Development of a defined minimal culture medium for *Prevotella copri* DSM 18205^{T}

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Master thesis

Development of a defined minimal culture medium for *Prevotella copri* DSM 18205^{T}

aventure

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Popular summary

Humans have been consuming probiotics without realising it during centuries through traditional fermented foods such as yoghurt and kefir. Later on we have found out that some of these bacteria can be beneficial for the prevention and treatment of conditions such as inflammatory bowel disease or allergies. However, what if probiotics could be used to target or even cure diseases? This is what the so-called Next Generation Probiotics (NGP) are aiming to do, and *Prevotella copri* is a candidate to become one. *P. copri* is a bacterium naturally present in the human gut, breast milk, as well as in sauerkraut and beer. But why is *P. copri* so interesting? Because this bacterium plays a beneficial role in glucose metabolism, since it is present in higher numbers in the gut of people who have an improved glucose tolerance after consuming fiber-rich foods. Thus, *P. copri* has a potential to target metabolic diseases such as type 2 diabetes or obesity. But before this can be a reality, we need to find a way to grow *P. copri* efficiently.

This project was carried out at the division of Biotechnology of Lund University in collaboration with Aventure AB. The final scope is to develop a vegan fermented product that contains *P. copri*, and this master thesis was focused on finding a suitable culture medium as a first step. *P. copri* is usually grown in complex culture media (such as PYG – Peptone Yeast Glucose), which means that a lot of ingredients are needed, and the exact chemical composition of the broth is unknown. Moreover, if the final product is going to be plant-based, the culture medium should also be free from animal-derived ingredients, which is unusual when growing this type of bacteria.

During this project we developed a defined minimal medium, in other words, a simple culture broth for *P. copri*, free from animal-derived ingredients. The experiments were carried out in small serum bottles and both the growth and the production of bacterial metabolites were monitored. The most important product by *P. copri* is succinate, which has a beneficial role in glucose metabolism. Different versions of the defined medium were developed, with and without plant-derived haemoglobin. Previous research has shown that hemin (a component found in haemoglobin) is required to grow *P. copri*, especially in defined medium. However, *P. copri* reached higher numbers and produced more succinate in the defined medium without haemoglobin, which was unexpected but good news for the aim of the project. Nevertheless, more work is needed in order to enhance the growth of *P. copri* in defined medium.

To sum up, this project shows that it is possible to grow *P. copri* in a minimal medium with no animal-derived ingredients, even when omitting hemin. As this hadn't been achieved before, it is a promising step for developing a vegan fermented product with *P. copri* in its formulation.

Abstract

The human gut microbiota is involved in the host's health and disease. There has been growing interest in the next generation probiotic candidate *Prevotella copri*, which plays a beneficial role in glucose metabolism. It is a Gram-negative obligate anaerobe that was first isolated from human faeces and which is present in plant-based fermented foods as well as in human breast milk. The aim of this project was to develop a minimal culture medium free from animal-derived ingredients, within the framework of potentially developing a fermented food product that contains *P. copri* in collaboration with Aventure AB.

The first phase of the project consisted of assessing the optimal pH condition as well as the efficacy of commercial horse serum in the complex medium PYG (Peptone Yeast Glucose) for growing *P. copri*. The second phase of the project involved the formulation of the DMMG (Defined Minimal Medium Glucose) as well as the evaluation of plant-derived haemoglobin (BvHb) as a substitute of commercial hemin in the culture medium.

The optimal pH for growing *P. copri* was 7.2, and the addition of horse serum was not indispensable for its successful growth in PYG. The DMMG was developed based on culture media previously formulated for *Bacteroides fragilis*, which rendered an acceptable biomass and succinic acid production. Moreover, the omission of hemin favoured the growth of *P. copri* in DMMG, which is inconsistent with previous studies. This finding suggests that more research is needed in order to elucidate the role of hemin in the cultivation of *P. copri* as well as to improve the formulation of the DMMG to enhance the biomass production.

Preface

As research on the field of probiotic bacteria increases, so does the interest of consumers when it comes to functional foods that contain these beneficial microorganisms. *Prevotella copri* is a candidate novel probiotic that plays a role in sugar metabolism. Previous research in the department of Food Technology and Nutrition at Lund University has shown that *P. copri* is present in plant-based fermented foods as well as in human breast milk, which support *P. copri*'s status as 'qualified presumption of safety' as a microorganism in food and feed. However, in order to culture this bacterium, several animal-derived ingredients are required in the culture medium.

This project was carried out at the division of Biotechnology at LTH as a collaboration with Aventure AB, a research-based company which develops functional foods. Following the market demands, the company has been considering the idea of developing a plant-based functional food product that contains *P. copri* which is beneficial in relation to glucose metabolism. The main goal of this master thesis was to successfully grow *P. copri* in a minimal medium free from animal-derived ingredients, which could eventually allow the scaling up of the project to obtain this bacterium in high numbers.

While carrying out my master thesis I received constant support and guidance from my supervisors Roya Sardari and Fang Huang, for which I am immensely grateful. I would also like to extend my gratitude to the head of the Biotechnology division Eva Nordberg for offering me this opportunity along with Olof Böök from Aventure AB. Special thanks to Leif Bülow and Simon Christensen for providing the plant-derived haemoglobin that was used in this project. I also want to thank Savvina Panagiota Leontakianakou, Alfredo Zambrano and Siri Norlander for their help with the HPAEC equipment, as well as Mahmoud Sayed for his advice with Gram-staining and microscopy. Finally, I cannot forget to thank my family, partner and friends for all the unconditional support during the scope of this project.

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Abbreviations

BvHb: *Beta vulgaris* Haemoglobin
DMMG: Defined Minimal Medium Glucose
DSMZ: German Collection of Microorganisms and Cell Cultures
EFSA: European Food Safety Authority
FAO: Food and Agriculture Organization
FDA: Food and Drug Administration
GMO: Genetically Modified Organism
HAB: Heme-Auxotrophic Bacteria
HPAEC-PAD: High-Performance Anion Exchange Chromatography coupled to Pulsed Amperometric Detection
HPLC: High-Performance Liquid Chromatography
NGP: Next Generation Probiotic
OD: Optical Density
P. copri: Prevotella copri
PYG: Peptone Yeast Glucose
SCFA: Short-Chain Fatty Acid
WHO: World Health Organization

1. Introduction

1.1. Background

The human gut microbiota plays an important role in relation to the host's health and disease. The term "probiotics" was originally referring to organisms or substances which contribute to intestinal balance, as opposed to antibiotics, which destroy microorganisms (Molin, 2013). Probiotics are defined by the WHO/FAO as live microorganisms which confer a scientifically proven health benefit on the host when administered in adequate amounts. It has been shown that probiotic bacteria can be beneficial in the prevention and treatment of disorders such as irritable bowel syndrome, inflammatory bowel disease, allergies and traveller's diarrhoea (Foerst and Santivarangkna, 2016).

Prebiotics are non-digestive food components that selectively favour the growth of beneficial microorganisms in the gut. The advantage of prebiotics is that if beneficial microorganisms are already present in the gut, it is much easier to add a dietary fibre in a food product than a living microorganism. The downside is that beneficial microorganisms need to be present in the gut to start with, and there is a chance that adverse microorganisms can also be stimulated. When dietary fibres reach the colon they are fermented by the gut microbiota, which mainly produce the beneficial short-chain fatty acids (SCFAs) propionic, butyric and acetic acid as metabolites (Molin, 2013).

The bacterium *Prevotella copri* was first isolated from human faeces and identified as a novel species by Hayashi *et al*. in 2007. It is a Gram-negative obligate anaerobe which can also be found in human breast milk and in plant-based fermented foods such as sauerkraut (Sairamesh, 2019). In a human study, this bacterium was found in increased proportions in subjects with an improved glucose metabolism after a 3-day consumption of barley kernel-based bread. This suggests that *P. copri* could have a beneficial effect on glucose metabolism and motivates the interest of this potential probiotic in relation to metabolic diseases such as diabetes (Kovatcheva-Datchary *et al*., 2015).

1.2. Isolation and evaluation of new probiotics

The majority of probiotics have been isolated from traditional fermented foods with a long history of use, which is a decisive factor for their safety evaluation (Chang *et al*., 2019). Many of the most well-known probiotic species are classified in the genera *Bifidobacterium* and *Lactobacillus*, which include more than 45 and 90 species, respectively (Foerst and Santivarangkna, 2016; Molin, 2013). An example of the taxonomic classification of two probiotic strains is shown in Table 1.

Table 1: Taxonomic classification of L. plantarum 299v *and* B. animalis BB12 *(Molin, 2013).*

Phylum	*Firmicutes*	*Actinobacteria*
Class	*Bacilli*	*Actinobacteria*
Order	*Lactobacillales*	*Bifidobacteriales*
Family	*Lactobacillaceae*	*Bifidobacteriaceae*
Genus	*Lactobacillus*	*Bifidobacterium*
Species	*L. plantarum*	*Bifidobacterium animalis*
Strain	*L. plantarum 299v*	*B. animalis BB12*

However, microorganisms other than bifidobacteria and lactobacilli are also being studied as potential probiotics. There is growing interest in the so-called next generation probiotics (NGP), which as opposed to traditional probiotics, are aiming to both prevent and treat specific diseases. The candidates for NGPs are mainly isolated from the human gut, which is also the case for *P. copri* (Chang *et al*., 2019; Hayashi *et al*., 2007).

Establishing the identity of a microorganism is the first step before carrying out any further studies such as phenotypic methods (like sugar fermentation profiles). It is important to identify the candidates at a strain level as many probiotic effects are strain-specific.

The identification of probiotic candidates is usually carried out by phylogenetic analysis based on the 16S rRNA gene. The next step consists of an *in vitro* selection of the strains in order to choose the ones with the appropriate properties, followed by *in vitro* safety assessments. In the case of *Bifidobacterium* and *Lactobacillus* strains, the EFSA includes them in their Qualified Presumption of Safety list because of the long history of safe consumption as well as the availability of epidemiological data (Foerst and Santivarangkna, 2016). This classification, which assesses the generic risk of microorganisms in food, is updated annually and provides an orientation for food business operators (Sairamesh, 2019). In other less studied species the following tests are carried out: detection of antibiotic resistance determinants and virulence genes as well as toxic metabolite production, among others (Foerst and Santivarangkna, 2016).

A previous master thesis executed at the Food Technology and Nutrition department of Lund University supports the classification of *P. copri*'s 'Qualified Presumption of Safety of microorganisms in food and feed' status by the European Food Safety Authority (EFSA), although further safety studies should be conducted in animals and humans (Sairamesh, 2019).

