UNIVERSITÀ
DEGLI STUDI
DI TRIESTE

UNIVERSITÀ DEGLI STUDI DI TRIESTE

XXXIV CICLO DEL DOTTORATO DI RICERCA IN

SCIENZE DELLA RIPRODUZIONE E DELLO SVILUPPO

Role of microbiota composition in mediating the effects of thiopurines in pediatric patients with Inflammatory Bowel Disease

Settore scientifico-disciplinare: BIO/14

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ANNO ACCADEMICO 2020/2021

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ABSTRACT

Inflammatory bowel disease (IBD) is characterized by alterations of the composition of the gut microbiota leading to a reduced diversity of bacterial species, referred as dysbiosis. In particular, *Enterobacteriaceae* family and *Pseudomonas* genus proliferate abundantly in the gut of IBD patients at the expense of other bacteria.

Thiopurine drugs, such as azathioprine (AZA), mercaptopurine (MP) and thioguanine (TG), are commonly used in the maintenance of remission in IBD but, despite their proven efficacy, some patients do not respond or develop adverse reactions.

As there is growing evidence of the role of bacteria in influencing the efficacy of therapies, the project aims to investigate *in vitro* if *Escherichia coli*, *Salmonella enterica*, *Klebsiella pneumoniae* (*Enterobacteriaceae* family) and *Pseudomonas aeruginosa* (*Pseudomonas* genus, *Pseudomonadaceae* family) interfere with cytotoxic effects of thiopurines.

In this context, the thesis has demonstrated:

- ✓ the role of *K. pneumoniae*, among the *Enterobacteriaceae* tested, and *P. aeruginosa* in reducing the cytotoxicity of MP and TG probably through internalization of these drugs, thus reducing their concentration;
- ✓ the role of *K. pneumoniae* catabolites, and in particular of compounds secreted by the bacterial strain during its logarithmic growth, in reducing the cytotoxicity of thiopurines.

This project could allow interesting information on the influence of candidate bacterial strains, representative of the mucosal gut microbiota of IBD patients, on thiopurine drugs in order to shed light on further factors of interindividual variability after treatment with these drugs and to achieve a personalized therapy.

RIASSUNTO

Le malattie infiammatorie croniche intestinali (MICI) sono caratterizzate da alterazioni della composizione del microbiota intestinale, o più precisamente, da una riduzione della diversità delle specie batteriche, definita come disbiosi. In dettaglio, la famiglia delle *Enterobacteriaceae* ed il genere *Pseudomonas* proliferano maggiormente nella flora intestinale di pazienti affetti da MICI a discapito di altre specie.

Le tiopurine, azatioprina (AZA), mercaptopurina (MP) e tioguanina (TG), sono farmaci comunemente usati nel mantenimento della remissione nelle MICI. Nonostante la loro comprovata efficacia, alcuni pazienti non rispondono al trattamento e/o sviluppano reazioni avverse.

Date le prove crescenti del ruolo dei batteri nell'influenzare l'efficacia delle terapie, questo progetto mira ad investigare *in vitro* se *Escherichia coli*, *Salmonella enterica*, *Klebsiella pneumoniae* (famiglia delle *Enterobacteriaceae*) e *P. aeruginosa* (genere *Pseudomonas*, famiglia *Pseudomonadaceae*) medino la citotossicità delle tiopurine.

In questo contesto, la tesi ha dimostrato:

- ✓ il ruolo di *K. pneumoniae*, tra le *Enterobacteriaceae* testate, e di *P. aeruginosa* nel ridurre la citotossicità di MP e TG probabilmente attraverso l'internalizzazione di questi farmaci, riducendone quindi la concentrazione;
- ✓ il ruolo dei cataboliti di *K. pneumoniae*, ed in particolare dei composti rilasciati dal ceppo batterico durante la sua crescita logaritmica, nel ridurre la citotossicità delle tiopurine.

Questo progetto potrebbe fornire interessanti informazioni sull'influenza di ceppi batterici candidati, rappresentativi del microbiota della mucosa intestinale di pazienti affetti da MICI, sulle tiopurine al fine di fare luce su ulteriori fattori alla base della variabilità interindividuale in seguito a trattamento con questi farmaci ed ottenere una terapia personalizzata.

1. INTRODUCTION

1.1 The gut microbiota

The human microbiota consists of 10-100 trillion of microorganisms that are present in human bodies; the gut microbiota is defined as the totality of microbes, such as bacteria, archaea, eukarya, viruses and protozoa, harbouring the gastrointestinal (GI) tract (Thursby & Juge, 2017). They are even more abundant than human cells: the number of gut bacteria exceeds 10^{14} and they reach the maximum density of 10^{12} microbial cells/mL in the colon (Adak & Khan, 2019).

The predominant phyla of the gut microbiota are Firmicutes and Bacteroidetes, representing almost the 90% of the bacterial population, followed by the other less abundant phyla Proteobacteria, Actinobacteria, Fusobacteria, Verrucomicrobia and Cyanobacteria (Adak & Khan, 2019). *Bacteroides* and *Prevotella* genera, belonging to Bacteroidetes phylum, and *Clostridium*, *Eubacterium* and *Ruminococcus*, belonging to Firmicutes phylum, represent most of the microorganisms of the gut (Adak & Khan, 2019).

The composition of the intestinal microflora varies along the GI tract in bacterial load and diversity of the species. The distribution is principally due to differences in physical features of the gut such as oxygen concentration and pH values, in nutrient availability and in the presence of antimicrobial peptides (**Figure 1.1**) (Donaldson et al., 2016). Indeed, facultative anaerobic bacteria are more present in the upper GI tract due to higher oxygen concentration, whereas strict anaerobic bacteria colonize preferentially the lower tract. Moreover, lower pH values and the abundance of nutrients and antimicrobial peptides limit the bacterial density in the upper GI tract in comparison with the large intestine.

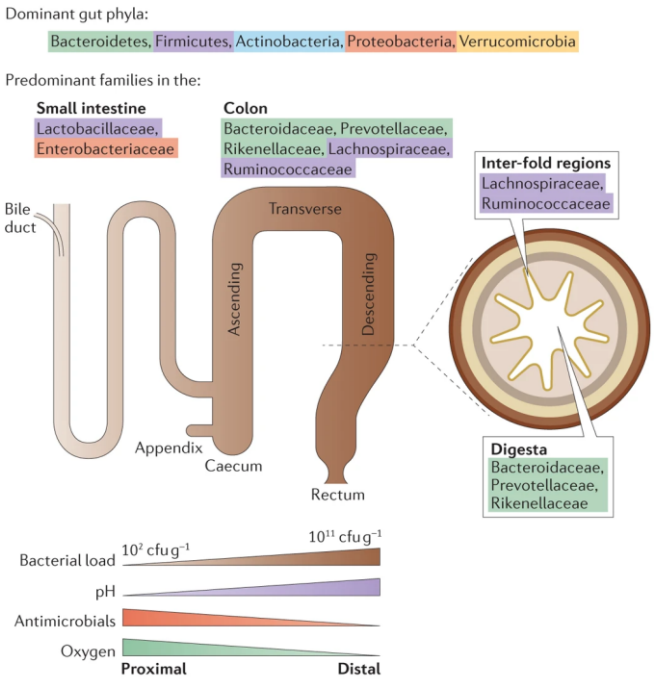

Figure 1.1 Bacterial load and distribution of the dominant phyla and families populating the small intestine and the colon according to pH values, presence of antimicrobials and oxygen concentration (Donaldson et al., 2016).

A limited number of bacterial species belonging to the genera *Streptococcus* (Firmicutes), *Actinomyces* (Actinobacteria), *Lactobacillus* (Firmicutes), *Staphylococcus* (Firmicutes) and *Prevotella* (Bacteroidetes) is present in the oesophagus (Hollister et al., 2014).

Given the characteristic pH of this district, the stomach is mainly the habitat of acid-resistant bacteria belonging to Firmicutes (*Streptococcus*, *Veillonella* and *Lactobacillus* genera), Bacteroidetes (*Prevotella* genus), Actinobacteria (*Rothia* genus) and Proteobacteria (*Neisseria* and *Haemophilus* genera) phyla (Nardone & Compare, 2015).

Short transit time, the influx of digestive enzymes and bile, and intermittent food substrate delivery make the small intestine a harsh environment for the microbial growth (Kastl et al., 2020). As a result, the bacterial density is lower and the microbial population is less diverse than in the large intestine (Kastl et al., 2020). Proteobacteria (especially *Enterobacteriaceae* family) is the most abundant phylum harbouring the small intestine, followed by Firmicutes (*Lactobacillaceae* family and *Clostridium*, *Staphylococcus*, *Streptococcus* genera) and Bacteroidetes (*Bacteroides* and *Prevotella* genera) (Donaldson et al., 2016; Kastl et al., 2020). Even if present in lower extent compared to the bacterial families and genera mentioned above, *Pseudomonas*, especially *Pseudomonas putida*, harbors the small intestine (Wang et al., 2005).

Clostridiaceae, *Lachnospiraceae*, *Ruminococcaceae*, belonging to Firmicutes phylum, and *Bacteroidaceae*, *Prevotellaceae* and *Rikenellaceae*, belonging to Bacteroidetes phylum, are the bacterial families that colonize in major part the large intestine (Donaldson et al., 2016; Kelly et al., 2017).

Moreover, the community of microbes differs also in the same district whether they colonize the mucosa or the lumen (**Figure 1.1**) (Donaldson et al., 2016; Eckburg et al., 2005; Vaga et al., 2020). In particular, the colonic microbiota of the inter-fold regions is enriched in *Lachnospiraceae* and *Rumicoccaceae* families; instead *Bacteroidaceae*, *Prevotellaceae* and *Rikenellaceae* abundantly proliferate in the colonic lumen (Donaldson et al., 2016).

The gut microbiota is commonly classified in the so-called enterotypes, clusters which comprise microbial populations with similar composition and metabolic behaviour: enterotype 1 is mainly represented by *Bacteroides*, enterotype 2 by *Prevotella* and enterotype 3 by *Ruminococcus* (Cheng & Ning, 2019). In detail, the first enterotype uses carbohydrates and proteins as energy source, the second enterotype digests fibres derived from plants and the third enterotype is able to degrade mucins of the mucus layers (Costea et al., 2018). Only long-lasting perturbations can cause switches of enterotypes, even if they are affected by the use of antibiotics and age (Cheng & Ning, 2019). To date, these clusters can be useful in association with diseases, with their progression and with susceptibility to them but, interestingly, since each enterotype has a different metabolic behaviour, they could be considered also in studies that correlate the gut microbiota profile to the metabolism of xenobiotics (Costea et al., 2018).

1.1.1 Factors influencing the gut microbiota

The composition of the gut microbiota can be influenced by many factors such as type of delivery, diet, age, pharmacological therapies, hormones, geographic location and diseases (**Figure 1.2**).

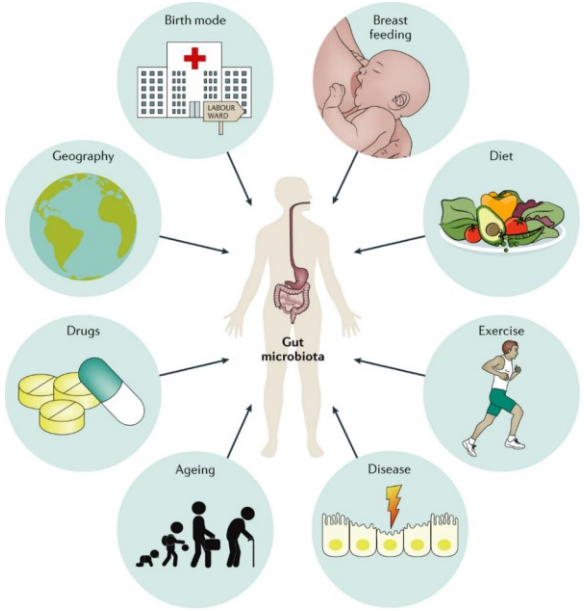

Figure 1.2 Elements influencing the gut microbiota composition (Quigley, 2020).

Since the GI tract is sterile at birth, the colonization starts right after in a way depending on the type of delivery (vaginal birth or caesarean delivery) and the infant's diet (breastfeeding or formula feeding) (D'Argenio & Salvatore, 2015; Vandenplas et al., 2020). During the first years of life, the gut microbiota undergoes a lot of modifications but, generally, the one of vaginally delivered and breast-fed infants is defined as the "healthy" microbiota (Lucafo et al., 2020; Rutayisire et al., 2016).

The proliferation of vagina-associated species belonging to *Lactobacillus* and *Prevotella* genera are associated with vaginal birth (Dominguez-Bello et al., 2010). Instead, the intestinal microflora of infants born from caesarean section is characterised by a lower diversity compared to infants born from a vaginal delivery and by the reduction of commensal bacteria at the expense of opportunistic pathogens associated with the hospital environment, including the genera *Enterococcus*, *Enterobacter* and *Klebsiella* (Shao et al., 2019).

Furthermore, regarding the infant's diet, breastfeeding contributes to develop a gut microbiota similar to that of adult healthy subjects contrary to formula feeding (Penders et al., 2006). Indeed, breast milk contains oligosaccharides which support the colonization and proliferation of commensal bacteria belonging to *Bifidobacterium* species, able to use those nutrients as carbon source, and hamper the growth of *Clostridium difficile* and *Escherichia coli* (Penders et al., 2006).

Approximately in the third year of life, once a diet based on solid food is introduced, the gut microbiota becomes more complex and adult-like (Bäckhed et al., 2015). Indeed, the introduction of fibres, carbohydrates and proteins during the weaning period allows to develop a more diverse intestinal microflora: levels of bacterial species belonging to *Bifidobacteriaceae*, *Clostridiaceae*, *Enterococcaceae*, *Lactobacillaceae* and *Enterobacteriaceae* decrease; whereas the ones included in the bacterial families *Lachnospiraceae*, *Ruminococcaceae*, *Veillonellaceae* and *Bacteroidaceae* increase (Laursen et al., 2017; Lucafo et al., 2020; Tanaka & Nakayama, 2017).

As previously described, the gut microbiota of adults and infants has not the same features. Furthermore, since age is one of the factors influencing the intestinal microflora, the gut microbiota of the elderly individuals differs also from the one of adult and infants. Indeed, it is characterised by a reduction in the bacterial richness and diversity and, more precisely, by a lower presence of *Bifidobacterium*, *Lactobacillus*, *Lachnospiraceae*, *Ruminococcaceae* and *Bacteroidaceae* and an abundance of opportunistic pathogens such as *Clostridium difficile* and *perfrigens* and *Enterobacteriaceae* (Nagpal et al., 2018).

Pharmacological therapies also alter the gut microbiota, both influencing its composition and its metabolic function (Vich Vila et al., 2020). The strongest evidence about this topic concerns antibiotics, but also other medications such as proton pump inhibitors, metformin, selective serotonin reuptake inhibitors and laxatives (Vich Vila et al., 2020; Weersma et al., 2020). In detail, on the basis also of the dosages and duration of treatment, drugs are able to alter the gut microbiota composition directly, changing the physiological conditions of the GI tract and of the mucosa, and indirectly, acting on genes and enzymes of pathways thus influencing bacterial metabolic functions (Vich Vila et al., 2020; Walsh et al., 2018). For instance, the oral bacterial microorganism *Streptococcus salivarius* is enriched in the gut after elevation of the physiological pH by proton pump inhibitors (Vich Vila et al., 2020).

Interestingly, the intestinal microflora results to be altered by hormones. For instance, Org and colleagues evidenced differences in gut microbial composition after gonadectomy in male mice and proved that there is no variation if 5α-dihydrotestosterone is administered (Org et al., 2016).

Geographic location is also a factor that impact on the gut microbiota composition, affecting diet and other environmental factors (Yatsunenko et al., 2012).

Variations in gut microbiota composition are frequently associated with diseases, especially gastrointestinal disorders, even if it is not already clear if it is one of the factors that lead to the onset of diseases or is characteristic of the disease (Fan & Pedersen, 2021; Khan et al., 2019).

1.1.2 Roles of gut microbiota in health

A mutualistic relationship between the gut microbiota and the host occurs: the gut microbiota has several roles contributing to the physiological functions of the host, which provides habitat and nutrients. The roles played by the intestinal microflora can be divided in three main categories: metabolic, protective and neuronal.

1.1.2.1 Metabolic functions

The metabolic functions of the gut microbiota consist in both catabolic and anabolic processes and, more precisely, include fermentation of undigested substrates such as dietary fibres and proteins, metabolism of harmful substances including xenobiotics and synthesis of substances useful for the host (Anderson et al., 2009).

Several types of oligosaccharides, resistant starch, non-starch polysaccharides, lignin and inulin are dietary fibres that the host is not able to metabolize and their digestion occurs thanks to various bacterial enzymes belonging to the class of glycosidases and polysaccharide lyases (Anderson et al., 2009). This process takes place in the large intestine, especially in the proximal colon, where there is a high availability of fibres and Bacteroidetes and Firmicutes phyla, responsible of the digestion, are abundant (Louis et al., 2010). The main products of the fermentation are gases such as carbon dioxide, alcohols such as ethanol and organic acids such as succinic and lactic acid and, noteworthy, short chain fatty acids (SCFAs) (Louis et al., 2010). SCFAs, mainly composed by acetate, propionate and butyrate, as well as being a source of energy and nutrition as substrates in gluconeogenesis and lipogenesis, have anti-inflammatory effects and anti-tumorigenic properties (Williams et al., 2017). Bacterial species of *Roseburia*, *Eubacterium rectale* and *Faecalibacterium prausnitzii* are SCFAs-producers (den Besten et al., 2013; Louis et al., 2010; Williams et al., 2017).

Microbial proteinases and peptidases are involved in the catabolism of proteins into amino acids leading to the subsequent decarboxylation in amines that can potentially be signalling molecules (Hollister et al., 2014).

Furthermore, bacterial strains belonging to *Clostridium*, *Lactobacillus*, *Enterococcus*, *Bifidobacterium* and *Bacteroides* genera have enzymes with hydrolase activity capable of the deconjugation of bile salts (Long et al., 2017).

Noteworthy, the gut microbiota is also capable of synthetizing essential vitamins, such as B and K group vitamins (LeBlanc et al., 2013). For instance, *Bifidobacterium bifidum* and *longum* subsp. *infantis* can produce vitamin B9; *Lactobacillales* synthetize vitamin B12 and bacteria of the genera *Bacteroides*, *Enterobacter*, *Veillonella* and *Eubacterium lentum* are vitamin K-producers (Aydin, 2017; LeBlanc et al., 2013; Thursby & Juge, 2017).

Host's metabolism of several minerals such as calcium, iron, magnesium, selenium, copper, zinc and silver is also influenced by gut microbiota: the intestinal microflora can impact on minerals bioavailability by incrementing their absorption (Skrypnik & Suliburska, 2018).

1.1.2.2 Protective functions

The gut microbiota exerts its protective role mainly contributing to the maturation of the immune system and enhancing the mucosal barrier (Adak & Khan, 2019).

Noteworthy, both innate and adaptive immune system are able to affect the gut microbiota and, at the same time, it can help in the immune system maturation and defend the host against opportunistic pathogens (Skrypnik & Suliburska, 2018). In a healthy individual, the host immune system develops an immune response against pathogens but not commensal bacteria because of its capability to distinguish between them (Lin & Zhang, 2017). Indeed, pattern recognition receptor (PRRs), mainly classified in Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain-like receptors (NOD-like receptors), are

present on the surface of intestinal immune cells, such as dendritic cells, and are able to distinguish pathogens from commensal bacteria through the binding to microbe-associated molecular patterns (MAMPs) and the differential signalling that allow the beginning of inflammatory responses in the case of pathogens (Lin & Zhang, 2017).

Mucus, antimicrobial peptides (AMPs) and IgA are respectively the first, second and third line of defence of the innate immune barrier (Adak & Khan, 2019).

Some bacteria such as *Lactobacillus planetarium*, *Faecalibacterium prausnitzii* and *Bacteroides thetaiotaomicron* are able to induce proliferation of epithelial goblet cells, responsible for the production of mucin glycoproteins that compose the mucus layer (Rooks & Garrett, 2016).

Interestingly, not only intestinal epithelial cells such as Paneth cells, goblet cells and enterocytes, but also some microorganisms of the genera *Lactococcus*, *Streptococcus* and *Streptomyces* are able to produce AMPs that exert their activities against opportunistic pathogens (Gallo & Hooper, 2012).

Dendritic cells promote the activation and differentiation of B cells in plasma B cells that are responsible for the production of secretory IgA that have the role of arresting the interaction of pathogens with the intestinal receptors, avoiding the translocation of microorganisms through the intestinal barrier (Min & Rhee, 2015). In this context, bacteria, such as *Bacteroides fragilis*, are capable of inducing B cells activation and, thus, the production of secretory IgA (Adak & Khan, 2019).

Furthermore, as previously mentioned, some bacterial strains of the intestinal microflora are able to digest dietary fibres producing SCFAs, microbial metabolites with anti-inflammatory effects (Williams et al., 2017). SCFAs exert their activity binding to their receptors and modulating the immune system through several mechanisms (Kayama & Takeda, 2015). For instance, among SCFAs, acetate, produced by *Bacteroides thetaiotaomicron* and *Bifidobacterium longum*, increases mucus secretion and enhances the integrity of the epithelial barrier and butyrate regulates pro-inflammatory cytokines secretion (Kayama & Takeda, 2015).

1.1.2.3 Neuronal functions

Since the gut microbiota is a part of the gut-brain axis, there is a bidirectional communication between the GI tract and the central nervous system (Mayer et al., 2015). On one hand, microorganisms can have a role in influencing the behaviour and the pathogenesis of some nervous diseases such as mood disorders, chronic pain and stress, Parkinson's disease (Mayer et al., 2015). On the other hand, the central nervous system can impact on the intestinal microflora modulating gut mobility, permeability, immune responses and secretion of acid, bicarbonates and mucus (Mayer et al., 2015).

1.2 Inflammatory bowel disease

Inflammatory bowel disease (IBD) is a multifactorial disease whose incidence rises year by year. Between 1990 and 2017 the number of cases worldwide increased from 3.7 to 6.8 million individuals and, from the 21st century, IBD has become a global disease (Alatab, 2020; Ng et al., 2017). However, the highest incidence of 0.3% occurs in Western countries such as Europe and North America (Ng et al., 2017).

Moreover, IBD incidence has been increasing also in children and almost 25% of patients that develop these disorders are <20 years (Rosen et al., 2015). Noteworthy, pediatric disease results to have an aggressive course, an extensive involvement and a rapid progression (Rosen et al., 2015).

IBD is characterized by chronic inflammation of the GI tract and comprises two main disorders, Crohn's disease (CD) and ulcerative colitis (UC) that have common features such as unknown etiology and extraintestinal manifestations but that can be distinguished by type of inflammation, disease location and symptomatology (Matsuoka et al., 2018). In particular, CD is characterized by a discontinuous inflammation involving the whole GI tract from the mouth to the anus (even if intestine and anus are the most frequently affected) and the entire thickness of the bowel from the mucosa to the serosa (Matsuoka et al., 2018).

Instead, UC presents a continuous inflammation extended from the rectum to the colon and mainly localized on the mucosa (Matsuoka et al., 2018).

Montreal classification is the most recent IBD classification that categorizes CD based on age of onset (<17 years, 17-40 years, >40 years), disease behaviour (non-stricturing and non-penetrating, stricturing, penetrating or perianal disease) and disease location (ileal, colonic, ileocolonic, isolated upper disease) (**Figure 1.3**) (Satsangi et al., 2006). UC classification is done on the basis of the severity of the disease or its extent in ulcerative proctitis (involvement of rectum), left sided or distal UC (involvement of a part of the colorectum up to the splenic flexure) and extensive UC or pancolitis (involvement of the rectum, sigmoid and descending colon up to the splenic flexure) (**Figure 1.4**) (Satsangi et al., 2006). The term indeterminate colitis (IC) is used in less of 20% of IBD patients when it is difficult to distinguish between CD and UC (Guindi & Riddell, 2004).

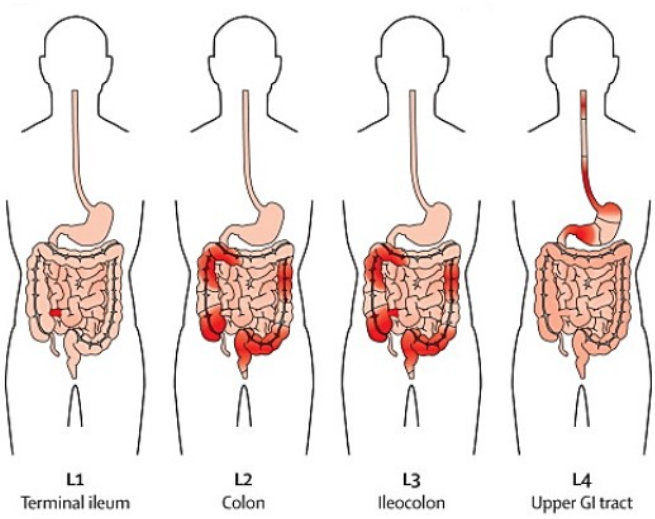

Figure 1.3 Classification of CD based on disease location. Readapted by (Baumgart & Sandborn, 2012).

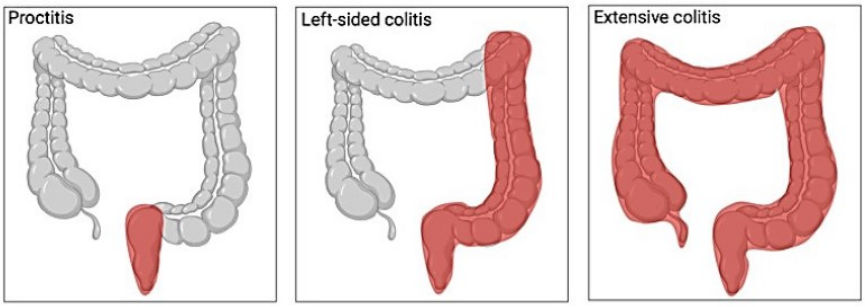

Figure 1.4 Classification of UC based on the extent of the disease. Readapted by (Yeshi et al., 2020).

1.2.1 Clinical manifestations, complications and diagnosis

Clinically, CD and UC disorders present similar symptoms such as diarrhea, hematochezia and abdominal pain although, as previously mentioned, site and depth of inflammation can differ. In addition, CD symptoms can also include nausea, vomit, malabsorption of vitamins and minerals, osteoporosis, hypoalbuminemia and post-prandial discomfort with cramps (Wilkins et al., 2011). Instead, patients with UC can also suffer from incontinence, tenesmus, increased bowel movements, weight loss, fatigue and anemia (Ungaro et al., 2017).

Up to 47% of IBD patients present also extraintestinal manifestations affecting various organs such as eyes, skin, joints, lungs, hepatobiliary tract and pancreas (Olpin et al., 2017; Rothfuss et al., 2006).

Narrowing of the bowel, known as strictures, and connection between the intestine and other organs like the skin, known as fistulae, are common complications of CD; pseudo polyps instead complicate the symptomatology of UC (Magro et al., 2013). Moreover, both CD and UC determine a higher risk of developing colorectal cancer (Lucafò et al., 2021).

Unfortunately, patients suffering from IBD undergo a chronic disease alternating periods of flares and of remission (Cosnes et al., 2011).

Endoscopy and colonoscopy are currently used for differential diagnosis of CD and UC (Kumar et al., 2019). Other non-invasive routine laboratory tests such as C-reactive protein, albumin, transaminase and erythrocyte sedimentation rate are frequently used to control the status of inflammation (Kumar et al., 2019).

1.2.2 Etiology

Although IBD etiology still remains unknown, the disease probably occurs because of a combination of genetic susceptibility, aberrant immune response of the host and environmental factors, as well as the interplay of gut microbiota; therefore, it can be considered a multifactorial disease (**Figure 1.5**) (Hold et al., 2014).

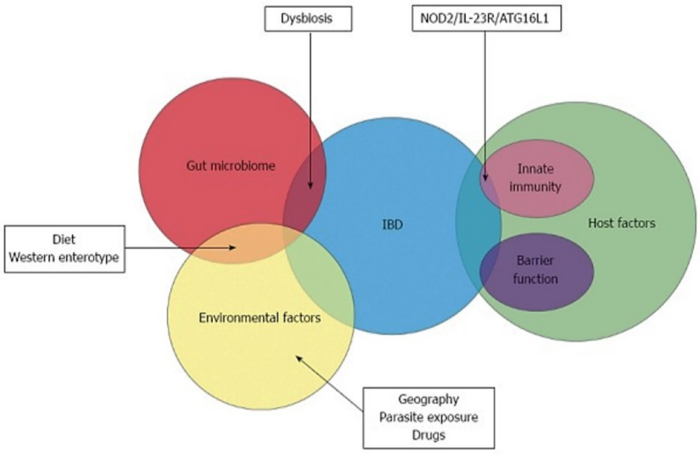

Figure 1.5 Role of gut microbiome, host and environmental factors in the etiology of IBD (Hold et al., 2014).

Advances in technologies for genetic tests have led us to better understand the contribution of genetic susceptibility of the host to IBD. Genome-wide association studies were helpful in identifying genetic biomarkers of the disease. Several studies associated 163 loci to IBD, of which 110 are linked both to CD and UC (Jostins et al., 2012). *NOD2* (nucleotide-binding oligomerization domain containing 2), encoding for a protein that is able to recognize muramyl dipeptide (MDP) in peptidoglycan of bacteria, was the first gene associated with CD susceptibility (Ogura et al., 2001). Other variants of the genes *ATG16L1* and *IRGM*, encoding for proteins related to autophagy, *IL-23R*, encoding for a subunit of the receptor of the pro-inflammatory cytokine IL-23, and *ECM1*, *CDH1*, *HNF4α* and *laminin B1*, genes implicated in mucosal barrier function, are also linked to higher risk to develop IBD (Thompson & Lees, 2011; Zhang & Li, 2014).

Dysfunctions of both innate and adaptive immune pathways contribute to an aberrant intestinal inflammatory response in IBD patients (Zhang & Li, 2014). There is evidence that the behaviour of cells mediating the innate immunity and the expression and the function of PRRs located on their surface and responsible for recognition of microbial antigens are altered (Bonen et al., 2003; Marks et al., 2006; Zhang & Li, 2014). Moreover, increased intestinal permeability and defects in mucus occur in IBD, leading to reduction in host defences against opportunistic pathogens (Michielan & D'Incà, 2015). Furthermore, regarding adaptive

immunity in IBD condition, abnormal responses driven by Th1, Th2 and Th17 are thought to cause intestinal inflammation in this disorder (Bamias & Cominelli, 2015; Cobrin & Abreu, 2005; Geremia & Jewell, 2012).

In this context, given the genetic susceptibility and the aberrant immune response of the host, environmental factors, such as hygiene, diet, smoking, pollution and the interplay of gut bacteria could have an important impact in patients suffering from IBD (Abegunde et al., 2016). A possible role of *Campylobacter* species, *Salmonella* species, *Escherichia coli*, *Listeria monocytogenes*, *Mycobacterium paratuberculosis* and many others has been suggested to be linked to IBD pathogenesis even if it is not still clearly proved (Axelrad et al., 2021). More likely and interestingly, IBD patients seem to develop a disturbed tolerance to microorganisms and thus to be more susceptible, both for genetic factors and immunological reactivity, not only to pathogens but also to commensal bacteria (Hold et al., 2014; Kayama & Takeda, 2012). Furthermore, bacterial penetration of intestinal mucosa and epithelium stimulation with subsequent cytokines production occur in IBD condition (Michielan & D'Incà, 2015).

1.2.3 Pediatric inflammatory bowel disease

As already mentioned above, pediatric IBD has an aggressive course, an extensive involvement and a rapid progression (Rosen et al., 2015). Furthermore, pediatricians are usually familiar with atypical presentations of IBD since 22% of children undergo perianal disease, anemia and growth failure, as well as extraintestinal disease (Rosen et al., 2015).

Monogenic IBD prevalently occurs in children. Nonetheless, no difference exists between the common risk genes of pediatric- and adult-onset IBD: what seems to be relevant is the higher burden of common risk variants and rarer variants with higher penetrance (Muise et al., 2012).

Noteworthy, the reduction of bacterial richness, described below and associated with the onset and with the disease itself, is a common feature of pediatric IBD patients, as well as adult ones (Lucafo et al., 2020).

1.3 Therapeutic approaches in IBD

To date, there is no curative pharmacological therapy for IBD and the treatment is aimed at improving symptoms and patients' quality of life. Therefore, the main objectives are inducing and maintaining the remission of active disease, achieving mucosal healing, avoiding relapses, hospitalization and surgery. Drugs commonly used in the treatment of this disorder are aminosalicylic acid, antibiotics, corticosteroids, thiopurines, methotrexate and biologic drugs such as TNF-α inhibitors, integrin receptor antagonists and interleukin antagonists. Generally, biologic drugs are used to induce and maintain the remission of IBD even if respectively in higher and lower dosages; corticosteroids are used in the induction because of their characteristic severe adverse effects, and thiopurine drugs in the maintenance of remission because of their slow onset of action (Adams & Bornemann, 2013; Cheifetz, 2013).

1.3.1 Thiopurines

Thiopurines, such as mercaptopurine (MP), its prodrug azathioprine (AZA) and thioguanine (TG) are immunomodulatory drugs widely used in the maintenance of remission of IBD, as previously mentioned, but also in the treatment of acute lymphoblastic leukemia, of rheumatoid arthritis and after organ transplant (Zaza et al., 2010).

These agents are purine analogous and act as antimetabolites to exert their cytotoxic activity through a complex pathway (**Figure 1.6**) (Franca et al., 2019). In particular, AZA can be converted in MP and S-methyl-4-nitro-5-thioimidazole through a non-enzymatic or enzymatic reaction involving glutathione S-transferase (GST). Once the conversion occurs, various solute carrier family transporters (SLCs) allow the internalization of MP in lymphocytes where it is metabolized by one anabolic enzyme, hypoxanthine phosphoribosyl transferase (HPRT) and two catabolic enzymes, xanthine oxidase (XO) and thiopurine methyl transferase (TPMT). The enzyme HPRT leads to the conversion of MP to thioinosine monophosphate

(TIMP), which is converted by inosine monophosphate dehydrogenase (IMPDH) to thioxanthosine monophosphate (TXMP) and then by guanosine monophosphate synthetase (GMPS) to thioguanine mono-, di- and triphosphate (TGMP, TGDP, TGTP). These, in turn, can be transformed in the corresponding thiodeoxyguanosine mono-, di- and triphosphate (dTGMP, dTGDP, dTGTP). The metabolites having TG as base are the so-called thioguanine nucleotides (TGN) and are responsible for the lympholytic effects of thiopurines (Franca et al., 2019). Furthermore, MP can be metabolized to the inactive compounds thiouric acid (TUA) and methyl-MP (MMP) by XO and TPMT respectively. TIMP can also be converted by TPMT to methyl thioinosine mono-, di- and triphosphate (MeTIMP, MeTIDP, MeTITP). Indeed, TPMT can methylate thiopurines both in the free base form and in the nucleotide form (Franca et al., 2019). The metabolites having the base of MMP are known as methylated derivatives (MMPN), are associated with hepatotoxicity and their generation competes with the production of TGN (Franca et al., 2019). TIMP undergoes other reactions which form thioinosine diphosphate (TIDP) and triphosphate (TITP) and eventually lead again to the formation of the same molecule, thanks to the enzymatic reaction of inosine triphosphate pyrophosphatase (ITPA).

TG undergoes a less complex metabolism and, after intracellular uptake, is directly converted in TGN through HPRT activity. However, this drug is not commonly used in the pharmacological treatment of IBD since it frequently leads to hepatotoxicity (Rulyak et al., 2003).

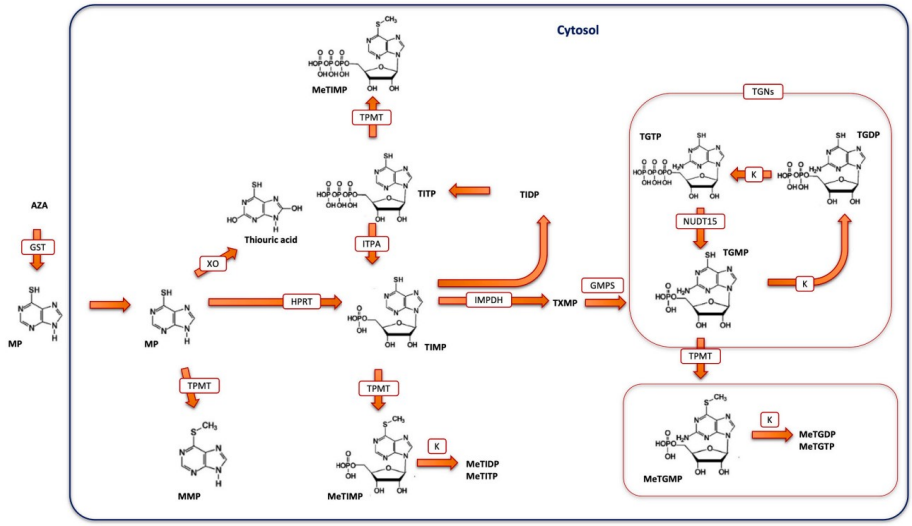

Figure 1.6 Thiopurine pathway (Genova et al., 2021).

As previously mentioned, thiopurines act as antimetabolites since they are purine analogous: the structural similarity of TGN to guanosine nucleotides allows their incorporation into DNA and RNA of lymphocytes disrupting nucleic acids replication by breaking their strands and leading to cell cycle arrest, apoptosis and inhibition of nucleotide and protein synthesis (Cara et al., 2004). More precisely, cell apoptosis is induced by TGTP, both incorporating into RNA and suppressing GTPase Rac1 activation through substitution of GTP, and dTGTP, incorporating into DNA and inhibiting DNA topoisomerase and ligase (Coskun et al., 2016). Additionally, MeTIMP is able to suppress the activity of phosphoribosyl pyrophosphate amidotransferase (PPAT), an enzyme that catalyses the first reaction of *de novo* purine synthesis (Lim & Chua, 2018).

Despite the proven efficacy of these drugs, some patients do not achieve satisfying therapeutic effects and develop adverse reactions; therefore up to 30% of IBD patients have to discontinue thiopurine therapy (Gonzalez-Lama & Gisbert, 2016).

Interindividual variability has been mainly related to genetic polymorphisms affecting TPMT activity. In detail, variant alleles such as *TPMT∗2*, *TPMT∗3A* and *TPMT∗3C*, associated with lower TPMT activity, lead to higher levels of TGN and therefore myelotoxicity (Franca et al., 2019). In this context, testing the genotype and the activity of TPMT, as well as monitoring TGN and MMPN levels in erythrocytes (therapeutic levels:

TGN 235-450 pmol/8×10^8 RBC and MMPN >5700 pmol/8×108 RBC), could help clinicians in adjusting the doses of thiopurines or in deciding to switch to another therapeutic option (Gonzalez-Lama & Gisbert, 2016).

Adverse effects of thiopurines can be both dose-independent and dose-dependent. The former are caused by idiosyncratic or allergic reactions and include nausea, rash, fever, flu-like illness, arthralgias, acute pancreatitis and hepatitis; the latter are the consequences of several factors, including genetic alterations in thiopurine metabolism enzymes, and present as myelotoxicity and hepatotoxicity (Gonzalez-Lama & Gisbert, 2016; Luber et al., 2019).

1.4 Association between the gut microbiota and inflammatory bowel disease

There is strong evidence of the association between the intestinal microbiota and IBD. On one hand, as previously mentioned, the gut microbiota plays its role in the pathogenesis of this disease; on the other hand, IBD is characterized by alterations in the composition of intestinal microflora.

The intestinal immune system of healthy individuals is tolerant towards antigens derived from the diet and from commensals and is activated in the presence of pathogens (Kayama & Takeda, 2012). However, in IBD in which the host is genetically susceptible to the disease and the immune system is aberrant, the balance between the gut microbiota and the immune system is lost because of an excessive response against non-pathogenic microbial organisms (Kayama & Takeda, 2012). In other words, microorganisms, defined as pathobionts, that usually are symbionts, can potentially become harmful and can overgrow only in determinate circumstances of altered genetic profile and defective immune system of the host promoting and enhancing IBD (Hold et al., 2014). In this context, the intestinal microbiota of patients suffering from IBD is enriched by pathobionts (Jochum & Stecher, 2020). For instance, Proteobacteria phylum, abundant in patients with this disorder, includes several pathobionts such as *Escherichia coli*, able to induce the production of pro-inflammatory cytokines and the release of the genotoxic compound colibactin, *Salmonella*, that disrupt the Th1/Th2 balance, *Klebsiella* species, capable of causing the progress of colitis, and *Pseudomonas* species, which cause epithelial damage after the release of its toxins (Nagao-Kitamoto & Kamada, 2017). Therefore, the interplay of the gut microbiota in the pathogenesis of IBD seems related to a mechanism of loss of tolerance towards several species belonging to the gut microbiota, even if several studies on animal models evidenced also a possible role of pathogens in the development and in the activity of this disease (Darfeuille-Michaud et al., 2004; Glassner et al., 2020; Rosenfeld & Bressler, 2010; Zhou et al., 2016).

Noteworthy, alterations of the composition of the gut microbiota leading to a reduced diversity and richness of bacterial species, referred as dysbiosis, occur in IBD even if it is not already clear if this status can be a cause or a consequence of the disease (Tamboli et al., 2004).

Several studies on both pediatric and adult patients with IBD evidenced a common increase in Proteobacteria phylum, especially *Enterobacteriaceae* family, in *Pasteurellaceae* and *Veillonellaceae* families, in *Fusobacterium*, *Streptococcus* and *Lactobacillus* genera and in *Ruminococcus gnavus* together with a reduction of bacterial species such as *Faecalibacterium prausnitzii*, *Blautia faecis*, *Akkermansia muciniphila* and of bacterial genera such as *Clostridium*, *Roseburia*, *Sutterella* and *Bifidobacterium* (Baldelli et al., 2021; Lewis et al., 2015; Lo Presti et al., 2019; Lucafo et al., 2020; Sartor & Wu, 2017; Schwiertz et al., 2010). These results were obtained examining fecal specimens and therefore they refer to the profile of luminal gut microbiota.

The profile of luminal gut microbiota differs from that of mucosal microbiota; unfortunately fewer studies refer to the latter because of the difficulties of obtaining biopsies since they request an invasive procedure (Lo Presti et al., 2019). According to Lo Presti and colleagues, the intestinal microflora populating the inflamed mucosa of IBD patients is enriched mainly in *Enterobacteriaceae* family; while *Rikenellaceae* and *Lachnospiraceae* families, *Bacteroides* and *Coprococcus* genera and the bacterial species *Parabacteroides distasonis* and *Faecalibacterium prausnitzii* are present in lower abundance in IBD patients compared to

healthy individuals (Lo Presti et al., 2019). Furthermore, *Pseudomonas* species are more represented in the mucosa of the ileum of pediatric patients with CD compared to healthy controls (Wagner et al., 2008).

Even if both CD and UC are related to dysbiosis, alteration of the gut microbial composition is more pronounced in CD patients and, interestingly, shifts in microbial composition seem to be involved in the course and in disease progression (Nishihara et al., 2021; Schirmer et al., 2018; Wills et al., 2014).

