

The effect of selenium on the gut microbiota and intestinal mucosa

Ilektra Takopoulou-Mavrona

Supervisors:

Dr. Hermie J.M. Harmsen

Gabriela Bravo Ruiseco

Dr. Hannah Rose Wardill

Ana Rita da Silva Ferreira

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ABSTRACT

The ensemble of microorganisms that inhabits the human gut, known as the microbiota, contributes significantly to health and disease. A disbalanced microbiota has been extensively linked with gut inflammation. Mucositis, a severe toxicity caused by chemotherapy for which there are limited therapeutic strategies, is characterized by gut inflammation and an alteration in the composition of microbiota. Potential therapeutic interventions through modulation of gut microbiota are examined in order to treat mucositis. Selenium is a trace element that has been shown to modulate gut microbiota composition and ameliorate intestinal mucositis. In this study, the effect of selenium on the growth of different gut bacteria as well as the effect on intestinal mucosa were tested. Our results showed that under strictly anaerobic conditions selenium had no effect on the growth of *Faecalibacterium prausnitzii*, *Bifidobacterium longum* and *Escherichia coli* and that it was toxic for *Blautia luti* and *Bacteroides thetaiotaomicron*. Under oxidative stress, selenium decreased the growth of *E.coli* and *B.thetaiotaomicron* but had no effect on the growth of *F.prausnitzii*, *B.longum* and *B.luti*. However, under semi/anaerobic conditions selenium was able to promote the growth of *F.prausnitzii*, *B.longum*, *B.luti* and *E.coli*. Moreover, selenium was able to prevent 5-FU cytotoxicity in T84 human colonic epithelial cells but had no proliferative effect. These results indicate that selenium can decrease potential pathogens under oxidative stress and can promote the growth of gut bacteria in conditions that mimic most successfully a healthy gut environment (semi/anaerobic). This preliminary study suggest that selenium might be an effective tool for treating mucositis but further animal and human studies must be conducted.

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INTRODUCTION

Microbiota in health

The human intestinal tract is the habitat of a large population of microorganisms including bacteria, fungi, archaea, protists and viruses, known as the microbiota. The most abundant representatives of this ensemble, the bacteria, count to approximately 10^{11} cells, giving a human cells/bacterial cells ratio of 1:1 (Milo et al, 2016; Yatsunenکو et al, 2012). Across the gastrointestinal tract, the colon is the most populated region. Bacteria reside in the lumen as well as the mucosa layer, which is in close contact with epithelial cells (Sekirov et al, 2010). Bacterial communities live in a mutualistic relationship with their host and they contribute significantly to health and disease.

In a healthy microbiota, anaerobic bacteria exceed in number aerobic and facultative anaerobic bacteria (Clemente et al, 2012). In a phyla level, Firmicutes and Bacteroidetes dominate while Proteobacteria, Verrucomicrobia, Actinobacteria, Fusobacteria and Cyanobacteria count much lower numbers (Qin, et al, 2010). A disruption of this equilibrium has been suggested to contribute to disease development (Tancrede 1992).

In the human gut, the microbiota serves many different functions. Gut microbes play an important role during metabolism. Bacterial fermentation occurring in the gut uses undigested dietary carbohydrates as main substrates to produce certain gases, biomass and short chain fatty acids (SCFAs), mostly acetate, propionate and butyrate (Topping DL, 1996). SCFAs have been linked to regulate gut health (Tan et al, 2014). Their anti-inflammatory and anti-oncogenic properties allow the suppression of microbial growth and contribute in the maintenance of colonic integrity (Tan et al, 2014). Bacteria can also synthesize vitamins and help in the absorption of nutrients (Burkholder and McVeigh, 1942).

Microbiota and immune system

Microbiota interact with the immune system in a bidirectional way. (Hand and Belkaid, 2014). On one hand, the immune system is responsible to preserve homeostasis between microbiota and the host by assuring the segregation of gut bacteria from epithelial cells and by enclosing invasive bacteria away from systemic immunity (Hooper et al, 2012; Johansson et al, 2008; Macpherson and Uhr, 2004). Moreover, the immune system can modulate the composition of microbial population, mainly through the secretion of antibacterial agents by epithelial cells (Salzman et al, 2003; Hooper et al, 2012). On the other hand, intestinal microbes influence immune responses. They provide resistance to colonization of pathogens, promote the activation and differentiation of immune cells in the gut and enhance the development of gut-associated lymphoid tissues (GALTs) (Kamada et al, 2013). In the bacterial population of microbiota, potential pathogens co-exist with other non-pathogenic bacteria and the host, but exogenous pathogens can also threaten gut equilibrium. It is suggested that microbiota can directly prevent the colonization of pathogens by consuming all the nutrients provided in the gut as well as indirectly by enhancing mucosal barrier function, by stimulating innate immune cells to secrete cytokines and by promoting activation of lymphocytes, especially Th17 and Treg cells and secretion of antibodies by B cells (Sommer and Bäckhed, 2013; Levy et al, 2016; Kamada et al, 2013). All these interactions characterizing the bifacial relationship between the immune system and the gut bacteria hold the key role in maintaining a healthy gut state.

Microbiota during gut inflammation

Under specific circumstances including genetic mutations, diet changes, excessive stress or infections, potential pathogens escape immune system's surveillance and dominate in the bacterial population, leading to a microbial imbalance known as dysbiosis (Honda and Littman, 2012). It is well known that a disturbed microbiota leading to dysbiosis will initiate and/or exacerbate inflammation in the gut. In a dextran sodium sulphate (DSS)-induced colitis model in mice, microbiota was found significantly changed (Hakkanson et al, 2015). Moreover, an altered microbiome has been linked with inflammatory bowel disease (IBD) in humans (Willing et al, 2009; Hold et al, 2014). Gut dysbiosis in inflammatory diseases is characterized by a lower bacterial diversity as a variety of different studies indicate (Mosca et al, 2016; Frank et al, 2007; Neut et al, 2002). IBD is characterized by a reduction of the bacteria belonging to the phylum Firmicutes, and a higher abundance of the phylum Bacteroidetes (Andoh et al, 2012). Especially, lower numbers of the butyrate-producing bacteria *Faecalibacterium prausnitzii* have been linked with the presence of the disease (Sokol et al, 2008; Sokol et al, 2009). Moreover, a specific strain of *F. prausnitzii* was found to improve inflammation in a DSS-induced colitis model (Rossi et al, 2015). In addition, a raise of the numbers of potentially pathogenic bacteria belonging to the family Enterobacteriaceae seems to trigger the inflammation causing IBD (Seksik et al, 2003). It is generally suggested that intestinal dysbiosis is characterized by a switch to a microbial community with a lower diversity accompanied by less obligate anaerobes and more facultative anaerobes (Lionel Rigottier-Gois, 2013). This conclusion has led to the hypothesis that a disrupted microbiota in the gut might be the cause of an influx of oxygen due to a damaged mucosa layer (Lionel Rigottier-Gois, 2013). It is proposed that blood enters the GI tract due to chronic inflammation occurring in IBD, so oxygen is released in the gut through hemoglobin (Lionel Rigottier-Gois, 2013). The oxygen influx might give a survival advantage to the facultative anaerobes over obligate anaerobes, leading to a microbial imbalance (Lionel Rigottier-Gois, 2013).

Microbiota and mucositis

While the microbiota has been most extensively linked with IBD, more recently, an appreciation has grown for its role in oncology due to the impact of cytotoxic treatment on its composition. Whilst highly efficacious, cancer therapies (chemotherapy and radiotherapy) are both unable to differentiate between rapidly dividing cells of the gastrointestinal tract and the highly proliferative cells of the tumor. This results in severe damage to the mucosal lining of the oral and gastrointestinal tract, resulting in severe inflammation, mucosal barrier breakdown and confluent ulceration (Peterson et al, 2011). Mucositis has been extensively studied in the oral cavity, however intestinal mucositis has waited a long time to be put on scrutiny (Keefe et al, 2004).

Intestinal mucositis is one of the most severe toxicities and a serious unsolved problem in oncology. Cancer patients with intestinal mucositis experience symptoms such as nausea, abdominal pain, vomiting and constipation or diarrhea (Keefe et al, 2004). As another unfortunate consequence, mucositis is further affecting the efficacy of the treatment as therapy has to be reduced or stopped. Moreover the stay in the hospitals is prolonged and the treatment costs are raised (Gibson and Keefe, 2006; Sonis, 1998). In addition to all that, patients have a low quality of life and they face increased chance of morbidity and mortality (Rubenstein et al, 2004). Despite extensive work by the Multinational Association for Supportive Care in Cancer, there are no universally accepted prophylactic interventions or

gold-standard therapeutic strategies. This highlights the complexity of this complication and the drug- and context-specific variations in its pathobiology. As such, there is great need to identify targetable underlying mechanisms to guide novel interventional development (Cinausero et al, 2017).

According to Sonis model, the pathogenesis of mucositis can be described in five steps; a) initiation, where DNA and non-DNA damage as well as reactive oxygen species (ROS) activation takes place, b) primary damage response, leading to generation of transcription factors such as NF- κ B and apoptosis, c) signal amplification, where inflammation and apoptosis are intensified, d) ulceration, characterized by lesions in intestinal mucosal barrier susceptible to bacterial colonization and considered the most symptomatic phase and finally e) the healing phase (Sonis, 2004). However, this model places little emphasis on the role of the microbiota during the pathogenesis of mucositis, only describing bacterial colonization and translocation at the site of mucosal injury. Touchefeu et al, 2014, pointed out the potential role of microbiota in the pathobiology of mucositis. Commensal bacteria can bind to Toll-like receptors (TLRs) and activate downstream NF- κ B signaling pathway, thus modulating repair of the mucosa layer. Moreover, they can control intestinal permeability and they can modulate mucosa layer function (Touchefeu et al, 2014). In addition, in a more recent review, it was investigated how the intestinal microbiota can influence the effectiveness and toxicity caused by chemotherapy. The "TIMER" model expands the Sonis model and suggests that microbiota participate in Translocation by controlling intestinal permeability, in Immunomodulation by binding to TLRs, promoting nF- κ B production and activating T-cells, in Metabolism of chemotherapeutic drugs producing toxic secondary metabolites, in Enzymatic degradation as an indirect metabolism of chemotherapeutic drugs that can lead to toxicity, and in Reduced diversity resulting in domination of potentially pathogenic bacteria (Alexander et al, 2017).

Several studies have shown that during chemotherapy-induced mucositis significant alterations in the microbial population occur (Touchefeu et al, 2014). Gastrointestinal mucositis caused by the chemotherapeutic agent 5-Fluorouracil (5 - FU) in rats was linked with lower numbers of *Enterococcus* spp., *Lactobacillus* spp. and *Streptococcus* spp. in the colon. In their fecal samples, it was detected lower abundance of *Lactobacillus* spp. and *Bacteroides* spp. and higher abundance of *Clostridium* spp. and *Staphylococcus* spp. and especially of *Escherichia coli* (Stringer et al, 2009). In another study, 5-FU treated rats had higher percentage of facultative anaerobic bacteria in the colon compared to controls (Von Bultzingslowen et al, 2003).

In a clinical study conducted in patients treated with high doses of chemotherapy, a significant alteration in the composition of fecal microbiota was observed (Montassier et al, 2014). In particular, patients with mucositis had a lower microbial diversity and a lower Firmicutes/Bacteroidetes ratio after chemotherapy. Specifically, numbers of anti-inflammatory *F.prausnitzii* and the probiotic species *Bifidobacterium* were reduced, while the potential pathogen *E.coli* was more abundant (Montassier et al, 2014). Similarly, a global decrease in bacterial load was observed in pediatric patients receiving chemotherapy. In particular, reduction of *Bacteroides* spp, *Bifidobacterium* spp, *F.prausnitzii* and *Clostridium* cluster XIVa was found in the patients compared to healthy subjects (van Vliet et al, 2009). Another study in cancer patients receiving chemotherapy also revealed an overall smaller population of intestinal microbiota, which however recovered after the treatment (Zwielehner J et al, 2011). Lower abundance of *Lactobacillus* spp, *Bifidobacterium* spp and

Clostridium cluster IV, including *F.prausnitzii* was accompanied by higher numbers of *C.difficile* and *E. faecium*, however it is difficult to interpret these findings due to the large inter-individual variation reported in this study (Zwiehler J et al, 2011).

Mucositis and probiotics

Given the large and growing body of evidence implicating the microbiota in mucositis development, probiotics have naturally received significant attention for their potential anti-mucositis efficacy. According to FAO / WHO definition, probiotics are “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (Hill C. et al, 2014). The International Scientific Association for Probiotics and Prebiotics recognizes that probiotics contribute to a healthy gastrointestinal tract and a robust immune system (Hill C. et al, 2014). Animal and human studies support that several probiotics such as *B. longum* 536 serve as anti-inflammatory agents and their administration in IBD patients is generally beneficial and safe, although their use should be dealt with caution because their molecular function has not been clarified yet (Plaza-Díaz et al, 2017) . Unfortunately, in the setting of mucositis, the efficacy of probiotics is highly variable and largely underwhelming. Critically, the use of probiotics in preclinical models of mucositis has been promising. For example, the VSL#3 probiotic formula proved to have a prophylactic effect when administered in rats treated with chemotherapy, decreasing symptoms such as diarrhea and weight loss (Bowen et al, 2007). Moreover, *Lactobacillus fermentum* decreased inflammation in 5-FU-induced mucositis in rats (Smith et al, 2008). Administration of some probiotic strains in 5 - FU treated mice has shown a beneficial effect (Yeung et al, 2015; Carvalho et al, 2018). *Bifidobacterium infantis* modulated immune response to improve chemotherapy - induced mucositis in a rat model (Mi H. et al, 2017). However, all these optimistic results are difficult to be translated into the clinical practice. A few studies have shown some positive outcomes. For example, during clinical trials in children under chemotherapy, the use of probiotic *Bifidobacterium breve* had a beneficial effect in gut microbiota (Wada et al, 2010). In another study, 5-FU treated patients were supplemented with *Lactobacillus rhamnosus* relieved from symptoms of mucositis (Osterlund et al, 2007). However, a recent big meta-analysis on the role of probiotics for regimen-induced diarrhea in cancer patients showed that there isn't any significant prophylactic effect of probiotics when translating into clinic. Better design of clinical studies including specific probiotics targeted to specific treatments or development of next generation probiotics is needed (Wardill et al, 2018).

In addition to the doubt of the efficacy of probiotics comes the limited number of bacterial species that can be used as such due to the fact that many of the obligate anaerobes living in the gut are not easy to cultivate in a laboratory environment. Although there has been a lot of progression in the culturing methods and novel sequencing techniques have revealed a great number of unknown commensal bacteria, a lot of effort needs to put in order new and better probiotics to be designed (Hill et al, 2017). The uncertainty of the ability of potential probiotic strains to survive through the GI tract and reach the large intestine adds as another limitation in the use of probiotics. New approaches concerning the use of probiotics as well as different ways of formulating a healthy gut microbiota should be considered.

Selenium in health and disease

Another way to control the gut microbiome is through diet, either by introducing prebiotics or modifying the levels of trace elements (Kasaikina et al, 2011). Prebiotics are “substrates

that are selectively used by a host microorganism to produce a health benefit” and they mainly refer to saccharides but also polyphenols and polyunsaturated fatty acids (Gibson et al, 2017). Trace elements, on the other hand, can also affect the microbiome. Levels of dietary Fe, for example, have been linked to changes in the composition of the gut microbiome (Tompkins et al, 2001).