The last step before moving on to *in vivo* pre-clinical and clinical studies consists of the *in vitro* selection of the best candidates that can potentially have a beneficial health effect. Different tests can be used depending on the application of the probiotic product. Some examples are tolerance to the gastrointestinal tract conditions, adhesion to the gut mucosa, inhibition of pathogens, interaction with the gut epithelia and ability of modulating the gut microbiota or immune modulation capability (Foerst and Santivarangkna, 2016).

Finally, the *in vivo* studies should provide the evidence for the health benefits of the probiotic on the host. Moreover, the dosage regimens as well as the duration of use that is recommended by the manufacturer should also be proven by these clinical studies (FAO/WHO, 2006).

1.3. Characteristics of *Prevotella copri*

P. copri is a non-spore forming, obligate anaerobic bacterium. It is rod-shaped, non-motile and Gram-negative (DSMZ GmbH, 2019). The table below presents its taxonomic classification.

Table 2: Taxonomic classification of P. copri *(DSMZ GmbH, 2019)*

Phylum	*Bacteroidetes*
Class	*Bacteroidia*
Order	*Bacteroidales*
Family	*Prevotellaceae*
Genus	*Prevotella*
Species	*Prevotella copri*
Strain	*P. copri* DSM 18205^T

Most human *Prevotella spp*. have been isolated from the oral cavity, but there are 4 species which inhabit the human large intestines, *P. copri* being the most abundant (Franke and Deppenmeier, 2018). Specifically, *P. copri* was first isolated from the faeces of a healthy 52-year old Japanese man. The type strain is DSM 18205, even though four other strains are included in these species (Hayashi *et al*., 2007).

Prevotella and *Bacteroides* are the two prevailing genera within the *Bacteroidetes* phylum in the human gut microbiome. Higher abundance of *Prevotella* is traditionally associated with a diet rich in fruits and vegetables, whereas the abundance of *Bacteroides* is linked to high-fat and protein-rich diets (De Filippis *et al*., 2019).

When it comes to food, *P. copri* has been detected in a variety of plant-based foods, such as sauerkraut, pickles, soy and pea drinks, even in beer. It has also been detected in dairy products like goat cheese and yoghurt drinks. In addition, *P. copri* has been found in human breast milk. These findings would support the classification of *P. copri* as 'Qualified Presumption of Safety (QPS) of microorganisms in food and feed' by the EFSA (Sairamesh, 2019).

1.4. Metabolism of *P. copri*

P. copri is an obligate anaerobe adapted to its environmental niche, where complex carbohydrates are frequently available. It possesses a large number of genes encoding enzymes for a wide range of dietary polysaccharides such as starch, xylans and pectin. Table 3 shows the carbohydrates metabolized by *P. copri*. These polymers are degraded into monosaccharides which subsequently enter the central carbon metabolism. The genome of *P. copri* encodes all the enzymes necessary for the EMP (Embden–Meyerhof–Parnas) pathway, which is the central process in its cytoplasmic sugar degradation (Franke and Deppenmeier, 2018).

Table 3. Carbon source utilization of P. copri *(DSMZ GmbH, 2019)*

Carbon source	Arabinose
	Cellobiose
	Glucose
	Lactose
	Maltose
	Raffinose
	Rhamnose
	Salicin
	Sucrose

The products of the carbohydrate fermentation are succinate, acetate and formate, which can be taken by other microorganisms in the gut (Figure 1). The fermentation equation can be summarised as:

22.65 glucose + 5.6 CO_2 → 22.7 succinate + 22.6 acetate + 5.5 formate

(Franke and Deppenmeier, 2018)

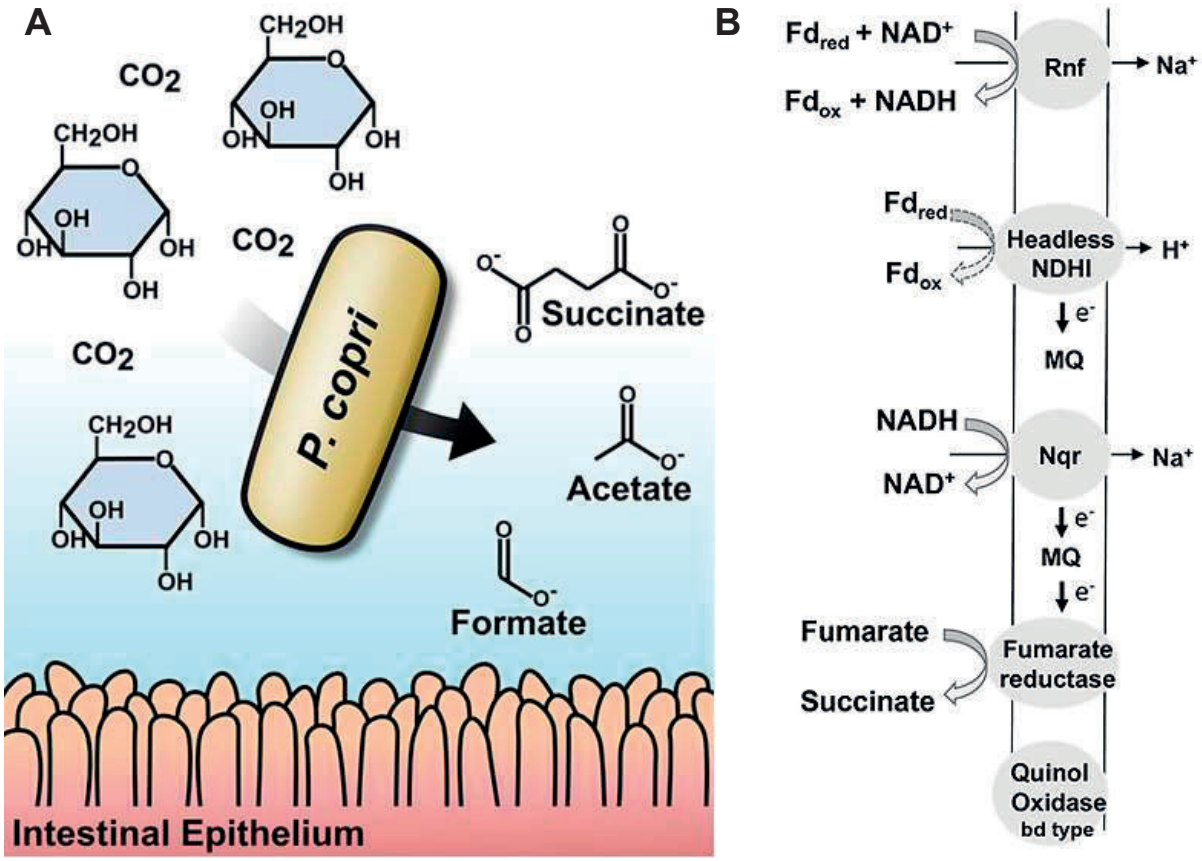

Figure 1. As a result of glucose fermentation, P. copri *produces succinate, acetate and formate (A). The respiratory chain of P. copri consists of five membrane-bound complexes, with fumarate as the terminal electron acceptor (B) (adapted from Franke and Deppenmeier, 2018).*

Microbial interactions with dietary polysaccharides and the resulting SCFAs are important energy and signalling molecules, the most important ones being butyrate and propionate (Figure 2).

Butyrate is a crucial energy source for the colonic epithelial cells; it plays a role in satiety, oxidative stress and it affects the intestinal barrier (Hamer *et al*., 2008).

Propionate has been shown to lower plasma fatty acids, which can lead to an improvement in insulin sensitivity. Moreover, propionate's beneficial effects are associated with a reduction in body weight, as it also inhibits food intake and prolongs satiety (Al-Lahham *et al*., 2010).

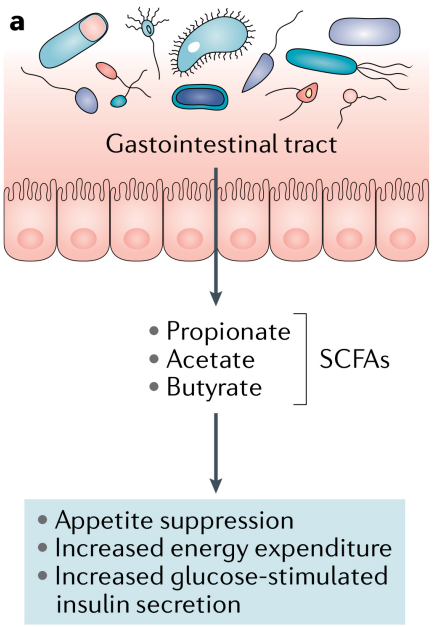

Figure 2. The SCFAs produced by the gut microbiota modulate the host's energy and glucose metabolism (Lau and Vaziri, 2019)

In the case of *P. copri*'s metabolites, acetate can reach the peripheral circulation and can also directly affect the adipose tissue, brain, and liver, inducing overall beneficial metabolic effects. A study showed that oral administration of acetate in an obese and diabetic strain of rats reduced weight gain and improved glucose tolerance. Furthermore, acetate can cross the blood-brain barrier and reduce appetite via a central homeostatic mechanism (Koh *et al*., 2016).

Regarding succinate, although it is unclear if it acts as a signalling molecule, it binds the receptor GPR91, which is expressed in dendritic cells. This fact suggests that microbially produced succinate may function as a signalling molecule which activates dendritic cells and could therefore modulate the intestinal inflammation. This was supported by a study showing that polyphenols in conjunction with a high-fat diet raised the succinate levels in the caecum and inhibited the growth and proliferation of colon cancer cells and angiogenesis (Koh *et al*., 2016).

Moreover, a study carried out on mice that were fed a fibre rich diet revealed that succinate was the highest carboxylic acid found in the cecum. They also found that dietary succinate improved the glucose and insulin tolerance in mice through intestinal gluconeogenesis (IGN), as shown in Figure 3 (De Vadder *et al*., 2016).