Variations in the composition of microorganisms cause unequivocally alterations of microbial metabolites in the gut (Heinken et al., 2021). For instance, since bacterial strains considered SCFAs producers, such as *Faecalibacterium prausnitzii* and *Roseburia* species, are reduced in IBD, these metabolites, especially butyrate, are expected to be present in lower concentrations in the gut of IBD patients (Ferrer-Picón et al., 2019). Moreover, according to Heinken and colleagues, the reduction of the bacterial richness in IBD determines also a lower diversity of secreted metabolites, especially those linked to sulphur metabolism; moreover, results of the functional and pathway-based analyses evidenced an increased potential to synthesize amino acids by Proteobacteria phylum (Heinken et al., 2021).

Besides bacterial dysbiosis, also alterations of the eukaryotic fungal microbiota, the mycobiome, and of the virus microbiota, the virobiota, are characteristic of IBD condition. In particular, fungi of *Candida* genus and *Caudovirales* bacteriophage families are more present in IBD patients compared to healthy individuals (Zuo & Ng, 2018).

1.5 Influence of gut microbiota and its metabolites on therapies

Growing evidence validates the role of gut microbiota in influencing the efficacy and toxicity of several drugs, although this research field is still quite underexplored because of the diversity of the gut microbiota and of the complexity of its relationship with the host.

Interestingly, the intestinal microflora can impact both on pharmacodynamics and pharmacokinetics of drugs even if, to date, the better reported effects refer to the latter and, more precisely, to metabolism (Saad et al., 2012). Indeed, after oral administration, several bacterial strains are able to interfere with the bioavailability of drugs internalizing and metabolizing drugs directly or indirectly by secretion of bacterial enzymes and metabolites able to modify the chemical structure of the compounds and by modulating the hepatic and intestinal expression and function of genes of the host (Björkholm et al., 2009; Clayton et al., 2009; Geller et al., 2017; Haiser et al., 2013; Oancea et al., 2017; Zimmermann et al., 2019). Activation, inactivation or detoxification are the modifications that drugs undergo through the gut microbial intervention (Zimmermann et al., 2019).

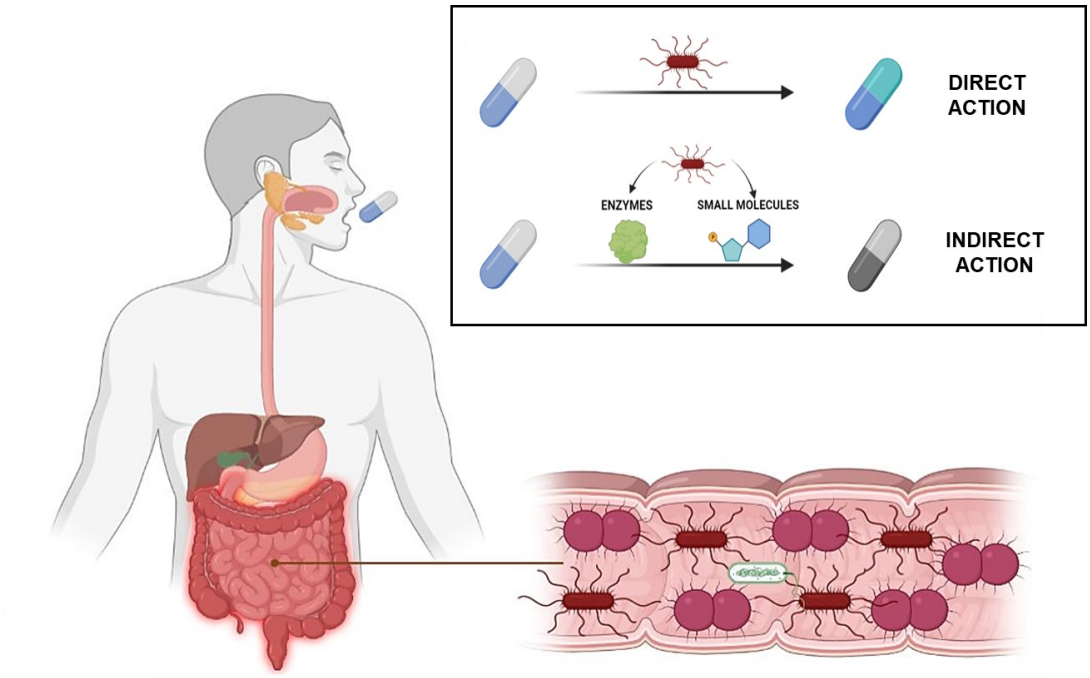

Figure 1.7 Main mechanisms through which the microbiota influences the efficacy of therapies. Readabted by (Franzin, Stefančič, et al., 2021).

Noteworthy, Zimmermann and colleagues highlighted the ability of 76 human gut bacterial strains to metabolize two thirds of the assayed commonly administered orally drugs (176/271) (Zimmermann et al., 2019). Another work by Geller et al. showed that, among intra-tumor Proteobacteria, bacterial species such as *Escherichia coli* and *Klebsiella pneumoniae*, that express the long isoform of the enzyme cytidine deaminase (CDD_L), can induce drug resistance and treatment failure inactivating the chemotherapeutic drug gemcitabine (Geller et al., 2017).

Furthermore, as previously mentioned, enzymes or metabolites released by gut bacteria are associated with drug resistance. Indeed, it is well known that bacterial secreted enzymes promote antibiotic resistance: microbial hydrolases, such as β-lactamases, catalyse the cleavage of β-lactam antibiotics inactivating penicillins, cephalosporins, carbapenems and monobactams; whereas, aminoglycoside antibiotics are substrate of nucleotidyltransferases, phosphotransferases and acetyltransferases (Liu et al., 2018). Enzymes can be secreted as they are or conveyed through bacterial extracellular vesicles (BEV) and thus BEV are associated to drug resistance (Kulkarni et al., 2015; Liao et al., 2015).

Furthermore, Clayton and colleagues described the role of the bacterial metabolite p-cresol, mainly produced by *Clostridium difficile*, in promoting O-sulfonation of acetaminophen and suggested its interplay in other reaction of O-sulfonation influencing the metabolism of other compounds such as minoxidil, tamoxifen and apomorphine (Clayton et al., 2009).

In this context, bacterial species belonging to gut microbiota could be involved, together with hosts' genetic diversity, in interindividual variability in response to treatment and further proofs of evidence could shed light on personalization of therapies on the basis of gut microbiota composition (Li et al., 2016).

1.5.1 Evidence on the influence of gut microbiota on thiopurines

The role of bacterial strains representative of the gut microbiota of IBD patients in mediating the efficacy and toxicity of thiopurines is still an unexplored field, even if the limited evidence available on this topic seems to indicate that thiopurines, maybe thanks to their analogy with purines, may be metabolized by intestinal bacteria or at least may be substrates of bacterial enzymes.

Interestingly, GST, previously described as the enzyme catalysing the conversion of AZA to MP and S-methyl-4-nitro-5-thioimidazole, results to be distributed widely among members of Proteobacteria, commonly increased in the gut microbiota of IBD patients (Vuilleumier & Pagni, 2002).

Furthermore, the bacterial orthologue of the human TPMT, previously described as the enzyme responsible for the methylation of thiopurines both in their free base and nucleotide form, was first isolated from *Pseudomonas syringae* (Cournoyer et al., 1998). Early reports evidenced also TPMT activity in *Pseudomonas aeruginosa*, *fluorescens* and *ovalis* (Krynetski & Evans, 2003). Indeed, bacterial TPMT is known to be involved in environmental detoxification processes as it methylates organic forms of selenium and provides resistance to the bactericidal compound tellurite (Krynetski & Evans, 2003; Ranjard et al., 2003).

The bacterial metabolism of TG was first investigated *in vitro* by Oancea and colleagues: the TGN metabolites were detected in bacterial isolates of *Escherichia coli*, *Bacteroides thetaiotaomicron* and *Enterococcus faecalis* after the exposure of their log phase cultures to TG. Also, metabolites are found after incubation of faecal samples of *Hprt*$^{-/-}$ mice with TG evidencing a bacterial biotransformation (Oancea et al., 2017).

Lastly, although in this case the metabolism by these bacterial strains has not been proved, Lazarević and colleagues proposed several bacterial species as possible candidates in thiopurines biotransformation on the basis of the research of enzymes involved in the pathway of thiopurine drugs (Lazarević et al., 2022). Potential metabolizer of thiopurines are *Enterobacter cloacae*, *Campylobacter concisus*, *Enterococcus faecalis*, *Bacillus subtilis*, *Bacteroides fragilis* and *vulgatus* (Lazarević et al., 2022).

2. AIMS

Alterations of the composition of the gut microbiota leading to a reduced diversity of bacterial species, referred as dysbiosis, occur in IBD even if it is not already clear if this dysbiosis is a cause or a consequence of the disease. In particular, bacterial species belonging to *Enterobacteriaceae* family and to *Pseudomonas* genus proliferate abundantly at the expense of other microorganisms in the intestinal microflora of pediatric IBD patients.

Thiopurines, such as mercaptopurine (MP), its prodrug azathioprine (AZA) and thioguanine (TG) are immunosuppressive drugs widely used in the maintenance of remission of IBD and, despite their proven efficacy, some patients do not achieve satisfying therapeutic effects and develop adverse reactions.

There is growing evidence of the influence of intestinal bacteria in influencing the efficacy of therapies: the gut microbiota can impact both on pharmacodynamics and pharmacokinetics even if, to date, the better reported effects refer to the latter and, more precisely, to metabolism. Indeed, several bacterial strains are able to interfere with the availability of drugs through internalization and metabolism of drugs; these modifications of drug pharmacokinetics can occur directly or indirectly by secretion of bacterial enzymes and metabolites able to modify the chemical structure of the compounds.

In this context, the aim of the project was to investigate the role of candidate bacterial strains representative of the gut microbiota of pediatric IBD patients, in particular *Escherichia coli*, *Salmonella enterica*, *Klebsiella pneumoniae* (*Enterobacteriaceae* family) and *Pseudomonas aeruginosa* (*Pseudomonas* genus), in mediating *in vitro* the cytotoxic effects of thiopurines.

In order to achieve the purpose of the project, the main objectives were:

- ✓ to evaluate the *in vitro* cytotoxic effects of a 72 hours treatment with thiopurines previously exposed to bacteria and their growth phase broths (GPBs) on immortalized cell lines;
- ✓ to measure thiopurines after their exposure to bacteria and their GPBs;
- ✓ to quantify thiopurine metabolites in lysates of cells treated with the drugs previously exposed or not to *K. pneumoniae* and *P. aeruginosa*;
- ✓ to evaluate the presence of thiopurines in bacteria after their exposure to drugs.

So far, no studies have explored the role of intestinal microbiota in interfering with the cytotoxicity of thiopurines. This project could allow interesting information on the influence of these bacterial strains, that usually adhere to mucosa and representative of the gut microbiota of IBD patients, on thiopurine drugs without an invasive approach and, together with subsequent studies on the luminal intestinal mucosa, would provide a new proof of evidence in thiopurine treatment in order to achieve a personalized therapy.

3. MATERIALS AND METHODS

3.1 Bacterial strains and culture conditions

E. coli ATCC 25922*, K. pneumoniae* ATCC 13883, *P. aeruginosa* ATCC 27853 and a clinical isolate of *S. enterica* were grown in Luria Broth (LB) at 37 °C under aerobic conditions, according to the microbiology laboratory standard procedures.

3.2 Antimicrobial activity test

Susceptibility of microorganisms to the thiopurines AZA, MP, TG, was preliminary evaluated by the "Broth Microdilution Susceptibility Test". Methylprednisolone (MTPD) and methotrexate (MTX) (Sigma-Aldrich, Milan, Italy), drugs used for the treatment of IBD were also tested. According to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (Weinstein, 2018) the test was performed in Mueller-Hinton broth in 96-well round-bottom microtiter plates on a final inoculum of 1-5 × 10^5 CFU/mL (CFU: colony forming units). Each plate included positive controls (bacterial strain without drugs), negative controls (medium only), and serial fourfold dilutions of each drug, ranging from 50 to 400 µM. The minimum inhibitory concentration (MIC) values were considered as the lowest concentration of drugs resulting in the complete inhibition of visible growth after 24 hours of incubation at 37 °C.

3.3 Exopolysaccharide (EPS) extraction

Fifty µL of dilution 1:100 of the overnight culture of *K. pneumoniae* was grown on cystine-lactose-electrolyte-deficient agar (CLED agar) for 3 days at 30 °C. Upon the formation of a thick patina, bacteria were scraped from the surface, collected into a tube and sent to the collaborating laboratory of molecular biomedicine of prof. Cescutti of University of Trieste in order to extract and purify the exopolysaccharide (EPS) as previously described (Cescutti et al., 2016).

3.4 *In vitro* exposure of drugs to bacterial strains, to their growth phase broths (GPBs) and to *K. pneumoniae* exopolysaccharide (EPS)

Bacterial strains grown overnight in LB were diluted 1:50 (*E. coli*, *S. enterica* and *K. pneumoniae*) or 1:10 (*P. aeruginosa*) in fresh LB, incubated at 37°C with shaking for about 2 (*E. coli*, *S. enterica* and *K. pneumoniae*) or 3 (*P. aeruginosa*) hours and diluted in Minimal Salts Medium M9 (Sambrook, 1989) at a final concentration of 10^7 CFU/mL. AZA, MP and TG (400 µM) were added to each bacterial suspension or to their growth phase broths (GPBs), obtained filtering bacteria from their log phase culture diluted in M9, for 4 h (*E. coli*, *S. enterica* and *K. pneumoniae*) or for 15 h (*P. aeruginosa*) at 37 °C.

MTPD was also exposed to *E. coli*, *S. enterica* and *K. pneumoniae* and MTX was incubated with *P. aeruginosa* as described above.

In addition, thiopurines were incubated with three serial dilutions (3.75 - 15 - 60 µg/mL) of *K. pneumoniae* EPS*,* extracted and purified as previously described (Cescutti et al., 2016). The range of concentration of EPS for the *in vitro* exposure were chosen on the basis of the ones estimated to be produced by microorganisms during incubation. At the end of the *in vitro* exposure, all samples were filtered and stored at – 80°C until their use for the tests indicated below.

3.5 Immortalized cell lines and cell culture

NALM6 B and JURKAT T cell lines were cultured in RPMI 1640 medium (Euroclone, Milan, Italy) containing 10% fetal bovine serum (FBS) (Sigma-Aldrich, Milan, Italy), 1% L-glutamine 200 mM (Euroclone, Milan, Italy), 1% penicillin 10000 UI/mL (Euroclone, Milan, Italy) and streptomycin 10 mg/mL (Euroclone, Milan, Italy) and incubated in a humidified atmosphere at 37 °C with 5% CO_2; cell passage was performed twice a week. Cell lines' authentication was performed by DNA fingerprinting analysis.

3.6 MTT cytotoxicity test

To compare the effects of the tested drugs incubated or not with bacteria, with their GPBs and with *K. pneumoniae* EPS, the cytotoxicity on cell lines, exposed to the treatments, was determined using a 3-(4,5-dimethyl-2thiazolyl) -2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay.

NALM6 and JURKAT cells were seeded at a density of 2×10^4 or 1×10^4 cells/well respectively in 96-well plates. Seven serial dilutions of thiopurines (ranging from 0.2 to 15 μM of AZA, from 0.3 to 20 μM of MP and from 0.08 to 5 μM of TG) exposed or not to bacteria or to GPBs were added to each well and incubated for 72 h. The cytotoxicity of thiopurines exposed to increasing concentrations of *K. pneumoniae* EPS on NALM6 and JURKAT cells was also evaluated with the same procedure and conditions described above.

In order to evaluate the specificity of the variation of cytotoxic effects of thiopurines, six serial dilutions of MTPD (ranging from 2×10^{-4} to 4 μM) exposed or not to *E. coli*, *S. enterica*, *K. pneumoniae* and their GPBs were used to treat NALM6 cells and seven serial dilutions of MTX (ranging from 4.4×10^{-3} to 5×10^{-2} μM) exposed or not to *P. aeruginosa* and its GPB were used to treat NALM6 and JURKAT cells as described above.

Viability was determined by adding 20 μL of MTT solution (5 mg/mL in phosphate buffered saline) (Sigma-Aldrich, Milan, Italy). Living cells reduce the yellow MTT to a blue formazan product. After 4 h of incubation at 37 °C, the formazan product was dissolved in 100 μL dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Milan, Italy) and the plates were read at 540 and 630 nm using the Automated Microplate Reader EL311 (BioTek Instruments, Vermont, USA). The percentage of cell viability was expressed as a ratio versus control.

3.7 Spectrophotometric analysis

To determine the concentration of thiopurines exposed or not to the bacterial strains, to their GPBs or to *K. pneumoniae* EPS and the concentration of MTX exposed or not to *P. aeruginosa*, absorbance peaks were analysed by UV spectrophotometry (280 nm for AZA, 320 nm for MP, 340 nm for TG and 370 nm for MTX) using UV-6300PC Double Beam Spectrophotometer (VWR, Milan, Italy). Calibration curves of each compound were done in order to quantify the amount of drugs in the samples.

3.8 Samples' preparation for the measurements of thiopurine metabolites in lysates and of thiodeoxyguanosine (dTGUA) in DNA of NALM6 and JURKAT cells

Three million NALM6 cells and 1.5×10^6 JURKAT cells were seeded in 50 mL tubes and treated with the most significant concentrations derived from MTT tests of AZA (15 μM), MP (2.5 μM) and TG (1.25 μM) exposed or not to *K. pneumoniae*. Timing of treatment depended on the type of experiment: for the measurements of thiopurine metabolites, cells were treated for 48 and 72 hours, while quantification of thiodeoxyguanosine (dTGUA) in DNA required a 72 hour-treatment. Also, NALM6 and JURKAT cells were seeded at the same cellular density mentioned before and treated for 72 hours with the most significant concentrations derived from MTT tests of MP (2.5 μM) and TG (2.5 and 1.25 μM) exposed or not to *P. aeruginosa*. At the end of the incubation, cells were counted by trypan blue dye exclusion assay. After centrifugation at 600 x g for 5 minutes at room temperature, the supernatant was removed and dried pellets were collected and stored at – 80 °C until the subsequent analyses.

3.9 Quantification of thiopurine metabolites by LC-MS/MS

The dried cellular pellets were added to a mixture of 250 μL of EDTA 50 mM, 15 μL of dithiothreitol solution 30 mg/mL and 10 μL of internal standard working solution (20 pmol/μL [$^2H_3$]MeTGMP, 60 pmol/μL [$^2H_3$]MeTGDP/ [$^2H_3$]MeTGTP, 100 pmol/μL [$^2H_3$]MeTIMP, 160 pmol/μL [$^2H_3$]MeTIDP/ [$^2H_3$]MeTITP, 40 pmol/μL [$^2H_4$]TGMP, 80 pmol/μL [$^2H_4$]TGTP/ [$^2H_4$]TGDP) (obtained by chemical synthesis as previously reported (Hofmann et al., 2012)), and vortex mixed. Proteins were denatured by heating for 5 min at 95 °C.

All samples were subsequently extracted by addition of 50 µL of methanol followed by the addition of 250 µL of dichloromethane with thorough mixing after each step. After centrifugation at 16100 x g for 20 min, 10 µL of the supernatant were used for liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis as described previously (Hofmann et al., 2012) on an Agilent 6495B triple quadrupole mass spectrometer (Agilent, Waldbronn, Germany) coupled to an Agilent 1290 Infinity II HPLC system with electrospray ionization mode in positive polarity. Chromatographic separation was carried out on a Biobasic AX column (2.1 × 50 mm, 5 µm particle size, Thermo Electron, Egelsbach, Germany) using water:acetonitrile 7:3 (v/v), with 10 mM ammonium acetate at pH 6 as mobile phase A and with 1 mM ammonium acetate at pH 10.5 as mobile phase B. The metabolites detected were TGMP (thioguanosine monophosphate), TGDP (thioguanosine diphosphate), TGTP (thioguanosine triphosphate), MeTGMP (methylthioguanosine monophosphate), MeTGDP (methylthioguanosine diphosphate), MeTGTP (methylthioguanosine triphosphate), TIMP (thioinosine monophosphate), TIDP (thioinosine diphosphate), TITP (thioinosine triphosphate), MeTIMP (methylthioinosine monophosphate), MeTIDP (methylthioinosine diphosphate) and MeTITP (methylthioinosine triphosphate). Metabolites' concentrations were expressed as pmol on million cells (pmol/Mio).

3.10 Quantification of dTGUA in DNA by LC-MS/MS

DNA was extracted from cellular pellets of NALM6 and JURKAT cells treated with thiopurines exposed previously or not to *K. pneumoniae* using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) and subsequently 1 µg of genomic DNA was digested with 15 U Degradase PlusTM (Zymo Research) for 16 h in a total volume of 30 µl. Samples were spiked with 6 µl of internal standard solution (4.2 pmol/µl [2H4]6-thioguanosine in water:acetonitrile 1:1 v/v) (obtained by chemical synthesis as previously reported(Hofmann et al., 2012)), mixed and centrifuged. Ten µl of the supernatant were used for LC-MS-MS analysis with a previously described method (Atreya et al., 2016) adapted on an Agilent 6495B triple quadrupole mass spectrometer (Agilent, Waldbronn, Germany) coupled to an Agilent 1290 Infinity II HPLC system with electrospray ionization mode in positive polarity. HPLC separation was achieved on a Poroshell 120 SB-C8 column (150 × 2.1 mm, 3 µm particle size, Agilent) using (A) 0.0075% formic acid in water and (B) 0.0075% formic acid in acetonitrile as mobile phases at a flow rate of 0.4 ml/min. Gradient runs started at 3.5% B, followed by linear increase to 5 % B from 2 to 6.9 min, increase to 80 % B to 7.2 min, remaining at 80 % B to 9 min, then re-equilibration. The mass spectrometer was operated in the multiple reaction monitoring (MRM) mode using the precursor/product ion pairs of *m/z* 284.1/168.1 and *m/z* 284.1/150.9 as quantifier and qualifier for dTGUA and *m/z* 304.1/168 for the internal standard [2H4]6-thioguanosine. Calibration samples were prepared using dTGUA (Selleck Chemicals, Planegg, Germany) in hydrolysis buffer in the concentration range from 0.1 to 25 pmol/sample. Calibration curves based on internal standard calibration were obtained by weighted (1/x) linear regression for the peak-area ratio of the analyte to the internal standard against the amount of the analyte. The concentration of the analyte in unknown samples was obtained from the regression line. Assay accuracy and precision were determined by analyzing quality controls that were prepared like the calibration samples. Values were expressed as percentage with respect to the control, i.e., considering the values of dTGUA in DNA of cells treated with drugs in M9 as 100%.

3.11 Sample's preparation and measurements of TPMT activity

In order to investigate the influence on TPMT activity of bacterial compounds released by *K. pneumoniae* during its growth (conditioned media), 5 x 10^6 NALM6 and 2.5 x 10^6 JURKAT cells were seeded in T175 flasks and exposed to RPMI medium (for the investigation of basal conditions), to M9 medium and to the *K. pneumoniae* conditioned media used in the previous tests. At the end of 72 hours of incubation, cells were counted by trypan blue dye exclusion assay. After centrifugation at 600 x g for 5 minutes at room temperature and removal of the supernatant, dried pellets were collected and stored at – 80 °C for high performance liquid chromatography coupled with UV detector (HPLC-UV) analyses. Subsequently, dried pellets were thawed, resuspended in phosphate buffer and frozen again at – 80 °C in order to enhance lysis of cells. On the day of the analysis, samples were thawed and sonicated in a water bath for 20 minutes to

achieve the complete lysis; then they were processed as previously described (Schaeffeler et al., 2004) and 25 µL were used for measurements of TPMT activity with the already established method (Schaeffeler et al., 2004). In detail, the method is based on the conversion of TG in methylthioguanine (MeTG). Measurements were further normalized to the protein concentration of the samples. Therefore, values were expressed as pmol of MeTG produced per hour normalized on µg of proteins.

3.12 Detection of thiopurines in *K. pneumoniae* pellets by HPLC-UV

Samples containing *K. pneumoniae* exposed or not to thiopurines were diluted 1:10 in fresh M9 and centrifuged at 12000 x g for 20 minutes at 4 °C to obtain bacterial pellets. Afterwards, bacteria were washed with phosphate buffered saline, centrifuged again at 12000 x g for 5 minutes at 4 °C and collected as dried bacterial pellets until HPLC-UV analysis. On the day of the analysis, bacterial pellets were lysed adding 100 µL of solution of acetonitrile and methanol (1:1) (Sigma-Aldrich, Milan, Italy) and incubating at -20 °C for 1.5 hours. Subsequently, bacterial debris were removed by centrifugation at 12000 x g for 20 minutes at 4 °C and the supernatant was placed under a nitrogen stream. Finally, the dried analyte was resuspended in MilliQ water (Merck Millipore, Milan, Italy) and 10 µL were injected in the instrument for the detection of AZA, MP and TG in *K. pneumoniae* pellets. Separation of the compounds of interest was achieved using a reverse-phase column Poroshell 120 SB-C8 (2.1 × 150 mm, 2.7 µm, Agilent Technologies, Milan, Italy). The mobile phases consisted of 0.0075% formic acid (Sigma-Aldrich, Milan, Italy) in water (eluent A) and 0.0075% formic acid in acetonitrile (eluent B) and the separation was performed using a gradient at a flow rate of 0.4 mL/min for 15 minutes. In particular, at time 0 the flow consisted in 100% eluent A that first linearly decreased at 96.5% (after 2 minutes) and then at 20% (after 7 minutes) until 10 minutes. The last 5 minutes, the column was reconditioned before the next injection. The column temperature was set at 30 °C. Regarding the spectrophotometric conditions, the detection wavelengths were set up at 280 nm, 320 nm and 340 nm (the peaks of absorbance of AZA, MP and TG respectively). Calibration curves were constructed in water by preparation a series of dilutions of the drugs (1, 2.5, 5, 10, 15 and 20 µM) after assessing that there was no signal interference of the matrix in the retention times of the analytes.

3.13 Detection of purines in *K. pneumoniae* GPB by HPLC-UV

Detection of purines guanine (G) and hypoxanthine (HP), analogous of thiopurines, was performed in samples containing *K. pneumoniae* GPB. Ten µL of pure sample was injected in the instrument. Chromatographic separation was achieved using the same conditions used for the detection of thiopurines in *K. pneumoniae* pellets by HPLC-UV using the reverse-phase column Poroshell 120 SB-C8 (2.1 × 150 mm, 2.7 µm, Agilent Technologies, Milan, Italy) (see paragraph above). Regarding the spectrophotometric conditions, the detection wavelengths were set up at 240 nm and 260 nm (the peaks of absorbance of G and HP respectively). Calibration curves were constructed in water by preparation a series of dilutions of the drugs (5, 10, 15, 20, 30 and 40 µM) after assessing that there is no signal interference of the matrix in the retention times of the analytes.

3.14 Metabolomic analysis of *K. pneumoniae* conditioned media

Metabolomic analyses were also performed on *K. pneumoniae* conditioned media derived from the *in vitro* exposures. In particular, preparation of the samples, LC-MS and LC-MS/MS analysis were readapted from a previously described method (Zimmermann et al., 2019). Chromatographic separation was achieved using the same conditions used for the detection of thiopurines in *K. pneumoniae* pellets by HPLC-UV using the reverse-phase column Poroshell 120 SB-C8 (2.1 × 150 mm, 2.7 µm, Agilent Technologies, Milan, Italy) (see paragraph above).

3.15 Statistical analysis

Statistical analyses were carried out using GraphPad Prism software (version 8). In particular, statistical significance was assessed by two-way ANOVA and Bonferroni's post-test for cytotoxicity tests. Welch's t test was carried out for UV analyses, for measurements of dTGUA in DNA and for TPMT activity. All the results are presented as mean ± standard error (SE) from 3 independent experiments. The significance threshold was set at 0.05.

Statistical analyses regarding the measurements of thiopurine metabolites were performed within R software (version 3.2.4). Data were analysed by fitting analysis of variance (ANOVA) models (aov function of the stats package), considering each metabolite concentration as the dependent variable and exposure time, drug used and the experimental condition (exposure of drugs to M9 or to *K. pneumoniae*) as independent variables (corresponding to a two-way ANOVA on a model: metabolite concentration ~ exposure time * drug used * condition). The significance threshold was set at 0.05. Furthermore, Welch's t test was carried out for comparing the total metabolite content of the experimental conditions.

Regarding metabolomics analyses, the MassHunter Qualitative Analysis Software (Agilent) with standard parameters were used for untargeted feature extraction. The MassHunter NIST 14 LC/MS/MS PCDL (Agilent) was used to recognise the compounds thanks to comparison between LC-MS/MS spectra. The abundance of compounds in the experimental conditions (*K. pneumoniae* exposed or not to AZA, MP or TG) were analysed through Welch's t test using R software (version 3.2.4) setting the significance threshold at 0.05. The data represented derived from 4 independent experiments.

4. RESULTS

4.1 Role of *K. pneumoniae* in mediating the *in vitro* effects of thiopurines

4.1.1 Bacterial susceptibility to drugs

The Broth Microdilution Susceptibility Test showed that even the highest concentration tested (400 µM) of AZA, MP, TG and MTPD did not affect the growth of *E. coli*, *S. enterica* and *K. pneumoniae.* Thus, 400 µM of each drug was chosen for the subsequent *in vitro* experiments.

4.1.2 Cytotoxicity of thiopurines exposed to candidate bacterial strains

MTT tests were performed in order to compare the cytotoxicity of thiopurines exposed or not to bacteria and thus evaluate a potential microbial biotransformation.

Cytotoxic effects on NALM6 and JURKAT cells after a 72-hour treatment with AZA, MP and TG exposed or not to *E. coli* and *S. enterica* are shown in **Figure 4.1 – 4.4**. ANOVA did not demonstrate a statistically significant difference.

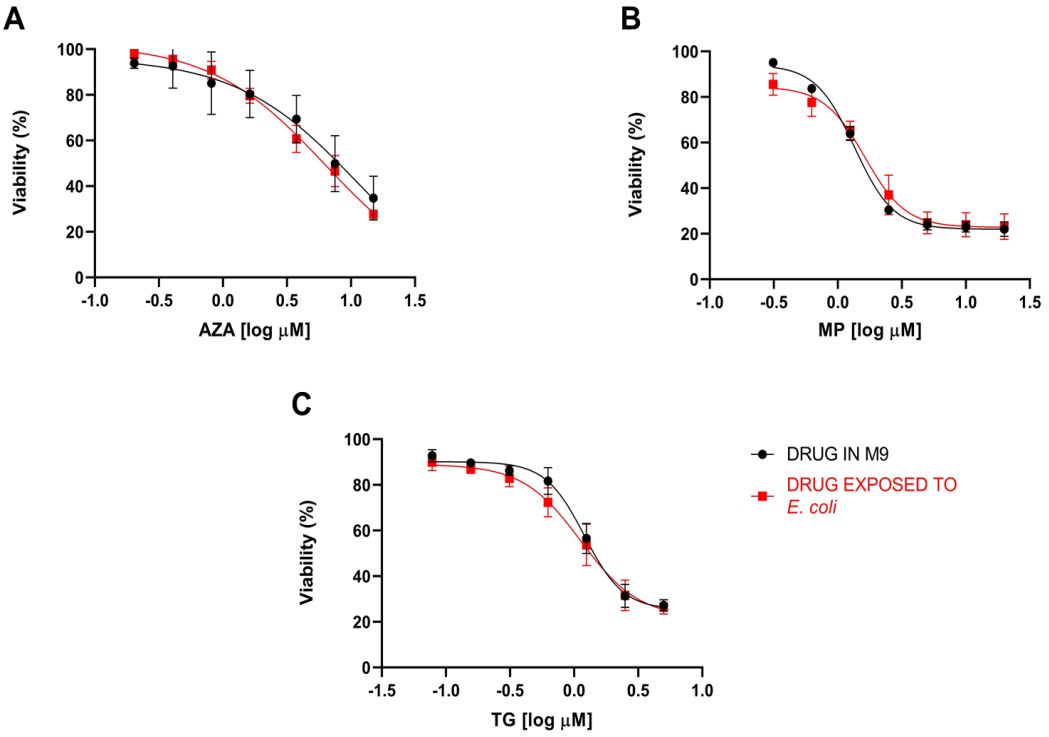

Figure 4.1. Evaluation of cytotoxic effects through MTT assay on NALM6 cells after treatment with several concentrations of AZA (**A**), MP (**B**) and TG (**C**) exposed to *E. coli* or not. Two-way ANOVA (drugs in M9 vs drugs exposed to *E. coli* ns).

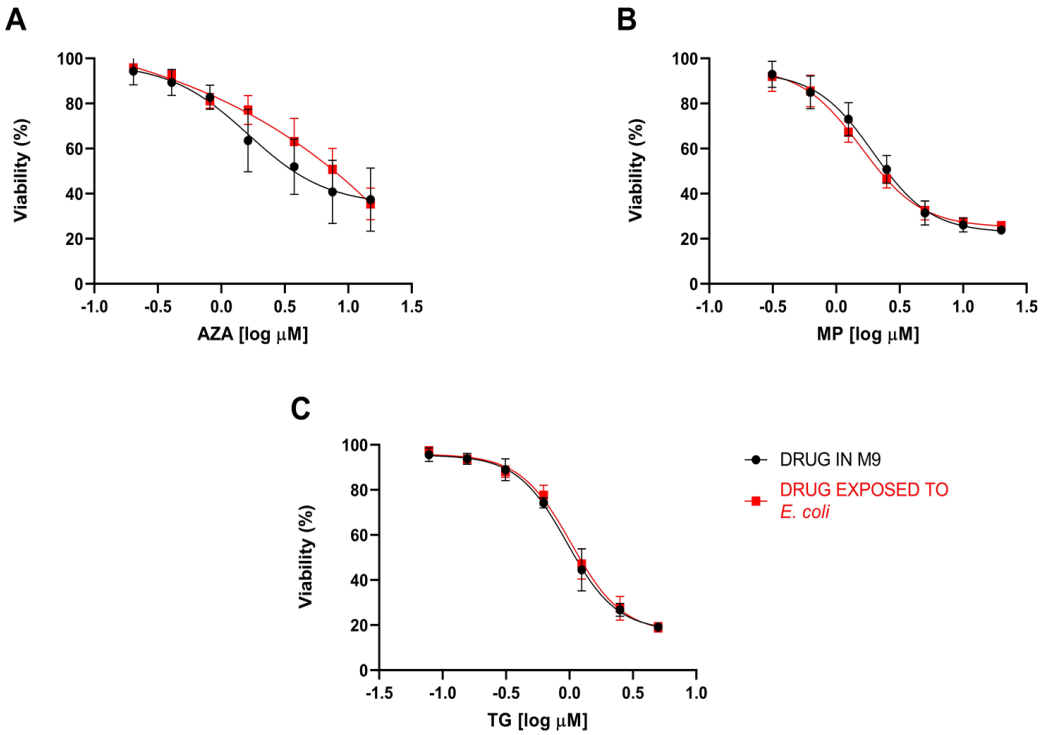

Figure 4.2. Evaluation of cytotoxic effects through MTT assay on JURKAT cells after treatment with several concentrations of AZA (**A**), MP (**B**) and TG (**C**) exposed to *E. coli* or not. Two-way ANOVA (drugs in M9 vs drugs exposed to *E. coli* ns).

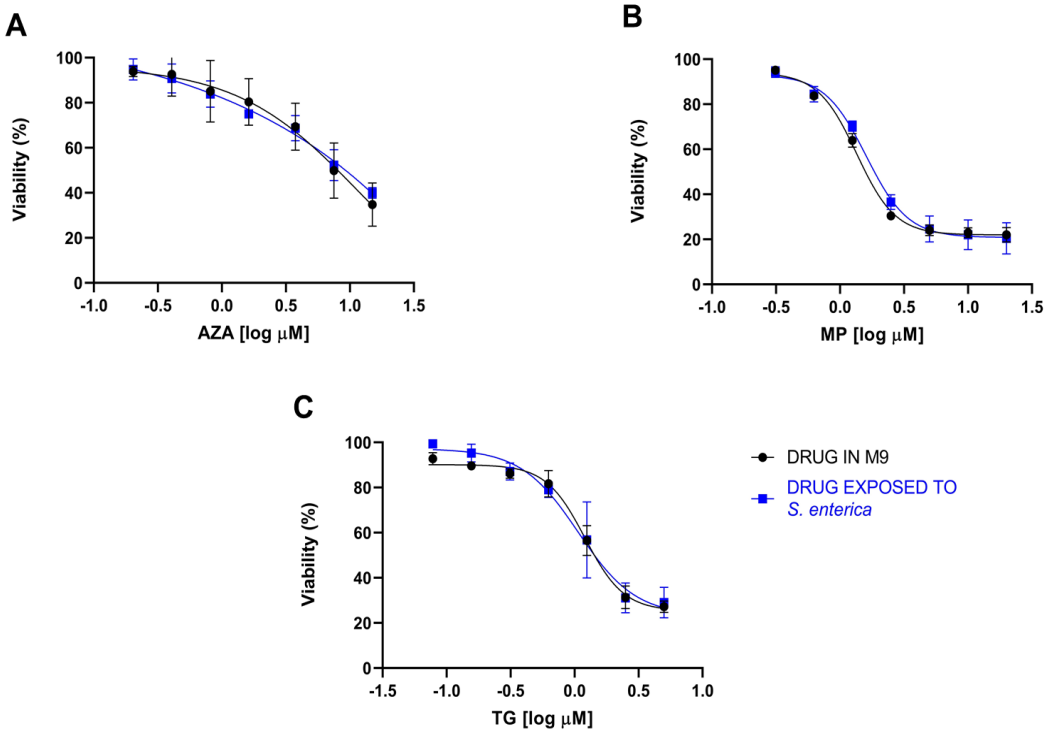

Figure 4.3. Evaluation of cytotoxic effects through MTT assay on NALM6 cells after treatment with several concentration of AZA (**A**), MP (**B**) and TG (**C**) exposed to *S. enterica* or not. Two-way ANOVA (drugs in M9 vs drugs exposed to *S. enterica* ns).

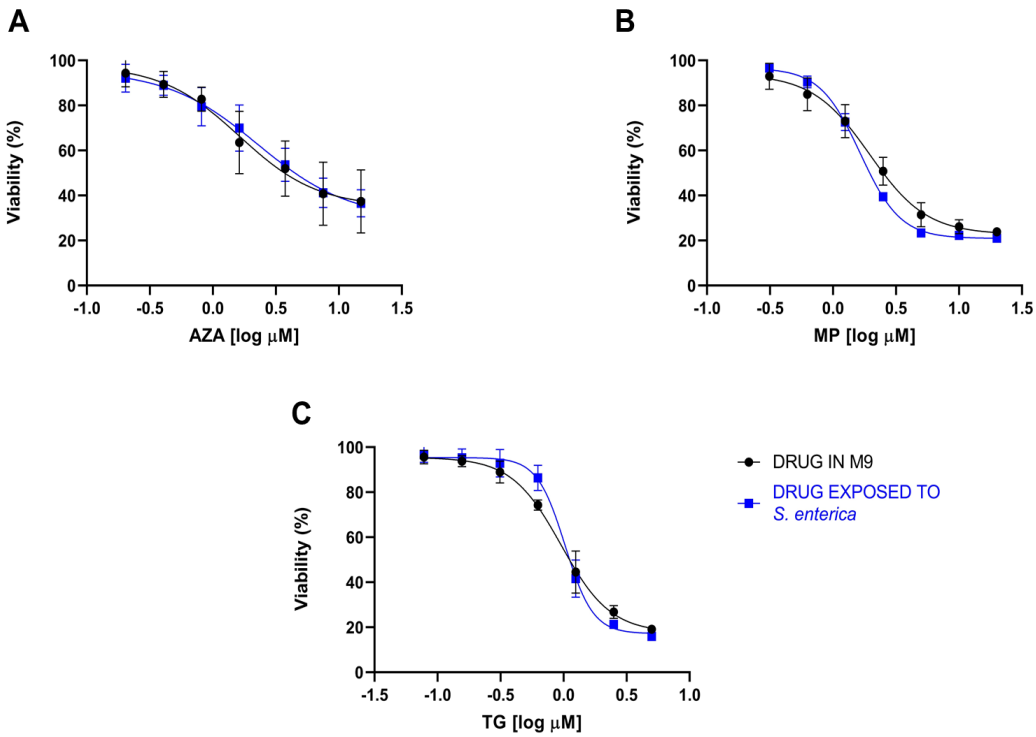

Figure 4.4. Evaluation of cytotoxic effects through MTT assay on JURKAT cells after treatment with several concentrations of AZA (**A**), MP (**B**) and TG (**C**) exposed to *S. enterica* or not. Two-way ANOVA (drugs in M9 vs drugs exposed to *S. enterica* ns).

Interestingly, the cytotoxic effects on NALM6 B cells (**Figure 4.5 A-B-C**) after a treatment of 72 hours with AZA, MP and TG were significantly lower after incubation with *K. pneumoniae* compared to the drugs not exposed to bacteria. In particular, at the highest concentration of AZA (15 µM) and at concentrations higher than 1.25 µM of MP and of TG, a significant difference between the toxicity of drugs exposed or not to the bacterial strain was observed (ANOVA with Bonferroni's post-test).

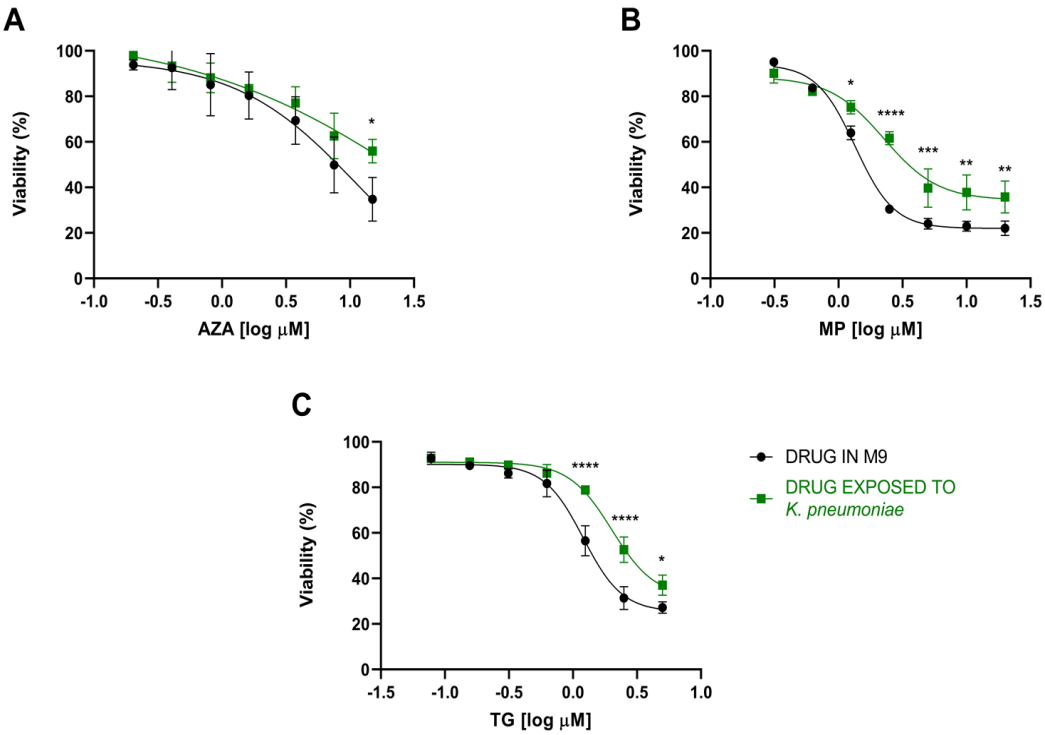

Figure 4.5. Evaluation of cytotoxic effects through MTT assay on NALM6 cells after treatment with several concentration of AZA (**A**), MP (**B**) and TG (**C**) exposed to *K. pneumoniae* or not. Two-way ANOVA (drugs in M9 vs drugs exposed to *K. pneumoniae* $p<0.01$, $p<0.0001$, $p<0.0001$ respectively). Bonferroni post-test (* = $p<0.05$ ** = $p<0.01$ *** = $p<0.001$ **** $p<0.0001$).