Selenium (Se) is a trace element very significant for human health. Foods with high content of selenium include meat, fish, cereals, Brazil nuts and mushrooms but it can also be found in lower amounts in several other fruit and vegetables (Kieliszek and Błażej, 2016). Selenium can be toxic if taken in large doses as excess amount of selenium can lead to a disease called “selenosis”, characterized by symptoms of hair loss, weakened nails, neurotoxicity and cardiovascular deficiencies (Vinceti et al, 2014). For adults, the Tolerable Upper Intake Level of Se is determined at 400µg per day (Institute of Medicine (US) Panel on Dietary Antioxidants and Related Compounds, 2000).

In the human body selenium is found most abundantly in the form of selenocysteine (Sec) as part of selenoproteins (Papp et al, 2007). Selenoproteins include glutathione peroxidase family (GPx), thioredoxin reductase family (TrxR) and iodothyronine deiodinases (IDD) (Tinggi, 2008). Selenoproteins mediate the redox reactions between sulfur and oxygen, so they are considered to have antioxidant activity (Tinggi, 2008; Hawkes and Alkan, 2010). Especially some GPs and TrxRs are very important to prevent damage in the cells from reactive oxygen species (Valko et al, 2006; Arner and Holmgren, 2000). Selenoproteins are important during DNA synthesis, they promote immune response, modulate inflammation and they are important for thyroid function (Arner and Holmgren, 2000; Schomburg and Köhrle, 2007). Selenium deficiency leads to a malfunctioning thyroid gland, while selenium supplementation seems to ameliorate Hashimoto’s symptoms (Schomburg and Köhrle, 2007). Selenium lowers the risk of certain cancer types (Rayman, 2012). However in high doses it might be toxic and increase the chance of tumorigenesis (Papp et al, 2007).

Selenium during inflammation

Selenium has an important role during inflammation and its antioxidant properties have been studied during colitis, IBD, and colon cancer. Insufficient levels of selenium have been detected in patients with IBD and low systemic selenium in the serum was associated with the severity of IBD (Fairweather-Tait et al, 2011; Castro Aguilar-Tablada et al, 2016). The four selenoproteins of GPx family (GPx1, GPx2, GPx3 and GPx4) that are expressed in the gut modulate inflammation and in particular, GPx2, GPx3 and GPx4 serve as antioxidants in the gut (Gîlcă-Blanariu et al, 2018). Selenium in the form of selenoproteins take part in signaling pathways involved in inflammation during IBD (Papp et al, 2007; Huang et al, 2012). In addition, the anti-inflammatory function of selenoproteins take place through NF-κB- and PPARγ-dependent pathways (Kaushal et al, 2014; Gîlcă-Blanariu et al, 2018). Kaushal et al, 2014 have demonstrated the role of selenium supplementation in the activation of M2 macrophages, which are considered to have anti-inflammatory effect (Kaushal et al, 2014).

In support of its anti-inflammatory actions, selenium supplementation relieved the symptoms of colitis in a DSS-mouse model and has been shown to minimize 5-FU induced mucositis in rats (Kaushal et al, 2014). Similarly, selenium was able to ameliorate oral mucositis and decreased to a small extent the severity of mucositis in cancer patients undergoing hematopoietic stem cell transplantation HSCT (Jahangard-Rafsanjani et al, 2013; Rodriguez et al, 2018).

Selenium and microbiota

As indicated previously, gut microbiota is closely related to inflammation in the gut and a potential impact of selenium in gut microbiota is worth investigating. Interestingly, selenium can modulate microbiota composition and intestinal microbes can also affect selenium levels in the human body (Kasaikina et al, 2011). Kasaikina et al, 2011 found that selenium supplementation in the diet of mice affects the bacterial colonization of the gut and increased the microbial diversity (Kasaikina et al, 2011). Especially, Parabacteroides were affected the most, but both Firmicutes and Bacteroidetes phyla being significantly altered (Kasaikina et al, 2011). Moreover, it was proposed that gut bacteria influences selenium and selenoprotein levels of the host (Kasaikina et al, 2011). These findings come to confirm a previous study in which bacteria were found to compete with the host for selenium in mice fed with selenium deficient diet (Hrdina et al, 2009). Bacteria use selenium to produce their own selenoproteins, and as a consequence the levels of available selenium for the host are decreased (Hrdina et al, 2009). In a recent study, the effects of selenium in intestinal barrier function and immune system was investigated. Interestingly, it was found that selenium supplementation in mice can improve gut barrier function and influence immune reactions through the modulation of gut microbiome (Zhai et al, 2018). In addition, Gangadoo et al, 2018 showed modification of gut microbiota and increased numbers of butyrate producing bacteria *F.prausnitzii* in poultry fed with selenium nanoparticles (Gangadoo et al, 2018). Finally, selenium – enriched probiotics promoted the growth of weaning piglets through alteration of gut microbiota (Lv et al, 2015).

This evidence has clearly shown that potential of selenium as a modulator of both inflammation and composition of the gut microbiota. Therefore, more studies focused on selenium should be performed.

Hypothesis and aim of the study

This study aims to:

- a. determine the effect of selenium on the growth of different gut bacteria under aerobic or anaerobic conditions
- b. Investigate the prophylactic and/or recovery effects of selenium on the viability of human epithelial cells (T84 cells) treated with the chemotherapeutic drug Fluorouracil (5-FU)

It is hypothesized that selenium supplementation will ameliorate the symptoms of intestinal mucositis via modulation of the gut microbiota and promotion of mucosal recovery. The long-term scope of the study is to improve current treatment of intestinal mucositis in cancer patients.

MATERIALS AND METHODS

Materials and reagents

MALDI-TOF microflex LT/SH, Bruker
MSP 96 polished steel BC target, Bruker
Matrix HCCA, portioned, Bruker
70% formic acid solution
Anaerobic workstation Whitley DG250
Anaerobic workstation Whitley A35
37° C O₂ incubator
Cell density meter CO 8000, Biowave
Previ Color Gram, bioMérieux
CH2 CHS Laboratory Optical Microscope, Olympus
Microscopy slides, Thermo Scientific™
Synergy™ 2 Multi-Detection Microplate Reader, Biotek
Microplate, 96-well, sterile, 2st/bag, Greiner bio-one
Captair smart 391, ductless filtering fume hood, Erlab
Cross Flow unit, Interflow
Clean Air CA / RSV 4 Biohazard cabinet, Interflow
xCELLigence, Real - Time Cell Analyzer S16, ACEA Biosciences, Inc
xCELLigence E-Plate 16, ACEA Biosciences, Inc
Cell culture flask T-150, VWR
Glass tubes
Eppendorf® Research® Plus Pipettes
Pipet tips, Gilson
10µl inoculating loops, sterile, VWR
Eppendorf® Safe-Lock tubes 1,5 mL
Screw Cap Micro Tube, 2 ml, PP, Sarstedt
T84 Human Colonic Adenocarcinoma cell line
YCFAG medium (see Appendix for protocol)
DMEM medium, Sigma - Aldrich
Sodium selenite ≥98%, powder, Sigma - Aldrich
5 – Fluoro-uracil (5-FU) ≥99% (HPLC) powder, Sigma - Aldrich
mColorpHast™ pH Test Strips, MilliporeSigma
PBS
Trypsin
Trypan blue

Strains and reagents

For this study, *Faecalibacterium prausnitzii* A2-165, *Bifidobacterium longum* spp *longum*, *Blautia luti* HTF-11B, *Escherichia coli* and *Bacteroides thetaiotaomicron* were used. Bacteria were grown in yeast extract, casitone, fatty acid and glucose (YCFAG) medium.

Characterization of -80° C glycerol stocks with MALDI – TOF technology

The effect of selenium was studied for five different bacterial strains. The bacteria studied were *Faecalibacterium prausnitzii*, *Bifidobacterium longum*, *Blautia luti*, *Escherichia coli* and *Bacteroides thetaiotaomicron*. In order to confirm the purity of the stocks, a small amount of bacteria were taken from -80° C glycerol stocks with a 10µl loop and put in a glass tube containing 3ml YCFAG. After 24 hours, the cultures were transferred into YCFAG agar plates with a 10µl loop. Next day, pure colonies were used and placed on spots in a 96 polished steel BC target. Two spots were prepared for the identification of each bacterium overlaid either with 1µL of matrix HCCA, portioned or with 1µL of 70% formic acid and 1µL of matrix HCCA. MALDI - TOF measurement was performed for the identification of the colonies.

Characterization of the growth of different bacterial species under treatment with selenium in aerobic and anaerobic conditions

To characterize the effect of selenium in their growth, bacteria were taken from glycerol stocks and inoculated in 3ml liquid YCFAG medium under anaerobic conditions. The bacteria were let grow for 24 hours and tested for purity with gram staining. Bacterial cultures were refreshed in YCFAG medium with a dilution of 1:100. After 18 hours, the bacteria were transferred to tubes containing YCFAG and selenium. Sodium selenite was diluted in MiliQ water until the concentration of 2mg/ml. The final concentrations of selenium used for the growth assays were 0µM (control), 10µM, 25µM and 100µM. The working cultures were prepared in duplicates in order to study the bacteria in both anaerobic (37° C anaerobic cabinet) and aerobic (37° C O₂ incubator) conditions. Each selenium condition was studied in biological triplicates. OD measurements were taken for each tube at time points 0 and 24 hours.

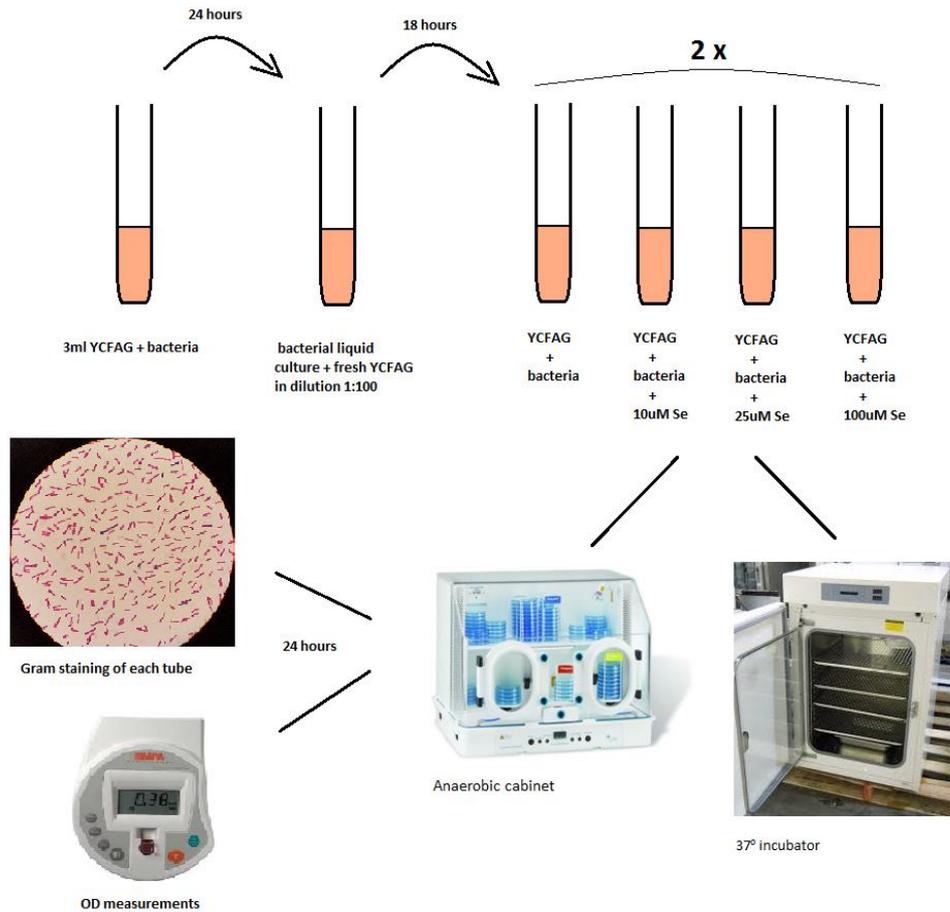


Figure 1. Overview of the experimental protocol for the characterization and study of the growth of different bacterial strains under supplementation with selenium.

Monitoring of microbial growth under selenium treatment using Biotek

The effect of different concentrations of selenium under semi-anaerobic conditions was evaluated by Biotek. In brief, glycerol stocks of the different strains were grown in YCFAG medium in the anaerobic cabinet. After 24 hours, the bacterial cultures were refreshed in new YCFAG medium in a dilution of 1:100. Next day, at approximately 18 hours, a 96-well plate was prepared, including different bacterial cultures in different selenium concentrations (Figure 2). Firstly, the working selenium solutions were prepared and the final concentrations of selenium tested were 0 μM (control), 10 μM, 25 μM and 100 μM. Bacteria were studied in biological triplicates and the different concentrations of selenium were tested as the technical triplicates. In order to keep the conditions anaerobic, a sealer was used to cover the 96-well plate and the plate was taken out of the anaerobic cabinet and placed in the Biotek machine. OD measurements at 600nm were taken every 30 minutes for 48 hours. Every 30 minutes, slight shaking of the plate also occurred. Growth curves were constructed using Prism graphpad.

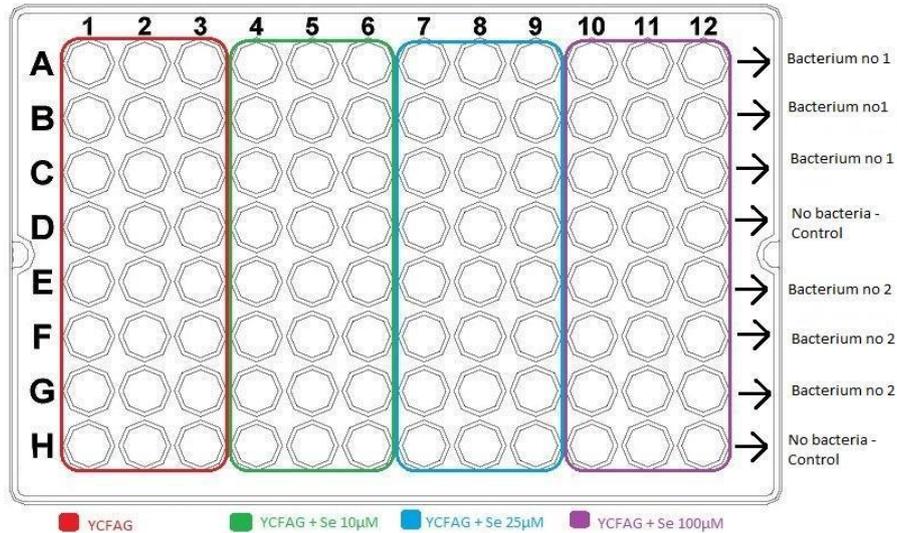


Figure 2. Layout of the 96-well plate destined for use in the Biotek. A 96 well plate was used for the Biotek assay and the bacteria was grown with or without the stimulation of selenium for 47 hours (initial $OD_{600} = 0.05$)

Study of T84 cells treated with 5-FU under selenium administration

To investigate the potential of selenium to prevent and/or recover damage by 5-FU treatment, T84 cells were grown in DMEM medium at 37° C and 5% CO² in T-150 flasks until they reached 80% confluency. The medium was removed from the flask and cells were washed with sterile PBS previously warmed up to 37°C. 3mL trypsin were added to the flask and cells were incubated for 10-15 minutes at 37°C. To stop trypsin reaction, warm DMEM medium was added to the culture and the resultant cell suspension was centrifuged for 5 minutes at 0.3rcf. The number of cells was counted using trypan blue and adjusted to 1×10^6 cells/mL. For the xCELLigence plate preparation, 10µL of the cells suspension were added to each well together with 90µL of DMEM medium. Three different plates were prepared. To study the T84 proliferation only, 100µL of medium were added to each well of the first plate which was placed on the xCELLigence for 48 hours at 37°C. 5µM, 10µM, 25µM, 50µM, 75µM and 100µM of selenium concentrations were tested. Medium in each well was removed and replaced by fresh DMEM at 24 hours. To study the effect of selenium in preventing 5-FU toxicity, 100µL of medium with or without supplementation of selenium (10µM or 25µM) were added to each well of the second plate which was placed on the xCELLigence for 24 hours at 37°C. After this period, the medium in each well was removed and replaced by medium containing 5-FU (150µM) and incubated for another 48 hours. Finally, to study the potential of selenium in recovering damage of 5-FU treatment, 100µL of medium were added to each well of the third plate. At 24 hours, the medium in each well was removed and replaced by medium containing 5-FU (150µM) and incubated for another 24 hours. After this period, cells were submitted to a post-treatment with different concentrations of selenium (5µM, 10µM and 25µM) and incubated for another 48 hours.