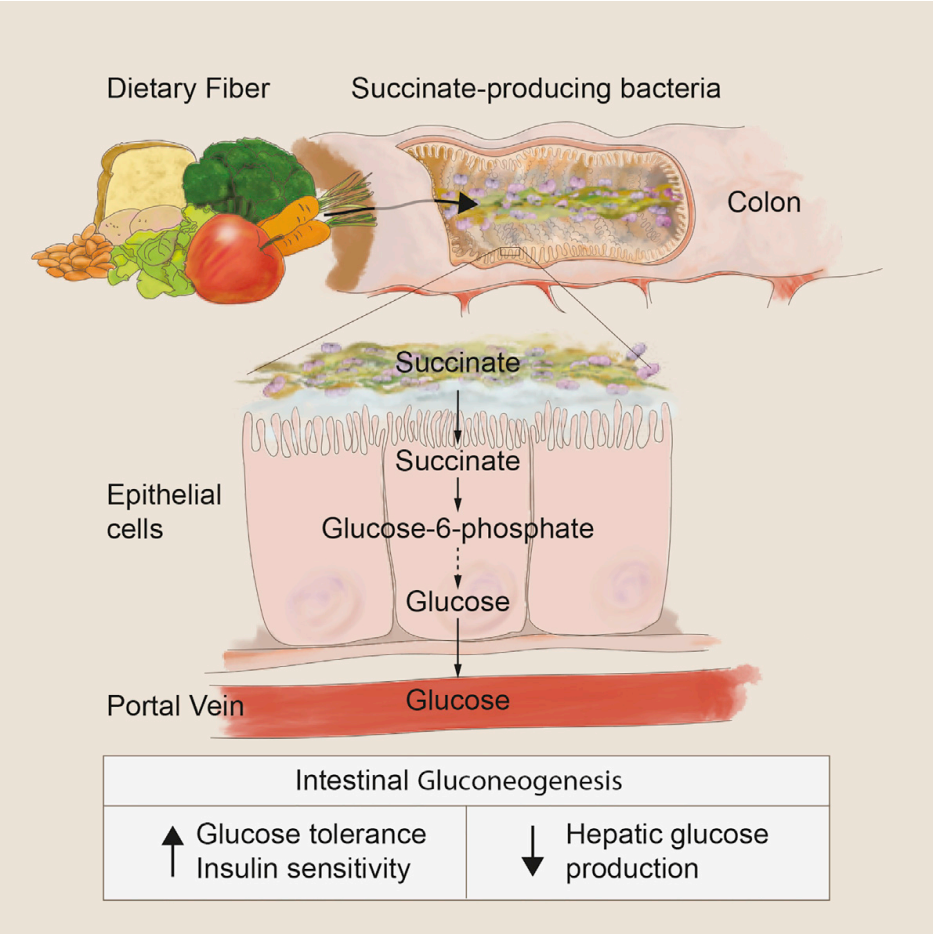

Figure 3. The succinate produced by microbial fermentation of dietary fibers induces metabolic benefits by functioning as an intestinal gluconeogenic substrate (De Vadder et al.*, 2016).*

In addition, succinic acid is an important building block for a wide range of chemicals. It has many applications, for instance as a surfactant, as an ion chelator and as an additive in the food and in the pharmaceutical industries (Ferone *et al*., 2019). As a food additive, succinic acid is used as a an acidulant and pH modifier, anti-microbial agent as well as a flavouring agent (Zeikus *et al*., 1999).

Succinic acid can be produced through the conversion of maleic anhydride from petroleum or through fermentation of renewable sources such as sugar beet (Figure 4) (Morales *et al*., 2016).

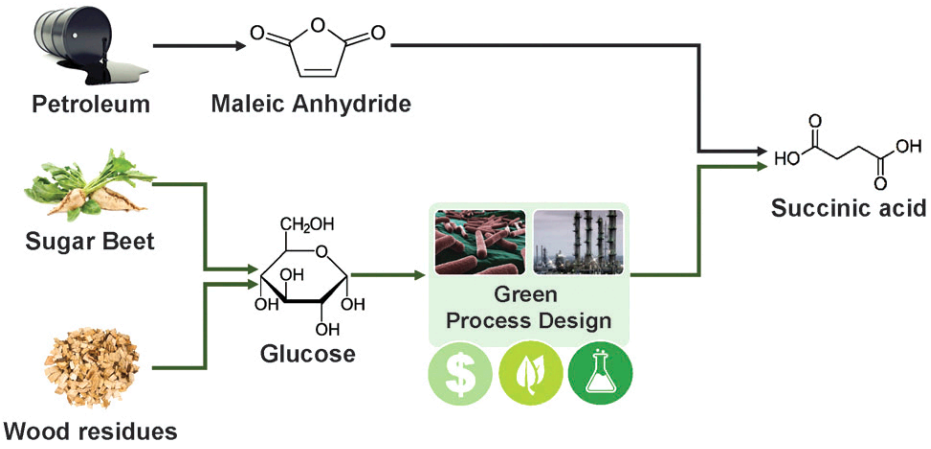

Figure 4. Production of succinic acid via the conventional petrochemical process and via the renewable resources (Morales et al.*, 2016).*

As of 2016, its market price lied between 2500 and 3000 USD per tonne. The raw material cost for maleic anhydride was 1300 USD per tonne of produced succinic acid, whereas the cost for sugar beet was 500 USD per tonne of succinic acid (Morales *et al*., 2016).

1.5. Cultivation of *P. copri*

1.5.1. Anaerobic conditions and redox potential

Obligate anaerobes are defined as microorganisms which are unable to utilize molecular oxygen for growth. While some anaerobes are only slightly inhibited by significant levels of oxygen (aero tolerant anaerobes), others die, or immediately stop growing, upon exposure to low levels of oxygen (strict anaerobes). *P. copri* is an obligate anaerobe, and as such it is crucial to retain anoxic conditions during all steps of its handling (DSMZ GmbH, 2020).

Most strict anaerobes require not only the absence of oxygen, but also a redox potential below -300 mV, which can be achieved by supplementing the culture medium with reducing agents. In the case of the PYG culture medium, cysteine-HCl x H_2O is added for this purpose, as well as resazurin as a redox sensitive dye. It is included in the medium in order to monitor the redox potential, it is generally non-toxic to microorganisms and it's effective at very low concentrations. Resazurin is dark blue in its inactive form after it has undergone an irreversible reduction step to resorufin, (which is pink at pH values near neutrality) it can go through a reversible reduction step where hydroresorufin is formed (which is colourless). The resorufin/hydroresorufin redox couple

is colourless below a redox potential of about -110 mV and becomes pink at a redox potential above -51 mV (DSMZ GmbH, 2020).

1.5.2. PYG culture medium

One of the recommended culture media for cultivation of *P. copri* is PYG, a complex medium that contains the following components:

Table 4. Components of the PYG culture medium and their function (DSMZ GmbH, 2020c; and Sairamesh, 2019).

Component	Concentration (per L)	Function
Trypticase peptone	5 g	Source of nitrogen and amino acids
Peptone	5 g	
Beef extract	5 g	
Yeast extract	10 g	Source of nitrogen, amino acids and vitamins
Glucose	5 g	Carbon source
K_2HPO_4	2 g	Buffer
Tween 80	1 mL	Growth enhancer
Cysteine-HCl x H_2O	0.5 g	Reducing agent
Resazurin	1 mg	Redox indicator
Salt solution	40 mL	Osmotic balance
Hemin	5 mg	Growth factor; commercially available from porcine or bovine origin (Merck, 2020a)
Vitamin K_1 solution	0.2 mL	Growth factor

1.5.3. Hemin

Hemin is an iron-containing porphyrin, which is present in a diverse group of proteins. It consists of a protoporphyrin IX ring with a ferric iron (Fe^{3+}) and a chlorine ligand, as shown in Figure 5. Hemin is the oxidised version of heme, which contains ferrous iron (Fe^{2+}) (VWR, 2020).

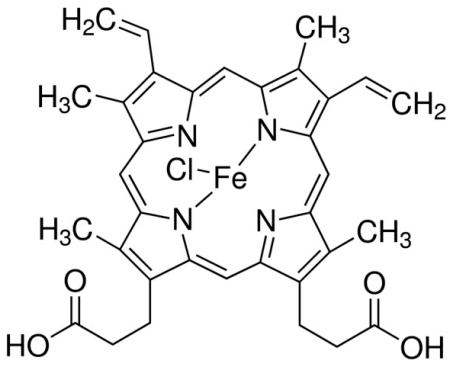

Figure 5. Hemin structure (Merck, 2020a).

Iron is a crucial cofactor implicated in many cellular processes in bacteria. Since ferric iron is poorly soluble, bacteria have developed several strategies in order to scavenge iron from their surroundings (Gruss *et al*., 2012). *Prevotella copri* is a heme-auxotrophic bacterium (HAB), since it cannot synthesize its own heme and therefore requires the addition of hemin to the culture medium (Gruss *et al*., 2012; Franke and Deppenmeier, 2018). In fact, amongst its genes, there is a putative hemin receptor (UniProt, 2020).

Both in prokaryotes and in eukaryotes, heme is implicated in oxidative metabolism at various levels, including oxygen transport, electron transport-dependent oxidative phosphorylation, oxidative stress response and detoxification. In the case of Gram-negative HAB, hemin is possibly required for the activation of fumarate reductase in order to generate energy through anaerobic respiration (Figure 1B) (Gruss *et al*., 2012).

In the human gut HAB are the major constituents, such as bacteroides, lactobacilli and enterococci. These bacteria obtain the heme from dietary animal and plant sources, after it is released from haemoproteins (like myoglobin and haemoglobin) thanks to proteolytic digestive enzymes. Other sources of heme are dead epithelial cells, faecal blood and the heme produced by enterobacteria (such as *E. coli*).

A well-established plant source of heme is leghaemoglobin, present in the root nodules of leguminous plants, like for instance soybean (Gruss *et al*., 2012). Leghaemoglobins have a critical role in the symbiotic nitrogen fixation mechanism of legumes, hence their classification as symbiotic haemoglobins. Besides oxygen transport (like their human analogue myoglobin), leghaemoglobins also have an oxygen scavenging role (Kundu *et al*., 2003).

Soy leghaemoglobin is used as a heme alternative by the California-based company Impossible Foods (Figure 6). In order to meet the production needs, they currently use a GMO version of leghaemoglobin, obtained through fermentation with genetically engineered yeast (Impossible Foods Inc., 2020). The GMO leghaemoglobin that Impossible Foods adds to their vegan products is approved by the FDA as it is generally recognised as safe (FDA, 2016).

Figure 6. Genetically engineered soy leghaemoglobin is a FDA-approved food ingredient used in some vegan meat substitutes (Impossible Foods Inc., 2020).

The plant haemoglobin that was used in this project (kindly provided by professor Leif Bülow and Simon Christensen) is a non-symbiotic haemoglobin from sugar beet (*Beta vulgaris*), denominated BvHb. It is the variant BvHb1.2, highly expressed in seeds during germination and implicated in nitrogen metabolism (Leiva Eriksson *et al*., 2019). Both mammalian myoglobins, leghaemoglobins and non-symbiotic haemoglobins contain the same identical heme moiety (FDA, 2016).