As shown in **Figure 4.6 B-C**, the cytotoxic effects on JURKAT cells after a 72-hour treatment with MP and TG were significantly reduced when the thiopurines were preincubated with *K. pneumoniae*. In particular, ranging from 1.25 to 2.5 μM for MP and from 0.6 to 1.25 μM for TG, a significant difference between drugs in M9 and the ones exposed to the bacterial strain was observed (ANOVA with Bonferroni's post-test). Furthermore, ANOVA evidenced a lower cytotoxicity after the exposure of AZA to the bacterial strain, even if Bonferroni's post-test did not underline a statistically significant difference at the different concentrations tested (**Figure 4.6 A**).

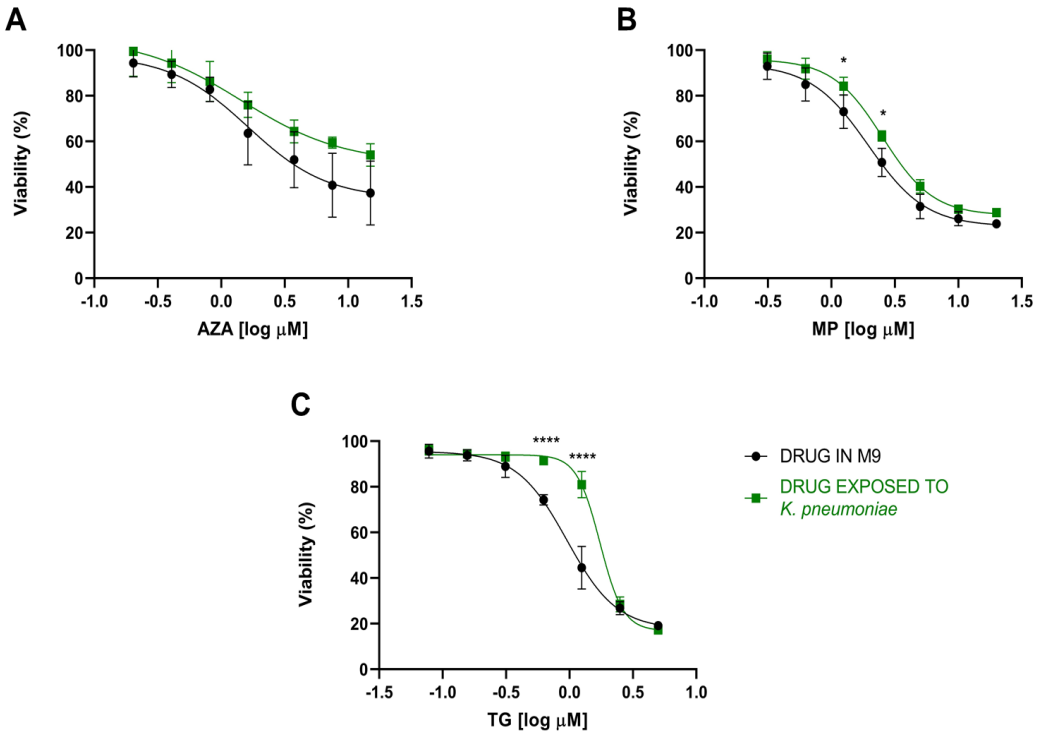

Figure 4.6. Evaluation of cytotoxic effects through MTT assay on JURKAT cells after treatment with several concentrations of AZA (**A**), MP (**B**) and TG (**C**) exposed to *K. pneumoniae* or not. Two-way ANOVA (drugs in M9 vs drugs exposed to *K. pneumoniae* $p<0.001$, $p<0.0001$, $p<0.0001$ respectively). Bonferroni post-test (* = $p<0.05$ **** = $p<0.0001$).

4.1.3 UV analysis evidenced a reduction of the concentration of thiopurines only after the exposure to *K. pneumoniae*

In order to identify variations in the concentration of thiopurines after the exposure to the bacterial strains, UV spectrophotometry analysis was performed.

UV analysis did not show a variation of concentrations of thiopurines exposed to *E. coli* and *S. enterica* (respectively **Figure 4.7 and 4.8**) in comparison with drugs in M9.

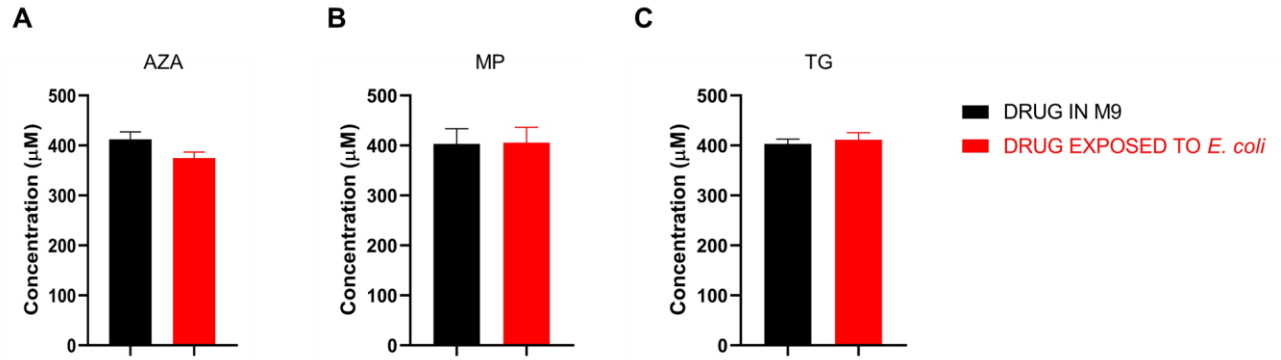

Figure 4.7. Concentration of AZA (**A**), MP (**B**) and TG (**C**) (means ± SE) exposed to *E. coli* or not. Welch's t-test ns.

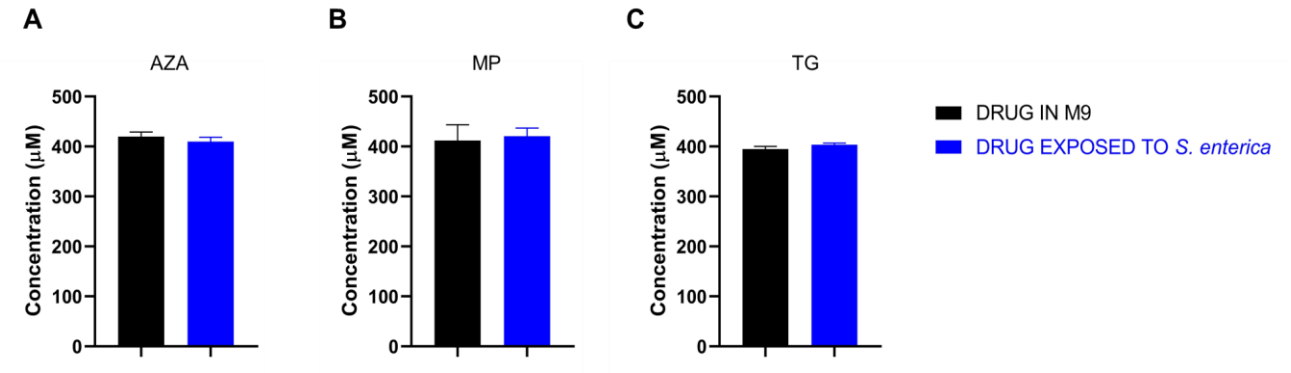

Figure 4.8. Concentration of AZA (**A**), MP (**B**) and TG (**C**) (means ± SE) exposed to *S. enterica* or not. Welch's t-test ns.

Contrary to what was observed for the other bacterial strains, after incubation of 400 µM of thiopurines to *K. pneumoniae*, the concentration of AZA, MP and TG significantly decreased compared to the drug in M9 (**Figure 4.9 A-B-C**). In particular, the reduction of the concentration in bacterial conditioned media was of 115.56 ± 20.76 µM for AZA, 110.56 ± 8.25 µM for MP and 116.10 ± 18.35 µM for TG.

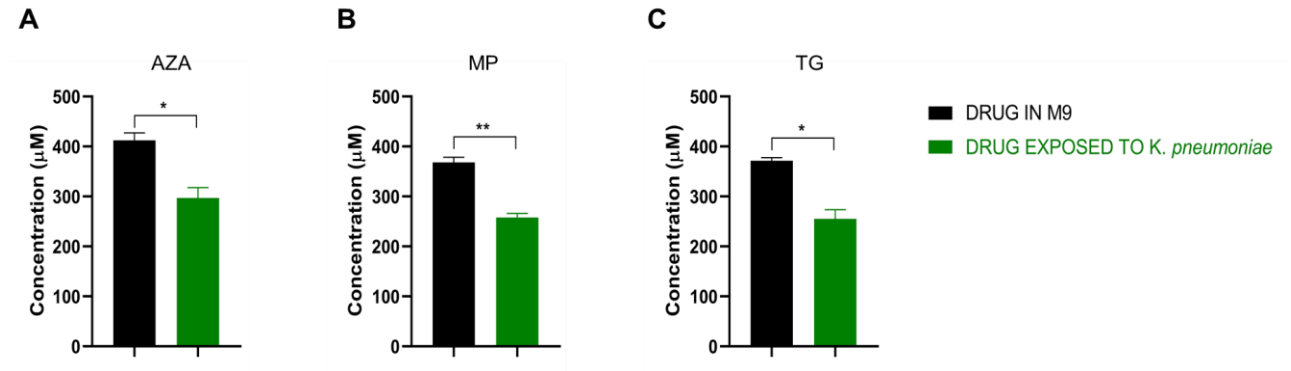

Figure 4.9. Concentration of AZA (**A**), MP (**B**) and TG (**C**) (means ± SE) exposed to *K. pneumoniae* or not. Welch's t-test (* $p<0.05$ ** $p<0.01$).

4.1.4 Effects on cytotoxicity of methylprednisolone exposed to *K. pneumoniae*, *E. coli* and *S. enterica*

Figure 4.10 A-B-C shows the cytotoxic effects on NALM6 cells after treatment of a 72-hour with methylprednisolone exposed or not to *K. pneumoniae*, *E. coli* and *S. enterica*. ANOVA did not underline any significant difference.

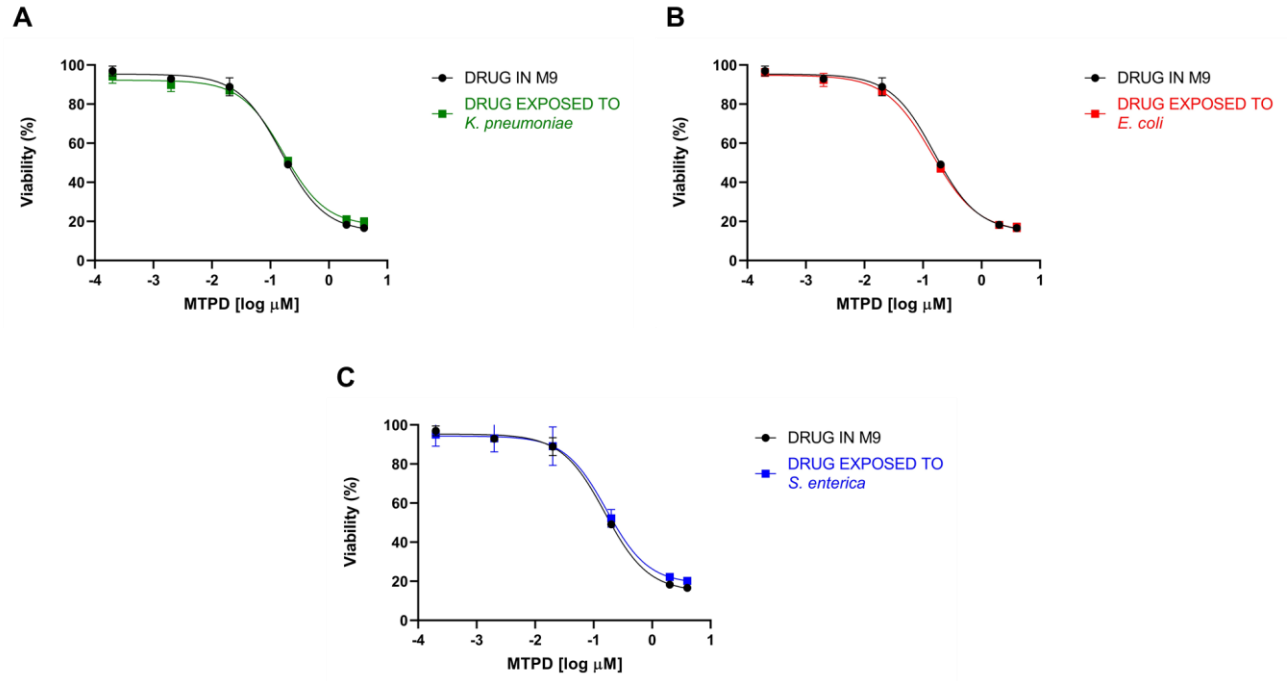

Figure 4.10. Evaluation of cytotoxic effects through MTT assay on NALM6 cells after treatment with several concentrations of methylprednisolone (MTPD) exposed or not to *K. pneumoniae* (**A**), to *E. coli* (**B**) and to *S. enterica* (**C**). Two-way ANOVA (drug in M9 vs drug exposed to bacteria ns).

4.1.5 Thiopurine metabolites were lower in NALM6 and JURKAT cells treated with drugs previously exposed to *K. pneumoniae*

Thiopurine metabolites were measured in NALM6 and JURKAT cells treated with AZA (15 µM), MP (2.5 µM) and TG (1.25 µM) exposed or not to *K. pneumoniae*. The concentrations tested were chosen on the basis of statistical significance of Bonferroni's analyses on cytotoxicity tests. The metabolites quantified were TGMP, TGDP, TGTP, MeTGMP, MeTGDP, MeTGTP, TIMP, TIDP, TITP, MeTIMP, MeTIDP and MeTITP.

As shown in **Table 4.1**, the concentration of total thiopurine metabolites was lower in NALM6 cells after treatment with AZA exposed to the *K. pneumoniae* ($p<0.05$ and $p=0.07$ for 48 and 72 hours respectively). Furthermore, TIMP was the most abundant metabolite in NALM6 both after a 48 and 72-hour treatment with AZA not exposed to bacteria, followed by MeTIMP and TGMP. On the contrary, treatment with AZA exposed to bacteria for 48 and 72 hours caused a modifcation of the thiopurine metabolite concentration ranking of this cell line: MeTIMP was the most abundant, followed by TIMP and TGMP.

Table 4.1. Average concentration of thiopurine metabolites (means ± SE) in NALM6 cells treated with 15 µM AZA exposed to *K. pneumoniae* (AZA Kp) or not (AZA M9) for 48 and 72 hours.

Metabolites	48 hours				72 hours			
	AZA M9		***AZA Kp***		***AZA M9***		***AZA Kp***	
	pmol/Mio	%	pmol/Mio	%	pmol/Mio	%	pmol/Mio	%
TGMP	92.03±11.20	24.55	54.77±7.24	21.38	129.26±24.82	18.64	54.32±12.18	14.22
TGDP	4.50±2.12	1.20	1.30±0.76	0.51	7.63±2.71	1.10	1.27±0.66	0.33
TGTP	21.75±2.69	5.80	6.90±1.57	2.69	33.80±5.30	4.87	5.74±1.22	1.50

MeTGMP	1.92±0.17	0.51	1.61±0.17	0.63	2.89±0.51	0.42	2.14±0.44	0.56
MeTGDP	0.17±0.09	0.05	0.00	0.00	0.42±0.22	0.06	0.15±0.15	0.04
MeTGTP	1.07±0.22	0.29	0.82±0.23	0.32	1.76±0.14	0.25	0.94±0.11	0.25
TIMP	161.20±14.29	43.01	76.66±7.75	29.92	293.32±51.78	42.29	83.20±16.55	21.78
MeTIMP	90.22±3.35	24.07	112.82±5.38	44.04	212.67±26.73	30.66	229.03±55.01	59.96
MeTITP	1.96±0.24	0.52	1.31±0.66	0.51	10.58±0.70	1.53	5.21±1.24	1.36
TOT.	374.81±26.36	100.00	256.21±21.34	100.00	693.54±97.43	100.00	382.00±85.82	100.00

Interestingly, JURKAT cells treated with AZA exposed to *K. pneumoniae* showed a reduction of the concentration of total thiopurine metabolites compared to the ones treated with the drug in M9 (**Table 4.2**) (p=0.29 and p<0.05 for 48 and 72 hours respectively). Moreover, MeTIMP was the most abundant metabolite in JURKAT cells treated with AZA and, contrary to what was observed for NALM6 cells, the thiopurine metabolites concentration ranking seemed not to change after treatment with the drug exposed to *K. pneumoniae*.

Table 4.2. Average concentration of thiopurine metabolites (means ± SE) in JURKAT cells treated with 15 µM AZA exposed to *K. pneumoniae* (AZA Kp) or not (AZA M9) for 48 and 72 hours.

Metabolites	**48 hours**				**72 hours**			
	AZA M9		***AZA Kp***		***AZA M9***		***AZA Kp***	
	pmol/Mio	%	pmol/Mio	%	pmol/Mio	%	pmol/Mio	%
TGMP	62.66±11.12	7.56	41.45±7.95	8.04	33.60±5.61	5.46	31.50±1.81	7.98
TGDP	10.19±2.41	1.23	3.44±0.73	0.67	4.31±0.65	0.70	1.50±0.16	0.38
TGTP	187.63±40.59	22.64	69.74±14.24	13.52	82.23±10.16	13.35	33.81±4.59	8.57
MeTGMP	2.76±0.78	0.33	1.36±0.21	0.26	2.00±0.04	0.32	1.27±0.10	0.32
MeTGDP	1.25±0.65	0.15	0.62±0.37	0.12	0.85±0.43	0.14	0.18±0.18	0.05
MeTGTP	12.04±3.88	1.45	8.15±1.49	1.58	9.68±0.52	1.57	5.27±0.29	1.34
TIMP	37.65±8.82	4.54	11.80±2.25	2.29	19.35±5.53	3.14	13.77±2.24	3.49
MeTIMP	501.13±151.96	60.47	368.33±59.76	71.43	452.92±21.95	73.53	298.66±26.51	75.70
MeTITP	13.40±4.15	1.62	10.75±2.55	2.09	10.99±0.64	1.78	8.56±1.06	2.17
TOT.	828.71±221.53	100.00	515.65±74.81	100.00	615.94±16.74	100.00	394.50±33.79	100.00

Overall, ANOVA assessed a significant decrease of TGMP, TGDP, TGTP, MeTGMP, MeTGTP and TIMP after treatment with AZA incubated previously with *K. pneumoniae* (p<0.01; p<0.001; p<0.001; p<0.01; p<0.05; p<0.001 respectively) (**Figure 4.11 - 4.14**, **4.16** and **4.17**). The metabolites TIDP, MeTIDP and TITP were not detected in none of the cell lines after treatment with AZA. Furthermore, comparing the metabolites generated after treatment of the cell lines, the nucleotides TGTP, MeTGDP, MeTGTP, MeTIMP and MeTITP were more abundant in JURKAT cells (p<0.001; p<0.05; p<0.001; p<0.001; p<0.001 respectively) (**Figure 4.13, 4.15, 4.16, 4.18** and **4.20**), while TGMP and TIMP were significantly higher in NALM6 cells (p<0.001) (**Figure 4.11** and **4.17**). Thiopurine metabolites after treatment with AZA did not differ depending on the timing of treatment, except for TGTP that was more abundant after a treatment of 48 hours compared to the 72 hours treatment (p<0.05) (**Figure 4.14**).

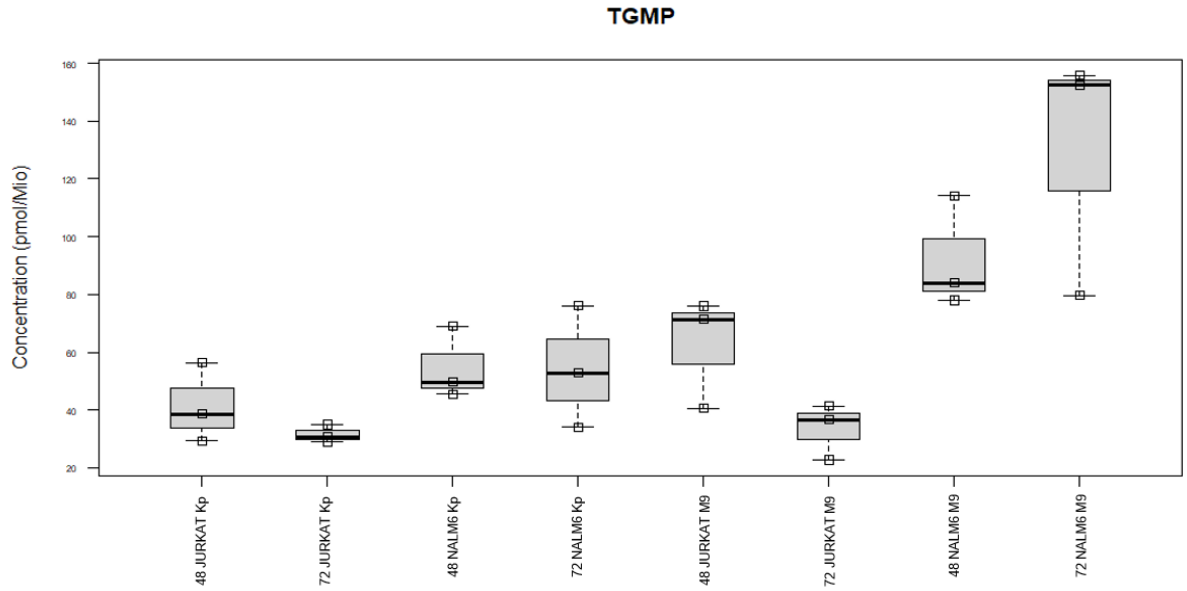

Figure 4.11. Concentration of the TGMP metabolite in NALM6 and JURKAT cells after treatment for 48 or 72 hours with 15 µM AZA in M9 or exposed to *K. pneumoniae*. ANOVA (treatment time ns; cell line $p<0.001$; experimental condition $p<0.01$).

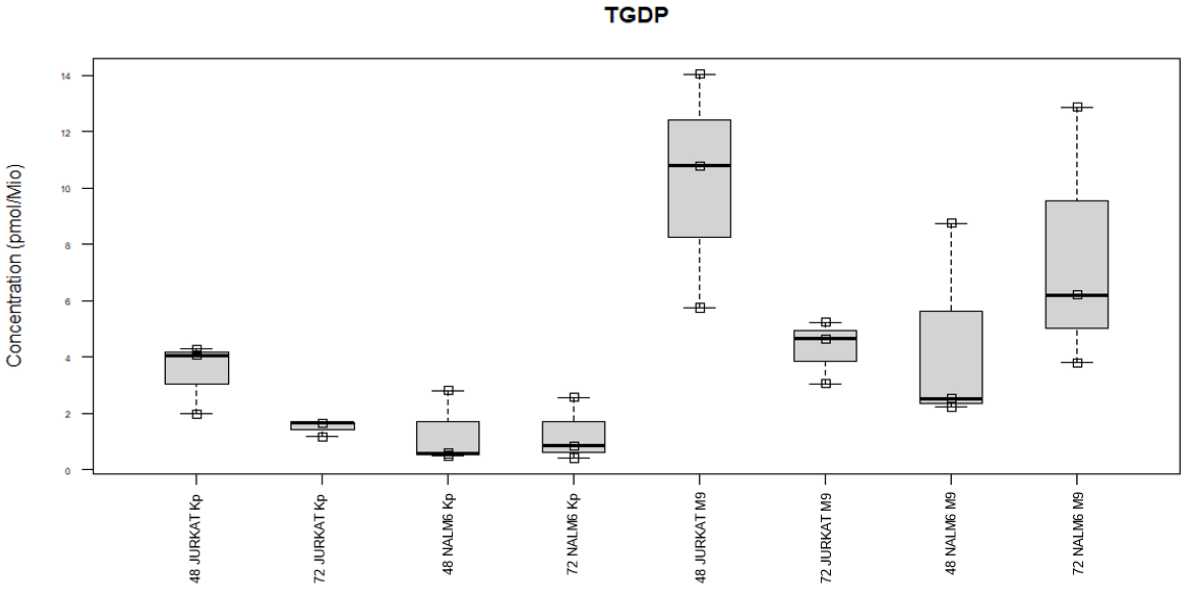

Figure 4.12. Concentration of the TGDP metabolite in NALM6 and JURKAT cells after treatment for 48 or 72 hours with 15 µM AZA in M9 or exposed to *K. pneumoniae*. ANOVA (treatment time ns; cell line ns; experimental condition $p<0.001$).

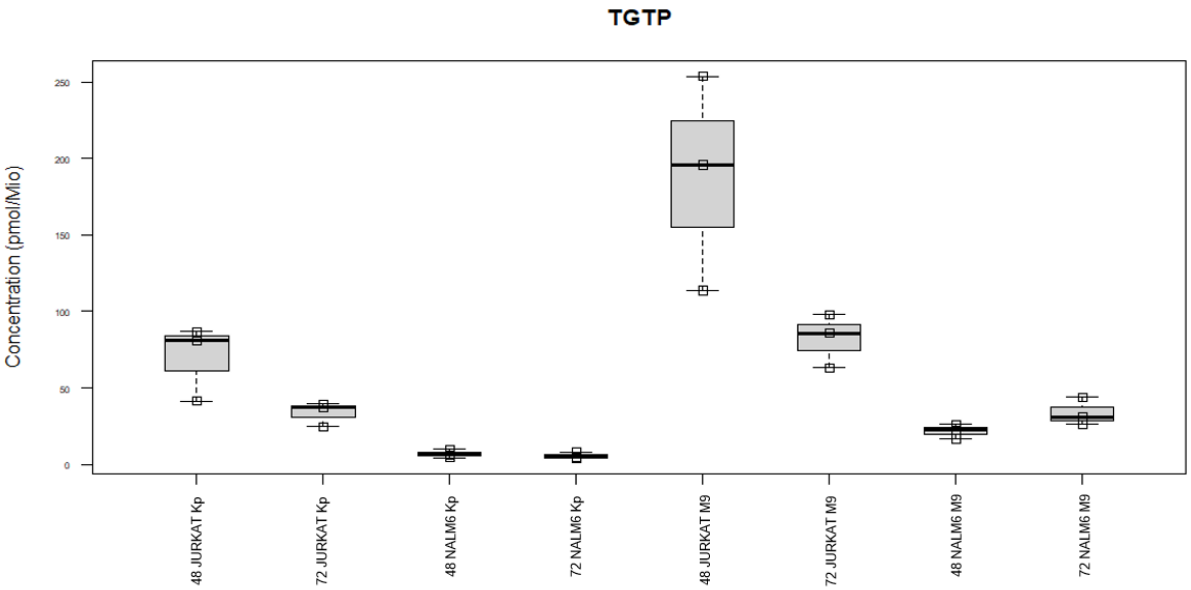

Figure 4.13. Concentration of the TGTP metabolite in NALM6 and JURKAT cells after treatment for 48 or 72 hours with 15 µM AZA in M9 or exposed to *K. pneumoniae*. ANOVA (treatment time $p<0.05$; cell line $p<0.001$; experimental condition $p<0.001$).

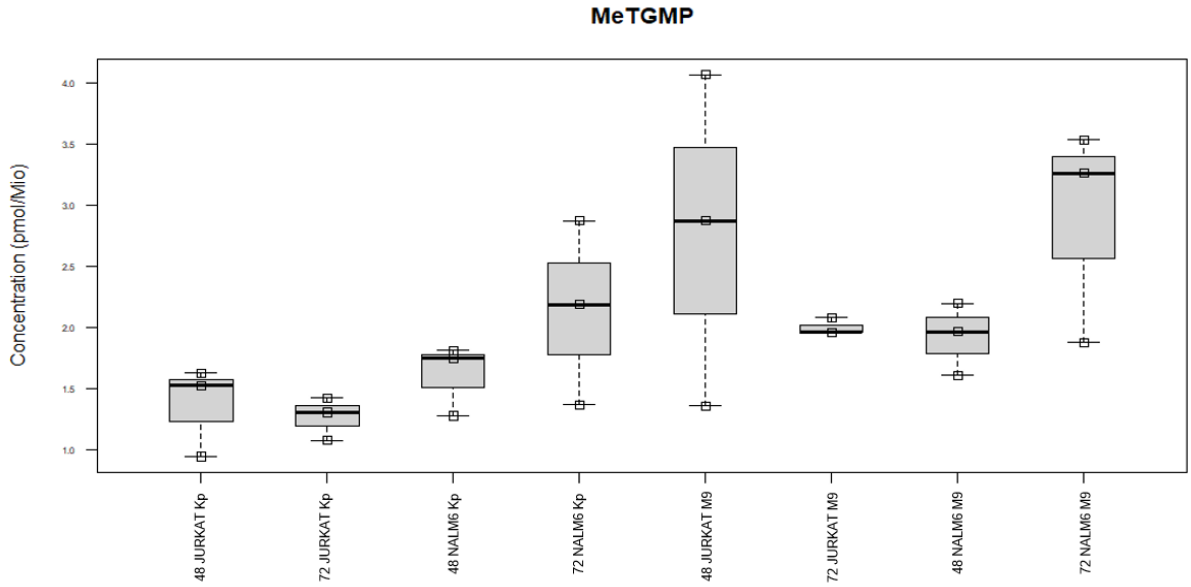

Figure 4.14. Concentration of the MeTGMP metabolite in NALM6 and JURKAT cells after treatment for 48 or 72 hours with 15 µM AZA in M9 or exposed to *K. pneumoniae*. ANOVA (treatment time ns; cell line ns; experimental condition $p<0.01$).

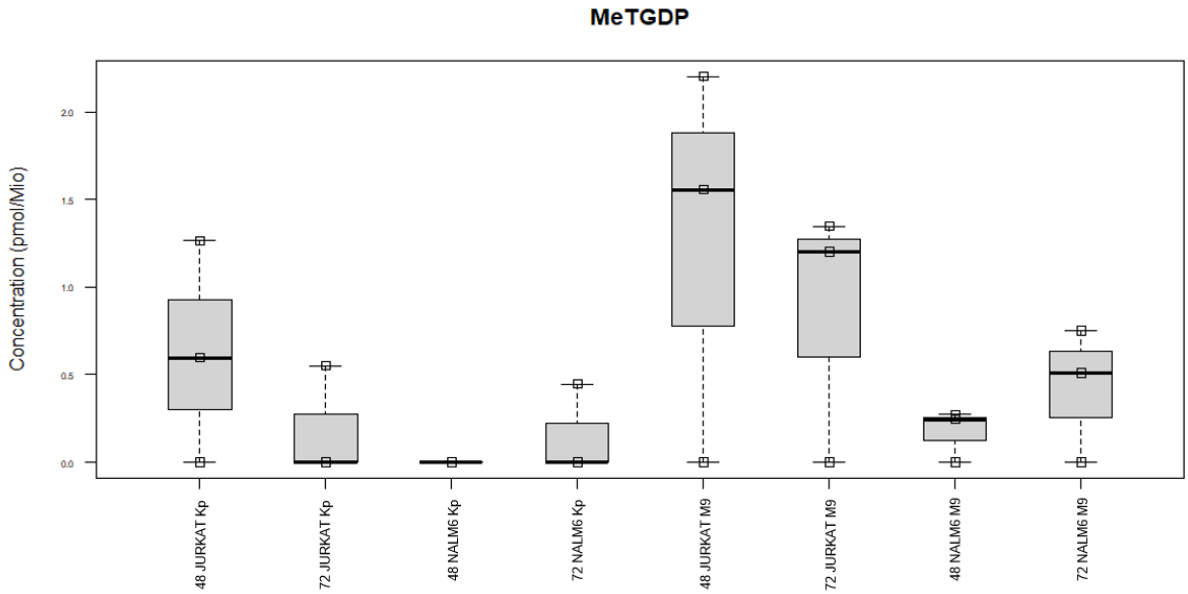

Figure 4.15. Concentration of the MeTGDP metabolite in NALM6 and JURKAT cells after treatment for 48 or 72 hours with 15 µM AZA in M9 or exposed to *K. pneumoniae*. ANOVA (treatment time ns; cell line $p<0.05$; experimental condition ns).

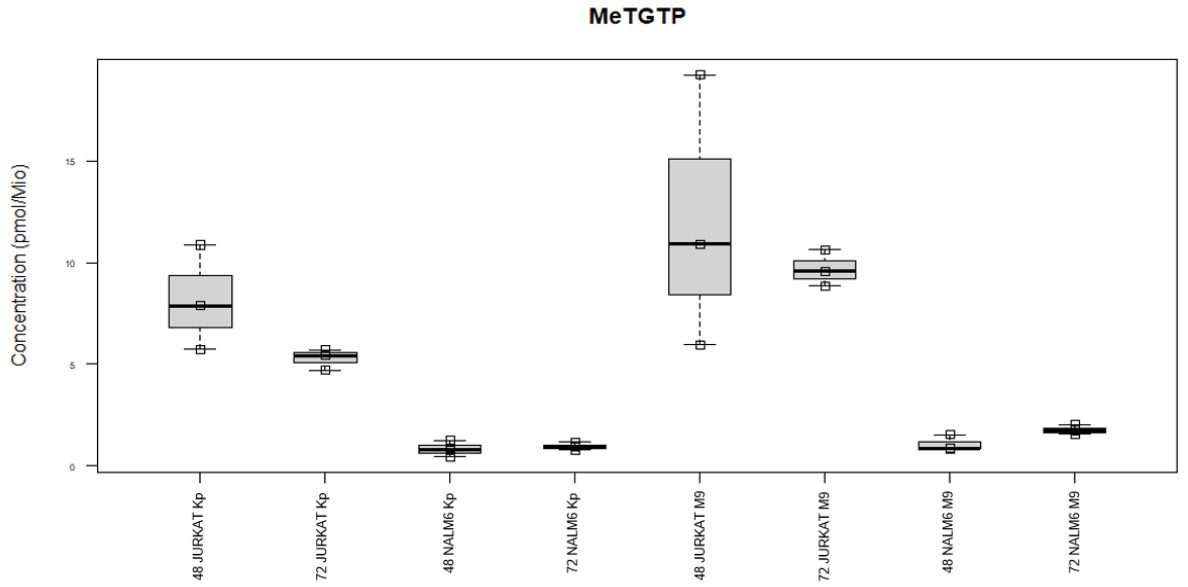

Figure 4.16. Concentration of the MeTGTP metabolite in NALM6 and JURKAT cells after treatment for 48 or 72 hours with 15 µM AZA in M9 or exposed to *K. pneumoniae*. ANOVA (treatment time ns; cell line $p<0.001$; experimental condition $p<0.05$).

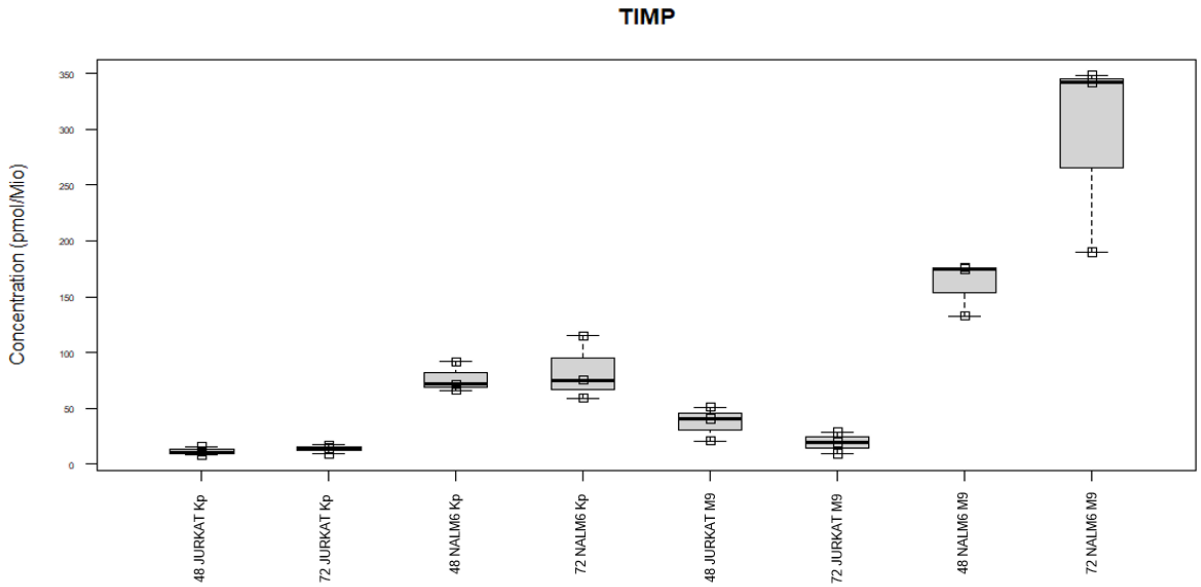

Figure 4.17. Concentration of the TIMP metabolite in NALM6 and JURKAT cells after treatment for 48 or 72 hours with 15 µM AZA in M9 or exposed to *K. pneumoniae*. ANOVA (treatment time ns; cell line $p<0.001$; experimental condition $p<0.001$).

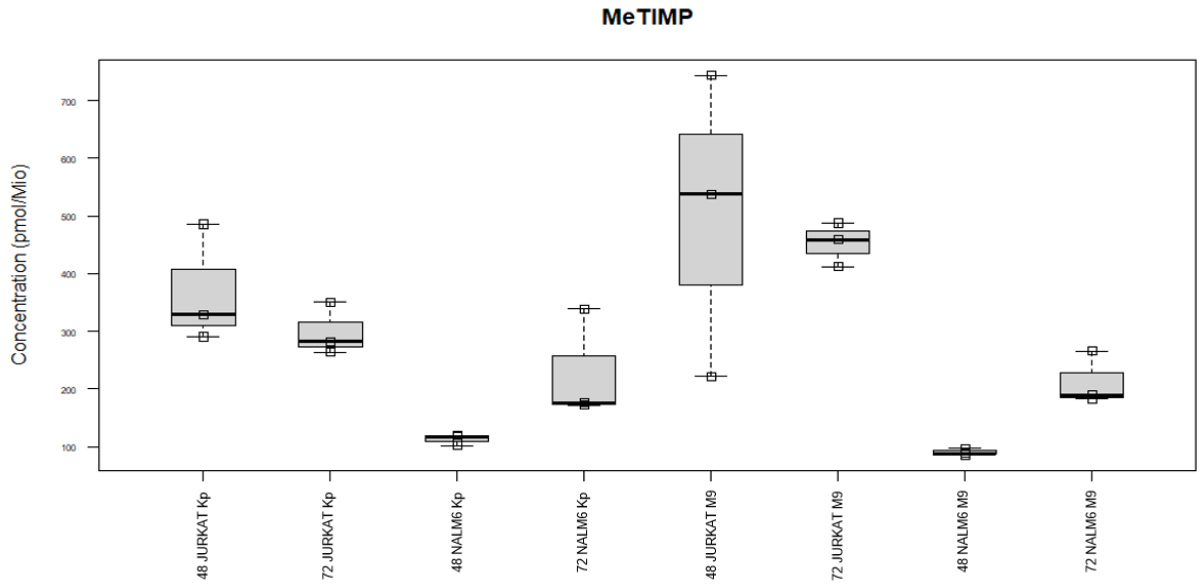

Figure 4.18. Concentration of the MeTIMP metabolite in NALM6 and JURKAT cells after treatment for 48 or 72 hours with 15 µM AZA in M9 or exposed to *K. pneumoniae*. ANOVA (treatment time ns; cell line $p<0.001$; experimental condition ns).

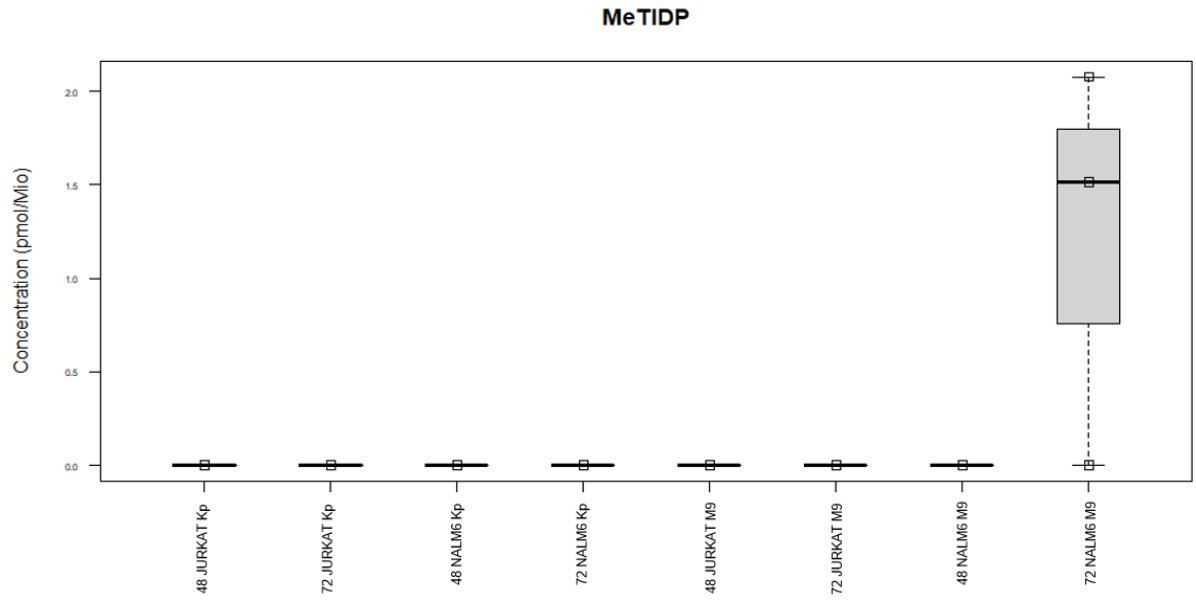

Figure 4.19. Concentration of the MeTIDP metabolite in NALM6 and JURKAT cells after treatment for 48 or 72 hours with 15 µM AZA in M9 or exposed to *K. pneumoniae*. ANOVA (treatment time ns; cell line ns; experimental condition ns).

