

**A STUDY OF ANTIMICROBIAL EFFECTS OF
REUTERIN BY LACTOBACILLUS REUTERI
DSM 17938 ON ESCHERICHIA COLI**



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ABSTRACT

Background

The widespread antibiotic resistance is an imminent problem in today's world. As the number of antibiotic resistance cases increase, it is inevitable to discover alternate treatment options. Probiotics could be one of the potentially important avenues which could be exploited to treat antibiotic resistance and other infections. The holistic approach of probiotics in exerting their beneficial characteristics not only makes it an attractive member in this regard, but also in providing essential nutrients to the host.

Results

Lactobacillus reuteri DSM 17938 is one of the most commonly used probiotics and so, the production of antimicrobial reuterin (3-hydroxypropionaldehyde) by the conversion of glycerol and its antagonistic effects on *E. coli* C600 were studied by co-culturing the microorganisms. Different growth media and analytical techniques such as HPLC, flow-cytometry were used to determine the qualitative and quantitative results of the reuterin production and its activity on the co-cultures. The experiments revealed that glycerol alone does not exhibit an inhibitory property on *E. coli*, but a concentration of 5 g/L induced the production of lethal amounts of reuterin in *L. reuteri* grown together with *E. coli* in SD4 medium with galactose. It was also found that glycerol concentrations beyond 10 g/L will lead to lethal doses of reuterin for *L. reuteri* too.

Conclusion

Thus, the work showed an optimal amount of glycerol concentration imparting antagonistic effect through the antimicrobial reuterin in co-cultures.

Results

Secondly, to study the exocellular electron transfer between two microorganisms, an attempt to design a modified reactor setup to explore the co-culturing possibilities was accomplished. The co-culturing possibilities of *L. reuteri* and *Prevotella copri* in Schaedler anaerobe broth yielded results that need to be studied in detail further.

Conclusion

More investigations are required to design a medium where both microorganisms can be adequately grown on.

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INTRODUCTION

Antimicrobial resistance (AMR) has been an emerging problem and is posed to be the biggest threat of this century. According to WHO, it has been defined as the ability of a microorganism to combat against the functionality of the antimicrobial substance towards it. This has caused serious concerns like inability to treat infections, their recurrence and the spread of diseases (WHO, 2017). “Antimicrobial resistance” is a broader term used when indicating the collective antagonistic response of all microorganisms such as bacteria, viruses, fungi and parasites to antimicrobials, while “Antibiotic resistance” is a specific term used only to describe the resistance of bacteria to antibiotics against them.

The golden era of antibiotics was during the 1930s to 1960s, when many antibiotics were discovered, ended abruptly when the antibiotic resistance surfaced up. Thereafter, the development, production and regulatory approval of antibiotics coupled with short-term efficacy proved costly for the pharmaceutical industry and it led the way to an uncertain future of global health (Nathan & Otto, 2014). A lot of factors that contribute to antibiotic resistance, but primarily, overuse or misuse of antibiotics and the lack of new antibiotics in the market have proven to be the main causal factors for antibiotic resistance (Prestinaci, 2005). Currently, even the so-called “last resort antibiotics” such as Carbapenams, Vancomycin are failing against multi-drug resistant bacteria and cause unavoidable deaths. Moreover, antibiotic resistance adds a substantial amount of burden on the social and economic lives of the people. Since we have already exhausted all the available potential antibiotics to treat drug resistant bacterial infections, alternate methods need to be developed. Although antimicrobial peptide elicitors (APEs) such as LL-37, histatin, psoriasin, dermicidin have been in the pipeline for the replacement of antibiotics, the limitations in producing and using them forces us to discover other already available sources (Ernesto, 2012). Probiotics could be considered as an excellent alternate to the antibiotics in treating the drug-resistant bacteria (Ventola, 2014).

Probiotics are defined as living bacteria that, when administered in adequate amounts, confer a health benefit on the host (WHO/FAO, 2002). Since the turn of the last century, the properties of probiotic bacteria have been exploited by the food industry and have been increasingly used as a supplementary nutrition in the form of functional foods. In addition, probiotics have been believed to exert a variety of health benefits in the host organism. They are shown to improve the gastrointestinal well-being such as colonic disorders, antibiotic-induced diarrhoea, reduction of symptoms of lactose intolerance etc. (Kechagia et al., 2013). Probiotics not only support to treat intestinal disorders but have also been shown to positively affect other organs by immune modulatory responses leading to producing bio-active metabolites (Chiang, 2012). Probiotics involve in alleviation or prevention of atopic diseases and allergies in infants, respiratory tract infections, urogenital infections, reduction of enzymes that promote cancer, treatment of ischemic heart diseases and auto-immune disorders like arthritis (de Vrese & Schrezenmeir, 2008).

Probiotic bacteria have been long studied for their ability to produce antimicrobial compounds such as lactic acid, bacteriocins and hydrogen peroxide apart from acetate, formate and ethanol. Among the probiotic bacteria, *Lactobacillus* and *Bifidobacteria spp.* are extensively studied and used. Certain strains of *Lactobacillus plantarum*, *Lactobacillus paracasei* and *Lactobacillus rhamnosus* have been shown to act against carbapenem-resistant *Enterobacteriaceae* by forming of antimicrobials (Chen et al., 2019). Reuterin

(3hydroxypropionaldehyde, 3-HPA) is one such broad-spectrum low molecular weight antimicrobial compound produced by *Lactobacillus reuteri* by the conversion of glycerol (Talarico, 1989).

The work performed here was to study the antimicrobial effects of *Lactobacillus reuteri* DSM 17938 on *Escherichia coli* C600 by reuterin. As an additional work, the growth of *Lactobacillus reuteri* DSM 17938 and *Prevotella copri* CCUG 58058T on Schaedler anaerobe broth was studied in order to explore the co-culturing possibilities to study the external electron transfer. Also, culturing of *L. reuteri* DSM 17938 in a modified Schaedler anaerobe broth was attempted.

BACKGROUND

Lactobacillus reuteri DSM 17938

Lactic acid bacteria (LAB) belong to a group of microorganisms which are ecologically and genetically diverse and include a wide variety of bacteria. *Lactobacillus reuteri* DSM 17938 (fig. 1, left), under phylum firmicutes and class Bacilli, is one of the organisms which is regarded as the most clinically studied and commercially used organism in food production (Giraffa, 2010). It is a gram-positive, rod-shaped, non-motile, micro-aerophilic, heterofermentative bacteria with several proven probiotic properties. *L. reuteri* was first identified and described by Kandler et al., in 1980. For the purpose of human use, it was isolated first from the breast milk of a Peruvian mother in 1990 and was designated as ATCC 55730. But in 2007, the strain was replaced by *L. reuteri* DSM 17938. It is a sister strain of *L. reuteri* ATCC 55730 and the two strains differed by the loss of two plasmids in *Lactobacillus reuteri* ATCC 55730 that were resistant to tetracycline and lincomycin. Otherwise, the genetic composition and all the other probiotic functions were comparable to each other. The commercial name for *L. reuteri* DSM 17938 today is *L. reuteri* Protectics and is owned by BioGaia AB (Biogaia, n.d).

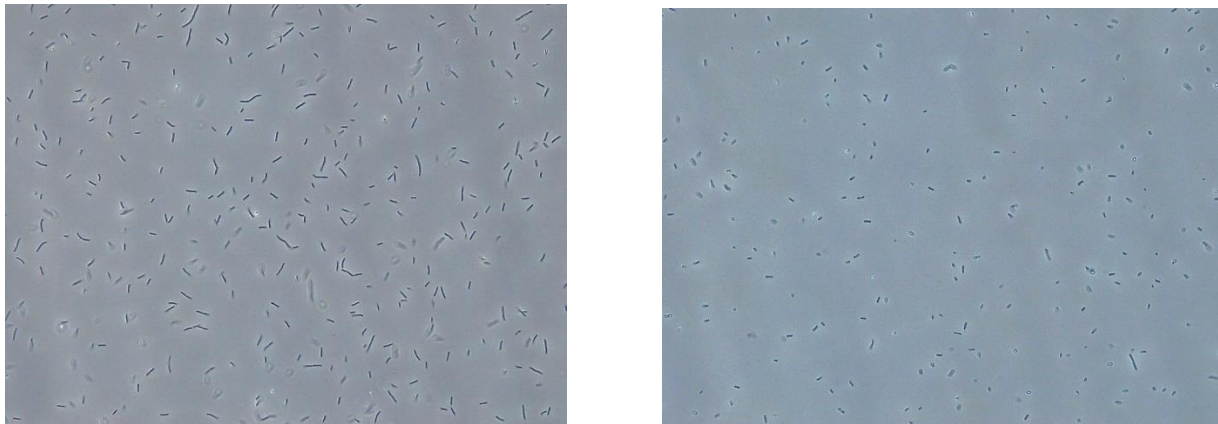


Figure 1: Microscopic images of *Lactobacillus reuteri* DSM 17938 (left) and *Escherichia coli* C600 (right) (Charlton John)

Escherichia coli C600

Escherichia coli strain C600 (fig. 1) is a gram-negative, non-spore forming, rod-shaped, facultative anaerobe mostly found in the lower-intestinal tracts of warm-blooded organisms (Tenaillon, 2010). It is a prototypical K-12 derived strain and one of the most commonly used laboratory strains for model studies. In the gut, *E.coli* is not found in abundance and much lower than other commensal bacteria, but it is the most common causal agent for a number of intestinal and extra-intestinal diseases. The pathogenic strain of *E.coli* carries virulence factors being responsible for virulence. They fall into many phylogenetic groups and the phylogenetic group B2 and D exhibit intestinal pathogenicity and cause infections of the gut (Clermont, 2000). *E.coli* causes urinary tract infections, enteric disease and sepsis or meningitis (Katouli, 2010) and is mainly treated with antibiotics.

Antibiotic Resistance

Antibiotics are classified into four different classes according to their mode of action against bacteria. Beta-lactam antibiotics like cephalosporins, penicillins, carbapenams act by interfering with the cell wall synthesis by inhibiting the enzymes required for the synthesis of

the peptidoglycan layer (McManus, 1997). Inhibition of protein synthesis is another mechanism through which the agents exert their antibacterial activity. Aminoglycosides and tetracyclines bind to 30S ribosomal subunit and chloramphenicol binds to 50S ribosomal subunit (Neu, 1998; McManus,1997). Another way is by the disruption of DNA synthesis by inducing breakage in the double-stranded DNA strands during replication by Fluoroquinolones. The fourth type of mechanism of action is of sulfanomides, trimethoprim by blocking the metabolic pathway for the production of certain important components like folic acid, which inhibits the DNA synthesis (Brunton *et al.*, 2006).