1.5.4. Horse serum

While this component isn't mentioned in the literature regarding the growth of *P. copri*, it has been successfully used in the cultivation of this bacterium in previous research at the Biotechnology department of Lund University, as advised by DSMZ. In fact, in the PYG preparation protocol, DMSZ recommend horse serum supplementation for *Pedobacter heparinus* and *Fretibacterium fastidiosum*. In the case of *Prevotella saccharolytica*, DSMZ recommends the addition of $5\%_{(v/v)}$ horse serum when cultivating it in chopped meat medium with carbohydrates (DSMZ GmbH, 2020b). Other studies support the beneficial effect of adding horse serum in the culture medium of *Bacteroidaceae* (Wahren and Holme, 1969) and specifically *Bacteroides forsythus* (Braham and Moncla, 1992).

Horse serum is used as a supplement in cell cultures (specially mammalian cells) since it contains growth factors and hormones (Merck, 2020d). While fetal bovine serum is widely used for *in vitro* cell cultures, horse serum contains more proteins and immunoglobulins than bovine serum (PAN-Biotech, 2020).

1.5.5. Defined minimal medium

A defined medium is composed of carefully measured ingredient concentrations, in other words, it is a medium where the exact chemical composition of each component is known (Merck, 2020b). At the same time a minimal culture medium typically consists of carbon and nitrogen sources, small amounts of inorganic ions (such as calcium, phosphorus, sulfur, potassium and magnesium) and buffer capacity.

On the other hand, complex media such as PYG additionally contain vitamins, minerals and growth factors to favour the growth of fastidious microorganisms like *P. copri* (Foerst and Santivarangkna, 2016).

Franke and Deppenmeier have previously achieved to grow *P. copri* in a defined minimal medium. They based their formulation on a culture medium used for the growth of *Bacteroides fragilis* (Varel and Bryant, 1974; see Appendix 1), although it contained casitone, which is from animal origin. In addition, Franke and Deppenmeier supplemented their medium with potassium butyrate, vitamin K_1, Wolfe's vitamin solution and hemin (Franke and Deppenmeier, 2018).

Furthermore, another paper also used a simplified version of Varel and Bryant's minimal medium for the growth of *Bacteroides fragilis* (Sperry *et al*., 1977). In this case the only animal-derived ingredient was hemin (see Appendix 2).

For this project, a modification of the minimal medium by Sperry *et al*. was used, in combination with some of the additions of Franke and Deppenmeier. Table 5 in section 3.1.2. describes the formulation of the DMMG that was used.

Figure 7. DMMG prior to autoclaving. The intense pink colour is due to the redox indicator resazurin, which becomes colourless after the heat treatment.

2. Objectives

In order to potentially develop a vegan functional food product that contains *P. copri* at the company Aventure AB, its growing conditions had to be investigated to be able to achieve an optimal fermentation without animal-derived elements in the culture medium.

In previous studies *P. copri* had been cultured in the complex medium PYG or other media that required at least one animal-derived component. This master thesis aimed to first assess the optimal pH condition as well as the efficacy of commercial horse serum in PYG for growing *P. copri*. The following phase of the project consisted of developing a defined minimal medium without animal-derived ingredients as well as the study of the efficacy of plant-derived haemoglobin as a substitute of commercial hemin in the culture medium.

3. Materials and Methods

3.1. Culture media preparation

3.1.1. PYG

The PYG culture medium was prepared according to the German Collection of Microorganisms and Cell Cultures' instructions (DSMZ GmbH, 2020c).

All the components, except for the glucose, cysteine-HCl x H_2O, the vitamin K_1 solution and the hemin solution were weighed and dissolved in distilled water, in a 1 L beaker stirred with a magnet. The culture medium was then distributed in two 500 mL bottles and these were microwaved for 10 minutes. The aim of this step was to remove as much dissolved oxygen as possible through boiling. The content of the bottles was subsequently transferred to a 1 L Erlenmeyer flask, which was placed in a box with ice and allowed to cool down while being bubbled with CO_2. The flushing with CO_2 is an important step, since *P. copri* is dependent on this gas for biomass formation (Franke and Deppenmeier, 2018).

The next step consisted of adding the cysteine-HCl x H_2O, the vitamin K_1 solution and the hemin solution, and after that the PYG medium was transferred to a 1 L beaker in order to adjust the pH. 8 N NaOH and 6 M HCl were used to increase or lower the pH, respectively. Then, the PYG was distributed in serum bottles, which were subsequently flushed with N_2 and sealed with butyl rubber stoppers and aluminium crimps (Figure 8). Finally, the bottles were autoclaved for 20 minutes at 121ºC and were kept refrigerated.

Figure 8. Bottling of the PYG culture medium.

Both the horse serum (Gibco, heat inactivated and bubbled with N_2) and glucose solution (filter-sterilised and bubbled with N_2) were added at the moment of inoculation in order to avoid unnecessary heat treatment. In the case of the glucose solution, the high temperatures of autoclaving could cause the Maillard reaction.

3.1.2. DMMG

Table 5 shows the composition of the DMMG medium that was developed for this project. It was based on the formulation by Sperry *et al*., with some modifications based on the DMMG used of by Franke and Deppenmeier.

Table 5. Composition of the DMMG.

Component	Mass concentration (per litre)
Glucose	5 g
$(NH_4)_2SO_4$	1 g
KH_2PO_4	0.9 g
NaCl	0.9 g
Cysteine-HCl x H_2O	0.5 g
Butyric acid	176 mg
$CaCl_2$	20 mg
$MgCl_2$ x $6H_2O$	20 mg
$MnCl_2$ x $4H_2O$	10 mg
Plant haemoglobin[1]	5 mg
$FeSO_4$ x $7H_2O$	4 mg
$CoCl_2$ x $6H_2O$	1 mg
Resazurin	1 mg

[1]BvHb1.2 (concentration: 83.25 mg/mL) kindly provided by professor Leif Bülow and Simon Christensen from the department of Pure and Applied Biochemistry at Lund University.

In addition, 200 µL of vitamin K_1 solution, 1 mL of Wolfe's vitamin solution were added. Table A6 of Appendix 3 shows the composition of Wolfe's vitamin solution.

As with the PYG preparation, the medium was boiled and cooled under CO_2. The butyric acid, cysteine-HCl x H_2O, Wolfe's vitamin solution as well as the BvHb (in some cases) were added

before the pH was adjusted to 7.2 with NaOH (50% in this case). The DMMG was finally distributed into serum bottles under N_2 prior to autoclaving. The glucose solution and in some cases the BvHb solution were added at the moment of inoculation.

3.2. Culture conditions

1 mL vials of *Prevotella copri* DSM 18205^T stock culture in PYG medium with 15% glycerol were kept at -80ºC. The stock culture was propagated once in 100 mL serum bottles of PYG (with 5 g/L of glucose and 5%$_{(v/v)}$ of horse serum); this was denominated the fresh inoculum. For each experiment, this fresh inoculum (with an OD between 4 and 5) was used to inoculate the serum bottles with different culture media and treatments (i.e. A, B and C, as shown in Figure 9).

Both the sugar solution, horse serum and in some cases the BvHb were added prior to inoculation (Figure 10). The bottles were incubated without agitation at 37°C and samples were harvested at times 0, 6, 12 and 24h. In order to minimize the oxygen exposure of *P. copri*, the injection and removal of samples was carried out using disposable syringes under aseptic conditions.

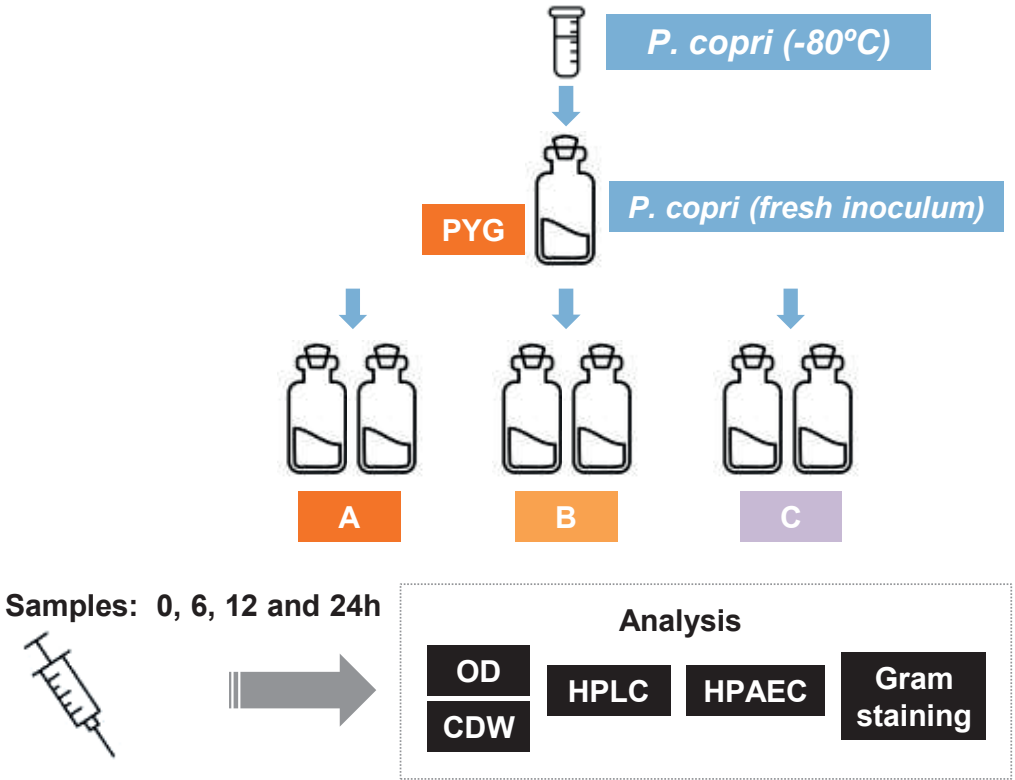

Figure 9. Schematic diagram of the general methodology that was used for this project.