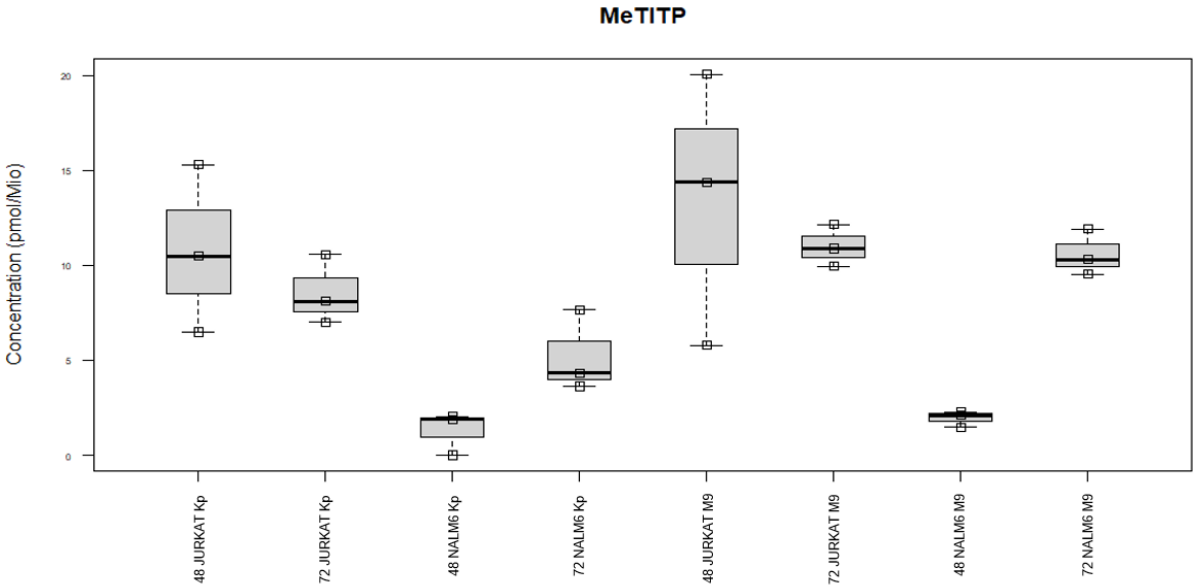

Figure 4.20. Concentration of the MeTITP metabolite in NALM6 and JURKAT cells after treatment for 48 or 72 hours with 15 µM AZA in M9 or exposed to *K. pneumoniae*. ANOVA (treatment time ns; cell line $p<0.001$; experimental condition ns).

Interestingly, the concentration of total metabolites was lower in NALM6 cells treated with MP exposed to *K. pneumoniae* (**Table 4.3**) ($p<0.05$). Also, TIMP, MeTIMP and TGMP were the most abundant metabolites after treatment with this drug at the different times and experimental conditions.

Table 4.3. Average concentration of thiopurine metabolites (means ± SE) in NALM6 cells treated with 2.5 µM MP exposed to *K. pneumoniae* (MP Kp) or not (MP M9) for 48 and 72 hours.

Metabolites	48 hours				72 hours			
	MP M9		***MP Kp***		***MP M9***		***MP Kp***	
	pmol/Mio	%	pmol/Mio	%	pmol/Mio	%	pmol/Mio	%
TGMP	99.77±7.16	23.23	50.70±3.19	46.62	116.02±10.63	34.24	74.54±20.04	45.82
TGDP	6.44±1.52	1.50	0.49±0.25	0.45	6.74±0.41	1.99	1.80±1.06	1.10
TGTP	24.96±1.60	5.81	4.90±1.10	4.50	20.66±4.42	6.10	9.30±3.26	5.72
MeTGMP	1.74±0.12	0.41	2.21±0.29	2.03	3.34±0.13	0.99	2.69±0.75	1.65
MeTGDP	0.09±0.09	0.02	0.00	0.00	0.62±0.32	0.18	0.17±0.17	0.10
MeTGTP	0.69±0.07	0.16	0.45±0.25	0.41	1.41±0.22	0.42	1.05±0.19	0.65
TIMP	204.29±49.39	47.56	15.16±2.70	13.94	60.97±19.48	17.99	23.93±3.05	14.71
MeTIMP	90.36±8.00	21.03	34.86±1.03	32.05	122.23±8.32	36.07	49.09±1.90	30.18
MeTITP	1.23±0.65	0.29	0.00	0.00	5.45±0.93	1.61	0.10±0.10	0.06
TOT.	429.57±62.85	100.00	108.76±7.54	100.00	338.85±43.07	100.00	162.67±25.85	100.00

The total amount of thiopurine metabolites was reduced after the treatment of JURKAT cells with MP exposed to *K. pneumoniae* (**Table 4.4**) (p=0.17 and p<0.01 for 48 and 72 hours respectively). As showed below, MeTIMP was the most abundant metabolite in this cell line treated with MP and the abundance of each metabolite did not vary depending on the timing of treatment and on the experimental condition.

Table 4.4. Average concentration of thiopurine metabolites (means ± SE) in JURKAT cells treated with 2.5 µM MP exposed to *K. pneumoniae* (MP Kp) or not (MP M9) for 48 and 72 hours.

Metabolites	48 hours				72 hours			
	MP M9		***MP Kp***		***MP M9***		***MP Kp***	
	pmol/Mio	%	pmol/Mio	%	pmol/Mio	%	pmol/Mio	%
TGMP	71.27±9.63	16.63	58.79±11.42	20.40	51.64±10.25	12.21	34.22±3.68	21.57
TGDP	7.91±1.18	1.85	3.76±1.12	1.31	4.90±0.44	1.16	0.95±0.48	0.60
TGTP	104.17±11.59	24.31	48.56±13.99	16.85	75.73±9.63	17.91	23.32±3.23	14.70
MeTGMP	1.91±0.40	0.45	1.37±0.68	0.48	1.07±0.54	0.25	0.69±0.35	0.44
MeTGDP	0.86±0.49	0.20	0.40±0.40	0.14	0.80±0.41	0.19	0.00	0.00
MeTGTP	7.36±2.11	1.72	5.85±2.18	2.03	7.80±0.63	1.84	3.14±0.80	1.98
TIMP	29.97±1.82	6.99	13.52±1.99	4.69	17.52±5.60	4.14	7.59±1.35	4.79
MeTIMP	200.19±33.43	46.72	155.86±36.79	54.10	259.29±22.69	61.32	88.74±27.29	55.93
MeTITP	4.86±0.75	1.13	0.00	0.00	4.10±2.14	0.97	0.00	0.00
TOT.	428.49±55.71	100.00	288.11±64.01	100.00	422.85±41.27	100.00	158.66±35.48	100.00

In detail, the thioguanine nucleotides TGMP, TGDP, TGTP and MeTGDP and the thioinosinic nucleotides TIMP, MeTIMP and MeTITP were significantly decreased after treatment of NALM6 and JURKAT cells with MP previously incubated with *K. pneumoniae* (p<0.05; p<0.001; p<0.001; p<0.05; p<0.001; p<0.001; p<0.001 respectively) (**Figure 4.21 - 4.23, 4.25, 4.27, 4.28** and **4.30**). The metabolites TIDP, MeTIDP and TITP were not detected in none of the cell lines after treatment with MP. Interestingly, similarly to what was observed also for AZA, the monophosphates TGMP, MeTGMP and TIMP were more present in NALM6 cells compared to JURKAT cells (p<0.001; p<0.01; p<0.001 respectively) (**Figure 4.21, 4.24** and **4.27**), whereas the triphosphates TGTP and MeTGTP and the monophosphate MeTIMP were higher in JURKAT cells (p<0.001) (**Figure 4.23, 4.26** and **4.28**). Moreover, similarly to what was observed also for AZA, only TGTP was more abundant after a treatment of 48 hours compared to the 72 hours treatment (p<0.05) (**Figure 4.23**).

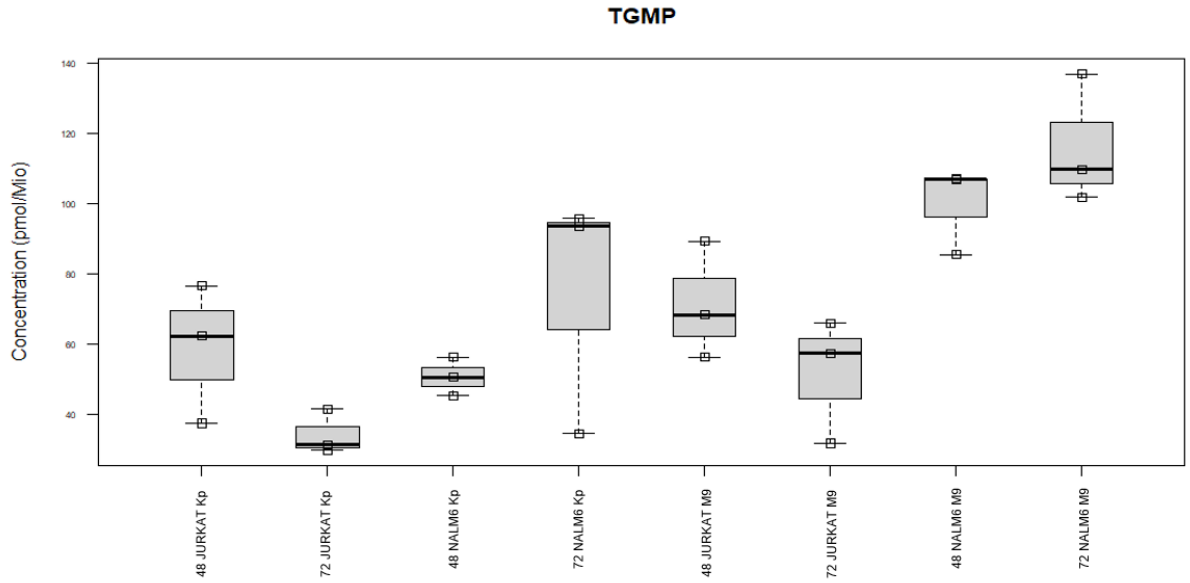

Figure 4.21. Concentration of the TGMP metabolite in NALM6 and JURKAT cells after treatment for 48 or 72 hours with 2.5 µM MP in M9 or exposed to *K. pneumoniae*. ANOVA (treatment time ns; cell line p<0.001; experimental condition p<0.05).

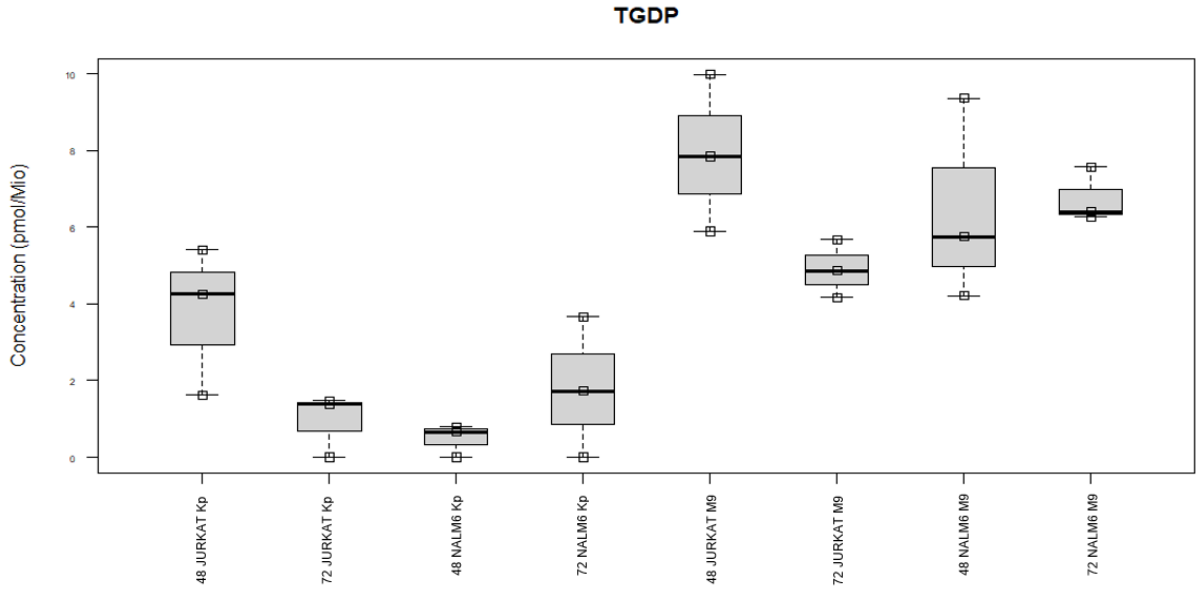

Figure 4.22. Concentration of the TGDP metabolite in NALM6 and JURKAT cells after treatment for 48 or 72 hours with 2.5 µM MP in M9 or exposed to *K. pneumoniae*. ANOVA (treatment time ns; cell line ns; experimental condition $p<0.001$).

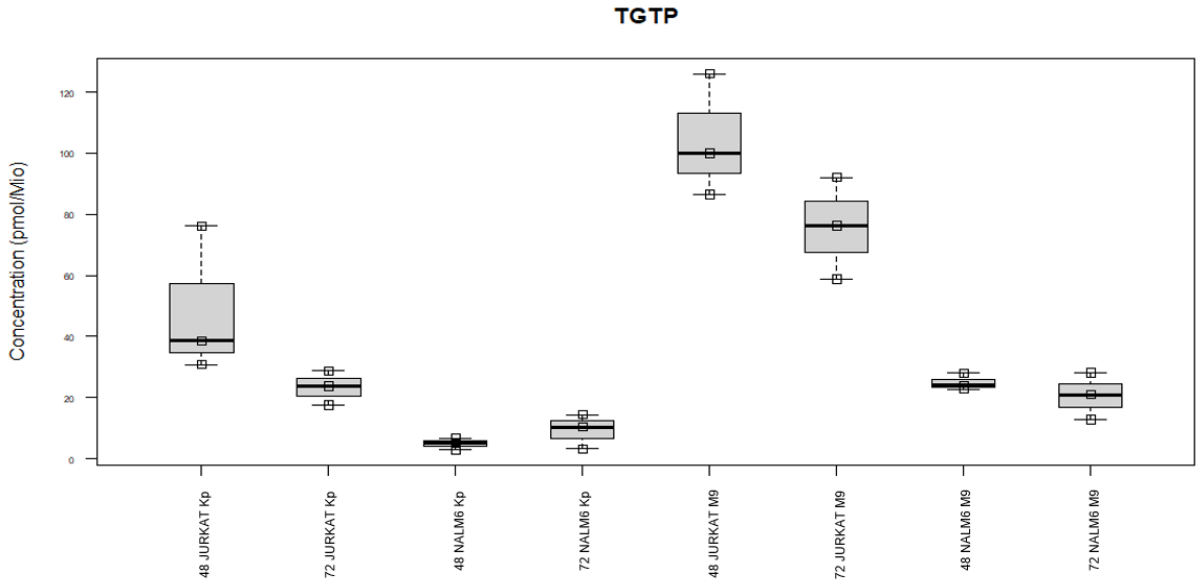

Figure 4.23. Concentration of the TGTP metabolite in NALM6 and JURKAT cells after treatment for 48 or 72 hours with 2.5 µM MP in M9 or exposed to *K. pneumoniae*. ANOVA (treatment time $p<0.05$; cell line $p<0.001$; experimental condition $p<0.001$).

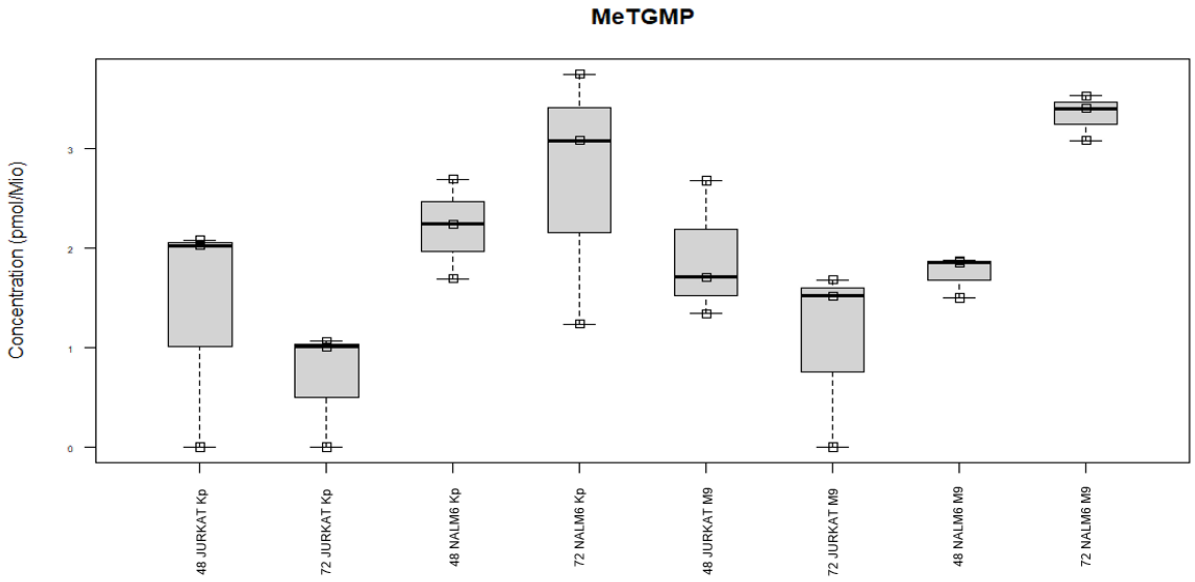

Figure 4.24. Concentration of the MeTGMP metabolite in NALM6 and JURKAT cells after treatment for 48 or 72 hours with 2.5 µM MP in M9 or exposed to *K. pneumoniae*. ANOVA (treatment time ns; cell line $p<0.001$; experimental condition ns).

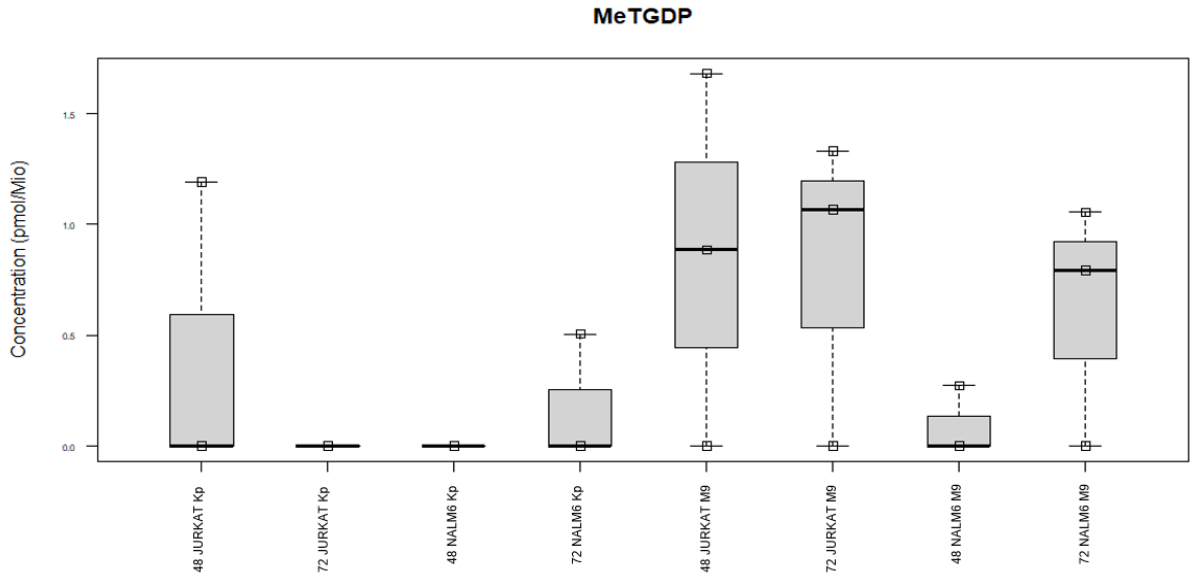

Figure 4.25. Concentration of the MeTGDP metabolite in NALM6 and JURKAT cells after treatment for 48 or 72 hours with 2.5 µM MP in M9 or exposed to *K. pneumoniae*. ANOVA (treatment time ns; cell line ns; experimental condition $p<0.05$).

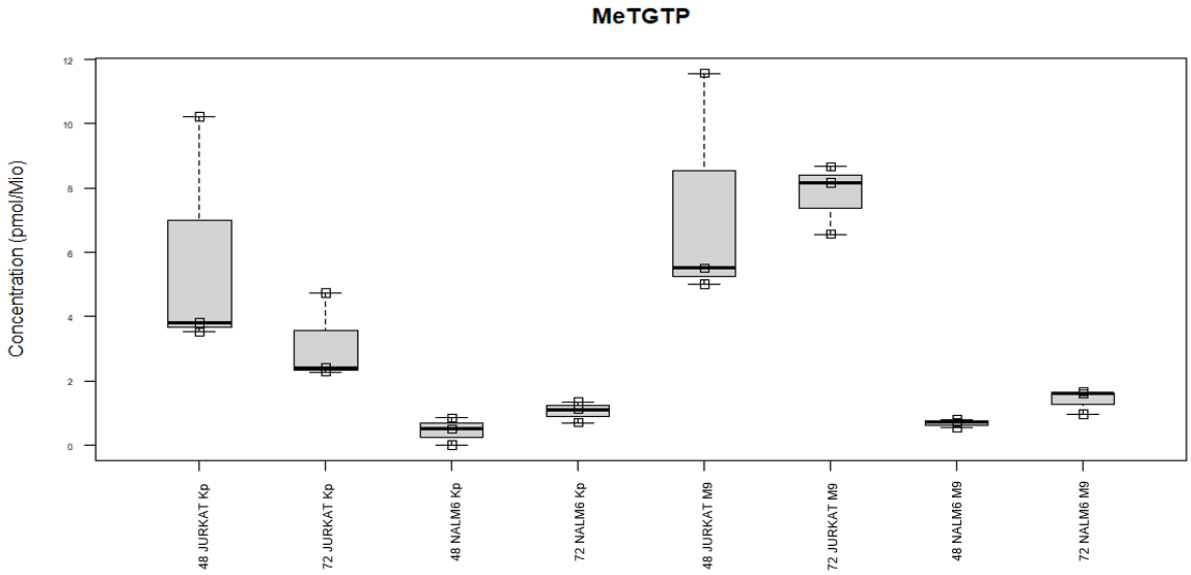

Figure 4.26. Concentration of the MeTGTP metabolite in NALM6 and JURKAT cells after treatment for 48 or 72 hours with 2.5 µM MP in M9 or exposed to *K. pneumoniae*. ANOVA (treatment time ns; cell line $p<0.001$; experimental condition ns).

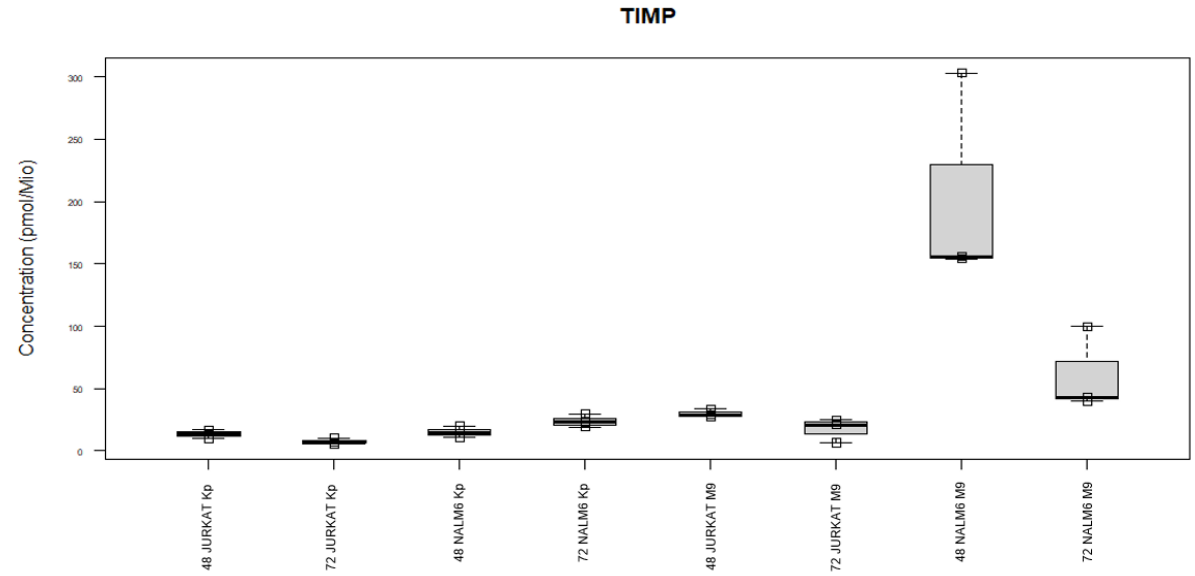

Figure 4.27. Concentration of the TIMP metabolite in NALM6 and JURKAT cells after treatment for 48 or 72 hours with 2.5 µM MP in M9 or exposed to *K. pneumoniae*. ANOVA (treatment time ns; cell line $p<0.001$; experimental condition $p<0.001$).

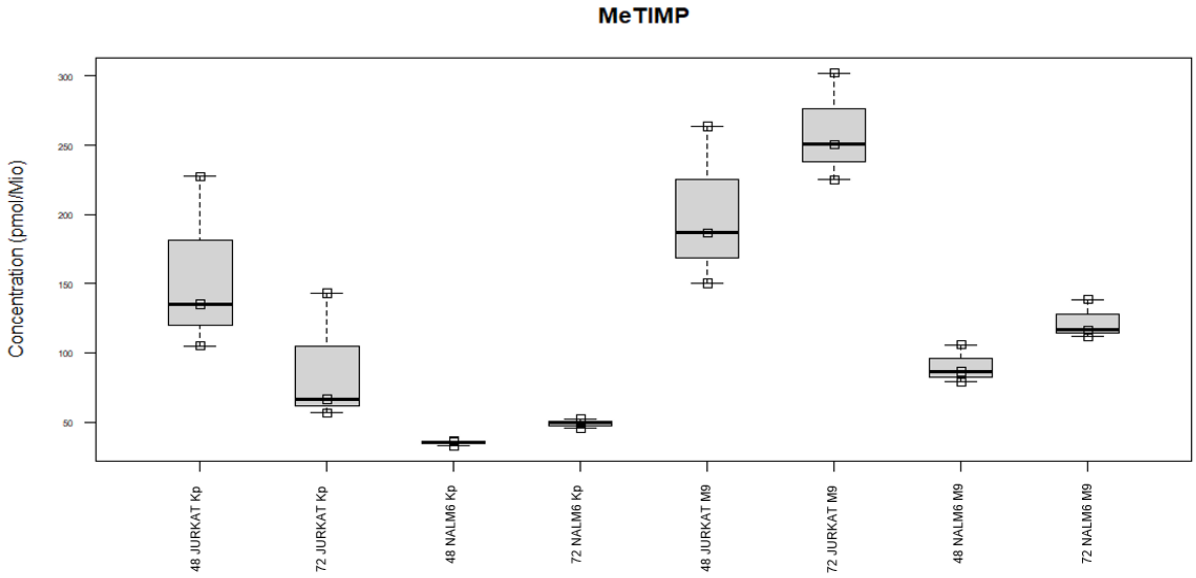

Figure 4.28. Concentration of the MeTIMP metabolite in NALM6 and JURKAT cells after treatment for 48 or 72 hours with 2.5 µM MP in M9 or exposed to *K. pneumoniae*. ANOVA (treatment time ns; cell line $p<0.001$; experimental condition $p<0.001$).

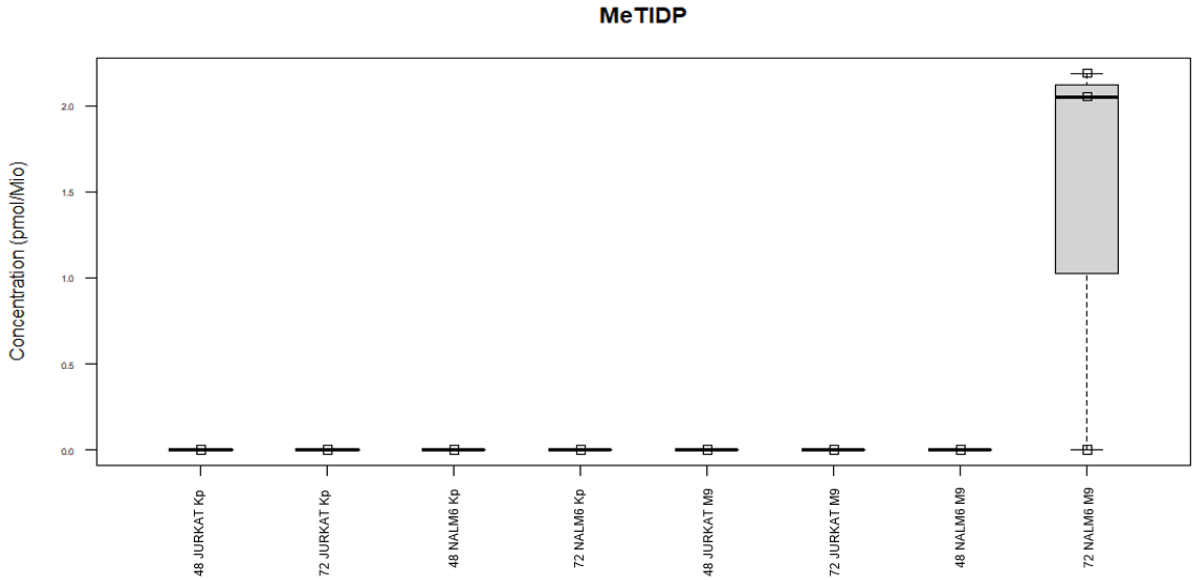

Figure 4.29. Concentration of the MeTIDP metabolite in NALM6 and JURKAT cells after treatment for 48 or 72 hours with 2.5 µM MP in M9 or exposed to *K. pneumoniae*. ANOVA (treatment time ns; cell line ns; experimental condition ns).

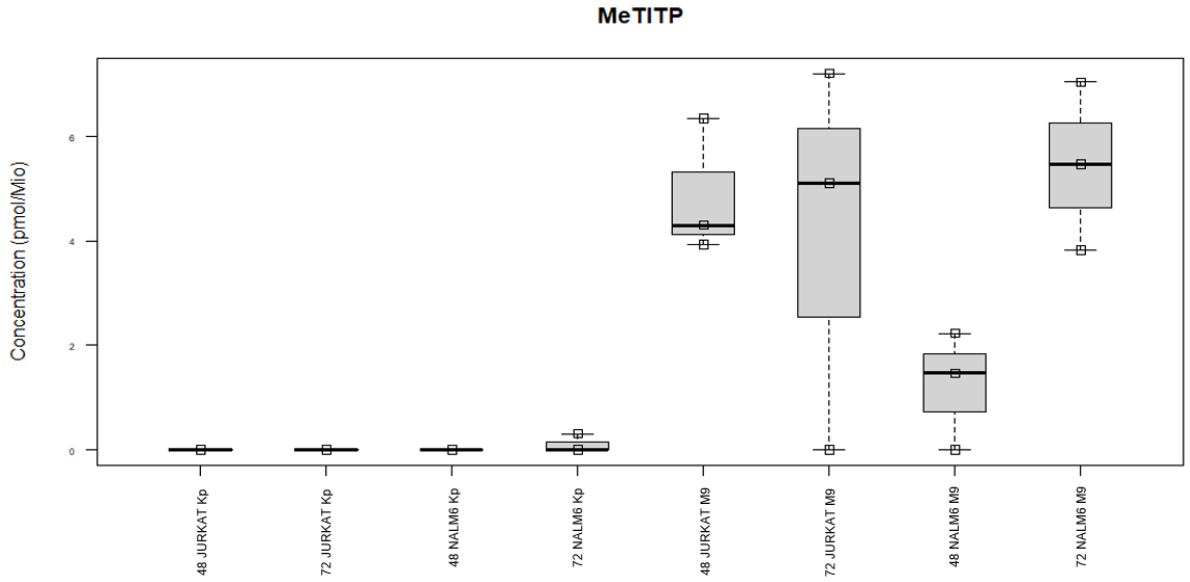

Figure 4.30. Concentration of the MeTITP metabolite in NALM6 and JURKAT cells after treatment for 48 or 72 hours with 2.5 µM MP in M9 or exposed to *K. pneumoniae*. ANOVA (treatment time ns; cell line ns; experimental condition $p<0.001$).

As shown in **Table 4.5**, treatment of NALM6 cells with TG previously exposed to *K. pneumoniae* caused a reduction in the total amount of thiopurine metabolites ($p<0.05$ and $p<0.01$ for 48 and 72 hours respectively). Interestingly, TGMP was the most abundant metabolite, followed by TGTP, in NALM6 cells after a 48 and 72-hour treatment with the drug exposed or not to the bacterial strain.

Table 4.5. Average concentration of thiopurine metabolites (means ± SE) in NALM6 cells treated with 1.25 µM TG exposed to *K. pneumoniae* (TG Kp) or not (TG M9) for 48 and 72 hours.

Metabolites	48 hours				72 hours			
	TG M9		***TG Kp***		***TG M9***		***TG Kp***	
	pmol/Mio	%	pmol/Mio	%	pmol/Mio	%	pmol/Mio	%
TGMP	311.93±46.41	66.21	77.08±18.44	83.85	214.87±3.49	71.16	66.87±13.50	80.76
TGDP	20.88±3.94	4.43	1.10±0.64	1.20	15.12±3.92	5.01	1.06±0.76	1.28
TGTP	107.60±9.26	22.84	7.90±3.09	8.59	50.37±4.25	16.68	6.96±3.18	8.40
MeTGMP	16.93±2.11	3.59	4.45±1.41	4.84	11.68±0.56	3.87	5.92±1.30	7.15
MeTGDP	2.73±1.41	0.58	0.16±0.16	0.17	2.66±1.38	0.88	0.12±0.12	0.14
MeTGTP	10.75±1.21	2.28	1.23±0.66	1.34	7.13±0.96	2.36	1.76±0.41	2.13
TIMP	0.30±0.16	0.06	0.00	0.00	0.10±0.10	0.03	0.11±0.11	0.13
TOT.	471.12±58.74	100.00	91.93±24.13	100.00	301.93±2.70	100.00	82.80±19.02	100.00

The trend of reduction of the concentrations of thiopurine metabolites after treatment of JURKAT cells with TG exposed to *K. pneumoniae* are shown in **Table 4.6** ($p=0.15$ and $p=0.06$ for 48 and 72 hours respectively). Moreover, TGTP was the most abundant metabolite after treatment for 48 and 72 hours with TG in M9; TGMP had the highest concentration compared to the other metabolites tested after treatment with TG exposed to the bacterial strain at both timing of treatment.

Table 4.6. Average concentration of thiopurine metabolites (means ± SE) in JURKAT cells treated with 1.25 µM TG exposed to *K. pneumoniae* (TG Kp) or not (TG M9) for 48 and 72 hours.

Metabolites	48 hours				72 hours			
	TG M9		*TG Kp*		*TG M9*		*TG Kp*	
	pmol/Mio	%	pmol/Mio	%	pmol/Mio	%	pmol/Mio	%
TGMP	39.37±5.63	29.19	42.92±10.67	52.44	36.82±3.39	40.29	29.32±4.93	67.98
TGDP	4.64±0.44	3.44	1.73±0.87	2.12	2.78±0.71	3.04	0.00	0.00
TGTP	73.27±4.31	54.32	29.89±10.57	36.52	40.85±9.45	44.69	11.27±3.21	26.13
MeTGMP	3.01±0.50	2.23	1.34±0.73	1.64	1.76±0.34	1.93	0.73±0.37	1.68
MeTGDP	1.41±0.78	1.05	0.00	0.00	0.95±0.48	1.04	0.00	0.00
MeTGTP	12.23±2.46	9.07	5.46±2.25	6.68	7.14±0.91	7.82	1.82±0.95	4.21
TIMP	0.96±0.49	0.71	0.50±0.50	0.61	1.09±0.55	1.20	0.00	0.00
TOT.	134.89±11.54	100.00	81.85±24.27	100.00	91.40±15.06	100.00	43.13±8.59	100.00

Noteworthy, according to ANOVA, the thionucleotides TGMP, TGDP, TGTP and their corresponding methylated compound MeTGMP, MeTGDP and MeTGTP were significantly decreased after treatment of NALM6 and JURKAT cells with TG exposed previously to *K. pneumoniae* (p<0.001; p<0.001; p<0.001; p<0.001; p<0.01; p<0.001 respectively) (**Figure 4.31 - 4.36**). The metabolites MeTIMP, TIDP, MeTIDP, TITP and MeTITP were not detected in none of the cell lines after treatment with TG. Interestingly, NALM6 cells presented higher levels of TGMP, TGDP, MeTGMP and TIMP in comparison to JURKAT cells (p<0.001; p<0.001; p<0.001; p<0.05 respectively) (**Figure 4.31, 4.32, 4.34** and **4.37**). Furthermore, the levels of TGMP, TGTP and MeTGTP were increased after a 48 hours-treatment (p<0.05; p<0.001; p<0.01 respectively) (**Figure 4.31, 4.33** and **4.36**).

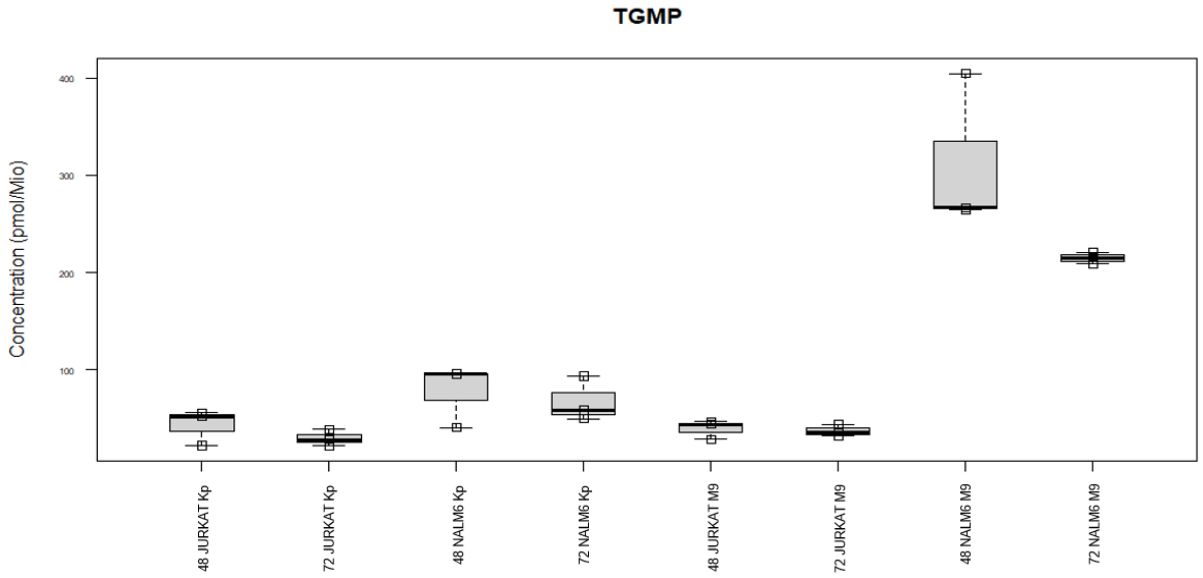

Figure 4.31. Concentration of the TGMP metabolite in NALM6 and JURKAT cells after treatment for 48 or 72 hours with 1.25 µM TG in M9 or exposed to *K. pneumoniae*. ANOVA (treatment time p<0.05; cell line p<0.001; experimental condition p<0.001).

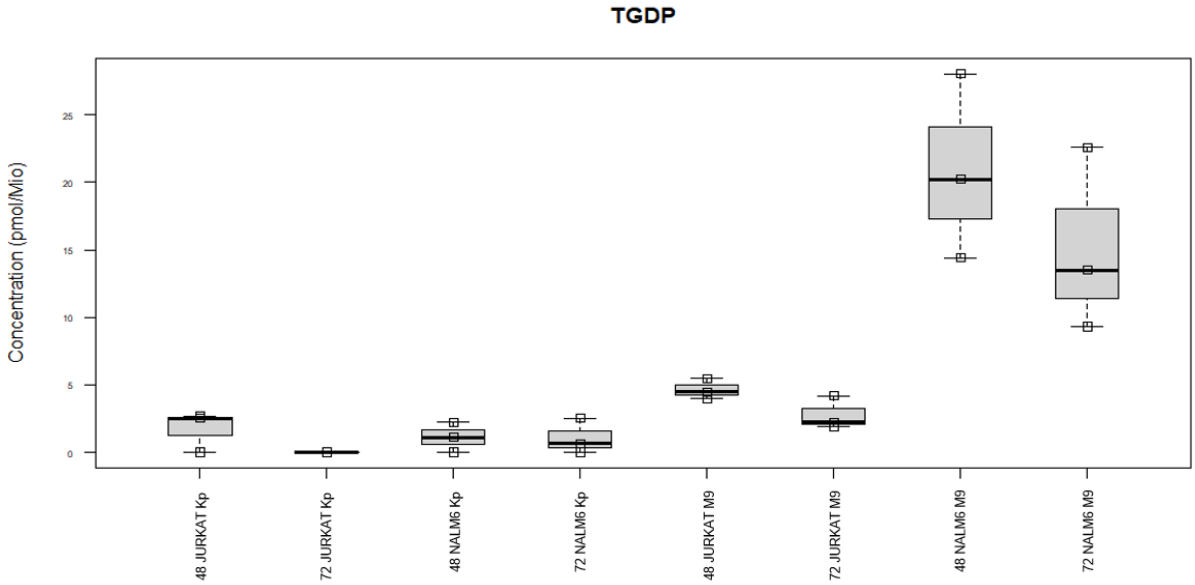

Figure 4.32. Concentration of the TGDP metabolite in NALM6 and JURKAT cells after treatment for 48 or 72 hours with 1.25 µM TG in M9 or exposed to *K. pneumoniae*. ANOVA (treatment time ns; cell line $p<0.001$; experimental condition $p<0.001$).

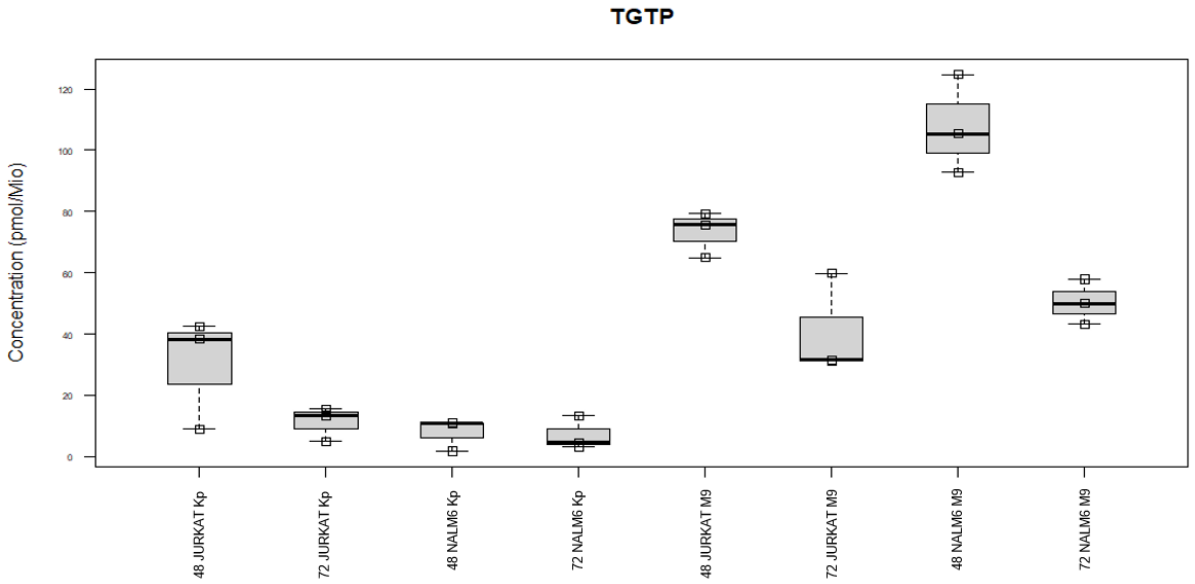

Figure 4.33. Concentration of the TGTP metabolite in NALM6 and JURKAT cells after treatment for 48 or 72 hours with 1.25 µM TG in M9 or exposed to *K. pneumoniae*. ANOVA (treatment time $p<0.001$; cell line ns; experimental condition $p<0.001$).

