THE UNIVERSITY
of EDINBURGH

The Role of Metabolism and Ecology in the Emergence of Microbial Communities

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For my parents

Abstract

Polymicrobial communities often show complex patterns of metabolic and ecological interactions, yet our understanding of how the properties of communities emerge from the metabolic rules of species interactions is still limited. A central feature of metabolic interactions within microbial communities is 'cross-feeding', where one species or lineage consumes the metabolic by-products of another. Cross-feeding bacteria excrete and consume a wide range of metabolites and this sets the stage for diverse intra- and inter-specific metabolic interactions. In this thesis, I use ecological and evolutionary theory to address a number of critical questions posed by cross-feeding bacteria, with a particular focus on the role played by microbial metabolism in driving the emergence and dynamics of microbial interactions. First, I explore the conditions that favour the emergence and maintenance of cooperative cross-feeding and show that the evolutionary outcome depends strongly on the shape of the trade-off curves between the costs and benefits of cooperation. Second, I investigate the origins of cross-feeding interactions via single lineage diversification and derive new predictions on the physiological mechanisms that may explain the stable coexistence of a cross-feeding polymorphism that evolved from a single clone. Third, I investigate what are the ecological consequences of cross-feeding metabolic interactions and demonstrate theoretically that a simple mechanism of trade can generate a diverse array of ecological relationships. Furthermore, I show the importance of the metabolic by-product properties in determining the ecological outcome. Fourth, I investigate how metabolic constraints of individual species shape the emergent functional and structural relationships among species. I show that strong metabolic interdependence drives the emergence of mutualism, robust interspecific mixing, and increased community productivity. Furthermore, I show that these emergent community properties are driven by demographic feedbacks. In general, these findings support the idea that bridging microbial ecology and metabolism is a critical step toward a better understanding of the factors governing the emergence and dynamics of polymicrobial interactions.

Lay Summary

Understanding the structure and functioning of polymicrobial communities is a major challenge in biology, as witnessed by the crucial yet largely unknown roles played by the human and soil microbiomes in human health and ecosystems functioning, respectively. Within polymicrobial communities, individual cells excrete and consume a wide range of metabolites and this sets the stage for metabolic interactions to occur between members of these communities. How do metabolic constraints influence the emergence of biological diversity (multiple clones) from simplicity (single clone)? What mechanisms underlie multispecies community function and spatial self-organization? How does cooperative cross-feeding evolve and is stable to invasion by cheats who pay no or less cost of cooperation but reap the benefits? I have used mathematical and computational approaches to address these questions. Specifically, I investigate how constraints of microbial metabolism generate the diversity of ecological and spatial relationships observed in microbial communities. Providing new insight into the mechanisms that underpin the emergence and maintenance of microbial communities is needed to be able to predict how these communities may be affected by environmental perturbations on both ecological and evolutionary timescales. This is important for the development of novel strategies that aim at improving human health as well as maintaining ecosystems functioning and services.

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Appendix

During the course of my PhD I contributed to another paper that does not appear as a chapter of my thesis. This paper appears as the following appendix.

A1. Eswarappa SM, Estrela S, and Brown SP (2012) Within-Host Dynamics of Multi-species Infections: Facilitation, Competition and Virulence. *PLoS ONE* 7(6): e38730.

Declaration

I declare that I composed this thesis myself, and that the work described here is my own except where explicitly stated.

Chapter 2

The work presented in this chapter was designed by Ivana Gudelj. I analysed the model and performed the numerical simulations, except for the evolutionary analysis that was performed by Ivana Gudelj. Ivana Gudelj wrote the subsequent manuscript with my input.

Chapter 3

I designed and led the work performed in this chapter with Ivana Gudelj's advice and supervision. I analysed the model and performed the simulations presented here and wrote the chapter.

This work has not been submitted for any other degree or professional qualification.

Sylvie Estrela, September 2013

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Publications

The following published papers have arisen from this thesis:

Estrela S & Gudelj I (2010) Evolution of Cooperative Cross-Feeding Could Be Less Challenging Than Originally Thought. *PLoS ONE* 5(11): e14121

Estrela S, Trisos CH, and Brown SP (2012) From metabolism to ecology: cross-feeding interactions shape the balance between polymicrobial conflict and mutualism. *The American Naturalist* 180: 566-576

Chapter 1

GENERAL INTRODUCTION

1.1. Summary

Polymicrobial communities often show complex patterns of metabolic and ecological interactions, yet our understanding of how the properties of communities emerge from the metabolic rules of species interactions is still limited. A central feature of metabolic interactions within microbial communities is 'cross-feeding', where one species or lineage consumes the metabolic by-products of another. Cross-feeding bacteria excrete and consume a wide range of metabolites and this sets the stage for diverse intra- and inter- specific metabolic interactions.

In this thesis I use ecological and evolutionary theory to address a number of critical questions posed by cross-feeding bacteria, with a particular focus on the role played by microbial metabolism in driving the emergence and dynamics of microbial interactions. I ask: How does cooperative cross-feeding evolve and resist invasion by cheats who do not pay the cost of cooperation (ch. 2[†])? How do stable cross-feeding polymorphisms evolve from a single lineage (ch. 3[†])? What are the ecological consequences of cross-feeding metabolic interactions (ch. 4)? How do metabolic constraints of individual species shape the emergent functional and structural relationships among species (ch. 5)? I use an integrative approach with the ultimate goal of bridging the gap between microbial metabolism and ecology.

In the remaining sections of this introduction, I take an overview of the key empirical and theoretical works in the field and outline the key questions addressed in this thesis. These concepts will be developed further in each chapter. The details on the methodologies used are provided in each chapter.

[†] Work performed at Imperial College London under Ivana Gudelj supervision (oct. 2009 - mar. 2011)

1.2. Cross-feeding interactions in the microbial world and the conflict-mutualism continuum

Microbial cells constantly modify their environment through the excretion of metabolic by-products, setting the stage for metabolic interactions**.** Of particular interest are cross-feeding interactions, where one organism uses metabolic by-products of others as energy or nutrient resources. When between two unrelated species who depend on each other to degrade a certain substrate, this is also known as syntrophy (Schink 1991). Cross-feeding plays a central role in maintaining ecosystem functioning (Stams 1994; Schink 2002; Pernthaler et al. 2008), promoting health (Samuel and Gordon 2006; Mahowald et al. 2009) as well as causing disease (Grenier 1992; Winter et al. 2010; Ramsey et al. 2011). Cross-feeding microbes can engage in multiple forms of metabolic interactions. They can exchange resources only or exchange resources for services. A notable and illustrative example is the interaction between the gram-positive bacteria *Streptococcus gordonii (Sg)* and the opportunistic oral pathogen *Aggregatibacter actinomycetemcomitans* (*Aa*) (fig. 1.1). While *Sg* rapidly consumes sugars (e.g. glucose), producing the metabolites lactic acid and hydrogen peroxide, *Aa* preferentially catabolizes lactate over high energy carbon sources when in the presence of *Sg* (Brown and Whiteley 2007). *Aa* is also capable of degrading hydrogen peroxide (H_2O_2), thus relieving *Sg* of oxidative stress (Ramsey and Whiteley 2009; Liu et al. 2011). Thus, *Sg* provides food to *Aa*, and in turn, *Aa* detoxifies *Sg*'s environment.

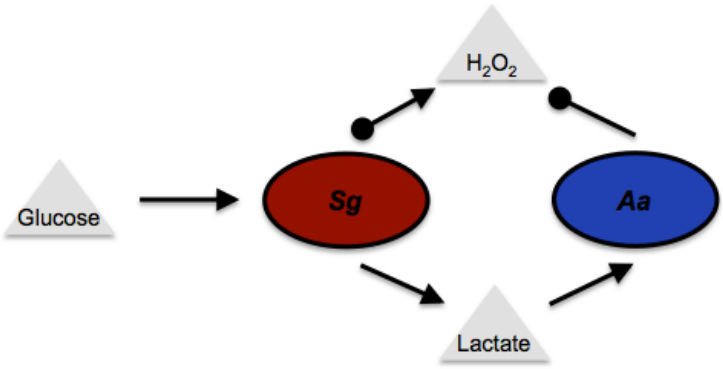


Figure 1.1. *Streptococcus gordonii* (*Sg*) and *Aggregatibacter actinomycetemcomitans* (*Aa*) engage in multiple forms of metabolic interactions. Schematic model of *Sg-Aa* metabolic exchange under aerobic conditions (Liu et al. 2011). Open arrows represent a positive effect, whereas oval arrows represent a negative effect upon the population or metabolite they are pointing toward.

This diversity of microbial metabolic relationships raises the question of how do metabolic interactions influence functional relationships among microbes. Cross-feeding is incidental when the metabolite excreted is a waste product of metabolism and therefore non-costly to produce at a basal level. In some instances, cross-feeding can be cooperative if species evolved specifically to benefit their partners (West et al. 2006). Moreover, cooperative cross-feeding can be costly if requiring an up-front investment cost to the producer, which may or may not be paid back by the partner species utilizing the metabolite (West et al. 2006; Bull and Harcombe 2009).

From an ecological perspective, although a species derives a benefit from feeding on another species' by-products, these benefits have to be weighted with the competitive costs of association (competition for space and/or nutrients). Hence, this balance of costs and benefits generates a variety of possible ecological outcomes, ranging from mutualism (when both species' growth rate is enhanced) through commensalism (when one species benefits with no effect on the other), to exploitation (when one species benefits at the expense of the other) and competition (when both species growth rate is reduced) (fig. 1.2) (Bronstein 1994; Connor 1995). Empirical evidence for this diversity of functional outcomes in cross-feeding relationships has been

accumulating in the literature, including mutually beneficial interactions (Samuel and Gordon 2006; Shou et al. 2007; Mahowald et al. 2009; Harcombe 2010; Hillesland and Stahl 2010), commensal interactions (Hansen et al. 2007), or exploitative interactions (Hansen et al. 2007; Jagmann et al. 2010).