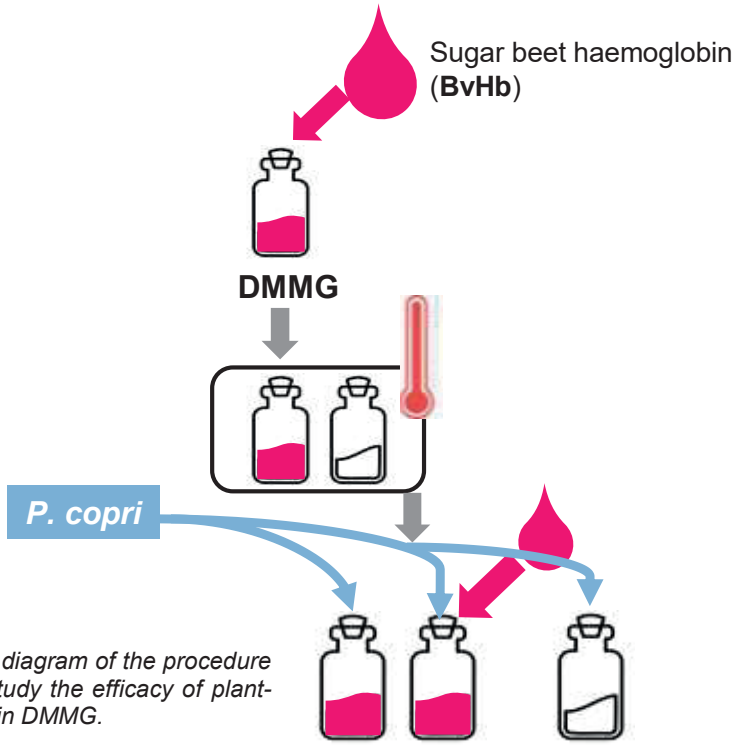

Figure 10. Schematic diagram of the procedure followed in order to study the efficacy of plant-derived haemoglobin in DMMG.

3.3. Determination of the optical density

In order to determine the cell mass (living and dead bacteria), 1 mL of cell culture sample was measured at 620nm using a Biochrom WPA Biowave II spectrophotometer. The blank was culture medium without cells.

3.4. Gram staining

In order to detect any contamination as well as to examine the cell morphology, Gram staining was performed with Sigma-Aldrich's Gram staining kit 77730-1KT-F according to the manufacturer, this protocol was followed:

1. Preparation of a slide smear: 1 mL of cell culture sample was centrifuged for a few seconds at 13500 RPM (using the Pulse function). The supernatant was discarded and a 1 µL inoculation loop was dipped in the pellet. A drop of water was placed on the glass slides, and the cells were transferred with the inoculation loop. The cells were then spread to an even thin film with the loop and the slides were placed on a heating plate in order to fix the smear.

2. With a dropper, the smear was flooded with the following reagents:
    2.1. Crystal violet solution, for 1 minute. Then the stain was poured off and the slide was gently rinsed with tap water.
    2.2. Iodine solution, for 1 minute, followed by rinsing (as explained above).
    2.3. The slide was then rinsed with the decolouring solution until the blue colour was washed away, followed by rinsing with tap water.
    2.4. Safranin solution, for 1 minute, followed by rinsing with tap water.
3. The excess water was shaken off the slides and they were allowed to air dry (Merck, 2020c).
4. The observation of the slides was carried out with a Leitz Laborlux K microscope, with the 100x lens (with immersion oil).

3.5. HPLC analysis

In order to determine the concentration of succinate, acetate and formate in the samples, HPLC (HPLC Ultimate-3000 RSLC, Dionex, Thermo Fisher Scientific, USA) was carried out. 1.5 mL of the harvested samples were centrifuged (13500 RPM, 5 min, 20°C). For each sample, 1 mL of supernatant was acidified with 20 µL of H_2SO_4 (20% v/v) for 20 minutes and subsequently centrifuged in order to precipitate any impurities (13500 RPM, 5 min, 20°C). The supernatant was carefully pipetted and transferred to the HPLC glass vials, which were kept at -20ºC.

1 mL of standard solutions of succinate, acetate and formate were prepared in ultrapure water, with the following concentrations: 0.5, 1, 1.5, 2, 2.5 and 3 g/L from 10 g/L stock solutions (Sigma-Aldrich). The standards were incubated with 20 µL of H_2SO_4 (20% v/v) for 20 minutes and subsequently centrifuged before being transferred to the glass vials and frozen.

Chromatographic separation was carried out at 40ºC using the analytical column Aminex HPX-87H (Bio-Rad, USA) and 5 mM H_2SO_4 as mobile phase, with a flow rate of 0.5 mL/min and an injection volume of 10 µL. Detection was monitored with an RI detector (Shodex, RI-101).

The measurements obtained from the standard solutions (area of the peaks, in µRIU*min) were plotted against the concentration values (in g/L) using Microsoft Excel. An equation was obtained for each metabolite (succinate, acetate and formate), after fitting the data using linear regression. The equations obtained had the format y=mx+b, where y is the area, x is the concentration, m is the slope of the line and b is the y-intercept (Figure 11).

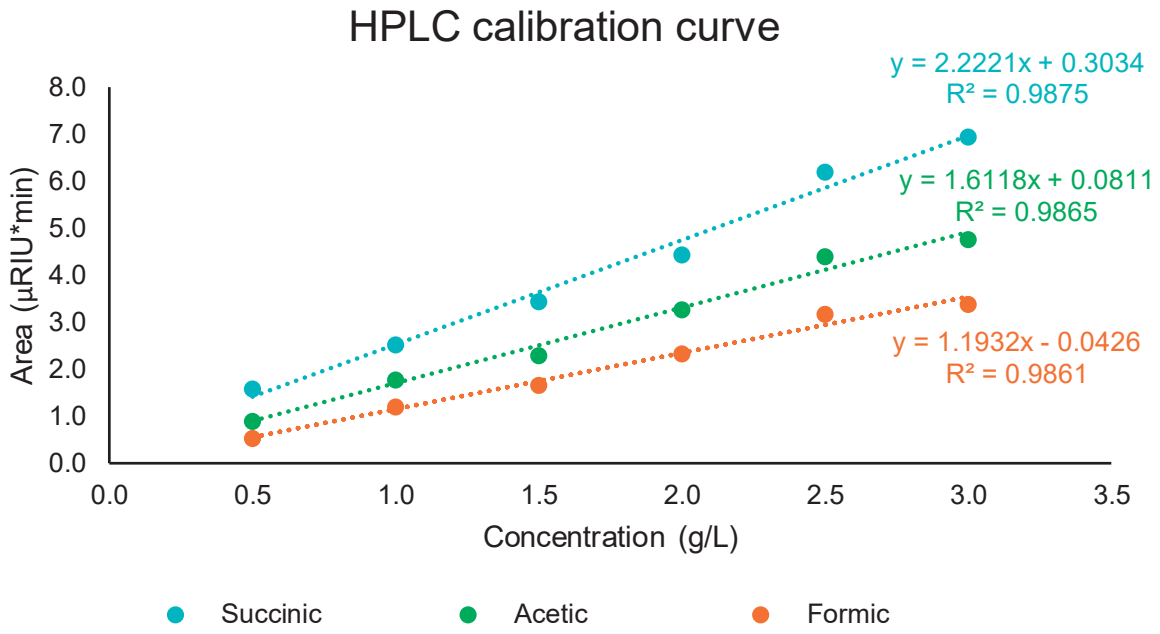

Figure 11. Calibration curve for the metabolites analysed with HPLC.

The measurements obtained from the HPLC analysis were interpolated using the equations in order to find out the concentration of each metabolite in each sample. For each batch of samples new standards were prepared and a new calibration curve was plotted.

3.6. HPAEC-PAD analysis

In order to monitor the sugar consumption of *P. copri*, HPAEC (system hardware model, Thermo Fisher Scientific, USA) was performed on the supernatant of each sample. The samples from times 0 and 6h were diluted 200x, whereas the samples from 12 and 24h were diluted 100x. The reason for this is the fact that after 12 and 24 h the amount of glucose in the culture medium is much lower because the bacteria have metabolized it, therefore there is no need to dilute it as much as at the beginning of the fermentation process. 2 mL of each diluted sample were transferred to the HPAEC plastic vials with a syringe which was connected to a 0.2 µm polypropylene filter. The vials were then kept at -20ºC.

2 mL of standard glucose solutions (D-(+)-glucose, Sigma-Aldrich) were prepared in ultrapure water, with the following concentrations: 5, 10, 20, 30 and 40 mg glucose/L. The standards were

filtered through 0.2 µm polypropylene filters when transferred to the HPAEC plastic vials, and kept at -20ºC.

The separation of glucose took place with a Dionex CarboPac PA20 analytical column. The mobile phase was obtained by the mix carried out with 3 pumps: pump A (ultrapure water), pump B (2 mM NaOH), and pump C (200 mM NaOH), with a flow rate of 0.5 mL/min. Detection took place with an ED40 electro-chemical detector.

Similarly to the procedure explained in the previous section, a calibration curve was plotted for glucose and an equation was obtained, which was used to calculate the glucose concentration in the samples. For each batch of samples new standards were prepared and a new calibration curve was plotted.

3.7. Cell dry weight

The aluminium boats used for this analysis (Figure 12) were labelled and kept in the oven at 80ºC overnight. The following day, these were allowed to cool down for 30 minutes in a desiccator and subsequently weighed in the analytical scale.

1.5 mL of cell samples were centrifuged at 13500 RPM for 5 minutes. The cell pellets were washed once with 1 mL of distilled water, centrifuged again and re-suspended in 500 µL of distilled water. The suspensions were transferred to the according aluminum boats that had been previously weighed and kept in the oven at 60ºC overnight. The following day the boats with the dried cell suspension were transferred to a desiccator to cool down for 30 minutes and subsequently weighed. The cell dry weight was obtained by subtracting the weight of the empty boat to the weight of the boat with the dried cells, divided by 1.5, obtaining the cell dry weight in grams of cells/mL of culture.

Figure 12. Aluminium boats used for determining the cell dry weight.

4. Results and discussion

4.1. Effect of the pH for the culture of *P. copri*

In order to verify if pH 7.2 is the optimal as suggested by the German Collection of Microorganisms and Cell Cultures (DSMZ GmbH, 2020c), *P. copri* was grown in PYG medium at pH values 4.5, 5, 5.5, 6, 6.5 and 7.2, with 5 g/L glucose and 5 $\%_{(v/v)}$ horse serum. As shown in Figure 13, the maximum amount of biomass, succinic acid production and glucose consumption was reached at pH 7.2.

