# Modelling the Role of Microbial Interactions in Kefir Community Dynamics

MSc by Research in Biological Sciences Royal Holloway, University of London

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# **Declaration of Authorship**

I, Julie Anne Rousseau, hereby declare that this thesis and the work presented in it is entirely my own. Where I have consulted the work of others, this is always clearly stated.

Julie Rousson

Signed: \_\_\_\_\_

Date: 14 January 2024

# Abstract

This document is an alternative format dissertation submitted for the degree of MSc by Research in Biological Sciences. Rather than a monograph, this format consists of a literature review (Part I) followed by the manuscript of a paper prepared for submission to a journal, in this case the Journal of the Royal Society Interface (Part II). The literature review sets the broader context for the research questions addressed in the paper.

A key goal of modelling in microbial ecology is to understand how community-level properties such as stability, resilience and adaptability emerge from the structure and composition of a community. My aim was to develop a minimal population dynamics model, using kefir for my case study, capturing the way that multiple aspects of metabolically-mediated interactions combine to influence the stability of the community in its ecological context. Kefir is intriguing due to its enduring viability despite short term structural variability and sensitivity to disturbance. Most mathematical modelling approaches simplify microbial interactions by using by using the mathematically tractable yet ecologically atypical model context of a chemostat. However, a different approach is required for kefir fermentation, a sequential batch process where primary resource replenishment is periodic and is accompanied by significant population disruption.

In my literature review (Part I), I outline some of the characteristics of kefir that make its enduring viability a puzzle. I review different modelling approaches and summarise potential sources of stability to model, such as a self-generated biofilm, spatio-temporally modulated interactions and diversity of metabolic roles.

For my project, described in the research paper (Part II), I developed a hybrid discrete and continuous dynamics model, driven by metabolic interactions modulated by spatiotemporal factors. The stability properties for kefir demonstrated using this hybrid model differ from those established in a chemostat. My results show that the discrete across-batch dynamics is the primary driver of long-term stability characteristics, despite the variable within-batch structure. I show how the model dynamics is modulated by the history of community composition preserved in the kefir biofilm, and argue that this mechanism provides a stability advantage for biofilm-forming communities. This approach could inform broader research into the dynamics of microbial communities subject to fluctuating resource availability.

# Preface

This project arose out of my interest in the design of complex adaptive systems. Ten years ago I was working as a consultant helping businesses to change their organisations to make best use of digital technology. I had spent the years since my undergraduate mathematics degree consulting across a broad range of industries, helping clients to make decisions in complex and uncertain situations. In the defence industry, I'd helped evaluate guidance and control systems for aircraft in operational contexts. In the environmental sciences I'd helped design management strategies to nurture new fishing industries alongside artisanal fisheries in developing countries. The maths was surprisingly similar.

In the business world, I'd developed a range of tools to help make decisions in various contexts on the cutting edge of digital technology. Organisations had to instil new ways of working, but the digital scene was changing faster and faster. Clients started asking 'how can we structure our organisation to be able to adapt to any change, even change we can't foresee?'.

That question triggered me to become interested in the notions of resilience, robustness and adaptability, how they relate, and how to design for them. At the same time, friends in systems engineering were grappling with how to design resilience into complex sociotechnical systems. Often, the bigger the engineered system, the more fragile it becomes. I started collaborating with systems scientists thinking about how systems properties like resilience emerge.

As it happened, during the pandemic years I started making kefir, a fermented milk drink a bit like yoghurt but a much more complex community. I noticed that resilience in kefir looked rather different to the resilience goals of typical engineered systems. I started reading and wondering about the organisational principles underpinning microbial systems. It seemed to me that engagement between engineers, business consultants and biologists could be fruitful. I started exploring the idea of building connections between research groups and realised I needed to understand the subject and the community better. One thing led to another, I enrolled at RHUL, and this literature review and research paper is a first step along that path.

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# **Part I: Literature Review**

## 1. Introduction

This 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 flavour and consistency 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 to humans are both 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.

The aim of this literature review is to draw together relevant insights and explain how these influenced the approach taken in the project. The project and its outcome are presented in Part II.

## 2. Microbial communities and their collective features

In 2020, the Philosophical Transactions of the Royal Society B carried a special issue entitled "Conceptual challenges in microbial ecology". The guest editors for the issue pointed out that, in order to predict an ecosystem's health or resilience, it is essential to understand the ecology of the microbial communities that play a role in it. They argued that the technical challenges that had previously constrained such research are now being met and descriptive studies are multiplying, but that this is exposing a lack of suitable conceptual frameworks for using the new data to understand microbial communities better (Prosser & Martiny, 2020).

Four key themes emerged in that special issue: the interplay between ecological and evolutionary processes; the need for more attention to the interactions between microbes; the importance of space for microbial communities, and the relationship between community composition, functioning and response to environmental changes. This review will focus primarily on the interactions between microbes and their impact on community endurance.

#### 2.1. Interactions within microbial communities

It is well known that many microbes live off the metabolic byproducts of other microbes. Microbes release a wide range of metabolites into the environment, for various reasons. Some may be metabolic waste products selectively released, or uncontrolled leakage of an intermediate metabolite. Enzymes are released to facilitate extracellular breakdown of complex biomolecules, and some of these may be lost to the environment. There may be anti-microbial compounds produced to inhibit others, or molecules mediating social interactions such as communication and cooperation.

The environment around a cell, referred to as the exometabolome, can provide a rich resource for other community members (Douglas, 2020). When a metabolite produced by one microbe is further metabolised by another, it is generally known as metabolic cross-feeding. There are some who only use this term if the interaction involves a two-way exchange of metabolites, and other authors set even more stringent requirements. A complex but inconsistent classification language has evolved to capture a range of nuance in the potential nature of metabolite-mediated interactions. These are reviewed by Smith *et al.* (2019), who advance a classification of types of cross-feeding that they suggest is internally consistent and consilient with majority use. Particularly problematic are commonly used terms such as cooperation, which are both polysemic and hard to defend from evidence. Here I will follow Smith *et al.* and use metabolic cross-feeding as a neutral general term, as defined above.

Cross-feeding is pervasive in microbial communities; in fact, there is an emerging perspective that "microbial communities are fundamentally decentralized and distributed metabolic systems, and as such, are driven by the interactions between cells and resources" (Gralka *et al.*, 2020). The metabolic networks can be complex, involving more interactions than can realistically be modelled. As a result, most current research focusses on pairwise interactions (Douglas, 2020). The limitations of this approach are illustrated by the work of Chang *et al.* (2023), who conducted hundreds of pairwise competition experiments with microbes that co-exist stably in a synthetic community, and showed that competitive exclusion occurred in the majority of pairs. They suggest that the assembly of a multi-species community does not require individual pairs to be able to coexist, and conclude that "multispecies coexistence is an emergent phenomenon".

Emergence is an important concept in microbial ecology, and it is therefore worth briefly discussing the broader concept of emergence in systems science.

#### 2.2. Microbial communities as systems

Aristotle first identified a system as something where "the whole is more than the sum of its parts", in contrast with "a mere heap". In the ensuing years many definitions of the system concept have been formulated, each grasping at some aspect of the idea. For example, Ludwig von Bertalanffy (1956) defined a system as "a set of elements standing in interrelations", and Anatol Rapoport (1968) defined a system as "a structure that functions as a whole in virtue of the causal interaction between its parts".

In 2018, a team of Fellows of the International Council on Systems Engineering (INCOSE), led by Hillary Sillitto, analysed the scope and context of more than a hundred definitions of 'system', with the aim to distil an encompassing definition. Their recommendation was to define a system as "an arrangement of parts or elements that together exhibit behaviour or meaning that the individual constituents do not". They also

presented a second definition speaking to the *nature* of systems, describing a system as "a persistent region of low entropy in physical or conceptual space-time". From this they propose that "systemness is the phenomenon that allows regions of organization to persist in a dissipative universe" (Sillitto *et al.*, 2018).

In the introduction to his edited volume "Organization in Biology", Mossio (2024) argues that organization has been a blind spot in biological thinking for the last century, and calls for "an explicit and specific notion of organization". The definitions above are significant to the current context in that they speak to questions about whether some multi-species groups of microbes have an identity as a whole, or are simply coexisting individual species. Most systems definitions highlight the role of organization in the answer.

A key characteristic of a system is that it has properties (and therefore behaviours) that the parts do not have by themselves. These are termed *emergent properties*, and are in contrast with *resultant properties*, which can be explained by summing those of the parts (Elder-Vass, 2005). The next section reviews emergent properties that are significant in microbial ecology.

