Interim Project Report

Modelling Microbial Cooperation Using Kefir as a Case Study

4-month project report for the
Degree MSc by Research
in Biological Sciences

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1. Introduction

This report outlines the status of my MSc project, as input to the 4-month project review to be held by mid-February. The purpose of this review is to ensure that the project is on track, gain focussed guidance from supervisors and adjust direction as needed.

The project was inspired by an ambition to discover organizational principles underlying the resilience and enduring viability of microbial communities. Resilience is an important property for any complex system to have in our rapidly changing world; it is therefore of interest to a broad spectrum of disciplines, from those engaged with the sustainability of natural systems to those designing and developing socio-technical ones.

The project draws on the example of kefir, a community of bacteria and yeasts that has been used to ferment dairy milk for thousands of years; besides prolonging the shelf-life of milk it is also valued for its apparent benefits to human health, and is currently widely used in milk consuming countries. The experience of artisanal producers shows that kefir is not robust, i.e. it does not resist change, in fact small changes in environmental conditions can drive significant change in the organoleptic properties of the fermented product, indicating changes in the community structure and metabolic processes. It is also not resilient in the traditional sense, in that it does not easily 'bounce back' to a former state after disturbance. Instead it displays something one might term 'labile resilience', settling readily into a new state, while remaining viable as a community. The resulting kefir milk product is likewise variable yet retains recognisable characteristics that ensure its enduring value to humans.

In systems dynamics terms the system is labile yet adaptive and produces an output that is variable within a broad range of possible states. Despite this, the *viability* of the system and the *value* of its output are robust. The fact that this can happen despite the absence of a centralised management structure is intriguing and suggests that it would be valuable to understand the mechanisms involved.

It is not possible within the scope of an MSc project to fully realise this ambition. However, I believe that it will be possible to generate useful insights and identify potentially fruitful lines of enquiry. Specifically, there did not seem to be much prior research aimed at understanding how the observed dynamics of kefir might arise. Therefore it was decided to begin by modelling the kefir system mathematically to see what we could learn about its capacity for enduring viability and resilience.

2. About kefir

Figure 1: Kefir grains

Kefir is a fermented milk product produced by inoculating milk with kefir grains, small rubbery structures that look like cauliflower florets. The kefir grains are comprised of a polysaccharide matrix and biofilm accommodating some 40-60 different species of bacteria and yeasts.

Traditionally, kefir grains are inserted into fresh dairy milk and fermentation takes place at room temperature over the next 24 hours, dropping the pH from ~6.7 to the kefir range of 4.6-4.2. After that period, the grains are recovered and inserted into a fresh batch of milk. The resulting kefir drink is either consumed directly or ripened for an additional period (Rattray & O'Connell, 2022). Besides prolonging the shelf life of the milk and removing pathogens, kefir is also traditionally associated with a range of positive health outcomes, see e.g. (Bengoa et al., 2019).

A significant amount of experimental kefir research has been carried out under the auspices of the food and pharmaceutical industries, seeking to validate kefir's reputed health benefits, manage its flavour and commercialise its production. Much attention has been directed at the microbes' metabolites and other compounds they synthesise, e.g. (Walsh et al., 2016). With the advent of modern methods for analysing mixed microbial genomes, this research has revealed much more detail about the microbial components of kefir and highlighted significant cross-feeding relationships between them, see e.g. (Blasche et al., 2021).

Kefir grains grow by about 5-10% during a typical 24 hr fermentation cycle. They only reproduce from kefir grains; if the constituent species are simply combined, no grains will form, nor will they form from kefir milk alone. In addition, product from grains does not scale consistently. In consequence, commercial kefir is actually a substitute made using a limited number of bacterial strains isolated from kefir.

Kefir is thought to have originated in the Caucasus mountains but is now found internationally. Samples from different countries typically show a common core set of primary bacteria but vary significantly in their secondary bacteria and yeasts, see e.g. (Walsh et al., 2016).

An important finding is that whereas the microbial composition of kefir grains is fairly stable over a 24 hour period, the relative proportions of different microbes in the kefir liquid change significantly over the same period. For example, Walsh et al show how the shift in dominant species in the liquid correlates with the decline in pH, reflecting both the pH lowering capability and the pH tolerance of various species (ibid).

Blasche et al suggest that cross-feeding relationships drive the sequence of microbes that emerge, with successive metabolic processes opening up new metabolic

niches for other species. (Blasche et al., 2021). Intriguingly, they show that the dominant species, *L. kefiranofaciens*, is unable to grow on its own in milk, yet has not so far been found anywhere else.