Antibiotic resistance against different antibiotics like chloramphenicol, beta-lactams, and aminoglycosides is conferred to gram-negative bacteria like *E.coli* by mobile genetic elements known as gene cassettes. The composition of a gene cassette (fig. 2) is a gene along with a downstream sequence which is known as a 59-base element (59-be). This 59-base element acts as a specific recombination site in the gene sequence. The gene cassettes are able to mobilize themselves into or out of a specific receptor site (*attI* site) by the help of a companion element called integron. The integron codes a site-specific recombinase (*IntI*) which facilitates the integration or excision of the cassettes (Hall RM, 1998).

There are more 200 different cassette-associated resistance genes and six different classes of integrons, with each class encoding a separate *IntI* integrase enzyme. The transcription of cassettes begins from the common promoter in the 5'-conserved region and the upstream cassettes reduce the distal gene expressions (Ploy *et al.*,2000).

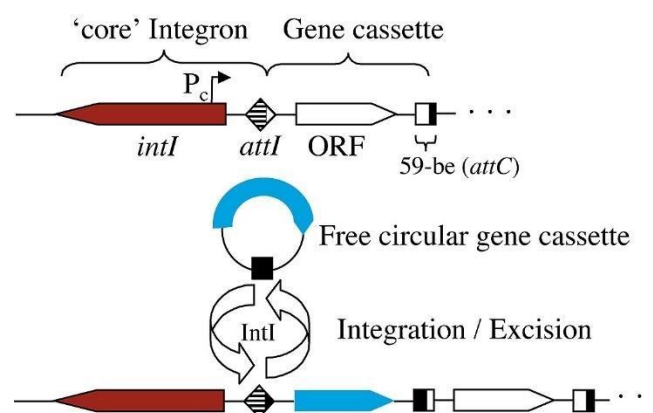


Figure 2: Integron in a simplified genetic structure (Koenig *et al.*, 2009)

Until recently, the non-pathogenic *E. coli* in the gastrointestinal tract was not found to have virulent genes, but now, they have been found to have a wide variety of multi-resistant genes which may act as an intermediate acceptor and donor of antimicrobial resistance to other pathogenic bacteria (Sanez *et al.*, 2004).

The Antimicrobial: Reuterin

Reuterin (3-hydroxypropionaldehyde) or 3-HPA is an antimicrobial compound produced by *L. reuteri*. It is a low-molecular weight, water-soluble, neutral, non-proteinaceous, broadspectrum antimicrobial produced as an intermediate during glycerol fermentation in *L. reuteri* under ambient physiological conditions such as anaerobic environment, temperature and pH (Talarico *et al.*,1990). While reuterin has a broad-spectrum antimicrobial activity against both gram-positive and gram-negative bacteria, it has been also shown to have anti-fungal,

antiparasitic and anti-carcinogenic activities (Dobrogosz and Lindgren, 1988; Dobrogosz et al., 1989). Due to its potential antimicrobial activity, it has been used in decontamination and preservation of meat in food industry (El-Ziney, 1999) and also in the production of milk and cheese to prevent the growth of *Listeria* and *E. coli* (El-ziney, 1998). As a probiotic, reuterin has been beneficial in exerting antimicrobial activity against enteric pathogens like *Clostridium difficile* in the gastrointestinal tract (Spinler et al., 2017). The consumption of probiotic lozenges with *L. reuteri* has also improved pregnancy gingivitis in women (Schlagenhauf, 2016).

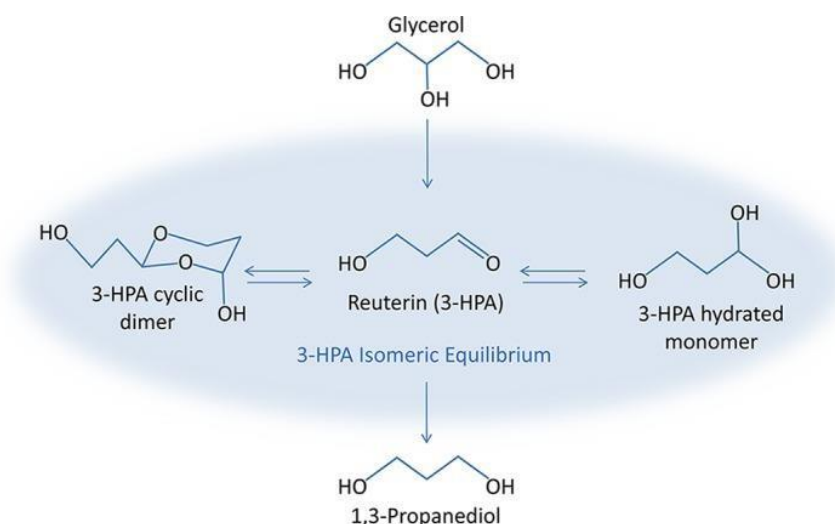


Figure 3: Reuterin synthesis pathway by glycerol fermentation (Spinler et al., 2017)

Reuterin is produced as an intermediate during the bioconversion of glycerol to 1,3-propanediol (fig. 3) which has been proposed to regenerate NAD⁺ from NADH to improve the biomass yield (Luthi-Peng et al., 2002). In the cell, it is metabolized in a metabolosome, a special bacterial compartment which may be owing to the toxicity of reuterin (Sriramulu et al., 2008). The conversion of glycerol to 3-HPA begins with the dehydration of glycerol in a coenzyme B₁₂-dependent catalysis by glycerol/diol hydratase (Fig. 3). The subsequent conversion of 3HPA into 1,3-PDO is ensured by the help of enzymes present in the propanediol-utilization (*pdu*) operon such as 1,2-propanediol oxidoreductase and a putative alcohol dehydrogenase. This, as mentioned above, produces a mole of NAD⁺ for every mole of 1,3-PD produced, which makes the cells use glycerol as an electron acceptor to re-oxidize NADH (Dishisha et al., 2014). Due to the reactive nature of the aldehyde group present in reuterin, it reacts with other compounds in aqueous solution and form other new compounds. It forms an HPA dimer by dimerization or HPA hydrate by hydration. It can also be dehydrated to form the toxic substance acrolein.

The mechanism by which reuterin exhibits its antimicrobial property remains difficult to determine because of the production of all the above-mentioned compounds. Though, two hypotheses remain as to how reuterin acts as an antimicrobial. The aldehyde group present in reuterin reacts with primary amines and thiol groups, which in turn inactivate the small molecules and proteins with these groups (Vollenweider & Lacroix, 2004). This can be attributed as to why reuterin has a broad-spectrum effect on pathogenic microorganisms. Another explanation could be because of the dimer form, which may block the enzyme ribonucleotide reductase. The similar structure of HPA dimer to that of a ribose sugar acts as a competitive inhibitor and blocks the enzyme required for the DNA synthesis. As the active site

of the enzyme contains a thiol group, it is difficult to determine the mechanism behind this antimicrobial activity (Schaefer et al., 2010).

Carbon Metabolism

L. reuteri

L. reuteri is a heterofermentative bacterium whose end products are lactate, acetate, ethanol and carbon dioxide. The glycolytic pathway begins by converting glucose into glucose 6-phosphate and production of metabolites by the main flux through the Phosphoketolase pathway (PKP) (Fig. 4). But the homofermentative LAB uses the Embden-Meyerhof-Parnas pathway (EMP) to produce ATP, NADH and pyruvate (Fig. 4). The pyruvate is later converted to acetyl CoA which enters the Tricarboxylic acid cycle. When compared with the EMP pathway, the PKP pathway in heterofermentative LAB is able to ferment pentose sugars but the resulting yield is poor. This could be rectified by adding an electron acceptor such as fructose and the redox imbalance could be overcome. This leads to the formation of an additional pathway where NAD(P)H can be reoxidized (Kandler, 1983). The flux through *L. reuteri* DSM 17938 follows mainly the PKP pathway and uses EMP pathway as a shunt (Årsköld et al., 2008).

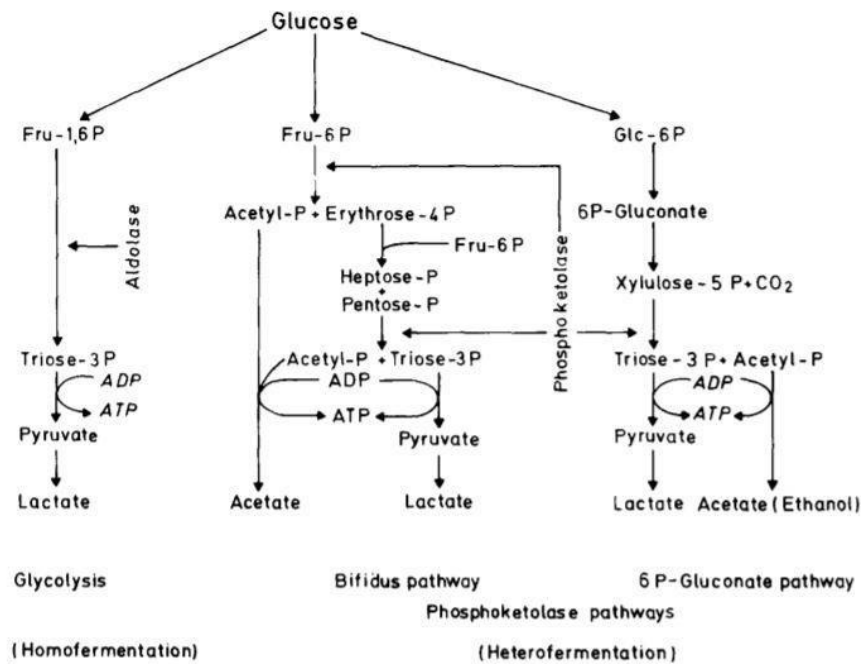


Figure 4: Glucose fermentation pathways in lactic acid bacteria- Schematic presentation (Kandler, 1983)

When the carbon source is galactose in both *L. reuteri* and *E.coli*, a permease takes it up and it follows the Leloir pathway (Fig. 5). There could be also another mechanism where the phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS) follows a different pathway where the enzyme D-galactose 6-phosphate isomerase converts D-galactose into the Tagatose-6P pathway (Fig. 5) (Bissett and Anderson, 1974)

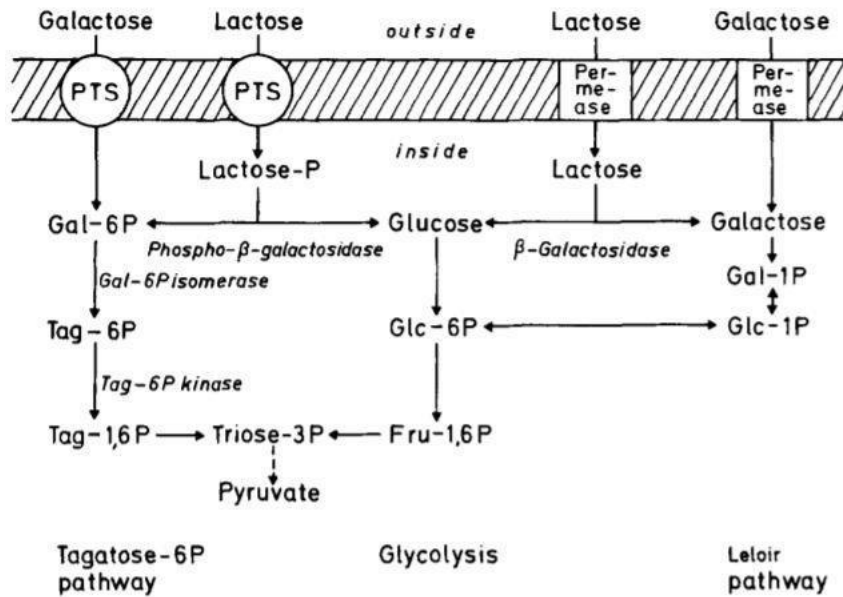


Figure 5: Leloir pathway and Tagatose- 6P pathway of galactose uptake- schematic presentation (Kandler, 1983)