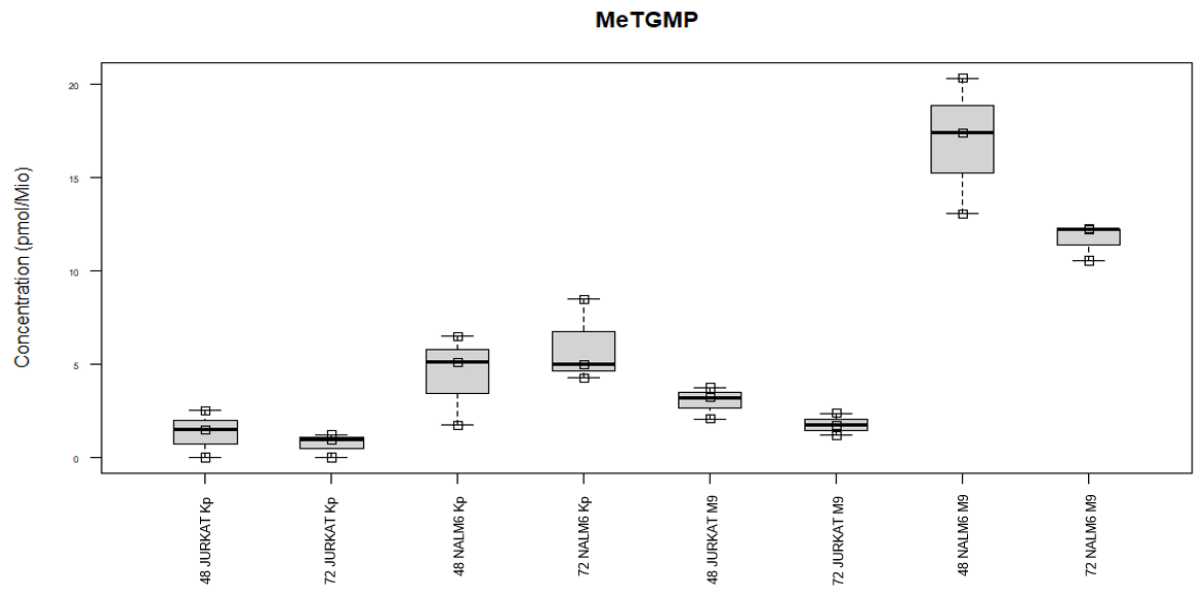

Figure 4.34. Concentration of the MeTGMP metabolite in NALM6 and JURKAT cells after treatment for 48 or 72 hours with 1.25 µM TG in M9 or exposed to *K. pneumoniae*. ANOVA (treatment time ns; cell line $p<0.001$; experimental condition $p<0.001$).

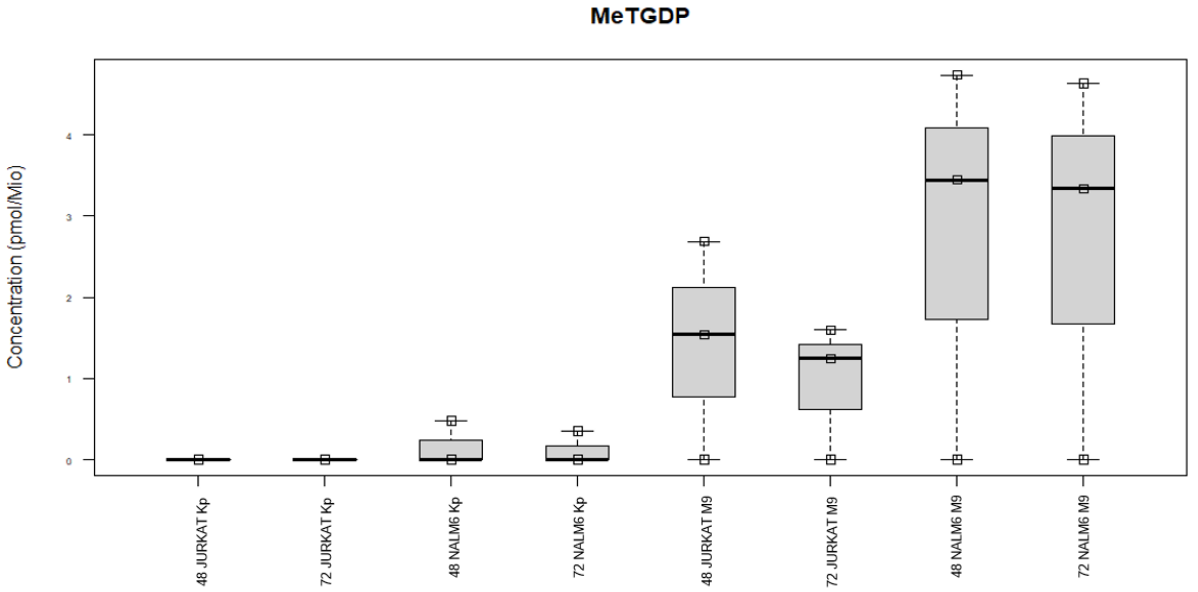

Figure 4.35. Concentration of the MeTGDP metabolite in NALM6 and JURKAT cells after treatment for 48 or 72 hours with 1.25 µM TG in M9 or exposed to *K. pneumoniae*. ANOVA (treatment time ns; cell line ns; experimental condition $p<0.01$).

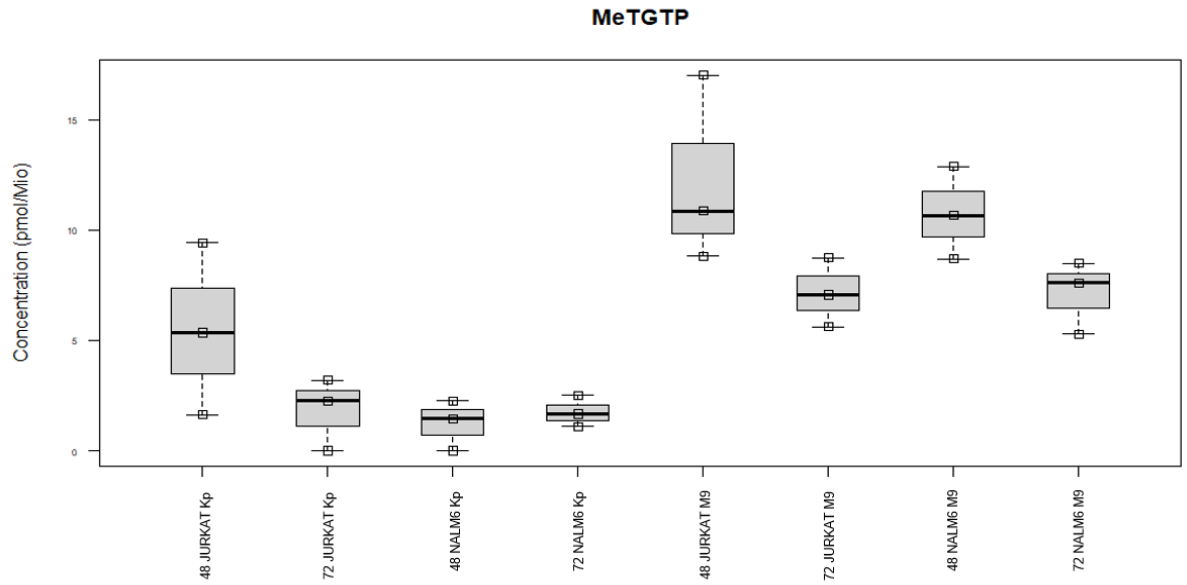

Figure 4.36. Concentration of the MeTGTP metabolite in NALM6 and JURKAT cells after treatment for 48 or 72 hours with 1.25 µM TG in M9 or exposed to *K. pneumoniae*. ANOVA (treatment time $p<0.01$; cell line ns; experimental condition $p<0.001$).

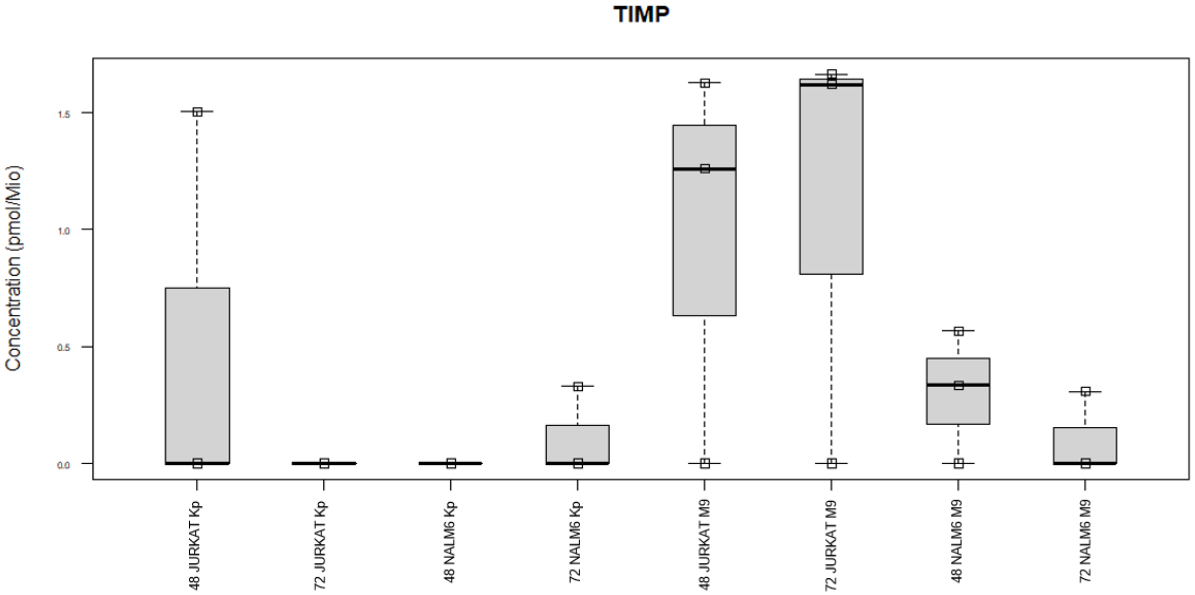

Figure 4.37. Concentration of the TIMP metabolite in NALM6 and JURKAT cells after treatment for 48 or 72 hours with 1.25 µM TG in M9 or exposed to *K. pneumoniae*. ANOVA (treatment time ns; cell line $p<0.05$; experimental condition ns).

4.1.6 Measurements of dTGUA in DNA of NALM6 and JURKAT cells treated with thiopurines previously exposed or not to *K. pneumoniae*

After a 72 hour-treatment of NALM6 and JURKAT cells with thiopurines exposed or not to *K. pneumoniae*, measurements of the dTGUA metabolite in DNA were performed. dTGUA concentrations reflect the incorporation of dTGTP in DNA and therefore the cytotoxic effects of thiopurines.

Comparing the concentration of dTGUA in DNA of NALM6 and JURKAT cell lines, the metabolite was more abundant in JURKAT cells' DNA (**Figure 4.38**).

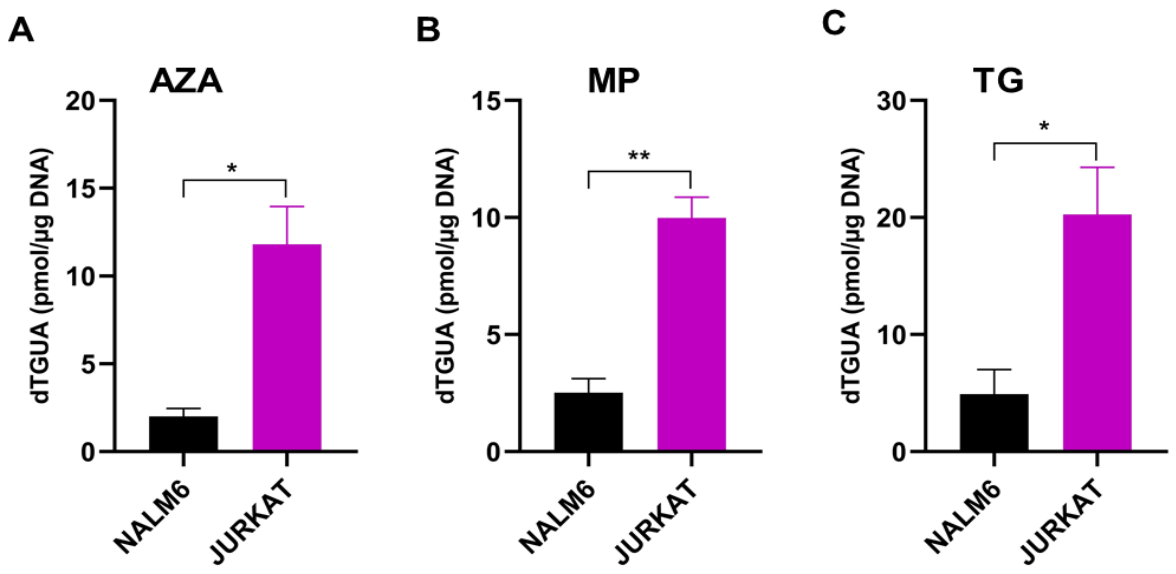

Figure 4.38. Concentrations of dTGUA (means ± SE) detected after 72-hour treatment of NALM6 and JURKAT cells with 15 µM AZA (**A**), 2.5 µM MP (**B**) and 1.25 µM TG (**C**) in M9. Welch's t test (* $p<0.05$ ** $p<0.01$).

Interestingly, as shown in **Figure 4.39 B-C**, the concentration of dTGUA incorporated in DNA after treatment for 72 hours with MP and TG previously exposed to *K. pneumoniae* was significantly lower compared to the one quantified in cells treated with the drugs in M9. Treatment of NALM6 cells with AZA previously exposed to bacteria reduced the levels of dTGUA in DNA, although the variation was not significant (**Figure 4.39 A**).

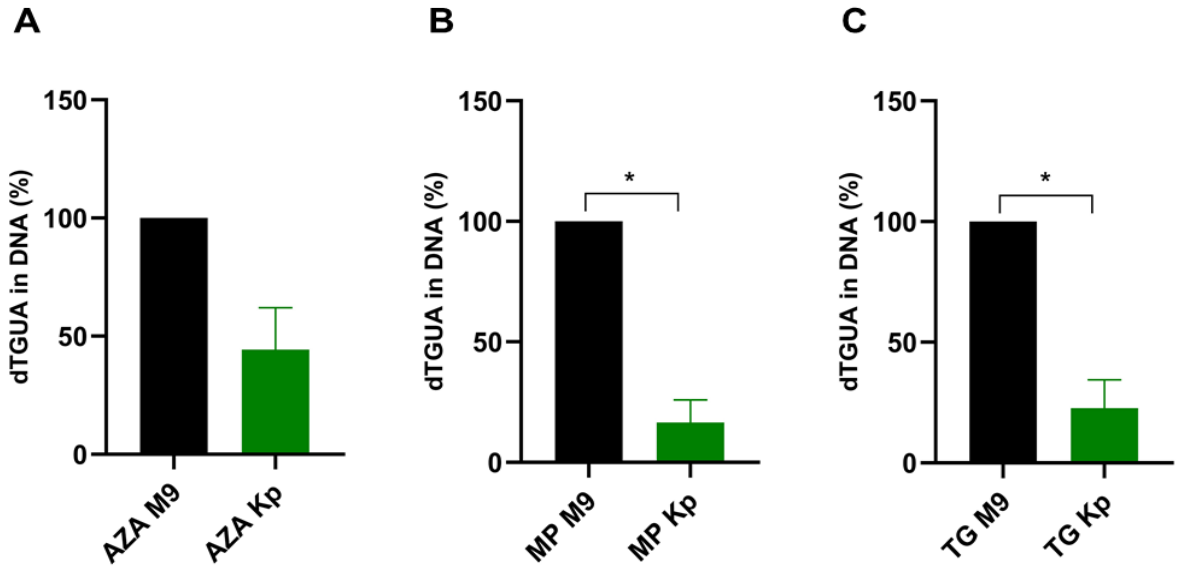

Figure 4.39. Concentrations of dTGUA (means ± SE) in the DNA, detected after a 72-hour treatment of NALM6 cells with 15 µM AZA (**A**), 2.5 µM MP (**B**) and 1.25 µM TG (**C**) in M9 (AZA M9/MP M9/TG M9) or exposed to *K. pneumoniae* (AZA Kp/MP Kp/TG Kp). Welch's t test ns.

Treatment of JURKAT cells with thiopurines exposed to *K. pneumoniae* determined lower levels of dTGUA incorporated in DNA compared to drugs in M9, used as control, although Welch's t test assessed a significant decrease only when cells were treated with TG exposed to bacteria (**Figure 4.40 A-B-C**).

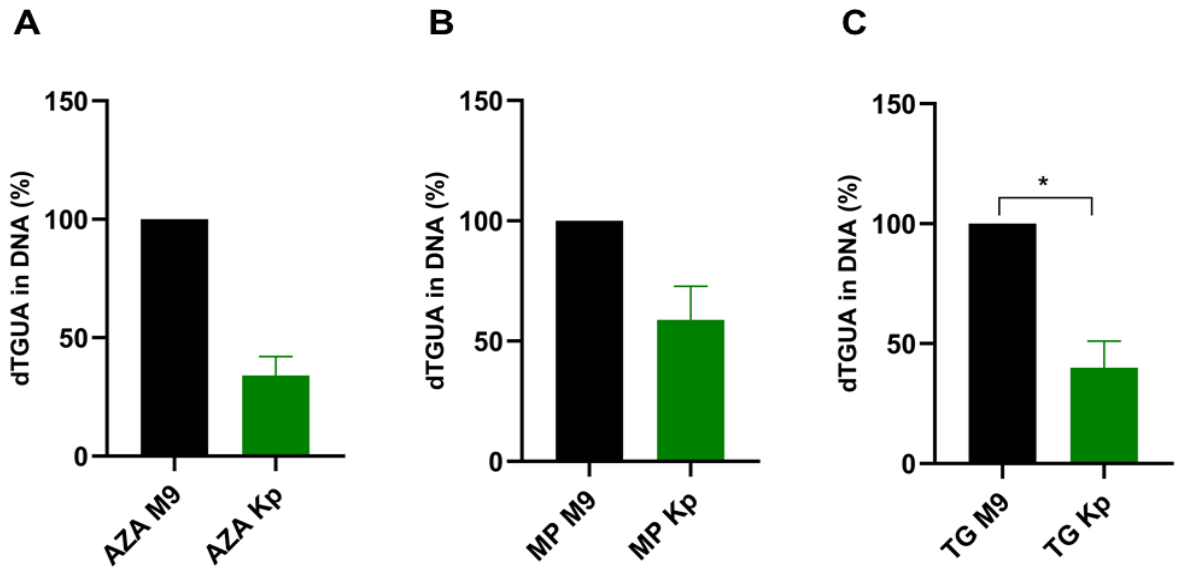

Figure 4.40. Concentrations of dTGUA (means ± SE) in the DNA detected after a 72-hour treatment of JURKAT cells with 15 µM AZA (**A**), 2.5 µM MP (**B**) and 1.25 µM TG (**C**) in M9 (AZA M9, MP M9, TG M9) or exposed to *K. pneumoniae* (AZA Kp, MP Kp, TG Kp). Welch's t test ns.

4.1.7 TPMT activity was not influenced by bacterial conditioned media

Since NALM6 cells treated with AZA exposed previously to *K. pneumoniae* presented higher levels of MeTIMP and in order to investigate if TPMT activity would be influenced by bacterial compounds released by the bacterial strain, the activity of the enzyme in NALM6 and JURKAT cells exposed to M9 and bacterial conditioned medium was measured.

Comparing the basal activity of the enzyme in the cell lines, TPMT activity was higher in NALM6 compared to JURKAT cells (**Figure 4.41**).

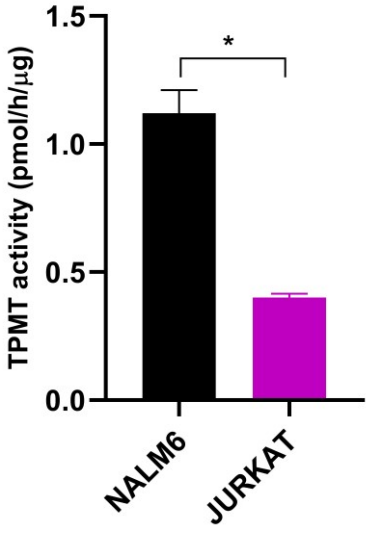

Figure 4.41. TPMT activity of NALM6 and JURKAT cells (means ± SE) in basal conditions. Welch's t test (* p<0.05).

As shown in **Figure 4.42**, there is no variation of TPMT activity in both cell lines exposed to M9 or to *K. pneumoniae* conditioned medium.

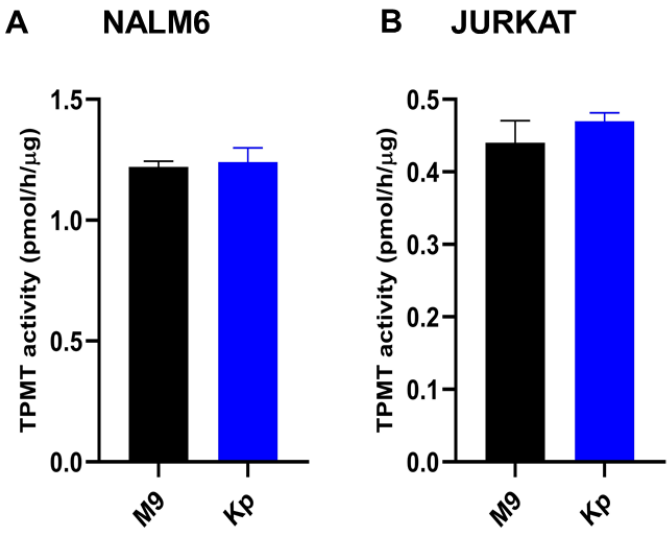

Figure 4.42. TPMT activity (means ± SE) of NALM6 (**A**) and JURKAT (**B**) cells exposed to M9 and in *K. pneumoniae* conditioned medium. Welch's t test ns.

4.1.8 Thiopurines detection in *K. pneumoniae*

In order to evaluate if thiopurines are internalized by *K. pneumoniae*, the drug amount inside bacterial cells was quantified after the *in vitro* exposure of *K. pneumoniae* to AZA, MP and TG (400 µM). As showed in **Table 4.7**, AZA was not detected in any sample; on the contrary, MP was detected in bacteria both after incubation with AZA and with MP, while TG was detected only after incubation with TG.

Bacterial samples	Concentration of AZA(µM)/bacterial pellet	Concentration of MP(µM)/bacterial pellet	Concentration of TG(µM)/bacterial pellet
K. pneumoniae exposed to AZA	0	2.71±0.10	0
K. pneumoniae exposed to MP	0	128.90±1.25	0
K. pneumoniae exposed to TG	0	0	142.85±1.35

Table 4.7. Average concentration of AZA, MP and TG (means ± SE) detected in *K. pneumoniae* pellets after *in vitro* exposure of the bacterial strain to thiopurines.

4.1.9 Metabolomic analyses on *K. pneumoniae* conditioned media

Metabolomic analyses were performed in order to investigate compounds differentially released by *K. pneumoniae* after exposure to AZA, MP and TG.

Results obtained from data acquired by LC-MS/MS both in positive (**Figure 4.43**) and negative (**Figure 4.44**) ionisation mode and from the comparison with spectra libraries underlined differences in extracellular metabolites secreted by *K. pneumoniae* exposed to AZA, MP and TG.

After exposure of the bacterial strain to AZA, nicotinic acid, N-carbamoyl aspartic acid and tryptophan were released in major quantity by *K. pneumoniae*, whereas adenosine, guanidinobutanoic acid, ketoglutaric acid and succinic acid were less present.

Moreover, tetrahydroharmine carboxylic acid, the dipeptide composed of L-proline and L-glutamic acid (Pro Glu), glutamate, tyrosine, phenylalanine, methionine, nicotinic acid, N-carbamoyl aspartic acid and orotic acid were released more abundantly in *K. pneumoniae* exposed to MP; instead, malic acid, phenyllactic acid, desmeninol, itaconic acid, hydroxyisovaleric acid and succinic acid were less present.

Lastly, *K. pneumoniae* exposed to TG presented higher levels of tetrahydroharmine carboxylic acid, glutamate, tyrosine, methionine, guanine, orotic and proprionic acid and lower levels of adenosine, phenyllactic acid, hydroxyisovaleric acid and ketoglutaric acid.

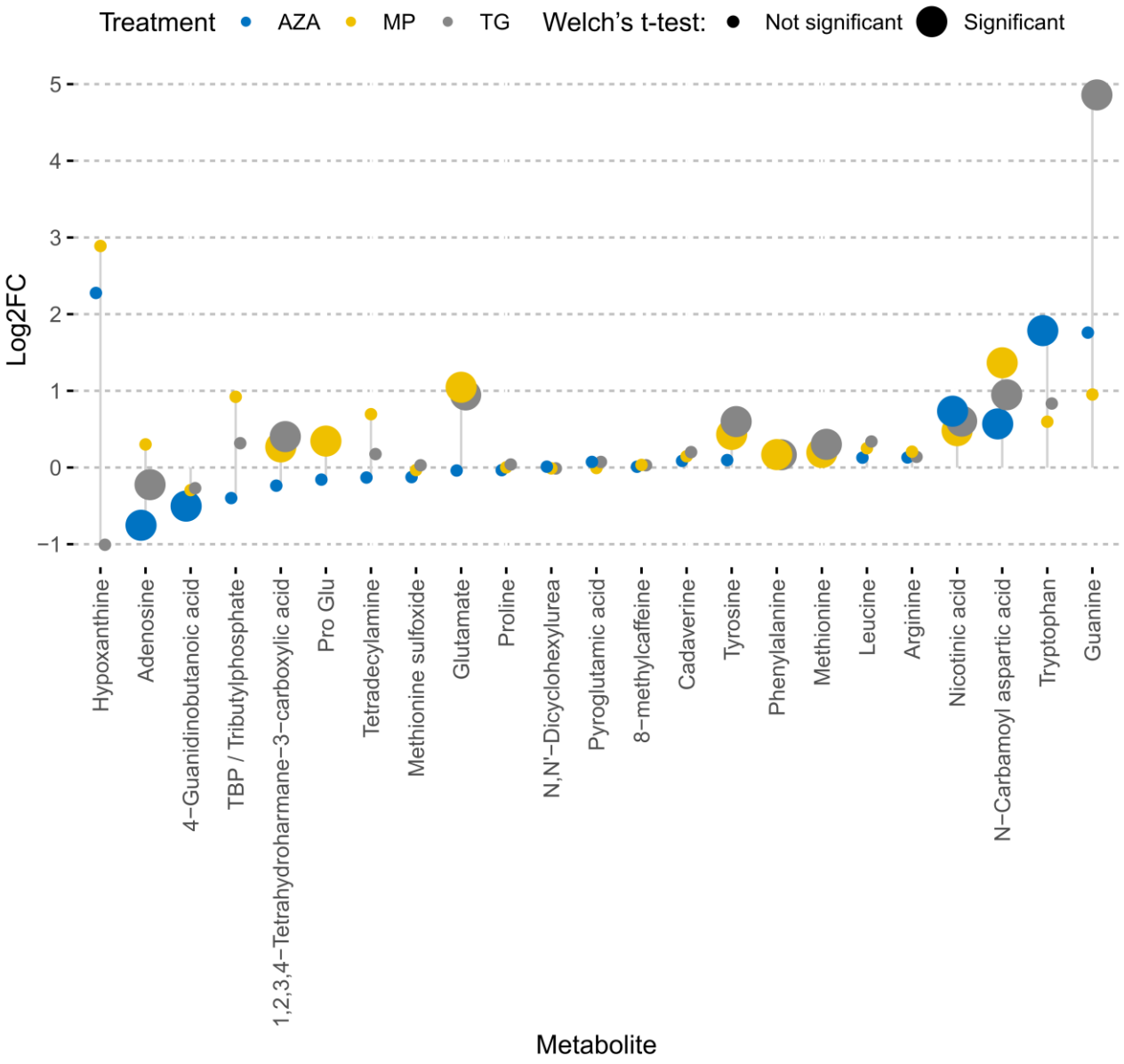

Figure 4.43. Results of metabolomic analysis of differences in extracellular metabolites secreted by *K. pneumoniae* exposed to AZA (blue), MP (yellow) and TG (grey). Data were obtained in positive ionisation mode. The points in the graph show the log2 fold change (Log2FC) calculated between the median of the untreated control group (*K. pneumoniae* not exposed to drugs) and the respective experimental condition (*K. pneumoniae* not exposed to AZA, MP or TG). Differences considered significant by Welch's t test (p<0.05) are shown with large dots, whereas the ones considered non-significant are shown with small dots.

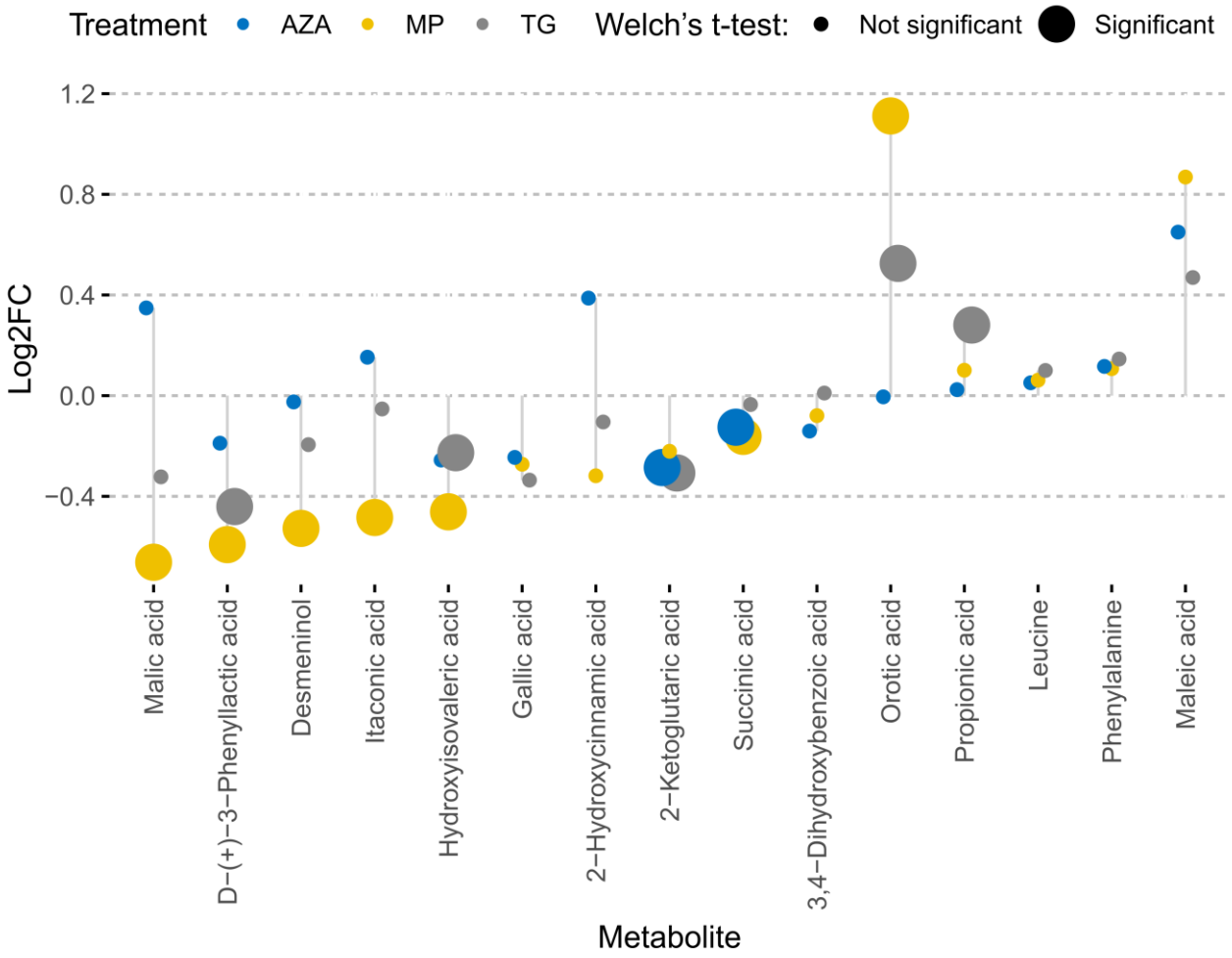

Figure 4.44. Results of metabolomic analysis of differences in extracellular metabolites secreted by *K. pneumoniae* exposed to AZA (blue), MP (yellow) and TG (grey). Data were obtained in negative ionisation mode. The points in the graph show the log2 fold change (Log2FC) calculated between the median of the untreated control group (*K. pneumoniae* not exposed to drugs) and the respective experimental condition (*K. pneumoniae* not exposed to AZA, MP or TG). Differences considered significant by Welch's t test (p<0.05) are shown with large dots, whereas the ones considered non-significant are shown with small dots.

4.2 Role of *K. pneumoniae* GPB in mediating the *in vitro* effects of thiopurines

4.2.1 Effects on cytotoxicity of thiopurines exposed to the GPBs of candidate bacterial strains

MTT tests were performed in order to compare the cytotoxicity of thiopurines exposed or not to bacterial GPBs and thus evaluate a potential interference of compounds secreted by bacteria.

Cytotoxic effects on NALM6 and JURKAT cells after a 72-hour treatment with AZA, MP and TG exposed or not to *E. coli* and *S. enterica* GPBs are shown in **Figure 4.45 – 4.48**. ANOVA did not demonstrate a statistically significant difference.

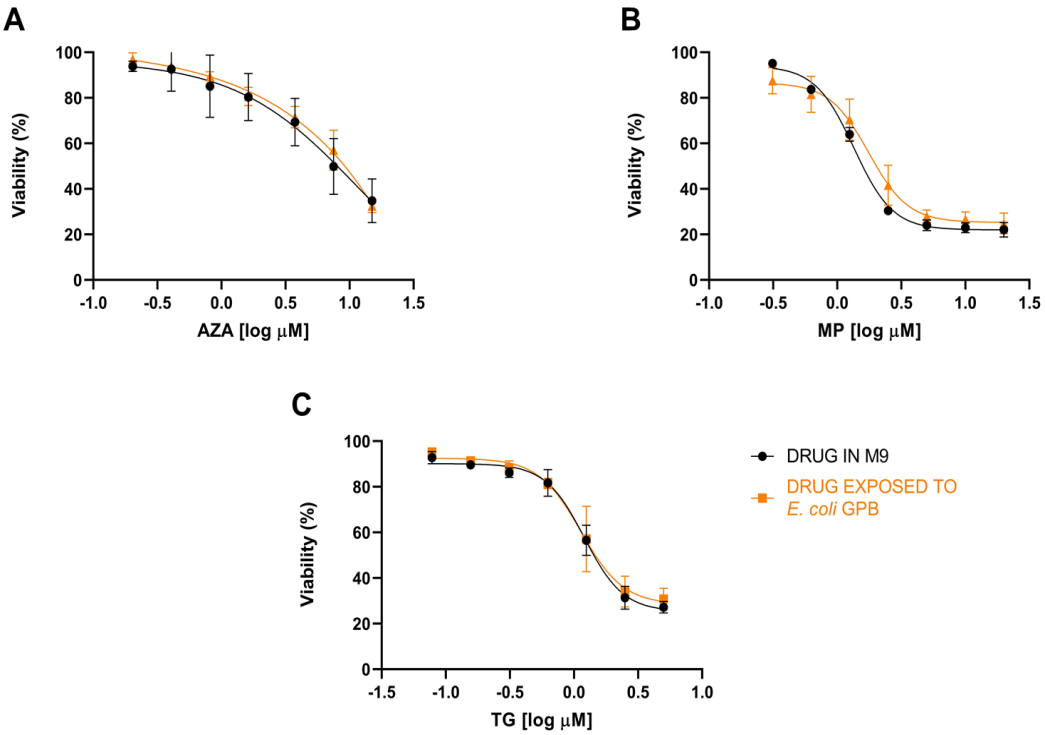

Figure 4.45. Evaluation of cytotoxic effects through MTT assay on NALM6 cells after treatment with several concentrations of AZA (**A**), MP (**B**) and TG (**C**) exposed to *E. coli* GPB or not. Two-way ANOVA (drugs in M9 vs drugs exposed to *E. coli* GPB ns).

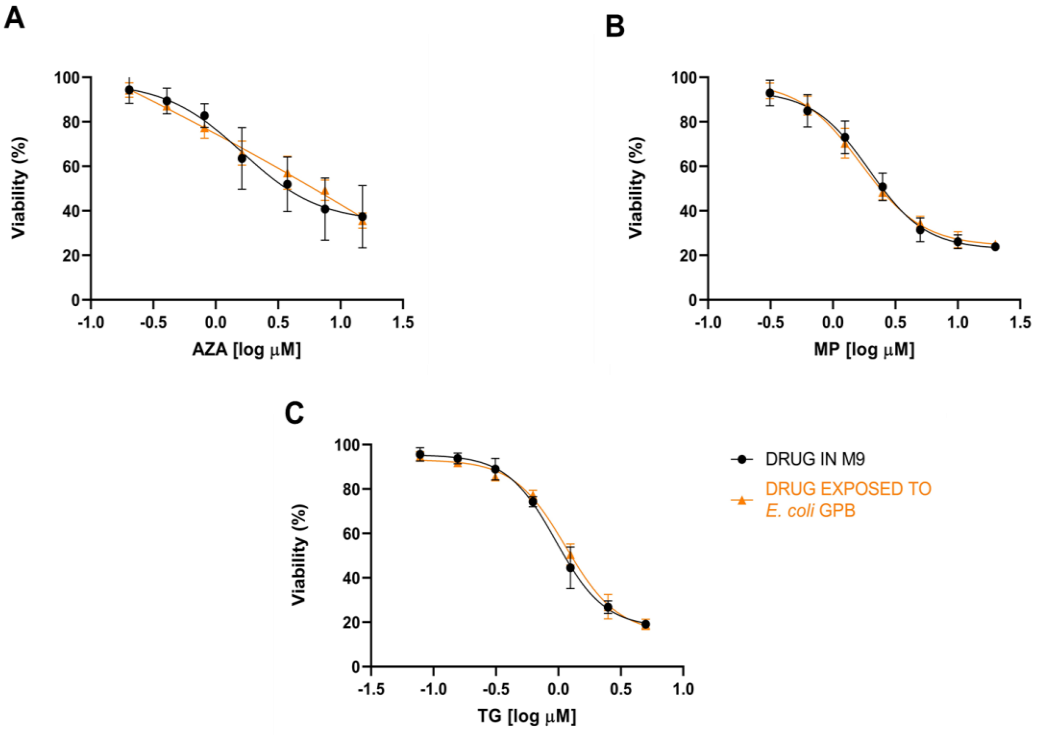

Figure 4.46. Evaluation of cytotoxic effects through MTT assay on JURKAT cells after treatment with several concentrations of AZA (**A**), MP (**B**) and TG (**C**) exposed to *E. coli* GPB or not. Two-way ANOVA (drugs in M9 vs drugs exposed to *E. coli* GPB ns).

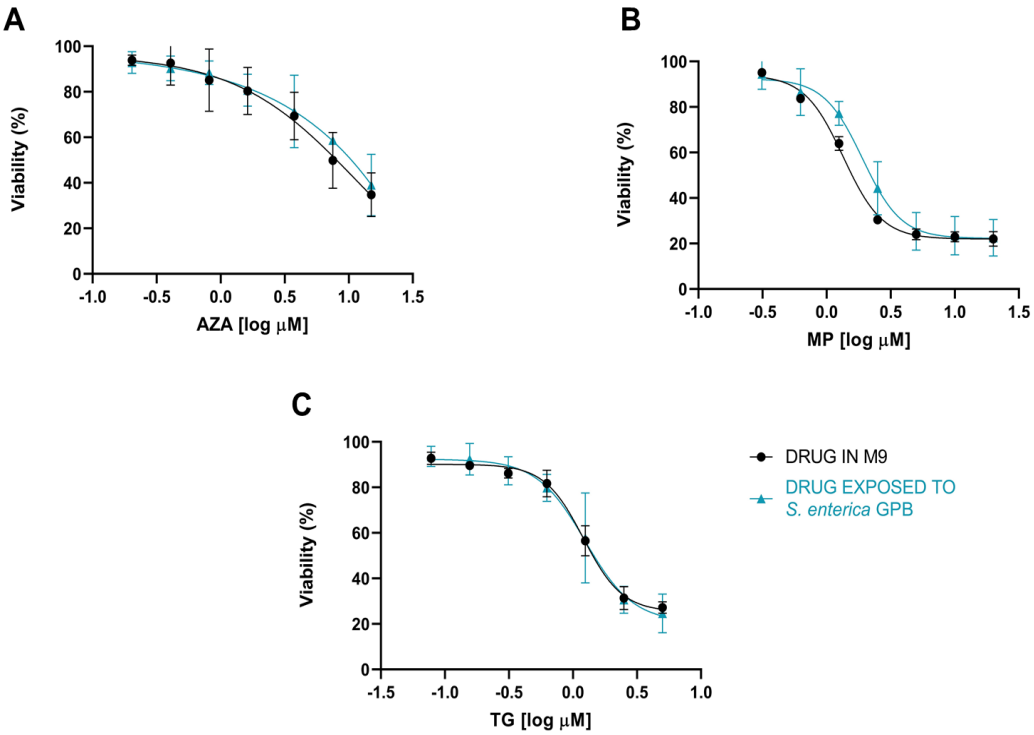

Figure 4.47. Evaluation of cytotoxic effects through MTT assay on NALM6 cells after treatment with several concentrations of AZA (**A**), MP (**B**) and TG (**C**) exposed to *S. enterica* GPB or not. Two-way ANOVA (drugs in M9 vs drugs exposed to *S. enterica* GPB ns).

