Regulation of gelatinase production for Enterococcus faecalis in different growth media

Regulering av gelatinaseproduksjon for Enterococcus faecalis i forskjellige vekstmedier

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1 Forord (Norwegian)

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3 Abstract

3.1 English version

Enterococcus faecalis is a bacterial species colonizing the colon of most mammals. One of its most important virulence factors is gelatinase, which is an enzyme able to hydrolyze various peptides. Earlier research indicates that when *E. faecalis* is grown in MRS there is close to no gelatinase activity, while when it is grown in GM17 the same strains can show gelatinase activity. There were also indications that the presence of skim milk in MRS would induce gelatinase activity (Sainz-Pardo 2011). This thesis has been dedicated to investigating if this holds true for a number of strains, as well as further investigating various factors in the two growth mediums that might influence the gelatinase activity of *E. faecalis,* and analyzing how this activity is affected. Developing a method of measuring gelatinase activity independent of optical density (OD) measurements was also a large part of the thesis.

One key difference between GM17 and MRS is that GM17 does not contain Tween 80. By allowing strains to grow at various concentrations of Tween 80 in GM17, one could observe that the gelatinase activity was negatively affected. It was established that Tween 80 had an effect on the production of gelatinase and not the activity itself.

It was also established that gelatinase biosynthesis activating pheromone (GBAP) produced in one strain grown in GM17 will positively affect the gelatinase activity of another strain when growing in MRS, causing increased gelatinase activity. As such some component(s) in MRS is (are) likely inhibiting the production of GBAP. This was further established by measuring the amount of GBAP produced by using the bioluminescent marker *lux* to quantify its transcription. It was found that barely no GBAP was produced in MRS, whereas significant amounts were produced in GM17. This strongly suggests that it is the GBAP production that is inhibited in MRS, by Tween 80 and possibly by other components, causing the reduced gelatinase production and thus reduced activity.

3.2 Norsk versjon

Enterococcus faecalis er en bakteriart som koloniserer tarmsystemet til de fleste pattedyr. En av de viktigste og mest kjente virulensfaktorene den innehar er gelatinase. Gelatinase er et enzym som hydrolyserer en rekke peptider. Tidligere forskning indikerer at selv om en stamme har høy gelatinase aktivitet i GM17, så har den ingen i MRS. Forskningen viste også tegn til at å tilsette skummet melk i MRS ville indusere gelatinaseaktivitet. (Sainz-Pardo 2011) Denne oppgaven er dedikert til å undersøke om dette stemte for en rekke stammer, i tillegg til å undersøke flere faktorer i de to vekstmediene som kan påvirke gelatinaseaktiviteten i *E. faecalis*, og videre å analysere hvordan dette påvirker aktiviteten. Også utvikling av en metode for å måle aktivitet uavhengig av optiske målinger var en viktig del av oppgaven.

En forskjell på GM17 og MRS er at GM17 ikke inneholder Tween 80. Ved å la ulike stammer vokse i GM17 under ulike konsentrasjoner av Tween 80, kunne man observere at gelatinaseaktiviteten ble påvirket negativt. Det ble også påvist at Tween 80 påvirker produksjonen av gelatinase og ikke selve aktiviteten.

Det ble videre påvist at gelatinase biosntese aktiverende feromon (GBAP) produsert fra en stamme grodd i GM17 kunne gi økt gelatinaseaktivitet for en annen stamme som vokser i MRS. Dette tyder på at det er GBAP-produksjonen som hemmes av komponenter i MRS.

Til slutt ble GBAP-produksjonen til diverse stammer målt ved hjelp av bioluminesens-markøren *lux,* som kunne brukes til å kvantifisere transkripsjonen til *gelE*-promotoren. Ved hjelp av denne fant man at bortimot ingen GBAP ble produsert i MRS, i motsetning til i GM17 hvor signifikante mengder ble produsert. Dette underbygger videre at det er noe i MRS, blant annet Tween 80 som fører til den reduserte gelatinaseproduksjonen og dermed også aktiviteten.

4 Introduction

Enterococcus faecalis is a species of gram-positive lactic acid bacteria native to the gastrointestinal tract common in both humans and animals which is occasionally able to cause various diseases to human beings. It is estimated to be responsible for up to 90% of all enterococcal hospital infections in humans (Kayaoglu & Orstavik 2004). Because of this, and its ability to gain resistance from antibiotics makes it a well known "hospital bacterium". *E. faecalis* infection has usually been treated with vancomycin, but due to increased resistance this is getting problematic (Giridhara Upadhyaya et al. 2009; Murray 1990). Its ability to survive harsh conditions will give it an advantage over other species (Kayaoglu & Orstavik 2004).

E. faecalis can host various virulence factors. Many of these occur in clustered parts of the genome, called pathogenicity islands (Giridhara Upadhyaya et al. 2009). These factors might be responsible for a diverse array of infections. Many infections often start as a urinary tract infection (UTI). This can lead to bacteremia (which can also occur separately from UTI), which again could spread the infection to other parts of the body, and in worst-case cause sepsis. *E. faecalis* responsible for 5-15% of all cases of bacterial endocarditis (Murray 1990). Endocarditis can occur without any prior reason, but is more likely to occur in individuals with drug addictions, heart disease or urinary tract infections (Murray 1990).

Another factor is Enterococcal Surface Protein (ESP), which is a protein associated with the cell wall, which may assist in UTIs, by assisting in adhesion to the bladder epithelium (Giridhara Upadhyaya et al. 2009). ESP is also associated with biofilm formation, and the gene coding for ESP is often found in stool isolates from patients suffering from endocarditis or bacteremia, but are rare in healthy subjects (Kayaoglu & Orstavik 2004).

Another surface protein is Aggregation Substance (AS). This will mediate connection between cells, inducing plasmid transfer in the form of conjugation, and the transfer of other virulence traits (Giridhara Upadhyaya et al. 2009). The presence of AS will also increase the cells ability to adhere to other cells and

extracellular matrix, such as human neutrophils. Adhesion to neutrophils has been shown to make them resistant to killing by neutrophils (Kayaoglu & Orstavik 2004). AS will also cause increased hydrophobicity in cell membranes. Cells with AS also seem to be resistant to phagocytosis, and thus possess increased resistance to the hosts' immune system (Giridhara Upadhyaya et al. 2009; Kayaoglu & Orstavik 2004). There is also speculation to whether AS can play a role in the cells ability to adhere to collagen, and as such promote endocarditis. The production of AS often depends on the uptake of so called "sex pheromones", and these pheromones thus facilitate conjugation (Kayaoglu & Orstavik 2004).

Some virulent strains of *E. faecalis* are also known to produce superoxide anion. This is an oxygen radical known to cause damage on an array of biological materials such as nucleic acids, lipids and proteins (Kayaoglu & Orstavik 2004). Superoxide anions are much more common in virulent strains (associated with bacteremia or endocarditis) than in non-virulent strains. Superoxide producing strains are also known to display increased survivability when grown in competition with other species (Kayaoglu & Orstavik 2004).

Yet another virulent factor of *E. faecalis* is cytolysin. Cytolysin is a unique hemolytic toxin in that it combines the effects of streptolysin S and Iantibiotic bacteriosins. It is able to signal and activate expression of itself, and is lethal to a large variety of cells, including both bacteria and cells of higher eukaryotic organisms (Coburn & Gilmore 2003). The bacteriocin component is active against a variety of gram-positive bacteria. There are indications that because of this activity, strains producing cytolysin will have an advantage over other bacteria, and can thus grow without competition, and more likely cause a more serious infection. This has been demonstrated *in vitro* (Coburn & Gilmore 2003). In higher organisms the cytolycin will attack the red blood cells and cause haemolysis, as well as other cells like neural tissue cells. This toxin is not specific against any certain species, and will also kill nematodes (Coburn & Gilmore 2003).

Antibiotic resistance can in itself be considered a virulent trait, as it will give strains possessing the resistance an advantage over other species when antibiotic treatment occurs, and thus enhance other virulent traits. This is also a

problem since so far, it is the only treatment for enterococcal infections (Alper ÇIFTCI 2009). Because of efficient use of horizontal gene transfer *in E. faecalis*, any advantageous mutations leading to resistance may easily be spread to other *E. faecalis* strains (Arciola et al. 2008; Murray 1990). Horizontal gene transfer has proven to be involved in dissemination of resistance to antibiotics like chloramphenicol, clindamycin, erythromycin, tetracycline, streptomycin and gentamicin (Murray 1990).

Virulent strains of *E. faecalis* will protect themself against antibiotics in various ways. Some can produce beta-lactamase, which can hydrolyze certain antibiotics, like penicillin, ampicillin and piperacillin. Protection against vancomycin is associated with an inducible cytoplasmic membrane-associated protein (Murray 1990). Because of this increased resistance, a combination of antibiotics is usually used when combating enterococcal infection in patients. Sometimes, due to some individuals surviving the first treatment, a second infection can occur, called a superinfection. As such, a different combination of antibiotics needs to be used for this second treatment. The strategy against *E. faecalis* infections so far has been to search for new antibiotics, but this will probably get increasingly difficult (Coburn & Gilmore 2003; Murray 1990; Upadhyaya et al. 2011). As vancomycin resistance is becoming increasingly common, the preferred antibiotic against *E. faecalis* is usually ampicillin, if the strain is susceptible. If not, combinations of ceftriaxone, oritavancin, gentamicin, fosfomycin, tigecycline and moxifloaxin have proved to be effective (Arias et al. 2010).

Production of biofilm can also be considered an antibiotic resistant trait, as it will physically prevent antibiotics and other defensive mechanisms from reaching the cells. It is also a virulent factor separately from this, in that it allows the producing strains to grow more efficiently. The production of biofilms also seems to be associated with expression of the *esp* gene, which encodes the enterococcal surface protein. However, there are likely other factors also influencing the production of biofilm (Arciola et al. 2008; Upadhyaya et al. 2011).

4.1 Gelatinase

One of the most studied and important virulence factors in the *E. faecalis* is gelatinase, which is the focus of this thesis (Gaspar et al. 2009). It is a metalloproteinase containing zinc, which can hydrolyze casein, hemoglobin, insulin, fibrinogen, collagen, gelatin, as well as various proteins/peptides (Giridhara Upadhyaya et al. 2009; Makinen et al. 1989). Gelatinase is not a specific protease to *E. faecalis,* and can be produced by a lot of different eukaryotic cells in in addition to bacteria. As such, gelatinase functions not just as a virulent factor, but is also part of physiological functions like altering and constructing tissue (Kayaoglu & Orstavik 2004).

Gelatinase is encoded by *gelE,* and experiments on the larvae of the moth species *Galleria mellonella* have shown that removal of this gene by molecular methods will significantly reduce its virulence (Gaspar et al. 2009).

Production of gelatinase in *E. faecalis* is controlled by the *fsrABCD* operon (*Enterococcus Faecali Regulator*), which controls production of *gelE* (gelatinase) in a quorum sensing regulation system. This pathway is depicted in in Figure 1. The expression of the fsr system and its regulon is regulated by the presence of extracellular gelatinase biosynthesis-activating pheromone (GBAP) (Teixeira et al. 2012), which is a cyclic peptide that consist of 11 amino acids, as shown in Figure 1. GBAP is encoded by *fsrD,* and processed be FsrB to the active GBAP and transport to the outside of the cell (Nishiguchi et al. 2009). When GBAP is at a sufficiently high concentration on the outside of the cell, it will phosphorylate the histidine kinase FsrC, which in turn will phosphorylate and activate FsrA, which is a response regulator that will activate the transcription of the genes in the Fsr regulon, including FsrBCD, causing more GBAP to be produced and the gelE operon to be transcribed (Ma et al. 2011). As such, a minimum of GBAP has to be built up outside the cell membrane before gelatinase can be produced (Galloway-Pena et al. 2011; Murray 1990; Nishiguchi et al. 2009; Teixeira et al. 2012). Some strains lack gelatinase production even if the *gelE* gene is present. In most of these cases, the strains lack the *fsrB* gene. This indicates that both genes work together to produce gelatinase (Galloway-Pena et al. 2011). There are also indications that the *fsr* system regulates other virulence factors in *E. faecalis*, like biofilm formation (Nakayama et al. 2006).

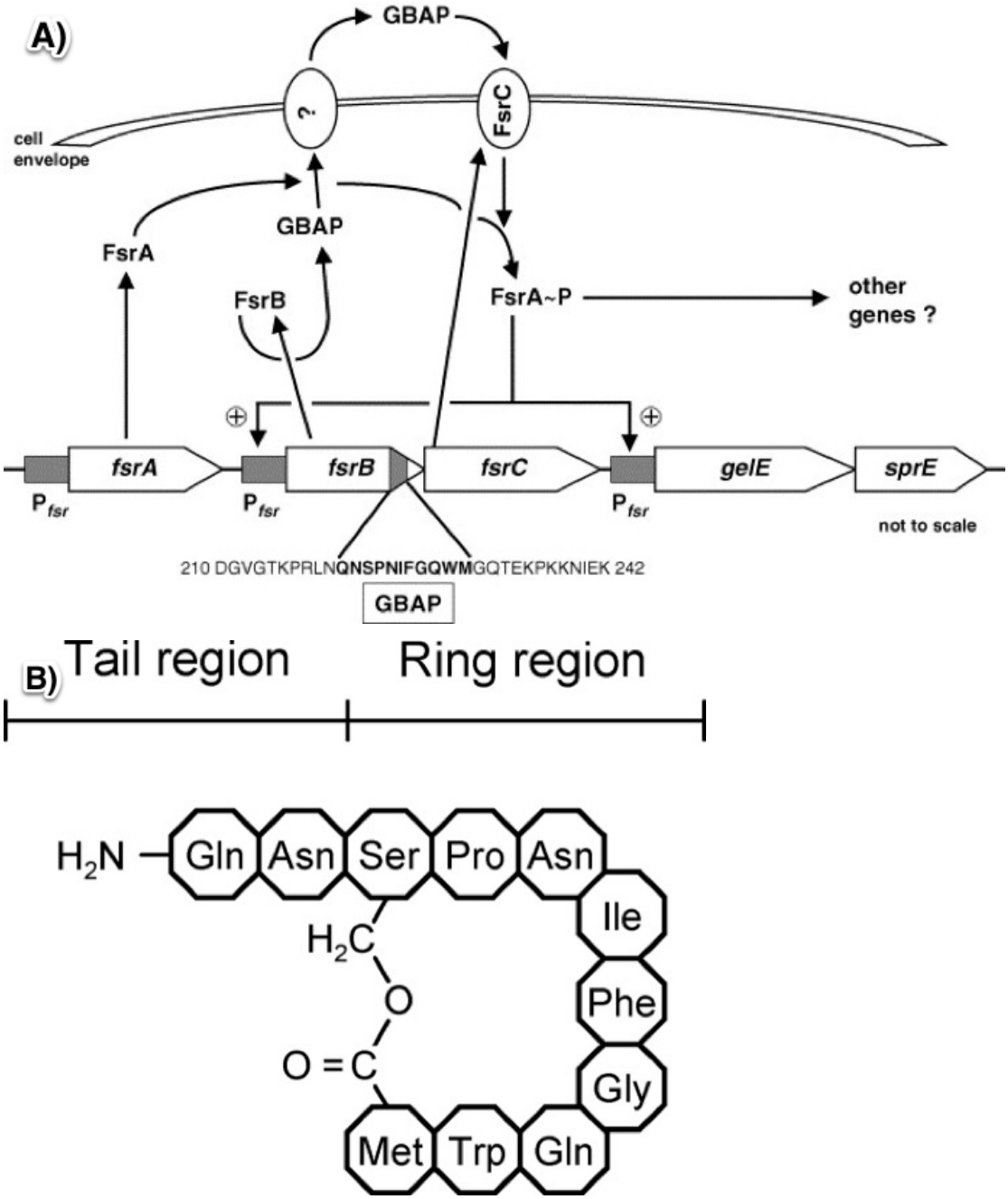

Figure 1: A) Regulation network for the *fsrABC* operon through GBAP, leading to *gelE*, which produces gelatinase in *E. faecalis*. (Podbielski & Kreikemeyer 2004) B) The structure of the Gelatinase Biosynthesis-activating pheromone (GBAP) (Nishiguchi et al. 2009)

4.2 Aim of the study

Geletinase activity in *E. faecalis* was found to be unstable in certain growth conditions. It was active in the GM17 growth medium but not in MRS, another type of growth medium. However, the gelatinase activity was recovered in MRS if skim milk was added. The aim of this thesis is to identify the factors that regulate the gelatinase production in the different growth conditions. In order to investigate this phenomenon further, a series of experiments were performed. Initially an analysis was performed showing how the same strain will yield different gelatinase activities when grown in the two different growth mediums. Following this, a series of experiments involving adding various factors to the two media to analyze how these factors influence the gelatinase production and/or activity.

