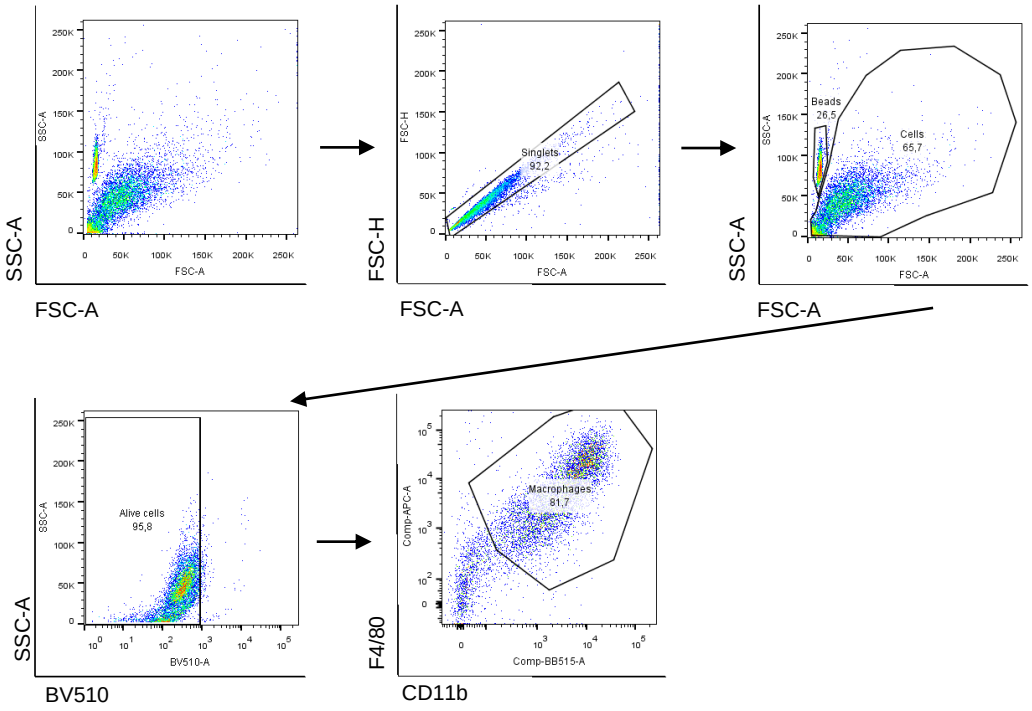
APPENDICES

Appendix 1: Gating strategies for *in vitro* assays

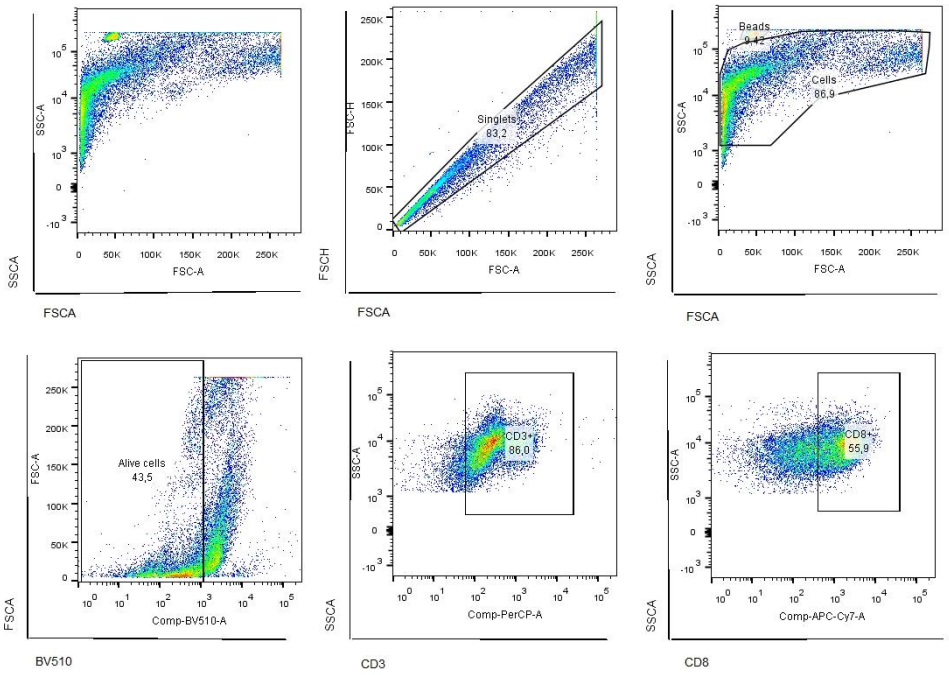