#### 2.3. Emergent properties of microbial communities

In his influential paper "What is microbial community ecology?", Konopka (2009) defined the emergent properties of a microbial community as "characteristics not identifiable by analysing the component organisms in isolation". A related definition is "any pattern or function that cannot be deduced as the sum of the properties of the constituent parts" (van den Berg *et al.*, 2022). These are compatible with the definition from systems science given above, the difference only being one of perspective; whereas the biology ones provide a way of recognising emergence in a natural system, the one from systems science, which was targeted at engineers, speaks to a situation where emergence is the planned outcome of a new design.

Examples of emergent properties of a microbial community fall in the categories of function (e.g. biochemical capabilities), viability (e.g. resilience after perturbation) and adaptability (e.g. stable coexistence). Systems science has identified a large number of such properties, collectively known as the 'systemic virtues', by analogy with the 'theoretical virtues' (Rousseau *et al.*, 2019).

Of particular interest for this project is the emergent property stability, with associated concepts of resistance (robustness), resilience and adaptability. In his chapter on complexity, diversity and stability, Justus (2008) makes clear the many and varied interpretations of these terms. It is noteworthy that all the definitions of ecological stability that he cites reference stability in terms of the composition of a community and the abundance of its members, specifically how effectively these are restored after perturbation. Variations are essentially differences in interpretation of the words 'abundance', 'effectively' and 'restored'.

Such an approach to stability is challenging for microbial community ecology. Horizontal gene transfer means that evolutionary change can occur over ecological timescales, with variations in taxa readily arising. Moreover, it is clear that the same role in a community can be played by different microbes, so replacement of one taxon with another does not necessarily destabilise the community. Microorganisms also have the ability to change their gene expression in response to changes in the environment, thereby changing their individual role as needed.

This fluidity has led to a shift in focus in microbial ecology towards thinking about stability of community *function* rather than composition. Community function is itself an

emergent property of a system, and usually refers to some biochemical capability of the system that could not be replicated by any of the individual species. More generally, Lemke and DeSalle (2023) suggest that diversity is usually not the only goal when trying to restore an ecosystem, and call for new methods of characterising them.

There are, in fact, indications that stability may be more directly linked to function than structure. For example, Smith *et al.* (2022) demonstrated experimentally that microbial communities could adapt to significant changes in temperature, but that this adaptation was driven by shifts in the species composition towards species with appropriate functional capabilities. Some of these shifts were to species that had been latent in the community, providing a reserve of functional diversity.

An underappreciated aspect of the emergence of properties in a complex system is that this is balanced by the submergence of some of the properties of some of the components of that system (Rousseau, 2018). In this case, the efficiencies and stability of the community are arguably supported by the loss of capability in individual strains. Machado *et al.* (2021) observe that there are clear advantages from division of labour, as evidenced by the higher abundance of cooperative communities and their ability to occupy more diverse habitats. Division of labour is well known within microbial biofilms, and possible mechanisms have been proposed whereby this might arise (Momeni, 2018; Smith *et al.*, 2019).

Many have suggested that microbial models could more usefully model functions than species, however a complication is that the relationship between community structure and function is still unclear. One of the most urgent questions raised in (Segrè *et al.*, 2023) is how community function arises from the functions of the members and the interactions between them.

In my research, I am using kefir as my case study community. The following sections will elaborate on how the general capabilities of microbial communities play out in the case of kefir.

#### 3. Kefir as a model microbial community

#### 3.1. Introduction to kefir and kefir research

Kefir is a fermented milk product produced by inoculating milk with kefir grains, small rubbery structures that look like cauliflower florets (Figure 1). The kefir grains are comprised of a biofilm accommodating some 40-60 different taxa of bacteria and yeasts, embedded in a self-produced exopolysaccharide matrix (EPS), kefiran.

To make kefir, grains are inserted into fresh dairy milk and left at room temperature (18 - 27°C) for 24 - 48 hours. Over this period, the pH drops to the characteristic 4.6 - 4.2 range and the milk becomes viscous and flavourful. The kefir product is then sieved to recover the



Figure 1: Kefir grains



Figure 2 : Kefir making process

grains, which are then inserted into a fresh batch of milk (Figure 2). The resulting kefir drink is either consumed directly or ripened for an additional period.

Kefir is effective at prolonging the shelf life of milk. Although the community is known to accept some new members, it is also highly effective at eliminating others, including human pathogens and microbes responsible for spoilage. Some of this action is due to the lowered pH, but kefir bacteria are also known to produce a broad range of targeted antibiotics (Rattray & O'Connell, 2022). It has also recently been found that kefir yeasts can produce a molecule that disrupts quorum sensing in some pathogenic bacteria (Malka *et al.*, 2021). Walsh *et al.* (2016) demonstrated that the significant diversity of microbes found in normal pasteurised milk can be almost entirely replaced by kefir microbes after 8 hours of fermentation.

In addition, drinking kefir has traditionally been claimed to confer significant health benefits to humans. In recent times, many studies have been conducted to investigate these claims and to try to identify potential mechanisms involved. Various authors have reviewed this research and highlighted findings suggesting benefits including antimicrobial activity, tumour suppression, wound healing, immunomodulation, anti-inflammatory and antiobesity action, cholesterol lowering, antioxidant effects, lactose tolerance improvement, alleviation of fatty liver and enhancement of intestinal bacterial flora, e.g. (Rosa *et al.*, 2017; Bengoa *et al.*, 2019; Farag *et al.*, 2020). Such health benefits may be ascribed to the presence of probiotic micro-organisms and/or metabolic products in the fermented kefir milk.

A significant amount of experimental kefir research has therefore 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 (Arslan, 2015; Tan *et al.*, 2020, 2022). Much attention has been directed at the microbes' metabolites and other compounds they synthesise. 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 metabolic interactions between them.

This research has resulted in a wide range of observational data about the kefir ecosystem, which is valuable for theoretical research. We will discuss the findings in the following sections.

#### 3.2. Kefir research approaches

Before turning to the outcome of academic research into kefir, it is worth highlighting some cautions about our ability to compare findings across different studies. There are, as yet, no formal protocols for conducting kefir research in academia, and each research group seems to define its own way of obtaining and preparing the kefir that they study.

For this review we will focus on research using kefir grains. It is worth highlighting that grains themselves are rarely used at an industrial scale, because they grow relatively slowly (about 10% per day), are sensitive to environmental conditions, produce inconsistent results and require costly processes (Nejati *et al.*, 2020). For this reason, commercially available 'kefir drinks' are typically made with a small subset of reference bacteria isolated from kefir, and no yeasts. Much research therefore focusses on such synthetic collections. Where grains are used in research, fermentation methods for producing kefir milk from grains vary widely.

Outside the academic context, there are groups of artisanal kefir makers who use social media to exchange their experiences with kefir-making, see e.g. (Kefir Facebook Group, 2023; Kefir Reddit Group, 2023). These makers regularly conduct informal experiments, whether planned or unplanned, and share their results. My own first foray into kefir-making was guided by this helpful community. The group is particularly enriched by those who share their personal knowledge of traditional methods that have been handed down in families for generations. Although this document is primarily focussed on the kefir *microbial* community, it is worth pointing out that the kefir community also contains a human. There is no mechanism for kefir to endure without one, and in fact the human in each system is the primary source of selection at community level. I have on a number of occasions inadvertently shifted culturing conditions sufficiently that the resulting kefir, although drinkable, was not particularly pleasant, and found myself unable to recover it to a better state. In this situation I discarded my grains and acquired new ones from a reliable source. Kefir making procedures have perhaps co-evolved with the grains in this manner.

Researchers ignore this body of knowledge at their peril. Although it can be cluttered with speculation, there is also valuable insight into the behaviour of the kefir community, in particular the sources of variability. In what follows I will reference information from artisanal makers where some apparent consensus has emerged, or where it suggests testable ideas.

Artisanal makers affirm that kefir is best made at temperatures in the range 18-27°C, with grain weight to milk volume ratios in the range 2-5%, in open jars and stirring 2-6 times in total over a typical 24 hour ferment, as affirmed in Bengoa *et al.* (2019). It is well known that the outcome is extremely sensitive to changes in these and other conditions, with a shift in microbial composition detectable as a change in flavour and viscosity of the kefir milk (Walsh *et al.*, 2016; Duran *et al.*, 2022). Surprisingly, academic studies report research using significantly different parameters, with temperatures up to 37°C and concentrations of 10% or more. The stirring regime is rarely reported, but automated stirring at 30-90rpm is not unknown. Whether culturing is aerobic or anaerobic is generally not mentioned.