5 Materials

5.1 Bacterial strains

Table 1: Bacterial strains used in this thesis

Bacterial Strain	Description	Source/Reference
LMGT 3563	*E. faecalis* Fly 1	
LMGT 3564	*E. faecalis* HIP11704 *(gelatinase negative)*	
LMGT 3567	*E. faecalis* T1	SS498
LMGT 3569	*E. faecalis* T3	Sapporo-109
DBH18	Gelatinase positive strain of *E. faecalis*	Loreto Gutiez Sainz-Pardo
MS182	V583 with pAT28[1)]	Margrete Solheim, unpublished
MS232	V583 with pAT28[1)] and overexpression of GBAP	Margrete Solheim, unpublished
MS234	V583 with *gelE* knocked out + and overexpression of GBAP + pAT28[1)]	Margrete Solheim, unpublished
MS253	OU510 with pAT28[1)]	Margrete Solheim, unpublished
MS275	OU510 with lux expression dependant on GBAP	Margrete Solheim, unpublished
S75	*E. faecalis* with independent lux expression	(La Rosa et al. 2012)
OU510	Pheromon negative, gelatinase negative strain *E. faecalis*	(Nakayama et al. 2006)
V583	First verified vancomycin restintant strain of *E. faecalis* in the US	(Sahm et al. 1989)

1) Plasmid with Spectinomycin resistance.

5.2 Growth Media

5.2.1 GM17

GM17 was made by mixing M17 broth with dH_2O, making a concentration of 37.25 g/l, before autoclaving at 121 °C for 15 minutes. Glucose was added to a final concentration of 0.4 % after autoclavation. For making plates, 15 g/l agar was added before autoclavation. 2x GM17 was made by doubling the M17 concentration to 74.5 g/l and the glucose concentration to 0.8 %. GM17 containing 10% Tween 80 was made by substituting 10% of the dH_2O with Tween 80.

5.2.2 M. R. S. (de man, Rogosa Sharpe)

MRS was made by mixing MRS broth with dH_2O, making a concentration of 52 g/l, before autoclaving at 121 °C for 15 minutes. For making plates, 15 g/l agar was added before autoclavation. 2x MRS was made by doubling the MRS concentration to 104 g/l.

5.2.3 Skim Milk

Skim milk was made by mixing Skim Milk Powder with dH_2O, making a concentration of 10%, before autoclaving at 121 °C for 5 minutes.

5.2.4 Todd-Hewitt Broth

TH was made by mixing Todd-Hewitt broth with dH_2O, making a concentration of 36.4 g/l, before autoclaving at 121 °C for 15 minutes. For making plates, 15 g/l agar was added before autoclavation. For TH plates containing skim milk, 15% of the dH_2O was substituted for 10 % Skim Milk, which was added after autoclavation.

5.3 Chemicals

Table 2: Chemicals used in the experiments

Chemical	Supplier
Agar	Merck
Ampicillin	
Chloramphenicol	Sigma
Erythromycin	Sigma
Ethanol 96% vol (GPR Rectapur)	VWR
Glucose	
Glycerol 85% (Emsure)	Merck
Hide-Remazol Brilliant Blue R	Sigma
Hydrocloric Acid	
M. R. S. Broth	Oxoid
M17 Broth	Oxoid
Skim Milk Powder	Oxoid
Spectinomycin	
Tetracycline	Sigma
Todd-Hewitt Broth	Oxoid
Trisma Base	
Tween 80	Sigma

5.4 Technincal Equipment

Table 3: Equipment used for the experiments.

Equipment	Model	Supplier
0.45 µm Sterile filter	Filtropur S 0.45	Millipore
96 well plate, Black	Nunc	Thermo Scientific
Autoclave	SC 500	Matachana
Automatic multichannel pipette	Finnipipette 50-300 µl	Labsystems
Automatic multichannel pipette	Finnipipette 50-300 µl	Thermo electronic corporation
Automatic Pipette	Physiocare Concept 0.5-10 µl	Eppendorf Research
Automatic Pipette	Physiocare Concept 10-100 µl	Eppendorf Research
Automatic Pipette	Physiocare Concept 20-100 µl	Eppendorf Research
Automatic Pipette	Physiocare Concept 100-1000 µl	Eppendorf Research
Automatic pipette	Finnipipette 0.5-5 ml	Thermo Scientific
Automatic pipette	Finnipipette 1-5 ml	Labsystems
Bioluminescent Imaging System	IVIS Lumina II	Perkin Elmer
Cell density meter for test tubes	Ultrospec 10	Amersham Biosciences
Centrifuge	Biofuge fresco	Heraesus
Cryogenic Vials	Nalgene Cryware 1.8 ml	Thermo Scientific
Disposable gloves	Nitrile XL Powder Free	VWR
Electronic pipette	Pipetus-akku	Hirschmann Laborgeräte
Electroscale	Mettler PM4600	Deltarange
Electroscale	XF-3200	Salter
Eppendorf tubes		
Fireboy	Plus V03	Integra
Fireboy	Eco	Tecnomara AG
Freezer	- 86 °C	Forma Scientific
Freezer	- 20 °C	Robo
Fridge	ER8892C 370 L	Electrolux
Glass flask	500 ml	Schott Duran
Glass flask	250 ml	Schott Duran
Glass flask	100 ml	Schott Duran
Glass test tubes	5 ml	Schott Duran
Glass test tubes	5 ml	
Ice Maker	KF85	Porkka
Inoculation loops	10 µl blue	Sarstedt
Inoculation loops	1 µl white	Sarstedt
Magnetic Stirrer	MR 1000	Heidolph

Magnetic Stirrer	MR 3001	Heidolph
Magnetic Stirrer	Ikamag RH	Janke & Kunkel IKA Labortechnik
Measuring Cylinder	100 ml	Brand Eterna
Measuring Cylinder	250 ml	Brand Eterna
Minishaker	MS2	IKA
Minishaker	Autovortex Mixer	Stuart
Minishaker	Vortex Genie 2	Scientific Industries
Nanodrop Spectrophotometer	ND-1000	Saveen Werner
Pietri Dish	9 cm	Heger AS
Pipette tips	For automatic pipettes supporting volumes up to 100-200 µl	VWR
Pipette tips	For automatic pipettes supporting volumes up to 1000 µl	VWR
Pipette tips	For automatic pipettes supporting volumes up to 10 µl	WVR
Pipette tips	Finntip: For automatic pipettes supporting volumes up to 5 ml	Thermo Scientific
Plastic centrifuge tube	50 ml Cellstar Tubes	Greiner bio-one
Plastic centrifuge tube	100 ml Cellstar Tubes	Greiner bio-one
Serological pipette	25 ml	Sarstedt
Spectrophotometer	UV-160	Shimadzu
Square pietri dish	100 mm	Sterilin
Sterile Bench		KEBO Production
Sterile Bench	TL2472	Holten
Stirring Magnet		
Test Tube Heater	SHT1	Stuart Scientific
Test tube Rack	6 X 12 tubes	Nalgene
Water Bath	U3	Julabo 6A
Water filter	Elix	Millipore
Water filter	Milli-Q	Millipore

6 Methods

A series of strains of *E. faecalis* was selected and used in many of these assays. The strains were selected based on their susceptibility against the antibiotics in the antibiotic milk plates (described on page 18) as well as their gelatinase activity and the presence of the *gelE* gene in their genome. Also, a strain without *gelE* and no observable gelatinase activity (LMG3564) was used in most of the assays as a negative control.

In addition to checking how milk affects the gelatinase activity, the difference in composition between GM17 and MRS has been observed, and the fact that there is a presence of Tween 80 in MRS but not in GM17 was noted.

To find if pre produced GBAP can induce gelatinase activity in MRS for *E. faecalis,* an experiment where this was done was performed.

Also, the difference in GBAP production in MRS and GM17 was measured in a final experiment.

6.1 Phenotypic and genotypic characterization of test strains.

Multiple strains of *E. faecalis*, as well as a few strains of *Enterococcus faecium* had to be tested for gelatinase activity and resistance against antibiotics. A BLAST (Basic Local Alignment Search Tool) search for their genome against the nucleotide sequence for *gelE* was also done, to ensure that the observed activity was a product of gelatinase. A simple assay was done to ensure this.

Each strain was grown in 5 ml GM17 overnight (18 hours) to ensure a fresh culture. Following this, 10 µl of each strain was applied to a pietri dish containing 1.5% milk, 1.5% agar and TH medium, before being incubated for 48 hours at 37 °C. The presence of gelatinase activity was then observed by seeing a clear zone in the milk around the colonies.

Each strain was also applied to each of a pietri dish containing 1.5% GM17 medium and 20 µg/ml Tetracycline, Erythromycin, Chloramphenicol or Ampicillin, to identify their resistance to these antibiotics. Finally, the genome for each strain was aligned against the nucleotide for *gelE* using BLAST. (Altschul et al. 1990)

6.2 Antibiotic and skim milk containing plates (ASM)

These ASM plates were developed for this thesis, and used in most of the assays in this thesis, to measure gelatinase activity. Inclusion of antibiotics was to prevent further growth.

They plates were composed of a mixture of 1.5% agar, 0.01M Tris HCl, 1.5% milk, 20 µg/ml Tetracycline, 20 µg/ml Chloramphenicol and 20 µg/ml Ampicillin. These antibiotics were chosen as they were found to be effective against the strains of *E. faecalis* which were used in most of the assays in this thesis. Room tempered skim milk and antibiotics were added after autoclavation. About 80 ml of this agar broth was poured into each plate. After the plate had solidified, wells in the agar were made by pushing a 4mm wide sterilized metal cylinder into the broth. Suction in the cylinder led to the cut out agar piece being picked up, forming a circular well in the agar. 25 µl soft agar of the same composition was deposited in each well, to prevent the sample from leaking under the agar.

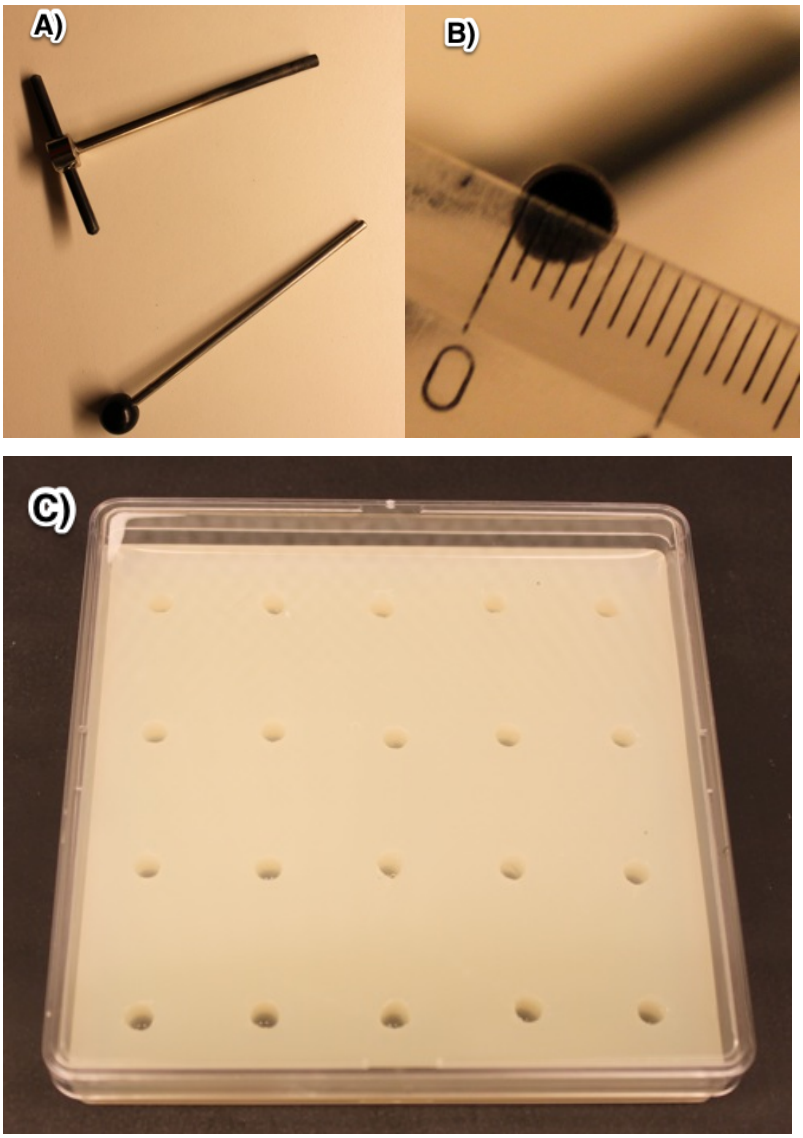

Figure 2: A) Tool used for creating wells in the agar. The upper part is hollow, and the lower part is used to push out agar from inside the hollow part. B) Demonstration of the width of the hollow part. C) Finished antibiotic milk plate with 5x4 wells.

6.3 MRS VS GM17

One of the main discoveries influencing my thesis were indications that gelatinase positive strains of *E. faecalis* that would show gelatinase activity in GM17, did not show this activity in MRS. There does not seem to exist much prior research on the subject of milk inducing gelatinase activity for enterococci. To verify that this held true for multiple strains, the following experiment was conducted.

The gelatinase positive strains were grown in GM17 overnight at 37 °C (17 hours) to ensure fresh cultures prior to the experiment. One tube of fresh MRS and one tube of fresh GM17 was inoculated (1% of total volume, 50 µl) per strain, and set to incubate at 37 °C for 9 hours. After incubation 70 µl from each sample was deposited to a separate well in an ASM plate. The plates were incubated for 48 hours at 37°C, before the gelatinase ativity was read as clear zones in the milk in the agar. This could be measured with a ruler.

6.4 Induction of Gelatinase activity in MRS by skim milk

To investigate if milk could induce gelatinase activity, the following assy was performed.