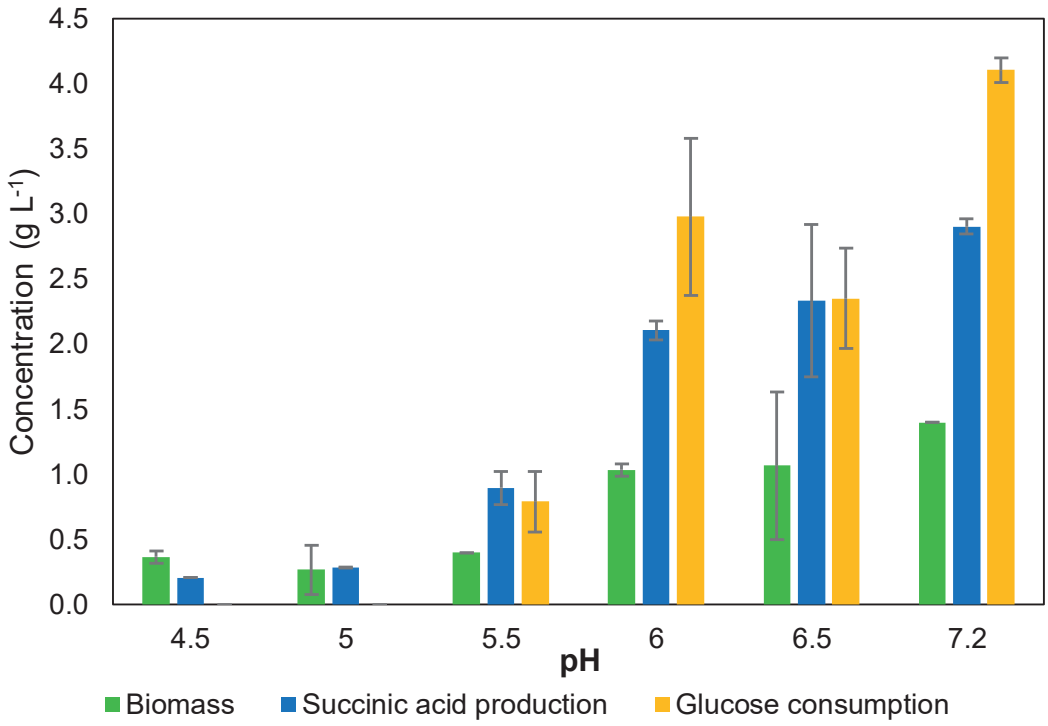

Figure 13. Effect of the pH on the biomass, succinic acid production and glucose consumption in PYG culture medium supplemented with horse serum.

The monitoring of metabolite production was focused on succinic acid, as it plays a role in glucose homeostasis (De Vadder *et al.*, 2016) and it constitutes an important building block for a wide range of chemicals (Ferone *et al.*, 2019).

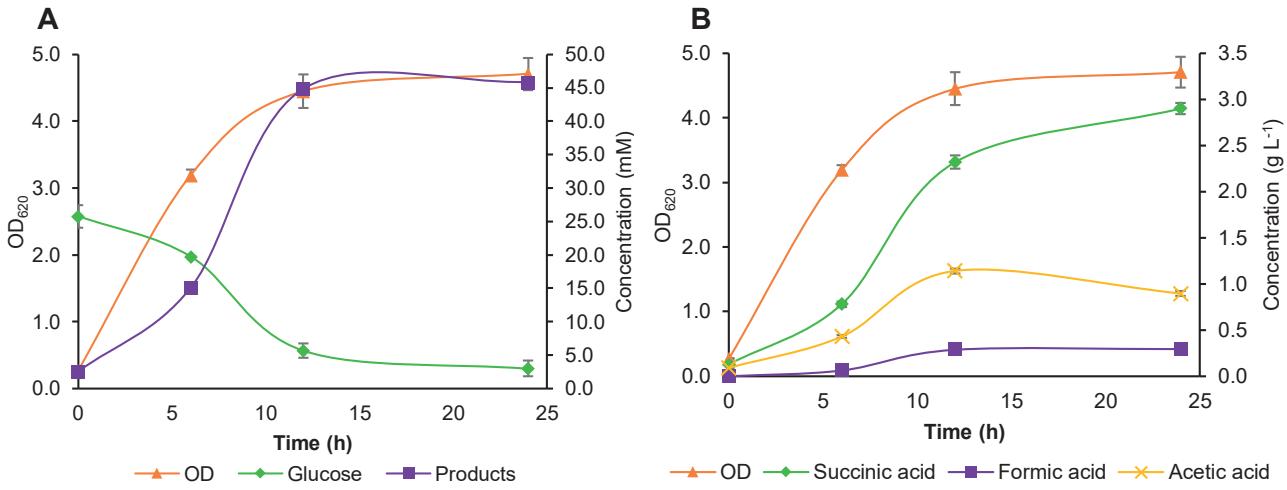

Figure 14. Growth, glucose consumption and total metabolite production (A) and metabolite profile (B) of P. copri *within 24h in PYG medium with horse serum at pH 7.2.*

Figure 14 summarises the growth, glucose consumption as well as the metabolite production of *P. copri*, which is the highest in comparison with the other pH values (data not shown). At pH 7.2, *P. copri* reached an OD of 4.71±0.24 after 24h and the metabolite concentration reached a maximum of 45.9±0.1 mM after around 12h of fermentation (Figure 14A).

Regarding the products from glucose fermentation (Figure 14B), the concentration of all three metabolites increases during the first 12h, stabilising afterwards. The most abundant product after 24h is clearly succinic acid (2.91±0.06 g/L), followed by acetic (0.896±0.025 g/L), and formic acid (0.293±0.003 g/L).

4.2. Effect of horse serum in PYG medium

In order to assess the efficacy of horse serum, *P. copri* was grown with (5%$_{(v/v)}$) and without it in PYG, as well as with and without glucose (5 g/L) as a negative control (Figure 15).

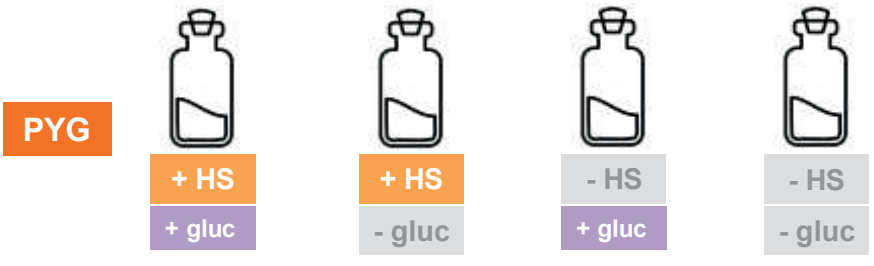

Figure 15. The different combinations used in order to determine the effect of horse serum in PYG.

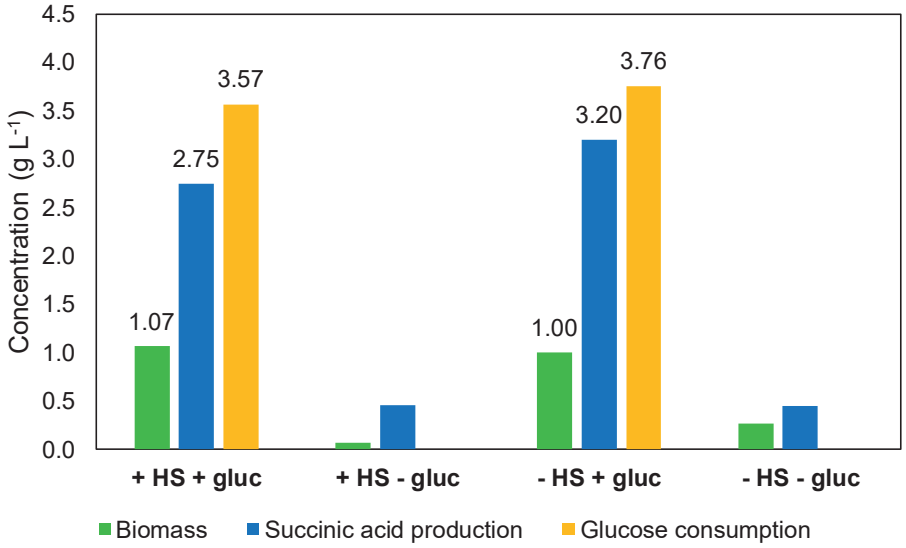

Figure 16. Effect of horse serum on the biomass, succinic acid production and glucose consumption in PYG culture medium

As shown in Figure 16, the omission of horse serum didn't have much of an impact on the three parameters presented. While the glucose consumption and the succinic acid production were unexpectedly higher when omitting horse serum, the biomass production was slightly higher with horse serum. The fact that there isn't a big difference between with and without addition of horse serum could partly be explained due to the presence of residual horse serum from the fresh inoculum. The final concentration of horse serum in the bottles that were meant to be free from horse serum was about 0.2%$_{(v/v)}$. Therefore, while this experiment should have been repeated with at least two propagations of the fresh inoculum, its results were promising for the next phase of the project.

4.3. Efficacy of BvHb in DMMG

4.3.1. DMMG vs PYG

In order to evaluate the efficacy of BvHb in DMMG, the first step was to compare PYG with DMMG, both containing the same animal-derived hemin. In both cases horse serum was omitted and glucose was added at a concentration of 5 g/L.

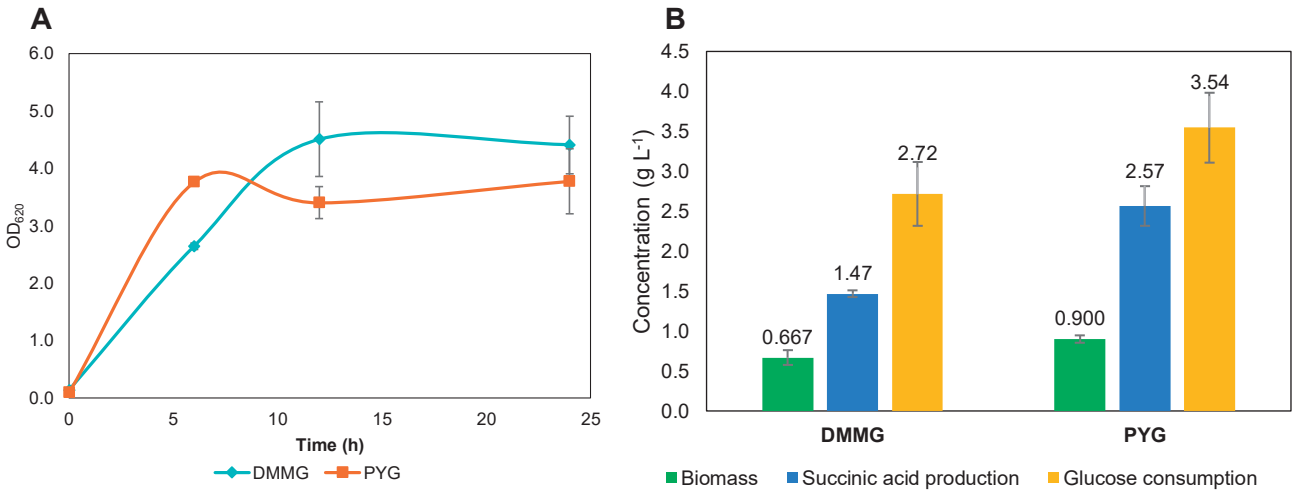

Figure 17. Comparison of the growth of P. copri *in DMMG versus PYG, both with animal-derived hemin (A) as well as the biomass, succinic acid production and glucose consumption in both culture media (B).*

Even though the maximum OD was higher when growing *P. copri* in DMMG (4.41±0.50) as compared to PYG (3.78±0.57), when looking at the relevant parameters shown in Figure 17B, PYG had a better outcome. The most substantial difference was in terms of succinic acid production, which was of 1.47±0.04 g/L in DMMG, while it reached 2.57±0.25 g/L in PYG. Regarding the biomass production, DMMG yielded 0.667±0.094 g/L, whereas with PYG 0.900±0.047 g/L were obtained. When comparing the results of Figure 17A with those in 17B, it is contradictory that *P. copri* reached a higher OD with DMMG but the biomass production was lower when compared with PYG. Both measurements take into account dead and alive cells, so the results should be consistent. One possible explanation could be that the morphology of the cells is slightly different in DMMG, which could affect the light scattering when measuring the OD.