DC Gating strategy



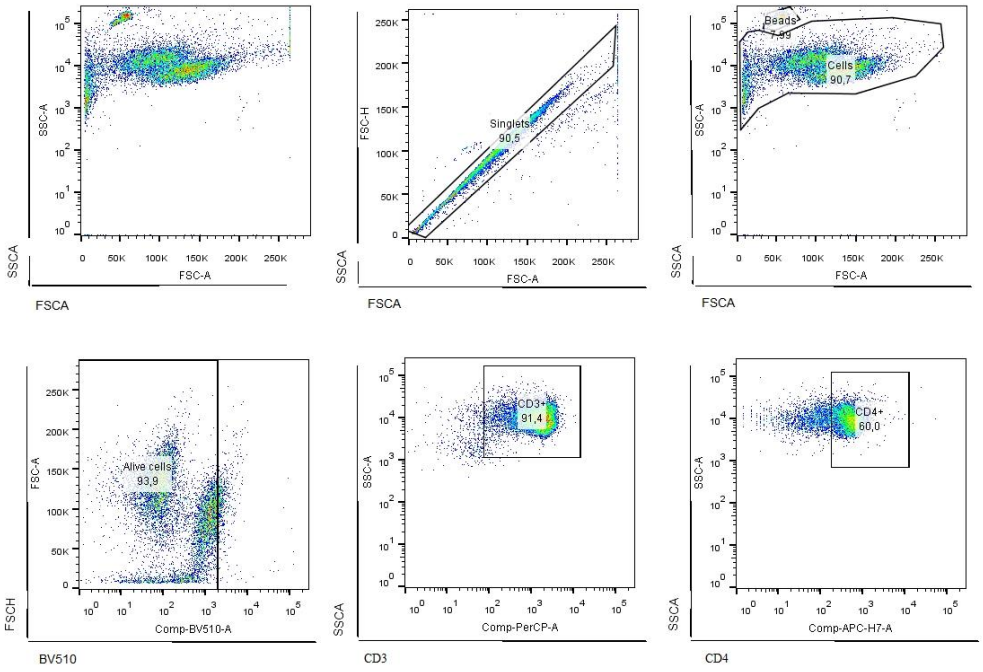
Macrophages Gating Strategy



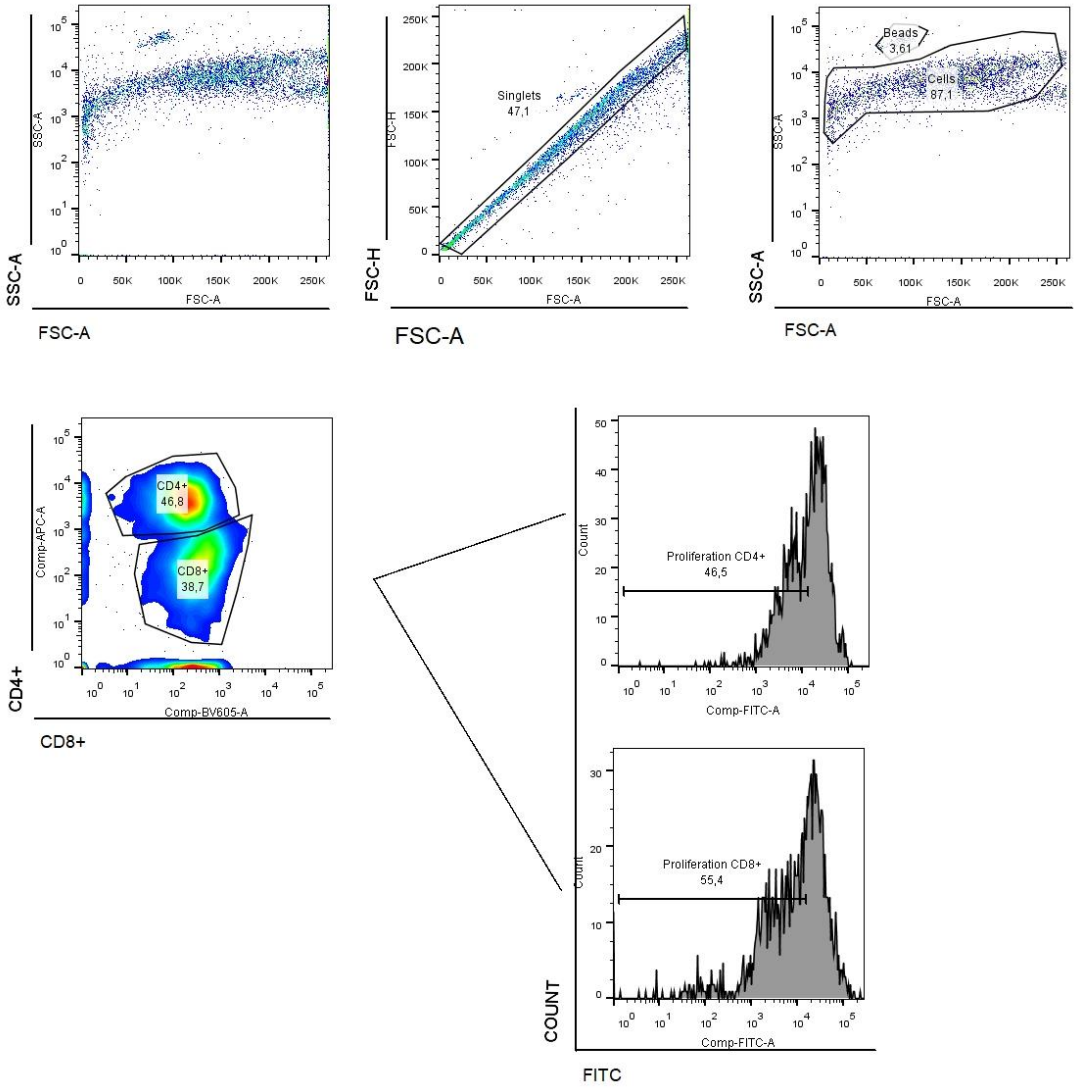
OT-I Lymphocytes gating strategy



OT-II Lymphocytes gating strategy



T lymphocytes proliferation assay gating strategy



Appendix 2: First *in vivo* assay, first mice processing.