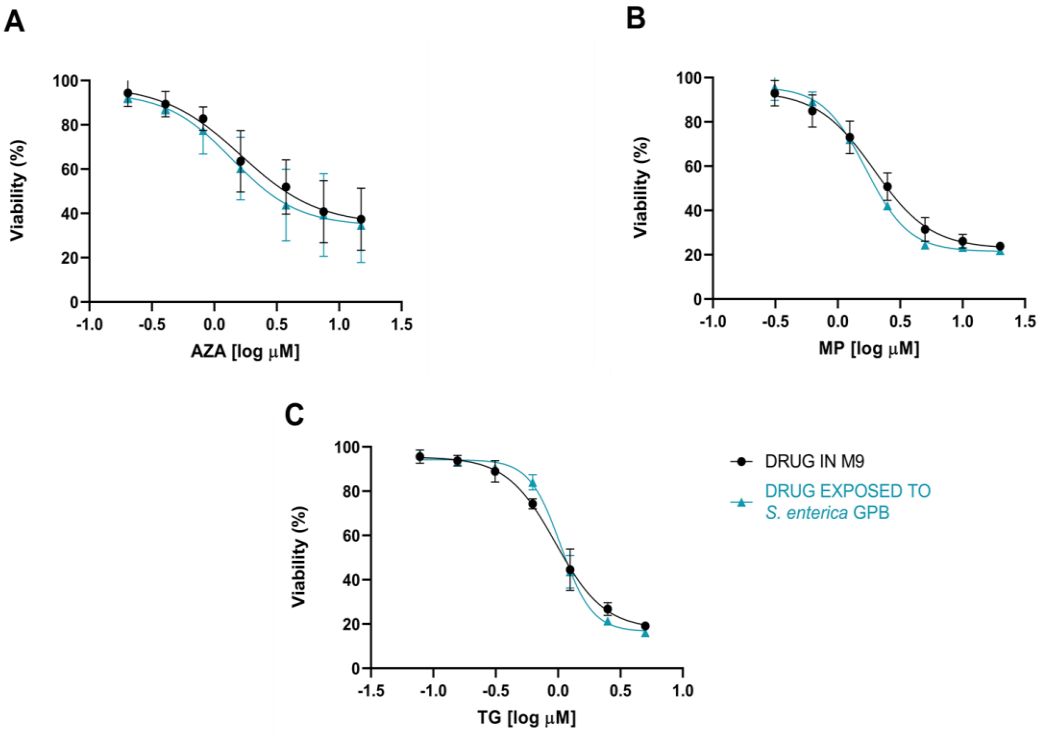

Figure 4.48. Evaluation of cytotoxic effects through MTT assay on JURKAT cells after treatment with several concentrations of AZA (**A**), MP (**B**) and TG (**C**) exposed to *S. enterica* GPB or not. Two-way ANOVA (drugs in M9 vs drugs exposed to *S. enterica* GPB ns).

Interestingly, the cytotoxic effects on NALM6 B cells (**Figure 4.49 A-B-C**) after a treatment of 72 hours with AZA, MP and TG were significantly lower after incubation with *K. pneumoniae* GPB compared to the drugs not exposed to GPB. In particular, at the concentration of 7.5 μM of AZA, at concentrations higher than 1.25 μM of MP and at the concentration of 1.25 μM of TG, a significant difference between the toxicity of drugs exposed or not to the bacterial strain was observed (ANOVA with Bonferroni's post-test).

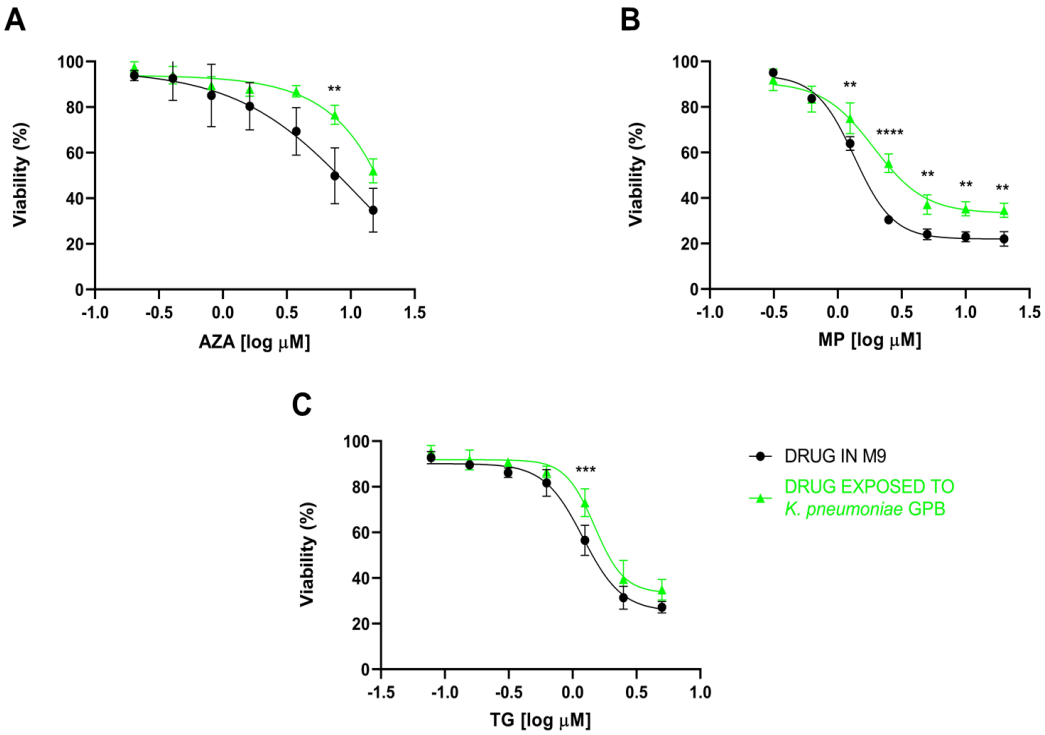

Figure 4.49. Evaluation of cytotoxic effects through MTT assay on NALM6 cells after treatment with several concentrations of AZA (**A**), MP (**B**) and TG (**C**) exposed to *K. pneumoniae* GPB or not. Two-way ANOVA (drugs in M9 vs drugs exposed to *K. pneumoniae* GPB $p<0.0001$). Bonferroni post-test (** = $p<0.01$ *** = $p<0.001$ **** = $p<0.0001$).

Furthermore, as shown in **Figure 4.50 B-C**, the cytotoxic effects on JURKAT cells after 72 hours-treatment with MP and TG previously incubated with *K. pneumoniae* GPB were significantly decreased compared to the control. In particular at the concentrations 2.5 and 1.25 μM of MP and at the concentration of 1.25 and 0.6 μM of TG, a significant difference between the toxicity of drugs exposed or not to the bacterial strain was observed (ANOVA with Bonferroni's post-test). As shown in **Figure 4.50 A**, there is a trend that indicates a lower cytotoxicity after the exposure of AZA to the bacterial strain, even if no statistically significant variation in cytotoxicity of the drug on JURKAT cells was highlighted.

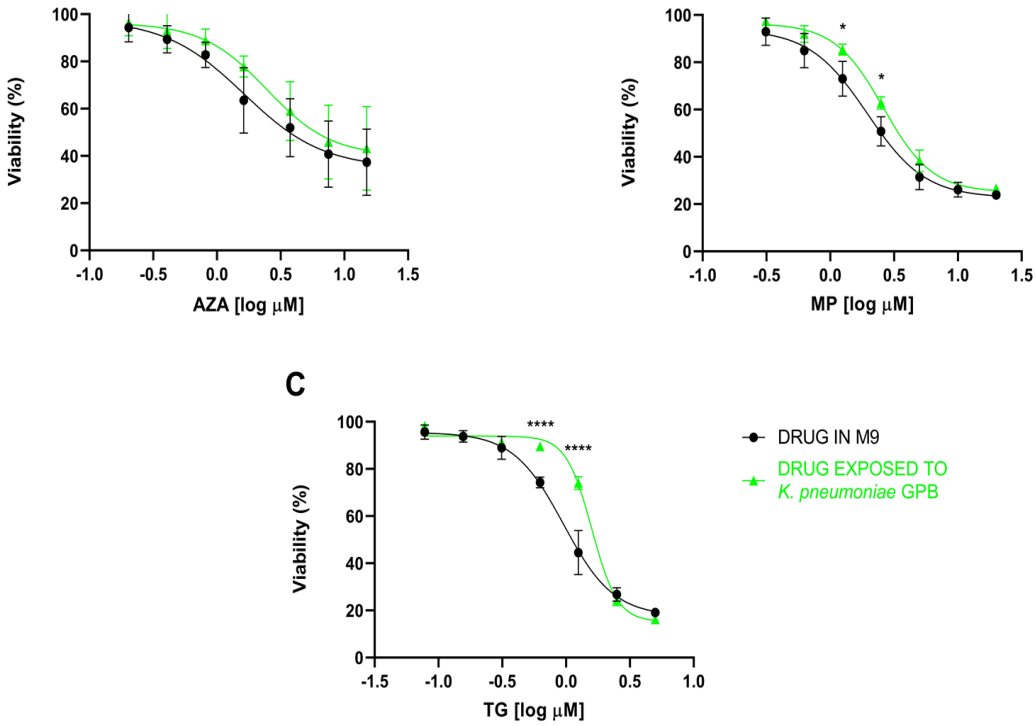

Figure 4.50. Evaluation of cytotoxic effects through MTT assay on JURKAT cells after treatment with several concentrations of AZA (**A**), MP (**B**) and TG (**C**) exposed to *K. pneumoniae* GPB or not. Two-way ANOVA (drugs in M9 vs drugs exposed to *K. pneumoniae* GPB ns, $p<0.0001$, $p<0.0001$ respectively). Bonferroni post-test (* = $p<0.05$ **** = $p<0.0001$).

4.2.2 UV analysis evidenced a reduction of the concentration of AZA only after exposure to *K. pneumoniae* GPB

UV analyses were performed in order to evaluate differences in concentrations of thiopurines after incubation of these drugs with bacterial GPBs.

UV analysis did not show a variation of concentrations of thiopurines exposed to *E. coli* and *S. enterica* GPBs (respectively **Figure 4.51 and 4.52**) in comparison with drugs in M9.

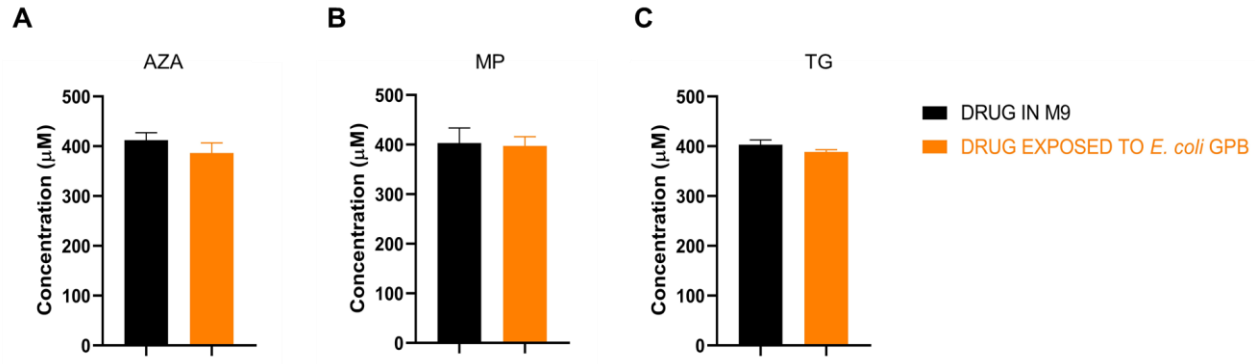

Figure 4.51. Concentration of AZA (**A**), MP (**B**) and TG (**C**) (means ± SE) exposed to *E. coli* GPB or not. Welch's t-test ns.

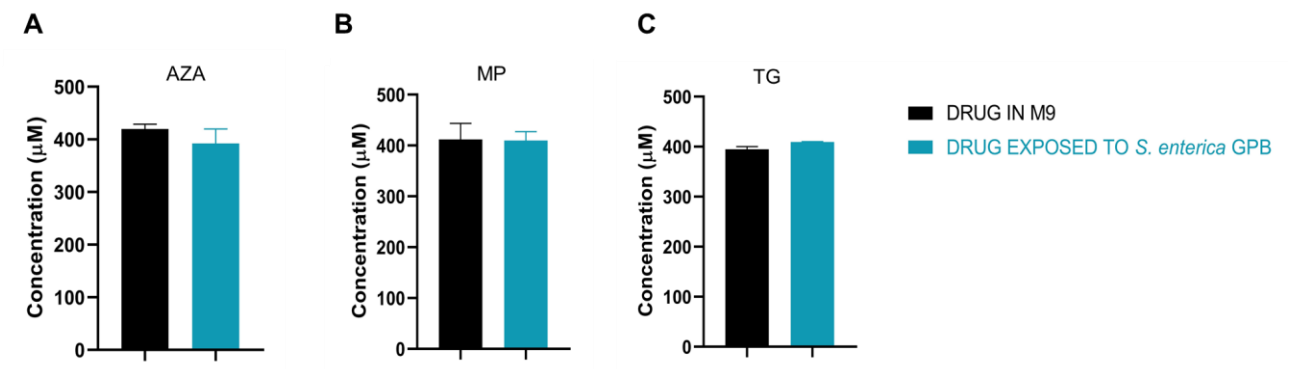

Figure 4.52. Concentration of AZA (**A**), MP (**B**) and TG (**C**) (means ± SE) exposed to *S. enterica* GPB or not. Welch's t-test ns.

As shown in **Figure 4.53 A**, the concentration of AZA significantly decreased after incubation with *K. pneumoniae* GPB compared to the drug in M9. Instead, the *in vitro* exposure to GPB did not determine a reduction of concentration of MP and TG (**Figure 4.53 B-C**).

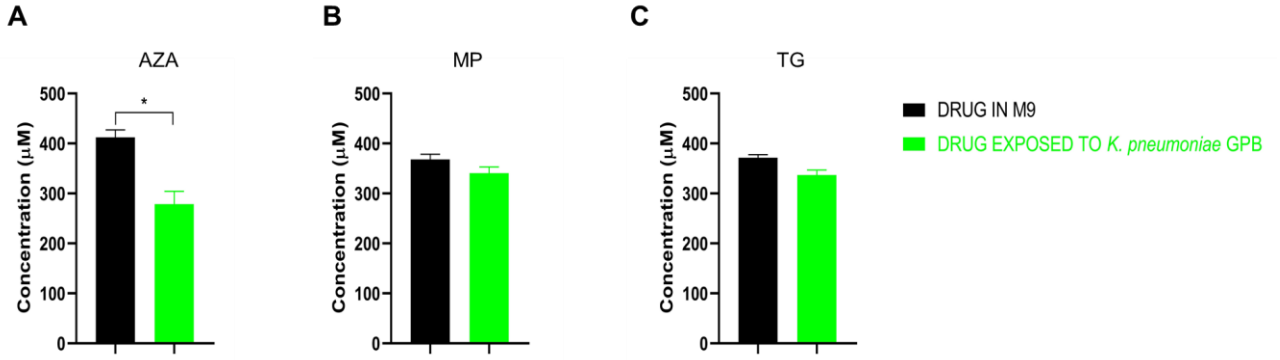

Figure 4.53. Concentration of AZA (**A**), MP (**B**) and TG (**C**) (means ± SE) exposed to *K. pneumoniae* GPB or not. Welch's t-test (* $p<0.05$).

4.2.3 Effects on cytotoxicity of methylprednisolone exposed to *K. pneumoniae*, *E. coli* and *S. enterica* GPBs

ANOVA did not underline any significant difference between the cytotoxic effects on NALM6 cells after treatment of 72 hours with methylprednisolone exposed or not to *K. pneumoniae*, *E. coli* and *S. enterica* (**Figure 4.54 A-B-C**).

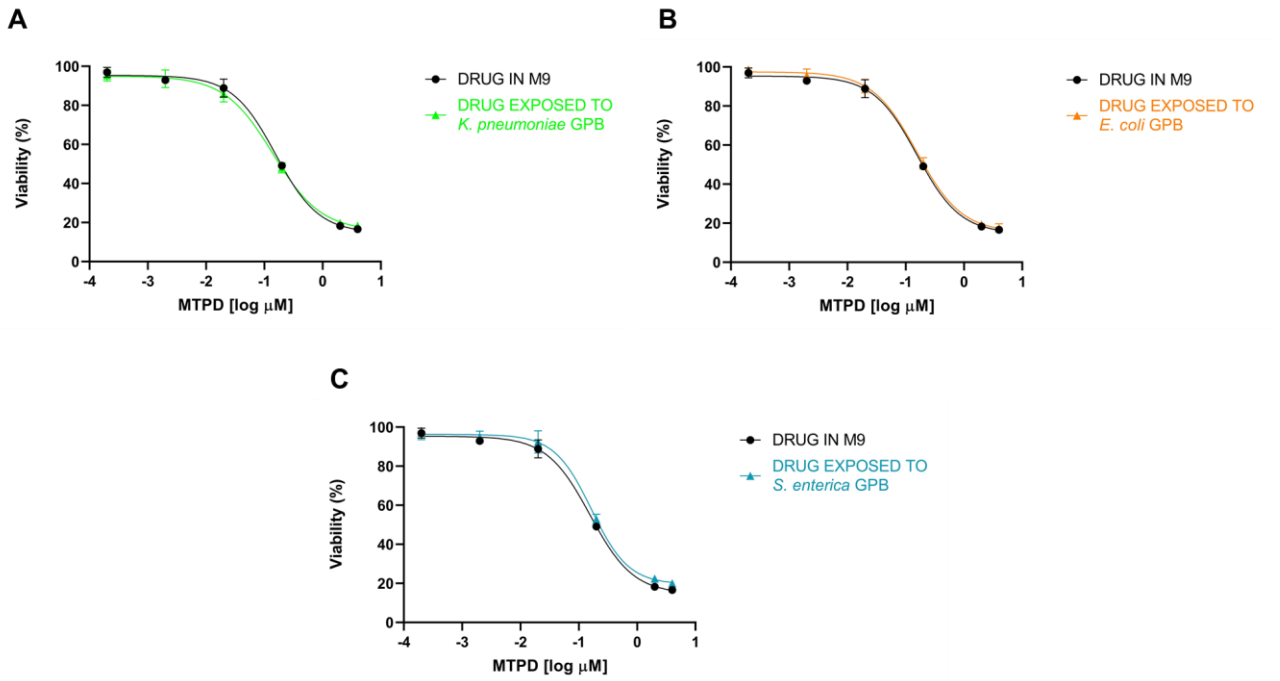

Figure 4.54. Evaluation of cytotoxic effects through MTT assay on NALM6 cells after treatment with several concentrations of methylprednisolone (MTPD) exposed or not to the GPBs of *K. pneumoniae* (**A**), to *E. coli* (**B**) and to *S. enterica* (**C**). Two-way ANOVA (drug in M9 vs drug exposed to GPBs ns).

4.2.4 *K. pneumoniae* EPS did not affect the cytotoxicity of thiopurines

In order to understand if *K. pneumoniae* EPS could interact with thiopurines reducing the cytotoxicity described above, *in vitro* exposure of thiopurines to *K. pneumoniae* EPS and subsequent cytotoxicity tests were performed.

The results evidenced that the incubation with several concentration of EPS did not affect the cytotoxicity of the thiopurines of NALM6 (**Figure 4.55**) and JURKAT (**Figure 4.56**) cells.

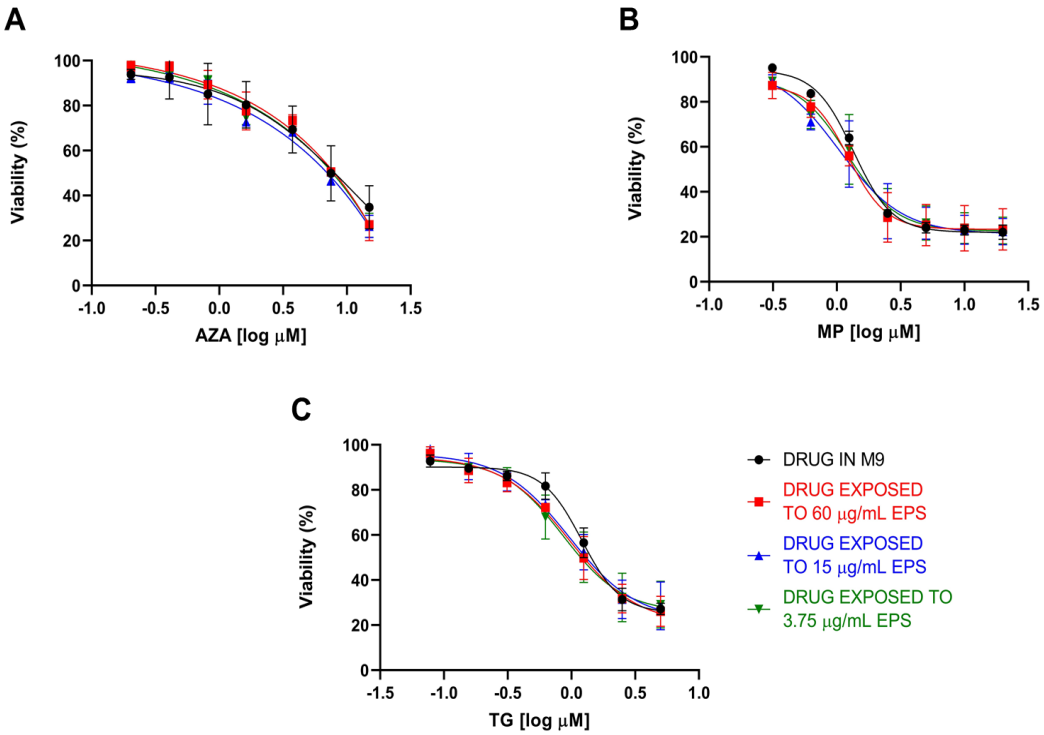

Figure 4.55. Evaluation of cytotoxic effects through MTT assay on NALM6 cells after treatment with several concentrations of AZA (**A**), MP (**B**) and TG (**C**) exposed or not to *K. pneumoniae* EPS. Two-way ANOVA (drugs in M9 vs drug exposed to increasing concentrations of EPS ns).

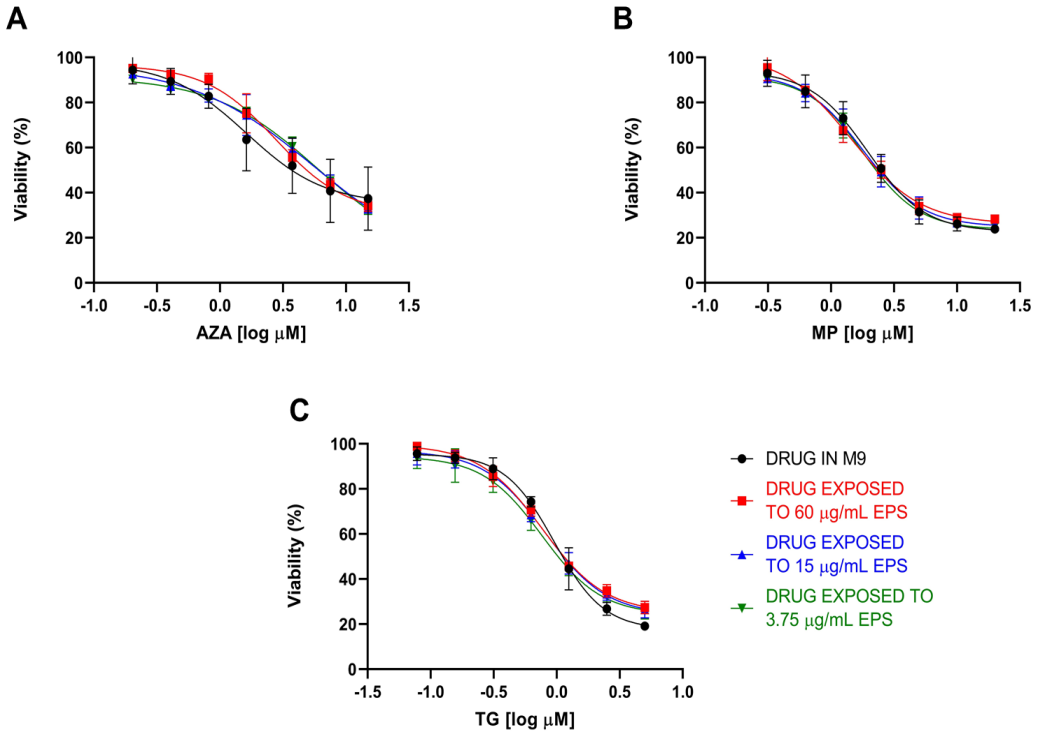

Figure 4.56. Evaluation of cytotoxic effects through MTT assay on JURKAT cells after treatment with several concentrations of AZA (**A**), MP (**B**) and TG (**C**) exposed or not to *K. pneumoniae* EPS. Two-way ANOVA (drugs in M9 vs drug exposed to increasing concentrations of EPS ns).

4.2.5 Exposure to *K. pneumoniae* EPS did not cause variation in the concentration of thiopurines

UV analysis did not show a variation of the concentration of thiopurines exposed to several concentrations of *K. pneumoniae* EPS (**Figure 4.57**) in comparison with drugs in M9.

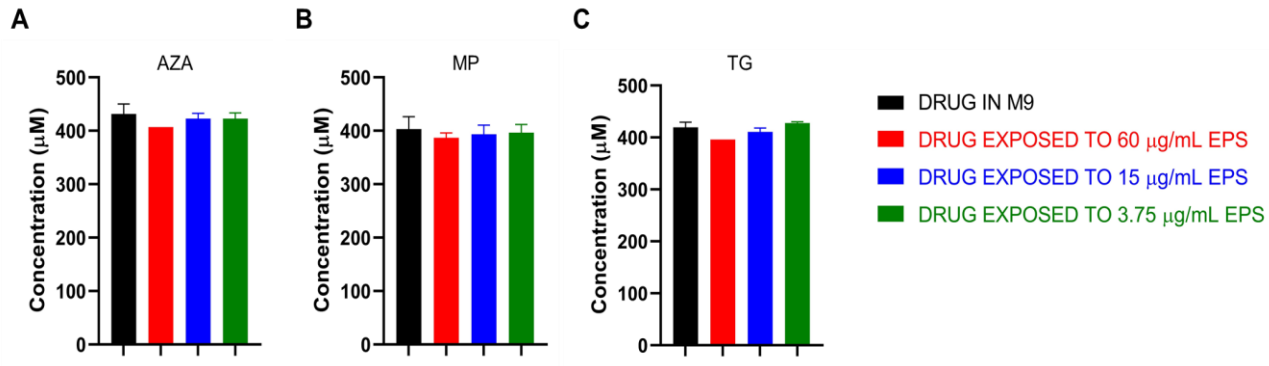

Figure 4.57. Concentration of AZA (**A**), MP (**B**) and TG (**C**) (means ± SE) exposed to *K. pneumoniae* EPS or not. Welch's t-test ns.

4.2.6 Detection of hypoxanthine (HP) in *K. pneumoniae* GPB

In order to evaluate the presence of purines, thiopurines analogous that could interfere with the cytotoxicity of these drugs, quantification of guanine (G) and hypoxanthine (HP) in *K. pneumoniae* GPB derived from the *in vitro* exposure was also performed. HP (9.3 ± 0.97 µM), but not G, was detected in *K. pneumoniae* GPB exposed or not to thiopurines.

4.3 Role of *P. aeruginosa* in mediating the *in vitro* effects of thiopurines

4.3.1 Bacterial susceptibility to thiopurines

The Broth Microdilution Susceptibility Test showed that even the highest concentration tested (400 µM) of AZA, MP, TG and MTX did not affect the growth of *P. aeruginosa.* Thus, 400 µM of each drug was chosen for the subsequent *in vitro* exposure.

4.3.2 Effects on cytotoxicity of thiopurines exposed to *P. aeruginosa*

MTT tests were performed in order to compare the cytotoxic effects of drugs in M9, used as control, and the ones of drugs exposed to *P. aeruginosa* or to its GPB and consequently to evaluate a potential microbial metabolism or interference.

The cytotoxic effects on NALM6 B cells after treatment of 72 hours with AZA, MP and TG exposed or not to *P. aeruginosa* and its GPB are shown in **Figure 4.58 A-B-C**. Interestingly, Bonferroni post-test underlined a statistically significant difference at the concentration of 2.5 µM of MP and starting from the concentration 1.25 µM of TG between drugs exposed to bacteria and drugs in M9.

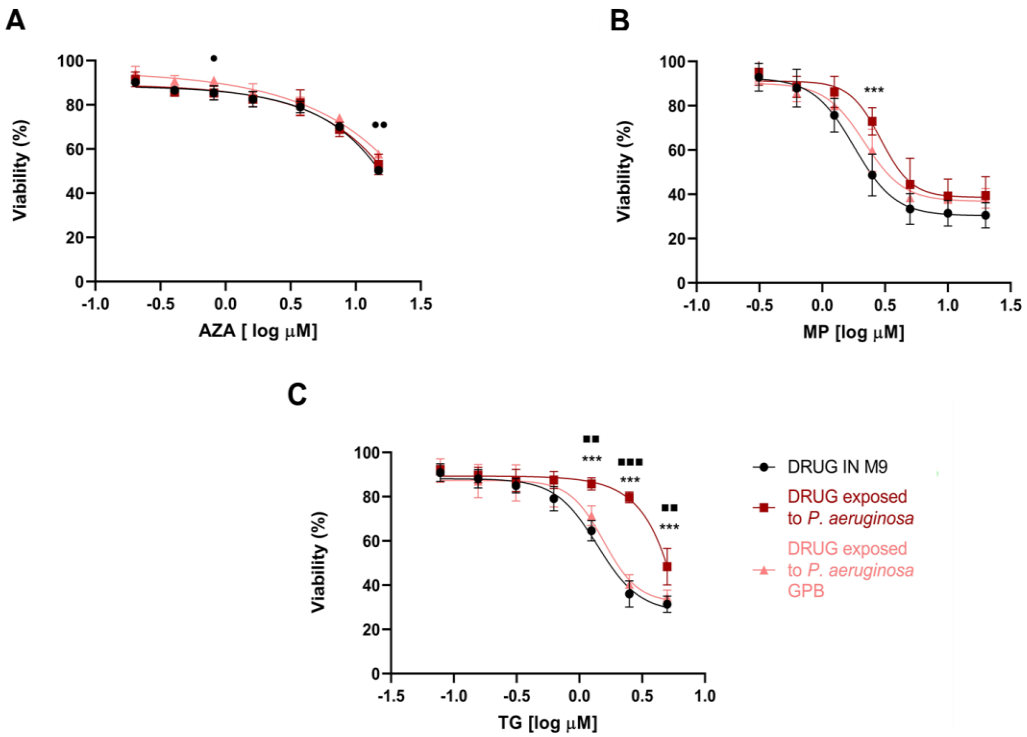

Figure 4.58. Evaluation of cytotoxic effects through MTT assay on NALM6 cells after treatment with several concentrations of AZA (**A**), MP (**B**) and TG (**C**) exposed or not to *P. aeruginosa* and to its GPB. Two-way ANOVA (drugs in M9 vs drugs exposed to *P. aeruginosa* vs drugs exposed to GPB $p<0.0001$, $p<0.001$, $p<0.0001$ respectively). Bonferroni post-test (*** = $p<0.001$ drugs in M9 vs drugs exposed to *P. aeruginosa*; ● = $p<0.05$ ●● = $p<0.01$ drugs in M9 vs drugs exposed to *P. aeruginosa* GPB; ▪▪ = $p<0.01$ ▪▪▪ = $p<0.001$ drugs exposed to *P. aeruginosa* vs drugs exposed to *P. aeruginosa* GPB).

The cytotoxic effects on JURKAT T cells after treatment of 72 hours with AZA, MP and TG exposed or not to *P. aeruginosa* and its GPB are shown in **Figure 4.59 A-B-C**. In particular, Bonferroni post-test assessed a statistically significant difference at the concentrations ranging from 1.25 to 5 µM of MP and starting from the concentration 1.25 µM of TG between drugs exposed to bacteria and drugs in M9.

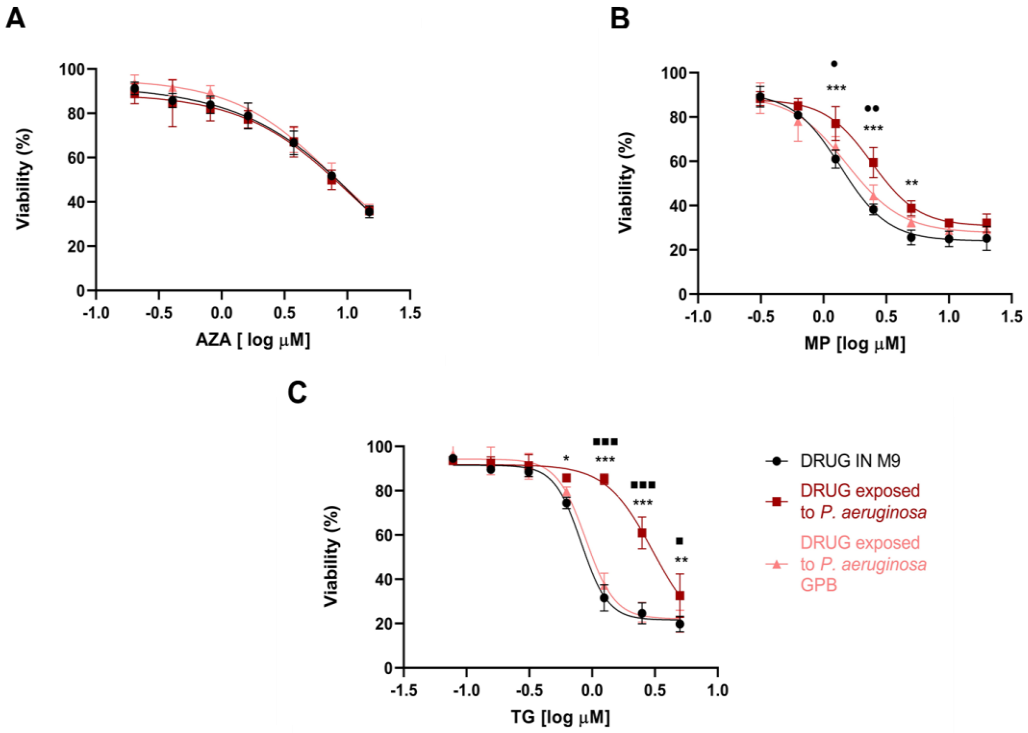

Figure 4.59. Evaluation of cytotoxic effects through MTT assay on JURKAT cells after treatment with several concentrations of AZA (**A**), MP (**B**) and TG (**C**) exposed or not to *P. aeruginosa* and to its GPB. Two-way ANOVA (drugs in M9 vs drugs exposed to *P. aeruginosa* vs drugs exposed to GPB $p<0.05$, $p<0.0001$, $p<0.0001$ respectively). Bonferroni post-test (** = $p<0.01$ *** = $p<0.001$ drugs in M9 vs drugs exposed to *P. aeruginosa*; ● = $p<0.05$ ●● = $p<0.01$ drugs in M9 vs drugs exposed to *P. aeruginosa* GPB; ▪ = $p<0.05$ ▪▪▪ = $p<0.001$ drugs exposed to *P. aeruginosa* vs drugs exposed to *P. aeruginosa* GPB).

4.3.3 Cytotoxicity of methotrexate exposed to *P. aeruginosa*

The cytotoxicity on NALM6 and JURKAT cells after treatment of 72 hours with methotrexate in M9 or exposed to *P. aeruginosa* is shown in **Figure 4.60 A-B**. In particular, ANOVA did not underline any statistically significant difference.

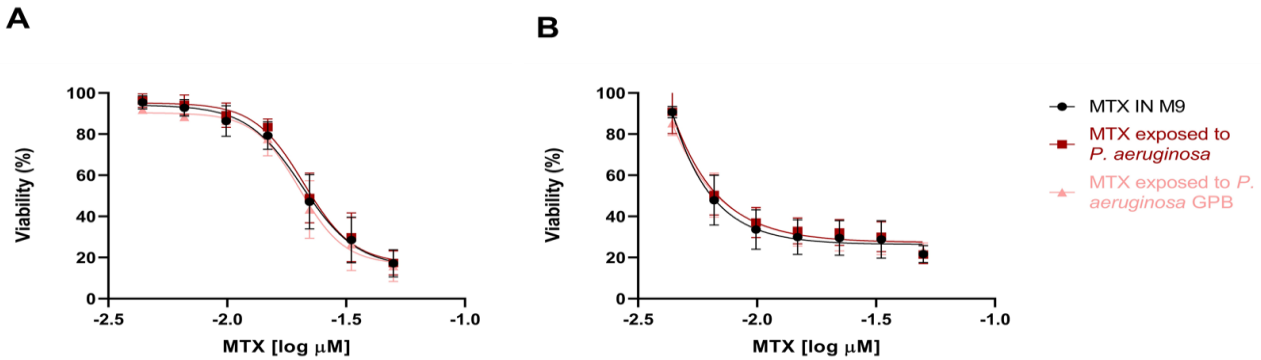

Figure 4.60. Evaluation of cytotoxic effects through MTT assay on NALM6 (**A**) and JURKAT (**B**) cells after treatment with several concentrations of methotrexate (MTX) exposed or not to *P. aeruginosa*. Two-way ANOVA (drug in M9 vs drug exposed to *P. aeruginosa* ns).

4.3.4 UV analysis evidenced a reduction of the concentration only of MP and TG after the exposure to *P. aeruginosa*

Noteworthy, as shown in **Figure 4.61 B-C**, only the thiopurines MP and TG were decreased in concentration after incubation with *P. aeruginosa*, but not with its GPB, compared to the control. Instead, the concentration of AZA did not vary based on the exposure to bacteria or to GPB (**Figure 4.61 A**).

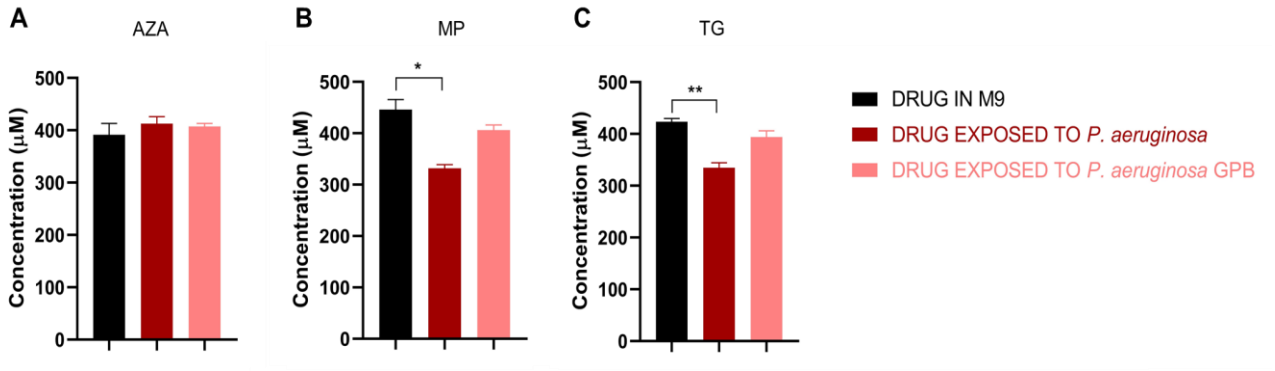

Figure 4.61. Concentration of AZA (**A**), MP (**B**) and TG (**C**) (means ± SE) exposed or not to *P. aeruginosa* and to its GPB. Welch's t-test (* $p<0.05$ ** $p<0.01$).

UV analysis did not show a variation of the concentration of methotrexate exposed to *P. aeruginosa* and its GPB (**Figure 4.62**) in comparison with drug in M9.

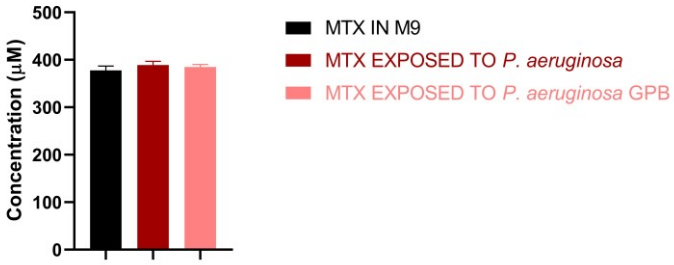

Figure 4.62. Concentration of methotrexate (MTX) (means ± SE) exposed or not to *P. aeruginosa* and to its GPB. Welch's t-test ns.

4.3.5 Thiopurine metabolites were lower in NALM6 and JURKAT cells treated with MP and TG previously exposed to *P. aeruginosa*

Thiopurine metabolites were measured in NALM6 and JURKAT cells treated with MP (2.5 µM) and TG (2.5 and 1.25 µM) exposed or not to *P. aeruginosa*. The concentrations tested were chosen on the basis of statistical significance of Bonferroni's analyses on cytotoxicity tests. The metabolites detected were TGMP, TGDP, TGTP, MeTGMP, MeTGDP, MeTGTP, TIMP, TIDP, TITP, MeTIMP, MeTIDP and MeTITP.

Interestingly, the concentration of total metabolites was lower in NALM6 cells treated with MP exposed to *P. aeruginosa* (**Table 4.8**) ($p<0.05$ and $p=0.12$ for 48 and 72 hours respectively). Furthermore, the thionucleotides TIMP, MeTIMP and TGMP were the most abundant at the different timings of treatment and experimental conditions.

Table 4.8. Average concentration of thiopurine metabolites (means ± SE) in NALM6 cells treated with 2.5 µM MP exposed to *P. aeruginosa* (MP Pa) or not (MP M9) for 48 and 72 hours.

Metabolites	48 hours				72 hours			
	MP M9		***MP Pa***		***MP M9***		***MP Pa***	
	pmol/Mio	%	pmol/Mio	%	pmol/Mio	%	pmol/Mio	%
TGMP	38.96±5.69	21.49	36.39±4.12	35.63	30.82±3.72	16.03	31.32±2.21	32.09
TGDP	1.06±0.46	0.58	0.16±0.16	0.16	1.17±0.35	0.61	0.00	0.00
TGTP	6.49±1.24	3.58	4.63±0.89	4.53	5.83±2.08	3.03	2.77±0.29	2.84
MeTGMP	0.56±0.28	0.31	0.85±0.10	0.83	0.49±0.04	0.25	0.82±0.16	0.84
MeTGDP	0.00	0.00	0.00	0.00	0.12±0.12	0.06	0.00	0.00
MeTGTP	0.73±0.16	0.40	0.52±0.27	0.51	0.59±0.37	0.31	0.73±0.11	0.75
TIMP	73.78±15.37	40.69	30.63±0.67	29.99	65.96±9.81	34.31	26.98±5.57	27.64
MeTIMP	59.73±3.14	32.95	28.95±4.47	28.35	87.27±31.65	45.39	34.97±2.76	35.83
TOT.	181.31±19.57	100.00	102.13±7.08	100.00	192.26±37.40	100.00	97.60±10.57	100.00

Ther trend of reduction of the total amount of thiopurine metabolites after treatment of JURKAT cells with MP exposed to *P. aeruginosa* for 48 and 72 hours is shown in **Table 4.9** (p=0.15 and p=0.12 respectively). Moreover, MeTIMP was the most abundant metabolite detected.

Table 4.9. Average concentration of thiopurine metabolites (means ± SE) in JURKAT cells treated with 2.5 µM MP exposed to *P. aeruginosa* (MP Pa) or not (MP M9) for 48 and 72 hours.