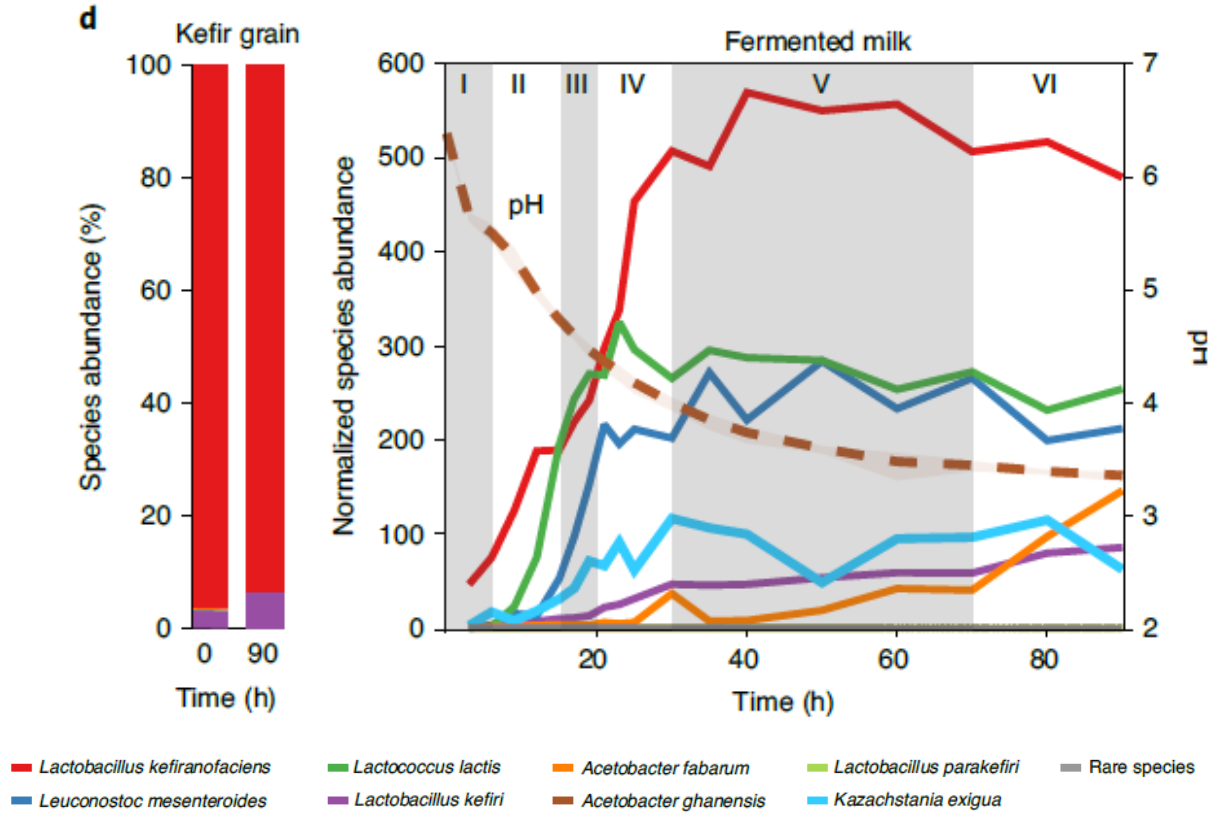


Figure 2: Normalised species abundance over a fermentation cycle, in the grain (left) and milk (right)

(reproduced from Blasche et al, 2021) (permission to be obtained if the graph is needed in the final publication)

Figure 2, reproduced from (Blasche et al., 2021) shows how the abundance of key species changes over the fermentation cycle. Note that a traditional fermentation cycle only lasts for 24 hours. It is striking that individual species start growing in a staggered fashion, with *L. kefiranofaciens* and *Lc. lactis* the first to increase in population. *L. kefiranofaciens* initially grows more slowly than the others, but still dominates the early period because of its dominance in the grains. *Lc. lactis* grows the fastest, reflecting the fact that it is individually well suited to the milk environment.

Blasche et al (ibid) therefore examine cross-feeding relationships between pairs of bacteria in milk-like and grain-like environments, and categorise the types of relationships as competitive or cooperative based on whether or not co-existence increases or decreases species growth and acidification of the environment. They suggest that their results show a form of cooperation amongst community members during the milk phase, contrasted with a more competitive interaction taking place within the grains.