E. coli

In *E. coli*, under anaerobic conditions, a complete TCA cycle is not performed. Instead, the enzymes are used to synthesize succinyl CoA and 2-oxoglutarate, which forms the oxidative and reductive chains of TCA cycle. These branches do not produce any energy but helps to maintain cell viability. In order for the glycolytic metabolites to keep flowing, the re-oxidation of NADH is essential. This happens by the fermentation of sugars to a wide variety of metabolites like lactate, ethanol, succinate along with acetate and formate (Wolfe, 2005). During anaerobiosis, where the availability of oxygen is severely limited, pyruvate is formed as a product from glycolysis. Lactate is produced by lactate dehydrogenase with a re-oxidation of NADH. The enzyme pyruvate formate lyase, *Pfl* converts pyruvate into formate and acetyl CoA after which formate is converted to hydrogen by formate hydrogen lyase (Fig.6). Acetyl CoA is either reduced to ethanol or oxidized to acetate. Acetate is synthesized through the PatAck pathway to gain an extra ATP. To maintain the redox balance, *E.coli* synthesizes other products from pyruvate such as succinate (Catalanotti *et al*, 2013) (Fig. 6).

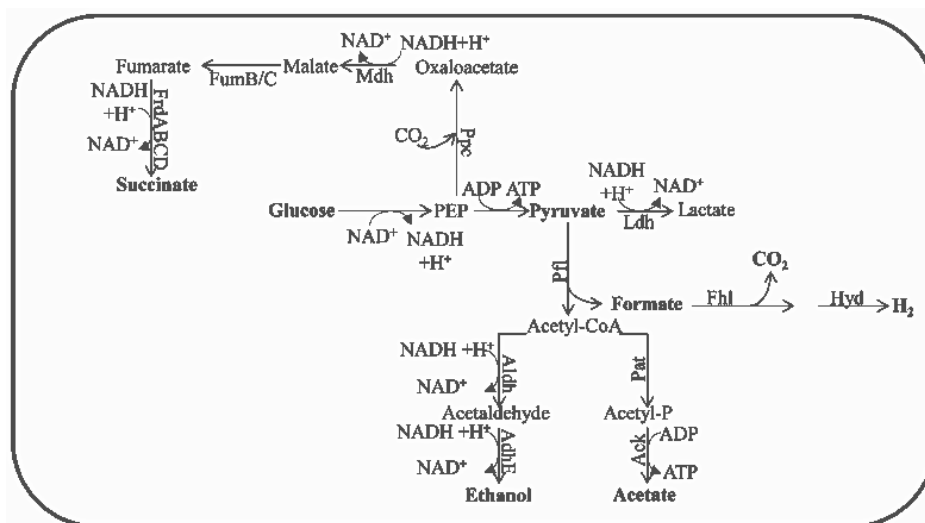


Figure 6: *E.coli* fermentation pathways (Catalanotti *et al.*, 2013)

When glycerol is present along with glucose, it is metabolized into pyruvate. This creates a higher amount of redox equivalents than with glucose. Thus, to eliminate this redox imbalance, succinate or ethanol is produced (Fig. 7).

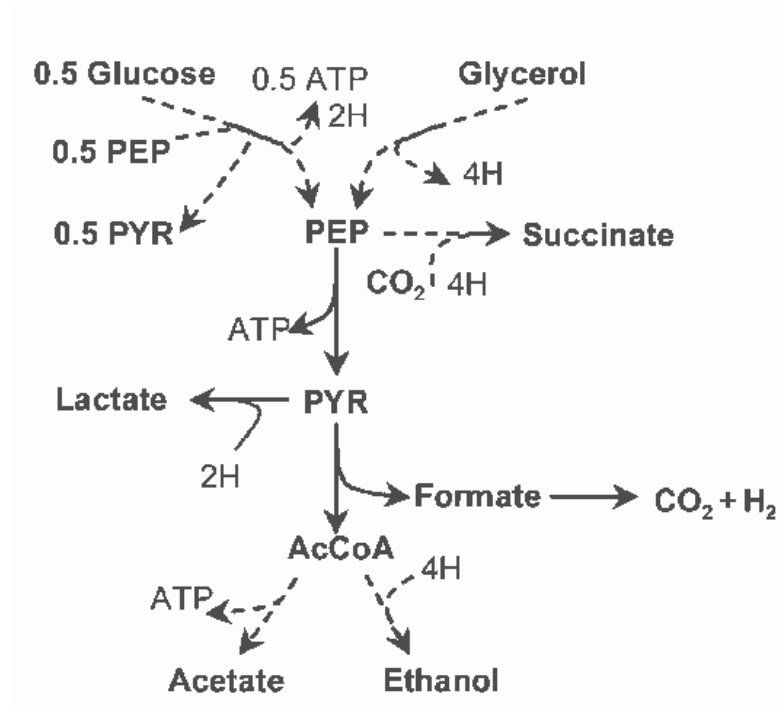


Figure 7: *E. coli* fermentation pathway of glycerol and glucose (Dharmadi et al., 2006)

MATERIALS AND METHODS

Cell culture of *L. reuteri* and *E. coli*

The microorganism *Lactobacillus reuteri* strain DSM 17938, whose antimicrobial properties have been studied was acquired from BioGaia AB, Stockholm, Sweden and *Escherichia coli* strain C600, a common laboratory strain was acquired from the Department of Applied Microbiology, Lund university, Sweden and were used in this study. A pre-culture of 25ml from both stock cultures was inoculated and grown anaerobically at pH 5.8 and at 37°C for 18h in a 50ml falcon tube. The pre-cultures of *L. reuteri* strain DSM 17938 of 100µL was first inoculated in filter sterilized De Man, Rogosa and Sharpe broth (MRS, Merck, Germany) and *Escherichia coli* strain C600 in Brain Heart Infusion (BHI, Bacto, USA) using Filtropur S 0.2µm (Sarstedt, Germany). Later, the harvested cultures were inoculated again in 1µL of 10X Tryptic Soy broth (Bacto, USA) or SD4 medium (see appendix) supplemented with 1g/L of Tween 80 (Sigma-Aldrich, Germany) in varying glycerol concentrations under the same conditions mentioned above in an Isothermal Calorimeter (Biometric AB, Sweden).

Bioreactor cultivation of *L. reuteri* and *P. copri*

The pre-culture of *L. reuteri* strain DSM 17938 was done by anaerobically inoculating the stock-culture stored at -80°C in Schaedler's anaerobic broth (Oxoid, UK). 50mL of medium was prepared using serum flasks with an addition of 50 µL of 1g/L of resazurin and sealed tightly with butyl rubber septum and aluminium crimp seal cap. It was then boiled and flushed with CO₂ gas (AGA AB, Sweden) using sterile needles simultaneously, until the resazurin indicated that there was no oxygen left. The prepared medium was then cooled down under CO₂ for another 15 min, while the pH was adjusted to pH 6.3 using 3M KOH. In order to maintain the pH and the anaerobic microenvironment, it was quickly flushed again with pure N₂ for 10 min and then autoclaved for 15 min at 1 bar at 121°C. The pre-culture of *Prevotella copri* strain CCUG 58058T (CCUG, Sweden) was prepared in a similar way to the *L. reuteri* strain DSM 17938 in the same medium. The pre-culture in MRS medium at pH 5.7 and 7.2 was also prepared in a similar manner but without any CO₂ and N₂ flushing.

The modified Schaedler anaerobe medium was prepared using the same medium using the above said conditions but with addition of components from MRS medium that were lacking in Schaedler broth. For 1L, 1.1 ml Tween 80, 2 g of di-potassium hydrogen phosphate, 5 g of sodium acetate, 0.2 g of magnesium sulphate and 0.04 g of manganese sulphate was added. During one of the fermentations of *L. reuteri* in Schaedler medium, 15 g of glucose was added to the prepared medium. *L. reuteri* DSM 17938 was inoculated in 50 mL of the modified medium (Table 3 in appendix) in the serum flask which was used for the fermentations.

After incubation of pre-cultures, the inoculum was inoculated in a calibrated and autoclaved bioreactor. The bioreactor used was a 3L single-glass walled bioreactor (Applikon, The Netherlands) connected with an ADI 1025 Bio-Console and an Applikon ADI 1010 control unit. The working volume was 1L of the specific media (MRS, SD4, Schaedler anaerobe medium and modified Schaedler anaerobe medium). In the fermentations where CO₂ was used, it was flushed into the reactor for 15 min at 0.5 L/min before the inoculation followed by N₂ to create an anaerobic environment. This was made sure that all the oxygen was removed.

The fermentation was carried out and the samples (6 mL) were collected every 30 min and the optical density (OD) was measured at absorbance 620 nm. The samples were then subjected to

ultracentrifugation at 13,200 rpm for 2 min at 4° C and the supernatant was saved for HPLC analysis in eppendorfs and stored at -20° C.

The cell dry weight was obtained by pre-weighing the filter paper and vacuum filtering 5 mL of sample and washing with water three times the amount of the sample. The filter paper was dried in a microwave oven at 350 W for 4 min and then weighed. The dry weight of the sample was calculated by subtracting the obtained weight by the weight of the filter paper and dividing it by the total volume of the sample.

Culturing of *L.reuteri* and *E.coli* co-cultures

The grown pre-cultures of *L. reuteri* and *E.coli* were inoculated again in media containing 10X TSB, 20 g/L glucose and/or galactose in white bottles with a glycerol concentration of 5 g/L, 10 g/L or 0 g/L, both separately and in co-cultures. They were immediately incubated at 37° C in an isothermal calorimeter and the heat generated by the cultures were recorded for at least 15 h.

Reuterin production

After the cultivation of cells in the bioreactor, the cells were harvested and centrifuged at 4000G for 5 min. The supernatant was discarded, and they were washed with PBS and 200mM of glycerol was added and incubated at 37° C for approximately two hours to stop the reaction proceeding further towards the production of 1,3-PD by aldehyde dismutation. It was then stored at 4° C. For some of the experiments in this work, an already prepared reuterin with a concentration of 64mM was used.