Metabolites	48 hours				72 hours			
	MP M9		***MP Pa***		***MP M9***		***MP Pa***	
	pmol/Mio	%	pmol/Mio	%	pmol/Mio	%	pmol/Mio	%
TGMP	19.04±9.59	9.48	4.78±0.48	13.36	8.75±1.98	7.49	6.36±0.78	16.85
TGDP	2.83±1.34	1.41	0.14±0.14	0.40	0.56±0.56	0.48	0.16±0.16	0.41
TGTP	39.97±18.56	19.91	6.53±1.36	18.23	9.87±1.87	8.44	7.03±0.87	18.61
MeTGMP	0.78±0.19	0.39	0.00	0.00	0.22±0.22	0.18	0.00	0.00
MeTGDP	0.82±0.28	0.41	0.00	0.00	0.33±0.33	0.28	0.00	0.00
MeTGTP	5.23±1.64	2.61	1.83±0.47	5.12	2.31±0.36	1.98	1.23±0.06	3.25
TIMP	8.43±4.09	4.20	1.66±0.66	4.64	3.55±1.83	3.04	1.47±0.39	3.91
MeTIMP	121.93±37.35	60.73	20.73±5.05	57.88	91.32±25.99	78.11	21.50±3.90	56.97
MeTITP	1.72±1.72	0.86	0.00	0.00	0.00	0.00	0.00	0.00
TOT.	200.77±74.47	100.00	35.81±6.11	100.00	116.92±31.42	100.00	37.74±5.38	100.00

Overall, ANOVA assessed a significant decrease of the nucleotides TGMP, TGDP, TGTP, MeTGDP, MeTGTP, TIMP and MeTIMP after treatment with MP incubated previously with *P. aeruginosa* (p<0.001; p<0.01; p<0.05; p<0.05; p<0.05; p<0.001; p<0.001 respectively) (**Figure 4.63-4.65, 4.67, 4.68, 4.69** and

4.71). The metabolites TIDP, MeTIDP and TITP were not detected in none of the cell lines after treatment with MP. Furthermore, comparing the metabolites generated after treatment of the cell lines, the nucleotides TGTP, MeTGDP and MeTGTP were more abundant in JURKAT cells (p<0.05; p<0.05; p<0.001 respectively) (**Figure 4.65, 4.67** and **4.68**), while MeTGMP and TIMP were significantly higher in NALM6 cells (p<0.001) (**Figure 4.66** and **4.69**). Moreover, thiopurine metabolites after treatment with MP did not differ depending on the timing of treatment.

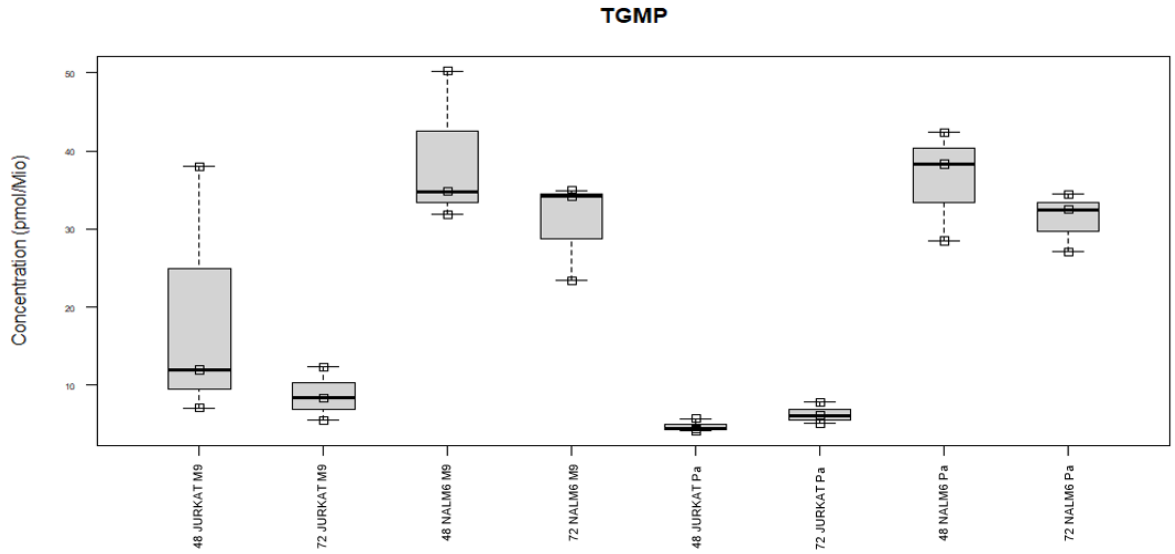

Figure 4.63. Concentration of the TGMP metabolite in NALM6 and JURKAT cells after treatment for 48 or 72 hours with 2.5 µM MP in M9 or exposed to *P. aeruginosa*. ANOVA (treatment time ns; cell line ns; experimental condition p<0.001).

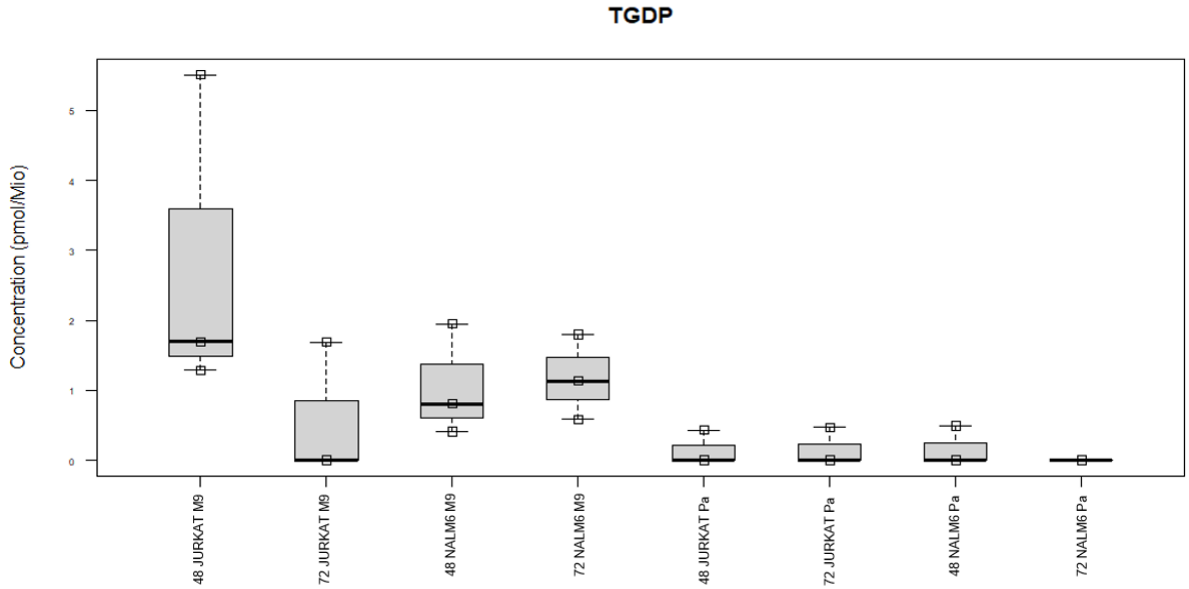

Figure 4.64. Concentration of the TGDP metabolite in NALM6 and JURKAT cells after treatment for 48 or 72 hours with 2.5 µM MP in M9 or exposed to *P. aeruginosa*. ANOVA (treatment time ns; cell line ns; experimental condition p<0.01).

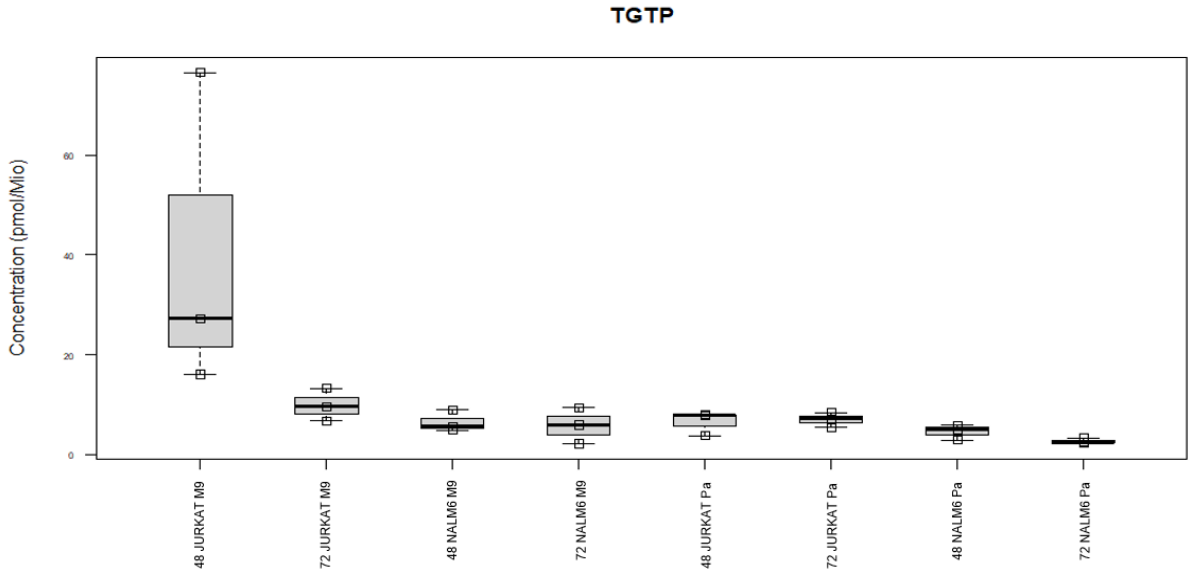

Figure 4.65. Concentration of the TGTP metabolite in NALM6 and JURKAT cells after treatment for 48 or 72 hours with 2.5 µM MP in M9 or exposed to *P. aeruginosa*. ANOVA (treatment time ns; cell line $p<0.05$; experimental condition $p<0.05$).

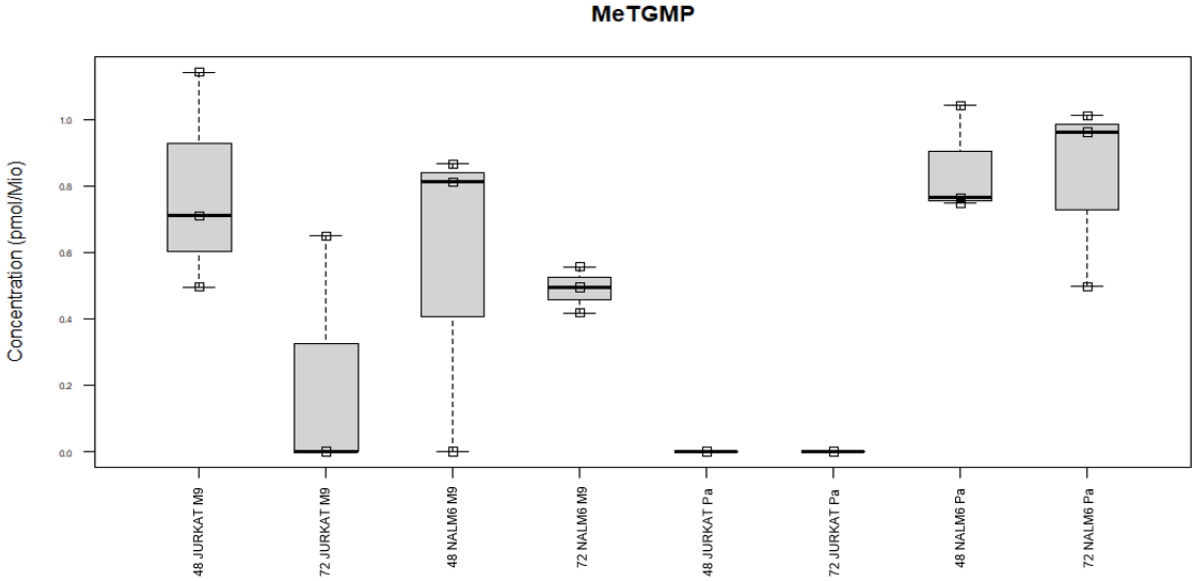

Figure 4.66. Concentration of the MeTGMP metabolite in NALM6 and JURKAT cells after treatment for 48 or 72 hours with 2.5 µM MP in M9 or exposed to *P. aeruginosa*. ANOVA (treatment time ns; cell line $p<0.01$; experimental condition ns).

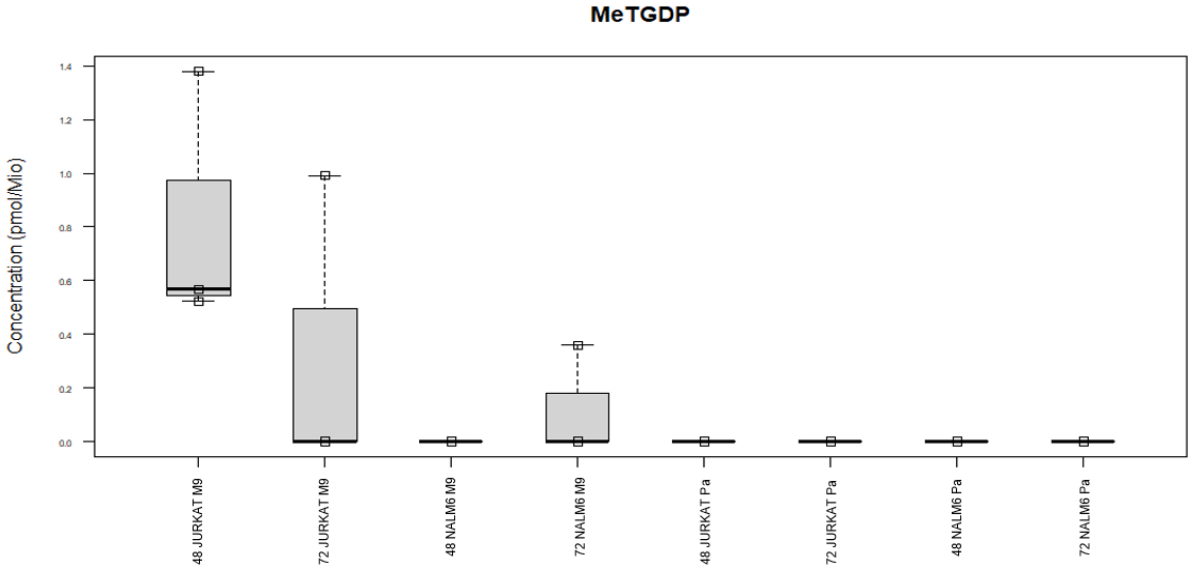

Figure 4.67. Concentration of the MeTGDP metabolite in NALM6 and JURKAT cells after treatment for 48 or 72 hours with 2.5 µM MP in M9 or exposed to *P. aeruginosa*. ANOVA (treatment time ns; cell line $p<0.05$; experimental condition $p<0.05$).

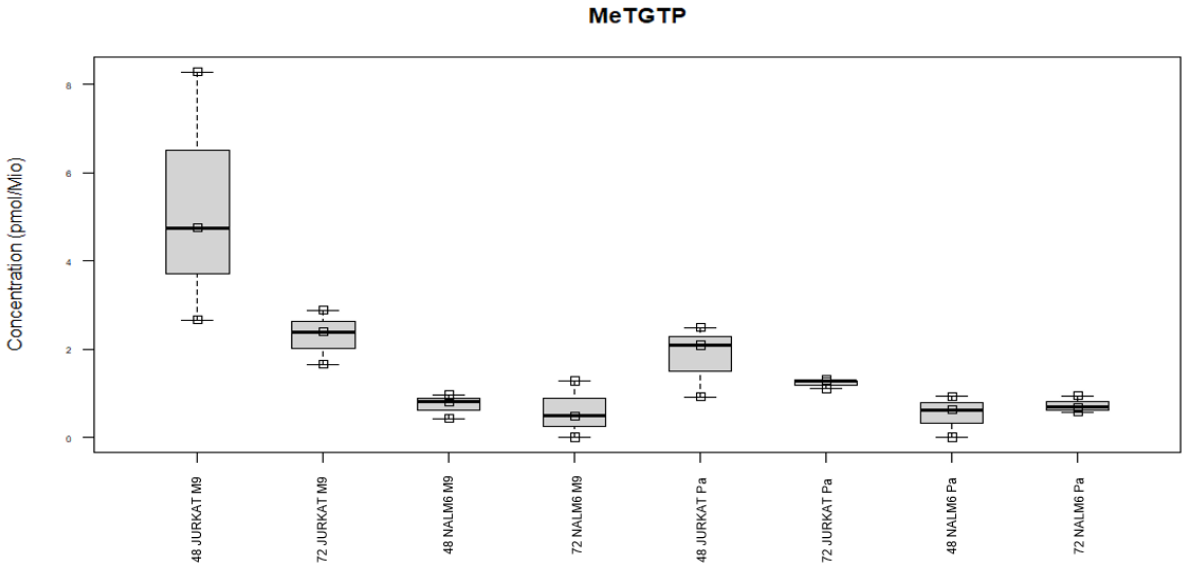

Figure 4.68. Concentration of the MeTGTP metabolite in NALM6 and JURKAT cells after treatment for 48 or 72 hours with 2.5 µM MP in M9 or exposed to *P. aeruginosa*. ANOVA (treatment time ns; cell line $p<0.001$; experimental condition $p<0.05$).

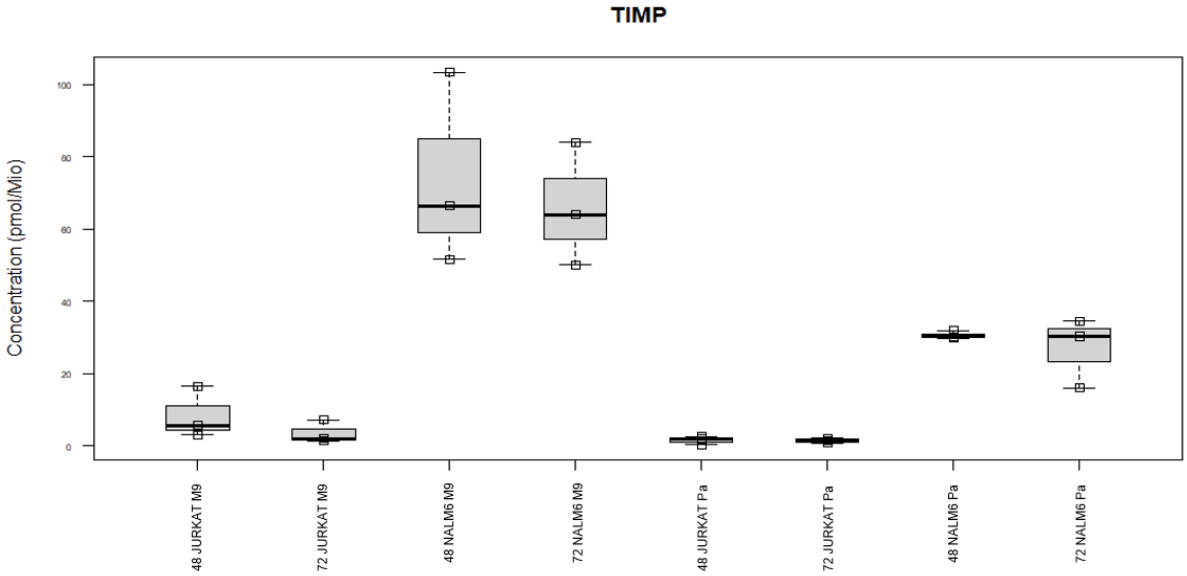

Figure 4.69. Concentration of the TIMP metabolite in NALM6 and JURKAT cells after treatment for 48 or 72 hours with 2.5 µM MP in M9 or exposed to *P. aeruginosa*. ANOVA (treatment time ns; cell line $p<0.001$; experimental condition $p<0.001$).

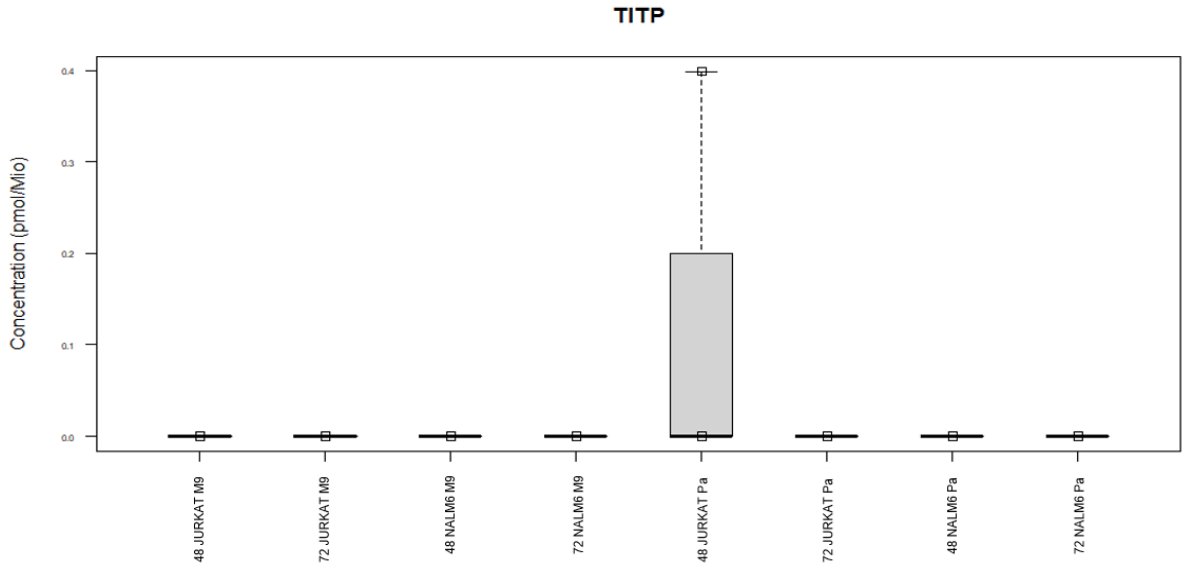

Figure 4.70. Concentration of the TITP metabolite in NALM6 and JURKAT cells after treatment for 48 or 72 hours with 2.5 µM MP in M9 or exposed to *P. aeruginosa*. ANOVA (treatment time ns; cell line ns; experimental condition ns).

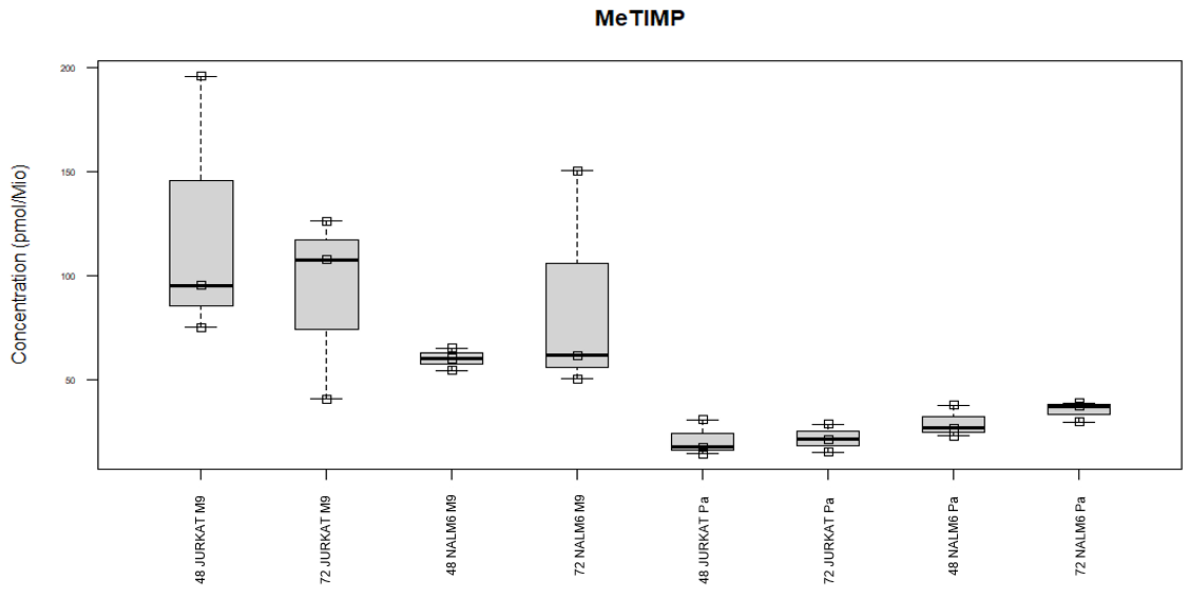

Figure 4.71. Concentration of the MeTIMP metabolite in NALM6 and JURKAT cells after treatment for 48 or 72 hours with 2.5 µM MP in M9 or exposed to *P. aeruginosa*. ANOVA (treatment time ns; cell line ns; experimental condition $p<0.001$).

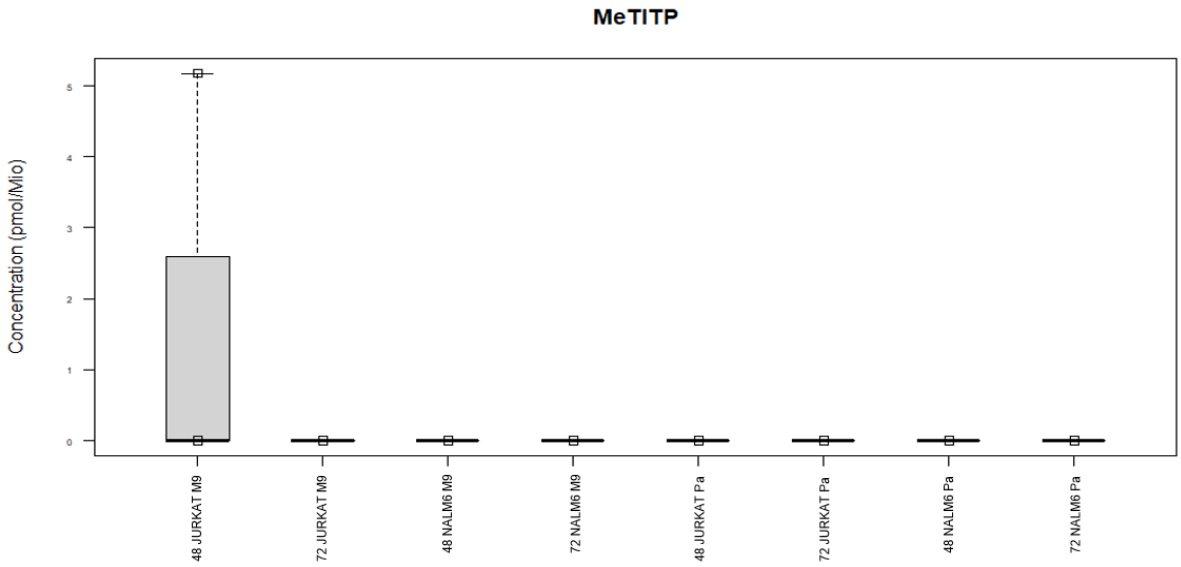

Figure 4.72. Concentration of the MeTITP metabolite in NALM6 and JURKAT cells after treatment for 48 or 72 hours with 2.5 µM MP in M9 or exposed to *P. aeruginosa*. ANOVA (treatment time ns; cell line ns; experimental condition ns).

As shown in **Table 4.10**, the concentration of total metabolites was lower when they were generated by NALM6 cells treated with 1.25 µM TG exposed to *P. aeruginosa* ($p<0.01$ and $p<0.01$ for 48 and 72 hours respectively). Noteworthy, TGMP was the most abundant metabolite after treatment of cells with drug exposed or not to bacteria both for 48 and 72 hours.

Table 4.10. Average concentration of thiopurine metabolites (means ± SE) in NALM6 cells treated with 1.25 μM TG exposed to *P. aeruginosa* (TG Pa) or not (TG M9) for 48 and 72 hours.

Metabolites	48 hours				72 hours			
	TG M9		***TG Pa***		***TG M9***		***TG Pa***	
	pmol/Mio	%	pmol/Mio	%	pmol/Mio	%	pmol/Mio	%
TGMP	106.61±4.32	79.77	33.27±8.28	90.43	73.24±4.69	78.20	29.34±5.44	89.74
TGDP	2.54±0.60	1.90	0.00	0.00	2.63±1.02	2.81	0.00	0.00
TGTP	16.59±1.54	12.41	1.85±0.97	5.04	11.56±4.42	12.34	1.95±1.00	5.96
MeTGMP	4.31±0.60	3.23	0.99±0.32	2.68	3.15±0.36	3.37	0.95±0.60	2.90
MeTGDP	0.86±0.19	0.64	0.00	0.00	1.06±0.16	1.13	0.00	0.00
MeTGTP	2.73±0.30	2.04	0.68±0.37	1.84	2.01±0.46	2.15	0.46±0.26	1.40
TOT.	133.64±2.15	100.00	36.78±9.73	100.00	93.65±10.84	100.00	32.69±6.96	100.00

The trend of reduction of total amount of thiopurine metabolites after treatment of JURKAT cells with 1.25 μM TG exposed to *P. aeruginosa* is shown in **Table 4.11** (p=0.06 and p=0.12 for 48 and 72 hours respectively). The metabolites TGMP and TGTP, followed by MeTGTP, were mainly present in cells treated with drug exposed or not to bacteria for 48 and 72 hours.

Table 4.11. Average concentration of thiopurine metabolites (means ± SE) in JURKAT cells treated with 1.25 μM TG exposed to *P. aeruginosa* (TG Pa) or not (TG M9) for 48 and 72 hours.

Metabolites	48 hours				72 hours			
	TG M9		***TG Pa***		***TG M9***		***TG Pa***	
	pmol/Mio	%	pmol/Mio	%	pmol/Mio	%	pmol/Mio	%
TGMP	8.87±1.79	36.09	4.95±1.14	62.35	10.28±3.42	57.30	4.23±0.98	72.90
TGDP	0.49±0.30	2.01	0.00	0.00	0.00	0.00	0.00	0.00
TGTP	10.15±2.32	41.29	1.79±1.23	22.60	5.11±1.01	28.48	1.12±0.73	19.31
MeTGMP	0.74±0.38	3.00	0.00	0.00	0.14±0.14	0.79	0.00	0.00
MeTGDP	0.44±0.23	1.79	0.00	0.00	0.26±0.26	1.45	0.00	0.00
MeTGTP	3.89±0.48	15.82	1.19±0.48	15.05	2.15±0.48	11.97	0.45±0.24	7.80
TOT.	24.57±4.75	100.00	7.94±2.81	100.00	17.94±5.09	100.00	5.80±1.93	100.00

Table 4.11. Average concentration of thiopurine metabolites (means ± SE) in JURKAT cells treated with 1.25 μM TG exposed to *P. aeruginosa* (TG Pa) or not (TG M9) for 48 and 72 hours.

Noteworthy, according to ANOVA, all the thionucleotides detected TGMP, TGDP, TGTP and their corresponding methylated compound MeTGMP, MeTGDP and MeTGTP were significantly decreased after treatment of NALM6 and JURKAT cells with 1.25 μM TG exposed previously to *P. aeruginosa* (p<0.001) (**Figure 4.73-4.78**). The metabolites TIMP, MeTIMP, TIDP, MeTIDP, TITP and MeTITP were not detected in none of the cell lines after treatment with TG. Moreover, NALM6 cells presented higher levels of the thioguanine nucleotides TGMP, TGDP, TGTP, MeTGMP and MeTGDP in comparison to JURKAT cells

(p<0.001; p<0.01; p<0.05; p<0.001; p<0.05 respectively) (**Figure 4.73-4.77**). Furthermore, the levels of TGMP and MeTGTP were increased after a 48 hours-treatment (p<0.01) (**Figure 4.73** and **4.78**).

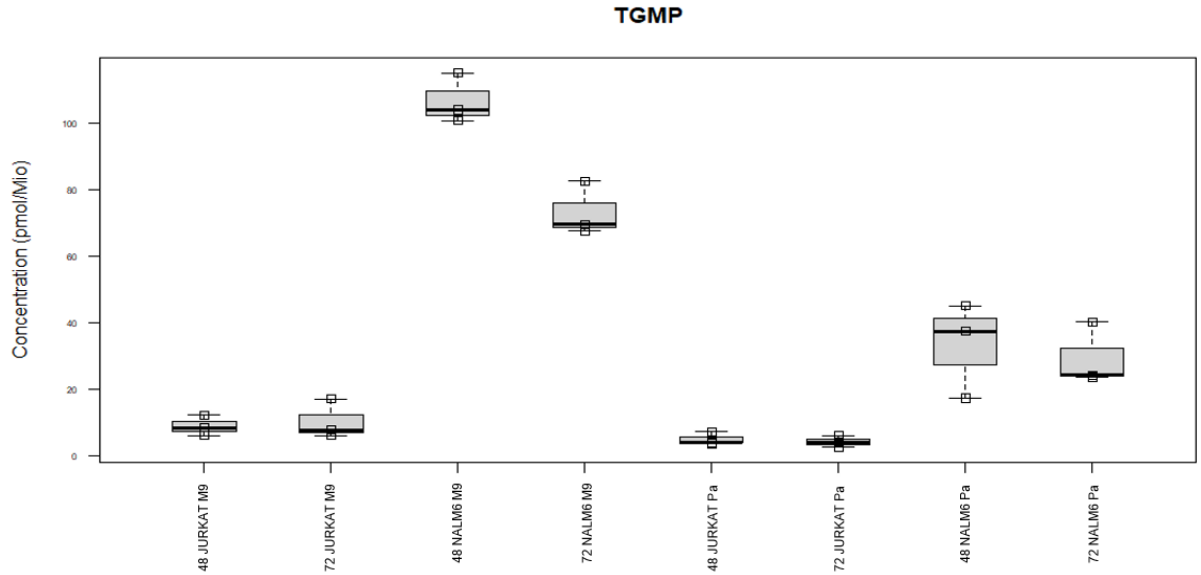

Figure 4.73. Concentration of the TGMP metabolite in NALM6 and JURKAT cells after treatment for 48 or 72 hours with 1.25 µM TG in M9 or exposed to *P. aeruginosa*. ANOVA (treatment time p<0.01; cell line p<0.001; experimental condition p<0.001).

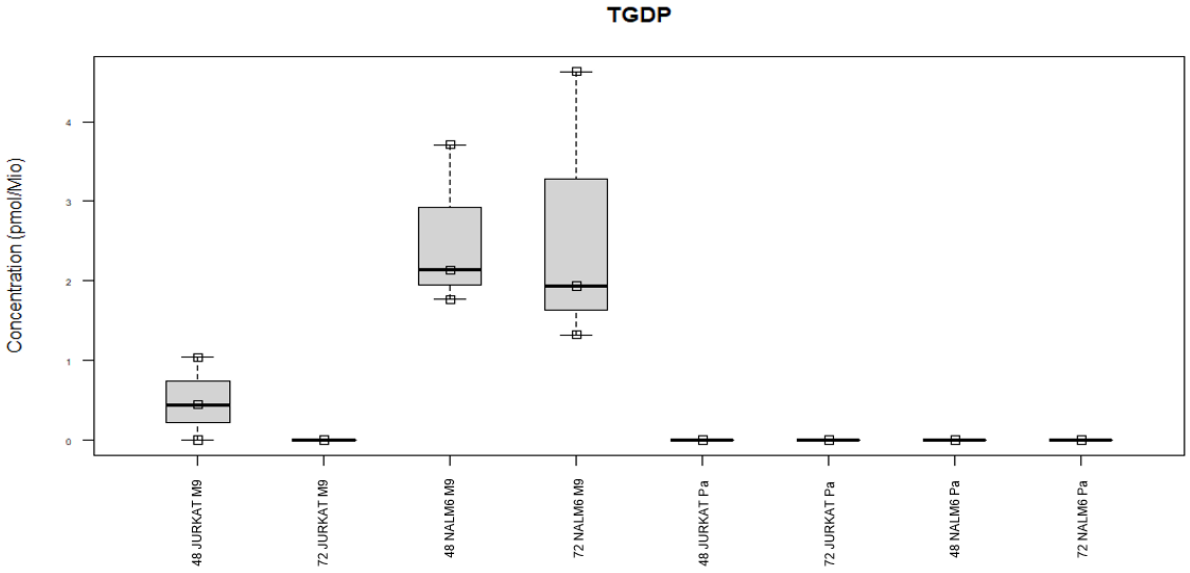

Figure 4.74. Concentration of the TGDP metabolite in NALM6 and JURKAT cells after treatment for 48 or 72 hours with 1.25 µM TG in M9 or exposed to *P. aeruginosa*. ANOVA (treatment time ns; cell line p<0.01; experimental condition p<0.001).

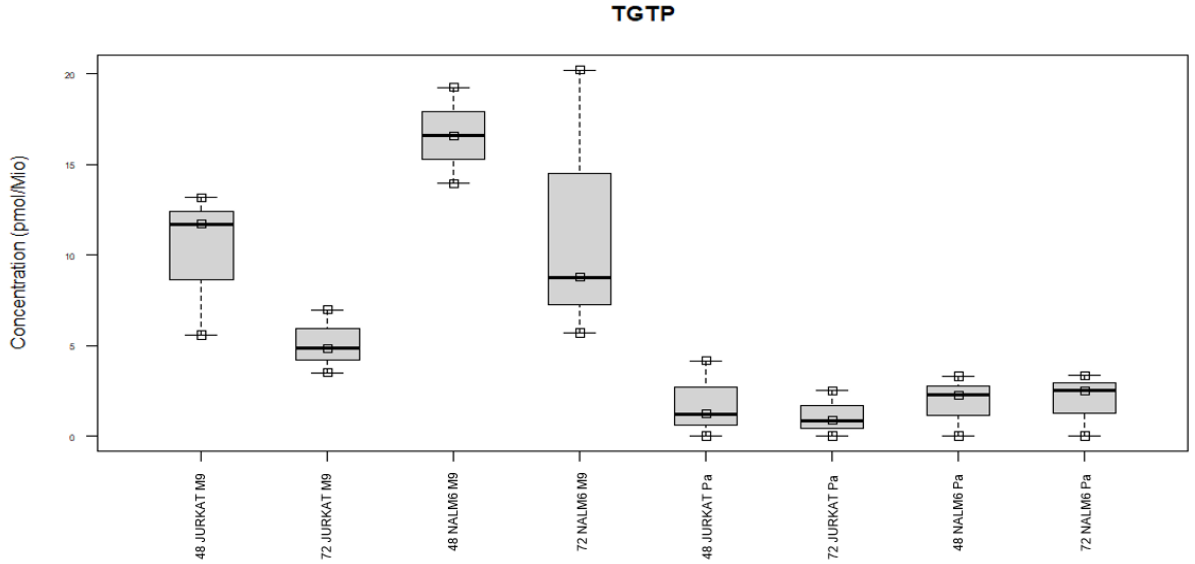

Figure 4.75. Concentration of the TGTP metabolite in NALM6 and JURKAT cells after treatment for 48 or 72 hours with 1.25 µM TG in M9 or exposed to *P. aeruginosa*. ANOVA (treatment time ns; cell line $p<0.05$; experimental condition $p<0.001$).

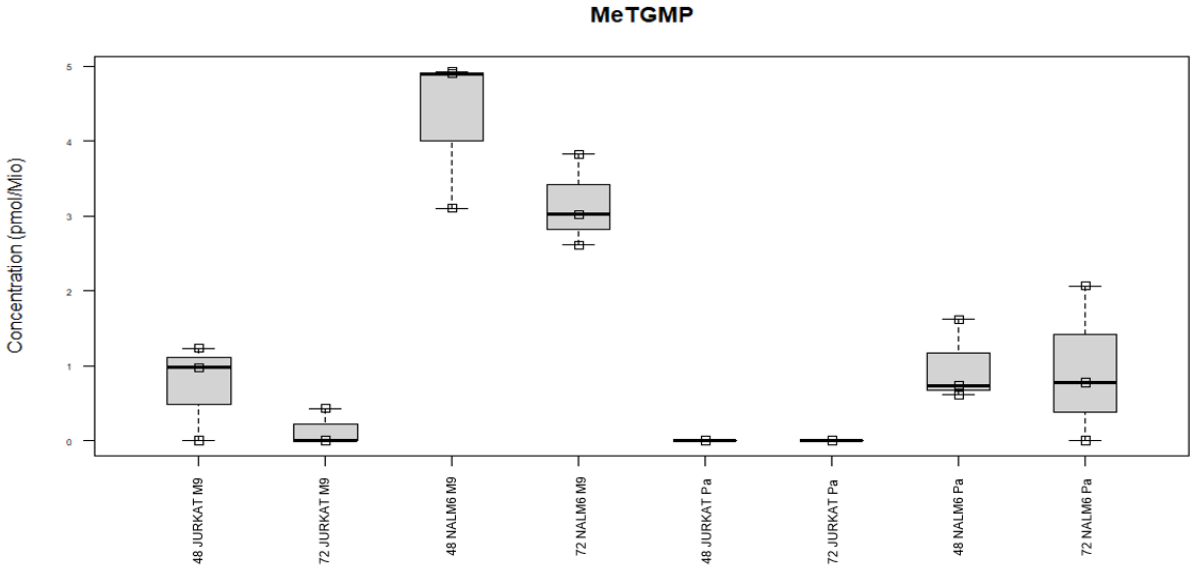

Figure 4.76. Concentration of the MeTGMP metabolite in NALM6 and JURKAT cells after treatment for 48 or 72 hours with 1.25 µM TG in M9 or exposed to *P. aeruginosa*. ANOVA (treatment time ns; cell line $p<0.001$; experimental condition $p<0.001$).

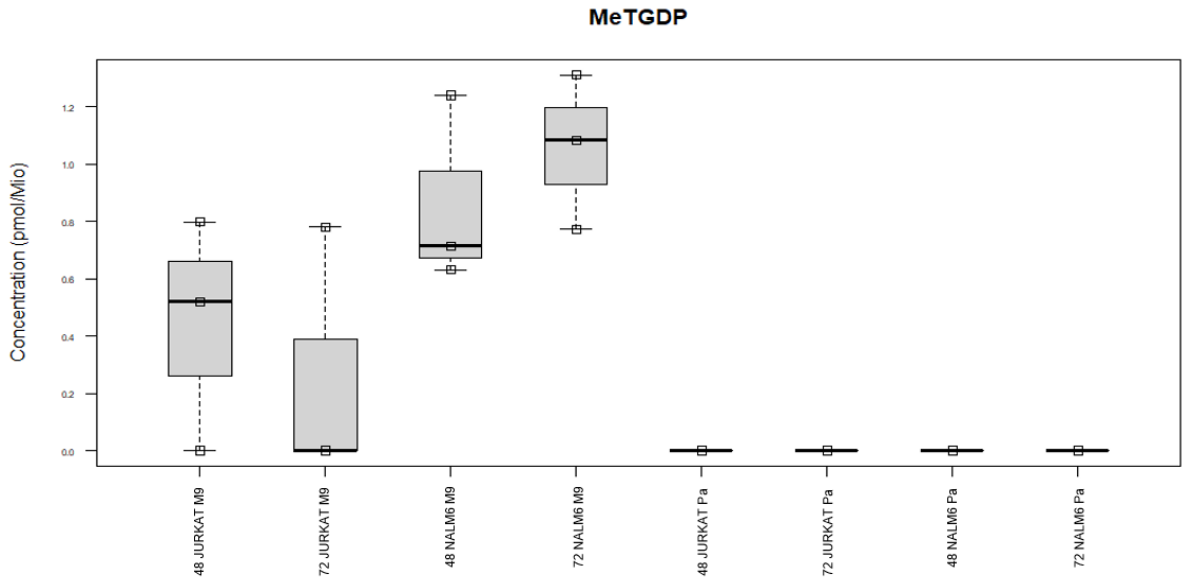

Figure 4.77. Concentration of the MeTGDP metabolite in NALM6 and JURKAT cells after treatment for 48 or 72 hours with 1.25 µM TG in M9 or exposed to *P. aeruginosa*. ANOVA (treatment time ns; cell line $p<0.05$; experimental condition $p<0.001$).