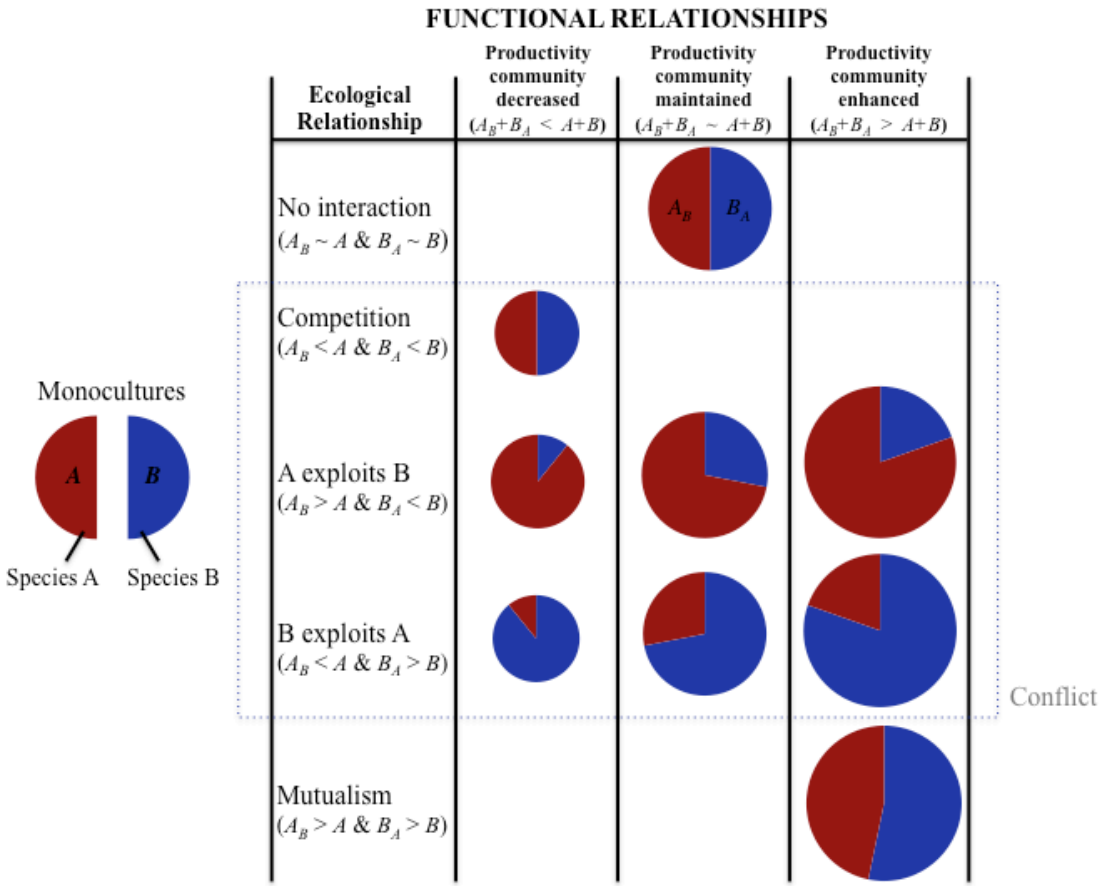


Figure 1.2. Schematic illustrating the potential functional relationships among two species. Competition leads to a decrease in community productivity. Mutualism leads to an increase in community productivity. Exploitation can result in either lower, similar, or higher community productivity. Productivity of species A in the absence (A) and presence (A_B) of species B. Productivity of species B in the absence (B) and presence (B_A) of species A.

Given the importance and ubiquity of cross-feeding interactions in the microbial world, this raises the critical question of how do cross-feeding interactions emerge in

the first place. In other words, what are the physiological, ecological, and evolutionary mechanisms driving the origin and maintenance of cross-feeding, and, what is in turn the role played by cross-feeding in the evolution of biological diversity (Rainey et al. 2000).

1.3. From one to many: the emergence and maintenance of diversity by cross-feeding

One of the first studies showing the important role played by cross-feeding for the emergence and maintenance of diversity was initiated by Helling and colleagues in the late 1980s (Helling et al. 1987). In a single-nutrient continuous environment, and from a single *Escherichia coli* clone, after a few hundreds of generations a cross-feeding polymorphism had evolved. Hence, these observations violated the ecological competitive exclusion principle (Gause 1934; Hardin 1960) that predicts that competition for a unique common limiting resource in a continuous environment cannot support species coexistence. Further experiments, revealed that the evolved *E. coli* polymorphism was mainly constituted by three cross-feeding genotypes: a glucose specialist, that exhibited increased rate of glucose uptake as well as increased rate of acetate excretion; an acetate specialist and cross-feeder, characterized by an enhanced ability to use acetate; and a glycerol generalist that had an enhanced ability to assimilate glycerol when compared with the ancestral and the other two evolved clones (Rosenzweig et al. 1994). The evolution of biological diversity from a unique founder genotype via cross-feeding is a common outcome of evolution experiments. Indeed, it has been observed that the stable coexistence of two *E. coli* strains in a serial transfer regime that had evolved during a long-term evolution experiment in *E. coli* (Turner et al. 1996; Rozen and Lenski 2000) was due to a cross-feeding interaction and a demographic trade-off in the strains ability to compete for a single rare/abundant limiting nutrient (Turner et al. 1996). When under starving conditions, stable coexistence of this polymorphism can be maintained by cannibalistic cross-feeding, such that the slow growing strain feeds on resources released from the dying (lysing) cells of the fast growing strain (Rozen et al. 2009).

Stable cross-feeding polymorphisms have also been observed in a seasonal environment with a mixture of two nutrients (glucose and acetate) (Friesen et al. 2004), and more recently in an evolved biofilm generated from a single clone of the opportunistic pathogen *Burkholderia cenocepacia* (Poltak and Cooper 2011). These empirical studies illustrate how the interplay between competition for common limiting resources and metabolic constraints set the stage for the evolution of resource partitioning and the emergence of cross-feeding polymorphisms among closely related genotypes. This supports the idea that both ecological opportunity and fitness trade-offs are essential for the emergence and maintenance of stable polymorphisms (Rainey et al. 2000).

From a theoretical perspective, the evolution of cross-feeding polymorphisms from a unique founder genotype in a continuous environment has been explored using metabolic control theory (Porcher et al. 2001); an adaptive dynamics framework (Doebeli 2002), and kinetic theory (Pfeiffer and Bonhoeffer 2004). For example, using an adaptive dynamics approach on a simple Michaelis-Menten model of bacterial growth, Doebeli (2002) suggested that evolution of a cross-feeding polymorphism was possible if there was a trade-off between the uptake efficiencies of the primary (limiting nutrient) and the secondary (waste product) resources, and this trade-off has positive curvature. Pfeiffer and Bonhoeffer (2004) proposed that cross-feeding may evolve in a continuous culture as a consequence of optimization principles in ATP-producing metabolic pathways, specifically by maximizing the rate of ATP production while minimizing the concentrations of enzymes and intermediates of the pathway. These theoretical studies have essentially focused their attention on the effect of metabolic trade-offs for the emergence of cross-feeding polymorphisms. While metabolic constraints are certainly important for the evolution of cross-feeding interactions, it will also depend strongly on ecological context. Such integration has been traditionally overlooked, leaving a large disconnect between microbial metabolism and ecology. There is therefore a need for theoretical approaches to bridge this gap and build an integrated mechanistic account of microbial community ecology.

1.4. Emergence of functional relationships from interspecific metabolic interactions in polymicrobial communities

The previous section describes the evolution, from a single clone, of cross-feeding interactions among closely related genotypes. However, it is now well acknowledged that microbes live predominantly in multi-species communities (Little et al. 2008), therefore setting the stage for metabolic interactions between species (distantly related genotypes).

While the importance of interspecific cross-feeding interactions has long been recognized by microbiologists (Mikx and Van der Hoeven 1975; Schultz and Breznak 1979; Grenier and Mayrand 1986; Stams 1994; Pelz et al. 1999; Schink 2002), only recently it has been of increasing interest to evolutionary biologists (Hansen et al. 2007; Shou et al. 2007; Bull and Harcombe 2009; Harcombe 2010; Hillesland and Stahl 2010; Lawrence et al. 2012). For example, Shou et al. (2007) engineered a synthetic system in yeast where two strains are auxotrophic for a different essential amino acid. The auxotrophic strains are only able to grow if the amino acid that they cannot synthesize is provided in the growth medium or supplied by the other partner- the two strains are therefore engaged in an obligate mutualistic relationship. The construction of this synthetic system has allowed to address a number of questions on the ecology and evolution of cross-feeding in yeast (Waite and Shou 2012; Momeni et al. 2013). Empirical evidence for the evolution of novel mutualisms between two distantly related species via cross-feeding comes from two independent works (Harcombe 2010; Hillesland and Stahl 2010). Harcombe (2010) experimentally evolved a novel cross-feeding mutualism between two bacterial species, *Salmonella enterica ser. Typhimurium* and an *E. coli* mutant, in a lactate minimal medium. While the *E. coli* mutant is unable to synthesize methionine, an essential amino acid for lactate degradation, *Salmonella* cannot grow on lactate but can produce methionine as a metabolic by-product. These complementary metabolic activities as well as the obligate nature of the interaction provide the foundations for the creation of a mutually beneficial interaction, where *Salmonella* produces methionine in exchange for an energy resource (acetate), produced by *E. coli*.

Methionine excretion by *Salmonella* was costly, and therefore cooperative. Furthermore, spatial structure as well as a preexisting mechanism of reciprocation were a necessary condition for the evolution of this cooperative and costly mutualism, thus in agreement with theory on interspecific cooperation (Trivers 1971; Sachs et al. 2004; Foster and Wenseleers 2006; West et al. 2007; Bull and Harcombe 2009). In a parallel study by Hillesland and Stahl (2010), an obligate mutualism between the archaeon *Methanococcus maripaludis* and the bacterium *Desulfovibrio vulgaris* was evolved. Unlike Harcombe's system, cross-feeding was not bidirectional but unidirectional, specifically food was traded for a permissive growth (i.e. by-product detoxification), and excretion of the waste-product was non-costly. A key message emerging from these two studies is the important role played by spatial structure in shaping the evolution of species interactions and community function. The links, however, between metabolic and ecological interactions (functional relationships), and how these shape species spatial organization (structural relationships) are still poorly understood.

1.5. Emergence of functional and structural relationships among microbial species

Knowledge of the mechanisms that influence the spatial organization of microbial biofilm communities has been growing in the empirical literature (reviewed in Elias and Banin (2012)). Within these multi-species communities, species can be spatially segregated, mixed, or organized in a layering pattern (fig. 1.3). Mechanisms that have been shown to influence species spatial organization within multispecies biofilms include: mixing species that have distinct monoculture structures (Murga et al. 1995); cross-feeding relationships (Nielsen et al. 2000; Christensen et al. 2002; Breugelmans et al. 2008; Brenner and Arnold 2011); or the detoxification of exogenous waste products (Cowan et al. 2000). In a rare study, Hansen et al. (2007) showed that selection of a two-species community in a spatially structured environment led to the evolution of an exploitative relationship from an initially commensal relationship. Furthermore, this shift in ecological relationship correlated

with a change in the species spatial relationship from initially segregated to a mantle-like pattern. This study highlights the central importance of spatial structure in shaping the evolution of species interactions, and in turn, the key role played by species interactions in affecting community function and structure.

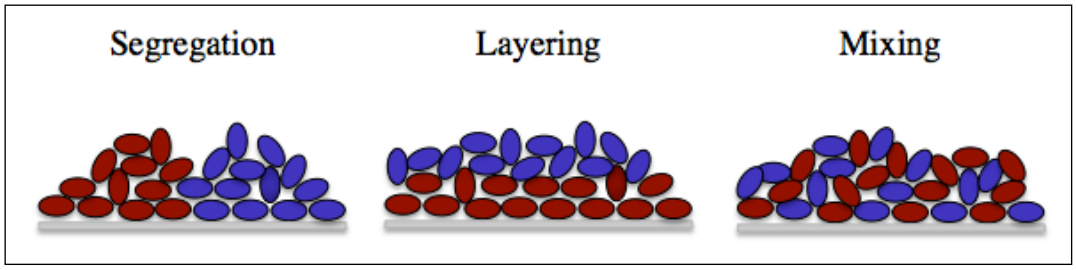


Figure 1.3. Spatial relationships among species. Segregation: the two species grow in spatially separated microcolonies. Layering: one species grows on top of the other (e.g. aerobic species over anaerobic species). Mixing: the two species are spatially intermixed.