Viability analysis of the cells

The viability of the cells was determined by using flow cytometry (BD Accuri C6 flow cytometer, USA) after the incubation of cocultures with glycerol and also before the inoculation and incubation in the isothermal calorimeter. A dye mixture of 6 µL of SYBR green (1X) and propidium iodide was added to 500 µL of sample diluted 1000 times. The concoction was incubated at 37° C in the dark and subsequently the analysis was carried out.

HPLC analysis of the metabolites

The metabolites such as reuterin (3-HPA), 1,3-PD, glycerol, glucose, galactose, lactate, acetate, and ethanol produced during the cultivation and co-culturing of *L. reuteri* and *E.coli* was analysed using High Performance Liquid Chromatography (HPLC). The separation was done at 30 °C on two Rezex™ ROA-Organic Acid H+ (8%) ion exclusion Phenomenex column in series (Bio-Rad, US) with a flow rate of 0.6 mL/min with a mobile phase of 5mM H₂SO₄. The separation was carried out by identifying and quantifying the metabolites using a HP1047A refractive index detector. The metabolites were identified by comparing the peaks due to the retention times of various compounds with the standard of each compound.

Antagonistic study by *L. reuteri*

The antimicrobial activity of reuterin produced during the cocultures of *L. reuteri* DSM 17938 and *E.coli* C600 from glycerol was determined by plating the culture in MRS and BHI plates, respectively. Several dilutions of both sugars, glucose and galactose of 10µL were plated in drop plate method and incubated at 37 °C for 24 h. The plates were then visually inspected for colonies and were counted in a colony counter.

Statistical analysis

The specific heat production rate (μ_{\max}) of *L. reuteri* and *E. coli* in the calorimeter was calculated from the heat production curve obtained. The least square method fitting a non-linear Gompertz-curve was used to calculate the μ_{\max} from the data points expressed in millivolts per min.

RESULTS

Selection of growth media for co-culturing of *L. reuteri* and *E.coli*

In order to co-culture both *L. reuteri* and *E. coli*, several media were tested. First, they were tested on MRS broth, an enriched media where only *L. reuteri* grew. Further tests on MRS plates revealed that *E. coli* grew in the MRS media, after 72 h, but the overall growth was not in par with that of *L. reuteri*. There were very few colonies, much below the countable range. BHI broth was also found to be unsuitable for co-culturing, as *L. reuteri* did not grow in BHI plates. Finally, the two organisms were inoculated in TSB medium but with an addition of Tween 80. Though the growth of *L. reuteri* and *E. coli* was not as high as in MRS broth and BHI broth, respectively. Co-culturing in SD4 with galactose was tried and was found to be suitable medium as well. Thus, TSB and SD4 medium were found to be the apt medium for the co-culture.

Growth of *L. reuteri* and *E. coli* at different pH in TSB broth

After the growth medium was selected, both *L. reuteri* and *E. coli* were grown at different pH in TSB in order to determine the optimum pH for the co-culture. When *L. reuteri* and *E.coli* were grown at pH 7.2, there was no growth observed with *L. reuteri*, but *E. coli* withstood the neutral pH (Fig. 8). This non-growth of *L. reuteri* was confirmed by HPLC analysis of the composition of supernatant of its culture, where it contained 2.201 g/L of glucose at the end of the cultivation as opposed to 2.273 g/L of glucose at the start. The growth was observed with both *L. reuteri* and *E. coli* grown at pH 5.8. Thus, pH 5.8 was chosen as an optimum for the coculture and the heat production rates for various (co-)cultures were measured (Table 1).

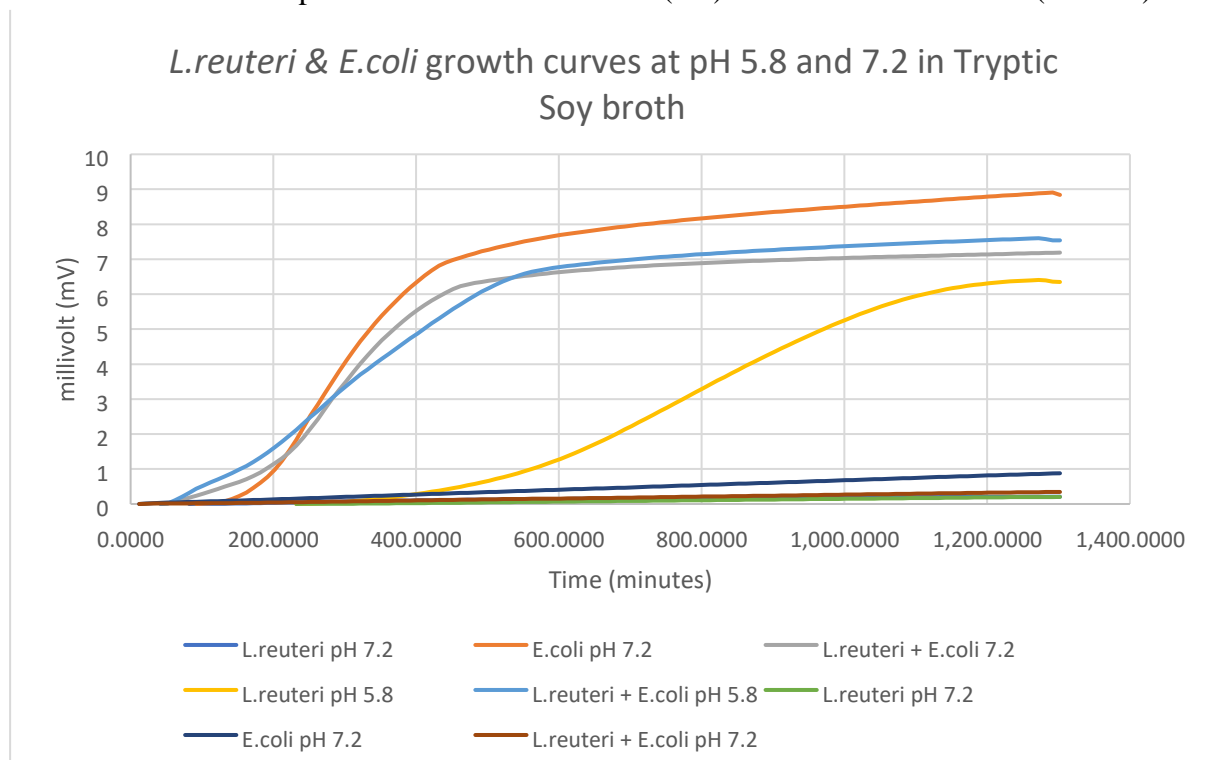


Figure 8: Growth of *L. reuteri* & *E. coli* in TSB at different pH conditions from isothermal calorimeter. Growth of *L. reuteri* at pH 5.8 (blue) and the growth of *E. coli* at pH 7.2 (orange)

Table 1: Maximum specific heat production rates (μ_{max}) obtained from isothermal calorimeter data for different glycerol concentrations in different media calculated using least square method fitting non-linear Gompertz-curve. These results correspond to Δ heat data.

Medium	Organism & glycerol (g/L)	μ_{max} (h ⁻¹) (Δ heat)
Tryptic Soy Broth (TSB) Glucose + Galactose 20 g/L	<i>L. reuteri</i> 0	0.002901
	<i>L. reuteri</i> 5	0.003252
	<i>L. reuteri</i> 10	0.002739
	<i>E. coli</i> 0	0.007123
	<i>E. coli</i> 5	0.003257
	<i>E. coli</i> 10	0.002978
	<i>L. reuteri</i> + <i>E. coli</i> 5	0.004366
	<i>L. reuteri</i> + <i>E. coli</i> 10	0.005035
Tryptic Soy Broth (TSB) Glucose 20 g/L	<i>L. reuteri</i> 0	0.002701
	<i>L. reuteri</i> 5	0.002714
	<i>L. reuteri</i> 10	0.002648
	<i>E. coli</i> 0	0.007034
	<i>E. coli</i> 5	0.003056
	<i>E. coli</i> 10	0.002992
	<i>L. reuteri</i> + <i>E. coli</i> 5	0.004646
	<i>L. reuteri</i> + <i>E. coli</i> 10	0.005172
SD4 (Semi-defined 4) Galactose 20 g/L	<i>L. reuteri</i> 0	0.002128
	<i>L. reuteri</i> 5	0.002439
	<i>L. reuteri</i> 10	0.006678
	<i>E. coli</i> 0	0.00166
	<i>E. coli</i> 5	0.001546
	<i>E. coli</i> 10	0.001753
	<i>L. reuteri</i> + <i>E. coli</i> 5	0.002569
	<i>L. reuteri</i> + <i>E. coli</i> 10	0.00911

Reuterin production by *L. reuteri*

The co-cultures were grown in different media with different carbon sources and were analysed by HPLC for reuterin (3-HPA) production. Both *L. reuteri* and *E. coli* were inoculated with different concentrations of glycerol to check the individual reuterin production in the presence of glycerol. The co-cultures were also inoculated with 5 g/L and 10 g/L of glycerol and reuterin production and its antagonistic effects on *E. coli* was measured. In Figures 9, 10 and 11, both *L. reuteri* and *E. coli* did not produce any reuterin in the absence of glycerol in the growth medium. In Figure 11, it was found that when grown in SD4 medium with galactose, the reuterin production was the highest and a concentration of 5 g/L killed all the *E. coli* in the coculture whereas *L. reuteri* survived. Also, all of the reuterin had been converted to 1,3-PD overtime. When the glycerol concentration was 10 g/L, both *L. reuteri* and *E. coli* in the cocultures were killed. It must be noted that no pure cultures of *E. coli* were killed at any concentrations of glycerol.

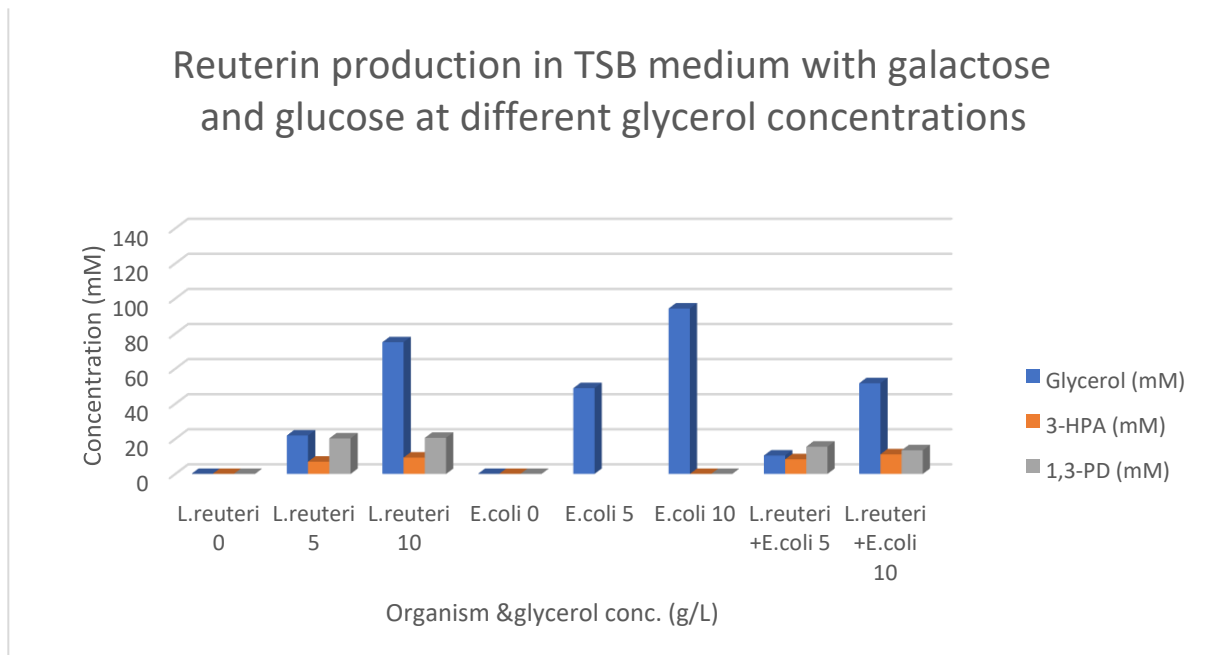


Figure 9: Reuterin (3-HPA) and 1,3-PD production and remaining glycerol in TSB medium with galactose and glucose at different glycerol concentrations measured by HPLC analysis.