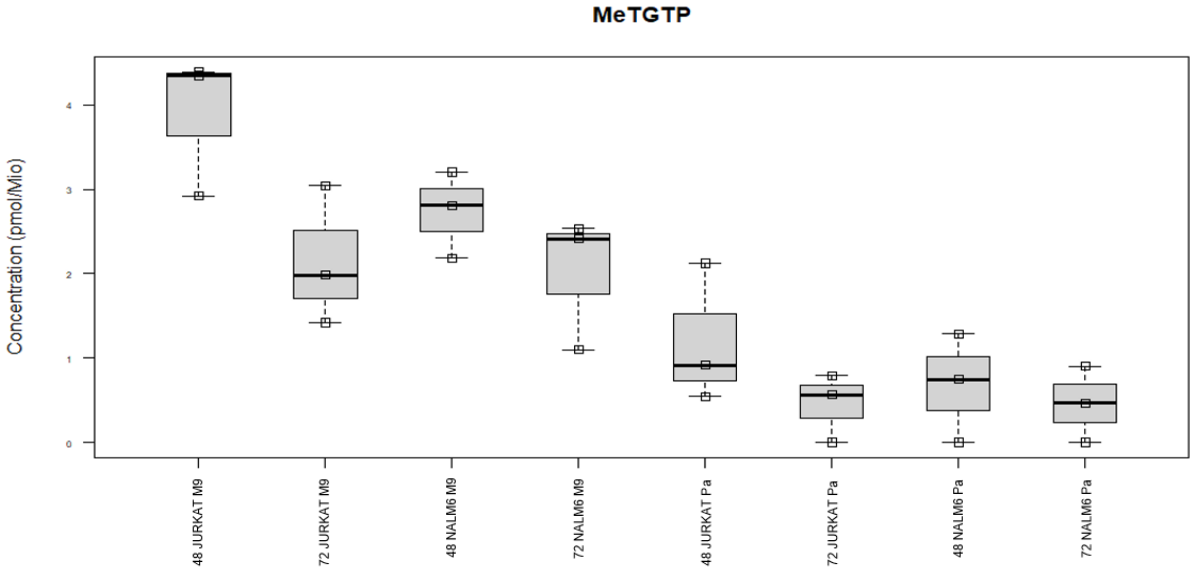

Figure 4.78. Concentration of the MeTGTP metabolite in NALM6 and JURKAT cells after treatment for 48 or 72 hours with 1.25 µM TG in M9 or exposed to *P. aeruginosa*. ANOVA (treatment time $p<0.01$; cell line ns; experimental condition $p<0.001$).

As shown in **Table 4.12** and similarly to what was observed in **Table 4.10** with a lower concentration of the same drug, thiopurine metabolites decreased after treatment of NALM6 with 2.5 µM TG exposed to *P. aeruginosa* ($p=0.09$ and $p<0.05$ for 48 and 72 hours respectively). Furthermore, TGMP was the most abundant metabolite after treatment of cells with the drug exposed or not to *P. aeruginosa* both for 48 and 72 hours.

Table 4.12. Average concentration of thiopurine metabolites (means ± SE) in NALM6 cells treated with 2.5 μM TG exposed to *P. aeruginosa* (TG Pa) or not (TG M9) for 48 and 72 hours.

Metabolites	48 hours				72 hours			
	TG M9		***TG Pa***		***TG M9***		***TG Pa***	
	pmol/Mio	%	pmol/Mio	%	pmol/Mio	%	pmol/Mio	%
TGMP	243.07±48.92	70.80	96.03±6.79	82.14	149.98±19.70	73.60	59.02±5.55	85.03
TGDP	10.18±4.56	2.96	1.32±0.54	1.13	7.60±0.72	3.73	0.63±0.33	0.91
TGTP	68.20±17.58	19.86	12.46±2.93	10.66	31.70±9.11	15.56	5.36±1.65	7.72
MeTGMP	10.56±2.45	3.07	4.27±0.41	3.66	5.87±0.85	2.88	2.60±0.12	3.74
MeTGDP	3.15±1.12	0.92	0.36±0.18	0.31	3.16±0.32	1.55	0.33±0.17	0.48
MeTGTP	8.19±0.66	2.39	2.46±0.35	2.11	5.46±1.84	2.68	1.48±0.22	2.13
TOT.	343.34±74.99	100.00	116.92±10.37	100.00	203.78±30.50	100.00	69.41±7.74	100.00

As shown in **Table 4.13**, JURKAT cells produced lower amount of thiopurine metabolites when treated with 2.5 μM TG exposed to *P. aeruginosa* (p=0.13 and p<0.05 for 48 and 72 hours respectively). Moreover, TGMP and TGTP, followed by MeTGTP, were the most abundant metabolites after treatment with the drug exposed or not to *P. aeruginosa* both for 48 and 72 hours, confirming previous data acquired treating cells with a lower concentration of drug.

Table 4.13. Average concentration of thiopurine metabolites (means ± SE) in JURKAT cells treated with 2.5 μM TG exposed to *P. aeruginosa* (TG Pa) or not (TG M9) for 48 and 72 hours.

Metabolites	48 hours				72 hours			
	TG M9		***TG Pa***		***TG M9***		***TG Pa***	
	pmol/Mio	%	pmol/Mio	%	pmol/Mio	%	pmol/Mio	%
TGMP	20.82±6.77	41.23	10.17±0.95	49.22	22.82±5.41	44.74	11.11±3.26	58.88
TGDP	1.20±0.71	2.37	0.18±0.18	0.85	1.28±0.09	2.50	0.23±0.23	1.24
TGTP	19.25±3.98	38.12	7.01±1.15	33.94	19.02±2.42	37.30	5.38±1.19	28.52
MeTGMP	1.49±0.39	2.94	0.43±0.24	2.10	1.07±0.03	2.10	0.00	0.00
MeTGDP	1.26±0.23	2.50	0.34±0.18	1.64	1.18±0.20	2.32	0.17±0.17	0.91
MeTGTP	6.31±1.10	12.49	2.53±0.34	12.24	5.63±0.37	11.04	1.97±0.42	10.44
TOT.	50.50±12.43	100.00	20.66±2.30	100.00	51.00±8.30	100.00	18.87±5.26	100.00

Interestingly, according to ANOVA, all the detected metabolites TGMP, TGDP, TGTP and their corresponding methylated compound MeTGMP, MeTGDP and MeTGTP were less abundant after treatment with 2.5 μM TG incubated previously with *P. aeruginosa* (p<0.001; p<0.01; p<0.001; p<0.001; p<0.001; p<0.001 respectively) (**Figure 4.79-4.84**). The metabolites TIMP, MeTIMP, TIDP, MeTIDP, TITP and MeTITP were not detected in any of the cell lines after treatment with TG. Furthermore, similarly to what was previously described, NALM6 cells presented higher levels of TGMP, TGDP, TGTP, MeTGMP and MeTGDP (p<0.001; p<0.01; p<0.01; p<0.001; p<0.01 respectively) (**Figure 4.79-4.83**). Moreover, a 48 hours-treatment determined higher concentrations of TGMP, TGTP and MeTGMP (p<0.05) (**Figure 4.79, 4.81** and **4.82**).

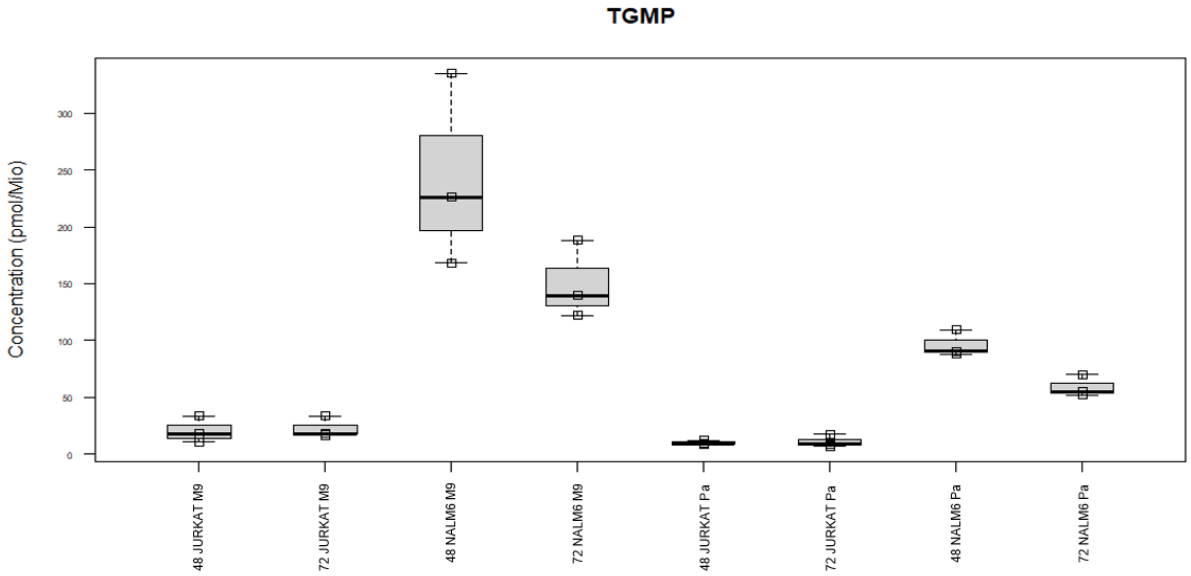

Figure 4.79. Concentration of the TGMP metabolite in NALM6 and JURKAT cells after treatment for 48 or 72 hours with 2.5 µM TG in M9 or exposed to *P. aeruginosa*. ANOVA (treatment time $p<0.05$; cell line $p<0.001$; experimental condition $p<0.001$).

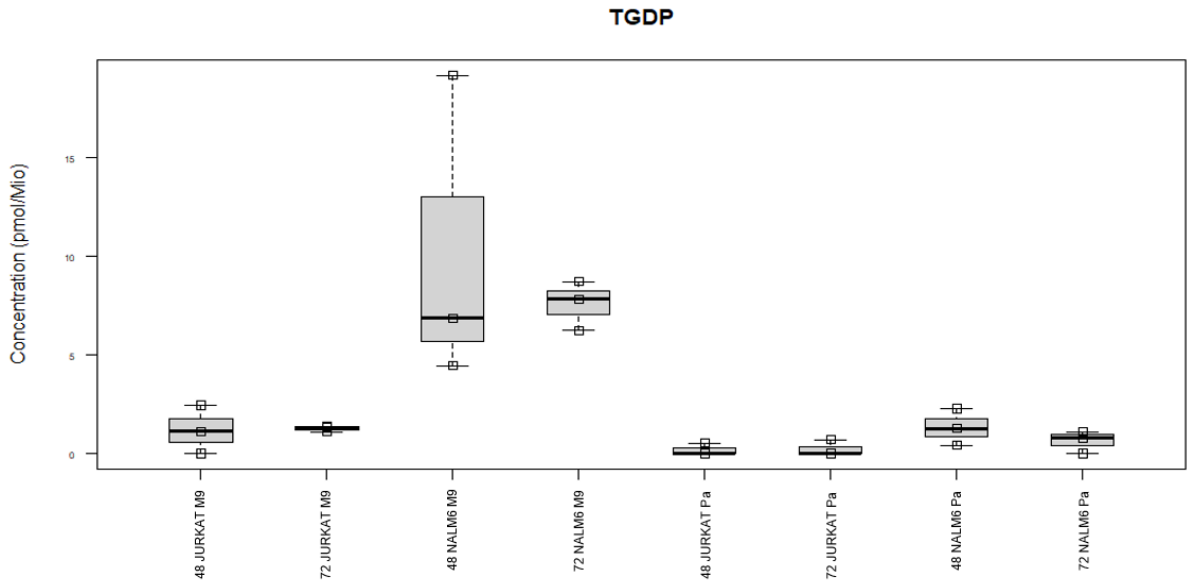

Figure 4.80. Concentration of the TGDP metabolite in NALM6 and JURKAT cells after treatment for 48 or 72 hours with 2.5 µM TG in M9 or exposed to *P. aeruginosa*. ANOVA (treatment time ns; cell line $p<0.01$; experimental condition $p<0.01$).

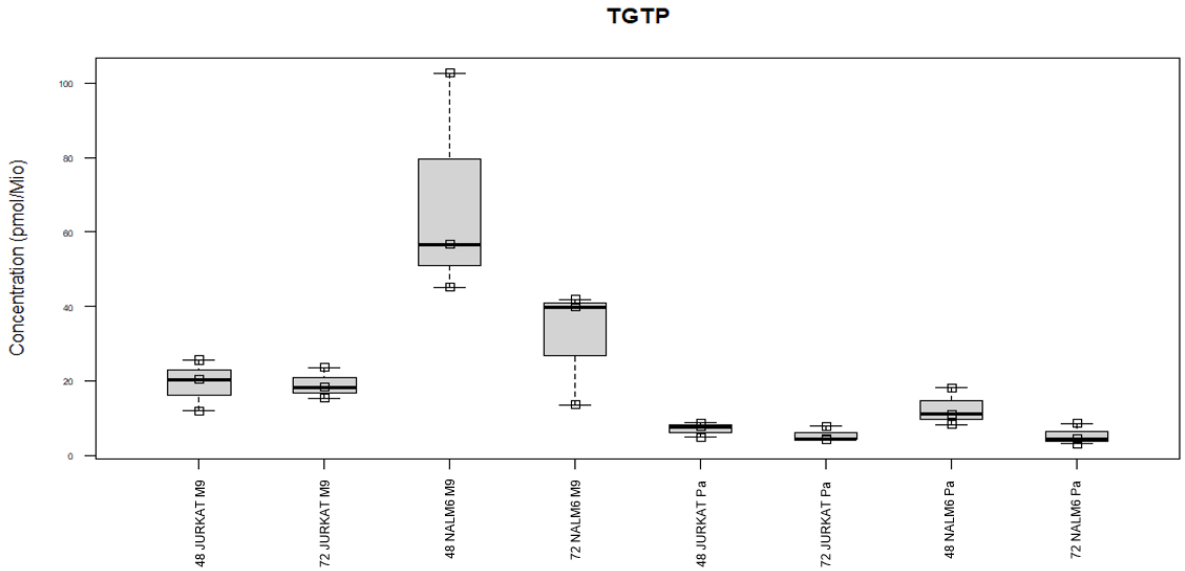

Figure 4.81. Concentration of the TGTP metabolite in NALM6 and JURKAT cells after treatment for 48 or 72 hours with 2.5 µM TG in M9 or exposed to *P. aeruginosa*. ANOVA (treatment time $p<0.05$; cell line $p<0.01$; experimental condition $p<0.001$).

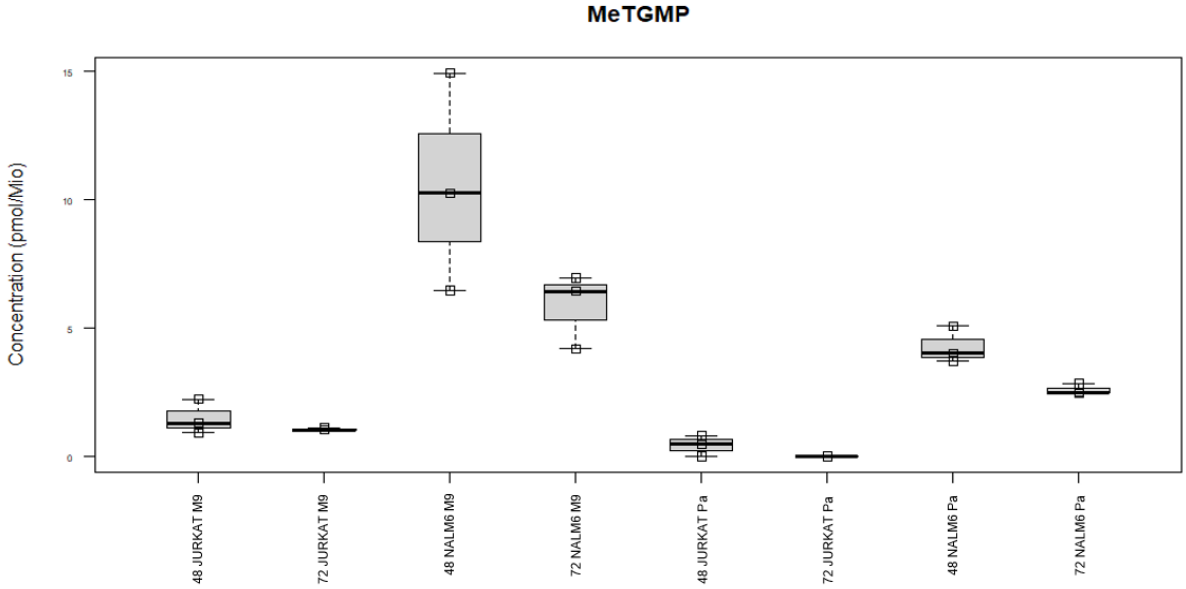

Figure 4.82. Concentration of the MeTGMP metabolite in NALM6 and JURKAT cells after treatment for 48 or 72 hours with 2.5 µM TG in M9 or exposed to *P. aeruginosa*. ANOVA (treatment time $p<0.05$; cell line $p<0.001$; experimental condition $p<0.001$).

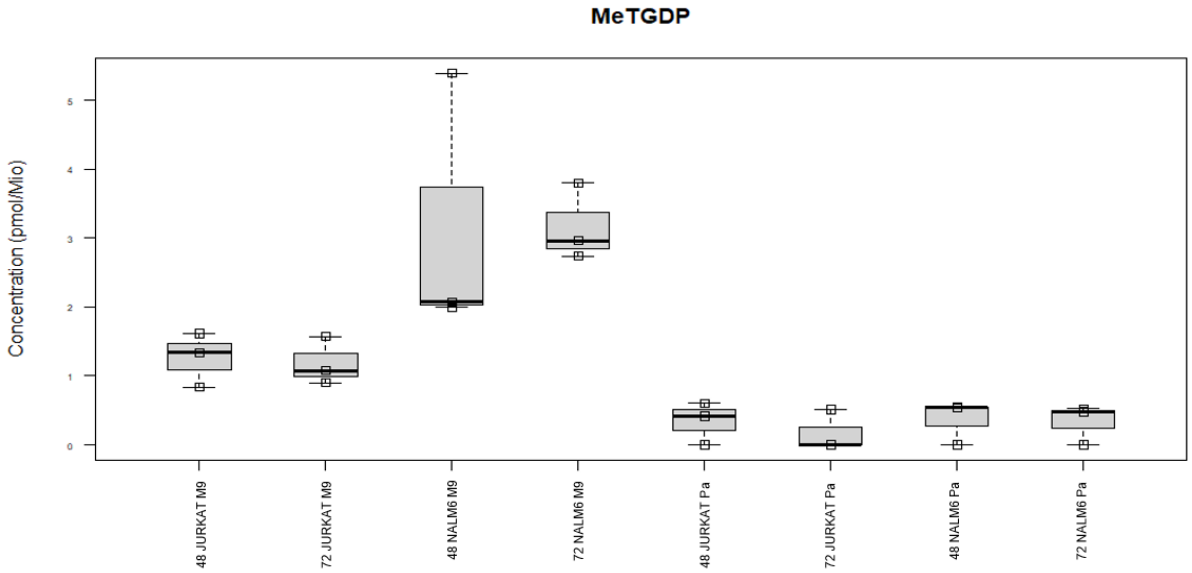

Figure 4.83. Concentration of the MeTGDP metabolite in NALM6 and JURKAT cells after treatment for 48 or 72 hours with 2.5 µM TG in M9 or exposed to *P. aeruginosa*. ANOVA (treatment time ns; cell line $p<0.05$; experimental condition $p<0.001$).

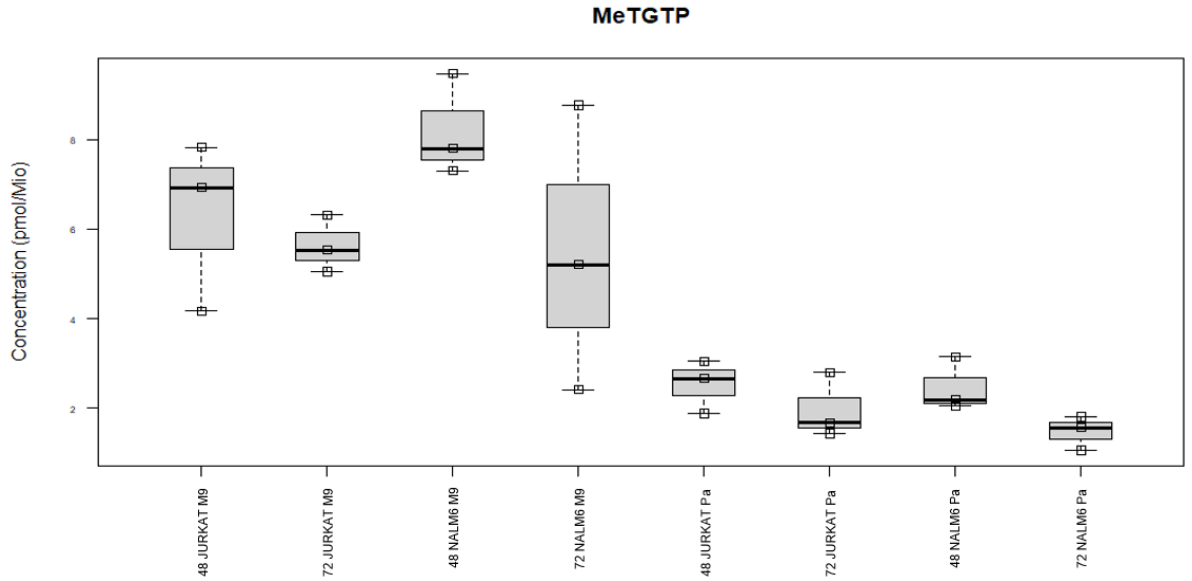

Figure 4.84. Concentration of the MeTGTP metabolite in NALM6 and JURKAT cells after treatment for 48 or 72 hours with 2.5 µM TG in M9 or exposed to *P. aeruginosa*. ANOVA (treatment time ns; cell line ns; experimental condition $p<0.001$).

5. DISCUSSION

5.1 Role of *Enterobacteriaceae* family and *Pseudomonas* genus in mediating the *in vitro* effects of thiopurines

Bacterial species belonging to *Enterobacteriaceae* family and *Pseudomonas* genus proliferate abundantly at the expense of other commensals in the gut microbiota of patients with IBD leading to a reduced diversity and richness of intestinal bacteria, the so-called dysbiosis (Khan et al., 2019; Lucafo et al., 2020; Sundin et al., 2017). Thiopurine drugs are commonly used in the maintenance of remission of IBD but, despite their proven efficacy, patients are prone to develop adverse effects and experience therapeutic failure (Franca et al., 2019; Konidari & Matary, 2014). Given the growing evidence of the influence of gut bacteria on drug efficacy (Geller et al., 2017), the present PhD project investigated *in vitro* the role of four bacterial strains belonging to *Enterobacteriaceae* family (*E. coli*, *S. enterica* and *K. pneumoniae*) and to *Pseudomonas* genus (*P. aeruginosa*) in mediating the *in vitro* effects of thiopurines.

5.1.1 Role of *K. pneumoniae*

The results obtained in the first part of the project revealed that thiopurine drugs do not exert an antimicrobial activity towards the bacterial strains tested *E. coli*, *S. enterica* and *K. pneumoniae*, contrary to what was previously observed with *Mycobacterium paratuberculosis* and *Campylobacter* species (Liu et al., 2017; Shin & Collins, 2008). Therefore, patients that undergo thiopurine therapy probably do not present a reduction of these bacterial species in their gut microbiota. Furthermore, based on this result, *in vitro* experiments were performed with a concentration of thiopurines which did not inhibit the bacterial growth and we could exclude bias due to bacterial susceptibility to drugs.

In the lymphocytic cell lines of NALM6 and JURKAT, the cytotoxicity of thiopurines was reduced after the incubation of these drugs with *K. pneumoniae*, instead of *E. coli* and *S. enterica* that did not affect cytotoxic effects. Previous evidence by Oancea and colleagues showed the ability of *E. coli* and other bacterial species to metabolize TG: thiopurine metabolites were detected in bacterial cultures after incubation with the drug (Oancea et al., 2017). However, the concentration of TG (1 mM) used for the experiments was higher than the one we tested and there were no data available that described the consequences on mammalian cells of the bacterial metabolism (Oancea et al., 2017). Noteworthy, in previous studies, intestinal cell line models, such as HCEC cells, were proposed as a suitable *in vitro* model for studying thiopurine intestinal cytotoxicity (Genova et al., 2021). Therefore, considering the reduction of the cytotoxic effects on the target immune cell models, it would be interesting to investigate if the same effects would be present after treatment of HCEC cells with drugs previously exposed to bacteria in order to reach new evidences about the role of microorganisms in reducing intestinal damage.*In vitro* exposure and subsequent cytotoxicity test were also performed on the synthetic glucocorticoid agent MTPD, a drug also used in IBD therapy that has a mechanism of action and a chemical structure different from that of the thiopurines. Since there is no variation in cytotoxicity between MTPD in M9 and exposed to bacteria, we concluded that the glucocorticoid was not subjected to biotransformation by any of the bacterial strains tested. A previous study showed that beclomethasone dipropionate, budesonide and prednisolone were metabolized by microorganisms obtained from faecal slurries of three healthy individuals losing their biological activity (Yadav et al., 2013). One of the mechanisms of biotransformation proposed was the hydroxylation at position 6 of the glucocorticoids (Yadav et al., 2013), a reaction that MTPD cannot undergo because of its chemical structure.

Moreover, incubation of thiopurines with *K. pneumoniae*, but not with *E. coli* and *S. enterica*, caused a reduction of the concentrations of thiopurines, indicating that the variation in cytotoxicity was due to a lower concentration of drugs in bacterial conditioned media. Based on cytotoxicity tests and UV analyses, we focused the subsequent experiments on *K. pneumoniae*.

Measuring thiopurine metabolites in NALM6 and JURKAT cells, another important information came out: thiopurine nucleotides decreased when cells were treated for 48 and 72 hours with the concentrations of AZA, MP and TG incubated before with *K. pneumoniae*. The result obtained suggested that the reduction of cytotoxicity after bacterial incubation is related with a lower presence of the metabolites of the drugs.

Noteworthy, analysing thiopurine metabolites after a fixed time of treatment (48 and 72 hours) a statistically different profiles of thiopurine metabolites between the cell lines was evident: this could be due to differences in the replication times and in the expression of enzymes involved in thiopurine pathway between the cell lines (Cowley et al., 2014; Schoene & Kamara, 1999). Differences of metabolite content depending on the timing of treatment, except for TGTP, were not highlighted, indicating that the nucleotides generated at an earlier time (48h) and at the same time (72h) of treatment used for cytotoxicity test could both be indicators of the final effect.

Interestingly, differences not only in the abundance but also in the percentage of each metabolite was highlighted after treatment of NALM6 cells with AZA exposed to bacteria. In detail, the concentration of the methylated nucleotide MeTIMP was higher after treatment with the thiopurine incubated with *K. pneumoniae*. Based on this result, we measured the activity of TPMT, enzyme responsible for the methylation of drugs in their free base and in nucleotide form (Franca et al., 2019), after incubating cells with *K. pneumoniae* conditioned media. Since TPMT activity seemed not to change after exposure to bacterial conditioned media, we concluded that there is no stimulation of the enzyme's activity by compounds released by this bacterial strain and hypothesized that the higher levels of MeTIMP in NALM6 cells treated with AZA exposed to bacteria could be due to stimulation by a bacterial secreted metabolite produced after exposure of *K. pneumoniae* to AZA or to S-methyl-4-nitro-5-thioimidazole, compound generated by the conversion of AZA to MP (Lazarević et al., 2022). Further experiments will be needed to understand better this result.

Measurements of dTGUA in DNA of cells were also performed because of its possible role as biomarker of thiopurine efficacy, alternative to the thiopurine metabolites TGN and MMPN: indeed, levels of dTGUA in DNA reflect the concentration of the metabolite dTGTP incorporated in DNA at the expense of deoxyguanosine triphosphate, inducing cell arrest and apoptosis (Toyonaga et al., 2021). Concentrations of dTGUA were lower in NALM6 and JURKAT cells treated with thiopurines incubated before with *K. pneumoniae*, suggesting that the exposure to this bacterial strain determined not only a lower metabolism but also a lower biological effect of drugs.

In this context, we investigated the intracellular environment of *K. pneumoniae* in order to understand if bacteria can internalize thiopurines. After exposing *K. pneumoniae* to a concentration of drugs, we succeeded in quantifying MP in bacteria after exposure to AZA and MP, whereas TG was detected after incubation of bacteria with TG. Therefore, the results obtained showed that *K. pneumoniae* is able to internalize the thiopurines MP and TG removing them to the bacterial conditioned media and determining a reduction of cytotoxicity when cells were treated with them. Indeed, when the preferred nitrogen source ammonia is limited, there is evidence that *K. pneumoniae* strains are able to assimilate the purines adenine and guanine to convert them in hypoxanthine and xanthine through deamination and subsequently metabolizing the compounds in uric acid, then in allantoin and finally in CO_2 and ammonia (de la Riva et al., 2008). As MP and TG are analogous of purines, the same catabolic pathway could be used in order to obtain nitrogen sources.

Furthermore, *K. pneumoniae* exposed to AZA presented low intracellular concentration of MP indicating that the bacterial strain is able to convert AZA to MP and S-methyl-4-nitro-5-thioimidazole through the activity of the enzyme GST or non-enzymatically through the action of nucleophiles. In particular, the scientific literature reported that some strains, among Proteobacteria phylum, have GST activity (Vuilleumier & Pagni, 2002). Unluckily, the reduction of concentration of AZA in bacterial conditioned media is not justified by the concentration of MP found inside bacteria and, therefore, we cannot exclude the contribution of other factors, such as bacterial secreted metabolites, in reducing the cytotoxicity of AZA. Further experiments will be needed to understand better the role of *K. pneumoniae* in interfering with the cytotoxicity of AZA.

Metabolomic analyses evidenced differences in the abundance of metabolites produced and secreted by *K. pneumoniae* in bacteria were exposed to AZA, MP and TG. In general, differences in substances attributable to tricarboxylic acid (TCA) cycle and in pyrimidine biosynthesis were evident.

For instance, metabolites such as malic acid, itaconic acid, ketoglutaric acid and succinic acid belonging to TCA cycle, were less present in media conditioned by *K. pneumoniae* exposed previously to thiopurines, whereas amino acids such as glutamate, tyrosine, phenylalanine, methionine and tryptophan, known to be substances involved in anaplerotic reactions of TCA cycle, were present in higher levels. Therefore, we speculated that, if substances released are proportional to intracellular metabolites, incubation with thiopurines slowed down TCA cycle in bacteria, determining lower concentration of compounds being part of it and higher concentration of compounds used to replenishing it. Indeed, since *K. pneumoniae* grew in M9 medium, there was a unique carbon source derived from glucose and thus glycolysis and TCA cycle pathway were used to generate energy and reducing power (Cabelli, 1955). Exposing thiopurines to *K. pneumoniae* determined novel sources of nitrogen for bacteria that could metabolize and substitute as alternative source to glucose contained in M9. Therefore, the results obtained could represent a proof of metabolism of thiopurines by *K. pneumoniae*.

Furthermore, metabolites belonging to *de novo* pyrimidine biosynthesis pathway such as N-carbamoyl aspartic acid and orotic acid, already known to be produced by several bacterial strains including *K. pneumoniae* (Lipowska et al., 2019), were present in higher extent in bacteria exposed to thiopurines. Interestingly, the synthesis of N-carbamoyl aspartic acid and orotic acid is reported to be stimulated by nitrogen sources: in particular, the formation of these compounds leading to *de novo* pyrimidine biosynthesis is initiated by ammonia presence (Visek, 1992). As previously mentioned, *K. pneumoniae* strains are able to metabolize purines in several step to CO_2 and ammonia (de la Riva et al., 2008) and we speculated they are able to convert thiopurines as well producing ammonia and stimulating *de novo* pyrimidine biosynthesis. Also this information could provide a further proof of the metabolism of thiopurines by *K. pneumoniae*.

5.1.2 Role of *K. pneumoniae* GPB

The second part of the project aims to investigate the role of GPB of *K. pneumoniae* in mediating *in vitro* the cytotoxic effects of thiopurines. Indeed, noteworthy, when the lymphocytic cell lines NALM6 and JURKAT were treated with drugs incubated previously with *K. pneumoniae* GPB, the cytotoxicity of thiopurines was significantly reduced, whereas the GPBs of *E. coli* and *S. enterica* did not affect drugs' cytotoxic effects at all. Since GPB was obtained filtering out bacteria from their logarithmic phase culture, we speculated that the cytotoxic effects of thiopurines could be influenced by compounds secreted by *K. pneumoniae* during its growth. To date, there is no evidence of compounds released by the bacterial strain of interest interfering with thiopurines' activity, nevertheless the *K. pneumoniae* metabolome, that arouses more and more interest also for the microbiological field and for its possible implication in clinic (Han et al., 2022; Rees et al., 2016; Wen et al., 2021), could provide important information about novel and bacterial-derived substances in mediating the cytotoxic effects of thiopurines.

In order to assess a specific and drug-dependent effect of *K. pneumoniae* GPB on thiopurine drugs, *in vitro* exposure and subsequent cytotoxicity tests were also performed on MTPD, a glucocorticoid synthetic agent used in IBD treatment having a chemical structure and mechanism of action different from the one of thiopurines. All the *Enterobacteriaceae* tested did not cause a variation in the cytotoxicity of this drug through the release of bacterial compounds. Indeed, the metabolism of MTPD is thought to be mostly mediated by 11β-hydroxysteroid dehydrogenases and 20-ketosteroid reductases (Mehta et al., 2022) and no data is available about the secretion of this type of enzyme by *K. pneumoniae* species. Moreover, as mentioned also in the first part of discussion, the chemical structure of MTPD could be not prone to be metabolized by the usual proposed mechanism of bacterial biotransformation that exploits mainly hydroxylation at position 6 of the glucocorticoids (Yadav et al., 2013).

In order to evaluate variation in the concentration of thiopurines, UV analyses were performed. The concentration of the thiopurine AZA, but not MP and TG, was lower after incubation with *K. pneumoniae* GPB. Therefore, the cytotoxic effects of MP and TG after exposure to bacterial GPB were not due to a lower concentration of drugs, as it is in the case of AZA exposed to *K. pneumoniae* GPB. Comparing these results with the ones obtained in the first part of the project regarding the UV analyses of thiopurines exposed to *K.*

pneumoniae, we concluded that the mechanism by which exposure to GPB causes a reduction in cytotoxicity could be different than the one previously observed with the incubation with bacteria. Furthermore, exposure to the GPBs of *E. coli* and *S. enterica* did not modify the concentration of drugs and therefore, given also the results on cytotoxicity, we focused the subsequent experiments only on the effects caused by *K. pneumoniae* GPB.

K. pneumoniae strains are capable to produce 77 different capsular exopolysaccharides (EPS) and, once released, EPS can form complexes with other compounds, such as antimicrobial peptides (Benincasa et al., 2016). In order to exclude the contribution of EPS of *K. pneumoniae* in the reduction of the cytotoxic effects of thiopurines, we incubated the drugs with increasing concentrations of the EPS produced by the bacterial strain used and then evaluated the cytotoxicity and the concentration of thiopurines. The results obtained did not evidence any variation of cytotoxicity and of concentration of drugs exposed to EPS in comparison with drugs in M9, used as control.

A previous study identified volatile molecules produced by the same strain of *K. pneumoniae* used for our experiments and released during its *in vitro* growth performing a two-dimensional gas chromatography coupled to time-of-flight mass spectrometry (GC×GC-TOFMS) (Rees et al., 2017). In particular, the compounds 3-methylhexane and 2,5-dimethyl-3-(2-methylpropyl)pyrazine were detected in the mid-exponential phase culture. Unluckily, there is no association in scientific literature between these compounds and thiopurines and the experimental design used was different from ours: analyses were performed after bacterial growth in LB, a different and more nutrient medium than M9 used for our *in vitro* exposures, that could induce *K. pneumoniae* to have many nutrient sources and thus a different and richer metabolome from the one of our experiments. Moreover, the authors were successful in detecting only volatile and non-polar compounds because of the characteristics of the analytical technique thus excluding polar compounds that could be also responsible of the variation of cytotoxicity previously described.

Interestingly, we were able to detect and quantify the purine hypoxanthine, an analogous of mercaptopurine, in *K. pneumoniae* GPB by HPLC-UV. Both purines and thiopurines are hydrophilic; therefore, they cannot permeate biological membranes by diffusion, and they use carriers to enter cells (Yao et al., 2011). Since the similarity of the chemical structure to that of thiopurines, we hypothesized that hypoxanthine, at the concentration detected of about 10 μM, could interfere with the uptake and the cellular availability of thiopurines during the treatment. For instance, previous works highlighted the affinity of binding of hypoxanthine to transporters such as SLC29A1 and SLC29A2 used by thiopurines for internalization in lymphocytes (Zaza et al., 2010) (Yao et al., 2011) (Yao et al., 2002). Furthermore, there is evidence that hypoxanthine could have also higher affinity than mercaptopurine for transporters such as SLC43A4 (Yamamoto et al., 2010). Further experiments such as co-treatment of thiopurines with 10 μM of hypoxanthine will be performed in order to understand if the purine secreted by *K. pneumoniae* has a role in mediating the cytotoxic effects of thiopurines.

5.1.3 Role of *P. aeruginosa*

Investigating the role of *Pseudomonas* genus, and in particular *P. aeruginosa*, in mediating the cytotoxicity of thiopurines was the purpose of the third part of the project.

As previously performed for *Enterobacteriaceae* strains, we evaluated whether thiopurines have an antimicrobial activity towards *P. aeruginosa*. Differently from other compounds (Shenoy et al., 2012), thiopurines do not inhibit the growth of *P. aeruginosa* and, based on this result, we exposed *in vitro* the highest concentration tested of thiopurines to *P. aeruginosa*.

Interestingly, the cytotoxic effects of the thiopurines MP and TG, but not AZA, were significantly reduced on the lymphocytic cell lines NALM6 and JURKAT after *in vitro* exposure of these drugs to *P. aeruginosa*. Contrary to what was previously observed with *K. pneumoniae* GPB, exposure to *P. aeruginosa* GPB did not cause variation in cytotoxicity of all thiopurines. The role of *P. aeruginosa* on the efficacy of thiopurines is still unknown, although previous evidences reported that *Pseudomonas* genus expresses the enzyme TPMT,

known to be involved in bacteria in environmental detoxification processes (Krynetski & Evans, 2003) (Cournoyer et al., 1998) and that could be also able to metabolize thiopurines converting them in the cytotoxic methylthioinosinic metabolites or in the inactive methylmercaptopurine (Franca et al., 2019).

In vitro exposure and subsequent cytotoxicity test were also performed on MTX, antifolate drug used in many inflammatory conditions such as IBD that has a mechanism of action and a chemical structure different from the thiopurines. Since there is no variation in cytotoxicity between MTX in M9 and exposed to bacteria and to GPB, we concluded that this drug was not subjected to biotransformation by *P. aeruginosa* or by bacterial compounds secreted by the bacterial strain. Previous evidence showed the ability of Proteobacteria, and in particular *Pseudomonas* genus, to metabolize MTX in the inactive metabolite 4-diamino-N-10-methylpteroic acid through the enzyme carboxypeptidase (Franzin, Stefancic, et al., 2021) (Buchen et al., 2005), even if there is no indication on the implication of bacterial biotransformation on the cytotoxicity of MTX.

Moreover, incubation of the thiopurine MP and TG with *P. aeruginosa* caused a reduction of the concentrations of thiopurines indicating that the variation in cytotoxicity was due to a lower concentration of drugs in bacterial conditioned media. We speculated that, as previously demonstrated for *K. pneumoniae*, MP and TG, thanks to their similarity in the chemical structure to hypoxanthine and guanine, could be internalized by this bacterial strain in order to be used as nitrogen source as purines (de la Riva et al., 2008). Therefore, we focused the subsequent experiments on the effect of *P. aeruginosa* on MP and TG.

Exposure to *P. aeruginosa* did not affect the concentration of AZA and MTX and, given also the cytotoxicity tests, we concluded that these drugs were not biotransformed by the bacterial strain. Furthermore, the exposure to bacterial GPB did not affect the concentration as well as the cytotoxicity of all drugs, suggesting that the *P. aeruginosa* metabolome could not interfere with the efficacy of the immunomodulators tested.

Noteworthy, thiopurine metabolites generated by NALM6 and JURKAT cells treated for 48 and 72 hours with MP and TG exposed previously to *P. aeruginosa* decreased significantly compared to what was observed for drugs not exposed, thus suggesting that the reduction of cytotoxicity is related to lower concentration of metabolites of the drugs of interest. As previously shown in measurements of thiopurine metabolites after treatment with drugs incubated with *K. pneumoniae*, the abundance of each thionucleotide varies, depending on the cell line: for instance, JURKAT cells present higher concentration of thioguanosine di- and triphosphate after treatment with MP and NALM6 cells generated higher concentration of thioguanine nucleotides. This could be due to the different replication times of each cell lines that influence the proliferation and the cytotoxicity and thus the metabolites produced after treatment with thiopurines (Cowley et al., 2014; Schoene & Kamara, 1999). Moreover, low differences of metabolite content, depending on the timing of treatment were evidenced; in particular cells presented higher concentration of thionucleotides after a treatment of 48 hours with TG.

In this context, *P. aeruginosa* has a role in reducing the cytotoxicity of MP and TG, even if the mechanism has not been elucidated yet. In the future, quantification of thiopurines and their metabolites inside bacteria would be performed in order to assess bacterial internalization and metabolism of these drugs.

6. CONCLUSION

In conclusion, the present study evidenced the role of *K. pneumoniae*, of its GPB and of *P. aeruginosa* in reducing the cytotoxic effects of thiopurines *in vitro*.

Regarding *K. pneumoniae*, we were successful in elucidating the causes of the reduction of the cytotoxic effects of the thiopurines MP and TG. The lower cytotoxicity was due to internalization of drugs by the bacterial strain depriving them from bacterial conditioned media and thus lowering the concentration of drug metabolites generated from cells treated with bacterial conditioned media. Instead, the reduction of the cytotoxic effects of AZA could be only partially explained by its conversion in MP and in the subsequent internalization of MP in bacteria.

Interestingly, we showed preliminary results on the effects of *K. pneumoniae* GPB on thiopurines: during their log phase growth bacteria could release substances interfering with cytotoxicity. Among the compounds belonging to *K. pneumoniae* metabolome, we detected the purine hypoxanthine and we hypothesized that this compound could compete with the same transporters of thiopurines lowering the availability of drugs for cells and reducing cytotoxicity.

P. aeruginosa resulted to be another bacterial strain capable of influencing the cytotoxicity of the thiopurine MP and TG. Although it has not been proved yet, our hypothesis to explain the lower cytotoxicity concerns internalization of these drugs by bacteria. Quantification of thiopurines and their metabolites inside bacterial pellets would be decisive to confirm or not our hypothesis.

However, *K. pneumoniae* and *P. aeruginosa* are only part of the species proliferating and constituting the gut microbiota of IBD patients and we focused our research only on these bacterial strains thus not replicating a more complex situation.

Nonetheless, this *in vitro* work allowed interesting results on bacteria that usually adhere to the intestinal mucosa without an invasive approach and, together with subsequent studies on the luminal intestinal mucosa of IBD patients, could provide a new proof of evidence in thiopurine treatment.

Future perspectives comprise studies of the association of strains of the faecal microbiota derived from pediatric IBD patients with thiopurines efficacy in order to identify new bacterial species with a role in the response to these drugs. This translational approach would provide new knowledge in this unexplored field in order to achieve a personalised therapy in IBD pediatric patients.

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