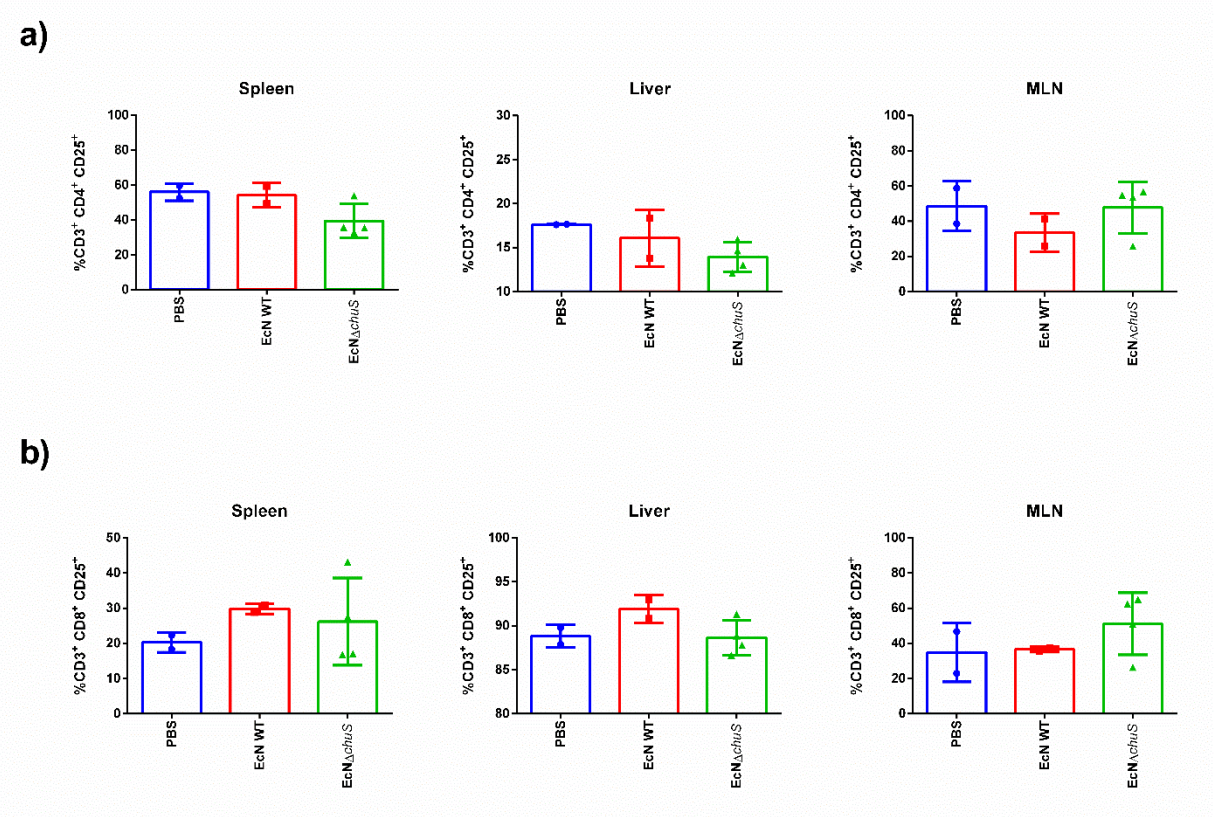


Figure S1. **T cells population after treatment with probiotic strains.** a) T $CD4^+$ cells of spleen, liver, and MLN. b) $CD8^+$ of spleen, liver, and MLN. Statistical analysis performed are One-Way ANOVA and Turkey's Multiple comparison test, for both tests $\alpha=0.05$.