While evolutionary ecology has traditionally assumed that population structure is a fixed environmental property (i.e. either structured or well-mixed), there has been a recent growing interest in regarding structuring of surface attached communities as an emergent property of collective bacterial behavior and demography (Nadell et al. 2010; Momeni et al. 2013). Of particular relevance to investigate questions at this interface are individual-based models (IBM) of biofilms, as these models use a bottom-up approach where the community structure and dynamics arise as an emergent property of the interactions between individual cells (Kreft et al. 2001). Despite this progress, there is still a significant gap in our understanding of how specific metabolic interactions shape the emergent spatial structure and function of microbial communities. To bridge this gap, further work is needed towards integrating molecular mechanism into emergent functional and structural properties of microbial communities.

1.6. Thesis Outline and aims

In chapter 2, I build on a simple analytical model (Bull and Harcombe, 2009) to explore the conditions that favour the emergence and maintenance of cooperative cross-feeding. I show that the evolutionary outcome of cooperative cross-feeding depends strongly on the shape of the trade-off curves between the costs and benefits of cooperation. This suggests that the evolution of cooperative cross-feeding is less challenging than previously thought.

In chapter 3, I use a systems-biology approach to investigate the origins of cross-feeding interactions via single lineage diversification. In particular, I derive new predictions on the physiological mechanisms that may explain the stable coexistence of a cross-feeding polymorphism that evolved from a single clone. I show that either a distinct resistance to waste product toxicity, or a distinct inhibition on a key enzyme of bacterial metabolism, may explain this stable coexistence. These findings provide new insights into the physiological mechanisms that may underpin the emergence and maintenance of diversity via cross-feeding interactions.

In chapter 4, I investigate what are the ecological consequences of cross-feeding metabolic interactions. I demonstrate theoretically that a simple mechanism of trade (food for detoxification) can generate a diverse array of ecological relationships in well-mixed populations, spanning mutualism, competition, and exploitation. Furthermore, the results highlight the importance of the metabolic by-product properties (toxicity and decay rate) in determining the conditions for mutualism. These results support the idea that bridging microbial ecology and metabolism is a critical step toward a better understanding of the factors governing the emergence and dynamics of polymicrobial interactions.

In chapter 5, I build on the findings in chapter 4 to investigate how metabolic constraints of individual species shape the emergent functional relationships and genetic structure of a spatially structured minimal two-species community. Using an individual-based modeling (IBM) framework, I show that strong metabolic

interdependence drives the emergence of mutualism, robust interspecific mixing, and increased community productivity. These emergent community properties are driven by demographic feedbacks, such that aid from neighbouring cells directly enhances focal cell growth, which in turn feeds back to neighbour fecundity. In contrast, weak metabolic interdependence drives conflict (exploitation or competition), and in turn greater interspecific segregation.

In Chapter 6, I summarise the key findings of this thesis and how they contribute to the understanding of the emergence and dynamics of microbial interactions. Furthermore, I discuss the potential implications of these findings for managing the health of the human microbiome, and suggest new avenues of research.

Chapter 2

EVOLUTION OF COOPERATIVE CROSS-FEEDING COULD BE LESS CHALLENGING THAN ORIGINALLY THOUGHT

This chapter is published as:
Estrela S & Gudelj I (2010) Evolution of Cooperative Cross-Feeding Could Be Less Challenging Than Originally Thought. *PLoS ONE* 5(11): e14121

2.1. Summary

The act of cross-feeding whereby unrelated species exchange nutrients is a common feature of microbial interactions and could be considered a form of reciprocal altruism or reciprocal cooperation. Past theoretical work suggests that the evolution of cooperative cross-feeding in nature may be more challenging than for other types of cooperation. Here we re-evaluate a mathematical model used previously to study persistence of cross-feeding and conclude that the maintenance of cross-feeding interactions could be favoured for a larger parameter ranges than formerly observed. Strikingly, we also find that large populations of cross-feeders are not necessarily vulnerable to extinction from an initially small number of cheats who receive the benefit of cross-feeding but do not reciprocate in this cooperative interaction. This could explain the widespread cooperative cross-feeding observed in natural populations.

2.2. Introduction

Cross-feeding between unrelated species, termed syntrophy, is the ability of one organism to use metabolites excreted by another organism (Pfeiffer and Bonhoeffer

2004). When this interaction involves a reciprocal exchange between the partners as a cooperative behaviour and not merely an exchange of waste products as a result of a selfish act, cross-feeding can be considered a mutualistic act known as reciprocal altruism (Trivers 1971) or reciprocal cooperation (Axelrod and Hamilton 1981; West et al. 2007). Such behaviour is common in the microbial world (Schink 2002; Velicer 2003; West et al. 2007; Marx 2009; Stams and Plugge 2009) and is of a fundamental importance to our understanding of microbial communities and their impact on the environment. A remarkable example can be found in the association between archaea and bacteria that couple methane oxidation with sulfate reduction, respectively. This syntrophic association has been estimated to involve the consumption of more that 80% of the ocean methane flux and is an important process needed to reduce the emissions of the green house gas methane from the ocean into the atmosphere (Hallam et al. 2004; Reeburgh 2007; Pernthaler et al. 2008). Syntrophic interactions are also known to play a key role in the degradation of xenobiotic compounds (Dejonghe et al. 2003) which is crucial for the minimization of surface and ground water contamination by pesticides. Other examples of syntrophy include interactions between fermentative bacteria and methanogenic archeon (Shimoyama et al. 2009); methanogens and ethanol fermenters (Bryant et al. 1967; Schink 1991) and between green-sulphur bacteria and the β-proteobacteria (Overmann and Schubert 2002).

While the importance of cross-feeding syntrophy is clear, what is less clear is how can a group of individuals who engage in such form of cooperative behaviour resist invasion by cheats who do not pay the cost of cooperation but reap the reward? A model exploring the conditions favouring the origin of cooperative cross-feeding between two microbial species was recently proposed by (Bull and Harcombe 2009). There the authors uncover some unintuitive constraints, namely that the benefit of cooperative cross-feeding applies only in the range of intermediate cell densities and is more easily selected when the cost of cross-feeding to the donor is low per benefit to the recipient and when the recipient already provides a large cross-feeding benefit to the donor. This finding is contingent on the existence of a trade-off between the cost to cooperators of performing an altruistic act and the benefit to the recipients towards whom the cooperation is directed. Such trade-off arises naturally from the

definition of a cooperative act because a cross-feeding cooperative individual sacrifices its intrinsic growth to benefit other species by facilitating their ability to grow. The authors also find that large populations of cooperative cross-feeders are vulnerable to exploiting genotypes (or cheats) who share the cross-feeding resources but do not reciprocate in the cross-feeding themselves.

In this paper we revisit the model presented in Bull and Harcombe (2009) and highlight a number of parameter regimes that tend to increase the window in which cooperation is favoured. Contrary to Bull and Harcombe (2009) we find that large populations of cross-feeders are not easily taken over and replaced by a small number of cheats. This result relies on the assumption that all types have the same carrying capacity. Subsequently we present an alternative evolutionary model that relaxes the assumption of equal carrying capacities and again show that replacement of cooperators by cheats is not the most common outcome of evolution.

2.3. Methods

Numerical simulations were performed using MATLAB. Parameter values for each illustration are provided in the figure legends.

2.4. Results

The mathematical model

In Bull and Harcombe (2009) the authors propose the following model of cross-feeding. Consider a spatially heterogeneous environment containing two separate local patches. Each patch contains a pair of clonal microbial populations interacting by cross-feeding in the following way. Patch 1 contains genotypes X and Y engaged in a cross-feeding syntrophy with X cross-feeding Y and Y cross-feeding X. Patch 2 contains genotypes X_c and Y whereby X_c receives a cross-feeding benefit from Y but does not reciprocate in the cross-feeding. Population dynamics of each patch are subsequently modeled as follows:

Patch 1 model:

Let *X(t)* and *Y(t)* denote densities of genotypes X and Y respectively, at time *t*. The rate of expansion of the X population is governed by:

1. *an intrinsic ability to grow* denoted by r_x;
2. *the per capita level of cross-feeding* described by $b_{yx}\frac{Y}{X+c_x}$ where b_{yx} represents a benefit to X per individual of type Y and c_x represents a damping constant that sets the cross-feeding resource proportional to *Y* when *X* is vanishingly small;
3. *crowding* implemented through a total carrying capacity *K* of the two microbial types.

Applying the same population expansion rules to type Y leads to the following system of equations

$$\begin{aligned}\frac{dX}{dt} &= X(r_x + b_{yx}\frac{Y}{X+c_x})(1-\frac{X+Y}{K}),\\ \frac{dY}{dt} &= Y(r_y + b_{xy}\frac{X}{Y+c_y})(1-\frac{X+Y}{K}),\end{aligned} \quad (1)$$

where r_y denotes the growth constant for the population of type Y, b_{xy} represents a benefit of cross-feeding to Y per individual of type X with the assumption that $b_{xy} = b_{yx}$. The parameter c_y denotes a damping constant that sets the cross-feeding resource proportional to *X* when *Y* is vanishingly small.