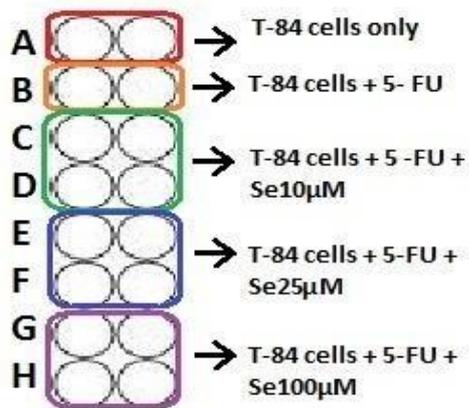


Figure 3. Example layout of 16-well plate destined for use in xCELLigence. 10 μ L of cell suspension with concentration 1×10^6 cells/mL were added to each well together with 90 μ L of DMEM medium. Different selenium and 5-FU treatments were then added to the cells. Incubation time fluctuated between 48 and 96 hours, according to different experimental setups.

Statistical analysis

Results from each experiment were statistically analyzed using Prism graph pad (version 5). Normality of sample data was checked using D'Agostino and Pearson omnibus normality test. A Tukey test was performed for normally distributed data and a Dunn's test for not normally distributed data.

RESULTS

Characterization of the growth of different bacterial species under treatment with selenium in aerobic and anaerobic conditions

To characterize the effect of selenium on the growth of different bacterial strains under oxidative stress, different concentrations of selenium (10 μM , 25 μM and 100 μM) were added to liquid bacterial cultures and the optimal density (OD) was measured. The graphs below show the ΔOD at 0 and 24 hours of each bacterial culture stimulated with selenium. The results were statistically analyzed using graph pad. Gram staining was performed to characterize the morphology and test the purity of every bacterial culture, except the ones that bacteria didn't grow.

The effect of selenium on the growth of Faecalibacterium prausnitzii

F.prausnitzii was able to grow both aerobically and anaerobically. However the growth was much lower in aerobic conditions when compared to anaerobic ones ($p=0,0002$). No differences were observed between the growth of control group and the ones supplemented with 10 μM , 25 μM and 100 μM of selenium in both conditions (Figures 4,5).

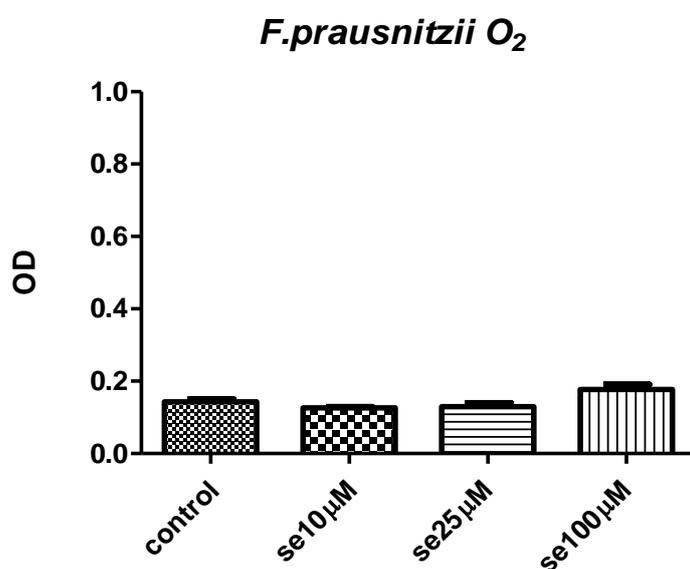


Figure 4. Low growth of *F.prausnitzii* in aerobic conditions. No differences in the growth were observed between control cultures and cultures incubated with 10 μM , 25 μM and 100 μM selenium.

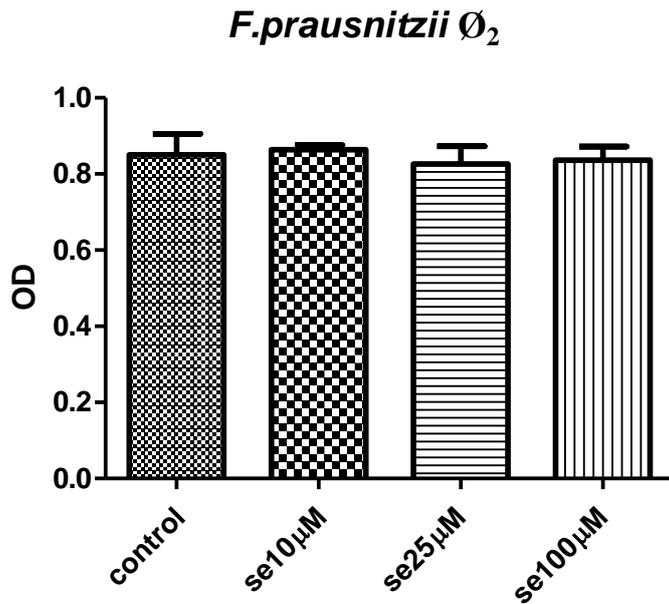


Figure 5. Growth of *F. prausnitzii* in anaerobic conditions. No differences observed between control cultures and cultures incubated with 10µM, 25µM and 100µM selenium.

No morphological differences of *F. prausnitzii* were observed in aerobic conditions (Figure 6). In anaerobic conditions chains of *F. prausnitzii* were observed under treatment with selenium (Figure 7). 100µM selenium caused formation of aggregates (Figure 7). Presence of selenium was observed by purple dots in the environment and on the bacteria, especially in the highest concentrated selenium cultures (Figures 6,7).

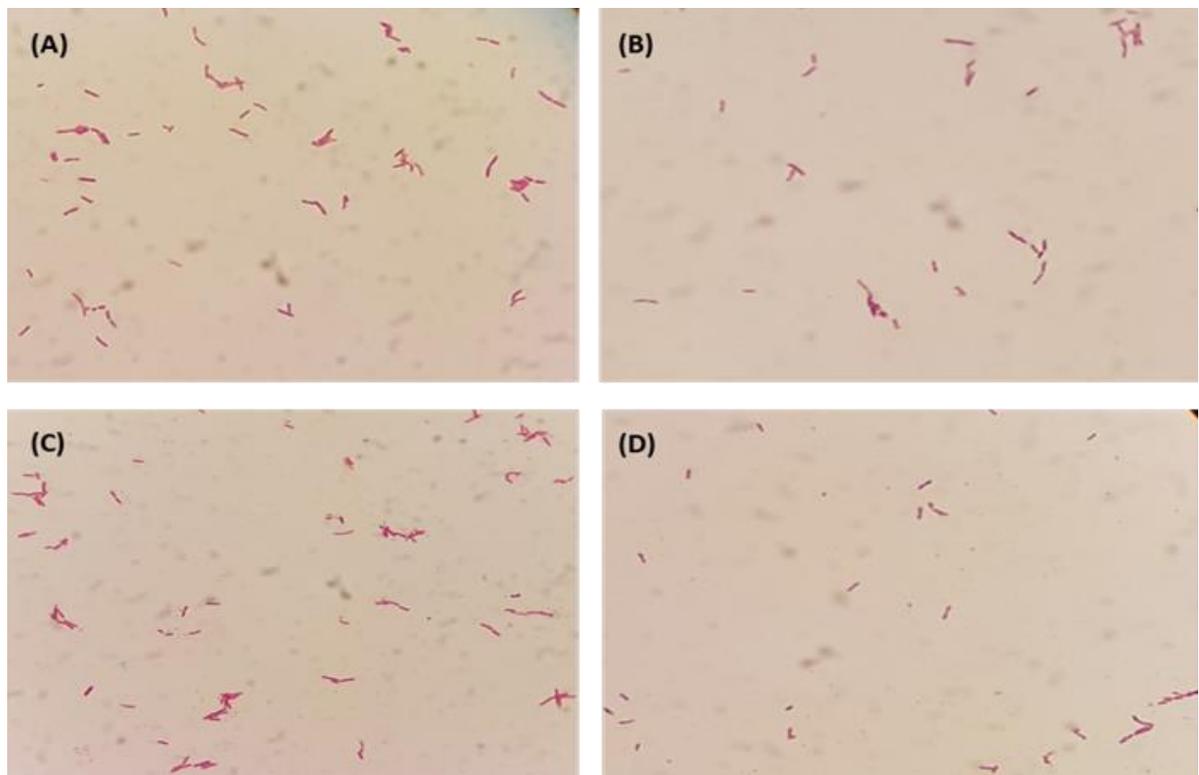


Figure 6. Optical microscopy (100X) of *F. prausnitzii* cultures after 24 hours of incubation with 0µM (A), 10 µM (B), 25 µM (C) and 100 µM (D) selenium in aerobic conditions. No morphological differences were observed. Selenium presence was obvious by the purple dots in the environment and on the bacteria, especially in 100µM selenium (D).

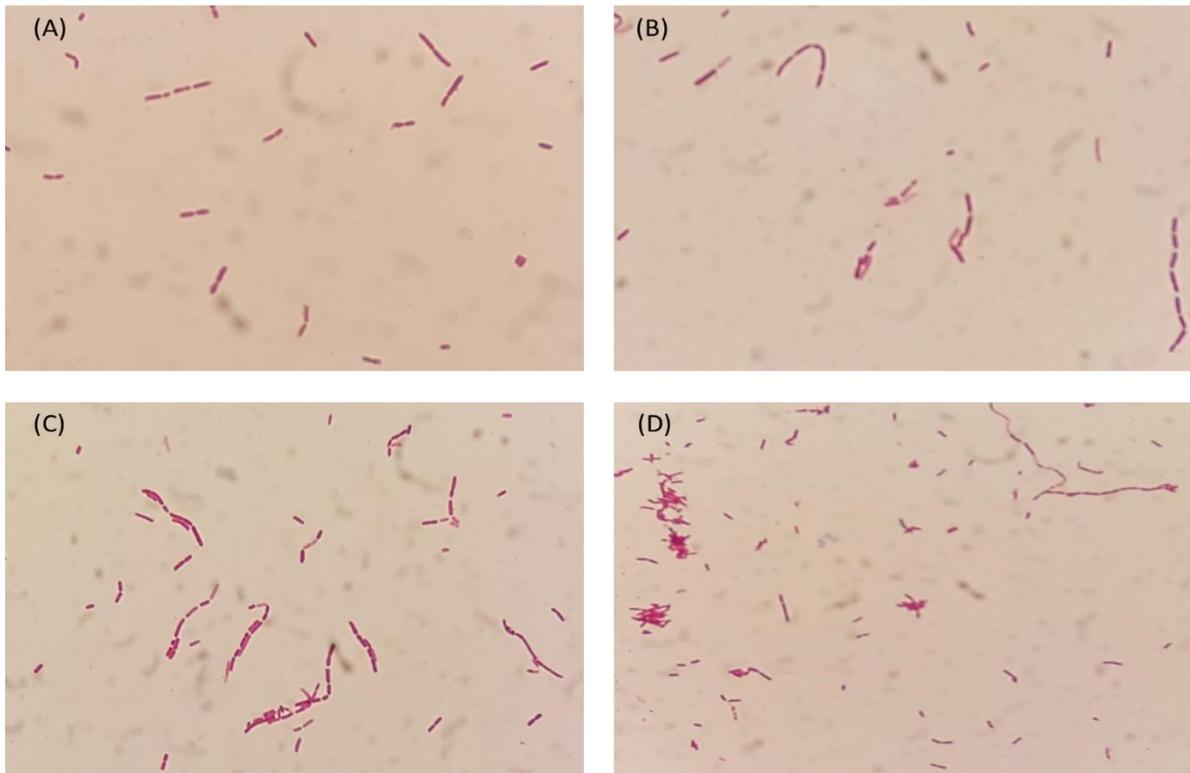


Figure 7. Optical microscopy (100X) of *F. prausnitzii* cultures after 24 hours of incubation with 0 μ M (A), 10 μ M (B), 25 μ M (C) and 100 μ M (D) selenium in anaerobic conditions. Chain formation (B),(C),D) and aggregation of bacteria (D) was observed in selenium supplemented cultures. Selenium presence was obvious by the purple dots in the environment and on the bacteria, especially in 100 μ M selenium (D).

The effect of selenium on the growth of Bifidobacterium longum

Similarly to *F. prausnitzii*, *B. longum* was able to grow in both aerobic and anaerobic conditions, however the growth was much lower in aerobic conditions compared to anaerobic ones ($p < 0,0001$). No differences between the growth of the control bacterial cultures and the ones supplemented with 10 μ M, 25 μ M and 100 μ M of selenium were detected in aerobic conditions (Figure 8). In anaerobic conditions a decreased growth was observed for the selenium supplemented cultures compared to the control, independently of the concentration of selenium. However, the observed differences were not found statistically significant (Figure 9).

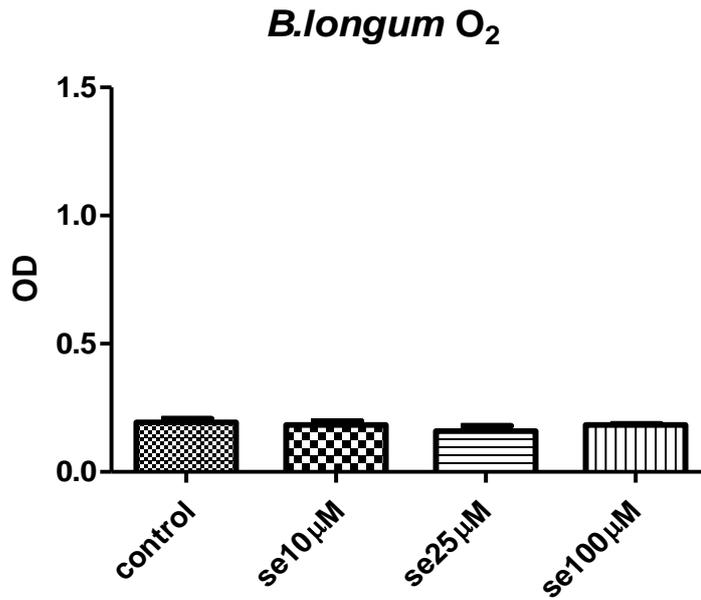


Figure 8. Low growth of *B.longum* in aerobic conditions. No differences were observed between control cultures and cultures incubated with 10µM, 25µM and 100µM selenium.