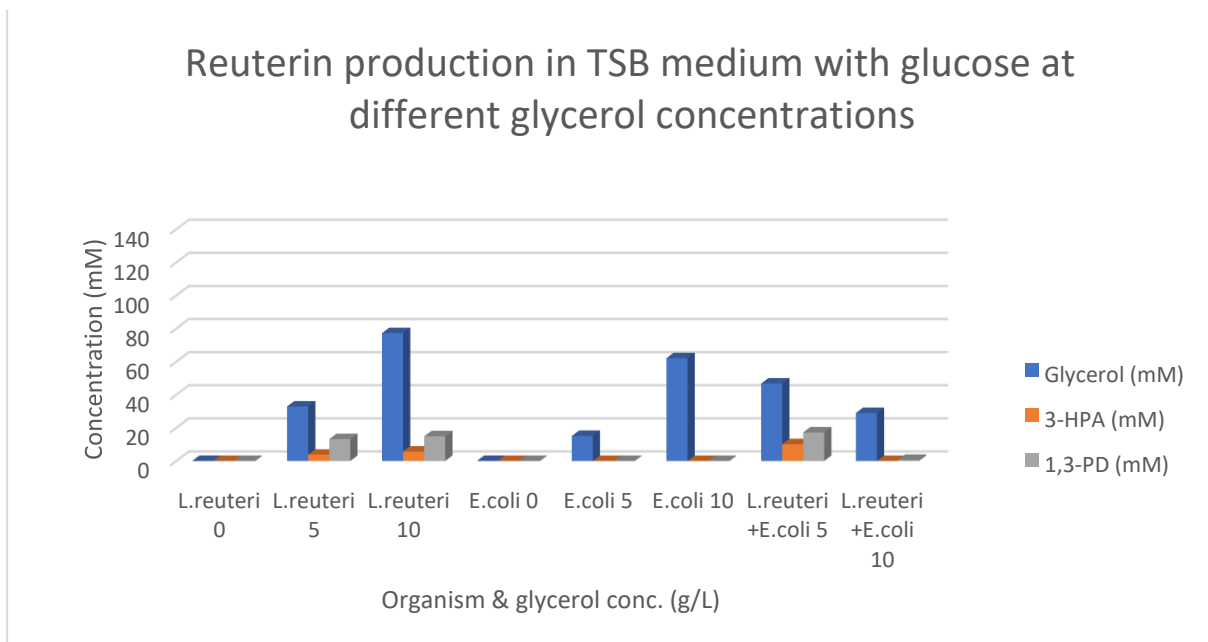


Figure 10: Reuterin (3-HPA) and 1,3-PD production and remaining glycerol in TSB medium with glucose at different glycerol concentrations measured by HPLC analysis.

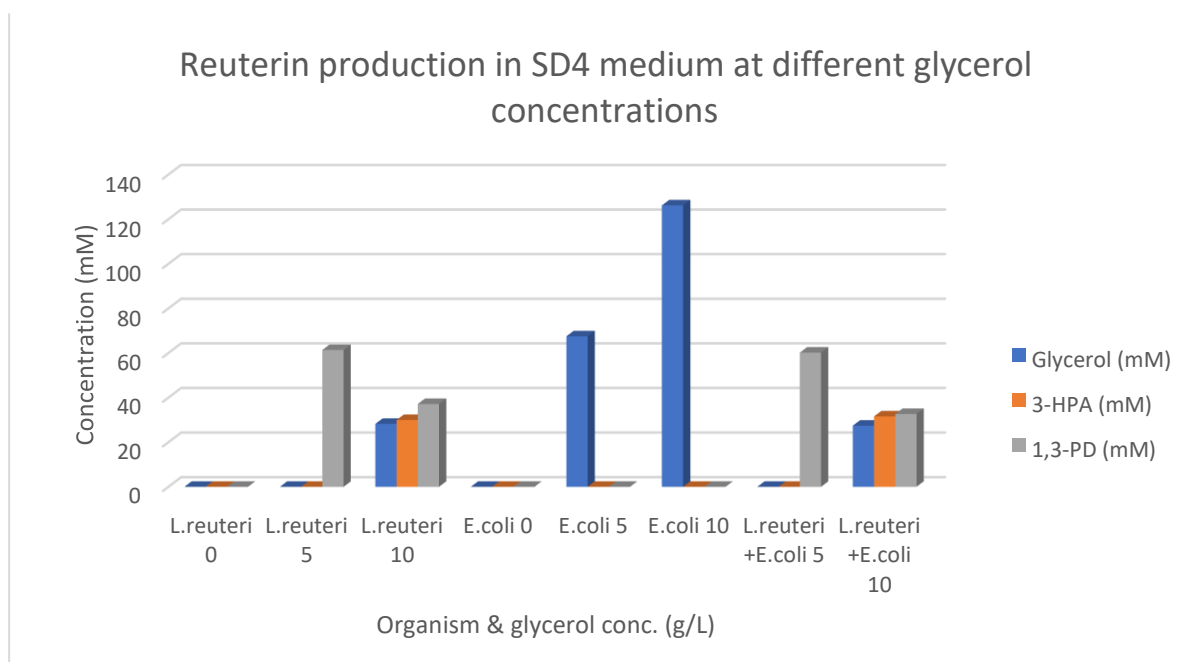


Figure 11: Reuterin (3-HPA) and 1,3-PD production and remaining glycerol in SD4 medium with galactose at different glycerol concentrations measured by HPLC analysis.

Antimicrobial activity of reuterin on *E.coli*

After inoculating and incubating the cell culture in the isothermal calorimeter, the cultures including both pure cultures and co-cultures of *L. reuteri* and *E. coli* were plated on MRS agar and BHI agar plates, respectively. The results from TSB medium (not shown here) indicated that not all the *E. coli* in the co-cultures were killed. Table 2 below shows the number of colony forming units (CFU) of each culture in SD4 medium with galactose. It can be clearly seen that at 5 g/L of glycerol concentration, all of the *E. coli* had been killed by the reuterin produced.

Table 2: Number of cfu of *L. reuteri* and *E. coli* after reuterin production by *L. reuteri* in SD4 medium with galactose. It can be seen that at 5 g/L of glycerol concentration (highlighted row) the *E. coli* in the co-culture is completely killed by the toxic effects of reuterin.

Organism & Glycerol concentration (g/L)	<i>Lactobacillus reuteri</i> (CFU)	<i>Escherichia coli</i> (CFU)
<i>L. reuteri</i> 0	1.10E+08	
<i>L. reuteri</i> 5	1.96E+07	
<i>L. reuteri</i> 10	0	
<i>E. coli</i> 0		8.30E+07
<i>E. coli</i> 5		9.20E+07
<i>E. coli</i> 10		1.02E+08
<i>L. reuteri</i> + <i>E. coli</i> 5	1.10E+08	0 (1.0E+00)
<i>L. reuteri</i> + <i>E. coli</i> 10	0 (1.0E+00)	0 (1.0E+00)

Carbon source utilization and metabolite production

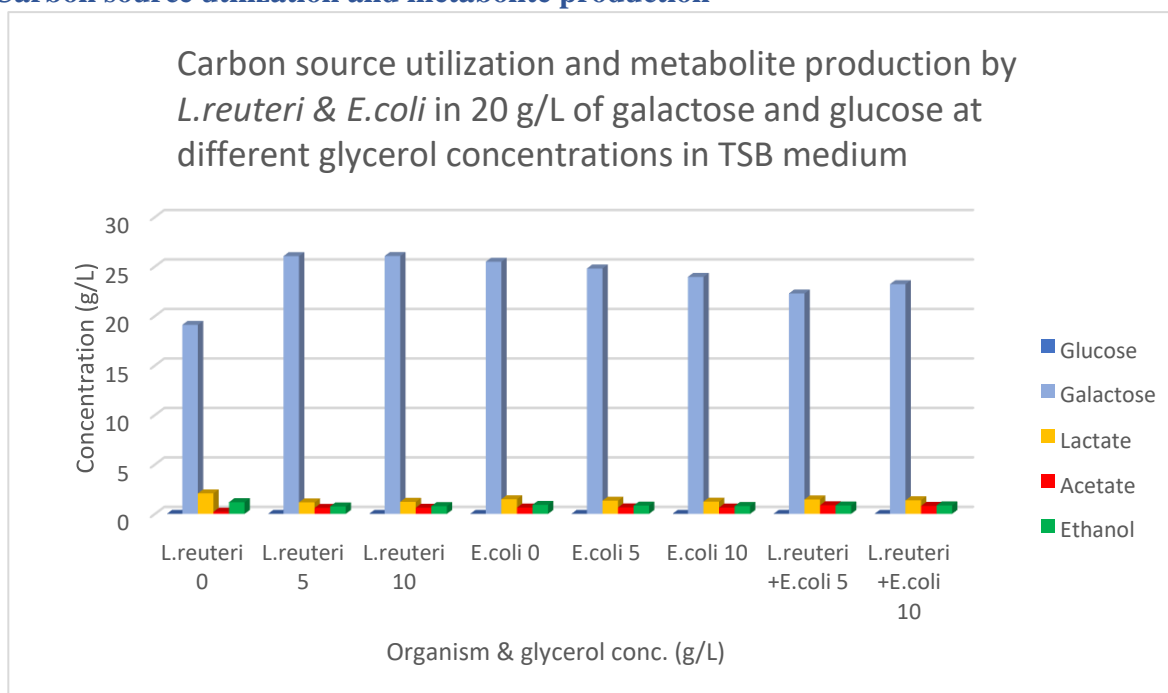


Figure 12: Carbon source utilization and metabolite production by *L. reuteri* and *E. coli* in 20 g/L of galactose and glucose mixture at varying glycerol concentrations in TSB medium measured by HPLC analysis.