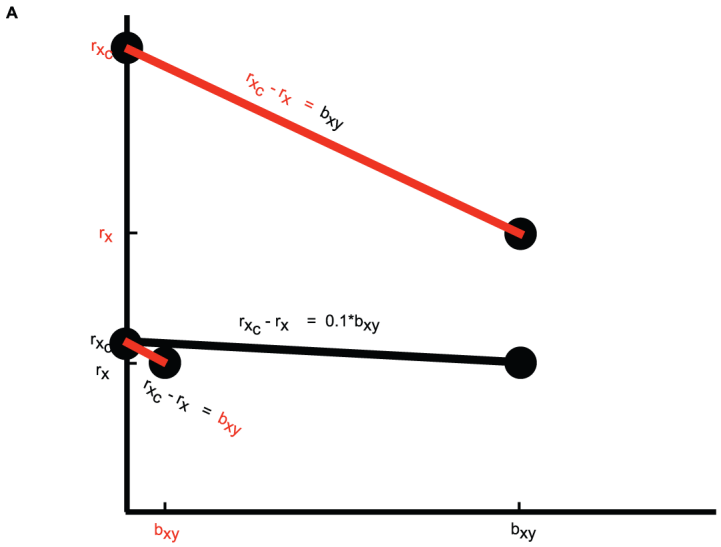
Patch 2 model

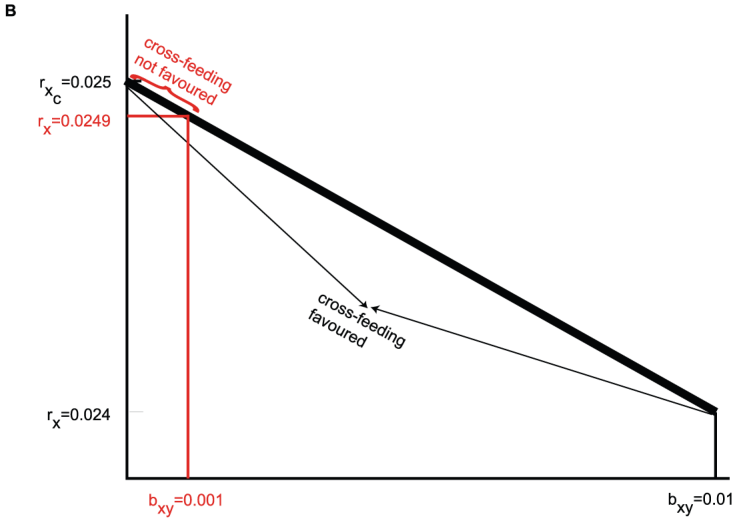
Let $X_c(t)$ denote the density of genotype X_c at time *t*. The model (1) can be adapted to describe interactions between X_c and Y as follows

$$\frac{dX_c}{dt} = X_c(r_{x_c} + b_{yx}\frac{Y}{X_c + c_{x_c}})(1 - \frac{X_c + Y}{K})$$
$$\frac{dY}{dt} = Yr_y(1 - \frac{X_c + Y}{K}) \quad (2)$$

where r_{x_c} denotes the growth term of non cross-feeder X_c with $r_y > r_{x_c}$ while c_{x_c} denotes the cross-feeding damping constant defined in a similar way as c_x in the model (1).

X_c can be viewed as a non-cooperative (or cheating) genotype. By definition a cooperative trait carries a cost to cooperator of performing an altruistic act while providing a benefit to the recipient towards whom the cooperation is directed. Just as in Bull and Harcombe (2009) we assume the existence of a trade-off between r_x and b_{xy} (as well as between r_y and b_{yx}) which means that a cross-feeding individual of a given type sacrifices its own growth to facilitate the growth of another type. Therefore, comparing model (1) and (2) we note that $r_{x_c} > r_x$ because X_c does not pay a cost of cooperation and that $b_{x_c y} = 0$ as X_c does not provide a cross-feeding benefit to Y and hence there is no bidirectional cross-feeding ($0 = b_{x_c y} < b_{xy}$). This forms a part of the cost/benefit trade-off and is illustrated in Figure 2.1.


A
r_{xc}
$r_{xc} - r_x = b_{xy}$
r_x
$r_{xc} - r_x = 0.1*b_{xy}$
r_{xc}
r_x
$r_{xc} - r_x = b_{xy}$
b_{xy}
b_{xy}


B
cross-feeding not favoured
r_{xc}=0.025
r_x=0.0249
cross-feeding favoured
r_x=0.024
b_{xy}=0.001
b_{xy}=0.01

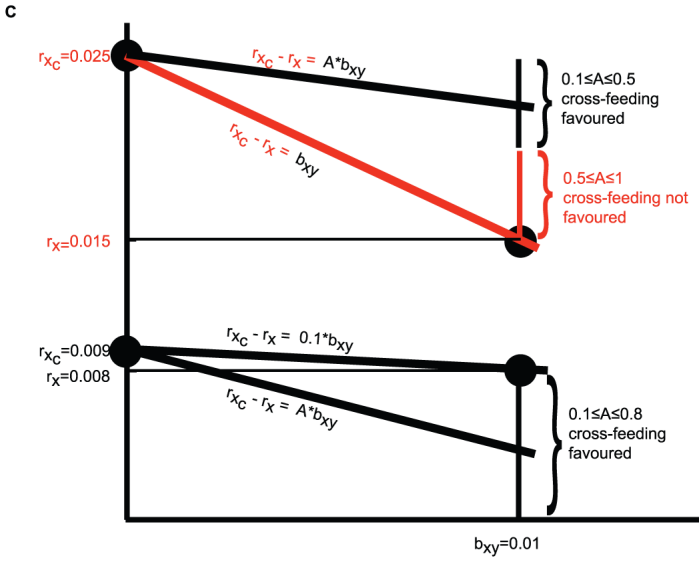

C
r_{xc}=0.025
$r_{xc} - r_x = A*b_{xy}$
0.1≤A≤0.5
cross-feeding
favoured
$r_{xc} - r_x = b_{xy}$
0.5≤A≤1
cross-feeding not
favoured
r_x=0.015
$r_{xc} - r_x = 0.1*b_{xy}$
r_{xc}=0.009
r_x=0.008
$r_{xc} - r_x = A*b_{xy}$
0.1≤A≤0.8
cross-feeding
favoured
b_{xy}=0.01

Figure 2.1. Trade-off between the cost of cooperation and the benefit to the recipient determines cross-feeding success. Whether cross-feeding is favoured at intermediate densities depends on (a) the slope of the trade-off function with cross-feeding more easily selected for shallow slopes; (b) the values of the cost ($r_{x_c} - r_x$) and the benefit (b_{xy}) of cross-feeding with cross-feeding more easily selected for high $r_{x_c} - r_x$ and b_{xy}; (c) the value of the intrinsic growth parameters (r_y, r_{x_c}, r_x) with cross-feeding more easily selected for low r_y, r_{x_c} and r_x. Throughout the figure black lines denote cases where cross-feeding is favoured while red lines denote cases where cross-feeding is not favoured.

The dynamics of model (1)

The cross-feeding model (1) has the following steady states

$(X^*,Y^*)=(0,0)$ and
$(X^*,Y^*)=(X,K-X)$ where $0 \leq X \leq K$.

The eigenvalues of the linearised system (1) around the zero state are $\lambda_1=r_x/r_y$ and $\lambda_2=1$ and since both $\lambda_1>0$ and $\lambda_2>0$ we conclude that (0,0) is an unstable steady state.

Therefore a small population $(X(0),Y(0))=(\varepsilon_1,\varepsilon_2)$ with ε_1 and ε_2 denoting positive constants near zero, will initially grow away from the zero steady state according to the following equation:

$$(X(t),Y(t)) = (\varepsilon_1 e^{\lambda_1 t}, \varepsilon_2 e^{\lambda_2 t}), \text{ for small } t. \qquad (3)$$

Subsequently the solution of (2) will approach one of the infinitely many steady states $(X,K-X)$ situated on the line segment $Y=K-X$. Which steady state it converges to cannot be determined with classical linearization techniques and will depend on the initial population sizes ε_1 and ε_2.

The dynamics of model (2)

Similarly the model (2) has the following steady states

$(X_c^*,Y^*)=(0,0)$ and
$(X_c^*,Y^*)=(X_c,K- X_c)$ where $0\leq X_c \leq K$.

The eigenvalues of the linearised model (2) around the zero steady state are $\lambda_1^c = r_{x_c} / r_y$ and $\lambda_2=1$ and since both $\lambda_1^c>0$ and $\lambda_2>0$ we conclude that (0,0) is an unstable steady state.

Therefore a small population $(X_c(0),Y(0))=(\varepsilon_1,\varepsilon_2)$ will initially grow away from the zero steady state according to the following equation

$$(X_c(t),Y(t)) = (\varepsilon_1 e^{\lambda_1^c t},\varepsilon_2 e^{\lambda_2 t}), \text{ for } t \text{ small.} \quad (4)$$

Subsequently the solution of (2) will approach one of the infinitely many steady states $(X_c,K-X_c)$ situated on the line segment $Y=K- X_c$. As for model (1), which steady state it converges to will depend on the initial population sizes ε_1 and ε_2.

Comparing the dynamics of models (1) and (2)

As in [17] the success of the non-cross feeding strategy is examined by comparing the cross-feeding genotype to the non cross-feeding genotype across the two patches. In other words starting with the same initial population densities $(X(0),Y(0))=(\varepsilon_1,\varepsilon_2)$ and $(X_c(0),Y(0))=(\varepsilon_1,\varepsilon_2)$ in patch 1 and patch 2 respectively, the $X(t)$ component of the solution of (1) representing densities of the cross-feeding strategy X is compared with the $X_c(t)$ component of the solution of (2) representing the density of the non cross-feeding strategy X_c.

From (3) and (4) it follows that

$$X(t) = \varepsilon_1 e^{\lambda_1 t} < X_c(t) = \varepsilon_1 e^{\lambda_1^c t}$$

for some small time t. Therefore as found in Bull and Harcombe (2009), at low population densities X_c always does better than X because $r_{x_c} > r_x$ and therefore $\lambda_1^c > \lambda_1$. This means that at low densities the cost of cooperation is not compensated by the benefit of cross-feeding.

Whether there exist a time interval for which the cross-feeding genotype does better than the non-cross feeding genotype ($X(t) > X_c(t)$) depends on a range of assumptions regarding the nature of the trade-off between the cost of cooperation and the benefit to the recipient, the initial population densities as well as the values of the intrinsic growth rates and/or the benefit of cross-feeding. For growth at intermediate densities the study presented in Bull and Harcombe (2009) generates the following results:

- BH1: When $b_{yx}>0$, selection always favours reciprocal cross-feeding from X to Y when $r_x \leq r_y$.
- BH2: Trade-offs with big gains in b_{xy} per decline in r_x enhance evolution of cooperation.
- BH3: Large b_{yx} enhance the evolution of reciprocity in the other direction from X to Y.

The above results have been generated by approximating non-linear dynamics with a linear model. In this paper we revisit BH1-BH3 for the non-linear models (1) and (2) assuming that each model has the same initial population densities of both genotypes ($\varepsilon_1=\varepsilon_2$). Our study shows that BH1 does not hold in general. As illustrated in Bull and Harcombe (2009), we find that cross-feeding from X to Y is favoured if the slope of the trade-off curve satisfies $\frac{r_x - r_{x_c}}{b_{xy}} = -0.1$, in other words if the cost of cross-feeding is 10% of the value of the benefit of cross-feeding, and if b_{xy} is sufficiently large (Figure 2.1a). In that case the cross-feeder X outgrows the non cross-feeder X_c for some intermediate time between the initial exponential growth and the final stationary phase (Figure 2.2a). However we find that changing the slope of the trade-off function has a profound effect on the above outcome. In particular we consider

the case where the slope of the trade-off function is changing from shallow (-0.1) to steep (-1). Decreasing the slope can be achieved either by lowering the benefit of cross-feeding (b_{xy}) or by increasing the cost of cooperation ($r_{x_c} - r_x$) (see Figure 2.1a). In both cases we find that the cross-feeders never outgrow the non cross-feeders i.e $X(t)<X_c(t)$ all $t>0$ (Figure 2.2b,c). Note that in the case where b_{xy} has been decreased (Figure 2.2b) the parameter b_{yx} was also altered so that $b_{xy}=b_{yx}$. Also note that in the case where the cost of cooperation has been increased (Figure 2.2c) the intrinsic growth rate of the Y genotype, r_y, is modified so that the assumption $r_y > r_{x_c} > r_x$ is upheld.