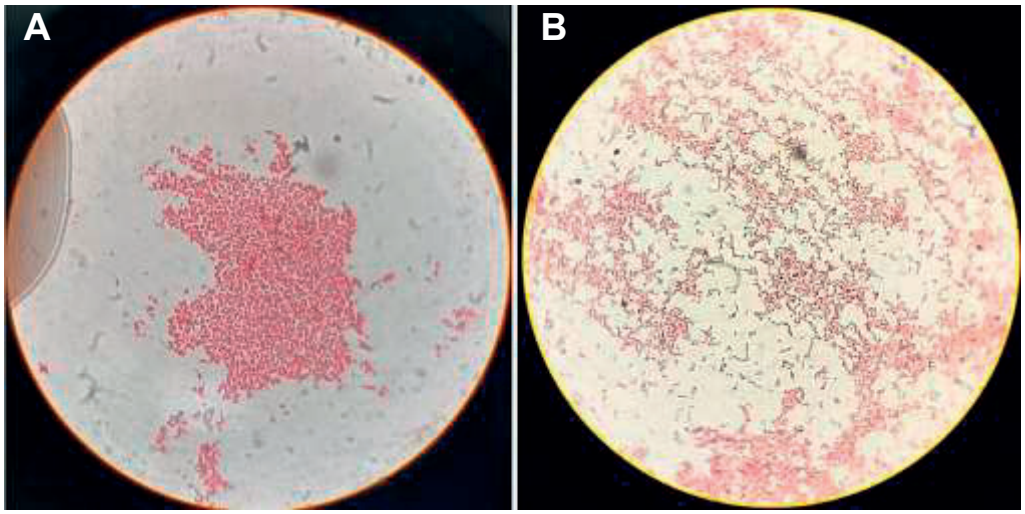

Figure 18. Microscope images (1000x) of P. copri *in PYG (A) and in DMMG (B) after Gram staining.*

In Figure 18, two samples of *P. copri* are shown after Gram staining. In the case of PYG (18A), the cells have an overall homogenous shape and present the expected colour for Gram-negative bacteria. These bacteria have a lower peptidoglycan content as well as a higher lipid content than Gram-positive bacteria. After the decolouration step in Gram staining, the lipid layer of Gram-negative cells is dissolved, so they no longer retain the violet dye. Thanks to the final step of counterstaining with safranin, only Gram-negative cells present a pink colour (Merck, 2020c).

In Figure 18B, the overall colour of the cells is pink, but there are several dark spots which could be interpreted as contamination (Gram-positive cocci). However, a more plausible scenario would be that the dark spots are due to the fact that the washing step with the decolourizing solution wasn't carried out thoroughly. In addition, the cells appear more irregular compared to PYG, which could be a consequence of the difference in composition of the media or the lack of specific components that are necessary for an optimal growth. Therefore, the differences in the overall morphology of the cells could have an impact on the light scattering and affect the OD readings.

4.3.2. DMMG with and without BvHb

DMMG was prepared without adding the animal-derived hemin solution. Instead, 5 mg/L of recombinant haemoglobin from sugar beet, BvHb, were added. Moreover, in one of the treatments, the BvHb was added pre-inoculation, so that it wouldn't be heat-treated during autoclaving.

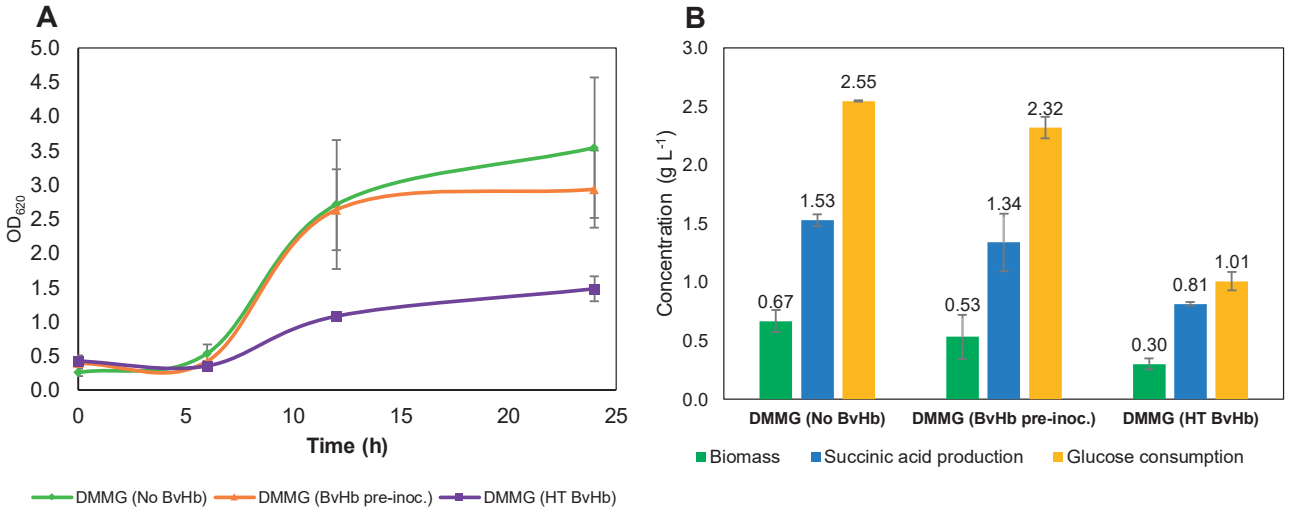

Figure 19. Comparison of the growth of P. copri *in DMMG with BvHb, heat-treated (HT) BvHb, and without BvHb (A) as well as the biomass, succinic acid production and glucose consumption with all three treatments (B).*

Figure 19A shows that the optimal growth in DMMG was achieved when BvHb was omitted, reaching an OD of 3.54±1.03. However, adding BvHb pre-inoculation didn't improve the growth of *P. copri* (maximum OD = 2.94±0.57), yet it was better when compared to the growth with heat-treated (HT) BvHb. Likewise, when comparing the parameters of interest (Figure 19B), the production of biomass and succinic acid were higher when the DMMG wasn't supplemented with BvHb.

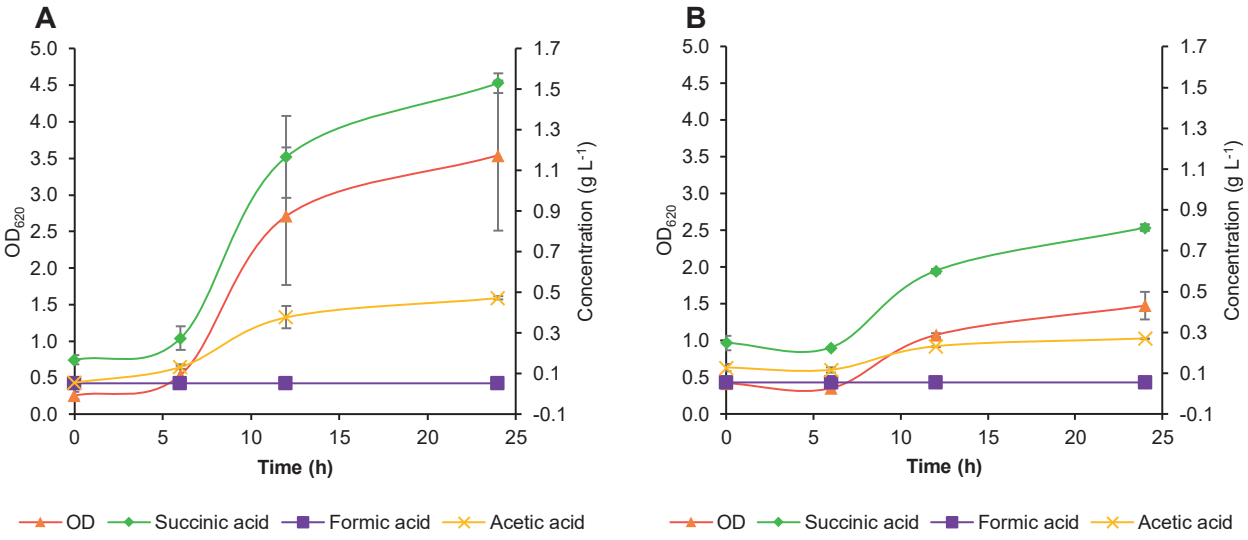

Figure 20. Comparison of the growth and the metabolite profile of P. copri *in DMMG without BvHb (A) and with heat-treated BvHb (B) in DMMG.*

When it comes to metabolite production (Figure 20), it is also evident that the final amount of succinic acid and acetic acid is higher in case of omitting BvHb in DMMG, reaching 1.53±0.05 g/L and 0.47±0.01 g/L, respectively. On the other hand, it seems that there is no production of acetic acid in either cases (with and without BvHb), as the amount detected remains stable through time and therefore it could be residual amounts from the fresh inoculum. Furthermore, there could be a component in DMMG (or lack thereof) that affects the metabolite profile, which is detrimental for formic acid production but favours the production of succinic acid.

When comparing the heat-treated BvHb and BvHb added pre-inoculation, there is a clear difference in terms of growth, biomass and succinic acid production (Figure 19B). According to the instructions of DMSZ for preparing PYG, the culture medium should be autoclaved after adding the hemin solution (DSMZ GmbH, 2020c). While it is true that BvHb consists of a whole protein that contains the hemin moiety, its heat treatment should denature it and release the hemin to the culture medium, as heme is released from haemoglobin when it is heated by cooking.