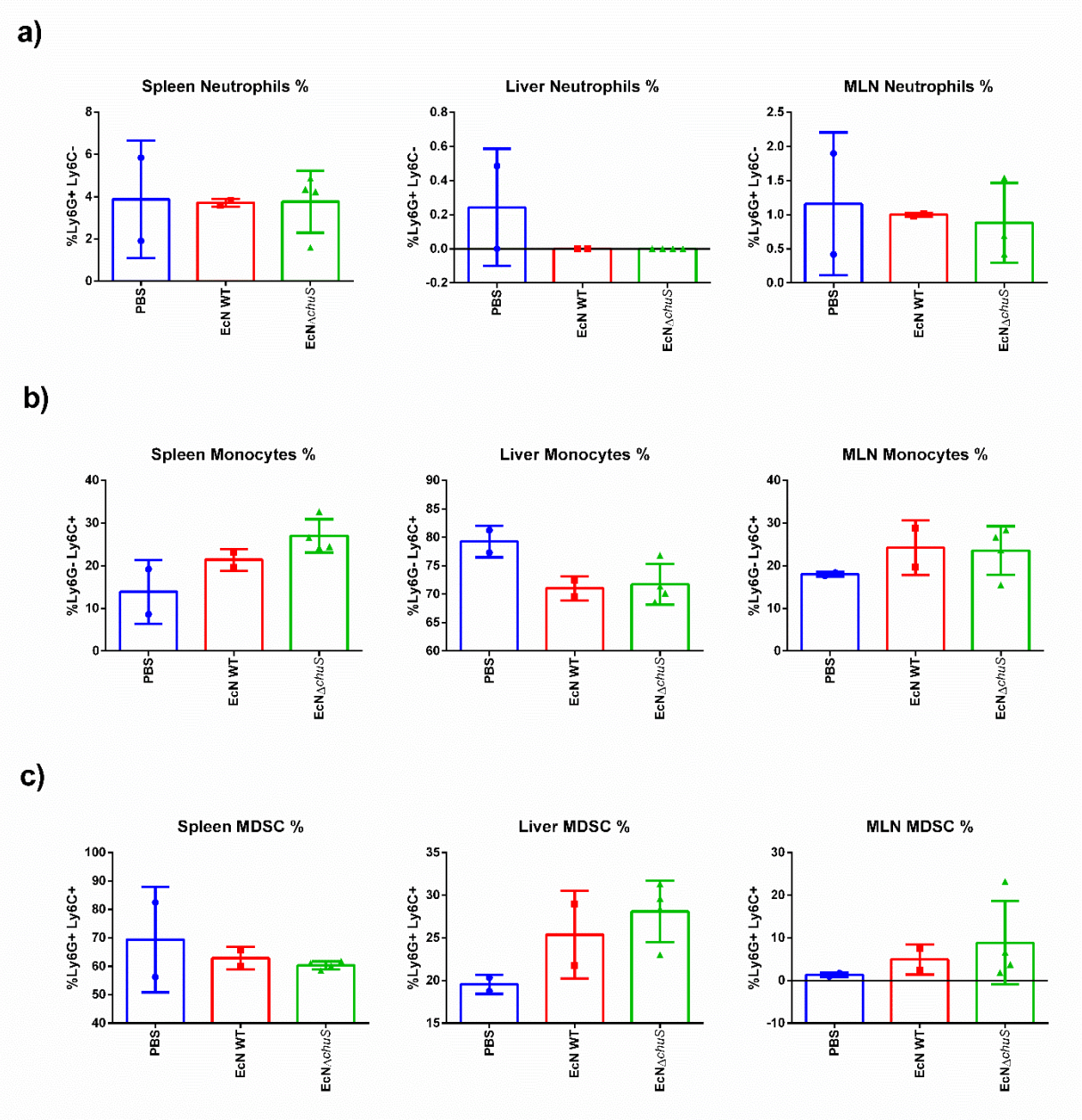


Figure S2. Modulation of Myeloid cells population after treatment with probiotic strains. a) Neutrophils of spleen, liver and MLN. b) Monocytes of spleen, liver and MLN. c) MDSC of spleen, liver and MLN. Data were analyzed with One-Way ANOVA and Turkey's Multiple comparison test, for both tests α=0.05.

Appendix 3:First *in vivo* assay, second processing.