Bacterial strains to be tested were grown overnight in MRS (18 hours). Following this, a series of 5 ml tubes was prepared for each strain, with the following medium composition: one with GM17, one with 2,5 ml water and 2,5 ml 2x MRS and one with 2,5 ml 10% milk and 2,5 ml 2x MRS. 2x MRS was used to account for the dilution that occurs when milk is added. One of each of these tubes were inoculated with 1% (50 µl) of the strain to be tested, before being incubated at 37 °C. 100 µl was taken out at several time points during the incubation and spread on a GM17 agar plates to measure growth. After 9 hours, 70 µl from each sample was added to wells in preproduced antibiotic milk plates. These plates was then incubated for 48 hours at 37°C, before the gelatinase activity was read as clear zones in the milk in the agar. This could be done by measuring with a ruler.

6.5 Inhibiting gelatinase activity in GM17 with Tween 80

6.5.1 Inhibiting production/activity of gelatinase with Tween 80

A GM17 broth containing 10% Tween 80 was made by substituting 10% of the water required for the broth with Tween 80 prior to autoclavation. After autoclavation 0.4% glucose was added as normal for GM17.

The GM17 broth containing Tween 80 was added at various concentrations to GM17, making 5 ml tubes of GM17 containing 0%, 0.1%, 0.2% 0.4%, 0.8%, 1.6%, 3.2% and 5% Tween 80.

Fresh overnight cultures of the strains to be tested were added at 1% inoculation (50 µl) to the tubes of GM17 with various concentrations of Tween 80 added.

The growth of the strains was measured at 3 and 5 hours spectroscopically at 620 nm. After 9 hours, 70 µl of the growing cultures were added to wells in antibiotic milk plates. After 48 hours the results were read by measuring clear zones in the milk with a ruler.

6.5.2 Inhibiting activity of gelatinase with Tween 80

A different assay was used to analyze if Tween 80 could affect the production of gelatinase or its function. Fresh overnight cultures of the strains were inoculated at 1% to GM17, and grown at 37°C for 9 hours. Following this, they were centrifuged at 13.000 rpm 4°C for 5 minutes. The resulting supernatants were filtered through 45 nm filters. 250 µl of the filtered supernatants were added to Eppendorf tubes containing 250 µl GM17 with various concentrations of Tween 80 to final concentrations of Tween 80 of 0%, 0.1%, 0.2% 0.4%, 0.8%, 1.6%, 3.2% and 5%. This was incubated at room temperature for 30 minutes, before 70 µl from each was added to wells in ASM plates. 70 µl from DBH18 growing in GM17 was included as a positive control. Plates were incubated for 48 hours at 37°C, before the gelatinase activity was read as clear zones in the milk in the agar. This could be measured with a ruler.

6.6 Activating gelatinase with pheromone (GBAP)

Because of how gelatinase is produced through the *fsrABCD* operon through the pheromone GBAP (see page 9), an experiment to see if adding GBAP to the growth medium would affect activity was performed.

Two cloned strains of V583 (MS232 and MS234) that carry a plasmid-encoded copy of *fsrD* preceded by the strong, constitutively expressed promoter p11 (pAT28p11*fsrD*) for over production of the pheromone GBAP was used for this. pAT28p11*fsrD* had been cloned into wild type V583 (MS232 and MS234) and a *gelE* deletion mutant constructed in a V583 background (MS234).

Spectinomycin was consequently added to the growing cultures at a concentration of 500 μg/ml to stabilize the plasmid. Strain MS232 was used as a negative control in these experiments. MS253 has a point mutation in *fsrD* which causes the loss of GBAP production and leads to the gelatinase-negative phenotype. In addition, the strain harbors an empty pAT28 plasmid. In these experiments, filtered supernatants from MS232, MS234 and MS253 were grown overnight (18h, 37°C) in GM17 with spectinomycin (500 μg/ml). Then they were reinoculated to fresh medium and grown to OD 1.0 (~ 4 hours for MS232 and MS234, ~ 6 hours for MS253). Following this, MS232, MS234 and MS53 were spun down and their supernatants sterile filtered through 45 nm filters. These supernatants were added in increasing (0% 10%, 20% and 40%) concentrations to tubes with MRS media and spectinomycin, before they were inoculated with 1% spectinomycin resistant, gelatinase positive strains of *E. faecalis* (MS182 and DBH18). After 9 hours at 37°C, 70 μl from each sample was added to wells in ASM plates. Plates were incubated for 48 hours at 37°C, before the gelatinase activity was read as clear zones in the milk in the agar. This could be measured with a ruler.

As controls, supernatants from overproducers of GBAP with and without gelatinase production were utilized, as well as supernatant from a strain with no production. Pure supernatant from the producers were also tested for gelatinase activity.

6.7 Quantifying the presence of pheromone GBAP by induction of *lux*

To find if the difference in gelatinase activity was due to inhibitions in GBAP productions or other factors, an assay measuring the GBAP produced in different conditions was constructed by using the luxABCDE reporter system (La Rosa et al. 2012). Strain MS275 is a gelatinase negative OU510 clone which has the *lux* operon connected to the *gelE* promoter from V583. This would cause bioluminescence whenever this promoter was active. As described in the introduction (section 4), this GBAP will activate this promoter. As OU510 does not produce GBAP itself, the *gelE* promoter of MS275 will only be active when GBAP is present, allowing for indirect quantification of GBAP.

The assay was done by sterile filtering the strains grown in both MRS and GM17 overnight and adding these at different dilutions to 96 well plates. Following this, an 100 times diluted overnight culture of the indicator strain (MS275) with the reporter system was allowed to grow in the presence of these supernatants, which would emit bioluminescence if GBAP was present. Table 4 shows contents of each well in the plate. Strains LMG3564 and MS253 are gelatinase negative and included as negative controls. In column 12, various concentrations of MRS and GM17 was used with another indicator strain, S75, which is another *E. faecalis* strain with the lux construct cloned in independent of *gelE,* allowing it to produce bioluminescence independently of GBAP. This was included as a positive control, and to analyze if MRS in the medium would affect bioluminescence. The plate with filtered diluted supernatant, fresh GM17 and 100x diluted indicator (MS275 for the samples, S75 for the positive control) would then be incubated for ten hours, with readings being made every 15 minutes with 4 minutes of exposure. The data was collected as photons per second.

Table 4: Schematic of the contents of wells in a 96 well plate for experiment 6.7.

Dilution	V583	DBH18	3564	3567	3563	3569	MS253	MS182	MS232	MS234		S75
4X	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12: 100% GM17
8X	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12: 75% GM17
16X	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12: 50% GM17
32X	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12: 0% GM17
64X	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12
128X	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12
256X	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12
0% SN	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12

7 Results

7.1 Optimalization of the gelatinase assay

A significant part of the time allotted to this thesis has been dedicated to developing methods of how to measure gelatinase activity and cell growth without using spectrophotometric readings. As mentioned, part of the thesis is about how milk affects the gelatinase activity of *E. faecalis* in MRS, and milk will interfere with OD measurements.

First, spectrophotometric methods were to analyze both gelatinase activity and growth. Growth would be measured at 620 nm, and gelatinase activity would be read at 595 nm. However, due to the fact that the growth mediums contained various concentrations of milk, the turbidity of the milk would scatter the light and interfere with the readings. Because of this fact, spectrophotometric growth measurements had to be replaced by direct plate counts over time. The original assay for measuring gelatinase activity was based upon taking out samples from the growing cultures at regular time points, and then centrifuging (10 min, 8000 rpm at 4 °C) and sterile filtering the supernatants (45 μm filters), and incubating for two hours while agitating at 37°C with Hide-Remazol Brilliant Blue R in 0.1M Tris HCl at 7.4 pH. This compound gives a bright blue color when hydrolyzed by gelatinase, and the activity can be read at 595 nm. However, this assay did not produce any usable results due to the skim milk interfering with the spectrophotometric readings.

A different approach was then undertaken. In this approach, the filtered supernatants were directly applied into wells of skim milk agar plates (1.5% milk, TH medium as the milk turned quickly blank in MRS and GM17 plates), and clear zones were observed around the wells containing samples with gelatinase activity. This assay was further improved upon addition of antibiotics to the plates. Antibiotics in the growth agar permitted application of living bacteria directly to the wells without need for filtersterilisation. To completely prevent growth, antibiotics were also added in the soft agar at the bottom of the wells. Finally, exchanging the growth medium in the plates with 0.01M Tris HCl buffer at pH 7.4 was a final improvement to further prevent growth on the plates and to

reduce the economic cost of the assay. The production of these plates is described in the Materials and Methods section. Through developing these plates, it was found that for most of the *E. faecalis* strains, and under most conditions, the time of growth of bacteria for maximum gelatinase activity was around 9 hours after a 1% inoculation at 37 °C. Because of that, the measurements of gelatinase activity are taken at 9 hours in this thesis.

7.2 Strain properties

Table 5: Data about the strains used in this thesis.

Strain[1]	Max ident[2]	Gelatinase activity[3]	Growth on Tet[4]	Growth on Ery[4]	Growth on CAM[4]	Growth on Amp[4]
3557	100%	+	+	-	-	(-)
3562	100%	+	+	+	-	(-)
3563	100%	+	+	+	-	+
3564	-	-	-	+	-	(-)
3565	100%	+	+	+	-	-
3566	100%	+	+	+	-	(-)
3567	100%	+	-	-	-	-
3569	100%	+	+	(+)	-	(+)
3571	100%	+	(+)	(+)	-	(-)
3572	99%	+				
3573	-	+	+	+	-	(-)
3574	-	-	+	+	-	+
3576	-	+	-	(-)	(+)	(-)
V583	100%	+	-	+	-	(-)
DBH18	100%	+	-	+	+	(-)

1) Denotes the name of the strain. 2) Denotes how well the strains genome aligns against the nucleotide sequence of *gelE from strain V583* in BLAST (Altschul et al. 1990) 3) Denotes wether or not any gelatinase can be observed clear zone around colonies on plates containing skim milk. 4) denotes if the strain grows on 20 µg/ml of Tetracycline, Erythromycine, Chloramphenicol and Ampicillin. + Indicates that it grows well, (+) indicates that it has reduced growth, (-) indicates that it barely grows at all, and – indicates no growth.

Based on the findings in Table 5, the antibiotics which to use in the ASM plates could be selected, as well as the strains to use for the various assays. Some strains have gelatinase-like activity but do not have the *gelE* gene. These strains are not *E. faecalis,* but rather *Enterococcus faecium*, which has a different mechanism to hydrolyze the milk.

7.3 MRS VS GM17

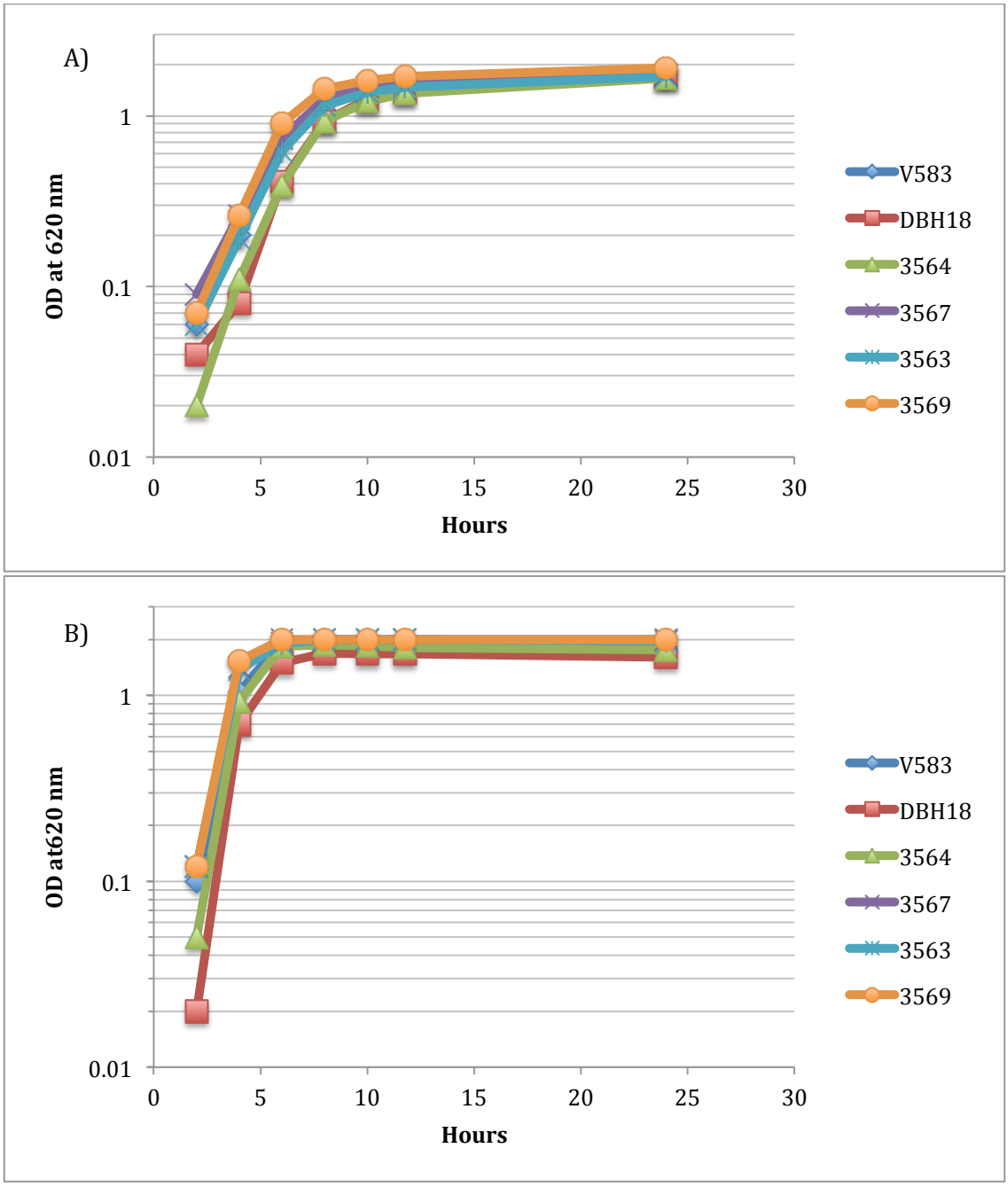

Figure 3: Growth of various strains of *E. faecalis* grown in MRS (A) and in GM17 (B), measured by OD at 620 nm.

Figure 3 shows that the growth is faster in GM17 than in MRS. Strains growing in MRS do however reach the similar OD levels over time. However, the fact that the growth is faster in GM17 than in MRS is likely not the reason why the gelatinase activity in MRS is lower. At the time of gelatinase activity measurement (after 9 hours), the cell density of the strains in MRS are similar to the cell density in GM17 and they are likely in the stationary growth phase.

Figure 4 shows that in MRS, most strains, even gelatinase negative strains (3564) have a clearance of around 6 mm. This indicates that in MRS, there is some components (probably proteases) that causes hydrolyzation of the milk. This was also found when developing the assay and ASM, as when the plates were made with milk and MRS, the milk would usually go blank overnight. For this reason, over 6 mm clear zone in the ASM plates is needed to verify any gelatinase activity for strains grown in MRS. With this in mind, no viable gelatinase activity can be observed for any of the test strains when grown in MRS in Figure 4.