Artisanal makers concur that any significant change to the kefir environment will result in a period of instability that will eventually settle. It is recommended that any dramatic change such as milk type or fermentation length is implemented in steps, 25% per week or more, with intervening periods to stabilise. There are many research projects that compare fermentation under different conditions, e.g. (Schoevers, 1999; Apar *et al.*, 2017), but I have never seen any mention of stabilisation steps between each.

The likelihood therefore is that each research project is working with a differently assembled kefir community. Researchers should thus be cautious when drawing general conclusions from across the literature. That said, although the community composition may vary, there are characteristics indicative of kefir that remain consistent. This suggests that these studies may be similar enough to inform research into community stability from a *functional* perspective, if not a structural one.

There have recently been increasing calls for closer coordination between experimental data collection (with associated method development) and mathematical model building (with associated theoretical research) (Widder *et al.*, 2016; Segrè *et al.*, 2023). It has been suggested that fermented foods are 'experimentally tractable microbial ecosystems' that could be valuable models for the processes in larger communities (Wolfe & Dutton, 2015). Given that kefir is small enough to be manageable, complex enough to be interesting, fast and easy to culture and functions naturally in a closed environment, I suggest that it provides an ideal community for which such a hybrid research approach could be productive.

## 3.3. Kefir community composition

The earliest modern historical records of kefir connect it with the Caucasus mountains, although traces of indicative genetic material have been found in early bronze-age China sites (Yang *et al.*, 2014). It was a closely guarded secret until the late 19thC when some grains were obtained by subterfuge and subsequently spread rapidly around the world.

Samples of kefir grains 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). In addition, Walsh *et al.* (2016) report that strains in a particular kefir are more closely related to each other than to strains from other kefirs. For a comprehensive review of the bacterial and fungal taxa found using high throughout sequencing on 25 kefir milks and associated grains from 8 distinct regions, see Marsh *et al.* (2013). Tibetan kefir grains may have a unique origin as they typically contain 200-300 microbial taxa, compared with 40-60 in European grains and their descendants worldwide. This includes a far greater diversity of fungal taxa (Liu *et al.*, 2019).

As Gralka *et al.* (2020) point out, periodic perturbations create opportunities for communities to reassemble. It is thus unsurprising that kefir populations in samples from environments with different culturing conditions vary significantly.

The kefir grains are an essential component in the fermentation process. Unlike yoghurt, kefir cannot be fermented by using the end product as a starter for the new batch; after a few transfers without grains, core microbes die out and the community collapses (Blasche *et al.*, 2021).

In addition, unlike vinegar mothers or kombucha scobys, if the constituent species are simply combined, grains will not form spontaneously, nor will they form from kefir milk alone (Walsh *et al.*, 2016). In an attempt to shed light on the possible origins of kefir, Motaghi *et al.* (1997) reported producing kefir-like grains in a goat-hide bag using pasteurized milk inoculated with sheep intestinal flora in a complex process spread over 12 weeks. They subsequently isolated 11 microbes from these kefir-like grains.

Grains reproduce readily from other grains however, growing about 5-10% during a typical 24 hr fermentation cycle. Wang *et al.* (2012) researched the cell surface properties of kefir microbes to formulate a theory about each's role in grain formation. They show that, of the core kefir microbes, *Lactobacillus kefiranofaciens* and yeast *Saccharomyces turicensis* are strong auto-aggregators at low pH, and *Lactobacillus kefiri* is a strong biofilm producer. None of the yoghurt microbes they investigated demonstrated these capabilities. They propose

that grains are formed when *Lb. kefiranofaciens* and *S. turicensis* co-aggregate to form small granules, to which *Lb. kefiri* attaches to produce the biofilm, and to which other microbes attach to form the grains. They suggest that co-aggregation is supported by electrostatic interactions between the positively charged, hydrophobic LAB and the negatively charged yeast at pH 4.2. The low pH may account for the fact that grain growth is reported to increase towards the end of a fermentation cycle, where pH is lowest (Blasche *et al.*, 2021).

Although grains appear roughly spherical, closer inspection shows that they are formed of a flat sheet that has expanded at multiple points into fractal-like bubbles on one side, thus causing the sheet to curl around the smoother side. The distribution of microbes within the grains is not uniform. Overall, microbial diversity is greater in the inside layers of the grain than the outer surface (Dobson et al., 2011). Short-chain lactobacilli such as the biofilm producer Lb. kefiri tend to be found near the surface of the grains, whereas long-chain



Figure 3: Section through a kefir grain showing microbes embedded in kefiran (© Science Photo Library C028/3314)

lactobacilli such as *Lb. kefiranofaciens* occupy the interior (Wang *et al.*, 2012). *Lb. kefiranofaciens* synthesises kefiran, the EPS matrix for the grains. It is known to be demanding in its growth conditions, for example it is strictly anaerobic, so it is unsurprising that it favours and indeed dominates the interior of the grains (Georgalaki *et al.*, 2021). Non-lactose-fermenting yeasts favour the deep layers of the grains, whereas the lactose-fermenting ones, which are in the minority, are found on the grain surface (Rattray & O'Connell, 2022).

This heterogeneity demonstrates the potential for specialised niches within the grain structure. The kefiran matrix may be a key contributor to this, given that physical environments with finer structure are known to sustain a wider variety of competitive species and can thus potentially stabilise microbial communities (Ursell, 2020). We will return to this topic later on.

## 3.4. Metabolic interactions between kefir microbes

Blasche *et al.* (2021) observed the dynamics of the core kefir species over a very long fermentation, sampling 18 times across this period (90 hours compared with the traditional 24 or 48 hr). They showed that the abundance of different microbes in the kefir liquid changes significantly over this period (see Figure 4), with the most rapid change around the usual 24 hour end point. They suggest that cross-feeding relationships drive the sequence of microbes that emerge, with successive metabolic processes opening up new metabolic niches for other species. It is striking that individual species start growing in a staggered fashion, with *L. kefiranofaciens* and *Lc. lactis* the first to increase in abundance. *L. kefiranofaciens* initially grows more slowly than the others, but as the manufacturer of the grain matrix, it

dominates the grains and therefore has a head start in the milk. *Lc. lactis* grows the fastest, reflecting the fact that it is individually well suited to the milk environment. In contrast, Blasche *et al.* (2021) demonstrated that *Lb. kefiranofaciens* is unable to grow on its own in milk, suggesting that it needs the metabolic support of the community to thrive.



Figure 4: Normalised species abundance over a fermentation cycle, in the grain (left) and milk (right) (reproduced from Blasche et al, 2021)

Walsh *et al.* (2016) confirm the dynamic nature of kefir fermentation, although measuring a more decisive shift in the latter part of the fermentation cycle towards the prominence of heterofermentative species such as *Leuconostoc mesenteroides*. They show how the change in dominant species in the liquid correlates with the decline in pH, and suggests that this reflects both the pH lowering capability and pH tolerance of various species.

Both Blasche *et al.* (2021) and Walsh *et al.* (2016) emphasise the microbial succession patterns they observe, and suggest that it results from microbes modifying the environment in such a way that makes it more suitable for others. Blasche *et al.* (2021) reported metabolomics evidence of cross-feeding, e.g. via metabolites that accumulated initially and then depleted later. Nejati *et al.* (2020) review types of interactions found in the categories bacteria-bacteria, yeast-yeast, and yeast-bacteria interactions.

Some of the kefir microbes can split lactose into glucose and galactose, but many cannot. Subsequent glycolysis by homofermentative lactic acid bacteria produces lactic acid, which causes the pH to drop rapidly and enables heterofermentative lactic acid bacteria to become active despite the temperature being suboptimal for them. Some microbes metabolise lactate (e.g. *A. fabarum, Lb. kefiranofaciens* and some yeasts), thus slowing the pH decline, to the benefit of the lactate producers (Walsh *et al.*, 2016). By the end of fermentation approximately half the lactose remains, showing that lactose itself is not a limiting resource

(Blasche *et al.,* 2021). These feedbacks illustrate the way in which metabolic interactions can serve to benefit multiple parties.

Turning to proteolysis, Blasche *et al.* (2021) report that a number of amino acids only started accumulating after 20 hrs, which is towards the end of a typical 24 hr fermentation. Likewise, significant growth in most of the community only started at the 20 hr mark, which they note coincides with the depletion of natural citrate in the milk. They speculate that citrate may interfere with the action of metalloproteases produced by microbes such as *Lc lactis*, that are needed for the initial splitting of complex milk proteins. Not all of the kefir microbes are capable of this step.