As mentioned above, the core bacterial species in kefir samples are fairly consistent but the yeasts and other bacteria can differ significantly. Kefir is known to adopt some local species into the community, while eliminating others. This suggests

that the 'metabolic cooperation' and 'niche partitioning' identified by Blasche et al may be based on functional capabilities rather than specific species. This view is supported by the identification of kefir gene families supporting relevant metabolic pathways, as described in (Walsh et al., 2016).

3. Research questions

There are a number of questions immediately raised by the above research.

1. Would the cross-feeding relationships suggested by Blasche et al give rise to the observed dynamic patterns during a fermentation cycle, where successive species rise in prominence in the milk?
2. What would the stability characteristics of such a dynamic system be when perturbed? Would the system converge towards an equilibrium state over multiple fermentation cycles?
3. Given that the species composition of the grains is relatively static during the 24hr fermentation and quite different from that of the milk, how is the grain population determined and maintained as new grains form and grow?

The current plan is to explore each of these questions to some degree, to discover a conceptual approach that has the potential to yield further insights.

4. Modelling approach

To begin addressing the first two questions, we decided to investigate how the observed dynamics of kefir might arise from simple metabolic interactions that combine non-linearly to produce complex outcomes.

From a modelling perspective, there are two distinct steps to be considered. During the milk fermentation cycle, which typically lasts 24 hours, there are significant dynamic changes that would be most appropriately modelled as an ecological system. During the milk replenishment step at the end of 24 hours, the grains, which will have grown, are moved to fresh milk with little or none of the liquid kefir transferred. To model the changes in grain composition over multiple fermentation cycles therefore requires an evolutionary approach.

For this reason, we decided to build a simple, functional-level model of the within-cycle dynamics in the kefir milk, nested within an across-cycle model of grain formation and transfer that will span multiple fermentation cycles. Conceptually, this nested structure is similar to that advocated by Loladze in his 2019 paper *Iterative chemostat: A modelling framework linking biosynthesis to nutrient cycling on ecological and evolutionary time scales* (Loladze, 2019). However for our model, the inner ecological system should be closed to resource replenishment or waste removal for the 24 hours, unlike the chemostat models typically used for microbial systems.

It is clear from the literature that the cross-feeding relationships between kefir microbes are extremely complex. This is exacerbated by the variable metabolic capabilities of individual microbes. At a conceptual level, however, it is possible to

identify key functional components to be modelled. At the start of a fermentation cycle, the milk system contains the resources lactose (milk sugar) and lactase (milk protein), kefir grains but no significant amount of kefir microbes yet. It also contains various other chemicals, e.g. citrate, which may inhibit the actions of some microbes, benefit others, and/or catalyse certain natural reactions.

The lactose is metabolised for energy, and results in the production of further catalysts and inhibitors, e.g. lactate from lactic acid, which inhibits some microbes and benefits others. Some microbes can metabolise lactose directly, others require it to be split (by others) into glucose and galactose before they can do so. Through the catalytic products of some microbes, lactase is split into shorter peptides and amino acids, which serve as common building blocks for population growth.

Figure 3 illustrates the relationships described above in system dynamics terms. Blocks indicate countable quantities of things. Arrows from block to block indicate that there is a process that depletes the one while proportionately increasing the other, as in a chemical reaction or metabolic process. The valve symbol on such an arrow indicates that the rate of this change is influenced by the amount of some other thing, e.g. a metabolic process occurring at a rate that depends on the quantity of microbes that employ it.