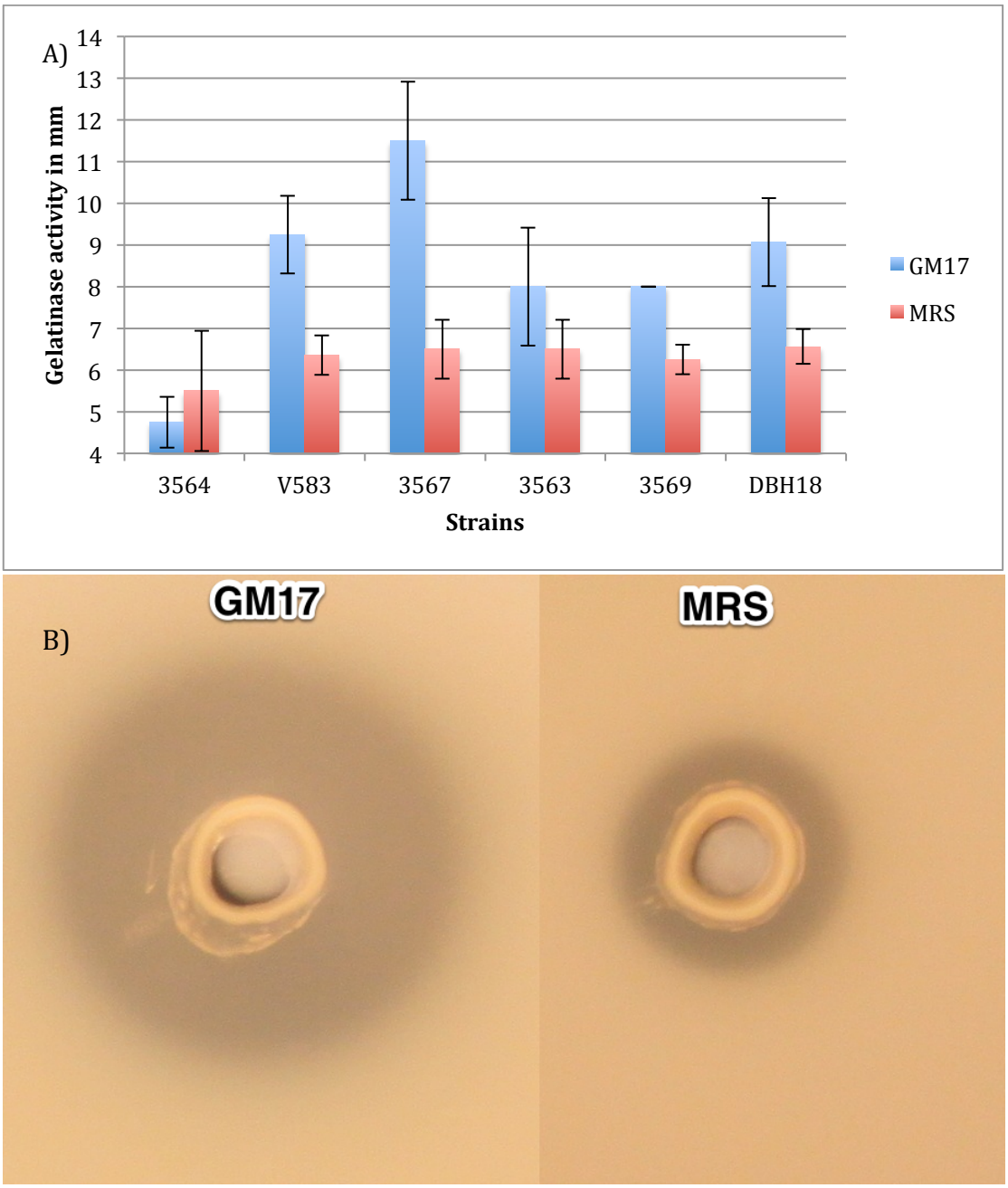

Figure 4: A) Gelatinase activity for various strains of *E. faecalis* in GM17 and MRS. A diameter of 6 mm is the minimum zone of detecting gelatinase activity for strains grown in MRS. B) Gelatinase activity for strain 3563 in GM17 (left) and in MRS (right), after being incubated at 37 °C for 9 hours. The white in the center is not growth, but the residue of dead cells.

7.4 Induction of Gelatinase activity in MRS by milk

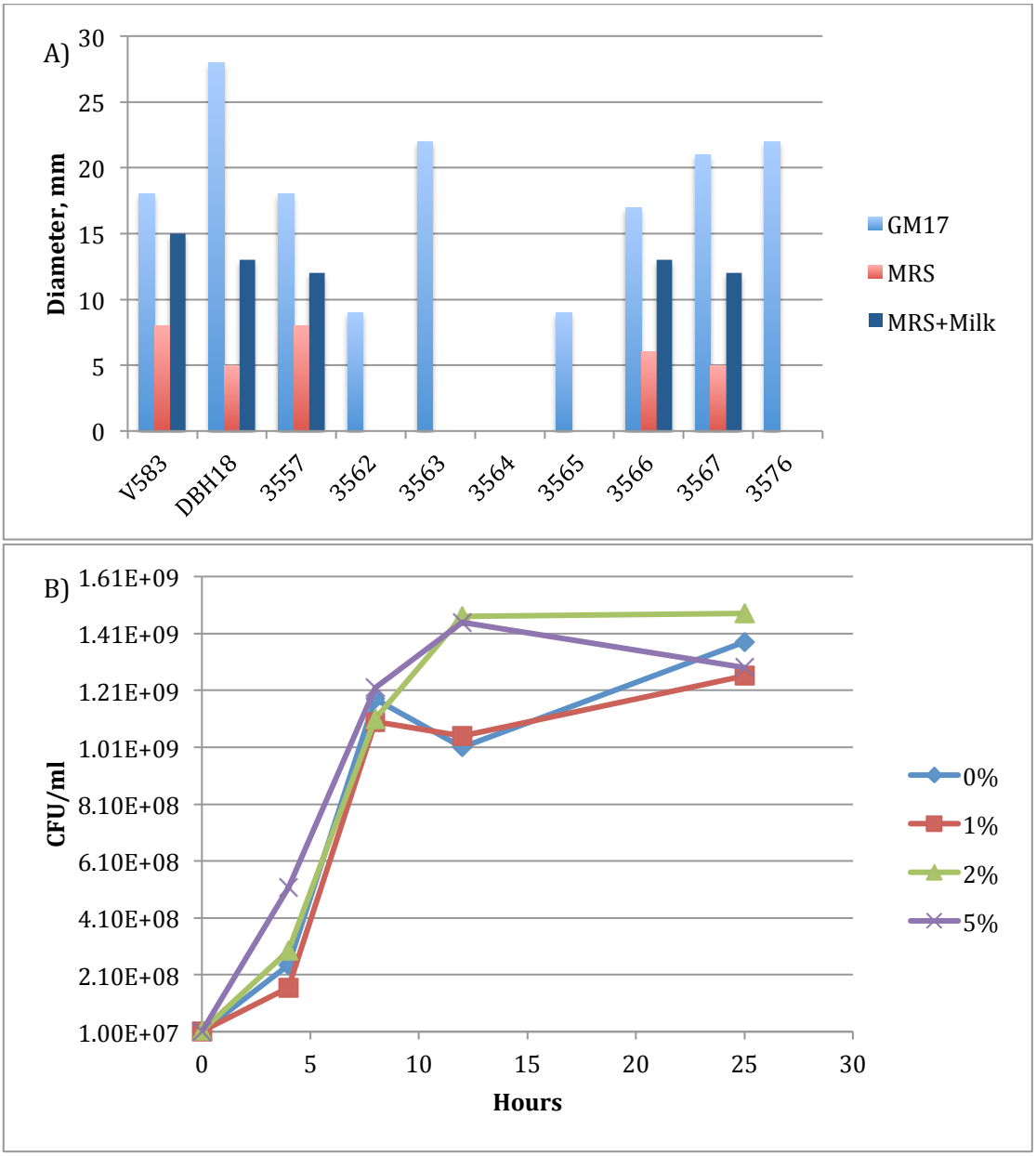

Figure 5: A) Gelatinase activity of various strains of *E. faecalis* in GM17, MRS and MRS with 5 % skim milk. B) The effect of skim milk (at 0, 1, 2, and 5%) added to MRS on the growth of strain DBH18.

When looking at how milk affects the gelatinase activity of *E. faecalis,* it is shown in Figure 5 A) all my strains have their highest gelatinase activity in GM17, less activity in MRS + 5% Skim milk and lowest activity in MRS. Some ingredients of MRS apparently inhibits gelatinase activity, while milk seems to induce gelatinase activity in MRS. How milk affects the growth of DBH18 is shown in Figure 5 B, and there does not seem to be a clear correlation between the growth and the addition of milk.

7.5 Tween as an inhibitor

As there was significantly lower gelatinase activity and bacterial growth in MRS compared to GM17 (Figure 4), the difference between the two media needed to be investigated. One key difference between GM17 and MRS is that MRS contains 0.1% Tween 80, whereas GM17 does not. Tween 80 (typical molecular structure shown in Figure 6), also known as polysorbate 80, is a hydrophilic non-ionic surfactant and is often used as an emulsifier. (Aizawa 2010) It is added in MRS to enhance the growth of lactic acid bacteria, many of which will convert Tween 80 to cyclopopane fatty acids and incorporate it into their membrane. (Partanen et al. 2001). Tween 80 is a detergent which might cause denaturation, and seems to be an obvious compound that might interfere with the production or the activity of gelatinase. Experiments on how addition of Tween 80 to GM17 would affect gelatinase were conducted.

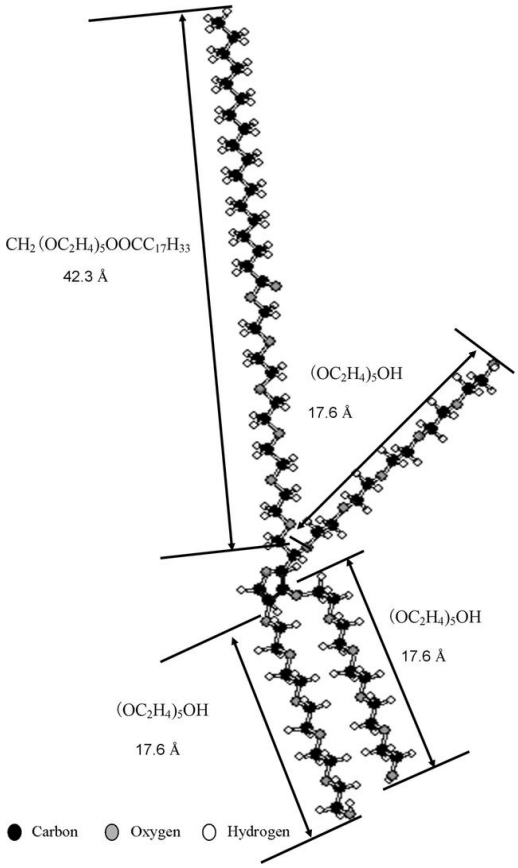

Figure 6: Molecular structure of Tween 80 (Aizawa 2010)

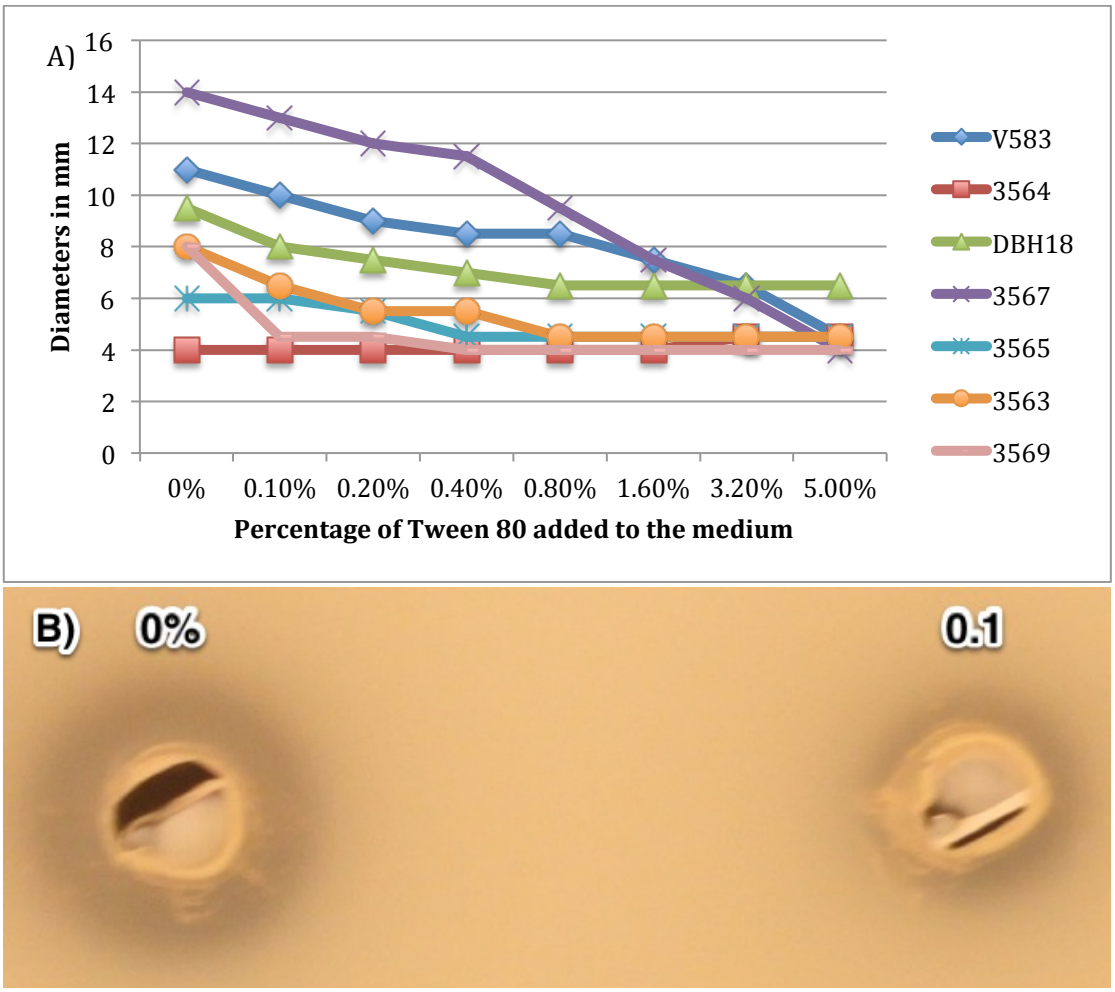

Figure 7: A) Gelatinase activity for various strains of *E. faecalis* after 9 hours, when grown in GM17 with increasing amounts of Tween 80 added to the medium. 4 mm is the minimum diameter of gelatinase detection for strains grown in GM17 B) Gelatinase activity for strain 3563 in GM17 (left) and in GM17 with 0.1% Tween (right), after being incubated at 37 °C for 9 hours. The white in the center is not growth, but the residue of dead cells.

As one can see in Figure 7, adding Tween 80 to MRS seems to inhibit gelatinase activity or the production of gelatinase in all of the wild type strains.