While many amino acids accumulate as expected, aspartate and glutamate do not, and any starting amount is rapidly depleted. This suggests these are key limiting resources for kefir microbes. As an illustration, Blasche *et al.* (2021) demonstrated that *L. mesenteroides* benefits from proximity to *A. fabarum*, which is known to synthesise aspartate. Yeasts also provide essential growth nutrients to bacteria, such as amino acids and vitamins (Arslan, 2015).

A peptidomic analysis of the protein metabolism of kefir microbes by Dallas *et al.* (2016) showed that fermentation was accompanied by changes in abundance of thousands of peptides derived from 27 milk proteins. They also found hundreds of protein fragments released either by kefir microorganisms or native milk proteases. These provide ample opportunity for cross-feeding, as illustrated by the large number of pairwise interactions identified in Blasche *et al.* (2021).

Blasche *et al.* (2021) report that the relative abundance of microbes in the grains changed much less, although they only measured it at the start and end of their 90 hour fermentation. Investigating this further, they examined pairwise interactions between microbes in milk and grain-like (milk agar) environments, and categorised the types of relationships as competitive or cooperative based on whether or not co-existence increases or decreases species growth or acidification of the environment, compared with a monoculture. They suggest that their results indicate a switch between cooperation amongst community members in the milk phase, and competitive interaction within the grains.

Blasche *et al.* (2021) suggest that because *Lb. kefiranofaciens* synthesises the grain it can maintain its dominance while 'carrying along' other members of the community to help metabolise the milk nutrients. This seems almost to imply an orchestrating role for *Lb. kefiranofaciens*. Georgalaki *et al.* (2021) see it otherwise, pointing out that *Lb. kefiranofaciens* is strictly anaerobic, so naturally tends to occupy the interior of the grain. These distinctions are important and touch on the issues to be covered in the next two sections.

## 3.5. Specialisation, versatility and redundancy in kefir

In the previous section, I mentioned that the dominant kefir species *Lb. kefiranofaciens* cannot thrive on its own in milk. Strangely, it has not been found anywhere else (Blasche *et al.*, 2021). It therefore depends on the rest of the community for its survival, and is known to be very particular about its culturing conditions. It is not expendable however, being the microbe that synthesises kefiran, the EPS at the heart of the grain. Equally important are the biofilm producers that create the rest of the grain. Given that the kefir community cannot persist in the absence of grains, these services are essential for the persistence of the ecosystem and are, notably, provided by specialist players.

The metabolic processes necessary to produce kefir are likewise distributed amongst taxa, although here more redundancy can be observed. Walsh *et al.* (2016) used whole metagenome sequencing to characterise the metabolic functions available within the kefir microbiome at different stages of fermentation. They noted for example that genetic pathways associated with carbohydrate metabolism were more prevalent in the early stages of fermentation, while pathways involved in amino acid metabolism were more prevalent in the latter stages of the cycle. Some of these pathways were associated with particular taxa and not with others – *Lb. kefiranofaciens* had far fewer pathways present than the yeast *S. cerevisiae*, for example. However, many pathways are available to multiple taxa. This type of redundancy ensures that the function can be executed across a wide variety of environmental conditions, as different microbes will have different tolerance limits.

Kefir also contains microbes that are versatile generalists. *Lc. lactis* can split both lactose and casein into metabolic precursors, and it can metabolise both glucose and citrate via multiple paths depending on conditions such as pH (Hugenholtz, 1993). It can grow and thrive perfectly well in milk on its own and is important for opening up metabolic opportunities for others.

Kefir is known to adopt local species into the community, while eliminating others, leading to the large geographical differences in kefir community composition. This suggests that the metabolic interplay is based on functional capabilities rather than specific species.

Section 2.3 discusses the manifest benefits to the community of division of labour, which requires specialization, as well as versatility, which requires redundancy and generalization. The question remains how these different roles emerge, coexist and rebalance as a result of the independent activities of the individual microbes.

## 3.6. Variability and stability in kefir

Given the evidence presented above, it is perhaps unsurprising that the flavour of kefir is hard to control. Kefir's flavour is strongly influenced by the aromatic metabolic products produced during its fermentation. These are determined by the species involved and the metabolic pathways in use, for which there are many options. The balance of the particular pathways in use varies throughout fermentation due to successive metabolic interactions, and they are highly sensitive to initial conditions and environmental factors.

Kefir fermentation is a batch process, so there is no resource influx and there is thus no non-trivial steady state for the batch. Artisanal producers report that if kefir is left unattended for too long, the grains will eventually dissolve or become discoloured and unrecoverable. The observed population changes in the milk thus represent non-equilibrium dynamics and cannot converge to a state of balance. When the kefir product is ready for consumption, the system will be interrupted while still in flux.

Some authors claim that the microbial composition of the grains does not change significantly during a fermentation, and therefore label the grains 'stable', e.g. (Garrote *et al.*, 1998; Blasche *et al.*, 2021). A true steady state throughout fermentation is implausible, given the dynamics in the milk and the permeable boundaries between the milk and grains. Blasche *et al.* only measured the start and end composition of the grains, so it is an open question whether there were dynamic changes in the grains between those points.

A more interesting question is whether the kefir system, as a whole, converges over multiple fermentation cycles to some repeatable fermentation pattern. This would arise if the grain population was the same at the beginning and end of the fermentation, despite any changes in between, so that the next cycle would unfold in the same way, providing external conditions remained constant.

The research projects discussed so far have focussed on the dynamics of a single fermentation cycle. Very few papers report investigating the dynamics across multiple cycles. De Almeida Brasiel *et al.* (2021) conducted daily fermentations for 30 days using the same grains, and used high-throughput sequencing to compare the kefir milk at the end of the 30 day period with a pool comprised of samples from each day. They observed that although the community composition of the two were similar, there were significant differences in the relative abundances at the end of the period, indicating daily population changes. They concluded that microbial community succession and quality changed over the 30 days. On the other hand, Vardjan *et al.* (2018) used grains from a commercial kefir manufacturer and continued their process for 10 weeks across dual laboratories. They reported remarkable consistency in the composition of the milk and grains, as well as the product quality, at the end of each batch.

These apparently contradictory results are both supported by reports from artisanal makers, who experience periods of day-by-day consistency suddenly disrupted by small changes in the weather, or seasonal changes in milk. For example, the nutrient content of summer milk from cows grazing on pasture is different to that of winter milk from cows fed on silage and leads to recognisable changes in the kefir product. The latter authors may simply have used grains already established in a long term stable state.

It is worth pointing out that, despite changes, the resulting product is always recognisably kefir, albeit with different aromatic notes and viscosity. If we were to consider functional stability of the community, where the function in question is to produce drinkable kefir, this system could arguably be considered stable. If the function is to produce kefir of a consistent flavour and mouthfeel, as industrial producers require, then making kefir from grains is not yet a sufficiently stable approach.

It is unclear whether these reports reflect an inherent instability in the kefir system over longer timescales, or simply the sensitivity of an equilibrium to initial conditions. Mathematical modelling is one of the key routes for investigating whether these different outcomes are plausibly explicable in terms of the longer term dynamics of the kefir system. Different approaches to such models are reviewed in the following chapter.

### 4. Approaches to modelling Kefir

#### 4.1. Modelling objective

As we have seen, kefir population dynamics unfolds over two timescales: the short-term within-batch fermentation dynamics and the long-term batch-to-batch dynamics. Indications are that the within-batch dynamics is highly variable, yet the batch-to-batch dynamics much less so. My objective is to develop a simple model of the kefir system that can provide insight into how these two observations could be reconciled. In particular, what would have to be true about the kefir system for it to endure over the long term, despite the variability and complexity of individual batches?

## 4.2. Requirements for such a model of kefir

Given the objective framed above, it is possible to identify the key aspects of the system to be modelled. Investigating the dynamics of the kefir system over a long timescale will

require a discrete-time model representing the microbial population in the grains from batch to batch. In the case of kefir, we also have the potential to model the underlying mechanics driving this discrete-time system.

The milk is completely replaced at the start of each batch; only the grains are transferred between batches. The grain state resulting from one batch becomes the initial state for the next, providing the starting conditions for the short-term within-batch dynamics. At the start of a batch, therefore, the grains contain all the microbes, and the milk contains all the resources.