After the cultivation of co-cultures in the isothermal calorimeter, the supernatant was harvested and was subjected to HPLC analysis to determine the amount of carbon source utilized during the growth and to estimate the metabolite production. Apart from the carbon sources such as glucose, galactose and a combination of galactose and glucose in different media used, lactate, acetate and ethanol were measured. The figures below showed that when the carbon source was a combination of galactose and glucose, both of the organisms preferred glucose as their carbon source. As a result, a large part of the galactose remained unused, whereas all of the glucose was utilized.

In Figure 12, in terms of metabolites, both *L. reuteri* and *E. coli* produced lactate the most which was followed by ethanol and acetate. The co-culture with 5 g/L of glycerol produced more acetate, whereas, the co-culture with 10 g/L of glycerol produce more ethanol than acetate. In Figure 13, the same metabolite production pattern was observed with lactate being the highest. But *E. coli* produced more acetate than ethanol. In Figure 14, when the culture was grown in SD4 medium, not all of the galactose used was consumed. While *L. reuteri* produced lactate and ethanol with some acetate, *E. coli* predominantly produced acetate except when there was no glycerol present. Also, the co-cultures produced more ethanol in 5 g/L and only acetate in 10 g/L of glycerol.

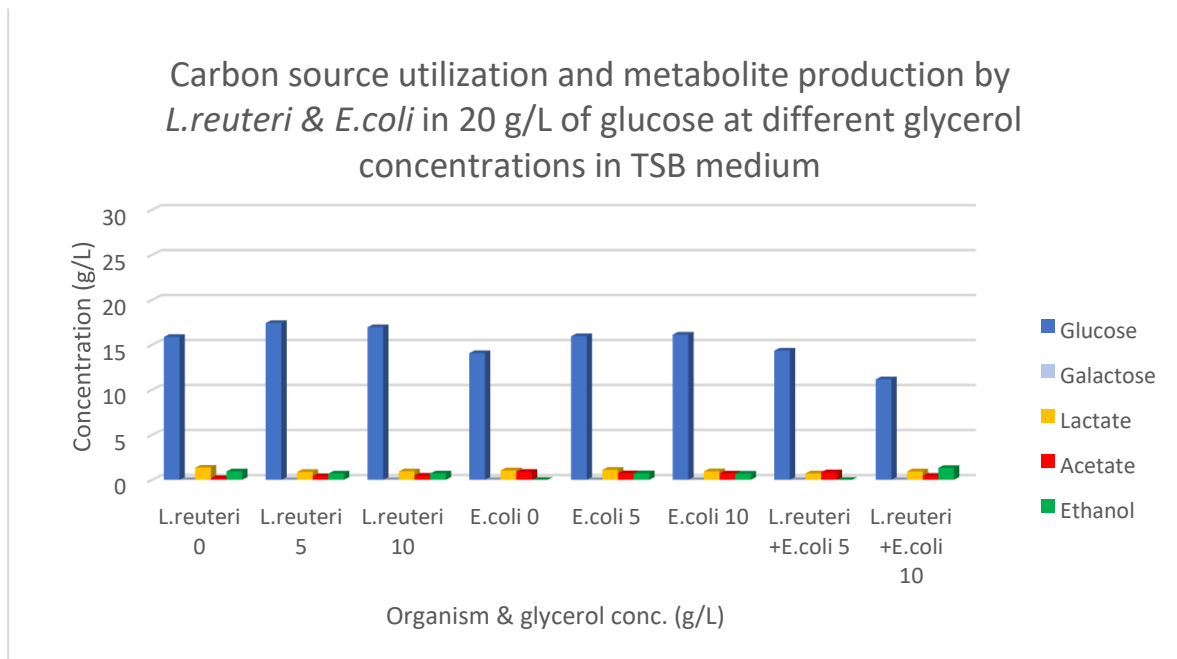


Figure 13: Carbon source utilization and metabolite production by *L. reuteri* and *E. coli* in 20 g/L of glucose at varying glycerol concentrations in TSB medium measured by HPLC analysis.

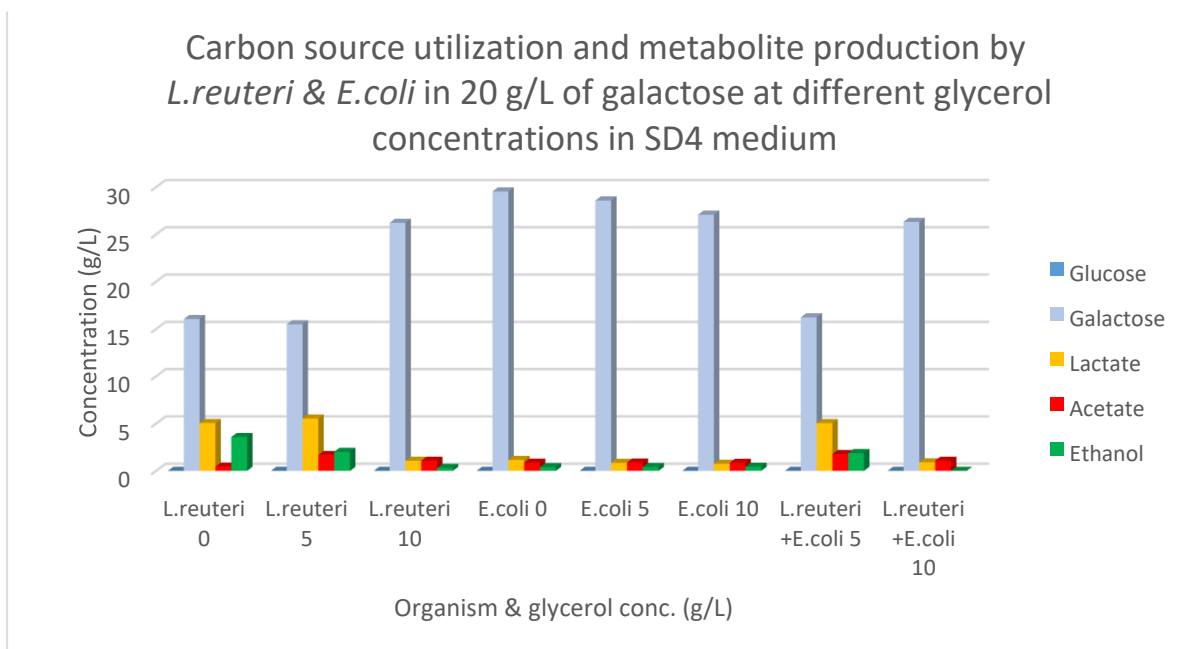


Figure 14: Carbon source utilization and metabolite production by *L. reuteri* and *E. coli* in 20 g/L of galactose at varying glycerol concentrations in SD4 medium measured by HPLC analysis.

Culturing of *L. reuteri* and *P. copri* in bioreactors

The additional work carried out as a part of this thesis was to check the growth of *L. reuteri* and *P. copri* in the bioreactor using Schaedler anaerobe broth. As the Schaedler anaerobe broth requires CO₂ sparged during the pre-culture to facilitate the growth of obligate anaerobes like *P. copri*, it was supplied as 0.5 bar for 15 min when boiling and cooling down of the media for pre-culture. But, when *L. reuteri* was pre-cultured, no CO₂ was sparged, and it was let to incubate for 48 h at 37°C. When the bioreactor was prepared and culture conditions were set according to the details mentioned in the Materials and Methods section except the CO₂ flushing, the culture grew logarithmically (Fig. 15) and rapidly and reached a μ_{\max} of 0.17 h⁻¹ with an average cell dry weight of 0.76 mg/mL.

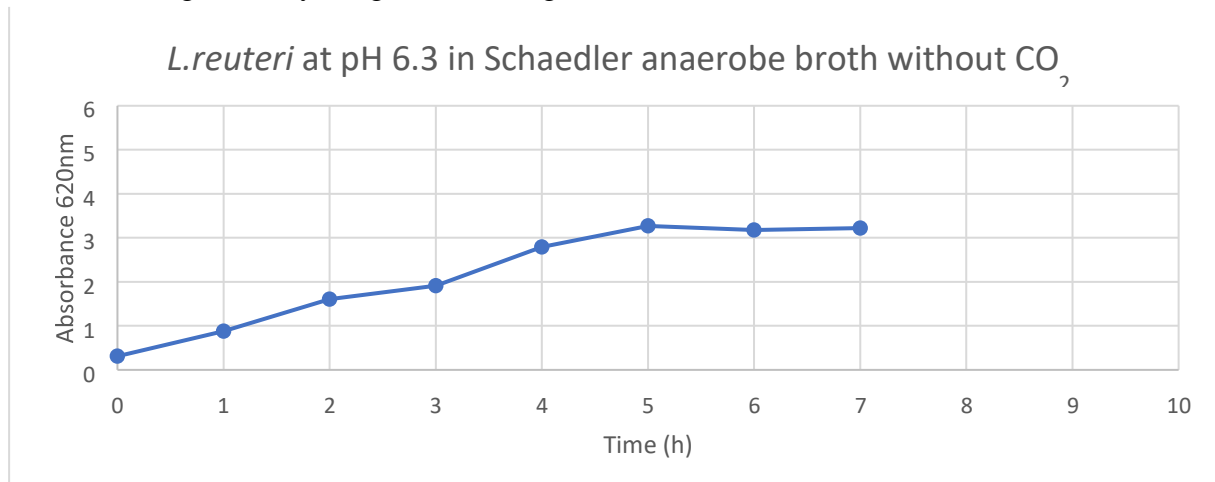


Figure 15: Fermentation growth curve of *L. reuteri* at pH 6.3 in Schaedler's anaerobe broth with no CO₂ sparging. A two-day old inoculum was used here.

The same experiment was repeated but with an overnight culture with CO₂ flushing inside the bioreactor for 15 min before inoculation. But the growth was very slow even after four h after which the cells started to reach the death phase(Fig 16).