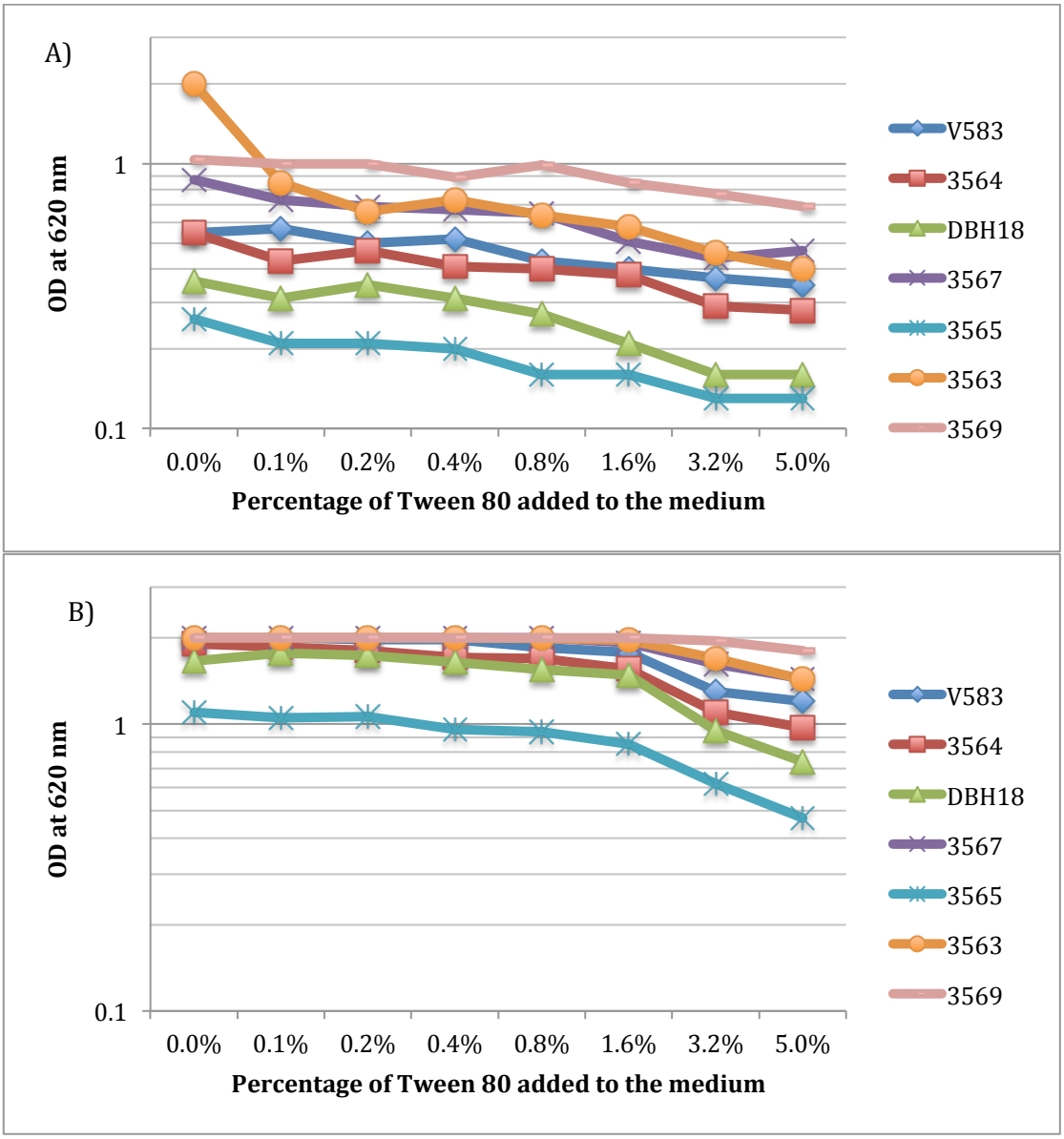

Figure 8: Growth of *E. faecalis* strains exposed to increasing amount of Tween 80 in GM17 after A) 3 and B) 5 hours.

Some reduced growth of E. faecalis strains was observed in GM17 in the presence of Tween 80 (Figure 8) However, the reduced gelatinase activity observed in Figure 7 was more dramatic and are apparently not due to the reduced growth observed in Figure 8. These findings indicate that Tween 80 has a big part in inhibting gelatinase activity in MRS.

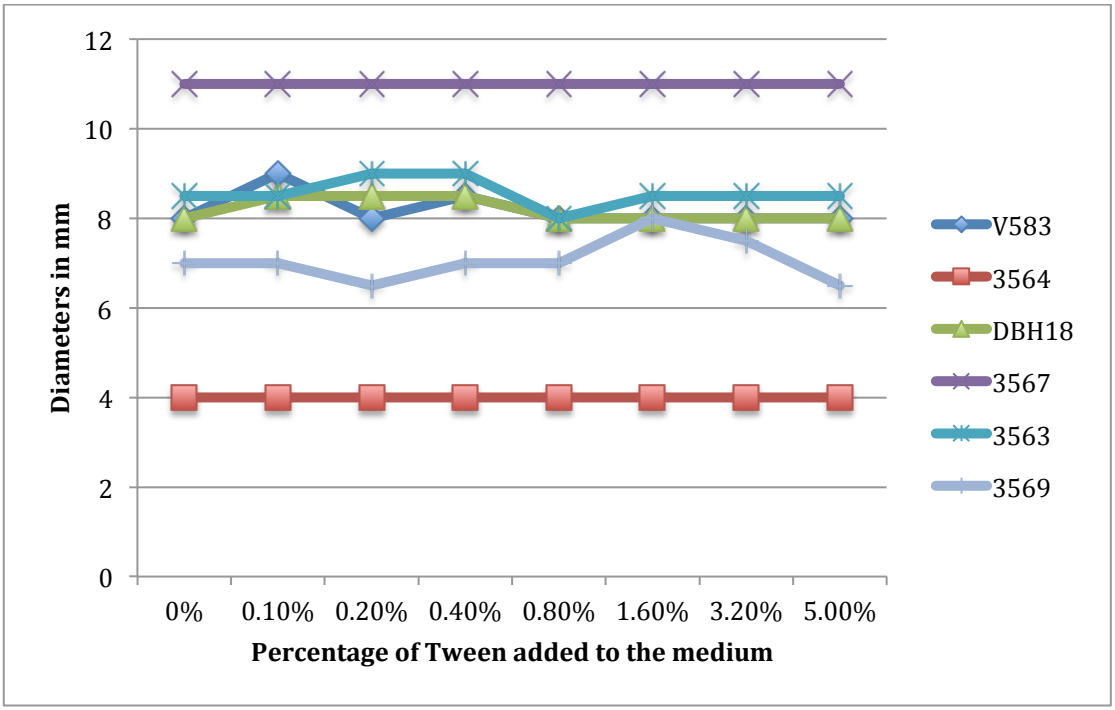

Figure 9: Gelatinase activity for supernatants of *E. faecalis* strains growth in GM17 for 9 hours at 37 °C before exposure to various concentrations of Tween 80 and being incubated at room temperature for 30 minutes. 4 mm is the minimum diameter for scoring gelatinase activity for strains grown in GM17.

The supernatant of gelatinase activity produced by various strains were tested in the presence of Tween 80. The results are presented in Fig 9 and shows that the gelatinase (zone of clearance in Figure 9) was not affected by the presence of Tween 80.

7.6 Activating gelatinase with pheromone (GBAP)

To further analyse the inhibition of gelatinase activity in MRS, an experiment where preproduced GBAP was added to a gelatinase positive strain growing in MRS was conducted. If this yielded higher activity than the same strain growing only in MRS, it would indicate that the inhibition by MRS is on the GBAP production, thus also inhibiting gelatinase production.

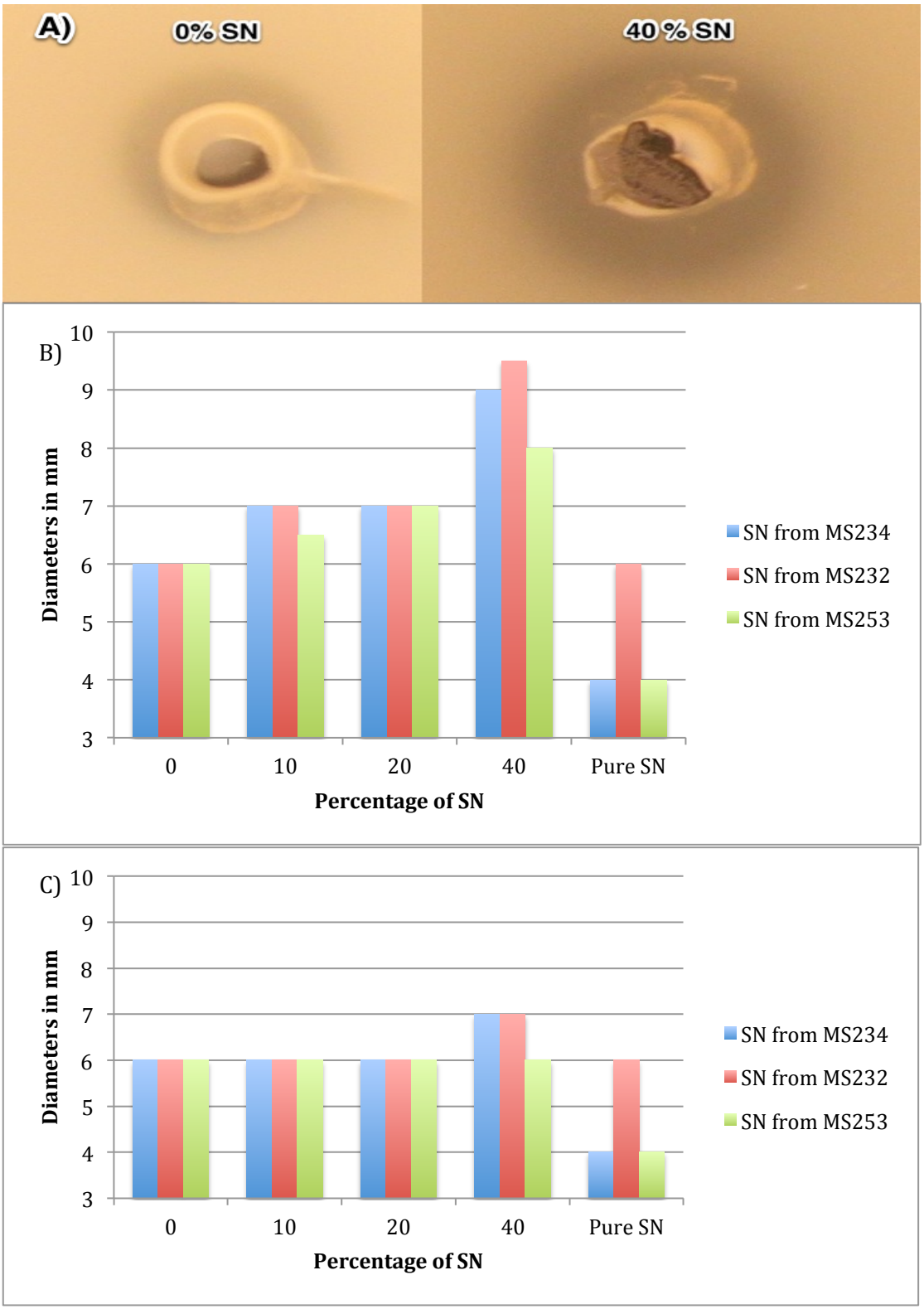

Figure 10: A) Gelatinase activity of MS182 grown in MRS for 9 hours with 0% supernatant (SN) from MS234 (left) compared with the same strain grown in MRS but with 40% supernatant from MS234 added to its growth medium (right). B) MS182 grown in GM17 with supernatants from various gene modified strains of V583 at various consentrations after 9 hours, and how this affects gelatinase activity. Pure SN is the supernatant alone without the MS182 growing in it. C) DBH18 grown in GM17 with supernatants from various gene modified strains of V583 at various consentrations after 9

hours, and how this affects gelatinase activity. Pure SN is the supernatant alone without the DBH18 growing in it.

Figure 10 A) shows how the clear zone in the agar increases for strain MS182 when growing in MRS with filtered supernatant, and thus GBAP from MS234 compared to growing in MRS without GBAP. Figure 10 B) shows the subtle increase of gelatinase activity for strain MS182 with increased proportion of supernatant from the gelatinase positive strains. The first negative control (supernatant from MS253, that has no genes encoding for either GBAP or gelE) also increases with increased ratio of supernatant. This might be due to the increase in GM17 ratio in the medium, which will lead to increased gelatinase activity (Figure 4) when *E. faecalis* strains are growing. The "pure SN" controls, which were sterile supernatants from MS232, MS234 and MS253 respectively and is not inoculated with any growing strain show that only strain MS232 has any gelatinase activity, which is expected as that is the only strain of the three able to produce gelatinase. One can observe similar, but less prominent results for DBH18 in Figure 10 C.

7.7 Observing presence of pheromone by induction of *lux*

To further verify the findings of Figure 10, the produced GBAP was attempted quantified by growing the strains in GM17 and MRS separately, and adding their sterile filtered supernatant to the growth medium of a strain (MS275) that would emit bioluminescence when its *gelE* promoter was activated by GBAP. After subtracting the highest value (40480 p/s) found in row H (see the contents of the wells and experimental setup in Table 4) for all measurements, and thus accounting for the background "noise" readings, the data could be visualized.

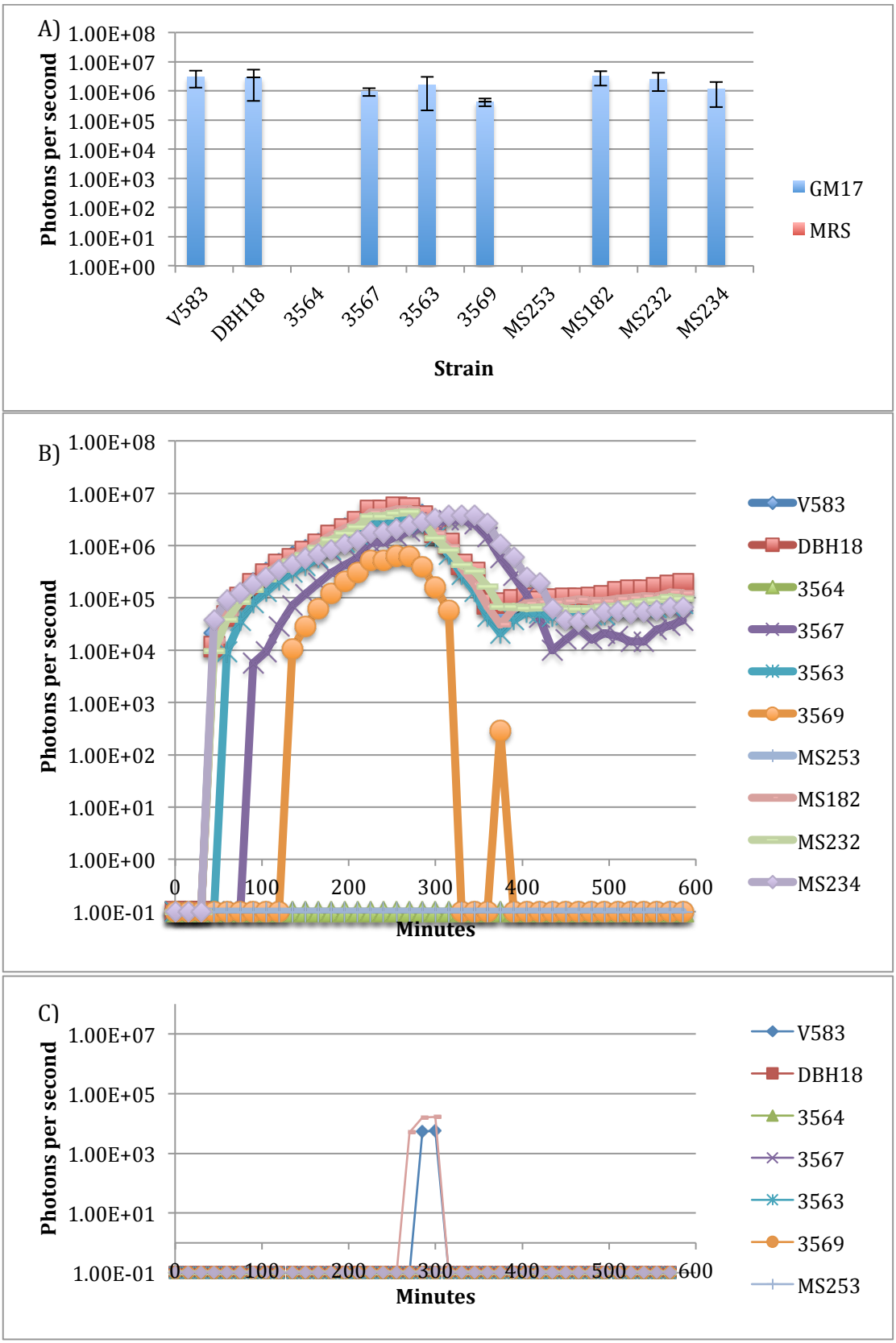

Figure 11: A) Comparison of bioluminescence in GM17 and MRS for the 4x diluted supernatants of various strains after 4 hours. B) Bioluminescence for the 4x diluted supernatants of various strains in GM17 over time. C) Bioluminescence for the 4x diluted supernatants of various strains in MRS over time.