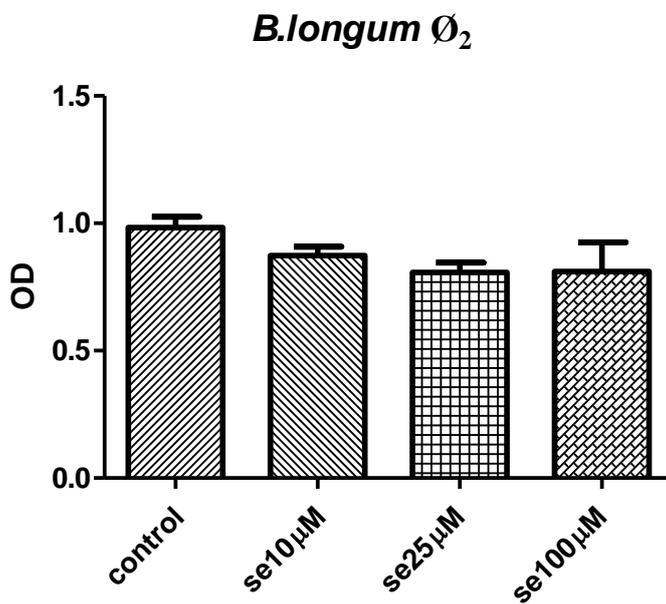


Figure 9. Growth of *B.longum* in anaerobic conditions. No significant differences were found between control cultures and cultures incubated with 10µM, 25µM and 100µM selenium.

B.longum formed aggregates when incubated with selenium both aerobically and anaerobically (Figures 10,11). Again, presence of selenium was observed by purple dots in the environment and on the bacteria, with a higher density in 100µM selenium cultures (Figures 10,11).

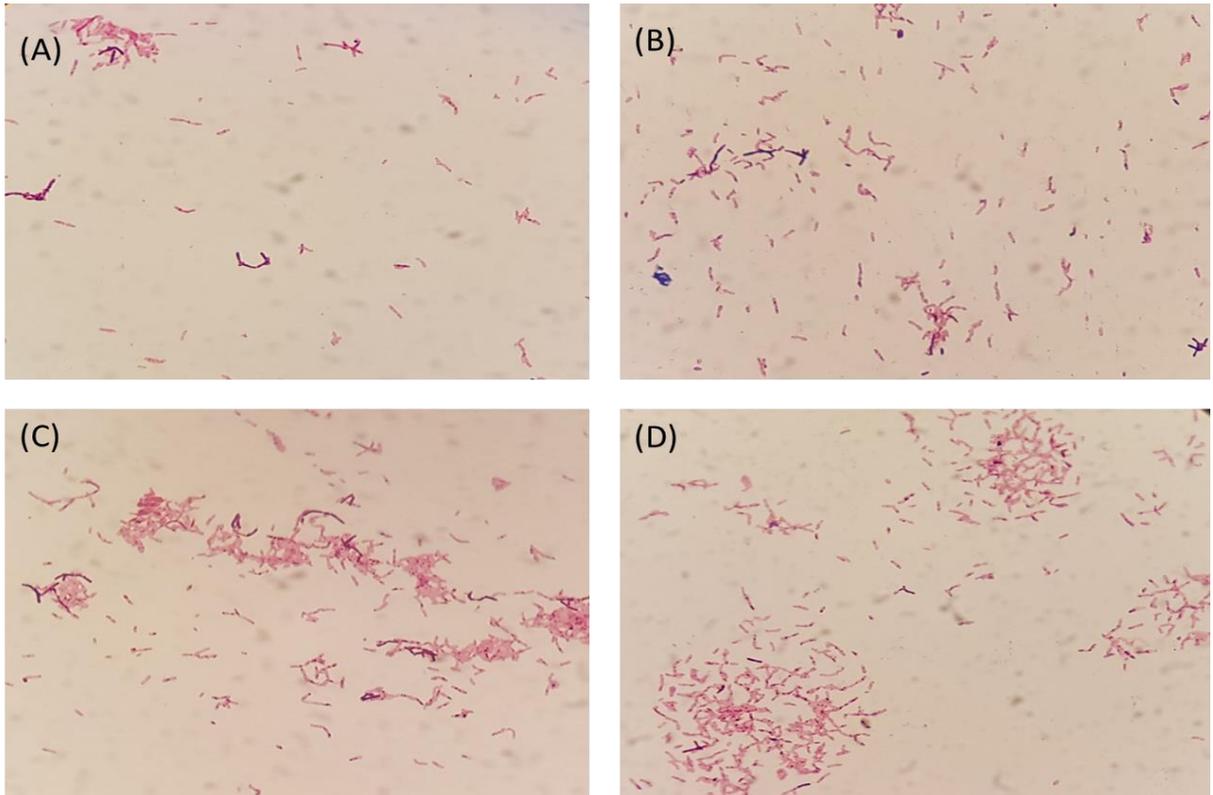


Figure 10. Optical microscopy (100X) of *B. longum* cultures after 24 hours of incubation with 0 μ M (A), 10 μ M (B), 25 μ M (C) and 100 μ M (D) selenium in aerobic conditions. Selenium provoked aggregation of *B. longum* (C)(D). Selenium presence was obvious by the purple dots in the environment and on the bacteria, especially in 100 μ M selenium (D).

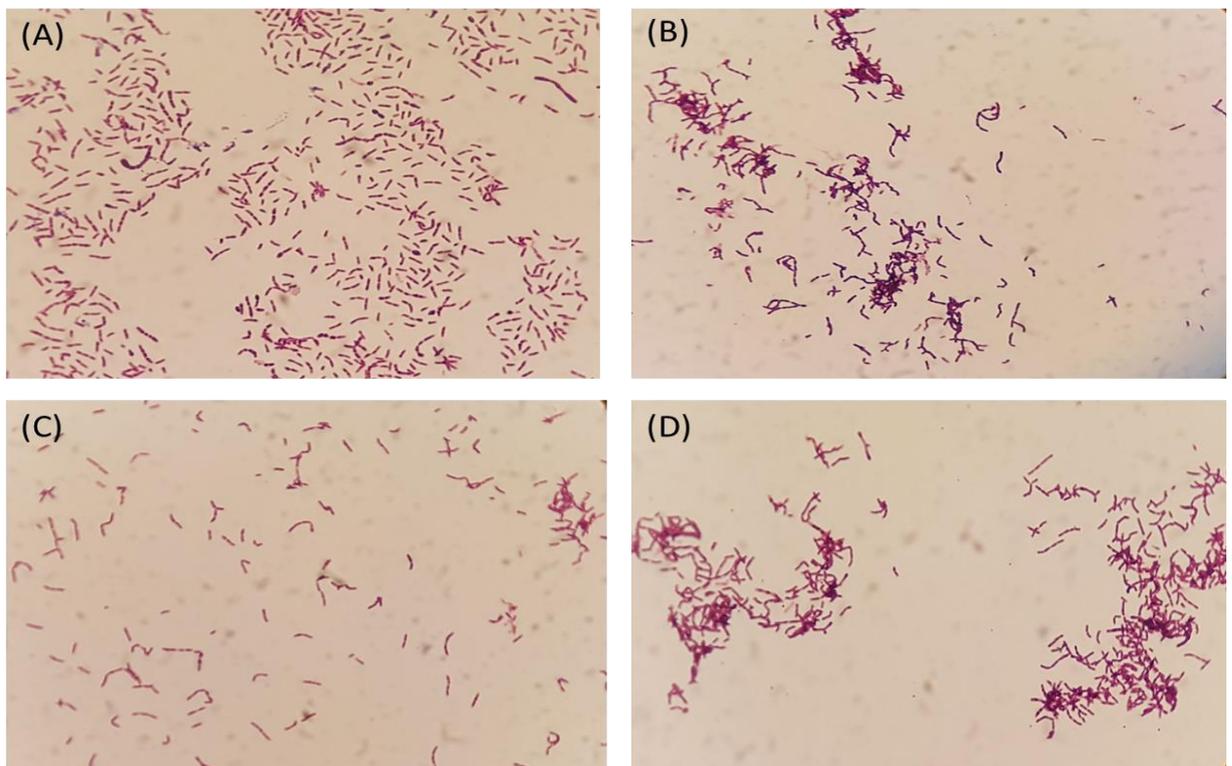


Figure 11. Optical microscopy (100X) of *B. longum* cultures after 24 hours of incubation with 0 μ M (A), 10 μ M (B), 25 μ M (C) and 100 μ M (D) selenium in anaerobic conditions. Selenium provoked aggregation of *B. longum* (B)(D). Selenium presence was obvious by the purple dots in the environment and on the bacteria, especially in 100 μ M selenium (D).

The effect of selenium on the growth of *Blautia luti*

B.luti was able to grow in anaerobic conditions but not in aerobic conditions (Figure 12). In anaerobic conditions supplementation with selenium was toxic for the bacteria and decreased their growth in a dose-dependent manner (Figure 13). 100 μ M selenium did not allow the bacteria to grow (Figure 13). The observed differences were found to be statistically significant ($p < 0,0001$).

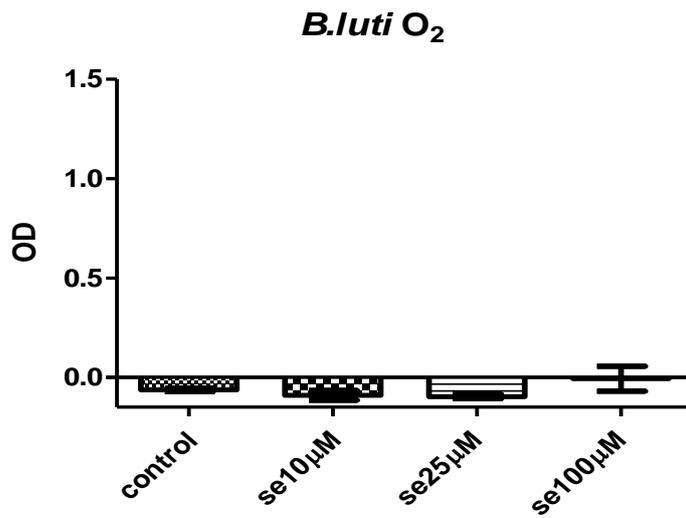


Figure 12. No growth of *B.luti* in aerobic conditions. No differences were observed between control cultures and cultures incubated with 10 μ M, 25 μ M and 100 μ M selenium.

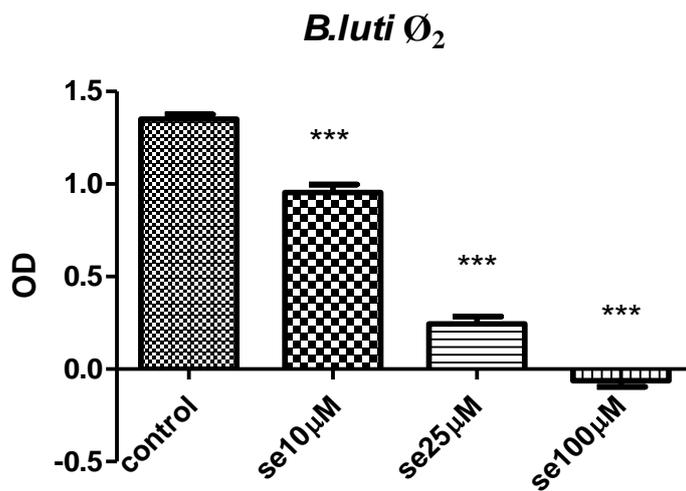


Figure 13. Toxic effect of selenium on the growth of *B.luti* in anaerobic conditions. Decreased growth of cultures incubated with 10 μ M and 25 μ M selenium. No growth of *B.luti* when incubated with 100 μ M. *** = $p < 0,0001$

Formation of chains of bacteria were observed in the presence of selenium in anaerobic conditions (Figure 14). Selenium presence was obvious by the purple dots in the environment and on the bacteria. Bacteria didn't grow in aerobic conditions.

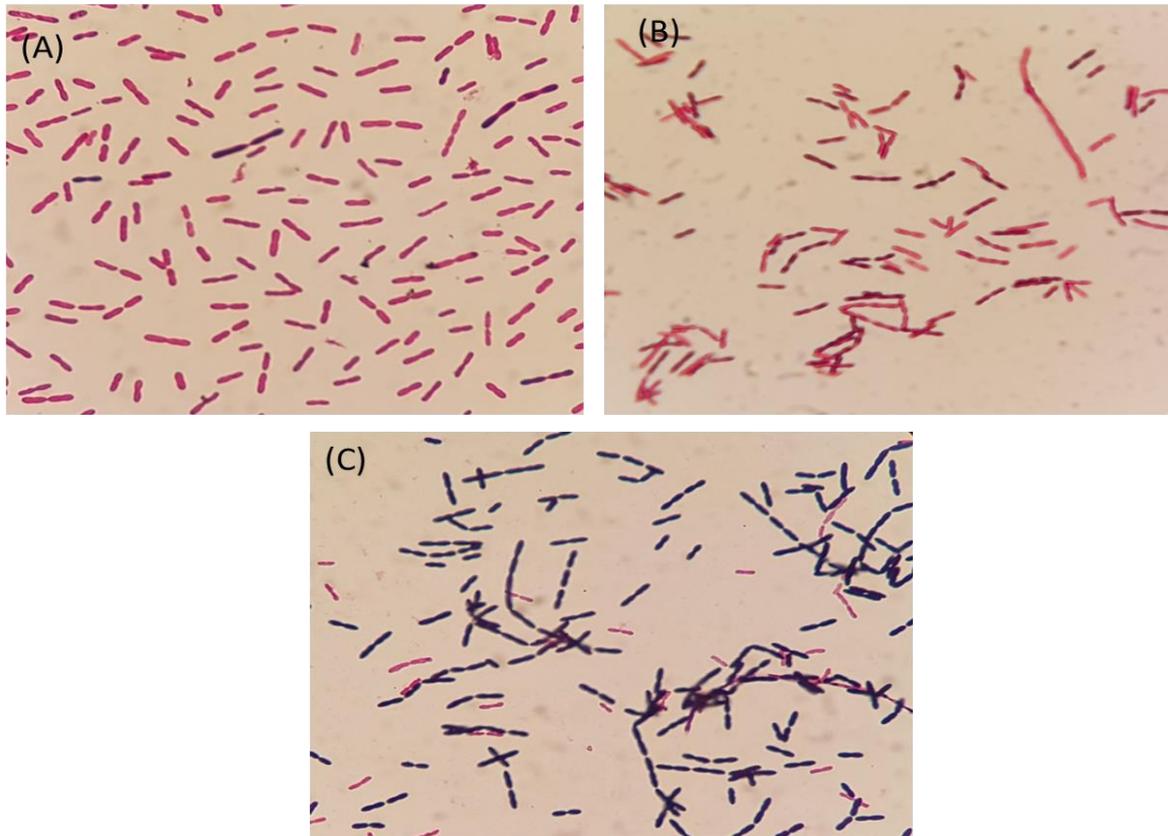


Figure 14. Optical microscopy (100X) of *B.luti* cultures after 24 hours of incubation with 0 μ M (A), 10 μ M (B) and 25 μ M (C) selenium in anaerobic conditions. *B.luti* could be stained both as gram-negative (A) and as gram-positive (B) bacterium. Chain formation was observed in the presence of selenium (B)(C). Selenium presence was obvious by the purple dots in the environment and on the bacteria.

The effect of selenium on the growth of Escherichia coli

E.coli was able to grow both in aerobic and anaerobic conditions. In aerobic conditions, supplementation with selenium lowered the growth of the bacteria, but the concentration did not affect the level of the decrease (Figure 15). The observed differences were statistically significant ($p=0,0084$). In anaerobic conditions there weren't any detectable differences (Figure 16).