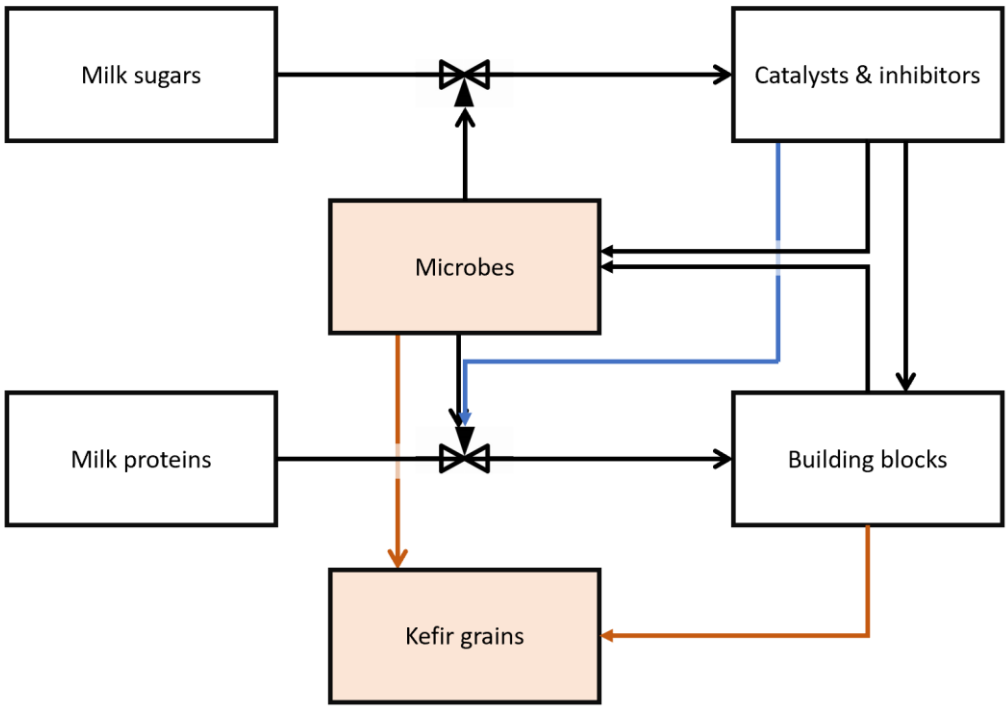


Figure 3: Conceptual model of kefir system dynamics

This diagram shows that grain formation can only happen under certain conditions, and suggests that those conditions need to be set up during the milk fermentation stage. This will be discussed further in the next sections.

Throughout the research project I will maintain a glossary of any key concepts I need to convey, where terms are used differently by different biologists. For example, the concepts to do with different types of cross-feeding and their implications are currently controversial. The nature of cooperation and mutualism versus co-existence is widely debated (Smith et al., 2019). In this report I refer to kefir as a community, but some prefer to use the term consortium. The underlying assumptions and implications of these differences need to be acknowledged.

5. Model of dynamics during milk fermentation cycle

Our plan is to build simple, modular models of key cross-feeding interactions that seem to unlock a metabolic opportunity for another species. These modules can then be joined up into increasingly complex models. We expect these will display more complex, non-linear behaviours. In particular we are looking to see whether such a model results in the observed dynamics.

During this exploratory phase, we began by writing a set of differential equations that appear to exhibit the observed behaviour. We subsequently simulated these equations using Silico, a visual Systems Dynamics modelling tool that makes it easy to see the dynamics of system states in different scenarios. This allowed us to explore the model empirically while exploring the equations algebraically.

In the following, lactose is considered an unlimited resource because it is typically not depleted during a normal fermentation cycle (Blasche et al., 2021).

5.1. Producing an inhibitor, e.g. Lactate production

This model captures an interaction exemplified by *Lc. lactis* metabolising lactose to produce lactate. These bacteria are inhibited by the lactate they produce, so their growth is constrained. Their dynamics can be modelled as

$$\dot{B_1} = (c_1 - b_1 A_1)\mathrm{B}_1$$

$$\dot{A_1} = a_1 \mathrm{B}_1$$

Where B_1 is the net biomass of bacteria, A_1 is the concentration of lactate and a, b and c are constants. When A_1 is low, B_1 will grow exponentially and drive an increase in A_1, but as A_1 increases it constrains the growth

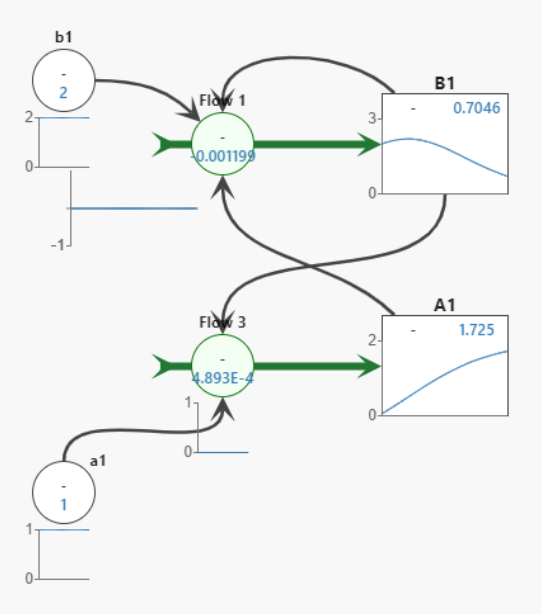


Figure 4: Bacteria producing a self-inhibiting metabolite

of B_1 and therefore also itself. The small graphs in Figure 4 show the changes in the bacterial population and lactate concentration respectively over simulated time.