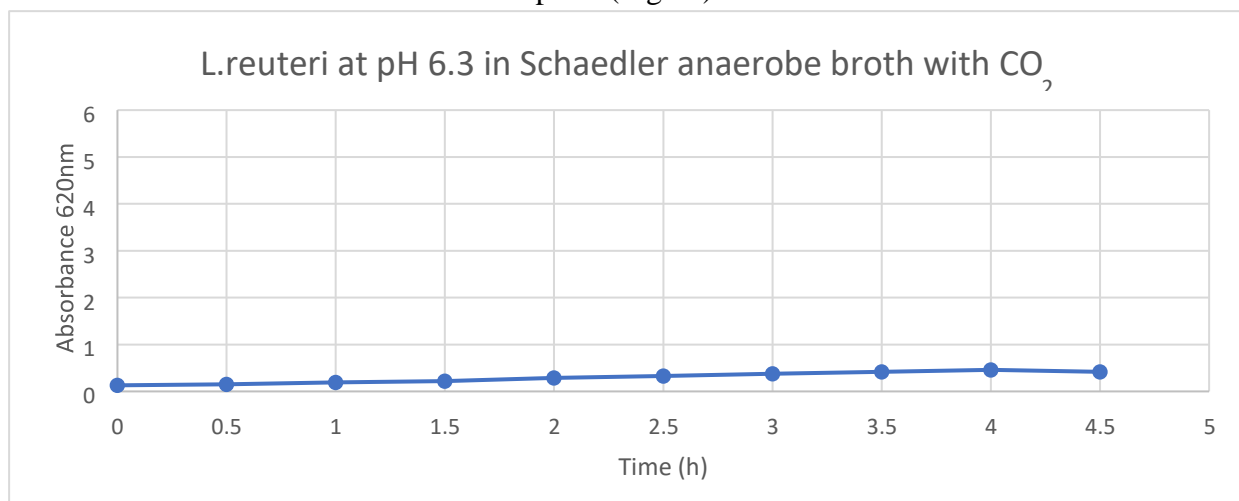


Figure 16: Fermentation growth curve of *L.reuteri* at pH 6.3 in Schaedler's anaerobe broth with CO₂ sparging.

L. reuteri was also attempted to grow in a modified Schaedler anaerobe broth. For that, the overnight pre-culture was inoculated in the bioreactor, which was not sparged with CO₂ before inoculation, but with nitrogen. The CO₂ was now sparged after 3 h of inoculation. After 30 min,

the slow growing cells started to reach a stationary phase and eventually the death phase (Fig. 17).

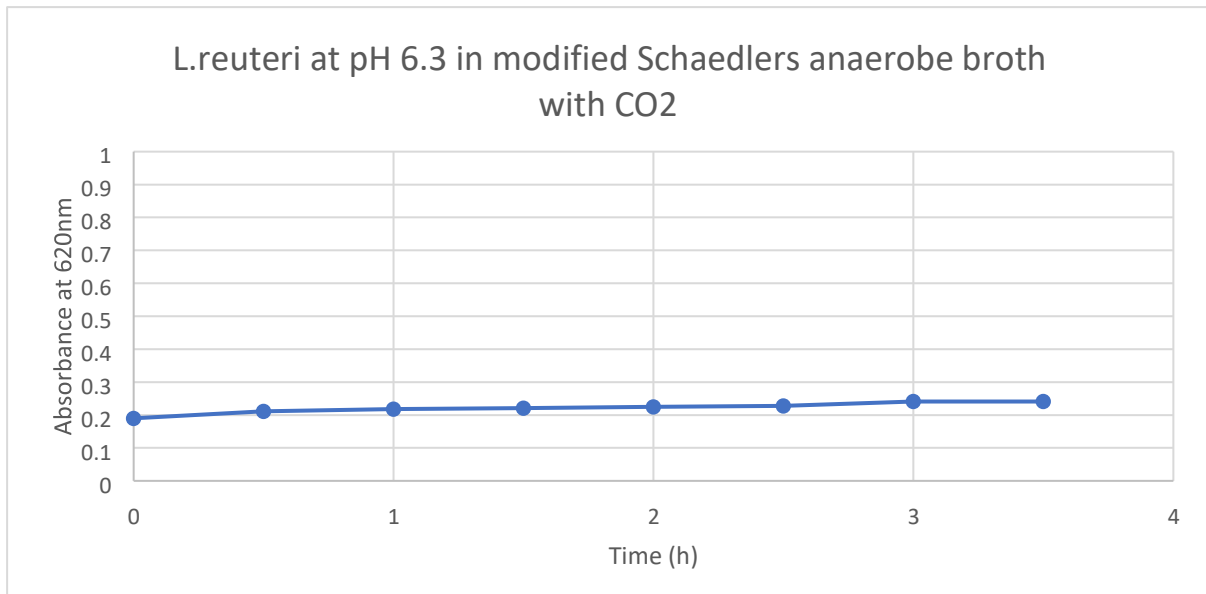


Figure 17: Fermentation growth curve of *L. reuteri* at pH 6.3 in modified Schaedler's anaerobe broth with CO₂ sparging.

P. copri was also cultivated in the bioreactor with the Schaedler's anaerobe broth at pH 7.2 and with same other fermentation parameters after growing the pre-culture in serum flasks. The cells in the bioreactor, after CO₂ sparging and inoculation, did not grow at all (Fig. 18). They rapidly started to die, and the optical density reached lower levels than the inoculum, meaning that there were eventually no cells in the medium left. The serum flask cultures of *L. reuteri* and *P. copri*, surprisingly, grew on the Schaedler's anaerobe media under anaerobic conditions, but when it was inoculated in the bioreactor in an anaerobic environment, they did not grow except for the two-day old inoculum of *L. reuteri*. Also, when the modified Schaedler anaerobe broth was added with 15 g/L of glucose with other additional compounds, the media turned brownish green and the pH started fluctuating. Thus, the inoculated cells stopped growing within 20 min and the fermentation was stopped immediately.

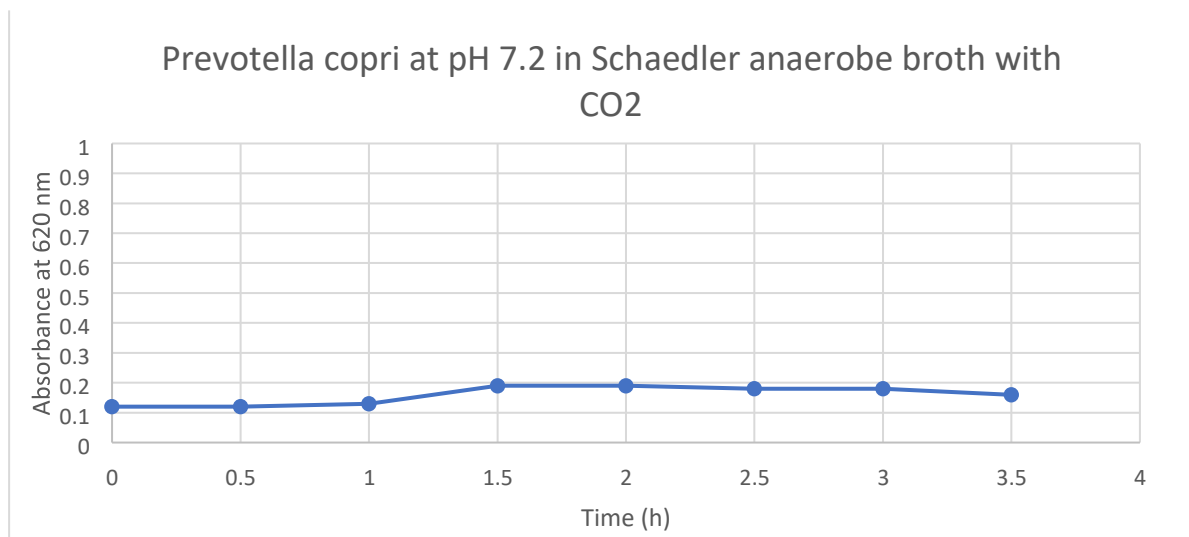


Figure 18: Fermentation growth curve of *P. copri* at pH 7.2 in Schaedler’s anaerobe broth with CO₂ pumping.

Modified reactor set up for co-culture

As co-culturing of bacteria in the fermenter requires two different species to be grown together, a cage, to hold one of the bacteria, was designed using a stainless-steel wire mesh (Fig. 19). The advantage of this design is that the bacteria can be inoculated inside a dialysis tubing in a medium in some respect different than the other in the same bioreactor. This prevents the mixing of two types of bacteria but allows only the desired components of a certain kD size diffuse through the selected dialysis tubing. This prototype was designed to study the extracellular electron transfer in *L. reuteri* and *P. copri* cocultures, which is under evaluation.



Figure 19: The prototype with a cage and dialysis tubing for co-culturing of *L. reuteri* and *P. copri* with media in the dialysis tubing and water inside a 3L single-walled glass fermenter.

DISCUSSION

The goal of this work was to study the effects of reuterin (3-HPA) produced by *L. reuteri* DSM 17938 in the presence of glycerol on *E. coli* C600. In addition, the possibilities of co-culturing *L. reuteri* and *P. copri* was also explored using the serum- flask cultures in Schaedler's anaerobe broth and fermentor. First, the medium selected for the co-culturing of *L. reuteri* and *E. coli* and their growth in different media has been discussed. Then, the reuterin production by *L. reuteri* at different glycerol concentrations and its antagonistic effects on *E. coli* along with the metabolites produced have been analyzed. Finally, the hypothesis of extracellular electron transfer between *L. reuteri* and *P. copri* and the experiments using Schaedler's anaerobe broth have been described.

The medium necessary for the co-cultures must contain all the required nutrients for the growth of both organisms. It is also important to note that previous researches recommend the use of medium that supports the growth and replication of food-borne microorganisms like *E. coli* when testing for their inhibition by a chemical compound like reuterin (Lopez-Malo et al., 2005). And so, initially, MRS medium was tested for its effects on the growth of *L. reuteri* and *E. coli*. The MRS medium was selected as it is a defined, rich medium with essential nutrients required for the growth of Lactic acid Bacteria (LAB) (Corry et al., 2003). While the growth of *L. reuteri* at pH 5.8 showed a μ_{max} of 0.47 h^{-1} in the bioreactor, the growth of *E. coli* was found to be much slower when grown on MRS agar plates. This was due to the high concentration of acetate and citrate present in the MRS medium which might have been fatal for *E. coli* ((Zhou et al., 2007; Over et al., 2009). So, the experiments revealed that, in order to attain an equal growth rate of both microorganisms, the common medium was either tryptic soy broth or SD4 medium. But the tryptic soy broth medium as such was not able to support the growth of *L. reuteri*, unless surfactant Tween 80 was provided to the medium. The latter is a source of oleate to metabolically active *L. reuteri* (Jacques et al., 1980). *E. coli* does not require Tween 80 as was exemplified by its growth in TSB medium without Tween 80 (Calix-Lara et al., 2012). Both TSB with Tween 80 and SD4 medium provided optimal growth conditions for the co-cultures and so they were grown in them at pH 5.8. Table 1 shows that all the cultures really grow in the isothermal calorimeter and none of them were non-viable when inoculated.

All cultures with *L. reuteri* produced reuterin in the presence of 5 g/L glycerol. Glycerol at a concentration of 10 g/L was also detrimental to *L. reuteri* (Table 2). The amount of reuterin produced at varying glycerol concentrations in glucose and galactose depends on the activity of catabolite repression element (CRE) sequence present in the genome of *L. reuteri* (Sriramulu et al., 2008). These regulatory elements are located upstream of *PduA* gene of *Pdu* operon and CRE is located downstream of the -10 promoter region of glycerol dehydratase locus. The carbon catabolite repression (CCR) occurs by a carbon catabolite control protein A (CcpA) by binding at CRE loci (van Pijkeren et al., 2012). Thus, with glucose, the CRE expression is active but not with galactose in SD4 medium where the concentration of reuterin was found to be up to 60.1 mM, all of which had been converted to 1,3-PD; the highest among the media tested.