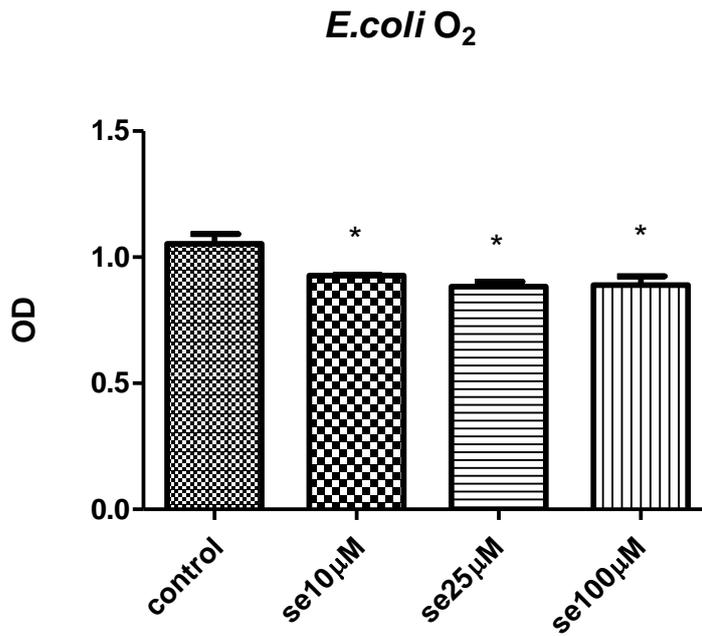


Figure 15. Growth of *E.coli* in aerobic conditions. 10µM, 25µM and 100µM selenium decreased the growth of *E.coli* in a dose-independent manner. * = p<0,05

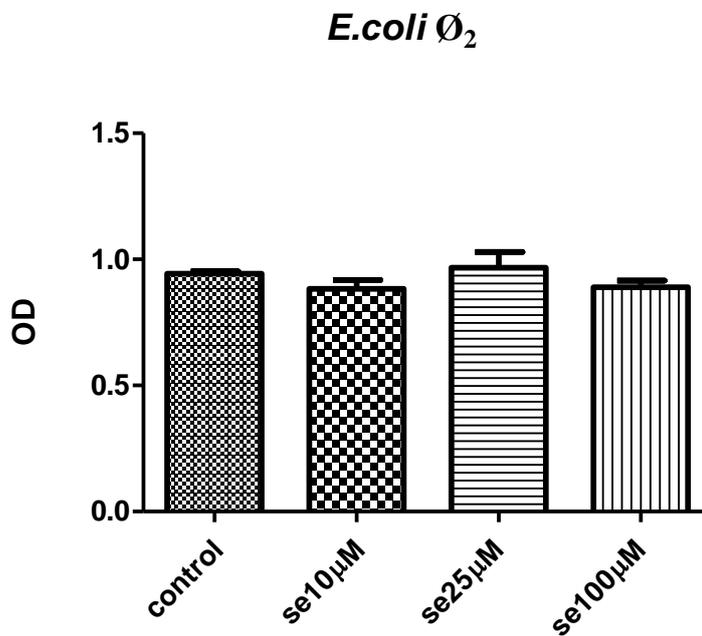


Figure 16. Effect of selenium on the growth of *E.coli* in anaerobic conditions. Selenium had no effect on the growth of *E.coli*.

No morphological differences were detected between control and selenium incubated cultures when gram staining was performed (Figures17,18). As previously observed, selenium presence was obvious by the purple dots in the environment and on the bacteria.

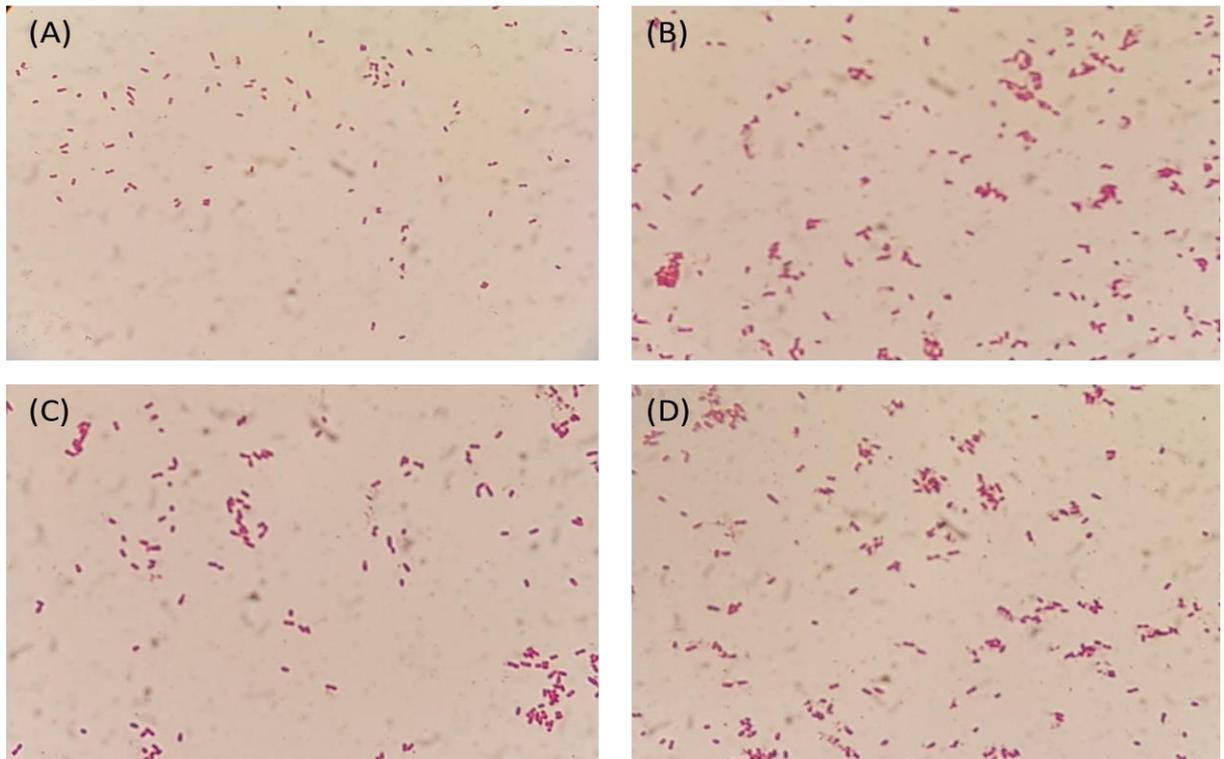


Figure 17. Optical microscopy (100X) of *E.coli* cultures after 24 hours of incubation with 0 μ M (A), 10 μ M (B) and 25 μ M (C) selenium in aerobic conditions. No morphological differences were observed. Selenium presence was obvious by purple dots in the environment and on the bacteria (B)(C)(D).

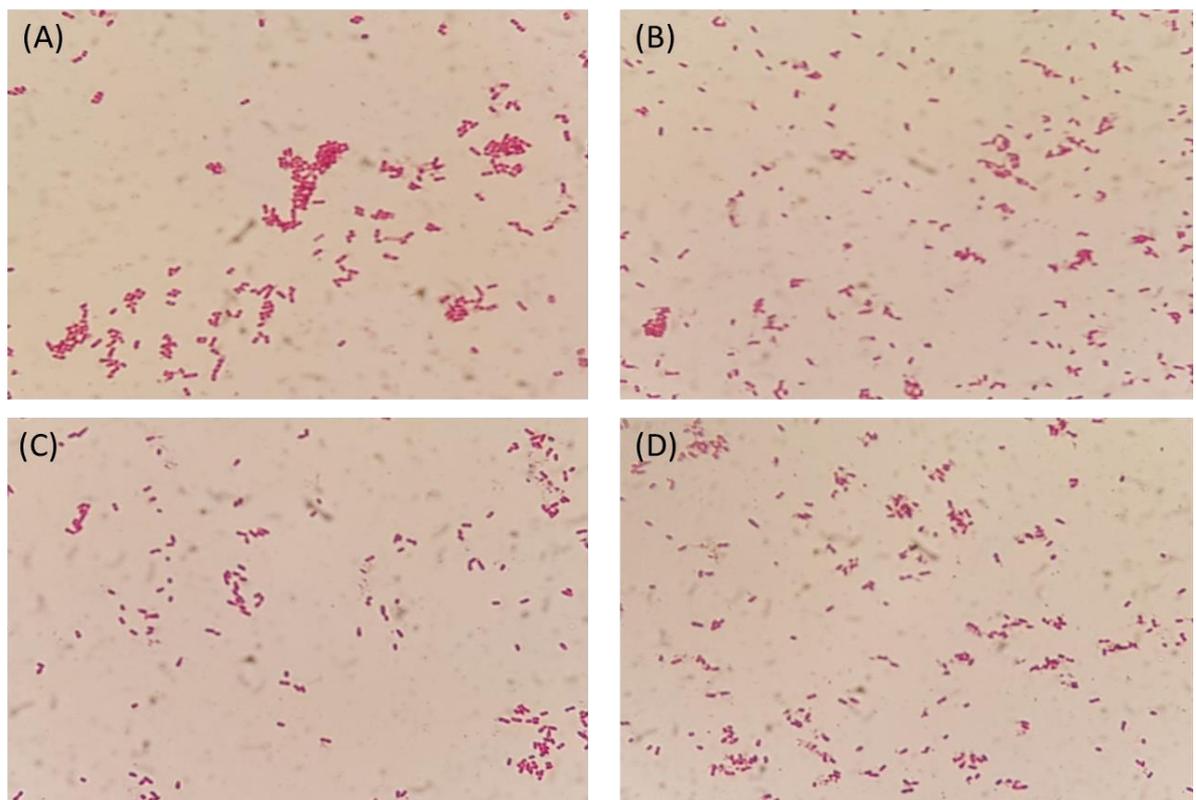


Figure 18. Optical microscopy (100X) of *E.coli* cultures after 24 hours of incubation with 0 μ M (A), 10 μ M (B) and 25 μ M (C) selenium in anaerobic conditions. No morphological differences were observed. Selenium presence was obvious by purple dots in the environment and on the bacteria (B)(C)(D).

The effect of selenium on the growth of *Bacteroides thetaiotaomicron*

B.thetaiotaomicron was able to grow both in aerobic and anaerobic conditions. However the growth was very low, especially in aerobic conditions. Selenium supplementation decreased the growth of the bacteria in both aerobic and anaerobic conditions. In aerobic conditions, the reduction was dose-independent (Figure 19). The observed differences were statistically significant ($p=0,0005$). In anaerobic conditions selenium in 10 μM lowered the growth of the bacteria while selenium in 25 μM and 100 μM inhibited the growth completely (Figure 20). The difference in the growth between the control and the selenium 10 μM supplemented cultures was not statistically significant in contrast with the difference detected between the control and the selenium 25 μM and 100 μM supplemented cultures ($p=0,050$).

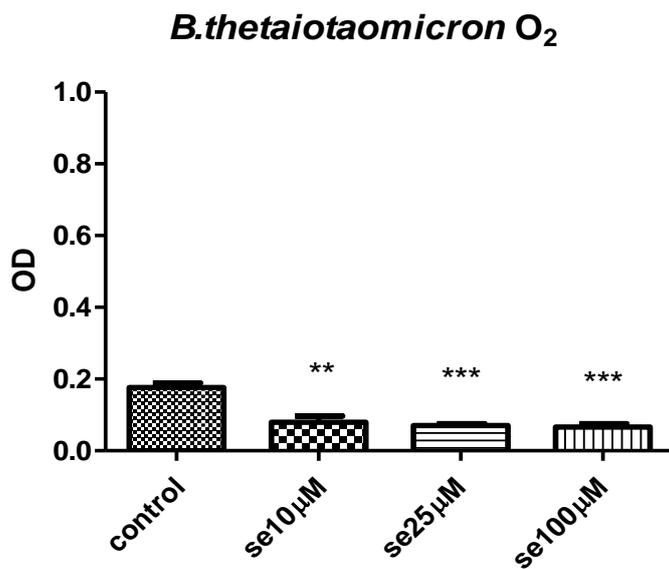


Figure 19. Growth of *B.thetaiotaomicron* in aerobic conditions. Selenium decreased the growth of the bacteria. ** = $p<0,001$, *** = $p<0,0001$

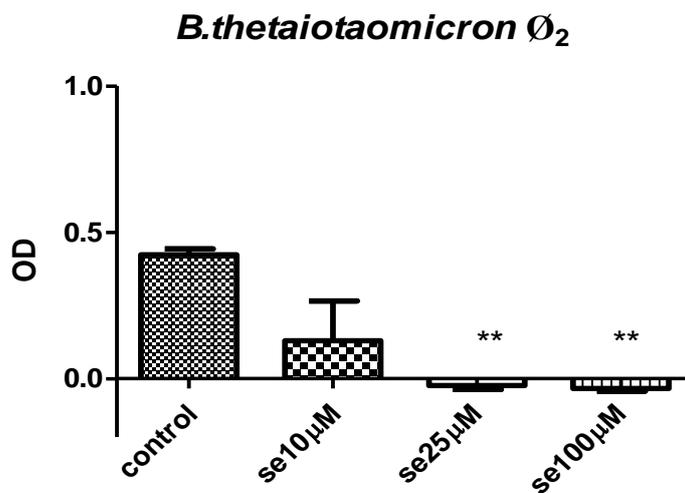


Figure 20. Growth of *B.thetaiotaomicron* in anaerobic conditions. 25 μM and 100 μM selenium inhibited the growth of the bacteria. ** = $p<0,001$

Gram staining was possible only for control cultures. Longer rods of bacteria were observed in aerobic conditions when compared to anaerobic conditions (Figure 21).

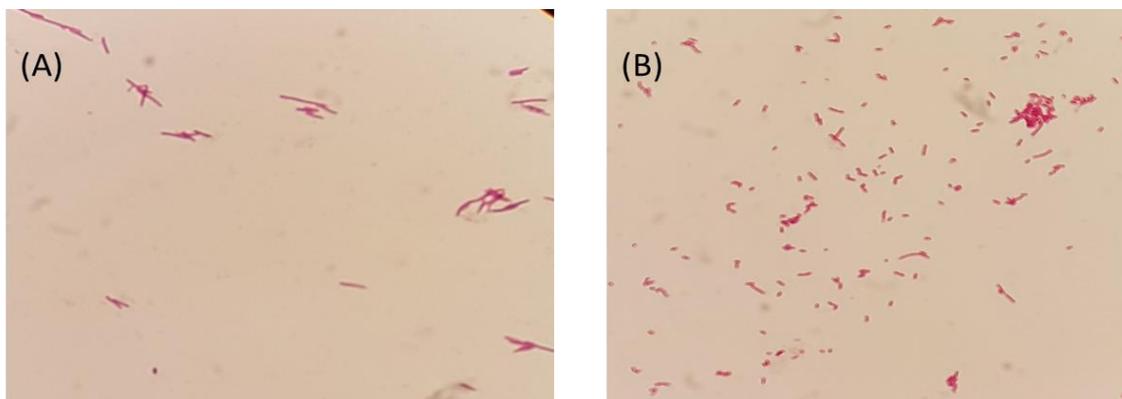


Figure 21. Optical microscopy (100X) of *B.thetaiotaomicron* cultures after 24 hours of incubation in anaerobic (A) and aerobic (B) conditions. Bacteria didn't grow in the presence of selenium.

Elemental selenium precipitation

After 24 hours of the bacteria growing with selenium, a red precipitate was observed in the tubes containing 100 μ M of selenium. This was most of the times obvious with gram staining in the form of purple dots in the environment but also on the bacteria. The red precipitate was the sodium selenite, the inorganic form of selenium that bacteria were treated, turning into red elemental selenium.

Selenium affects microbial growth in semi-anaerobic Biotek experimental conditions

In order to monitor the microbial growth under selenium treatment, bacterial cultures were grown for 48 hours and the OD values were measured at 600 nm every 30 minutes. The experiment took place in semi- anaerobic conditions for every bacterial species except *E.coli* for which it took place also in aerobic conditions. The graphs and statistical analysis were made using Prism graph pad.