5.2. *Consuming a limited resource, e.g. Citrate metabolism*

This model is exemplified by *Lc. lactis* metabolising citrate and depleting it. Since citrate is the only resource modelled in this scenario, once it is depleted the population of bacteria declines.

$$\dot{B}_2 = (b_2 A_2 - c_2)\mathrm{B}_2$$

$$\dot{A}_2 = -a_2 \mathrm{B}_2$$

A_2 cannot be negative so in the model it is artificially constrained so that $A_2 \geq 0$.

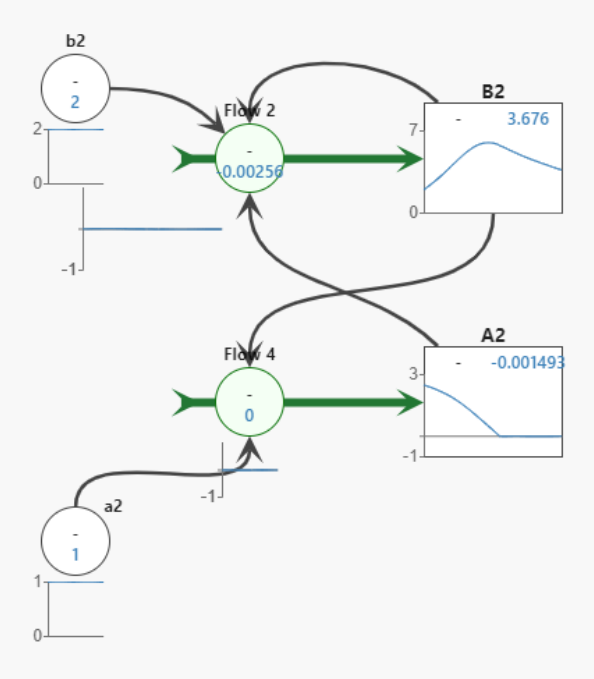


Figure 5: Bacteria consuming a limited resource

5.3. *Exploring these two equations*

The nature of these equations is unusual and differs from what would be expected from a Lotka-Voltera type model. Note that the rate of change of resource A does not depend on its current value. My supervisor showed that the phase plots for these equations do not converge towards an equilibrium point, instead displaying a parabolic form.

In response to concerns about the parameterization of the model, my supervisor predicted mathematically that the shape of these curves would be independent of the constants and initial conditions. I ran a number of simulations with different parameter values to confirm this.

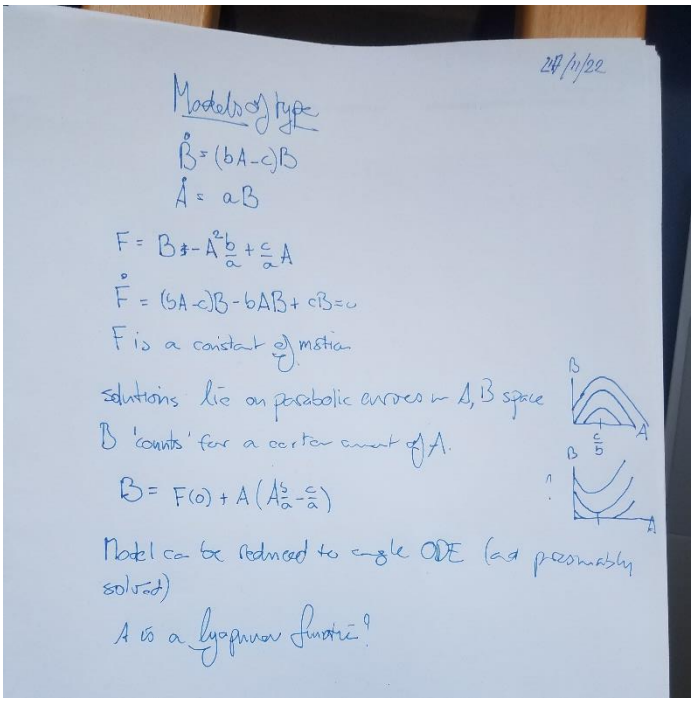


Figure 6: Analysis of phase plots for the initial pair of equations

The mathematics suggest the presence of a constant of motion in this system. It could be an interesting option to investigate this further to understand its implications.