Whether the cross-feeding is favoured at intermediate densities is not solely determined by the slope of the trade-off function. For example retaining the shallow slope of -0.1 but changing the benefit of cooperation indicates that a small benefit (and therefore a small cost) of cross-feeding is less likely to favour the cross-feeding (Figure 2.1b). While this finding again contradicts BH1 it is in agreement with the result BH3 given that we assume that $b_{xy}= b_{yx}$.

The result BH2 states that shallow trade-offs enhance the evolution of cooperation. While our findings agree with BH2 our results show that depending on the r and b parameter values, steep trade-offs can also promote the evolution of cooperation. For example the lower the values of r_{x_c} and r_x (and by definition r_y), the steeper the angle of the trade-off for which the cross-feeding is favoured at intermediate densities (Figure 2.1c). Keeping b_{xy} fixed Figure 1c illustrates that when r_{x_c} =0.009 and r_x=0.008 the cross-feeding is favoured for trade-off slopes satisfying $(r_{x_c} - r_x) \le 0.8 b_{xy}$. However, when r_{x_c} =0.025 and r_x=0.015 cross-feeding is favoured for less steeper slopes $(r_{x_c} - r_x) \le 0.5 b_{xy}$. Note that when $r_{x_c} - r_x \ge 1 \cdot b_{xy}$ the cross-feeding is never favoured.

Reducing the initial population densities for both models (1) and (2) can lead to a dramatic change in the outcome from cross-feeding being favoured at intermediate

densities (Figure 2.3a) to cross-feeders never outgrowing the non cross-feeders (Figure 2.3b). Similar results have been observed in Bull and Harcombe (2009).

We also note that changing the slope of the trade-off relationship has an impact on the final population densities. For example comparing the outcomes of Figures 2.2a and 2.2b it can be seen that decreasing the benefit of cross-feeding leads to lower final population sizes of both X and X_c genotypes. This could be explained in the following way. Decreasing the benefit of cross-feeding lowers the impact of cross-feeding on population growth and therefore growth of different genotypes is dominated by their intrinsic ability to grow. Given that $r_y > r_{x_c} > r_x$ the Y genotype dominates the dynamics of both model (1) and (2) resulting in a smaller final population sizes of both X and X_c. Similarly, by comparing the outcomes of Figure 2.2a and 2.2c it can be seen that an increase in the cost of cooperation also results in lower final population sizes of both X and X_c. In this case an increase in the cost of cooperation was achieved by increasing $r_y - r_x$ and $r_y - r_{x_c}$ so that again the genotype Y dominated the dynamics of both model (1) and (2) resulting in a smaller final population sizes of both X and X_c.

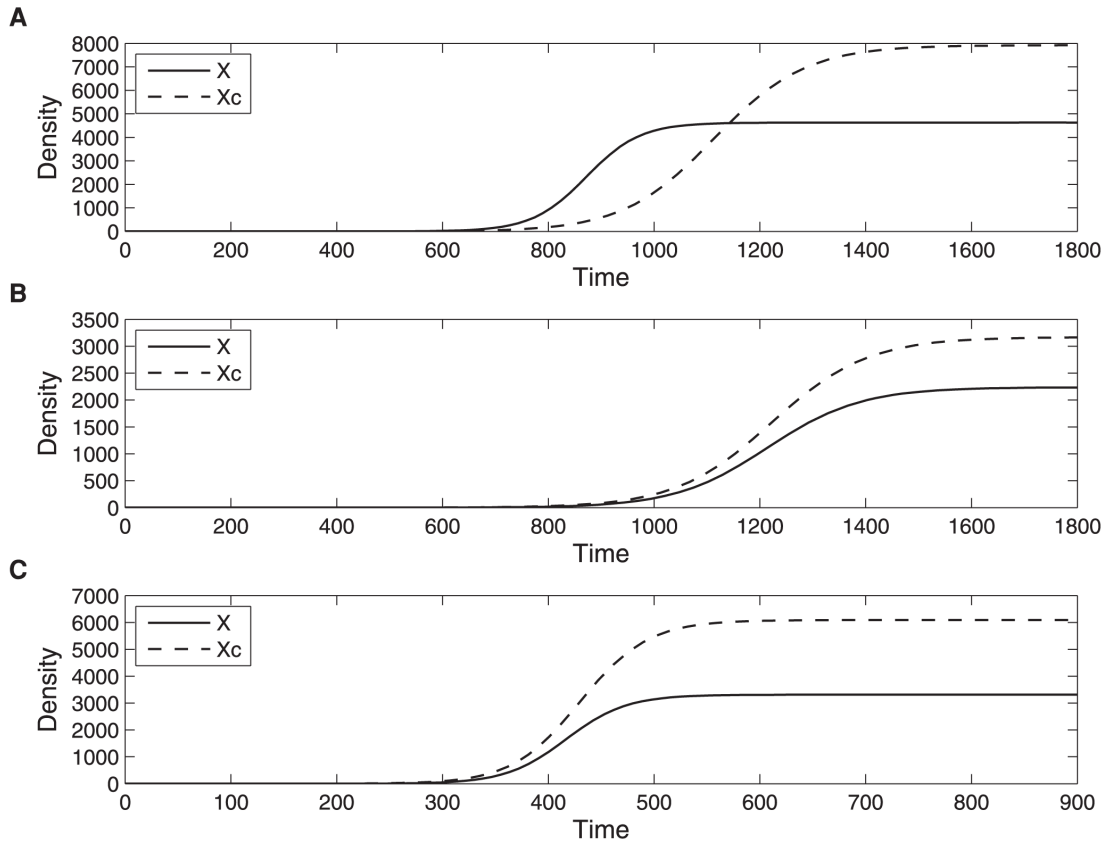


Figure 2.2. Simulation of two-species population growth for the model (1) and model (2). In the case of model (1) type X and Y cross-feed each other and in the case of model (2) X_c doesn't cross-feed Y but Y cross-feeds X_c. Here we plot $X(t)$ solution of (1) (full line) together with $X_c(t)$ solution of (2) (dashed line) with (a) r_y=0.011, r_x=0.008, r_{x_c} =0.009, b_{xy}=b_{yx}=0.01; (b) r_y=*0.011*, r_x=0.008, r_{x_c} =0.009, b_{xy}=b_{yx}=0.001; (c) r_y=0.03, r_x=0.015, r_{x_c} =0.025, b_{xy}=b_{yx}=0.01. For both simulations of model (1) and (2) and in all three cases presented here K=10000, $c_y = c_x = c_{x_c} = 1$ and ε_1=ε_2=0.01.

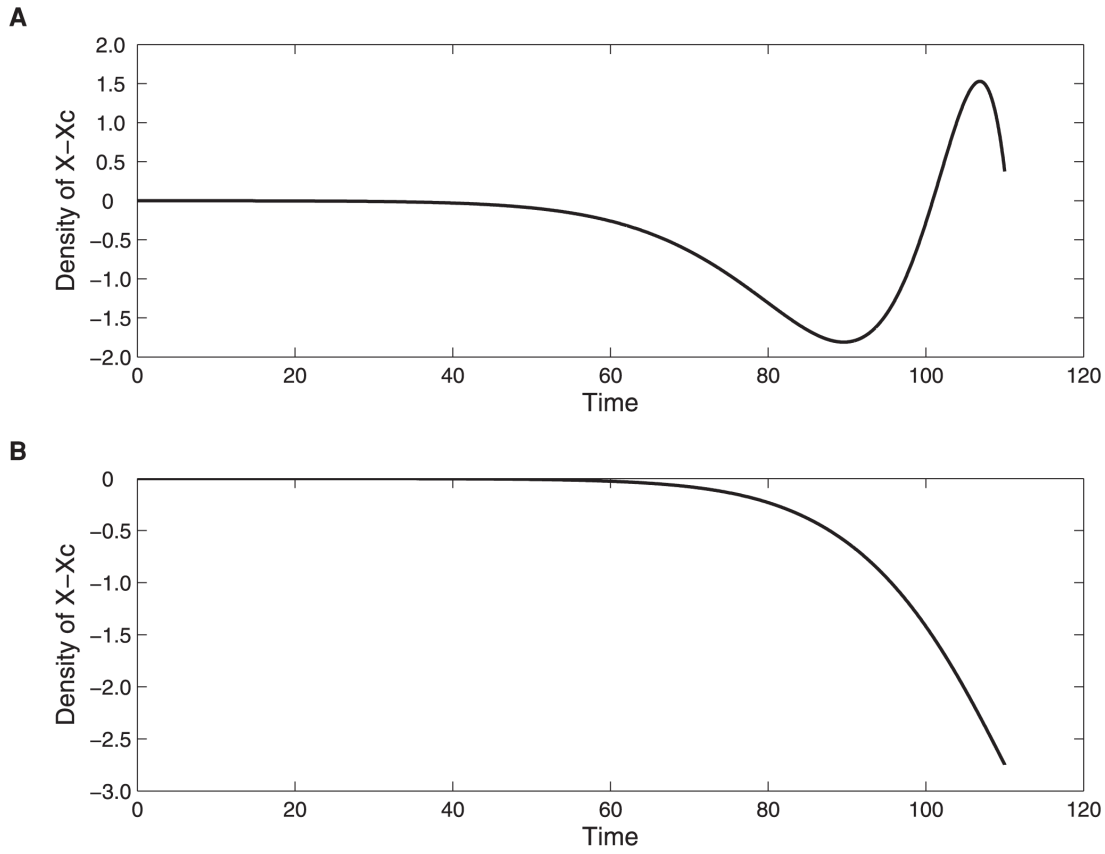


Figure 2.3. Initial population densities influence whether the cross-feeding will be favoured. For both simulations of model (1) and (2) r_y=0.11, r_x=0.088, r_{x_c}=0.09, b_{xy}=b_{yx}=0.01, K=10000 and $c_y = c_x = c_{x_c} = 1$. (a) cross-feeding is favoured for initial conditions ε_1=ε_2=0.01; (b) cross-feeding is not favoured for initial conditions ε_1=ε_2=0.001.

Evolutionary dynamics

Competition between cheats and cooperators

So far we have been considering a scenario where pairs of interacting microbial genotypes engaging in different levels of cross-feeding grow in two isolated patches or colonies Bull and Harcombe (2009). One could envisage a situation where at some point the populations will become large enough so that other types could migrate or could arise by mutation. This immediately raises the following question. What would

happen to the equilibrium dynamics in patch (1) if a small amount of a cheating genotype X_c is introduced either through migration from patch 2 or through mutation in genotype X? To answer this question model (1) can be adapted as in Bull and Harcombe (2009) to include an equation for the cheating genotype X_c:

$$\begin{aligned}
\frac{dX}{dt} &= X(r_x + b_{yx}\frac{Y}{X + X_c + c_x})(1 - \frac{X + X_c + Y}{K}) \\
\frac{dY}{dt} &= Y(r_y + b_{xy}\frac{X}{Y + c_y})(1 - \frac{X + X_c + Y}{K}) \\
\frac{dX_c}{dt} &= X_c(r_{x_c} + b_{yx}\frac{Y}{X + X_c + c_x})(1 - \frac{X + X_c + Y}{K})
\end{aligned} \qquad (5)$$

We are interested in the dynamics of (5) given the initial conditions $(X(0),Y(0),X_c(0)) = (X^*,Y^*,\varepsilon)$, where (X^*,Y^*) is a non-zero steady state of model (1) and

ε is a small constant. Such initial conditions denote the fact that a small population of non-cross feeding cheats has been introduced into patch 1 after its resident population has reached an ecological equilibrium.