F. prausnitzii growth curves show that this bacterium grew better with the presence of 10 μ M selenium supplementation when compared to the control (YCFAG medium only). 25 μ M of selenium decreased the growth of the bacteria while 100 μ M of selenium inhibited the growth completely (Figure 22).

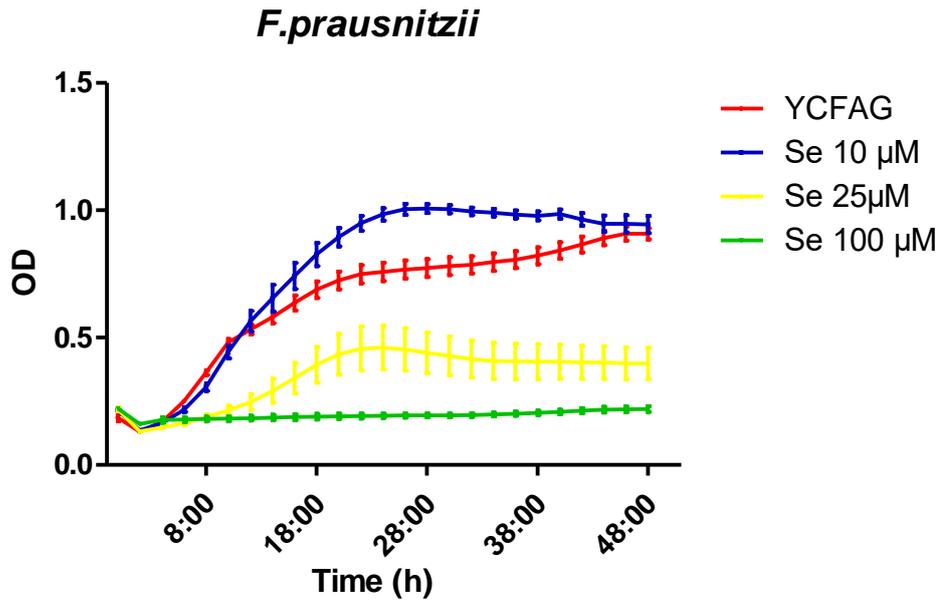


Figure 22. The effect of selenium on the growth of *F.prausnitzii*. Bacterial cultures of *F. prausnitzii* were prepared and different concentrations of selenium were added. Optimal density was measured by Biotek for 48 hours, under semi/anaerobic conditions. 10μM selenium promoted the growth of *F.prausnitzii*. 25μM selenium lowered the growth of *F.prausnitzii* while 100μM selenium inhibited the growth completely. Maximum OD values of each curve not statistically significant. Data from biological and technical triplicates.

Selenium promoted the growth of *B.longum* but independently of selenium dosage. However, there was a small delay in reaching maximum growth of the bacteria supplemented with 25μM selenium and an even bigger delay in the growth of bacteria supplemented with 100μM selenium when compared to 10μM selenium (Figure 23).

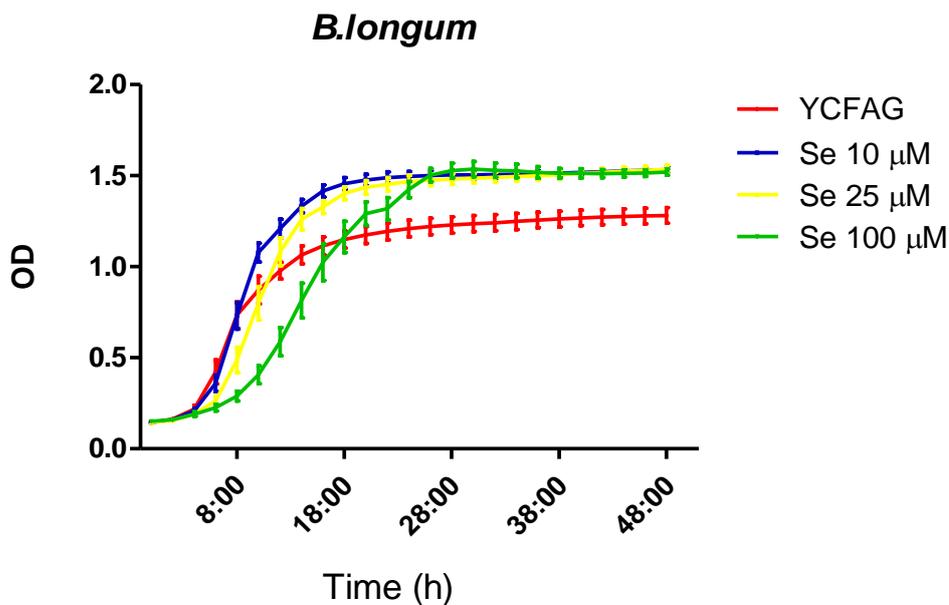


Figure 23. The effect of selenium on the growth of *B.longum*. Bacterial cultures of *B.longum* were prepared and different concentrations of selenium were added. Optimal density was measured by Biotek for 48 hours, under semi/anaerobic conditions. Maximum OD values were higher in selenium treated bacteria compared to the control. The differences observed however were not found statistically significant. Data from biological and technical triplicates.

B.luti was positively affected by 10 μ M and 25 μ M of selenium in the medium. Both the growth curves are higher compared to the control. Bacteria fed with 25 μ M of selenium reached the maximum growth later in comparison to 10 μ M of selenium and control. 100 μ M of selenium was toxic for the bacteria and inhibited the growth completely.

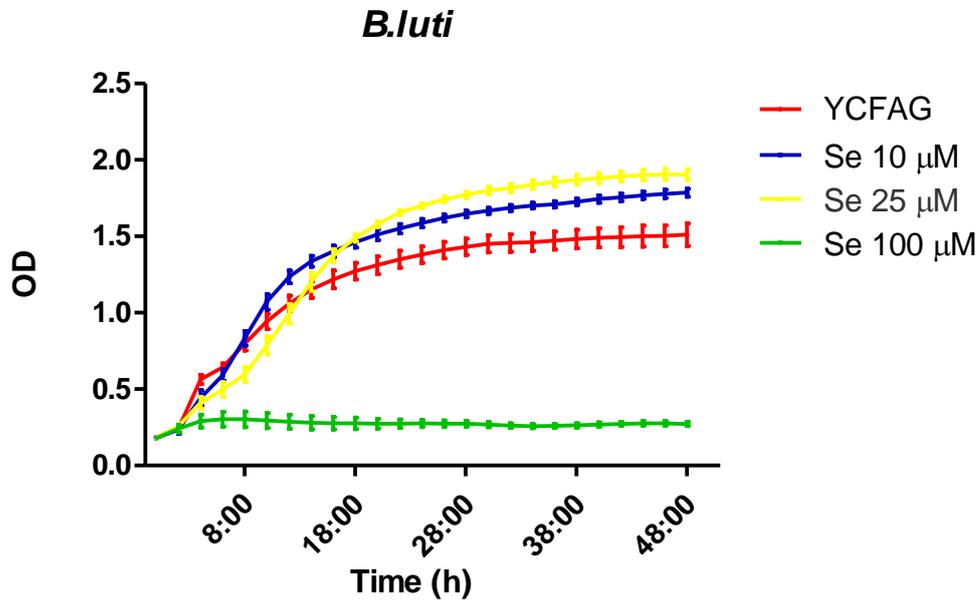


Figure 24. The effect of selenium on the growth of *B.luti*. Bacterial cultures of *B.luti* were prepared and different concentrations of selenium were added. Optimal density was measured by Biotek for 48 hours, under semi/anaerobic conditions. 10 μ M and 25 μ M of selenium promoted the growth of *B.luti* while 100 μ M selenium was toxic for bacteria. The differences in maximum OD values were not statistically significant. Data from biological and technical triplicates.

In anaerobic conditions, *E.coli* had a huge growth advantage when it was supplemented with selenium, independently of the concentration of selenium (Figure 25). In aerobic conditions, selenium didn't affect the level of the growth of *E.coli*. The same growth pattern was observed for every different condition, however selenium delayed reach of maximum growth (Figure 26).

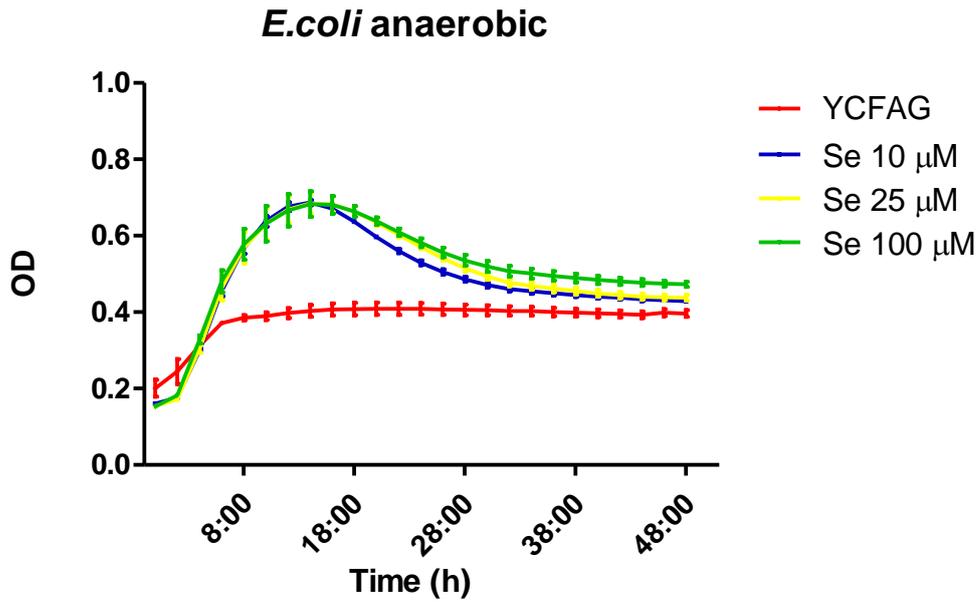


Figure 25. The effect of selenium on the growth of *E. coli* in anaerobic conditions. Bacterial cultures of *E. coli* were prepared and different concentrations of selenium were added. Optimal density was measured by Biotek for 48 hours, under semi/anaerobic conditions. Selenium promotes the growth of *E. coli* but not in a dose-dependent manner. The maximum growth was higher for every concentration of selenium compared to the controls. However the differences were not found to be significant. Data from biological and technical triplicates.

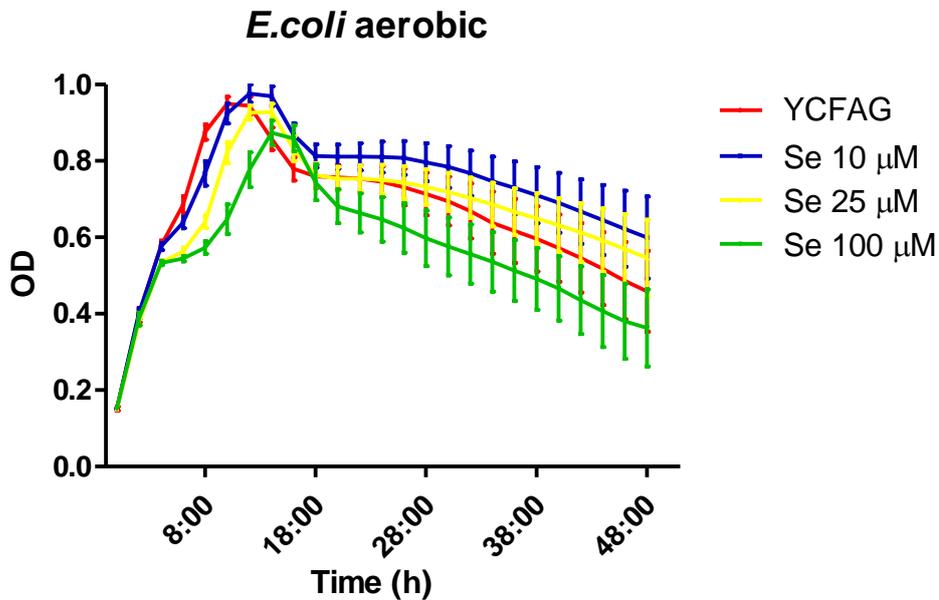


Figure 26. The effect of selenium on the growth of *E. coli* in aerobic conditions. Bacterial cultures of *E. coli* were prepared and different concentrations of selenium were added. Optimal density was measured by Biotek for 48 hours, under semi/anaerobic conditions. Selenium did not affect the level of the growth, but caused a delay in reaching maximum growth in a dose-dependent manner. Level of maximum growth did not differ between the different conditions. Data from biological and technical triplicates.

Selenium might be able to prevent 5-FU cytotoxicity but does not affect T84 proliferation

The effect of selenium in T84 proliferation was studied alone but also before and after 5-FU administration. The graphs below show the T84 viability curves under the different treatments and conditions. The bar charts present the final cell index of each experiment. The data from the final cell index were analyzed statistically using PRISM graph pad.

Selenium supplementation did not offer any proliferative advantage in T84 cells. 50 μ M, 75 μ M and 100 μ M concentrations of selenium proved to be toxic for T84 cells, but not 5 μ M, 10 μ M and 25 μ M of selenium (Figures 27,28).

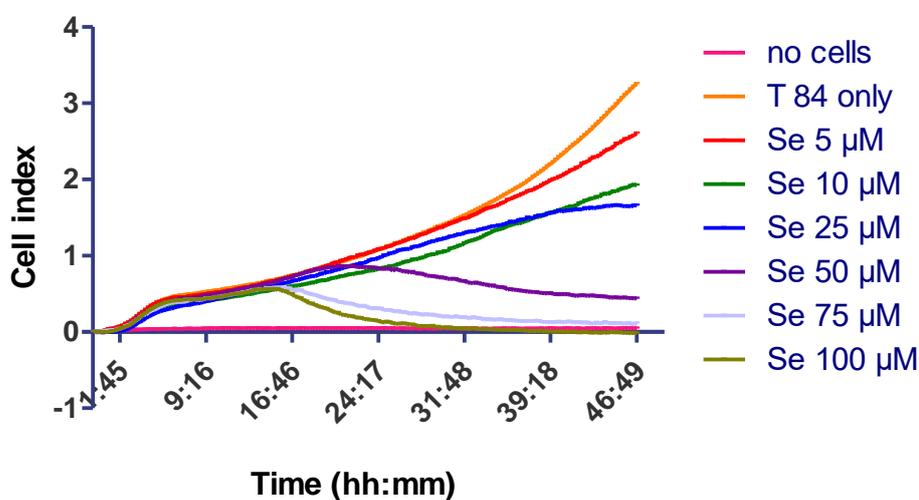


Figure 27. The effect of selenium on the proliferation of T84 cells. T84 cells were grown for 47 hours until they have reached full confluency. Selenium was added at 0 hour and cell viability was measured by xCELLigence. Selenium supplementation did not offer any proliferative advantage in T84 cells. Selenium in 50 μ M, 75 μ M and 100 μ M concentrations proved to be toxic for T84 cells, but not 5 μ M, 10 μ M and 25 μ M of selenium.