5.4. Combining 5.1 & 5.2, e.g. Lc lactis producing lactate and consuming citrate

In the following model, we combine the previous two models to represent a bacterium such as Lc lactis that metabolises both citrate and lactose, depleting the citrate and producing lactate that is self-inhibiting.

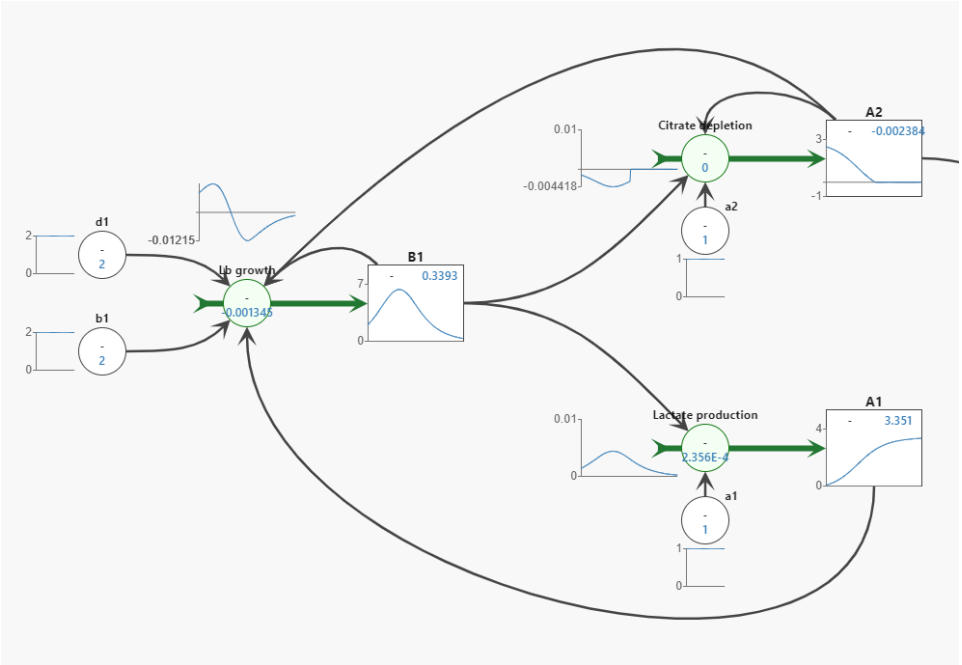


Figure 7: One bacterium consuming two resources, one of which is limited and the other results in an inhibiting metabolite

The relevant equations are as follows:

$$\dot{B_1} = (c_1 - b_1 A_1 + d_1 A_2)\mathrm{B}_1$$

$$\dot{A_1} = a_1 \mathrm{B}_1$$

$$\dot{A_2} = -a_2 \mathrm{B}_1 * \max(A_{2,}0)/A_2$$

In this case, the growth boost from citrate simply declines as it is depleted, while the inhibiting effect of lactate increases as before. This results in a combined time series as shown in Figure 7.

5.5. Removal of an inhibitor, e.g. Lc lactis benefiting Lb. kefiranofaciens by consuming citrate

This model is the first to explore a mutual interaction between two species. Blasche et al note that amino acid production does not start significantly until the citrate is depleted. They suggest that this is due to the inhibiting effect of citrate on the ability of *Lb. kefiranofaciens* to split lactase into peptides and amino acids. *Lb. kefiranofaciens* can metabolise lactose but displays a reduced growth rate initially that accelerates with time.