The within-batch dynamics can be represented by a continuous-time model reflecting both the dynamics in the milk and in the grains. The model must also reflect the interplay between the milk and the grains. Various metaphors have been used for this, most notably a 'basecamp' from which microbes 'emerge to colonize the milk' (Blasche *et al.*, 2021), or a 'fortress' (Flemming *et al.*, 2016). Grains could also be viewed as a refuge, a germline or even a factory with different 'assembly-line' processes operating in different micro-niches within the grain structure. Each of these metaphors could lead to a different model of the grain-milk interaction. I would argue that the (mostly pairwise) research done so far does not yet permit confident discrimination between them, however, it is perhaps more likely that all of those perspectives apply in some measure simultaneously. One caveat is that kefir microbes are non-motile, therefore microbial movement between milk and grain is likely to be largely diffusive in nature. Figure 3 also shows how tightly microbes can be embedded within the grains' kefiran matrix.

The literature discussed in earlier sections suggests that within the continuous-time model, metabolic interactions between microbes should be the primary drivers of system dynamics, e.g. (Gralka *et al.*, 2020). From a modelling perspective it is also useful to note that the majority of metabolites in the exometabolome come from living cells rather than lysis (Douglas, 2020). The milk proteins and lactose are not replenished during a single fermentation batch.

In this study we are interested in the qualitative characteristics of the dynamics within and across batches. We are more concerned with the comparison between dynamics under different initial conditions or with different parameters, than in the absolute values of the states calculated for any single instance. For this reason, parameters need not be collated from the literature for this project.

It is worth highlighting a point made by Chang (2005) about the difference between a steady state and a thermodynamic equilibrium. Although both result in concentrations of species remaining unchanged over time, biological systems are open systems and to keep them in a steady state requires a constant supply and removal of materials from the system. Such systems will, for example, contain concentration gradients, whereas a system in equilibrium will necessarily be closed and homogenous (Chang, 2005, 228). That said, many ecologists equate the two, and some go further in defining equilibrium in terms of a probability distribution of points corresponding to steady states, as discussed in (Mittelbach & McGill, 2019, 69). In the current work we will be focussing on steady states more than equilibria.

## 4.3. Modelling species dynamics

Standard ecological models such as those described in ecology textbooks, e.g. (Mittelbach & McGill, 2019), have to be adapted for use with microbial communities. A

treatment tailored for microbial ecology can be found in Kirchman (2018), along with detail on the biochemical processes that affect interactions of microbes with each other and with their environment. Van den Berg *et al.* (2022) provide an extensive review of different approaches to modelling microbial communities, particularly with a view of predicting emergent properties.

The growth of a single microbial species is typically modelled with a growth rate that reduces with population density in some way. This provides a natural limit to a population. Logistic growth corresponds to a linear relationship between growth rate and density.

For interacting species, the classic Lotka-Volterra approach models pairwise interspecies interactions in terms of the effect of each species on the growth rate of the other (Hofbauer & Sigmund, 1988). This effect is captured via interaction coefficients that can reflect positive or negative effects such as competition or predation. Lotka-Volterra models have been generalised in many ways, e.g. to accommodate more than two species, and in this form have found use in microbial models (van den Berg *et al.*, 2022). However resources are only indirectly captured in this type of model, represented in terms of their effect on species growth rates)

A more direct approach is the MacArthur consumer-resource model (MacArthur, 1970), where the abundance of relevant resources is explicitly modelled along with the population of species. In this instance, species growth rates are limited by the proximity and availability of the resources they need. This type of model allows for different timescales between resources and consumers, which enables the introduction of time lags and heterogeneity effects. For example, a Monod function is often used to incorporate spatial distance, by effectively modulating a microbe's growth rate with the probability that it will encounter the resource needed for that growth, e.g. (Posfai *et al.*, 2017). This equation has the same form as the Michaelis-Menten equation, which relates the speed of a chemical reaction to the likelihood of the chemical being in sufficient proximity to its catalyst (Chang, 2005).

In classic consumer-resource models, resources accumulate through their own growth. These can be extended, however, to include resources released as byproducts of the consumption of nutrients, e.g. (Marsland *et al.*, 2019). It has been shown that species– metabolite interaction modelling captures the dynamics of microbial communities more effectively than direct species–species modelling (Brunner & Chia, 2019).

Another approach is trait-based modelling. In this case, the community is defined by phenotypic traits rather than taxa, and the models describe how trait combinations respond to environmental variables and influence them in their turn. This approach is appealing as it encapsulates a focus on function. However, trait-based models are not designed to investigate interactions between community members; they focus more on interactions between community members and the environment (van den Berg *et al.*, 2022). So they are less obviously suitable for the current project.

#### 4.4. Modelling metabolic constraints

For the purpose of this project, some form of consumer-resource model adapted for cross-feeding seems to be the most appropriate. However, one of the limitations of this type of model is the assumption that community members have a fixed metabolism.

Finite resources lead to tradeoffs during microbial growth. For example, respiration results in more energy from glucose than fermentation does, but it takes longer to produce it. It can thus be more advantageous to use fermentation when resources are sufficiently

abundant that efficiency is not a factor. Most microbes are able to execute multiple metabolic strategies and can shift between them if the environment makes it beneficial to do so. For example, there are four possible pathways used by lactic acid bacteria to metabolise citrate, resulting in distinct aroma outcomes in kefir. *Lactococcus lactis* can utilise all of these, with preferences depending on pH and the availability of other nutrients (Hugenholtz, 1993).

Bachmann *et al.* (2016) demonstrate that tradeoffs in strategies affecting yield, growth rate and substrate affinity can arise from a common mechanism involving tradeoffs in allocation of resources. These typically involve the allocation of resources between protein synthesising activities within the cell and the production of public goods such as catalysts external to the cell.

Various authors have explored the effects of such tradeoffs on the features of ecosystems. One approach is to model a fixed enzyme budget that can be applied in different ways, representing different metabolic strategies. For example, Posfai *et al.* (2017) model the tradeoff between growth from utilising a nutrient with the cost of producing it. Taillefumier *et al.* (2017) consider the production of enzymes for transporting required resources directly into the cell, or for transporting available resources and converting them into required ones, or some combination of the two. Li *et al.* (2020) extend this type of model by including tradeoffs relating to how microbes modify their environment, and develop general procedures for assessing metabolic strategies. In the models used, there is no crossfeeding between community members and resources are supplied via a constant resource flux. All three studies examine how the tradeoffs in question influence species fitness and thus focus on the steady state conditions of those that endure.

Another limitation of consumer-resource models is that they typically do not allow for dynamic interactions between community members. Marsland *et al.* (2019) extend such a model to include metabolic cross-feeding, with such interactions being environment-dependent. Here, a fixed energy budget creates the constraint required to ensure stoichiometric realism in the conversion of one nutrient into another. Their study situation reflects a chemostat in which they investigate the impact of nutrient supply rates on community composition and metabolite flux between members.

Calabrese *et al.* (2021) presents research using a nonequilibrium thermodynamics framework to show that mass and energy conversion in microbial metabolism and growth are tightly coupled by scaling laws. These relate the thermodynamic efficiency to the electron donor uptake rate and the growth yield. This underscores the view that 'the emergence of communities with a trophic structure could be a consequence of the design principles of cellular metabolism' (Gralka *et al.*, 2020).

The above modifications provide useful examples of ways in which consumer-resource models could be adapted for modelling the kefir batch fermentation stage. It is notable though that all the studies reviewed so far ultimately model microbial communities in a chemostat context with a constant supply of resources and waste removal, that can therefore establish a steady state. Such research tends to ignore the dynamics that occurs immediately after a perturbation, and focuses only on the characteristics of the steady state achieved. There are many situations in nature for which this is an appropriate approach. However, in the case of kefir batch fermentation, the community is forever in the process of reassembly, to accommodate the way they themselves have changed their environment. My project's model and simulation will need to accommodate this situation.

#### 4.5. Modelling spatio-temporal effects

There are significant differences between the grain and milk environments that will influence community assembly. These change over time as the microbes themselves modify their environment.

Kefir grain is comprised of a biofilm assembled around a matrix built from the EPS kefiran. The structure of biofilms is very complex, with heterogeneous clusters of cells and channels for circulating liquid. The EPS matrix enables the emergence of localized gradients within the film that provide habitat diversity, captures nutrients present in the liquid and retains enzymes that predigest nutrients externally to cells (Flemming *et al.*, 2016).