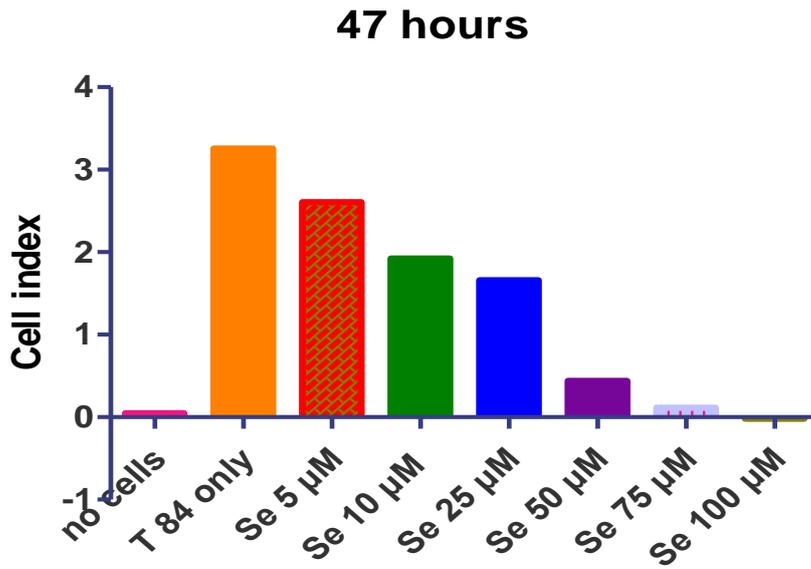


Figure 28. T84 cell index at 47 hours after growing with different concentrations of selenium. Negative effect of selenium in T84 cell proliferation in a dose-dependent manner. Statistical analysis was not possible due to low number of data.

10µM of selenium supplementation 24 hours before 5-FU administration was able to prevent 5-FU cytotoxicity as the viability of T84 cells was the same between control (T84 only) and 10µM selenium treated cells. However 25 µM of selenium did not affect the viability of 5-FU treated cells (Figure 29,30).

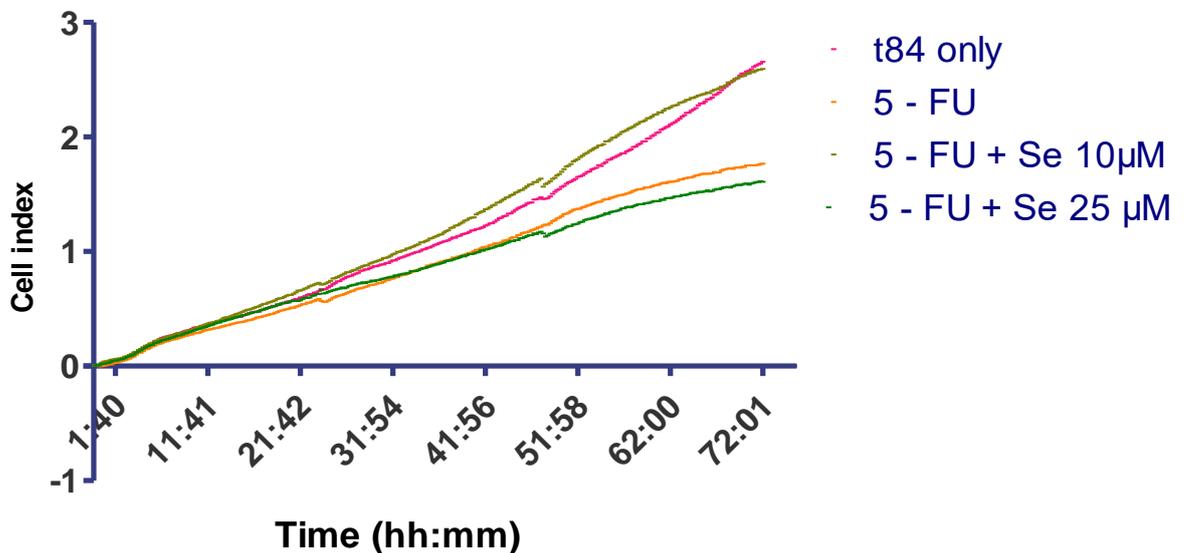


Figure 29. Effect of pre-treatment of selenium in 10µM and 25µM on T84 cells viability in 5-FU treated T-84 cells. Selenium was added at 0 hours and 5-FU was added at 24 hours. Cell viability was measured by xCELLigence for 72 hours. 10µM selenium promoted the T84 cell proliferation.

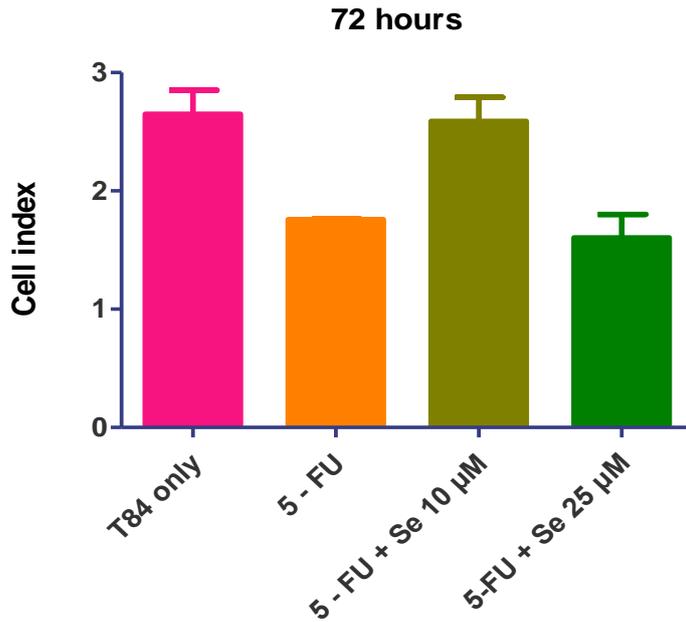


Figure 30. Cell index of pre-treated with 10µM and 25µM of selenium 5-FU treated T84 cells at 72 hours. Index of cells pre-treated with 10µM selenium is similar with cells growing without 5-FU and higher than 5-FU treated cells. However, the differences are not statistically significant

Selenium was able to give a slight proliferative effect compared to the 5-FU only treated cells (Figures 31,32). However, the differences in cell index at 96 hours between the different groups were not statistically significant.

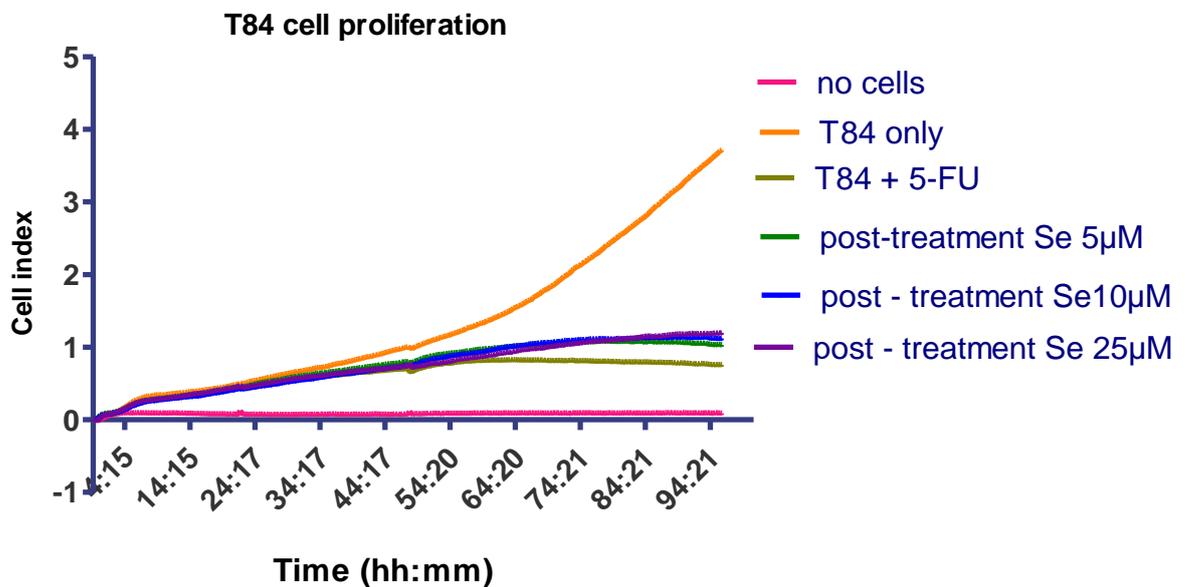


Figure 31. Effect of treatment of selenium on T84 cells viability, 24 hours after 5-FU administration. T84 cells were treated with 5-FU at 24 hours and supplemented with 5µM, 10µM or 25 µM of selenium 24 hours after 5-FU administration. Cell viability was monitored for 96 hours overall.

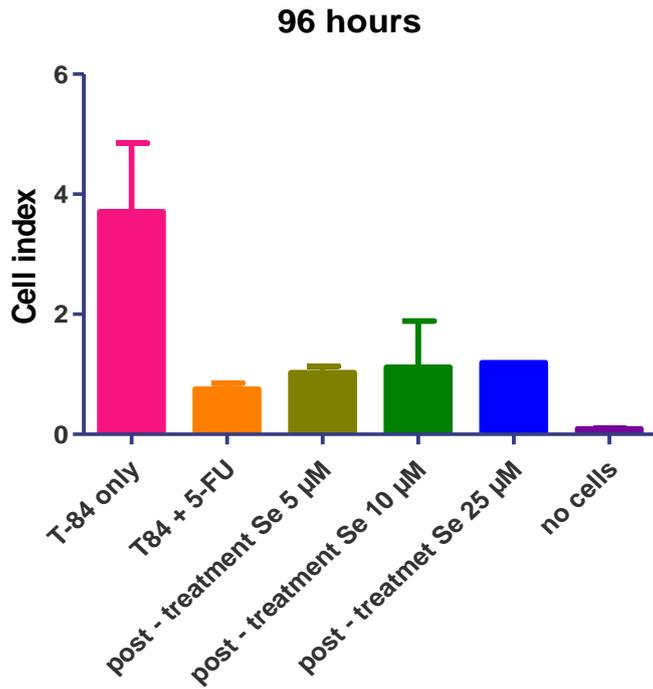


Figure 32. Effect of post-treatment of 5 μ M, 10 μ M and 25 μ M of selenium in cell index of 5-FU treated T84 cells at 96 hours. Slightly higher cell index of selenium supplemented cells compared to 5-FU treated cells. No significant differences were found.

DISCUSSION

Gastrointestinal mucositis is a very severe and most common side effect of chemotherapy/radiotherapy and patients face a plethora of very painful symptoms which lead to less efficient cancer treatment and a higher chance to morbidity and mortality (Sonis, 2004). Unfortunately, treatment of mucositis remains a big challenge as there is not any accepted treatment protocol. Therefore, it is very important to investigate novel therapeutic strategies which will ameliorate the symptoms and/or prevent mucositis.

In the last few decades, evidence has shown that the composition of the gut microbiota plays a major role in the development of mucositis. Microbes present in the gut significantly contribute to health and disease and mucositis has indeed been linked with a disbalanced microbiota (Alexander et al, 2017). As it was expected, traditional probiotics (eg. *Bifidobacterium infantis*) have been tested as a new therapeutic approaches. However, most of them have failed when translated to the clinics (Wardill et al, 2018). Nevertheless, several studies have shown that the manipulation of the gut microbiota might be an efficient way to overcome mucositis symptoms. Additionally, the development of new generation probiotics might also prove helpful in the treatment of mucositis.

The present project is focused on the trace element selenium and its potential role as a therapeutic approach to treat mucositis. Previous studies have shown that selenium supplementation attenuates oral mucositis in cancer patients as well as it ameliorates chemotherapy-induced mucositis in rats. Moreover, together with probiotics, selenium supplementation resulted in less intestinal damage in mice (Jahangard-Rafsanjani et al, 2013; Lee et al, 2017; Qiu et al, 2019). In this study, the growth of different gut bacteria was investigated under supplementation with selenium. Five different bacterial strains, *Faecalibacterium prausnitzii*, *Bifidobacterium longum*, *Blautia luti*, *Escherichia coli* and *Bacteroides thetaiotaomicron* were tested. This cluster of bacteria was carefully selected as they represent different groups of important bacteria present in the human gut. *F. prausnitzii* is a butyrate-producing bacterium and one of the most abundant bacterium in the human gut. *B. longum* is a widely used probiotic strain, *B. luti* represents 30% of the total gut microbial population, *E. coli* inhabits the gut as a very small population but is also a potential pathogen and *B. thetaiotaomicron* represents the phylum of Bacteroidetes (Sokol et al, 2008, Qiu et al, 2019; Eren et al, 2015; Tenaillon et al, 2010; Xu et al, 2003). We considered this group an indicative sample of a gut microbial population without ignoring the fact that it is an extremely limited amount of different bacterial strains compared to the number of the different species that normally inhabit human gut.

F. prausnitzii was able to grow in anaerobic conditions but its growth was compromised under aerobic conditions (Figures 4,5). This confirms the fact that even though *F. prausnitzii* is an obligate anaerobe, it can tolerate to some extent the presence of oxygen (Khan et al, 2012). When the bacteria were grown in glass tubes in 3 ml YCFAG medium, selenium had no effect on their growth for every concentration that was examined (10 μ M, 25 μ M and 100 μ M). However, selenium in 10 μ M concentration was able to promote the growth of *F. prausnitzii* when using Biotek. Selenium in 25 μ M slowed down the growth while selenium 100 μ M was toxic for the bacteria, as they didn't grow at all (Figure 22). Morphological observation with gram staining indicated the presence of selenium as purple dots in the environment but also on the bacteria stimulated with 100 μ M of selenium (Figure 6,7). Aggregation of the bacteria in the same concentration was observed in anaerobic

conditions. Moreover, in anaerobic conditions, the bacteria formed chains in the presence of the selenium (Figure 7).

Similar to *F.prausnitzii*, there was a significantly lower growth of *B.longum* in aerobic conditions compared to the anaerobic ones (Figures 8,9). *B.longum* is an obligate anaerobe however according to the results, it seems that it can tolerate oxygen (Yuan et al, 2006). Selenium showed no effect on the growth of *B.longum* in aerobic conditions but in anaerobic conditions, the presence of selenium was medium toxic and slowed down the growth of the bacteria (Figures 8,9). However the results were not statistically significant. The growth of *B.longum* was promoted when treated with selenium in every concentration using Biotek. However in the higher the concentration there was a delay for the growth to reach the maximum level (Figure 23). Selenium provoked aggregation of the bacteria, especially in the highest concentration in anaerobic conditions as observed with gram staining (Figure 10). The presence of the selenium was detectable through the purple dots appeared in the environment and on the bacteria (Figures 10,11).

B.luti was able to grow in anaerobic conditions but couldn't grow at aerobic conditions, indicating that it is a strict anaerobe without tolerance to oxygen (Figures 12,13). Selenium supplementation had toxic effect for this bacterium when grown in tubes in anaerobic conditions (Figure 13). Specifically, the highest the concentration, the lower the growth, with 100 μ M selenium inhibiting the growth completely. Interestingly, when grown in a sealed 96 well-plate and monitoring the growth, selenium was able to promote the growth of the bacterium in concentrations 10 μ M and 25 μ M (Figure 24). There was a delay to reach the growth maximum level for 25 μ M compared to 10 μ M selenium treated bacteria. 100 μ M of selenium was toxic for *B.luti* and it didn't grow. Gram staining showed chain formation of *B.luti* as a result of the presence of selenium (Figure 14).

E.coli is a facultative anaerobic bacterium which grows better in aerobic than anaerobic conditions (Palmer C et al, 2007). Our results can indeed confirm this growth difference (Figures 15,16). Selenium supplementation slowed down the growth of *E.coli* in aerobic conditions, however there was no effect on the growth of the bacteria in anaerobic conditions in glass tubes (Figures 15,16). The differences were not significant. Interestingly, when this bacterium was grown in sealed 96 well-plate its growth was highly enhanced in the first 24 hours compared to the control and it was not dependent on the concentration of selenium (Figure 25). When the same bacteria where tested for their growth in completely aerobic conditions in a 96 well-plate, then they grew much higher compared to the anaerobic conditions, but selenium had no effect on their growth (Figure 26). Similarly to previous observations, growth of selenium treated bacteria was slightly delayed, and the delay was dose-dependent. No morphological differences were observed with gram staining between controls and selenium supplemented bacteria both for aerobic and anaerobic conditions (Figures 17,18). However the presence of selenium was obvious by purple dots (Figures 17,18).