Apart from the zero steady state the model (5) has infinitely many steady states satisfying the equation $X + Y + X_c = K$. As with models (1) and (2) the local stability of these steady states cannot be determined from simple linearization techniques. Numerical simulations indicate that for an initial condition (X^*,Y^*,ε) the model (5) will converge to a steady state $(X^*-\delta_1,Y^*-\delta_2,\delta_1+\delta_2)$ where δ_1 and δ_2 are small constants (Figure 2.4). This means that once established the cooperator genotype is not necessarily vulnerable to exploitation by the cheating genotype. Instead the cheat remains in the population but at low levels, close to the initial value ε.

A similar observation can be made for the case where a small amount of cooperator genotype is introduced into patch 2 whose resident genotypes have reached an ecological equilibrium.

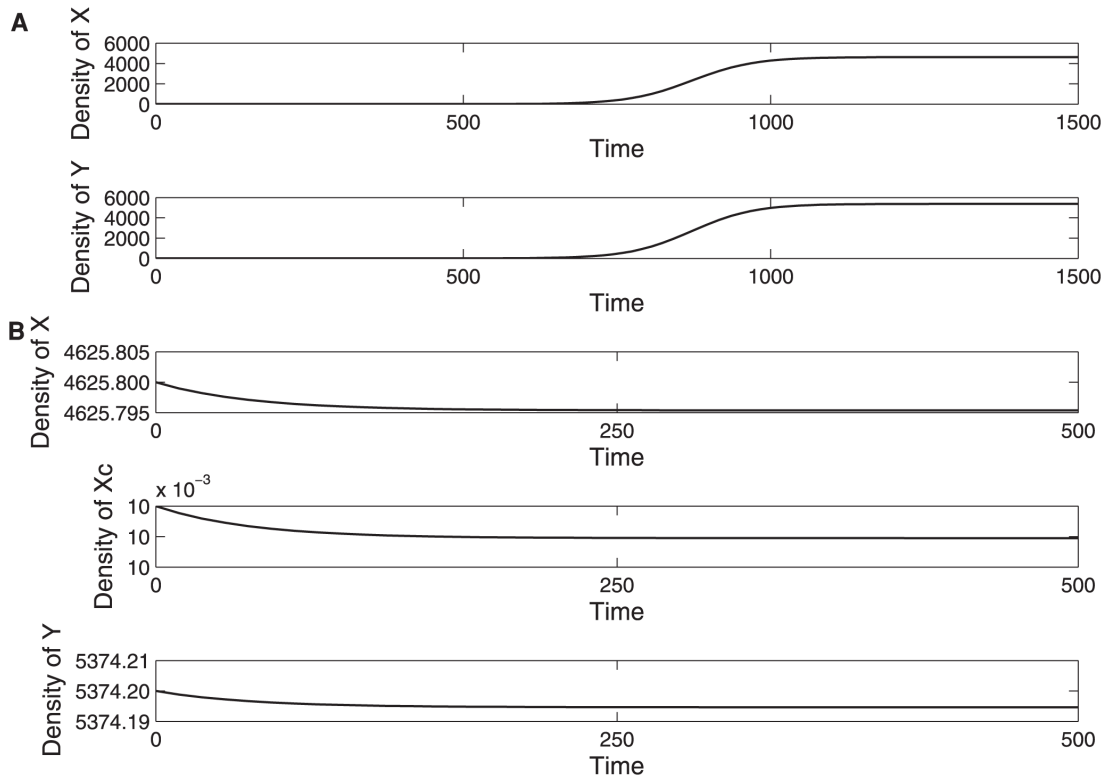


Figure 2.4. Evolutionary dynamics where mutants do not invade (a) Numerical simulations of the model (1) with r_y=0.11, r_x=0.08, b_{xy}=b_{yx}=0.01, K=10000 and $c_y = c_x = c_{x_c} = 1$. The figure shows an initial population $X(0)$=$Y(0)$=0.01 converging to a steady state (X^*,Y^*). (b) Numerical simulations of the model (5) where a small amount of non-cross feeder ($X_c(0)$=0.01*)*, is introduced into the steady state population (X^*,Y^*). The figure shows that the cross-feeder X is not vulnerable to invasion by non-cross feeder X_c. In addition to the above parameters r_{x_c} =0.09.

Evolution of cooperation

The competition model (5) assumes that all interacting types have the same carrying capacity, which in practice might not always be the case. In fact a cross-feeding between unrelated species often involves organisms that specialize on different resources. One such example is the interactions between two mutant strains *Escherichia coli* and *Salmonella enterica ser. Typhimurium* described in Harcombe (2010). Both strains were grown in lactose but *Salmonella* is not able to utilize lactose as an energy resource and instead uses a metabolite (acetate) excreted by *E.*

coli. On the other hand, *E. coli* can only degrade lactose in the presence of the amino acid methionine, which is synthesized by *Salmonella* but not by *E. coli*.

Motivated by Harcombe (2010) we alter the assumptions in (5) in order to explore general conditions for the evolution of cooperative cross-feeding. We begin by assuming that there is no interspecific competition for resources between the two cross-feeding types X and Y. This assumption is motivated by the fact that *E. coli* and *Salmonella enterica ser Typhimurium* do not utilize the same limiting nutrient as energy source and therefore do not compete for the same resource. For simplicity we also assume that the benefit of cross-feeding is simply proportional to the density of the individuals of the type providing nutrients. Therefore the cross-feeding interactions between X and Y can now be written as:

$$\begin{aligned} \frac{dX}{dt} &= X(r_x + b_{yx}Y)(1-\frac{\beta X}{K_x}), \\ \frac{dY}{dt} &= Y(r_y + b_{xy}X)(1-\frac{Y}{K_y}). \end{aligned} \qquad (6)$$

where β is the parameter describing the intraspecific competition amongst individuals of type X while K_x and K_y denote carrying capacities of X and Y respectively. The above system (6) has a trivial (0,0), two semi-trivial (K_x/β,0), (0, K_y) and the non-trivial steady state (K_x/β,K_y). While the trivial and both semi-trivial steady states are unstable, the non-trivial steady state is stable (see Appendix A).

We choose b_{xy} , the benefit of cross-feeding to Y per individual X, as the evolving trait belonging to a one-dimensional phenotypic trait space [0, b_{xymax}]. This phenotype can be viewed as an investment made by X into cooperation so that individuals with b_{xy}=0 do not invest into cooperation while individuals with b_{xy}=b_{xymax} invest maximally into cooperation. We assume that there will always be a biologically feasible maximum to any investment.

We now consider the effect of adding a mutant type X_m with phenotypic characteristic b_{xym} to the system (6) that is at the non-trivial steady state $(K_x/\beta, K_y)$. The evolution of the benefit of cross-feeding to Y per individual X (b_{xy}) is governed by the following three trade-offs:

1. The trade-off between investment into cooperation (b_{xy}) and an intrinsic ability to grow (r_x) is denoted by $r_x = f(b_{xy})$, which is a decreasing function of b_{xy}.
2. We also assume an asymmetric competition between the resident type X and a mutant type X_m, whereby increased investment into cooperation results in an increased competitive ability. This can in part be justified by the inevitable existence of structure with a given environment. For example *Salmonella* strains that produce large amount of methionine could have a larger amount of acetate in their neighbourhood (created by *E.coli* through cross-feeding) than the *Salmonella* types producing less methionine. Therefore we define a function $\beta(b_{xy} - b_{xym})$ describing the effect of the mutant strategy b_{xym} on the resident strategy b_{xy} which is a decreasing function of $b_{xy} - b_{xym}$.
3. Finally we assume the existence of a trade-off between the investment into cooperation and the carrying capacity K_x, where the carrying capacity is now a decreasing function of b_{xy}, denoted by $K(b_{xy})$. This assumption is motivated by the known inhibitory properties of methionine (Lawrence et al. 1968) so that an increased investment into cooperation leads to the over production of methionine which in turn leads to a reduction in the carrying capacity of the cooperating producer.

The equations for the new (mutated) system are given by:

$$\begin{aligned}
\frac{dX}{dt} &= X(f(b_{xy}) + b_{yx}Y)(1 - \frac{\beta(0)X + \beta(b_{xy} - b_{xym})X_m}{K(b_{xy})}), \\
\frac{dY}{dt} &= Y(r_y + b_{xy}X + b_{xym}X_m)(1 - \frac{Y}{K_y}), \qquad (7) \\
\frac{dX_m}{dt} &= X_m(f(b_{xym}) + b_{yx}Y)(1 - \frac{\beta(0)X_m + \beta(b_{xym} - b_{xy})X}{K(b_{xym})}).
\end{aligned}$$

The fitness of the invading mutant X_m is the largest eigenvalue of the system (7) at the steady state $(K_x/\beta,K_y,0)$ (see (Rand et al. 1994)), and is denoted by $\lambda_{b_{xy}}(b_{xym})$ which takes the following form

$$\lambda_{b_{xy}}(b_{xym}) = (f(b_{xym}) + b_{yx}K_y)(1 - \frac{\beta(b_{xym} - b_{xy})K(b_{xy})}{K(b_{xym})\beta(0)}).$$

For a discussion of the notion of fitness see (Metz et al. 1996). The invader's success will depend on its fitness in the following way: an invader with phenotypic characteristic b_{xym} when initially rare will be able to invade the resident population with phenotypic characteristic b_{xy} if $\lambda_{b_{xy}}(b_{xym}) > 0$. Alternatively, if $\lambda_{b_{xy}}(b_{xym}) < 0$, the invading population will die out. A phenotypic value for which the local fitness gradient is zero is called an 'evolutionarily singular strategy' (Metz et al. 1996), in our case denoted by b^*. According to Metz et al. (1996) and Geritz et al. (1998), at a singular strategy several evolutionary outcomes are possible. A singular strategy can: lack convergence stability and therefore act as an evolutionary repellor; be both evolutionarily and convergence stable and therefore be the final outcome of the evolution (also called 'continuously stable strategy'); and, finally, be convergence stable but not evolutionarily stable, in which case it is called a 'branching point'. These classifications are based on the assumption that, away from a singular strategy, the principle of mutual exclusion holds so that, after a successful invasion, the nearby invading population takes over and replaces the resident population. However, in a small neighbourhood of a singular strategy, the successful invasion by a nearby mutant can, under certain conditions, result in the coexistence of the invader and of the resident type populations (Geritz et al. 1998).

Here the outcome of the evolution of cooperation is investigated in a manner similar to the one described in Kisdi (1999). The results are summarized in the Table 2.1 and detailed calculations are presented in Appendix B.