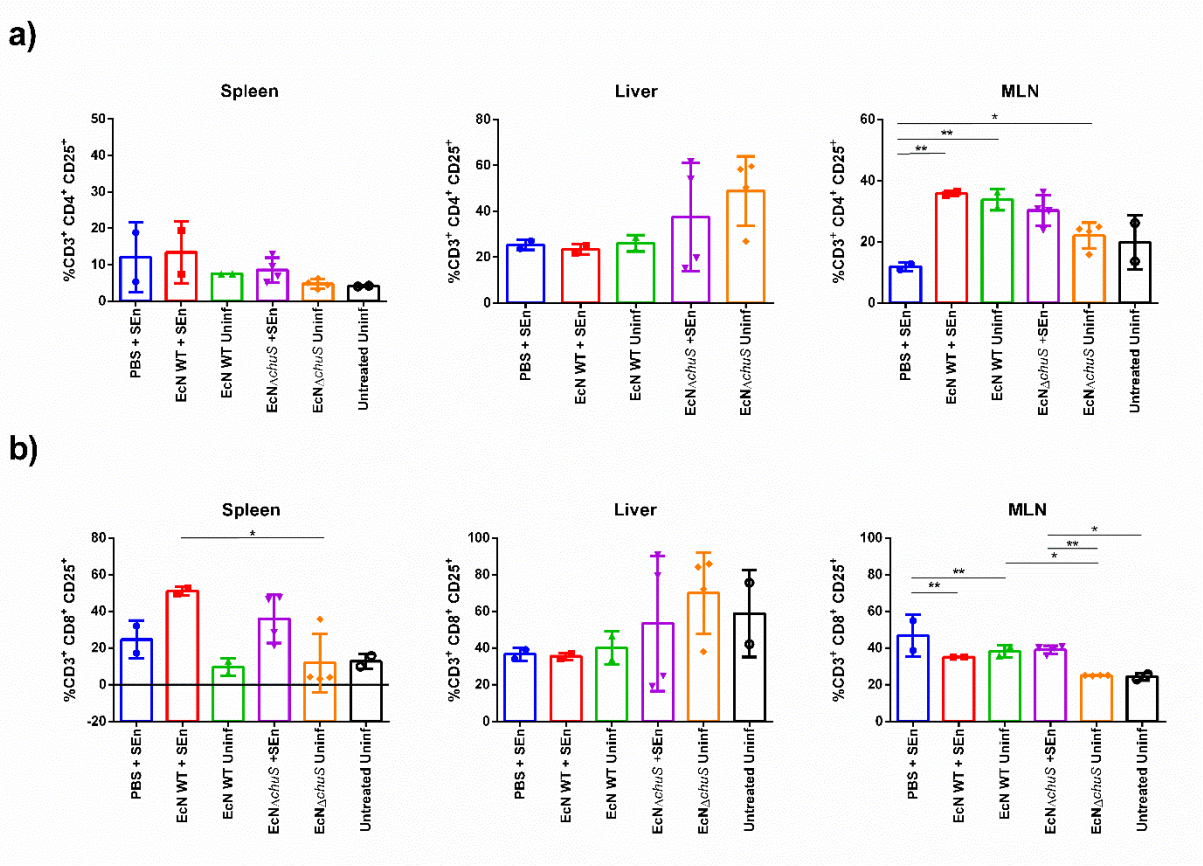


Figure S3. T cells population 48 hours post infection with SEN. a) T CD4$^+$ cells of spleen, liver, and MLN. b) CD8$^+$ of spleen, liver, and MLN. Statistical analysis performed are One-Way ANOVA and Turkey's Multiple comparison test, for both tests α=0.05. MLN data differences were found statistically significant, P value= 0.0025 for T CD4$^+$ and 0.0006 for T CD8$^+$. Also, Spleen Data for T CD4$^+$, P value=0.0198.