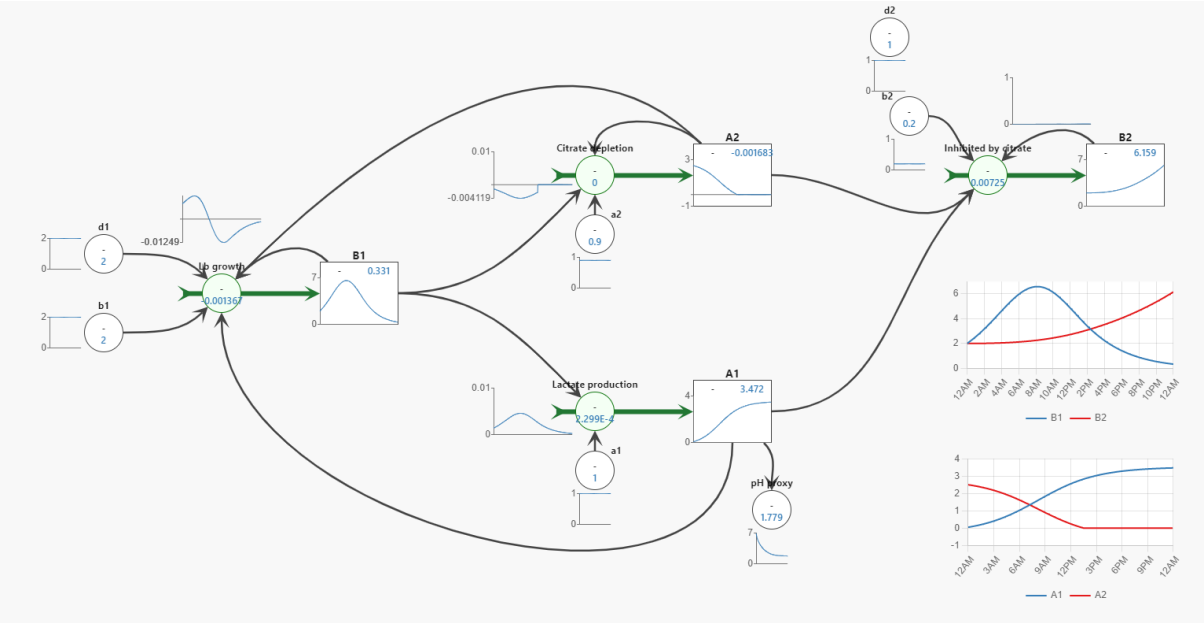
This model is still a work in progress. Below is a first attempt at the equations and simulation for this scenario. However it does not yet include a constraining factor for *Lb. kefiranofaciens*, nor the beneficial effect on it of lactate, nor the inhibiting effect of its lactose metabolism on *Lc lactis* due to the additional lactate generated.

$\dot{B_1} = (c_1 - b_1A_1 + d_1A_2)B_1$

$\dot{A_1} = a_1\mathrm{B}_1$

$\dot{A_2} = -a_2B_1 * max(A_{2,}0)/A_2$

$\dot{B_2} = (\mathrm{c}_2 + b_2A_1 - d_2A_2)\mathrm{B}_2$ *** this is still WIP



The next step in this project will be to complete this model.

6. Model of grain formation

To model the transfer of microbes from batch to batch, we need to model the process of grain formation. Reviewing the relevant literature has revealed insights that will be helpful in due course when building this model.

Wang et al investigated the surface properties, coaggregation abilities and biofilm forming capabilities of the key microbes in kefir in order to develop a proposal on how grains form. Their findings suggest that

> "…grain formation begins with the self-aggregation of Lb. kefiranofaciens and S. turicensis to form small granules. At this point, the biofilm producer, Lb. kefiri, then begins to attach to the surface of granules and co-aggregates with other organisms and components in the milk to form the grains. On sub-culturing, more organisms attach to the grains resulting in grain growth." (Wang et al., 2012).

They illustrate this process using the diagram in Figure 8. They point out that the self-aggregation process only starts at low pH (~4.2), which agrees with the finding

from (Blasche et al., 2021) that grain growth was low until the 24 hr point, at which pH ~= 4.

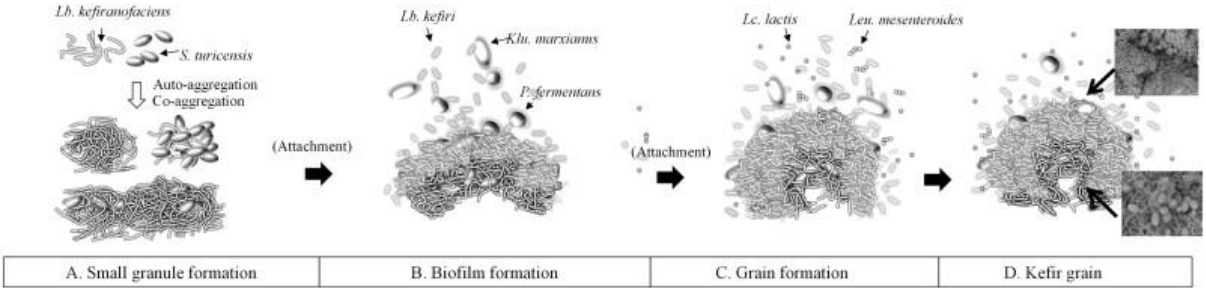


Figure 8: Grain formation (illustration from Wang et al (2012))

This means that the microbial mix at the end of the 24 hr cycle will have the greatest impact on grain composition. In fact, we used these findings from (Wang et al., 2012) to prioritise the species to focus on for the milk modelling.