The *E. coli* cell population decreased in the presence of both *L. reuteri* and glycerol concentration of 5 g/L and higher induced that was due to reuterin production. A complete killing was only observed in SD4 medium with galactose. The highlighted row in Table 2 shows that *E. coli* cells in the co-culture were killed and the plating of cells yielded no colonies. This

may be because of the increased expression of genes that encode for proteins that contains thiol groups in the presence of reuterin. In addition to that, many genes that are responsible for the oxidative stress could also have been overexpressed. This was found when the gene *bhs* that is involved in stress response and biofilm formation and *yhD* that encodes an aldehyde reductase which protects cells from harmful reactive oxygen species was overexpressed during reuterin treatment (Schaefer et al., 2010). *L. reuteri* was affected at 10 g/L (109 mM) of glycerol with a reuterin concentration of more than 67mM in SD4 medium with galactose. But the inhibition in TSB medium occurred when the reuterin concentration was much lower than 30mM in glucose and at 30mM in glucose and galactose mixture. These data are not in line with previous researches where a concentration between 30 to 50 mM of reuterin was sufficient to inhibit *L. reuteri* (Cleusix et al., 2007) and a concentration of 8.5mM or higher was enough to inhibit *L. reuteri* (Ortiz-rivera et al., 2017). The contrasting results can be attributed to the strain differences in the researches performed. The other two researches used *L. reuteri* ATCC 55730 and *L. reuteri* ATCC 53608, while *L. reuteri* DSM 17938 was used in the current work. Among the metabolites produced during the growth (Fig. 12,13 and 14), lactate was the highest in all the cultures irrespective of the medium or the organism. The co-cultures differed only in producing acetate or ethanol. The inhibition could have been due to the mixture of fermentation products (Calix-Lara et al., 2012), but the non-inhibition in 5 g/L of glycerol in TSB medium provides evidence that acetate or lactate concentrations were not involved in the killing of *E. coli*. Also, Figure 20 (see appendix) gives confirmation that glycerol itself is not responsible for the inhibition where the cells grew logarithmically until all the substrate was utilized.

In *P. copri*, the organism is not capable of producing propionate from succinate as it lacks genes encoding methylmalonyl-CoA mutase, methylmalonyl-CoA epimerase and succinate: methylmalonyl-CoA transferase (Franke and Deppenmeier, 2018). The hypothesis is that during co-culturing with *L. reuteri*, the electrons can be donated from NADH oxidation through mediators to *P. copri*, which the organism requires for CO₂ fixation to produce succinate by fumarate reductase with production of ATP. The hypothesis is that vitamin B2 and vitamin K may act as electron carriers (mediators) between *L. reuteri* and *P. copri*. Figure 19 shows the modified reactor setup designed for this reason to co-culture both *L. reuteri* and *P. copri* in the bioreactor using a dialysis bag to separate the two cultures but allowing exchanges of small molecules. The cultures did not grow except for one (Fig. 15) and the troubleshooting that needs to be done has been described below:

Troubleshooting

The growth of *L. reuteri* and *P. copri* in Schaedler anaerobe broth in the serum flasks under anaerobic conditions, and the non- growth of the same in the bioreactor may be attributed to one or several factors. These factors need to be re-checked to find what obstructed the growth of these bacteria in the bioreactor. Some of them have been mentioned below:

1. The media could have lacked a few essential components needed for growth.
2. The Schaedler anaerobe broth is light sensitive and the light could have denatured the media in the bioreactor as the serum flasks were incubated in the dark.
3. The static incubation could be a reason for why they grew in the serum flasks and not in a dynamic mode in the bioreactor.
4. The metal in the bioreactor might had affected growth. It may be essential to verify whether the metal was made of 316L grade stainless steel.

5. The gas flow (N₂) might have had traces of oxygen which should have been removed by passing it through a heating tube with copper wire before sparging into the bioreactor.
6. Diffusion limitation could have been another explanation why the cells did not grow in the bioreactor.
7. Head space sparging of CO₂ instead of sparging through the medium could have had detrimental effects on the cells.
8. The delayed inoculation after the sterilization could have denatured the important media components during the overnight resting period.

The only growth of *L. reuteri* (Fig. 15) in Schaedler anaerobe broth in the bioreactor occurred when the two-day old pre-culture was used as an inoculum could have been an anecdotal event. The experiment needs to be repeated several times to find out the robustness.

CONCLUSION

In conclusion, the co-culturing of *L. reuteri* and *E. coli* in a common medium with glycerol at 5 g/L have proved that *L. reuteri* has antagonistic effects on the survival of *E. coli* by producing the antimicrobial compound reuterin or 3-hydroxypropionaldehyde. Though the production of reuterin is higher in higher concentrations of glycerol, it is absolutely necessary to preserve *L. reuteri* for the continued production of reuterin and avoid the toxic effects of reuterin. Thus, a higher concentration, greater than 5 g/L should not be used in the co-cultures with *E. coli*. The SD4 medium and galactose helps greatly in the growth and production of reuterin when compared with TSB medium substituted with Tween 80 and so, SD4 can be an excellent medium for co-cultures. As mentioned earlier, the fermentations of *L. reuteri* and *P. copri* in Schaedler's anaerobe broth as performed should be troubleshoot and found out what hindered the growth of the bacteria in both normal and modified Schaedler anaerobe broth.

After analyzing and evaluating all the data, the primary aim of the thesis, the inhibition of growth of *E. coli* C600 by the probiotic *L. reuteri* DSM 17938, has been achieved and confirmed by the production and activity of antimicrobial substance reuterin. This opens up one of the alternate ways to tackle the antibiotic-resistance menace that is rampant around the world in this century.

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APPENDIX

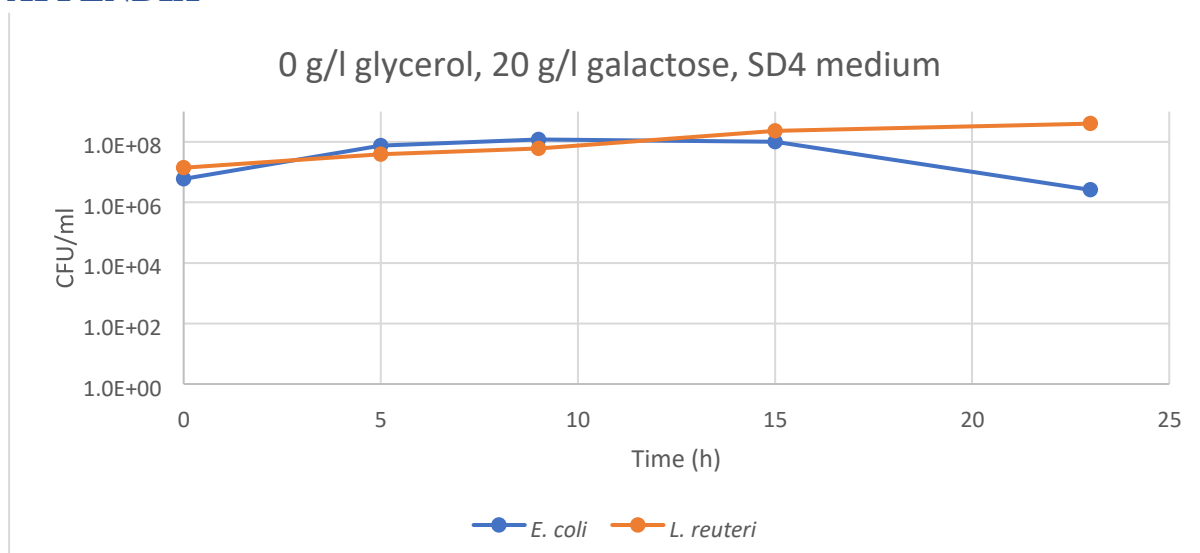


Figure 20: Growth profiles of *L. reuteri* DSM 17938 and *E. coli* C600 in the absence of glycerol in SD4 medium with galactose as the substrate.

Table 3: Modified Schaedler's anaerobe broth

Schaedler anaerobic broth pH 6.3	Amount (g/L)
Tryptone Soya broth	10
Special peptone	5
Yeast extract	5
Glucose	5
Cysteine HCl	0.4
Haemin	0.01
Tris buffer	0.75
Tween 80	1
di- Potassium hydrogen phosphate	2
Sodium acetate	5
Magnesium sulphate	0.2
Manganese sulphate	0.04

SD4 Medium:

All the prepared solutions were sterile filtered unless otherwise specified. The vitamin solution was stored at -20°C and trace element and UAG solution was stored at 4°C.

1L of SD4 medium consists of the following recipe:

- **Sugar solution**
25 g of galactose dissolved in 200 mL of H₂O and autoclaved.
- **Salt solution**

K_2HPO_4 (2.5 g), KH_2PO_4 (2.5 g), $MgSO_4 \cdot 7H_2O$ (0.25 g), NaAc (0.5 g), and $MnCl_2 \cdot 2H_2O$ (0.013 g) dissolved in 200 mL of H_2O . The pH of the solution was adjusted to 5.5 with 5M H_2SO_4 and autoclaved.

- **Tween 80** (1 mL)
- **Casamino acid solution**
5g of casamino acids dissolved in 50 mL of H_2O
- **Amino acids solution**
Reduced glutathione (0.01 g), L-Alanine (0.06 g), L-Serine (0.2 g) L-Tryptophan (0.03 g), L-Asparagine (0.2 g), L-Cysteine (0.17 g), and L-Glutamine (0.2 g) dissolved in 50 mL of H_2O
- **UAG solution**
Uracil (0.4 g), Adenine (0.2 g), Guanine (0.2 g) and NaOH (50%) (0.24 g) in 30 mL of H_2O
- **Vitamin solution**
10 mL of the solution consists of Biotin (10 mg/L), Pyridoxal – HCl (206 mg/L), Folic acid (10 mg/L), Riboflavin (98 mg/L), Nicotinic acid (98 mg/L), Thiamine – HCl (101 mg/L) Ca-Pantothenate (95 mg/L), and P-Aminobenzoic acid (10 mg/L) dissolved in H_2O . Folic acid and Riboflavin dissolved first in 1N NaOH
- **Trace element solution**
1 mL added from a 100 mL solution prepared by dissolving EDTA (1.5 g) and $ZnSO_7 \cdot 7H_2O$ (0.45 g) in 50 ml of H_2O , and after adjusting the pH to 6.0, $MnCl_2 \cdot 2H_2O$ (0.1 g), $CoCl_2 \cdot 6H_2O$ (0.03 g), $CuSO_4 \cdot 5H_2O$ (0.03 g), $Na_2MoO_4 \cdot 2H_2O$ (0.04 g), $CaCl_2 \cdot 2H_2O$ (0.45 g), $FeSO_4 \cdot 7H_2O$ (0.3 g), H_3BO_3 (0.1 g), and KI (0.01 g) were added and the pH was adjusted to 6.0 after each addition.