These characteristics of biofilms have particular significance for community dynamics in the light of the changes that occur during fermentation. As the pH drops, certain metabolic pathways become more or less viable. Therefore, pH gradients within the grain create micro-niches in which different communities will assemble and different metabolic steps may predominate. Varying oxygen levels in the grain will similarly enable aerobic and anaerobic processes within relatively close proximity. The fact that the grain matrix can trap extra-cellular enzymes reduces the cost of maintaining an adequate concentration of them and so increases the value of a niche.

It is thus likely that grain microbes may generally be in closer proximity to the resources they need than milk ones, with correspondingly greater metabolic efficiency. This suggests that more metabolic paths may be viable at the same time within the grains than in the more homogeneous milk environment, leading to a broader active time window for each grain microbe. Moreover, if a Monod function is used to model spatial effects, as discussed in 4.3, the affinity between a given microbe and resource pair will be influenced by such considerations and be different in the grain and milk environments. Some authors recommend modelling biofilms using fractal mathematics, and have proposed that Michaelis-Menten kinetics be adapted to reflect 'fractal kinetics' by including a spatially-modulated time-dependence in the value of the affinity constant, e.g. Savageau (1995).

Given that the grains and milk hold differently constituted communities, the exchange of microbes and resources between the two environments will be highly significant in any model. In an extensive discussion of diffusion in biofilms, Stewart points out that diffusion is the predominant mode of transport within cell aggregates. He goes on to show how increasingly complex aspects of biofilms can be accounted for with diffusion limitation and diffusion-based concentration gradients. He argues that although water channels can carry nutrients deep into a biofilm, this does not necessarily allow access to the interior of cell clusters. He concludes that, barring some exceptions, most biofilm properties can be modelled as diffusion effects (Stewart, 2003).

The relative diffusivity of entities in the two media is important. Being smaller, resources will diffuse in and out of the grains more readily than microbes. This implies that at the start of a batch, the initial metabolic activity will predominantly be in the grains. Newly forming grains will be populated from both grain and milk microbes throughout the ongoing reassembly of the milk community.

#### 4.6. Discrete-time models

In many ecological systems, the output at the end of a growing season affects the initial conditions for the next one or even subsequent ones. For example, in temperate areas, leaves

fall from trees at the end of a growing season and release nutrients into the soil, which affects plant growth in subsequent seasons.

Loladze (2019) points out that this type of process is not accommodated by classical chemostat models. He introduces a modelling framework that incorporates an 'iterative chemostat' dynamic system where the final state feeds into the next iteration. In his application example he starts with a simple chemostat model of the growth of plankton using nitrogen and phosphorous, and adds a feedback loop representing the recycling of these elements following the seasonal death and decay of the plankton. By linking this to core biogenic processes he demonstrates how this type of model enables ecosystem-scale questions to be explored in a rigorous way (Loladze, 2019). Loladze's framework comprises a continuous mechanistic model of the system dynamics punctuated by annual changes in nutrient supply based on prior population levels.

Ives *et al.* (2000) added periodic mortality, such as might be seen in winter, to a simple predator prey model iterating over a longer period. They showed that the long term dynamics, while complex, still displayed regularity and its stability behaviour was amenable to analysis. That said, periodic mortality produced high variability in the population, even when the mortality itself was not highly variable. The year-to-year population dynamics depended strongly on the relative periodicity of mortality events to predator-prey oscillations (Ives *et al.*, 2000).

If a real system of this type were sampled for annual population changes, it would result in a discrete time-series. Various discrete-time population models have been introduced to fit such datasets, e.g. the Hassell model (Hassell, 1975). Discrete-time models have been well studied are well known for being able to produce extremely complex dynamics, even when the underlying equation is quite simple (Hofbauer & Sigmund, 1988, 35).

Traditionally, these models were determined phenomenologically and were effectively used as heuristics. Since then, however, a number of authors have explored ways to derive them from ecological first principles, e.g. (Royama, 1992; Geritz & Kisdi, 2004; Brännström & Sumpter, 2005). They found that these well known models were in fact special cases of more complex, generalised models punctuated by discrete events. This type of derivation provides an intuitive biological grounding for heuristic models.

Royama (1992) derived the Ricker model from first principles for a single species by assuming discrete generations of identical individuals are randomly distributed over a uniform resource environment, and modelling births as a function of the proximity of other individuals.

Later, Brännström & Sumpter (2005) generalised this approach by deriving a model incorporating competition, specified as either scramble or contest competition, and spatial distribution, either clustered or uniform. In the case of scramble competition over uniform resources, their model reduced to Royama's derivation of the Ricker model. Other special case combinations of distribution or competition type reduced to the Hassell, Beverton-Holt and Skellam models.

Geritz & Kisdi (2004) derived another more generalised model that involved population structure (eggs and adults) as well as interactions between consumers and resources. Adding the separation into within-year and between-year dynamics, they could derive various one-dimensional discrete-time models, and by assuming different types of continuous dynamics within a year, they could also derive the Ricker, Hassell and Beverton-Holt models. They go on to examine the factors that underpin overcompensation or undercompensation in the between-year dynamics. They point out that if the within-year dynamics is such that the input-output relation is non-monotonous, e.g. due to structured populations or interacting species, this will give rise to overcompensation between years, which can lead to complex dynamics e.g. limit cycles or chaotic behaviour.

For our purposes, it is useful to note that in the Brännström & Sumpter model, the initial conditions of a period are a function of the end state of the prior period, whereas in the Geritz & Kisdi model, the initial conditions are determined by an accumulation of factors (egg-laying) over the entire period. Given the role kefir grains play as a refuge, our kefir model will be closer to the latter.

#### 5. Potential sources of stability and endurance

The models considered in section 4.6 explain how periodic ecological events can cause extremely complex variability between periods, particularly due to overcompensation in one period for changes in the prior one. However, this work also suggests that despite this variability, there may be an underlying stability in the dynamics that could drive long-term convergence of the discrete between-period states, if all else remains equal.

As discussed in section 4.5, the kefir community has distinctive spatio-temporal characteristics that influence the community structure. These have the potential also to influence its long-term viability.

Firstly, the literature shows that kefir is a complex community with many metabolitemediated interactions between its members, and that these interactions are contingent on metabolic pathways that are sensitive to time-dependent environmental conditions. From this literature, I infer that the kefir community contains both metabolic generalists (species with multiple metabolic options) and specialists (species with rare functions) and that there is a degree of functional redundancy (duplication) in most specialisms (multiple species with the same rare function). Specialists will likely be most efficient in their own niche, but generalists have metabolic fallback options to reduce their vulnerability to changes affecting their primary pathways. At community level, specialisation has the effect of preserving the cohesion of the community, providing efficiency benefits to generalists who could, in principle, manage on their own. Redundancy is useful in unstable environments, so that a function can be preserved even when conditions fluctuate. Generalists have a broader niche so provide continuity across different contexts.

In consequence, the kefir community has many different ways of consuming the resources in the milk. Any perturbation in environmental conditions could potentially shift one microbe's metabolic options, so triggering a cascading change in behaviour throughout the community, with corresponding shifts in metabolites and favouring a different community composition. Food producers have found kefir flavour hard to control due to the variable production of aromatic metabolites. This confirms that community assembly is highly sensitive to changes in environmental conditions, both externally and internally generated. It remains to be investigated whether that sensitivity might contribute to instability in the longer term dynamics.

Secondly, kefir is characterised by the presence of the grain biofilm, without which it cannot endure. Yoghurt-like serial batch ferments use a sample of the product as the starter for the next batch, so the starter holds a snapshot of the microbial distribution in the milk at the time of transfer. In kefir, microbes are likely to be exchanged between grain and milk

throughout the fermentation. The grain composition at the time of transfer is notably different to that of the milk.

This mechanism, and the other aspects of biofilms discussed in section 4.5, imply that the grains preserve microbes that might not be active in the milk at the time of transfer. This ensures that a greater diversity of microbes are available to provide the range of metabolic options described above. It is not implausible that this pool of diversity could impact the ongoing viability of the community.

On the other hand, the kefir system in its ecological context has properties that add complexity, if not instability, to the long-term dynamics. Firstly, there is periodic mortality as the entire milk population is removed and the grains are abruptly moved from an environment at pH 4.6 to one at a pH of 6.7 containing very different resources. Secondly, the absence of a constant external resource supply means that the population of individual taxa may grow and then decline, so the mapping from start to end of a batch may not be monotonic. Thirdly, the within-batch dynamics involves an interacting population, which ensures that the dynamics of one batch affects the dynamics of the following batch, not just its initial conditions. These characteristics were all highlighted in section 4.6 as potential sources of instability in discrete mathematical models.