Table 2.1. Possible evolutionary singularities (b^*) with different functional forms of K and β.

	β concave near 0 ($\beta''(0)<0$)	β linear near 0 ($\beta''(0)=0$)	β convex near 0 ($\beta''(0)>0$)
K concave near b^* ($K''(b^*)<0$)	Branching point or CSS	CSS	CSS
K linear near b^* ($K''(b^*)=0$)	Branching point	Degenerate	CSS
K convex near b^* ($K''(b^*)>0$)	Branching point or repellor	Branching point or repellor	Branching point; repellor or CSS

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Under what conditions the cheating type X_m that does not invest into cooperation and hence have b_{xym}=0, outcompetes the resident type X that has a non-zero investment into cooperation namely b_{xy}>0? From Table 2.1 it follows that this is only possible when K is a convex function near the singular strategy b^*(see Figure 2.5a left for an example). In that case the singular strategy could be a repellor which means that if the benefit to Y of the resident population X, b_{xy}, is less than b^*, the system will evolve towards the population where there is no benefit to Y from X. On the other hand if $b_{xy}>b^*$, the system will evolve towards the population where Y receives a maximal possible benefit from X (Figure 2.5a right).

In all of the remaining cases the following outcomes are possible. The singular strategy b^* is a continuously stable strategy (CSS) which implies that an initially monomorphic population of type X with the trait b_{xy} remains monomorphic throughout the course of evolution with a non-zero investment into cooperation, b^*, representing the final outcome of evolution. Alternatively b^*could be a branching point whereby an initially monomorphic population becomes dimorphic in the vicinity of b^*. In this case the outcome of evolution is a population containing two or more phenotypes with varying degree of investment into cooperation.

Table 2.1 shows that convex K does not always imply that the singular strategy b^* is a repellor. Under certain conditions (see Appendix B) it can also be a branching point (Figure 2.5b) or a CSS. Therefore the instances where a cheat phenotype with

b_{xy}=0 outcompetes and replaces a cooperating phenotype with b_{xy}>0 could be viewed as relatively rare.

However, given that the carrying capacity trade-off is motivated by the inhibitory properties of methionine (Lawrence et al. 1968) we argue that a concave K illustrated in Figure 2.5c left would be more appropriate as there is a threshold concentration of methionine above which the carrying capacity decreases. In this case the singular strategy is never a repellor and therefore cheats never outcompete and replace cross-feeding cooperators (Figure 2.5c right).

In this section we have classified a variety of evolutionary outcomes with respect to persistence of cooperation that depend on the shape of the *K* and *b* trade-offs. While there are many experimental evolutionary studies on microbial cooperation that have acknowledged the existence of different outcomes when a cooperative population is invaded by a mutant with a different investment into cooperation (Velicer et al. 2000; Griffin et al. 2004; MacLean and Gudelj 2006; Diggle et al. 2007; Gore et al. 2009; MacLean et al. 2010) very little is still known about the conditions that favour the evolution of cooperative cross-feeding between species. Pioneering work on the experimental evolution of novel cooperation between two cross-feeding species has been an important step towards a better understanding of the factors that enable interspecific cooperation in a cross-feeding interaction (Harcombe 2010). But as highlighted by the author in Harcombe (2010) there is still "a lack of clear explanation of the mechanisms necessary for the evolutionary origin of cooperation, particularly between species". Further experimental studies are needed to shed light on this important problem.

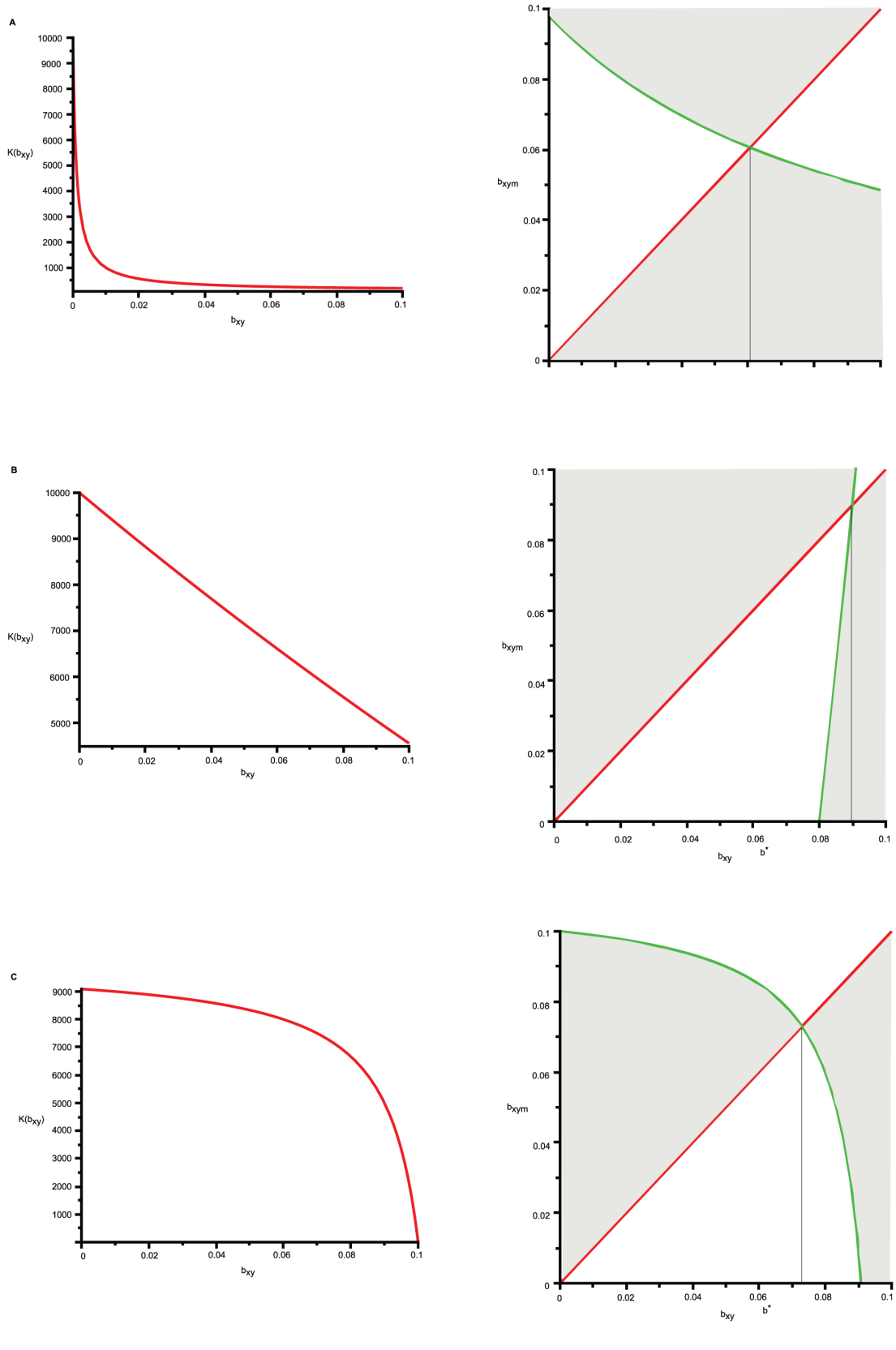
A
K(bxy)
bxy
bxym
B
K(bxy)
bxy
bxym
b*
C
K(bxy)
bxy
bxym
b*

Figure 2.5. Evolutionary outcomes. The left hand side of the figure represents examples of different trade-offs while the right hand side of the figure gives the corresponding pairwise invisibility plots, PIPs, (Geritz et al. 1998). The shaded area indicate the combinations of b_{xy} and b_{xym} for which the fitness of the mutant, $\lambda_{b_{xy}}(b_{xym})$, is positive. In all cases $f(x)$=0.025-0.16x, $\beta(x)$=1-(x+0.1)/0.2 while $K(x)=\alpha_1(x-\alpha_2)/(x-\alpha_3)$. (a) the left hand side shows a steep convex K function with α_1=100, α_2=-0.1 and α_3=-0.001 with a corresponding PIP on the right hand side illustrating that in this case b^* is a repellor; (b) the left hand side shows a shallow convex K function with α_1=-50000, α_2=0.2 and α_3=-1 with a corresponding PIP on the right hand side illustrating that in this case b^* is a branching point; (c) the left hand side shows a concave K function with α_1=10000, α_2=0.1 and α_3=0.11 with a corresponding PIP on the right hand side illustrating that in this case b^* is a CSS.

2.5. Discussion

When the cost of cross-feeding to the donor ($r_{x_c} - r_x$) is greater or equal to the benefit to the recipient (b_{xy}) cooperation is never favoured. Indeed, by definition a reciprocal interaction provides a direct fitness benefit to the cooperators and this suggests that a cooperative trait will only be selected if the benefit to cooperate is higher than its cost. Additionally, this also reflects the fact that an individual that doesn't pay the cost of cooperation in the short term will not gain the benefit of cooperation in the long term (West et al. 2007).

Previous theoretical results indicate that the cross-feeding is more easily selected when its cost to the donor is low per benefit to the recipient, in other words $(r_{x_c} - r_x)/b_{xy}$ is sufficiently small (Schaffer 1978; Foster and Wenseleers 2006) and when the recipient already provides a large cross-feeding benefit to the donor, in other words when b_{xy} is sufficiently large (Bull and Harcombe 2009). Our study recovers the same outcomes (Figure 2.1a,b) but in addition we obtain results that are at odds with those presented in Bull and Harcombe (2009) in the case of growth at

intermediate densities. Before summarising the differences in outcomes we note that they come about due to the fact that while we study the non-linear system (1) the results in Bull and Harcombe (2009) are obtained using a linear approximation of (1). Contrary to Bull and Harcombe (2009), we find that the cross-feeder does not always outgrow the non cross-feeder when the benefit of cross-feeding to X per individual of type Y is $b_{yx}>0$ and $r_x \leq r_y$ (Figure 2.2b,c). In addition to Bull and Harcombe (2009) we find that steep trade-offs can also promote the evolution of cross-feeding (Figure 2.1c). Namely, a decrease in the intrinsic growth rates increases the range of values of $(r_{x_c} - r_x)/b_{xy}$ for which the cross-feeding is favoured. This is explained by the fact that when intrinsic growth rates are low compared to the benefit of cross-feeding, the cross-feeding term dominates the overall growth of microorganisms and therefore the cost of cross-feeding is not required to be too low for the cross-feeding to be favoured. Surprisingly, our model indicates that in some cases cross-feeding is favoured even if the cost to the donor is up to 80% of the value of the benefit to the recipient. This seems to suggest the following. Firstly, if populations have high intrinsic growth rate and are therefore less dependent on the cross-feeding interactions to grow, cross-feeding interactions are less favoured. Secondly, an increase in synergic benefit of cooperation should result in cooperation being more easily selected for (West et al. 2006).