Myoglobin and soy leghaemoglobin are denatured at 74ºC and 64ºC, respectively (FDA, 2016). However, the autoclaving process takes place at 121ºC, which could lead to the degradation of BvHb's hemin. However, the autoclaving should also degrade the animal-derived hemin that was added to PYG, as the heme moiety is the same for myoglobin and non-symbiotic haemoglobins (FDA, 2016). So either there was a component in the animal-hemin solution that protected it from being degraded during autoclaving, or there was a factor in BvHb that after autoclaving negatively affected the growth of *P. copri*.

The fact that both succinic acid and biomass production are higher in DMMG without BvHb than in DMMG with pre-inoculation BvHb contradicts what has previously been established in the literature. As a heme-auxotrophic bacterium (HAB), *P. copri* requires the addition of hemin to the culture medium since it cannot synthesize its own (Franke and Deppenmeier, 2018; DSMZ GmbH, 2020c). Still, BvHb contains hemin in its structure but might not be as readily available for *P. copri* as when hemin solution is added to the culture medium.

A simpler explanation could be a likely oxygen contamination when adding the BvHb pre-inoculation, which could have negatively affected the growth of *P. copri*. However, this risk was always present as the glucose solution was added pre-inoculation in every experiment. Even though *P. copri* is an obligate anaerobe, it has been demonstrated that under microaerophilic conditions *P. copri* can generate additional ATP thanks to the presence of a cytochrome bd quinol oxidase in its respiratory chain (Figure 1B). For the activation of this cytochrome oxidase, the presence of hemin is required (Gruss *et al*., 2012), so as long as there is hemin in the culture medium, some oxygen contamination could even be beneficial. On the other hand, if the strictly anaerobic conditions are maintained during fermentation, a lack of hemin may not be a problem, which could explain why *P. copri* had an improved growth in DMMG without hemin.

However, similarly to what could have happened with the horse serum free PYG experiment, traces of hemin could have been transferred to the DMMG bottles when inoculating *P. copri* from the fresh inoculum. Ideally, these experiments should be repeated with several serial transfers to make sure there are no traces of hemin. In addition, it is possible that some residual hemin could have been present in the rubber stoppers, since it has been shown that they can retain heme if they have previously been used for experiments with a complex medium (Sperry *et al*., 1977)

Nonetheless, these results are promising for the final scope of developing a defined minimal medium free from animal-derived ingredients. As shown in Figure 21, in this project *P. copri* was

grown in DMMG without hemin, which wasn't achieved in previous studies. The reasons why these results are different require further investigation, especially when it comes to the role of hemin in the DMMG.

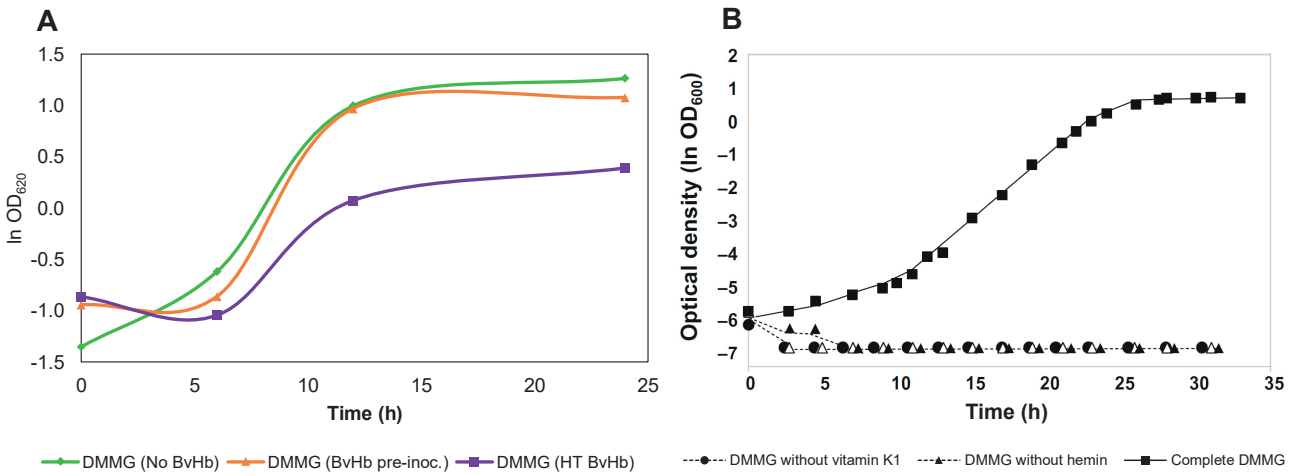

Figure 21. Comparison of the growth of P. copri *in DMMG with BvHb, heat-treated (HT) BvHb, and without BvHb as the natural logarithm of the optical density versus time (A). Growth of* P. copri *in DMMG with and without animal-derived hemin and vitamin K_1 (adapted from Franke and Deppenmeier, 2018)* (B).

5. Conclusion

In this project, *P. copri* was successfully cultivated in a defined minimal medium without supplementation with animal-derived components. While the biomass and succinate production weren't as high as those achieved with the complex medium PYG, this acomplishment was substantially more successful than previous research in terms of growth (Franke and Deppenmeier, 2018). The plant haemoglobin BvHb was added to DMMG instead of commercial hemin, but it had a negative impact on the growth of *P. copri*. Therefore, more research needs to be carried out in order to elucidate the role of hemin in the DMMG.

If the hypothetical fermented product developed by Aventure AB would contain alive *P. copri* as a probiotic, the viability of the bacteria in DMMG should be examined, besides the biomass production.

Last but not least, more research is needed in order to elucidate the molecular mechanisms by which *P. copri* can target or prevent a specific metabolic condition in order to be considered a next generation probiotic.

6. Future perspective

Besides the suggestions mentioned in the conclusions, it would be interesting grow *P. copri* with carbon sources other than glucose, as well as at higher concentrations to see if the biomass production is improved or if it affects the metabolite profile. In addition, according to the final scope of developing a plant-based fermented product, it would be necessary to study the growth of *P. copri* in a complex matrix, for example oat-based. Scaling up to 1 L bioreactor as well as co-culturing *P. copri* with another bacterium that metabolizes succinic acid would also be relevant.

Regarding hemin, it would be interesting to use leghaemoglobin instead of BvHb, and in both cases add a heat treatment step before adding the haemoglobin pre-inoculation, so it can denature and possibly make its heme moiety available in the DMMG. Moreover, adding leghaemoglobin or BvHb to DMMG at different concentrations would help to determine if there is an issue with inhibition or toxicity.

Another significant line of research would be to study the effect of vitamins in the DMMG, as they are heat-treated by autoclaving. Similarly to what was done with BvHb, vitamins could be added prior to inoculation to see if there is an impact in the biomass production or metabolite profile.

Lastly, other methods to determine growth and viability should be tested. While optical density and cell dry weight indicate bacterial proliferation, they do not provide information about the physiology because both dead and viable cells are represented in the experimental measures.

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8. Appendices

Appendix 1: DMMG by Varel and Bryant for the growth of *Bacteroides fragilis*

Table A1. DMMG (Varel and Bryant, 1974)

Component	Percentage
Glucose	0.5 (w/v)
Mineral solution	5 (v/v)
Hemin solution	0.0001 (w/v)
Resazurin solution	0.0001 (w/v)
VFA solution	0.45 (v/v)
B vitamin solution	0.5 (v/v)
$FeSO_4$ x $7H_2O$	0.0004 (w/v)
$(NH_4)_2SO_4$ (6 mM)	5 (v/v)
Casitone (vitamin-free)	0.2 (w/v)
Cysteine-HCl x H_2O (2.5% solution)	2 (v/v)
Na_2CO_3 (8% solution)	5 (v/v)

Table A2. Mineral solution for Varel and Bryant's DMMG.

Component	Mass concentration (per L)
KH_2PO_4	18 g
NaCl	18 g
$CaCl_2$ x $2H_2O$	0.53 g
$MgCl_2$ x $6H_2O$	0.4 g
$MnCl_2$ x $4H_2O$	0.2 g
$CoCl_2$ x $6H_2O$	0.02 g

Table A3. VFA (Volatile Fatty Acid) solution for Varel and Bryant's DMMG.

Component	Volume (per 100 mL)
Acetic acid	36 mL
Isobutyric acid	1.8 mL
n-valeric acid	2 mL
DL-2 methylbutyric acid	2 mL
Isovaleric acid	2 mL

Table A4. B vitamin solution for Varel and Bryant's DMMG.

Component	Mass concentration (per 100 mL)
Thiamin-hydrochloride	20 mg
Calcium-D-pantothenate	20 mg
Nicotinamide	20 mg
Riboflavin	20 mg
Pyridoxine-hydrochloride	20 mg
p-aminobenzoic acid	1 mg
Biotin	0.25 mg
Folic acid	0.25 mg
Vitamin B_{12}	0.1 mg

Appendix 2: DMMG by Sperry *et al*. for the growth of *Bacteroides fragilis*

Table A5. DMMG (Sperry et al.*, 1977)*

Component	Mass concentration (per L)
Glucose	5 g
Na_2CO_3	4 g
$(NH_4)_2SO_4$	1 g
KH_2PO_4	0.9 g
NaCl	0.9 g
Cysteine-HCl x H_2O	0.5 g
$CaCl_2$ x $2H_2O$	26.5 mg
$MgCl_2$ x $6H_2O$	20 mg
$MnCl_2$ x $4H_2O$	10 mg
$FeSO_4$ x $7H_2O$	4 mg
$CoCl_2$ x $6H_2O$	1 mg
Resazurin	1 mg
Vitamin B_{12}	10 µg

The amount of hemin used in their experiments ranges from 0.5 to 100 ng/mL.

Appendix 3: Wolfe's vitamin solution

Table A6. Wolfe's vitamin solution (ATCC, 2016)

Component	Mass concentration (per L)
Pyridoxine HCl	10 mg
Thiamine HCl	5 mg
Riboflavin	5 mg
Nicotinic Acid	5 mg
Calcium pantothenate	5 mg
p-Aminobenzoic acid	5 mg
Thioctic acid	5 mg
Biotin	2 mg
Folic acid	2 mg
Vitamin B_{12}	0.1 mg
Potassium phosphate monobasic	900 mg

The components were weighed and mixed to distilled water and the solution was subsequently filter-sterilised with 0.2 µm polypropylene filters.