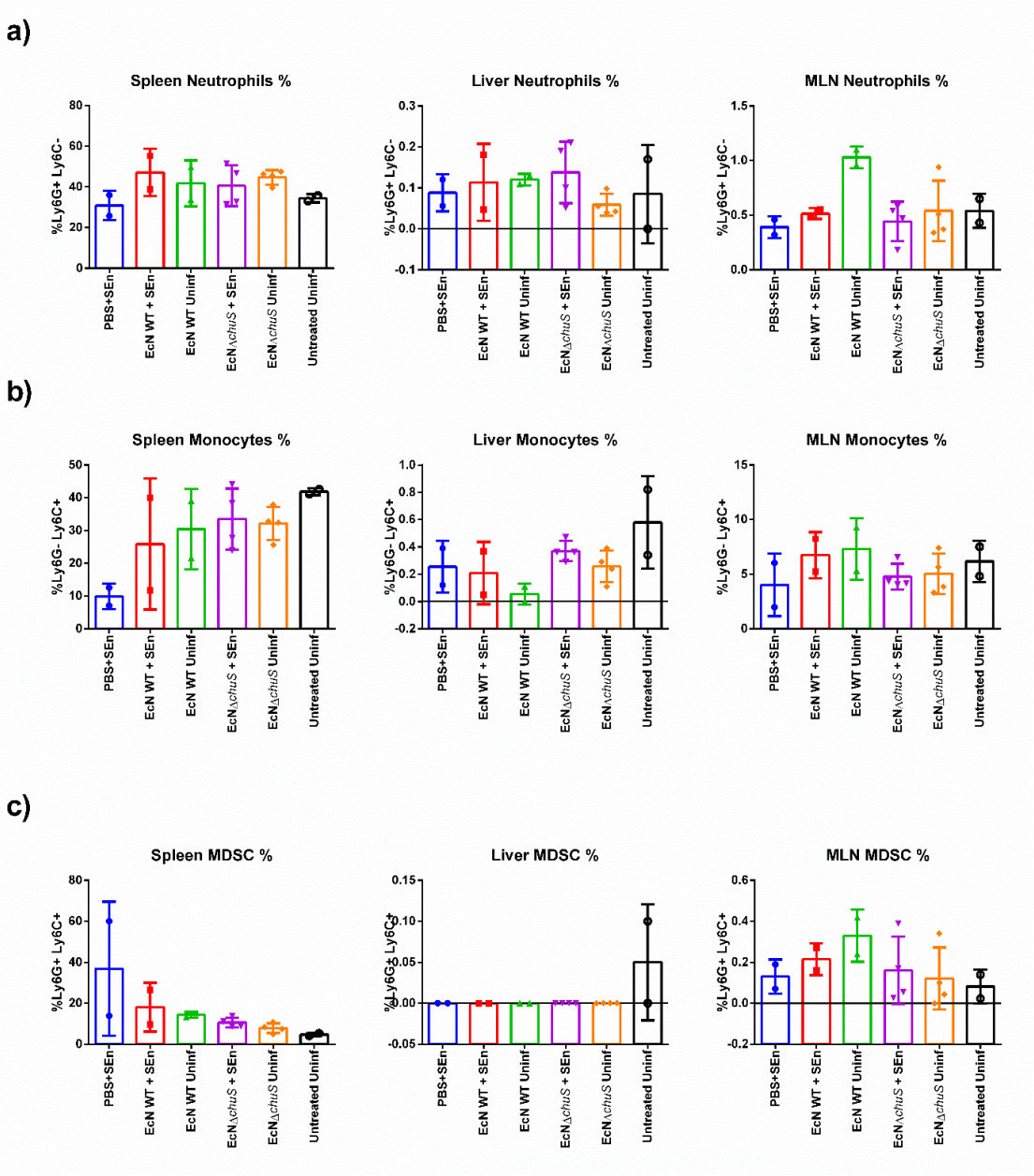


Figure S4. Modulation of Myeloid cells population 48 hours post infection with SEN. a) Neutrophils of spleen, liver and MLN. b) Monocytes of spleen, liver and MLN. c) MSDC of spleen, liver and MLN. Data were analyzed with One-Way ANOVA and Turkey's Multiple comparison test, for both tests α=0.05. Only MLN data of TIP DC were statistically significant. P value=0.025.

SCIENTIFIC PRODUCTION

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C. Pardo-Roa, G. A. Salazar, L. Noguera, F. J. Salazar-Echegarai, I. Coronado, C. E. Palavecino, C.A. Riedel, M Alvarez-Lobos, A.M. Kalergis, S.M. Bueno. Effect of *Salmonella* pathogenicity island excision in early stages of infection in mice. XXXVIII Congreso Chileno de Microbiología. 22-25 de noviembre 2016. Valdivia, Chile.

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