All of the factors discussed in this section play a role in the stability of the community, whether reinforcing or counteracting. A key question, therefore, is how these various factors interconnect to facilitate the long-term endurance of kefir, and to what extent this mechanism might be replicated in other microbial communities. The question of what drives the enduring viability of kefir therefore remains, but we now have much more detailed understanding of the considerations, and can formulate a more nuanced and actionable set of questions for investigation.

#### 6. References

- de Almeida Brasiel, P.G., Dutra Medeiros, J., Barbosa Ferreira Machado, A., Schuchter Ferreira, M., Gouveia Peluzio, M. do C. & Potente Dutra Luquetti, S.C. (2021) Microbial community dynamics of fermented kefir beverages changes over time. *International Journal of Dairy Technology*, 74, 324–331.
- Apar, D.K., Demirhan, E., Özel, B. & Özbek, B. (2017) Kefir Grain Biomass Production: Influence of Different Culturing Conditions and Examination of Growth Kinetic Models. *Journal of Food Process Engineering*, **40**, e12332.
- Arslan, S. (2015) A review: chemical, microbiological and nutritional characteristics of kefir. *CyTA Journal of Food*, **13**, 340–345.
- Bachmann, H., Bruggeman, F.J., Molenaar, D., Branco dos Santos, F. & Teusink, B. (2016) Public goods and metabolic strategies. *Current Opinion in Microbiology*, **31**, 109–115.
- Bengoa, A.A., Iraporda, C., Garrote, G. I. & Abraham, A.G. (2019) Kefir micro-organisms: their role in grain assembly and health properties of fermented milk. *Journal of Applied Microbiology*, **126**, 686–700.
- van den Berg, N.I., Machado, D., Santos, S., Rocha, I., Chacón, J., Harcombe, W., Mitri, S. & Patil, K.R. (2022) Ecological modelling approaches for predicting emergent properties in microbial communities. *Nature Ecology & Evolution*, 6, 855–865.
- von Bertalanffy, L. (1956) General system theory. General Systems, 1, 1-10.

- Blasche, S., Kim, Y., Mars, R.A.T., Machado, D., Maansson, M., Kafkia, E., Milanese, A., Zeller, G., Teusink, B., Nielsen, J., Benes, V., Neves, R., Sauer, U. & Patil, K.R. (2021) Metabolic cooperation and spatiotemporal niche partitioning in a kefir microbial community. *Nature Microbiology*, 6, 196–208.
- Brännström, Å. & Sumpter, D.J.T. (2005) The role of competition and clustering in population dynamics. *Proceedings of the Royal Society B: Biological Sciences*, **272**, 2065– 2072.
- Brunner, J.D. & Chia, N. (2019) Metabolite-mediated modelling of microbial community dynamics captures emergent behaviour more effectively than species–species modelling. *Journal of The Royal Society Interface*, **16**, 20190423.
- Calabrese, S., Chakrawal, A., Manzoni, S. & Van Cappellen, P. (2021) Energetic scaling in microbial growth. *Proceedings of the National Academy of Sciences*, **118**, e2107668118.
- Chang, C.-Y., Bajić, D., Vila, J.C.C., Estrela, S. & Sanchez, A. (2023) Emergent coexistence in multispecies microbial communities. *Science*, **381**, 343–348.
- Chang, R. (2005) *Physical Chemistry for the Biosciences*, 2005th edition. University Science Books, Sausalito, Calif.
- Dallas, D.C., Citerne, F., Tian, T., Silva, V.L.M., Kalanetra, K.M., Frese, S.A., Robinson, R.C., Mills, D.A. & Barile, D. (2016) Peptidomic analysis reveals proteolytic activity of kefir microorganisms on bovine milk proteins. *Food Chemistry*, **197**, 273–284.
- Dobson, A., O'Sullivan, O., Cotter, P.D., Ross, P. & Hill, C. (2011) High-throughput sequence-based analysis of the bacterial composition of kefir and an associated kefir grain. *FEMS Microbiology Letters*, **320**, 56–62.
- Douglas, A.E. (2020) The microbial exometabolome: ecological resource and architect of microbial communities. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **375**, 20190250.
- Duran, F.E., Özdemir, N., Güneşer, O. & Kök-Taş, T. (2022) Prominent strains of kefir grains in the formation of volatile compound profile in milk medium; the role of Lactobacillus kefiranofaciens subsp. kefiranofaciens, Lentilactobacillus kefiri and Lentilactobacillus parakefiri. *European Food Research and Technology*, **248**, 975–989.
- Elder-Vass, D. (2005) Emergence and the Realist Account of Cause. *Journal of Critical Realism*, **4**, 315–338.
- Farag, M.A., Jomaa, S.A., Abd El-Wahed, A. & R. El-Seedi, H. (2020) The Many Faces of Kefir Fermented Dairy Products: Quality Characteristics, Flavour Chemistry, Nutritional Value, Health Benefits, and Safety. *Nutrients*, **12**, 346.
- Flemming, H.-C., Wingender, J., Szewzyk, U., Steinberg, P., Rice, S.A. & Kjelleberg, S. (2016) Biofilms: an emergent form of bacterial life. *Nature Reviews Microbiology*, **14**, 563–575.
- Garrote, G.L., Abraham, A.G. & Antoni, G.L.D. (1998) Characteristics of kefir prepared with different grain[ratio]milk ratios. *Journal of Dairy Research*, **65**, 149–154.
- Georgalaki, M., Zoumpopoulou, G., Anastasiou, R., Kazou, M. & Tsakalidou, E. (2021) Lactobacillus kefiranofaciens: From Isolation and Taxonomy to Probiotic Properties and Applications. *Microorganisms*, **9**, 2158.

- Geritz, S.A.H. & Kisdi, É. (2004) On the mechanistic underpinning of discrete-time population models with complex dynamics. *Journal of Theoretical Biology*, **228**, 261–269.
- Gralka, M., Szabo, R., Stocker, R. & Cordero, O.X. (2020) Trophic Interactions and the Drivers of Microbial Community Assembly. *Current Biology*, **30**, R1176–R1188.
- Hassell, M.P. (1975) Density-Dependence in Single-Species Populations. *Journal of Animal Ecology*, **44**, 283–295.
- Hofbauer, J. & Sigmund, K. (1988) *The Theory of Evolution and Dynamical Systems: Mathematical Aspects of Selection,* Cambridge University Press, Cambridge England ; New York.
- Hugenholtz, J. (1993) Citrate metabolism in lactic acid bacteria. *FEMS Microbiology Reviews*, **12**, 165–178.
- Ives, A.R., Gross, K. & Jansen, V.A.A. (2000) Periodic Mortality Events in Predator–Prey Systems. *Ecology*, **81**, 3330–3340.
- Justus, J. (2008) *Complexity, Diversity, and Stability. A Companion to the Philosophy of Biology,* pp. 321–350.
- Kefir Facebook Group (2023) The Official UK Fermentation Group | Facebook. https://www.facebook.com/groups/theofficialukfermentationgroup.
- Kefir Reddit Group (2023) Kefir, the healthful cultured milk (and water) product. *https://www.reddit.com/r/Kefir/*.
- Kirchman, D.L. (2018) *Processes in Microbial Ecology*, 2nd edition. Oxford University Press, Oxford, United Kingdom.
- Konopka, A. (2009) What is microbial community ecology? The ISME Journal, 3, 1223–1230.
- Lemke, M. & DeSalle, R. (2023) The Next Generation of Microbial Ecology and Its Importance in Environmental Sustainability. *Microbial Ecology*, 85, 781–795.
- Li, Z., Liu, B., Li, S.H.-J., King, C.G., Gitai, Z. & Wingreen, N.S. (2020) Modeling microbial metabolic trade-offs in a chemostat. *PLOS Computational Biology*, **16**, e1008156.
- Liu, W., Zhang, M., Xie, J., Wang, H., Zhao, X., Chen, B. & Suo, H. (2019) Comparative analyses of microbial community diversities of Tibetan kefir grains from three geographic regions. *International Journal of Dairy Technology*, **72**, 536–544.
- Loladze, I. (2019) Iterative chemostat: A modelling framework linking biosynthesis to nutrient cycling on ecological and evolutionary time scales. *Mathematical Biosciences and Engineering*, **16**, 990–1004.
- MacArthur, R. (1970) Species packing and competitive equilibrium for many species. *Theoretical Population Biology*, **1**, 1–11.
- Machado, D., Maistrenko, O.M., Andrejev, S., Kim, Y., Bork, P., Patil, K.R. & Patil, K.R. (2021) Polarization of microbial communities between competitive and cooperative metabolism. *Nature Ecology & Evolution*, 5, 195–203.
- Malka, O., Kalson, D., Yaniv, K., Shafir, R., Rajendran, M., Ben-David, O., Kushmaro, A., Meijler, M.M. & Jelinek, R. (2021) Cross-kingdom inhibition of bacterial virulence and communication by probiotic yeast metabolites. *Microbiome*, **9**, 70–70.