B.thetaiotaomicron couldn't grow well in both aerobic and anaerobic conditions. However, under anaerobic conditions it was also possible to see a reduction in the growth of selenium-stimulated bacteria when compared to controls (Figure 20). Selenium lowered the growth of *B.thetaiotaomicron* in aerobic conditions, without differences on the level of the decrease between the three concentrations of selenium (Figure 19). In anaerobic conditions was also observed a decreased growth with 10 μ M selenium (Figure 20). 25 μ M and 100 μ M selenium

inhibited the growth completely proving that is toxic for the bacteria (Figure 20). It wasn't possible to grow *B.thetaiotaomicron* in a sealed 96 well-plate.

Our results show that selenium in strictly anaerobic conditions had either no effect (*F.prausnitzii*, *B.longum*, *E.coli*) or it was toxic for some bacteria (*B.luti*, *B.thetaiotaomicron*). In aerobic conditions selenium had no effect on *F.prausnitzii* and *B.longum* but decreased the growth of *E.coli* and *B.thetaiotaomicron*. Anaerobic conditions are more likely to imitate the environment that exist in the healthy gut which is characterized by anoxic conditions and domination of obligate anaerobes (Lionel Rigottier-Gois, 2013). On the other side, aerobic conditions are seem to imitate the environment in gut inflammation as there is a rise in the oxygen levels and domination of facultative anaerobes (Lionel Rigottier-Gois, 2013). Even though these two situations can mimic the two different environments in the gut, we must take into consideration that these two conditions represent two extreme situations as a healthy human gut is not 100% anoxic and an inflammatory gut is not 100% oxygenated. Under anaerobic conditions, selenium was not able to offer any protective effect that could be reflected by an increase on the growth of *F. prausnitzii*, *B. longum* and *B. luti*. Since a toxic effect of selenium on the growth of *B. luti* was observed, the question of how the selenium supplementation could affect the composition of the gut microbiota was raising. Our results show that most likely, supplementation of low doses of selenium will not affect the composition of the gut microbiota. However, higher concentration seem to have a contradictory effect, making selenium supplementation a challenging therapeutic approach. In aerobic conditions, selenium had no effect on the growth of *F. prausnitzii* and *B. longum*. Interestingly, this trace element had a toxic effect on *E. coli* and *B. thetaiotaomicron* (Figures 15,19). Although they are both commensals of human gut, they can also become potential pathogens under specific conditions (Wexler, 2007; Tenaillon et al, 2010). Our results might therefore indicate that supplementation with selenium in a gut inflammatory situation could lower the number of facultative anaerobes and potentially pathogens *E.coli* and *B.thetaiotaomicron*, which may give growth advantage to obligate anaerobes and other commensal bacteria. Therefore microbial population might be restored via the increase of the ratio of obligate anaerobes towards facultative anaerobes and aerobes.

The growth of all different bacteria (*F.prausnitzii*, *B.longum*, *B.luti*, *E.coli*) was increased by 10 μ M selenium when monitored in a 96-well plate which was sealed to reassure semi-anaerobic conditions. 25 μ M of selenium positively affected the growth of *B. longum*, *B. luti* and *E. coli* but lowered the growth of *F. prausnitzii* (Figures 22,23,24,25). 100 μ M of selenium increased the growth of *B. longum* and *E. coli* but it was toxic for *F. prausnitzii* and *B. luti* as they did not grow when compared to controls. At a first glance, the results from the previous experiments seem contradictory. However, anaerobic conditions between glass tubes and a sealed 96 well-plate differ from each other, as the latter allows a small influx of oxygen and thus explaining the different results. Moreover, the different amount of medium and bacteria used between the experiments can also influence the outcome of our study. Trace amounts of oxygen incoming in the 96-well plate might actually help create better conditions that mimic a healthy gut environment. It is known that there is an oxygen gradient in human gut with the most anaerobic conditions occurring inside the gut lumen and most oxygenated ones occurring in the gut mucosa stemmed from oxygen diffusion out of the gut epithelium (Khan et al, 2012). Specifically, *F. prausnitzii* which was once considered extremely sensitive to oxygen is actually able to grow in gut mucosa where there is influx of oxygen by using an extracellular electron shuttle (Khan et al, 2012). It is therefore a positive outcome that selenium was able to promote growth of the beneficial bacteria *F.*

F. prausnitzii, *B. longum* and *B. luti* in these experimental conditions. However, selenium also enhanced the growth of *E. coli* in the same conditions, and actually to a greater extent compared to the other bacterial strains (Figure 25). Therefore, it is difficult to interpret these results and claim a clear positive effect of selenium in the microbial population. What is clear however, is the fact that selenium influences the growth of these gut bacteria and we can conclude that selenium supplementation will alter the composition of microbiota. Whether this modulation will be towards a higher abundance of beneficial bacteria such as *F. prausnitzii*, *B. longum* and *B. luti* or also potential pathogens such as *E. coli* remains to be investigated. Further animal and human studies must be conducted in order to answer this complicated question.

Due to the great advantage that selenium gave to *E. coli* during the Biotek experiments, we hypothesized that there is a mechanism in which *E. coli* uses selenium to exploit the trace amounts of oxygen that exist in these experimental conditions and benefits from it. To test this hypothesis, we performed the same experiment but in aerobic conditions. *E. coli* was able to grow better in aerobic conditions. However, selenium did not offer any growth advantage to the bacterium. These observations confirm the hypothesis that *E. coli*, which is a facultative anaerobe and can benefit from oxygenated environments, uses selenium to exploit oxygen and grows better.

In the direction of understanding the mechanism by which selenium works, the observation of the red elemental selenium precipitation, indicated that there is a transformation of sodium selenite, the form of inorganic selenium that was provided to the bacteria, to elemental selenium. There is evidence that several bacteria can metabolize sodium selenite and produce red elemental selenium as a process of detoxification (Stolz et al, 2006; Javed et al, 2015; Staicu and Barton, 2017). According to our observations, it is possible that this is also the case for the commensal gut bacteria. This conclusion is also emphasized by the fact that the growth curves constructed using Biotek revealed a delay to reach the maximum growth (but not in the level of the growth) for the bacteria supplemented with selenium, and the delay was proportionate to the concentration of selenium. This delay was probably a result of a detoxification process of sodium selenite by the bacteria. However, we have to take into consideration that there is a possibility that sodium selenite turning into elemental selenium was due to the reduced environment that the culturing medium created as this transformation can also occur chemically (Kessi and Hanselmann, 2004).

Selenium supplementation was also studied for its prophylactic and/or recovery potential on T84 cells, a model colonic cell line to study epithelial barrier function, which were treated with the chemotherapeutic drug 5-FU (Devriese et al, 2017). First of all, selenium alone was studied to test the proliferation of T84 cells and for that, several concentrations of selenium were used (5 μ M, 10 μ M, 25 μ M, 50 μ M, 75 μ M and 100 μ M). The cell viability was monitored for 47 hours. Selenium in the concentrations of 5 μ M, 10 μ M and 25 μ M lowered cell proliferation but not in a toxic level. However, high concentrations of selenium were toxic for T84 cells (Figures 27,28). These observations are in agreement with the effect that selenium had on the growth of gut bacteria. Indeed, we observed that, while 10 μ M and 25 μ M selenium had a positive effect on most of the bacteria tested, 100 μ M resulted in toxic effects. The observed differences in the cell index were not analyzed statistically due to low number of data. However, the fact that selenium was not toxic for the cells allowed to further proceed the experiments and test selenium's prophylactic effect on 5-FU treated cells. Our results suggest that treatment with 10 μ M of selenium 24 hours before 5-FU

administration allowed the prevention of cytotoxicity in T84 cells (Figure 29). The prophylactic effect was not detected with 25 μ M of selenium. The difference however in cell index between 5-FU only treated cells and 10 μ M plus 5-FU treated cells at 72 hours was not statistically significant (Figure 30). This is probably due to the fact that the number of collected data was low. Selenium was also studied as a post-treatment, 24 hours after 5-FU administration (at 48 hours of the experiment running). The effect of selenium was studied for 48 hours after 5-FU treatment. Although there was a slightly higher proliferative effect of selenium-treated cells compared to 5-FU-treated cells, the differences were not statistically significant (Figures 31,32). Moreover, the proliferation of untreated T-84 cells was much higher compared to all the other groups.

The above mentioned results suggest that selenium in low concentration, especially 10 μ M, was able to prevent 5-FU cytotoxicity in this experimental setup. It is hypothesized that this is due to the anti-oxidant activity of selenium and not due to its proliferative effect. If it was for the last one, then selenium would have given a proliferative advantage to T84 cells both alone or after 5-FU administration. The potential preventive effect of selenium is a promising therapeutic strategy in the context of mucositis. Selenium supplementation of cancer patients before chemotherapy might be able to prevent or ameliorate the symptoms of chemotherapy-induced gastrointestinal mucositis. However, this suggestion must be taken into consideration with caution as the role of selenium on cancer has not been yet elucidated. Although for many years selenium supplementation was considered effective in the prevention and treatment of many different cancers, a recent study showed that there is no actual beneficial effect of selenium supplementation during cancer (Clark et al, 1996, Chen et al, 2013, Vinceti et al, 2018) On the contrary, some findings underline that selenium supplementation might actually increase the risk of prostate cancer (Lippman et al, 2009). The results of this study show a potential preventive effect of selenium during chemotherapy – induced mucositis but it is critical to define the optimal selenium dosage considering the contradicting results of selenium supplementation for the prevention of cancer. Moreover, it is important to repeat this series of experiments in order to have the necessary data to do a proper statistical analysis that will lead to a clear conclusion.

The present study suggests that selenium has an important role to play on the gut microbiota and on the gut mucosa layer. It is likely that selenium supplementation in mucositis patients which is characterized by gut inflammation might decrease the number of potential pathogens *E.coli* and *B.thetaiotaomicron*. This might favor the ratio of obligate anaerobes/facultative anaerobes (via the decrease of *E.coli*) and lower the ration of Firmicutes/Bacteroidetes (via the decrease of *B.thetaiotaomicron*). Moreover, it is possible that selenium supplementation in low concentration might favor the growth of butyrate producing bacterium *F.prausnitzii*, of probiotic species *B.longum* and the beneficial commensal *B.luti* in a healthy gut. However, this might be also accompanied by an increase in the number of *E.coli*. Moreover, these observations might prove useful in the use of selenium as an enrichment in traditional (*B.longum*) but also next generation (*F.prausnitzii*) probiotics to help the beneficial bacteria colonize the gastrointestinal tract. A potential preventive effect of selenium in mucosal damage caused by chemotherapeutic agent 5-FU suggest that selenium supplementation might have a beneficial effect in preventing mucositis in cancer patients. This preliminary study indicate that selenium might have a potential therapeutic role in treating mucositis and gut inflammation in general, however further investigation is needed to draw conclusions about the role of selenium in the modulation of microbiota during inflammation and especially mucositis.

As a future research, it is suggested that the growth of more bacterial species is studied under treatment with selenium, as there are hundreds of other commensals living in the human gut and have not been the subject of this study. In addition, it would be useful to create co-cultures of bacteria and investigate the effect of selenium on the growth of these bacteria and the modulation of their population. Co-cultures are more indicative of what actually might happen in a population of microorganisms with selenium such as gut microbiota. Furthermore, co-cultures of bacteria and T84 cells with selenium supplementation using Hoxban system might reveal how gut epithelial cells and microbiota interplay with each other but also how they metabolize selenium (Sadaghian Sabadad et al, 2015). It has been found that bacteria and the host compete for selenium incorporation into selenoproteins (Kasaikina et al, 2011). Supernatants of selenium supplemented bacteria can also be studied on their effect on T84 cells proliferation or 5-FU treated T84 cells. In this way it can be tested if bacteria produce certain metabolites when consuming selenium that might be beneficial for T84 cell proliferation. Finally, it is very important to pass from in vitro studies to animal and human studies. This is the only way that we can draw conclusions about the effect of selenium on gut microbiota composition and gut inflammation as it is very different how actually selenium works in a highly complex biological system such as the human gut.

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Appendix

YCFAG medium

Materials

Dry components

	Chemical	Grams (per Liter)
1	Casitone	10
2	Yeast extract	2.5
3	NaHCO ₃	4
4	K ₂ HPO ₄	0.45
5	KH ₂ PO ₄	0.45
6	NaCl	0.9
7	MgSO ₄ · 7 H ₂ O	0.09
8	CaCl ₂ · 2 H ₂ O	0.12
9	Sodium acetate	2.7
10	Glucose	4.5
11	Cysteine	1
12	(Bacteriological Agar no. 1)	15

Fluids

	Chemical	Volumes (per Liter)
1	Resazurin 0.02%	5 mL
2	Hemin 0.2%	5 mL
3	Pink Vitamin Mix	1 mL
4	Yellow Vitamin Mix	1 mL
	SCFA's	
5	Propionate	600 µL
6	Isobutyrate	100 µL
7	Isovalerate	100 µL
8	Valerate	100 µL
9	1M HCl (autoclaved)	5-10 mL

Method

Preparation of Resazurin and Hemin

Resazurin (0.02%)

Dissolve 0.01g to 50ml Milli-Q water

Hemin (0.2%)

Dissolve 0,1g to 50ml Milli-Q water

Preparation of the vitamin mixes

1 x	1 x	1000x	1000x (100ml)	Vitamins mix	
$\mu\text{g}/100\text{ ml}$	$\mu\text{g}/\text{ml}$	$\mu\text{g}/\text{ml}$	(g)		
1	0,01	10	0,001	biotin	Pink Mix
1	0,01	10	0,001	cobalamin	
3	0,03	30	0,003	<i>p</i> -aminobenzoic acid	
5	0,05	50	0,005	folic acid	
15	0,15	150	0,015	pyridoxamine	
5	0,05	50	0,005	Thiamine-HCl	Yellow mix
5	0,05	50	0,005	Riboflavin	

- Take 70 ml of Milli-Q water
- Add all the vitamins of pink or yellow mix accordingly
- Adjust pH to slightly alkaline by adding few drops of 1 M NaOH until all the vitamins dissolved
- Adjust the pH around 7.4
- Adjust the volume 100 ml

Preparation of the YCFAG medium

- Weigh dry components 1-10
- Place the bottle on a hot stirring plate with a magnet
- Add the dry components 1-10 and Resazurin to a 500 mL bottle with 300 mL of Milli-Q water
- Heat up until almost boiling with the caps slightly unscrewed. Shake the bottles several times during procedure.
- Place the bottle back on the hot plate
- Add dry component 11 and fluids 1,2,3 and 5-8
- For making agar plates also add component no. 12
- Check pH and set the pH to approximately 6.0 with 1M HCl
- Add milli-Q water to a volume of 1 Liter
- Autoclave with a slightly opened cap
- Close the cap directly after autoclaving

For broth medium

- Let the bottle cool down
- Place the bottle under a hood
- Add yellow vitamin mix
- Check the pH with pH paper

- Set the pH to 6.5 with autoclaved 1M HCl or autoclaved 1M NaOH if needed

For agar plates

- Place the bottle in a 55°C incubator for 30 minutes to cool down
- Add yellow vitamin under a hood with flow set at 70
- Check the pH with pH paper
- Set the pH to 6.5 with autoclaved 1M HCl or autoclaved 1M NaOH if needed
- Pour the plates under the hood
- Let them solidify for 15-30 minutes
- Turn the plates and let them dry for 30 minutes
- Wrap the plates up in plastic bags for storage or place the plates ready to use in the anaerobic cabinet

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