In Figure 11 A, one can observe the difference in the promoteractivity in the various strains after 4 hours. There is also a comparison between whether the strain was grown in GM17 or MRS. No bioluminescence, and therefore no GBAP production was observed for any strains in MRS. There was also no GBAP production in strain 3564 or MS253. These strains have no gelatinase activity and are used as negative controls. This is also displayed in Figure 12 A), where a visual picture shows the bioluminescence of the strains after four hours. One can barely observe any bioluminescence at all for the strains grown in MRS, and significantly more for strains grown in GM17, even when diluted thoroughly. In figure Figure 12 B the differentiation of how another bioluminescent strain (strain S75), with a different promoter is affected by being grown in MRS compared to GM17. The measured values decreases with higher ratio MRS, but this observation can not be responsible for all of the reduction one can observe in Figure 11, as it shows that one will barely find any bioluminescence at all in strains grown in MRS. The lower measurements might be due to the reduced growth associated with MRS for *E. faecalis* strains (Figure 3). Also, the top dilution (row A in Table 4) has the highest ratio of MRS in the plates, with 75% GM17 and 25% MRS. The ratio of MRS is halved for each row, meaning the bioluminescence should not be affected more than the red line (75% GM17) in Figure 12 B on the least diluted samples. As such, the bioluminescent abilities of the *lux* operon marker is likely only affected minimally, and it is the GBAP production that is influenced in the strains, accounting for most of the reduced bioluminescence observed.

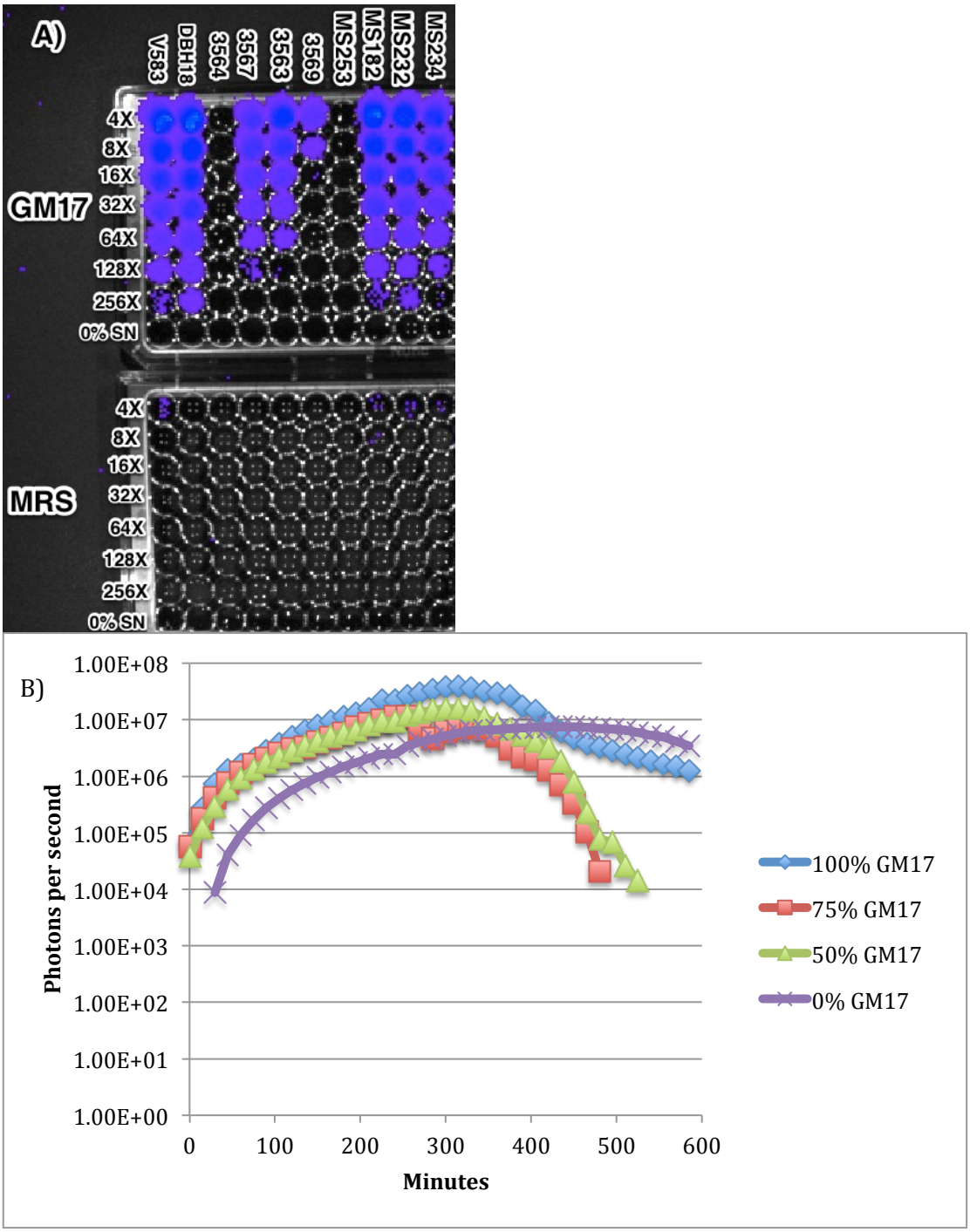

Figure 12: A) Visual comparison bioluminescence from various strains in GM17 (top) and in MRS (bottom) after 4 hours. Taken by the Ivis Lumina II with 4 minutes exposure. B) Bioluminescence of strain S75 when growing in different ratios of GM17 and MRS

8 Discussion

In this chapter, the individual experiments will be discussed, as well as conclusions derived from these and suggestions for that could be done based on the findings in this thesis

8.1 MRS VS GM17 and the role of skim milk

The difference in gelatinase activity between *E. faecalis* strains grown in MRS and GM17 is prominent, as demonstrated in Figure 4. The growth of the strains were also affected, but the decline in growth (Figure 3) was much less affected than the reduction of gelatinase activity, as the gelatinase activity observed in GM17 was almost completely abolished in the MRS medium.

Early gelatinase measurements performed in the thesis seemed to indicate that milk could induce gelatinase activity in MRS (Figure 5). However, these findings have been troublesome to reproduce in new batches of skim milk powder. Also, as the gelatinase activity measured by the diameter of the zones of turbidity clearance in Figure 5 is a lot higher than the values in Figure 4, they cannot really be trusted due to reproducibility. The experiments giving data about skim milk in Figure 5 was done in the beginning of the time in the lab, before the assay had been fully developed. As the time allotted to a master thesis is limited, priority has been given to working on other experiments and not to the milk induction experiments, focusing more in the mechanism behind gelatinase activity inhibition in MRS. The data is however still included to demonstrate the findings that inspired the thesis, even though the focus moved elsewhere, due to lack of reproduction of the gelatinase induction by skim milk.

8.2 Tween

As described in the introduction, the different results obtained between experiments performed in GM17 and MRS media were investigated further to find out what caused the variation in gelatinase activity. The gelatinase activity results obtained by strains of *E. faecalis* when grown in the presence of Tween 80 in the GM17 medium are shown in Figure 7 and Figure 8. In Figure 7 one can observe that all strains decrease their gelatinase activity as the concentration of

Tween 80 increases. However in Figure 8 one can observe that the growth of the strains were also impacted by the presence of Tween 80. However, the growth was only greatly influenced at higher concentrations of Tween 80 (Fig 8A and B), while concentrations as low as 0.1 % of Tween 80 caused significant decrease in gelatinase activity (Figure 7A). As 0.1% is the amount of Tween 80 present in MRS, this indicates that the presence of Tween 80 is only partly influencing the production or efficiency of the gelatinase enzyme. Figure 9 suggests that Tween 80 does not have an effect on enzymatic activity of gelatinase, as there is no real decline in activity with the presence of Tween 80 in the gelatinase suspension. One can probably conclude that Tween 80 had no effect on the enzymatic activity of gelatinase, but did apparently have a significant effect on the production of gelatinase. Tween 80 did not account for the reduced activity that is observed between MRS and GM17 (Figure 4). There must be other factors differentiating between growth in GM17 and MRS media that influence the production or activity of gelatinase.

8.3 Pheromone Induction

It is known that expression of gelatinase activity is regluated through a two component regulatory quorum sensing system. By using different *E. faecalis* mutant derivatives, missing gelatinase (MS234), an peptide pheromone (GBAP) overproducing strain (MS232), a pheromone (GBAP) negative and gelatinase negative mutant (MS253) and adding filtered supernatants from these to strains growing in MRS, one would find if strains would produce gelatinase in MRS if GBAP was added to the medium. If the strains would produce gelatinase in these conditions, it would indicate that MRS inhibited the production of GBAP but not necessarily the production of gelatinase.

The findings presented in Figure 10 indicate that one can induce multiple strains of *E. faecalis* to produce gelatinase activity with the pheromone from another strain. The reason we see such less induced gelatinase activity by GBAP in DBH18 compared to in MS182 (Figure 10) is probably because the growth of DBH18 is lower, as it is only partly resistant to the spectinomycin present in the supernatants. As there is gelatinase production in strain MS232, one can observe

that the supernatant from MS232 yields higher gelatinase activity than the supernatant MS234. This is expected, as the supernatant from MS32 has gelatinase, as one can observe in the "pure SN" columns in Figure 10. The fact that adding GBAP to strains growing in MRS will induce gelatinase activity indicates that the inhibition of activity present in MRS is not linked to inhibition of gelatinase function, but to the production of GBAP, leading to the inhibition of gelatinase, as production of gelatinase is dependent on GBAP.

8.4 Observing presence of pheromone by induction of *lux*

The results of the final experiment of the thesis (experiment 7.7) indicated that that the GBAP pheromone production inhibited the gelatinase production in MRS, as no (or very little) pheromone was produced in MRS in the gelatinase positive strains (Figure 11 and Figure 12). However, the GBAP production did not have a direct relationship to the gelatinase activity observed in Figure 4. The strains with the highest GBAP production were not the strains that have the highest observed gelatinase activity. The reduced GBAP production in MRS is likely the product of Tween 80 inhibition as well as inhibition from other compounds that differentiates from GM17.

8.5 Suggestions for future work

Many of the experiments in this thesis should be repeated to ensure that the results are viable, reproducable and statisticly improved. However, due to the limited time available this was not possible. The antibiotic milk plates can also be further developed, for example by comparing data obtained by other of measuring gelatinase, as at this point, it is not very accurate. Also, measuring the pheromone production under more circumstances by induction of lux should be done under different conditions, like by adding Tween 80 at various concentrations to strains growing in GM17, or skim milk to strains growing in MRS before filtering supernatants and allowing them to grow in the presence of the indicator strain MS275. As MRS seems to inhibit production of GBAP, there is reason to believe that Tween 80 inhibits its production, too, and it would be interesting to research this further. Further experiments on the difference in composition between GM17 and MRS needs to be done, as the Tween 80 does

not account for all of the reduced activity observed for strains growing in MRS. There must be other factors present in the mediums also influencing the GBAP production which should be investigated further. This can be done by adding ingredients from MRS to GM17, like dipotassium hydrogen phosphate or triammonium citrate.

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10 Appendix

10.1 Observing presence of pheromone by induction of lux

Raw data from the experiment. Contents of wells described in Table 4.

Minutes	0	15	30	45	60	75	90	105	120	135
A1	-860.3	7596	29740	62110	95630	141100	229700	339700	494900	663800
A2	4393	3187	21430	52170	86320	141600	222800	323000	477100	594100
A3	247.7	1791	4259	913.1	-2764	2036	4121	-513.8	155.9	3109
A4	2537	3346	6248	10060	15790	25160	46060	49730	68580	112900
A5	-25.6	3783	15520	37440	49860	76700	120800	171200	253300	346800
A6	6175	2428	4729	8791	11090	14440	25140	25710	31750	51200
A7	5917	1983	2770	1180	3923	1538	460.2	1677	4235	4437
A8	395.8	5043	27800	55890	90910	137400	223400	331500	485800	639300
A9	3223	9040	17720	50280	79260	124900	183100	267500	386700	530700
A10	17880	6922	26160	78400	129800	163300	222600	284900	375300	483300
A11	6406	13590	-2473	-3012	-3289	-2048	-2413	-2646	-5021	-338.2
A12	-753.4	-3142	1273	-2520	-2931	-5254	-3074	18000	-5230	-4474
B1	1613	3863	16790	28720	50610	75050	125500	180400	287100	349300
B2	3123	11490	15930	35140	57940	88340	136400	205400	259300	352700
B3	2733	9875	89.96	1218	140.5	-1929	1972	-505.1	2256	2375
B4	4162	1362	4122	13690	12440	26370	37340	54140	80820	133300
B5	4977	5519	6675	16510	22480	43480	56430	80530	110700	155200
B6	3348	3680	3487	4281	3896	4610	7046	6722	4123	7936
B7	3626	2786	576	1028	2163	3903	5688	530.2	-1617	-2220
B8	3835	4722	11720	26030	42660	67540	103100	161200	214500	281500
B9	4066	7573	12160	25800	38690	60410	93330	137300	178100	258500
B10	4043	3409	12920	24020	41980	72060	117300	177600	269000	363600
B11	1251	956	-576.6	-2962	-2857	-1457	-1114	-6066	-2532	-498.9
B12	-3135	4299	1949	3250	-2329	-1882	-2676	-1550	-6239	-1739
C1	2566	3575	8234	20510	34440	50560	76160	99790	179300	196800
C2	2117	4887	9662	26860	40190	69770	97660	150600	187000	247000
C3	867	-1398	870.5	1853	3497	3258	3066	599.1	-2264	2839
C4	1047	168.7	7352	2703	15180	20780	50020	69180	60410	104000
C5	2025	4575	1127	9936	13890	18460	24990	36640	37060	63780
C6	6174	3386	5221	4661	1368	3192	1513	1186	232.4	6835
C7	1880	4747	3609	1468	1549	969.4	759.2	2589	-1627	2121
C8	4403	3789	6495	17730	22640	44060	61980	88040	113300	173600
C9	3931	2689	10420	18860	32620	49600	70870	101700	135400	184100
C10	624.6	3900	6215	11890	17260	30460	53730	86740	139200	184400
C11	3048	-1610	1738	2621	-2415	2163	-853.8	-2395	-745.6	-1425
C12	2805	3445	-825.9	-425.1	631.1	963.9	983.8	2569	-407.9	3115
D1	2108	7113	6601	13190	18260	25300	35090	75950	68360	96850
D2	5968	7240	7909	23130	28810	48040	73360	105900	117400	166800
D3	3241	1836	1710	1316	-79.2	303.9	2415	-2904	18.64	1639