- Marsh, A.J., O'Sullivan, O., Hill, C., Ross, R.P. & Cotter, P.D. (2013) Sequencing-Based Analysis of the Bacterial and Fungal Composition of Kefir Grains and Milks from Multiple Sources. *PLOS ONE*, **8**, e69371.
- Marsland, R., Cui, W., Goldford, J., Sanchez, A., Korolev, K. & Mehta, P. (2019) Available energy fluxes drive a transition in the diversity, stability, and functional structure of microbial communities. *PLOS Computational Biology*, **15**, e1006793.
- Mittelbach, G.G. & McGill, B.J. (2019) *Community Ecology*, 2nd edition. OUP Oxford, Oxford, United Kingdom; New York, NY.
- Momeni, B. (2018) Division of Labor: How Microbes Split Their Responsibility. *Current Biology*, **28**, R697–R699.
- Mossio, M. (2024) *Introduction: Organization as a Scientific Blind Spot. Organization in Biology* History, Philosophy and Theory of the Life Sciences. (ed. by M. Mossio), pp. 1–22. Springer International Publishing, Cham.
- Motaghi, M., Mazaheri, M., Moazami, N., Farkhondeh, A., Fooladi, M.H. & Goltapeh, E.M. (1997) Kefir production in Iran. *World Journal of Microbiology and Biotechnology*, **13**, 579–581.
- Nejati, F., Junne, S. & Neubauer, P. (2020) A Big World in Small Grain: A Review of Natural Milk Kefir Starters. *Microorganisms*, **8**, 192.
- Posfai, A., Taillefumier, T. & Wingreen, N.S. (2017) Metabolic Trade-Offs Promote Diversity in a Model Ecosystem. *Physical Review Letters*, **118**, 028103.
- Prosser, J.I. & Martiny, J.B.H. (2020) Conceptual challenges in microbial community ecology. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **375**, 20190241.
- Rapoport, A. (1968) *General System Theory. The International Encyclopedia of Social Sciences* (ed. by D.L. Sills), pp. 452–458. Macmillan, New York, NY.
- Rattray, F.P. & O'Connell, M.J. (2022) *Kefir. Encyclopedia of Dairy Sciences (Third Edition)* (ed. by P.L.H. McSweeney) and J.P. McNamara), pp. 438–445. Academic Press, Oxford.
- Rosa, D.D., Dias, M.M.S., Grześkowiak, Ł.M., Reis, S.A., Conceição, L.L. & Peluzio, M.D.C.G. (2017) Milk *kefir* : nutritional, microbiological and health benefits. *Nutrition Research Reviews*, **30**, 82–96.
- Rousseau, D.N. (2018) Three General Systems Principles and Their Derivation: Insights from the Philosophy of Science Applied to Systems Concepts. Disciplinary Convergence in Systems Engineering Research (ed. by A.M. Madni), B. Boehm), R.G. Ghanem), D. Erwin), and M.J. Wheaton), pp. 665–681. Springer International Publishing, Cham.
- Rousseau, D.N., Billingham, J.A. & Calvo-Amodio, J. (2019) Systemic Virtues as a Foundation for a General Theory of Design Elegance. *Systems Research and Behavioral Science*, **36**, 656–667.
- Royama, T. (1992) *Population process models. Analytical Population Dynamics* Population and Community Biology Series. (ed. by T. Royama), pp. 140–168. Springer Netherlands, Dordrecht.
- Savageau, M.A. (1995) Michaelis-Menten mechanism reconsidered: implications of fractal kinetics. *Journal of Theoretical Biology*, **176**, 115–124.
- Schoevers, A. (1999) Mass Cultivation and Activity of Kefir Grains.

- Segrè, D., Mitri, S., Shou, W., Süel, G.M., Mizrahi, I., Kelly, L., Rebolleda-Gómez, M., Ratzke, C., Ogbunugafor, C.B., Schwartzman, J.A., Kryazhimskiy, S., Leventhal, G.E., Kost, C. & Bell, T. (2023) What do you most want to understand about how collective features emerge in microbial communities? *Cell Systems*, 14, 91–97.
- Sillitto, H., Martin, J., McKinney, D., Griego, R.M., Dori, D., Krob, D., Godfrey, P., Arnold, E.
  & Jackson, S. (2018) Systems Engineering and System Definitions, Version 1.0, Issued 14 December 2018, International Council on Systems Engineering.
- Smith, N.W., Shorten, P.R., Altermann, E., Roy, N.C. & McNabb, W.C. (2019) The Classification and Evolution of Bacterial Cross-Feeding. *Frontiers in Ecology and Evolution*, 7.
- Smith, T.P., Mombrikotb, S., Ransome, E., Kontopoulos, D.-G., Pawar, S. & Bell, T. (2022) Latent functional diversity may accelerate microbial community responses to temperature fluctuations. *eLife*, **11**, e80867.
- Stewart, P.S. (2003) Diffusion in Biofilms. Journal of Bacteriology, 185, 1485–1491.
- Taillefumier, T., Posfai, A., Meir, Y. & Wingreen, N.S. (2017) Microbial consortia at steady supply. *eLife*, **6**, e22644.
- Tan, K.-X., Chamundeswari, V.N. & Loo, S.C.J. (2020) Prospects of kefiran as a food-derived biopolymer for agri-food and biomedical applications. *RSC Advances*, **10**, 25339– 25351.
- Tan, L.L., Tan, C.H., Ng, N.K.J., Tan, Y.H., Conway, P.L. & Loo, S.C.J. (2022) Potential Probiotic Strains From Milk and Water Kefir Grains in Singapore—Use for Defense Against Enteric Bacterial Pathogens. *Frontiers in Microbiology*, **13**, 857720.
- Ursell, T. (2020) Competitive ecosystems are robustly stabilized by structured environments. 2020.03.09.983395.
- Vardjan, T., Mohar Lorbeg, P. & Čanžek Majhenič, A. (2018) Stability of prevailing lactobacilli and yeasts in kefir grains and kefir beverages during ten weeks of propagation. *International Journal of Dairy Technology*, **71**, 51–60.
- Walsh, A.M., Crispie, F., Kilcawley, K., O'Sullivan, O., O'Sullivan, M.G., Claesson, M.J. & Cotter, P.D. (2016) Microbial Succession and Flavor Production in the Fermented Dairy Beverage Kefir. *mSystems*, 1, e00052-16.
- Wang, S.-Y., Chen, K.-N., Lo, Y.-M., Chiang, M.-L., Chen, H.-C., Liu, J.-R. & Chen, M.-J. (2012) Investigation of microorganisms involved in biosynthesis of the kefir grain. *Food Microbiology*, **32**, 274–285.
- Widder, S., Allen, R.J., Pfeiffer, T., Curtis, T.P., Wiuf, C., Sloan, W.T., Cordero, O.X., Brown, S.P., Momeni, B., Shou, W., Kettle, H., Flint, H.J., Haas, A.F., Laroche, B., Kreft, J.-U., Rainey, P.B., Freilich, S., Schuster, S., Milferstedt, K., van der Meer, J.R., Groβkopf, T., Huisman, J., Free, A., Picioreanu, C., Quince, C., Klapper, I., Labarthe, S., Smets, B.F., Wang, H. & Soyer, O.S. (2016) Challenges in microbial ecology: building predictive understanding of community function and dynamics. *The ISME Journal*, 10, 2557–2568.
- Wolfe, B.E. & Dutton, R.J. (2015) Fermented Foods as Experimentally Tractable Microbial Ecosystems. *Cell*, **161**, 49–55.

Yang, Y., Shevchenko, A., Knaust, A., Abuduresule, I., Li, W., Hu, X., Wang, C. & Shevchenko, A. (2014) Proteomics evidence for kefir dairy in Early Bronze Age China. *Journal of Archaeological Science*, **45**, 178–186.

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