7. Next steps

The following next steps are envisaged for the research:

a) Complete the inhibitor removal model described in 5.5
   i. Add the missing parts identified
   ii. Add a further mutually beneficial interaction exemplified by the interaction between *Lb. kefiranofaciens* and *Leuconostoc mesenteroides*, which cannot split lactase itself and so benefits from the peptides and amino acids made available. Introducing *Lc. mesenteroides* requires modelling the inhibiting effect of acetic acid on *Lc lactis*, a feedback that will increase the non-linearity of the model.
   iii. Consider adding a model of pH reduction with optimal pH ranges for key microbes.

b) Build and integrate a yeast dynamics model. Non-lactose fermenting yeasts such as *Saccaromyces cervisiae* auto-aggregate with *Lb. kefiranofaciens* to kick-start grain formation, so will be important for the future evolutionary-scale model.

c) Characterise the milk model dynamics

d) Build a grain model & integrate (nest) the 2 models

e) Explore overall dynamics & stability

f) Assess significance of outcomes

It is possible that simulation will have to move from Silico to R as the model becomes more complex.

Figure 9 shows a draft timeline for the research aspects of the project, alongside the parallel timelines for the presentation, poster session and report writing. It will be helpful to discuss the viability of this timeline during our review meeting.

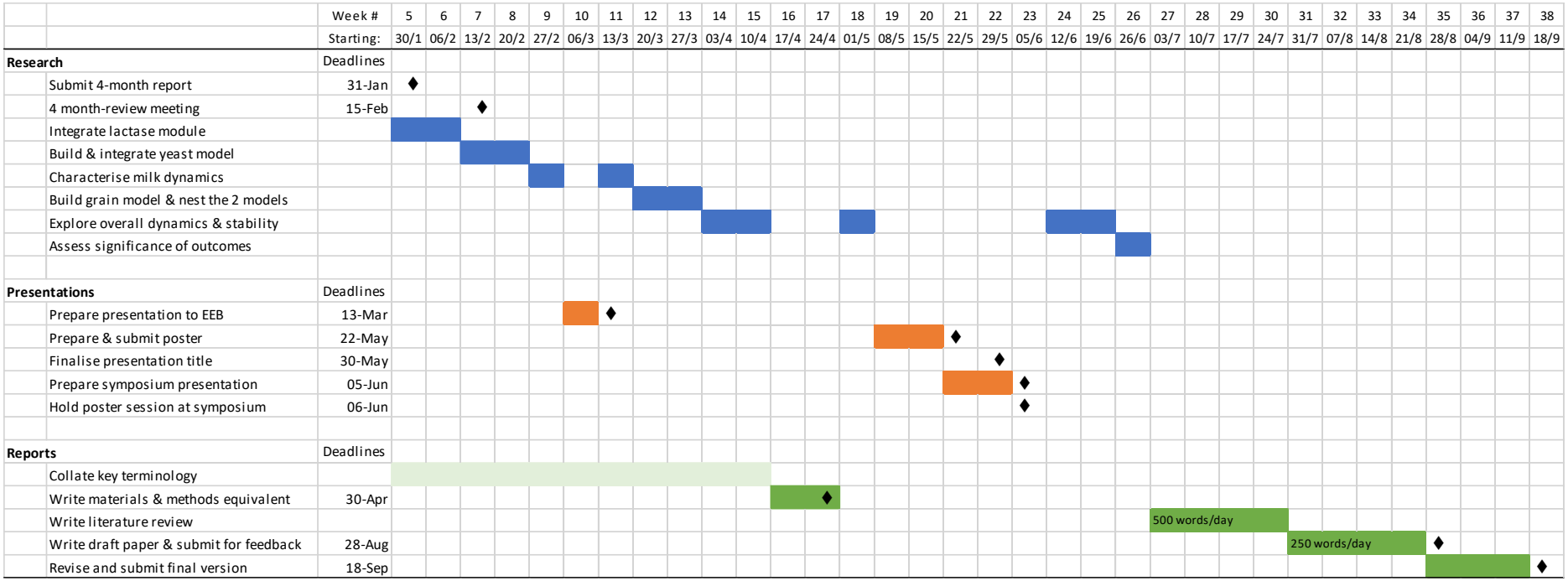


Figure 9: Draft Project Timeline

8. Acknowledgements

I'd like to express my appreciation to my supervisor, Prof Vincent Jansen, for his insight and thoughtful guidance, and to my advisor, Dr Cristina Garcia, for her advice and encouragement.

9. References

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