D4	961.3	4473	1687	3324	7892	10080	13600	18630	20870	38970
D5	6061	4868	4115	5788	5364	9691	17390	16580	17300	26780
D6	2409	4479	4876	3538	2878	5151	3097	693	-1471	5680
D7	3768	4428	4140	3250	3598	4673	4621	3352	-1694	991
D8	1766	622.6	3730	12130	13120	24540	36380	50370	66220	90650
D9	3859	2254	1500	9533	14580	21270	35890	47440	63800	84990
D10	1052	3262	673.5	7817	10630	13750	17290	27380	33760	63770
D11	-972.9	1507	1589	1530	385.3	946	721.6	-1709	-1354	746.5
D12	-185.8	-218.5	-2761	-2871	-1654	-1745	2818	-567.7	-4816	-337.3
E1	2409	2524	2249	7000	8959	16560	19880	24320	25240	45200
E2	2416	2846	3750	10270	20130	19980	35410	47590	54240	68030
E3	2239	2953	229.6	3269	-28.1	3414	1871	240.4	-2956	-1146
E4	2022	2973	3636	6635	4014	6519	5803	5069	5287	11110
E5	814.8	2714	1201	1294	2684	1953	5287	2330	2144	4319
E6	8019	2733	7300	4330	2976	3183	2216	1197	-2286	-710.8
E7	2902	4282	3205	4039	2478	1762	2677	-538.9	-997.8	3046
E8	4795	3221	5955	3829	8861	18320	19620	26260	25000	40590
E9	1692	2480	3843	7121	10430	12710	18210	21120	30120	46730
E10	1609	4479	413.4	6765	6499	8942	12790	13300	12100	23090
E11	2368	893.3	-1012	1759	-848.1	1215	-1496	-3151	-4379	-641.7
E12	-2644	1021	133.4	-419.5	2567	-644.1	-1945	-3357	-5069	-3824
F1	1635	1958	930	1494	5412	8063	10600	9972	11880	21850
F2	3999	2151	3618	4345	9238	13830	18170	19970	22620	29200
F3	4568	1281	4663	2560	3878	2454	3266	2398	636.6	1572
F4	2162	2187	2950	3417	2805	2400	3829	1392	2449	4758
F5	3429	854.4	653.7	5365	370.5	715.6	5466	1979	1476	4366
F6	3688	2010	2628	6741	3397	1899	3664	2131	512.1	6091
F7	1601	3462	3352	1087	2528	1323	2260	-1707	136.9	1981
F8	-941.2	-35.26	329.8	1208	3875	7828	8124	7911	3956	13960
F9	2813	3449	4235	3404	3930	4336	7037	8514	6661	12930
F10	2682	3971	1964	1606	4382	3201	5647	6530	1102	9019
F11	451.3	-809.5	2852	-1893	-68.65	-2795	362.6	-1559	-669.9	-1378
F12	-3890	-162.3	-2979	609.1	-392	189.7	-1688	-3567	-1583	-2655
G1	684.8	698.8	-84.83	2500	2250	5337	2199	1427	-1554	4133
G2	2448	-4.64	3693	5238	6676	7656	8308	5085	2543	10170
G3	4828	3135	4030	3746	4459	2316	2557	-582.7	6.025	2775
G4	997.5	1854	2195	1256	1438	1915	3249	311.2	-5.203	3782
G5	2751	1471	2260	2517	1533	3428	2698	-1530	-2013	1465
G6	1892	5536	3156	3534	3351	2366	1838	921.4	-902	1092
G7	-262.9	2385	2150	4481	2126	-937.4	4077	-1368	-1081	-688.2
G8	366.7	1217	-854.2	-428.9	2930	3118	3362	67.31	1720	4218
G9	3477	310.6	1182	-807.7	2667	2245	4657	437.1	3552	5086
G10	2363	1287	-134.1	-362.6	3493	3179	2531	3677	3148	2593
G11	-2894	-365.7	202.9	-260.7	2716	1459	746.7	-705.4	-1204	146.1
G12	-3762	-1938	2093	-1345	3232	1039	-605.8	8514	-979.8	-642.8
H1	453.6	1221	1221	-22.86	2897	220	-538.3	-1793	-3922	1845
H2	2350	1755	1755	1748	313.2	643.3	964.9	-874	-492.4	3028
H3	3424	549.1	549.1	2533	1936	4815	2919	387.3	-118.4	1310

H4	4587	5974	5974	4269	3602	4278	9925	1369	1920	4277
H5	5490	6624	6624	3499	3442	3608	5024	1841	427.3	4034
H6	3290	-163.9	-163.9	3725	4631	3646	3803	29.02	-410.1	3838
H7	1669	461	461	-1663	-187.3	-2800	1588	-1800	3.898	-428.2
H8	946.6	383	383	1621	-732	-1661	724.8	-2267	-2216	-1050
H9	6641	203.9	203.9	3348	1262	287.5	4346	-215.6	1731	460.7
H10	-1588	1320	1320	1983	1819	-410.6	1312	-1148	-1739	2483
H11	-2214	-1240	-1240	1369	-1615	-2080	433.7	-1392	-4103	-1405
H12	2238	-1582	-1582	2173	804.2	-368.8	-4774	-4542	-3300	32.39

Minutes	150	165	180	195	210	225	240	255	270	285
A1	859600	1146000	1567000	2192000	2815000	4421000	4421000	5070000	5310000	3967000
A2	802900	1103000	1576000	2126000	2880000	4683000	4683000	5399000	5191000	3699000
A3	3681	5103	7399	11380	7312	17910	17910	22760	22170	21600
A4	157400	231500	340900	464800	624700	1178000	1178000	1527000	1912000	2231000
A5	451200	622600	869000	1183000	1650000	2699000	2699000	3163000	3331000	2777000
A6	68890	101200	161400	248100	346500	561200	561200	676300	661400	436600
A7	9950	6406	13170	17980	17740	33060	33060	37600	39700	33800
A8	837700	1148000	1602000	2140000	2774000	4337000	4337000	4957000	5054000	3701000
A9	697900	958300	1321000	1765000	2287000	3704000	3704000	4260000	4426000	3240000
A10	604400	724200	877800	1075000	1285000	1808000	1808000	2101000	2481000	2866000
A11	-432.4	1697	-351.2	-3231	-3624	1120	1120	3020	223.7	4371
A12	-2799	-272.6	2815	-624	-2299	3967	3967	-3446	2714	2222
B1	477200	705900	988700	1391000	1826000	2346000	2843000	3123000	2689000	1551000
B2	466400	695700	985700	1360000	1830000	2392000	2979000	3370000	2587000	1435000
B3	2382	4581	4257	6363	8265	14190	15650	21050	18250	20690
B4	185000	244500	342200	496200	670400	908200	1197000	1556000	1828000	1882000
B5	215700	300700	440200	635900	860300	1139000	1425000	1549000	1402000	930100
B6	12150	19660	30770	44060	64920	87680	101300	121500	111000	68650
B7	-19.19	3447	7370	2063	8092	14370	13880	12390	15610	6507
B8	404200	613900	855600	1158000	1516000	1887000	2329000	2597000	2015000	1113000
B9	363900	517200	740700	1036000	1339000	1730000	2058000	2253000	1695000	955000
B10	467300	561900	686900	862500	1071000	1312000	1539000	1821000	2086000	2224000
B11	-1881	-1597	3950	2874	3829	1215	4753	1764	4183	2579
B12	-1453	-189.4	2652	-1570	-1969	1462	383.1	2032	3500	1289
C1	272200	410100	589800	806600	1101000	1428000	1428000	1781000	1252000	687700
C2	321500	508500	699800	978900	1365000	1787000	1787000	2174000	1532000	778400
C3	2964	8224	3667	7038	7468	9158	9158	15230	11670	8705
C4	120400	183400	265600	365200	500500	683000	683000	1040000	1016000	747300
C5	90840	131500	198100	287200	392800	534600	534600	640200	509100	261500
C6	4114	3465	12350	9761	13990	16480	16480	26240	23570	16300

C7	1376	6243	6808	7388	6463	6416	6416	12550	8445	8095
C8	239600	368200	512200	718500	949300	1217000	1217000	1529000	1042000	562000
C9	279600	428100	606800	800900	1088000	1406000	1406000	1826000	1286000	686800
C10	272000	361400	482000	617700	783400	994700	994700	1364000	1463000	1165000
C11	1264	-511.4	295	-905.1	1534	3061	3061	5692	4695	3629
C12	1880	2350	3779	511.7	2986	11740	11740	3053	4562	2445
D1	146300	220100	309500	426400	591300	769300	769300	895400	595200	298700
D2	215600	312800	443600	626300	843300	1138000	1138000	1315000	907000	456600
D3	4118	2949	3796	2195	3465	2665	2665	7913	6916	5525
D4	55780	81350	123500	192900	243600	335700	335700	440300	362300	207000
D5	37080	58700	87480	131200	182900	234200	234200	291100	210400	120200
D6	5286	1571	6393	6692	7775	9490	9490	9136	11060	5278
D7	3316	3113	5544	5380	4389	5688	5688	8965	7655	6670
D8	132700	210500	300900	422800	574600	767200	767200	932600	571400	302600
D9	124200	187300	281000	388300	542100	688400	688400	861000	577200	309700
D10	91500	137400	184700	254400	342000	448700	448700	536100	375100	203000
D11	1783	438.5	3394	369	2211	1384	1384	898.6	4258	2007
D12	3655	-49.93	-629	1002	4457	2594	2594	2851	6758	2024
E1	67840	95020	148000	200700	284800	362100	362100	439500	254200	154400
E2	95220	146900	218500	303300	431800	588200	588200	609700	360300	213900
E3	-350.4	1190	2542	-679.3	4488	3507	3507	5129	5764	4303
E4	20190	29710	43670	61300	95120	112600	112600	167200	144900	148400
E5	9862	19750	26760	38550	55170	75440	75440	83440	63200	39460
E6	4452	5022	2213	4300	5093	7315	7315	6783	5559	7909
E7	2139	4236	3390	3677	3579	3977	3977	8091	3411	5178
E8	54740	86420	125400	173000	248100	332100	332100	387200	241100	137900
E9	65620	101700	145100	212200	295500	373000	373000	451000	272400	166100
E10	31280	50880	74410	109300	148700	185000	185000	220600	146100	79160
E11	8691	524.4	849.8	-1838	4301	3186	3186	3372	4146	-1082
E12	692.1	1236	946.7	-283.7	8166	731.8	731.8	3517	3255	27910
F1	28140	36020	60660	93300	132600	165900	205400	208400	117800	74160
F2	44700	62690	97500	132200	188500	250300	290000	269000	151700	95480
F3	2943	1701	4364	3870	3943	5013	3547	5250	6142	8102
F4	7534	9641	13970	24330	31440	36930	45740	46180	34650	22840
F5	3117	1994	6397	9895	23870	17640	24960	24790	19740	12360
F6	1578	5921	5460	4461	4918	4180	2728	3624	5183	1749
F7	2584	1937	3567	1734	3404	3500	5208	4390	3642	4589
F8	19210	30680	48860	68640	101000	135200	155900	149900	96540	53800
F9	19090	29730	41110	58310	81680	111300	133500	129200	111600	51420
F10	11960	13090	28590	35150	52360	65500	70220	67730	46990	33090
F11	-347.9	-845.8	114.4	609.9	1188	-1879	-250.8	595.4	-1247	-160.3
F12	-1523	995.8	-371.1	900.8	4342	1643	3894	2266	-1520	253.2
G1	8249	7373	13210	19860	24530	36250	36250	37460	24990	15600
G2	15380	21020	33350	49890	68890	99060	99060	86740	47830	32700
G3	925.8	3610	3328	5421	5383	2533	2533	7656	4933	5450
G4	1457	5242	9026	6551	12220	13630	13630	14590	9968	8146

G5	2631	3306	3627	7905	7659	7600	7600	8379	7590	7782
G6	1955	2304	1515	2430	7774	3103	3103	7052	5537	4376
G7	3498	-739.6	3661	670.4	4461	1446	1446	3244	4403	5081
G8	7515	8374	15720	20500	29000	35610	35610	43390	28400	16390
G9	6717	13890	17680	26040	38450	50400	50400	58190	38070	20160
G10	7096	6461	6892	15150	15730	19100	19100	20470	13240	12370
G11	754.8	-1199	2113	710.5	1515	1724	1724	2034	4503	677.8
G12	1312	-3669	3264	4330	4698	325.1	325.1	3527	5555	3147
H1	-748.2	-2104	2427	2214	-711.1	2725	804.5	2548	1926	4054
H2	27730	-722.5	6217	2033	4964	5423	5166	5530	5493	4681
H3	4749	1414	2566	4034	2179	2289	2668	5033	6395	6816
H4	4970	2993	7214	2777	7097	5643	4663	5190	4526	7102
H5	2938	4705	4195	7100	4256	4034	4907	7340	6391	5607
H6	2627	1786	4844	4591	5146	4199	3191	5449	6000	2741
H7	1477	-282.2	1483	1510	1924	808.2	-170.3	3074	2458	4774
H8	4509	1734	269.9	2675	2685	404.8	2541	4920	3843	3599
H9	2542	1985	3072	2760	4664	856	2984	7183	6477	4130
H10	1996	921.3	1268	2462	1419	3124	2628	1196	2367	4595
H11	1061	-480.9	2224	-642.3	1543	1263	2720	789.5	4701	2009
H12	2954	-493.2	2541	4243	3124	5030	1957	2510	4892	4279

	300	315	330	345	360	375	390	405	420	435
A1	1751000	890200	388400	240800	110300	92290	113300	117300	108300	116000
A2	1838000	1133000	470700	349800	116600	120100	130800	138200	125700	133800
A3	27330	25310	24510	19250	18240	9392	1143	-381.7	-2315	2132
A4	2777000	3092000	3120000	2627000	1512000	614900	300900	155600	81760	50510
A5	1499000	703900	319000	169600	84000	61360	78860	92490	90780	84340
A6	198200	97090	31870	23500	17850	40770	30770	32430	27710	26260
A7	16440	14000	7627	8180	8018	10810	5778	6988	2418	7218
A8	1714000	969100	405300	222400	108700	71760	103100	119700	123900	123100
A9	1420000	861100	464600	362800	206300	107400	107300	104200	106000	108800
A10	3330000	3806000	3892000	3821000	2703000	1101000	634100	286900	228900	101000
A11	489.6	25.26	1459	766.8	229.5	3559	-2235	-3151	-4265	-809.6
A12	-341.5	585	37.3	1612	-3323	6544	-2750	-1814	-4670	220.2
B1	882900	402800	147600	103600	39420	50930	72790	76810	77750	82890
B2	859600	405200	150500	118000	55890	55600	79800	87330	93510	99020
B3	12120	10910	5644	6131	10650	2721	2817	769.1	1614	-563.6
B4	1603000	1005000	513200	266300	86970	65510	25470	23350	39760	50930
B5	498700	258000	84540	63270	28520	31560	52990	49770	51060	50420
B6	38210	21600	5680	9094	14170	13730	9165	18670	5919	9890
B7	7788	8511	6344	2503	3096	7805	3172	2053	2374	3527
B8	690600	309200	109300	90250	37960	41610	63410	80350	76490	74890
B9	612300	279800	107800	85310	40370	46900	58010	67510	66800	62880
B10	2136000	1379000	638000	372400	188200	115900	75580	53180	45880	55680
B11	506	1437	1777	-980.8	925	3229	-394	-1857	-2651	-945.7
B12	1089	570.9	-3519	-285.4	141	2641	-548.1	-2546	-5121	2462
C1	454000	182100	71370	63520	30190	40370	57980	65030	60280	65530

C2	522600	215900	96130	71120	28760	43660	70190	79000	73740	83150
C3	5664	3844	4988	-1034	4823	8745	4479	2439	-1005	3599
C4	422500	253800	103800	37880	32250	18390	19830	29200	33770	35940
C5	180400	70000	29950	21750	12240	22040	25830	28240	29800	28320
C6	13440	8292	8324	7539	13390	12680	11680	12210	8085	10130
C7	11840	6102	4411	6587	6409	9762	4629	4189	1375	3263
C8	357200	145800	65020	52920	25760	38820	54950	61770	64030	56860
C9	482900	193100	81070	63190	31400	40980	58290	67880	60580	69320
C10	609900	356400	185600	80460	85060	49940	44810	56570	31800	33140
C11	3195	790.8	-565.6	143.4	2726	5429	603.1	-1513	-2783	2839
C12	-16.2	1005	1887	1592	3268	4378	-717.8	2547	-3144	2428
D1	196200	75760	38700	25090	23400	32440	37540	45050	38420	41880
D2	288400	124200	62950	43370	24140	42240	58190	63410	62760	66910
D3	5433	4843	1462	3084	8007	7825	4235	7580	550.9	6647
D4	142300	65230	26730	25360	11610	15580	15160	24610	19170	19100
D5	75220	31240	16820	15110	8396	18060	18730	16310	15230	14050
D6	8025	7864	6619	6429	4859	9371	7197	7412	2690	3664
D7	4707	5759	3247	5887	6812	7946	5672	6394	2629	5462
D8	197300	81350	33670	21240	13220	25930	38820	42550	34830	39100
D9	200700	86920	38480	27230	17350	27230	38540	37920	35230	40890
D10	135900	64390	34910	34230	22210	26330	28940	25270	20610	24850
D11	551.3	1288	-1644	-367.5	6055	4490	1579	-2628	-4758	-539.6
D12	1259	-1437	429.8	-2034	1551	6032	-199.7	-1375	-1733	-898.3
E1	90160	43060	18470	15520	12690	22050	24720	25520	18790	22930
E2	129800	50310	33400	18310	12880	27340	35870	38570	33690	38770
E3	5677	6902	5344	4922	9520	10460	4919	6997	2519	2585
E4	148600	145100	177800	195500	219800	87040	130500	145800	85060	43770
E5	27080	11870	7849	5073	10490	12350	10850	11130	2709	12600
E6	6679	3120	1992	5870	10020	7964	5723	4074	4069	4330
E7	7546	8025	4260	5816	7352	10760	6497	4962	3823	4591
E8	79180	43190	20030	17450	14140	21980	26030	26140	23800	21400
E9	99100	43650	19900	16070	15030	25430	23740	24260	25870	23840
E10	61360	31890	15630	13550	14920	22320	18000	15450	15690	16470
E11	4149	1754	699	8.611	2542	5816	278.1	104.2	-3762	532.4
E12	-250.8	-1716	-2262	2291	2133	3472	-699.1	-1189	-1882	-1662
F1	45940	18310	14420	7078	10950	15950	13660	15940	9720	15600
F2	54770	27960	19950	14030	12380	17460	24230	20820	18860	16660
F3	7128	1935	4156	5204	9722	9708	6473	4101	2583	3389
F4	17840	11250	7591	5756	6867	10570	11450	5044	3826	4867
F5	10940	5780	8290	6362	9564	10520	5471	4879	1757	6298
F6	5059	7156	4292	3196	10090	11150	6352	7041	3042	2836
F7	5121	4329	6815	4617	8158	8556	5386	4431	2476	5158
F8	40330	14490	6700	4456	5984	6826	10690	10410	8929	11380
F9	38380	16630	12340	7282	8424	13220	13690	11360	6719	9533
F10	25330	12580	7832	10030	26250	15220	6785	9239	6835	4510
F11	2150	634.2	-1036	-96.66	975.9	4123	-729.1	-1341	-782.6	-173.9
F12	1890	493.3	-428.1	-1810	1505	2792	1098	-474.1	-2534	1946
G1	12000	8950	4270	7520	9375	10420	6459	4122	3573	4259

G2	19360	11320	11720	7934	12120	13000	11530	11600	9044	12770
G3	6992	8968	8303	9082	10900	10580	7687	6623	5884	7614
G4	7103	3447	4606	4246	9962	11960	7525	6443	4809	3123
G5	7958	3976	6555	5077	8753	11700	7181	4655	6061	6142
G6	6691	5478	6212	5050	5529	8942	5848	7233	8390	2006
G7	4781	3406	3176	4209	8201	6278	4715	6319	-291.1	1260
G8	15650	5260	5631	4701	10320	11370	6713	9102	5614	5977
G9	17450	10050	5834	4225	8171	10970	10480	5272	5946	4909
G10	10380	8319	4868	10500	8552	9844	4178	9203	2979	4895
G11	3133	-594.4	3287	1252	4753	5076	-1976	1415	18760	-1848
G12	5547	2328	3830	2545	2542	1850	452.1	2474	-706	-23.36
H1	4598	3573	3023	5088	5692	5679	208.8	4213	228.2	4380
H2	7862	5297	8129	8594	5137	9224	5621	3478	1167	4280
H3	9845	4964	7799	6628	10810	7236	5877	5604	4522	4162
H4	6562	9541	6718	7877	8801	11940	5557	8469	7129	5629
H5	10700	6613	4507	7227	9024	11280	7233	8046	3261	6400
H6	7515	5253	5748	4235	8982	9712	4783	3067	3014	5586
H7	4615	3190	3320	4791	4811	7533	1912	6108	-188.6	5681
H8	6143	5389	4318	5942	6480	6664	4255	6158	2001	1012
H9	8606	4237	5237	7490	10710	13410	6564	4844	3434	4145
H10	5059	3692	5408	4476	6956	7828	3781	2494	118.1	-1124
H11	2924	3681	-2745	3580	3495	6117	1090	1514	-1097	-1524
H12	3306	4880	3145	592.9	1320	6403	1470	432.8	744.8	1506

Minutes	450	465	480	495	510	525	540	555	570	585
A1	120500	118800	121700	124400	126500	132700	142100	147900	161000	145200
A2	135900	138200	142300	147200	168600	180600	182800	195700	211700	227800
A3	1418	3031	-242.2	731.1	1263	2801	6639	718.6	7782	1601
A4	56330	65740	56450	61320	58840	54980	55250	65190	70030	77600
A5	87860	87690	86720	87900	97930	104900	104600	106200	111100	116700
A6	25780	20470	15460	22880	19590	17800	19450	16800	25850	21330
A7	5408	2913	-425.5	5705	6793	26.48	3042	2329	3958	5778
A8	127300	135100	128200	128100	135800	140400	149000	146300	166500	162700
A9	101700	101400	101900	106800	108300	116500	125000	130500	142400	137400
A10	78510	75450	81380	93490	96610	91630	95620	97490	106800	108000
A11	-2133	-3484	-4383	-1417	1638	-2547	-874.3	-5464	-429.2	2343
A12	-5105	-3572	-3272	279.1	-2015	-4721	-1801	-2424	1227	114.3
B1	80950	91890	86660	85410	82910	82770	83490	82730	84750	82010
B2	104200	95550	89460	102700	108000	103400	99980	99220	99020	99260
B3	360.4	-506.9	-2317	3080	2813	1620	-45.98	-342.5	1624	3144
B4	51120	48620	45420	52780	46010	43190	45130	44800	49530	47840
B5	50990	46750	44970	51890	42770	39260	51250	41110	43090	41000
B6	8536	4524	4877	9304	6776	4025	7104	4054	7504	5403
B7	1982	1232	-1046	6285	4975	-179.8	2872	-733.8	735.2	2653
B8	71530	76800	71410	74540	73630	77350	68630	65330	68500	66090
B9	70110	66440	60790	61120	64760	62170	71140	61980	63820	61050
B10	49180	52760	51690	56900	58210	56530	55200	48050	56750	55010

B11	-1193	-1077	-2432	1740	1707	-4157	-2003	-393.5	-912.2	822.6
B12	-5280	-374.6	-7244	474.8	-2504	-3728	-2234	-731.6	1835	3827
C1	62260	58370	54580	56020	52870	51490	49840	50840	47840	50490
C2	78630	74210	75900	73250	75450	67060	70510	68630	71330	59810
C3	688.1	3308	2073	3196	4720	5091	4318	5052	3260	3463
C4	32740	27530	30500	31350	23470	24050	23380	31860	21620	18740
C5	26060	25440	19650	26210	21760	17790	16250	14810	17380	13490
C6	8495	4664	3187	6103	4838	2737	3826	1940	-96.02	3873
C7	2071	1296	-867.5	4571	4139	-824.3	1024	927.4	-1398	2614
C8	51910	52180	46060	53960	50660	46310	44950	44610	42780	37170
C9	63850	63040	54380	57630	53300	48310	49970	47030	47460	41520
C10	40960	33230	32670	33610	27000	22790	21400	14390	17370	16320
C11	-38.14	-188.1	-2815	2888	1556	-519.7	-492.4	-3151	1240	1632
C12	435.6	1323	-2529	3774	1437	-2849	-3016	-3274	4179	911.9
D1	34700	34970	35690	32720	26010	24470	27490	22810	21590	25210
D2	64310	61140	52800	50480	55630	52400	50760	51030	49490	45560
D3	5433	3714	-690.5	7000	6406	3209	3978	1723	3645	1154
D4	14910	11290	8339	12040	11290	10980	12110	11960	8758	9494
D5	16040	9940	13430	13990	10710	9816	11340	7341	9545	6779
D6	5415	3030	3226	5545	5147	3055	2241	1762	1987	4175
D7	2436	-755.1	977.2	3050	3085	4051	1901	2789	6438	1580
D8	35910	36060	26630	29160	32800	23300	24750	19640	22220	16510
D9	30260	29390	29570	29000	27900	25480	25170	20180	26140	19070
D10	19670	14330	12070	12630	11470	9153	15280	18230	23350	26100
D11	-1293	1200	-3890	-775.2	1251	-2000	779.2	-732.7	1487	1604
D12	-1133	-2601	-5122	1744	1927	-693.1	-2717	1811	860	1252
E1	19410	18910	18220	15690	18170	13320	12820	11310	12710	7849
E2	32170	30020	26240	35020	25320	23530	22800	20060	18310	13430
E3	2234	603.6	-442.5	29880	890.8	902.3	271.2	7488	-814.4	992.6
E4	20210	14110	5089	10610	8899	4353	6871	5717	4683	9726
E5	4188	4112	1657	3952	3491	-745.7	4556	4382	3102	-479.8
E6	4135	1800	-184.9	2729	3939	1024	208.5	2282	3375	1666
E7	4921	2490	1722	3322	2592	2143	519.5	2590	805.3	1002
E8	19470	15840	16330	17800	13780	14150	9743	11880	10070	9736
E9	20920	18080	15550	17840	17030	13060	9625	12740	12910	14310
E10	10180	10070	9150	12150	14210	19570	25730	35950	50880	63110
E11	-1601	411.4	-923.5	432.1	-4227	-1434	-2369	-369.5	1594	1259
E12	-2243	-3674	-5892	603.8	902.6	-2932	-1127	1272	2070	-282.6
F1	12750	7413	6302	8336	11130	7021	7726	8623	5232	5950
F2	19310	17790	14380	16270	16140	14280	13300	9717	7696	8114
F3	2728	1971	2575	2833	-372.7	1220	3177	490.2	5254	3701
F4	5061	4342	1401	4669	4176	694.5	531.2	388.1	2079	2174
F5	3839	2960	-1602	3540	2793	299.9	2644	1086	1043	2557
F6	2696	1423	3584	4784	2431	1764	1925	2351	3741	3080
F7	2205	1877	1980	1297	5058	-2321	584.3	3986	2806	1247
F8	11400	4459	3113	4212	5154	3030	3097	2265	3717	-287.8
F9	7547	5144	5201	15740	5836	4596	7277	3726	4533	2746
F10	4681	6006	3739	9709	7265	2556	-1023	4608	5220	1483

F11	-1614	-1267	-1726	-681	162	-384.2	-557.4	-1622	504.4	-2175
F12	-1977	13570	-4005	300.7	-739.3	243.9	-4452	-4573	1414	-384.9
G1	4114	4321	1826	2035	3168	3312	2305	2021	14640	881.1
G2	5356	3725	7997	6536	10260	6943	5438	6113	3600	3309
G3	3106	13.8	1777	2545	4916	1626	4273	4648	3165	119.7
G4	5096	3081	-873	3488	1432	-817.7	2105	1379	450.6	1818
G5	2940	3055	1280	3923	4780	1904	4536	500.7	2022	2019
G6	-595.3	688.1	3462	1894	2438	3718	369	425.3	3225	495.8
G7	5379	801.2	790.5	1985	1180	770.7	2253	2717	4274	226.9
G8	2084	2389	1471	2685	4818	1653	2383	-174.5	2319	169.9
G9	4057	3421	1178	4094	4159	1896	3468	1855	3341	1475
G10	3183	1971	-1197	3061	4058	68.79	1996	652.8	3050	2341
G11	446	-650.3	-1723	-2421	4054	-997	1287	-428.5	886.7	-340.7
G12	1674	-1464	-2957	1675	1726	125.3	-1446	543.7	828.1	1931
H1	-174.2	-2760	-2760	-958.8	-934.5	-611.9	-1817	-1642	-841.6	524.8
H2	2303	-269.5	-269.5	1255	2471	362.1	667.4	-394	2293	639.6
H3	1915	579.3	579.3	3876	1768	3910	4911	1396	4112	5015
H4	2925	5173	5173	4025	4017	3296	1662	2681	3993	4474
H5	1783	2367	2367	4565	2929	881	4899	2881	4088	4226
H6	269.9	2114	2114	2862	1170	3531	1326	1850	1958	3724
H7	1778	-465.9	-465.9	464.1	-179.4	-643.5	3565	-95.9	-583.7	2048
H8	1605	2358	2358	-2265	2016	-1472	1572	-200.4	819.6	108.1
H9	2894	853.3	853.3	1679	2268	-1140	3680	2376	4122	2123
H10	-152.6	959.7	959.7	-959.5	-1557	-1227	-66.91	-1460	842.1	-1141
H11	-2394	-2794	-2794	-1695	-2438	-2486	-1459	-1764	-524.5	-574.9
H12	-1806	-618.1	-618.1	1358	-1722	436.3	1767	928.1	2627	-1680