The advantage of cross-feeding is also known to change with initial population densities of interacting microorganisms (Bull and Harcombe 2009). In addition we find that a reduction in the initial population densities can lead to a dramatic change in the outcome from cross-feeding being favoured at intermediate densities to cross-feeders never being able to outgrow non cross-feeders.

In evolutionary terms, our study reveals a result different to that reported in Bull and Harcombe (2009). We find that once a population of two cross-feeders has been established in a spatially isolated colony, the large populations of cross-feeders are not vulnerable to small numbers of exploiting genotypes that arise through migration or mutation and who share in the cross-feeding resources but do not reciprocate in cross-feeding themselves. However, this result relies on the assumption that all

microbial types have the same carrying capacity. Subsequently we considered a more general evolutionary model assuming that X and Y utilize different resources and therefore have different carrying capacities, (Harcombe 2010). Motivated by Lawrence et al. (1968) we also introduced the following additional trade-offs: an increased investment into cooperation results in an increased competitive ability but a decreased carrying capacity. We find that an exploiting genotype that does not reciprocate in cross-feeding can take over and replace the resident cooperator genotype only in certain cases when the carrying capacity trade-off is convex. Given that such trade-off is motivated by the inhibitory properties of methionine (Lawrence et al. 1968) we argue that a concave trade-off illustrated in Figure 2.5c would be more appropriate as there is a threshold concentration of methionine above which the carrying capacity decreases. Our results indicate that a concave trade-off between investment into cooperation and carrying capacity is most likely to give rise to populations containing a single phenotype that has a non-zero investment into cooperation.

In conclusion our results have a number of important messages. Firstly, the shape of the trade-off between the cost and benefit of cooperation has a profound effect on the success of cross-feeders (cooperators) in comparison to non cross-feeders (cheats). In other words whether cross-feeding is favoured or not depends on whether the cost to the donor decreases slower or faster than the benefit to the recipient. This is in accordance with both classical (Levins 1962; Levins 1968; Schaffer 1978) and recent (Boots and Haraguchi 1999; de Mazancourt and Dieckmann 2004; White and Bowers 2005; Mealor and Boots 2006; Gudelj et al. 2007) theoretical work showing that the precise form of the trade-off curves crucially determines the outcome of evolution. Therefore in order to deepen our understanding of the evolution of cooperative cross-feeding, it is extremely important to obtain precise estimation of the shape of the cost/benefit trade-off. Elucidating the shape of a trade-off relationship in general is something that has so far proven to be particularly challenging. However, due to their large population sizes, short generation times and known genetic structure microorganisms present an ideal system with which to

experimentally study the nature and form of trade-off relationships (Bohannan et al. 2002; Jessup and Bohannan 2008).

Secondly, we have demonstrated that the impact of the trade-off between the cost and the benefit of cross-feeding varies with different environments. For example, in the environments where the intrinsic growth rates of microbes under consideration are higher than the benefit of cross-feeding, cooperative behaviour is favoured only for sufficiently shallow trade-offs. However, in the environments where the intrinsic growth rates are lower than the benefit of cross-feeding, cooperation behaviour is favoured for a large range of trade-off slopes.

Finally, when considering the evolution of cross-feeding we found that if all interacting individuals have the same carrying capacity a small population of cheats could not invade an already established population of cooperating cross-feeders. If we assume that cross-feeding species specialize on different resources and hence have different carrying capacities the outcome of evolution depends on the shape of the trade-off between investment into cooperation and competitive ability and the trade-off between investment into cooperation and carrying capacity. The most common outcome of evolution is either polymorphism where the evolving population contains two or more genotypes with varying degree of cooperation or monomorphism where the evolving population contains a single phenotype that makes a non-zero investment into cooperation. This further demonstrates that cross-feeding could be viewed as a robust interaction, a result that accords with a large number of cross-feeding examples readily observed in nature.

2.6. Appendices

Appendix A

Steady states of system (6) can be found by solving the following set of equations:

$$0 = X(r_x + b_{yx}Y)(1 - \frac{\beta X}{K_x}),$$
$$0 = Y(r_y + b_{xy}X)(1 - \frac{Y}{K_y}). \quad \text{(A1)}$$

for X and Y. It is easy to see that there are four steady states $(X_1^*,Y_1^*)=(0,0)$, $(X_2^*,Y_2^*)=(K_x/\beta,0)$, $(X_3^*,Y_3^*)=(0, K_y)$ and $(X_4^*,Y_4^*)=(K_x/\beta,K_y)$. The Jacobian matrix of (A1) takes the form

$$J(X,Y) = \begin{pmatrix} (r_x + b_{yx}Y)(1 - \frac{2\beta X}{K_x}) & b_{yx}X(1 - \frac{\beta X}{K_x}) \\ b_{xy}Y(1 - \frac{Y}{K_y}) & (r_y + b_{xy}X)(1 - \frac{2Y}{K_y}) \end{pmatrix}$$

evaluated at $(X,Y)=(X_i^*,Y_i^*)$ where $i=1..4$. For the trivial steady state we have

$$J(0,0) = \begin{pmatrix} r_x & 0 \\ 0 & r_y \end{pmatrix},$$

hence the trivial steady state is unstable. Similarly for the semi-trivial steady states we have

$$J(\frac{K_x}{\beta},0) = \begin{pmatrix} -r_x & 0 \\ 0 & r_y + b_{xy}\frac{K_x}{\beta} \end{pmatrix} \text{ and } J(0,K_y) = \begin{pmatrix} r_x + b_{yx}K_y & 0 \\ 0 & -r_y \end{pmatrix},$$

from which we conclude that both semi-trivial steady states are also unstable. Finally for the non-trivial steady state we have

$$J(\frac{K_x}{\beta},0) = \begin{pmatrix} -(r_x + b_{yx}K_y & 0 \\ 0 & -(r_y + b_{xy}\frac{K_x}{\beta}) \end{pmatrix},$$

and therefore the non-trivial steady state is stable.

Appendix B

Consider the rare mutant strategy X_m in the resident population X. The mutant increases in number of its growth rate

$$\lambda_{b_{xy}}(b_{xym}) = (f(b_{xym}) + b_{yx}K_y)(1 - \frac{\beta(b_{xym} - b_{xy})K(b_{xy})}{K(b_{xym})\beta(0)})$$

is positive (see system of equations 7) while a mutant with negative growth rate dies out (see Geritz et al. (1998) and Kisdi (1999) for details). The resident population has zero growth rate ($\lambda_{b_{xy}}(b_{xy}) = 0$) at equilibrium population density $X(b_{xy})=K(b_{xy})/\beta(0)$. It follows that a mutant strategy b_{xym} that is slightly larger than b_{xy} can invade and replace the resident if the fitness gradient

$$\left.\frac{\partial\lambda_{b_{xy}}(b_{xym})}{\partial b_{xym}}\right|_{b_{xym}=b_{xy}} = (f(b_{xy}) + b_{yx}K_y)(\frac{K'(b_{xy})}{K(b_{xy})} - \frac{\beta'(0)}{\beta(0)})$$

is positive; mutants with $b_{xym} < b_{xy}$ can invade if the fitness gradient is negative. Repeated invasions and substitutions result in directional evolution until the population reaches an evolutionary singularity where the fitness gradient is zero. The singular strategy b^* can subsequently be classified in the following way. According to Geritz et al. (1998) if

$$\left[\frac{\partial^2\lambda_{b_{xy}}(b_{xym})}{\partial b_{xym}^{\,2}}\right]_{b_{xym}=b_{xy}=b^*} = (f(b^*) + b_{yx}K_y)(-\frac{\beta''(0)}{\beta(0)} + \frac{K''(b^*)}{K(b^*)}) < 0 \quad \text{(B1)}$$

the singular strategy is evolutionary stable. If

$$\frac{d}{db_{xy}}\left[\left.\frac{\partial\lambda_{b_{xy}}(b_{xym})}{\partial b_{xym}}\right|_{b_{xym}=b_{xy}}\right]_{b^*} = (f(b^* + b_{yx}K_y)(\frac{K''(b^*)}{K(b^*)} - \left(\frac{K'(b^*)}{K(b^*)}\right)^2) < 0 \quad \text{(B2)}$$

the singular strategy is convergence stable. Therefore if (B1) and (B2) hold the singular strategy is a continuously stable strategy (CSS); if (B2) holds but (B1) does not so that

$$-\frac{\beta''(0)}{\beta(0)} + \frac{K''(b^*)}{K(b^*)} > 0 \quad \text{(B3)}$$

the singular strategy is an evolutionary branching point. Finally if (B2) does not hold

so that

$$\frac{K''(b^*)}{K(b^*)} - \left(\frac{K'(b^*)}{K(b^*)}\right)^2 > 0 \quad \text{(B4)}$$

the singular strategy is a repellor.

Chapter 3

METABOLIC BASIS OF A CROSS-FEEDING INTERACTION OF AN EVOLVED POLYMORPHISM IN *ESCHERICHIA COLI*: A RECONCILIATION BETWEEN EXPERIMENTS AND THEORY

3.1. Summary

Cross feeding is the ability of one organism to feed on the metabolic by-product of another organism. Although cross-feeding interactions are ubiquitous in nature, our understanding of how it evolved in the first place is still limited. Here, we use a systems-biology approach to investigate the origins of cross-feeding interactions via single lineage diversification. In particular, we derive new predictions on the physiological mechanisms that may explain the stable coexistence of a cross-feeding polymorphism that evolved from a single clone. We show that either a distinct resistance to waste product toxicity, or a distinct inhibition of a key enzyme of bacterial metabolism, may explain this stable coexistence. These findings provide new insights into the physiological mechanisms that may underpin the emergence and maintenance of diversity via cross-feeding interactions.

3.2. Introduction

Cross feeding, termed syntrophy when between unrelated species, is the ability of one organism to use incompletely oxidized metabolites excreted by another organism as energy resource. This interaction plays a key role in the microbial world (Schink 2002; Marx 2009; Stams and Plugge 2009). Indeed, cross-feeding interactions have been of major influence on ecosystems such as for the reduction of green house gas methane (Hallam et al. 2004; Reeburgh 2007; Pernthaler et al. 2008), the degradation

of xenobiotic compounds (Dejonghe et al. 2003), as well as in microbial gut communities (Belenguer et al. 2006). While cross-feeding interactions are well-documented, little is still known about the mechanisms that enable their stable coexistence. This also raises the question of how cross-feeding organisms that depend on another organism to grow can evolve and be maintained instead of the evolution of one unique competitor that is able to degrade the primary resource completely. Furthermore, experimental evolutionary studies in bacteria have shown that cross-feeding is one of the mechanisms that allows for the emergence and maintenance of diversity in single-limited continuous environments. This observation contrasts with the competitive exclusion principle (Gause 1934; Hardin 1960) that predicts that competition for the same limiting resource in a continuous environment cannot support species coexistence, but instead, one of the competitors will be selected while the others will be excluded. Also, the evolution of a stable polymorphism involving cross-feeding has been clearly demonstrated in both continuous culture (Helling et al. 1987; Rosenzweig et al. 1994; Treves et al. 1998) and in serial culture (Turner et al. 1996).

Theoretical models on the evolution of cross-feeding have suggested that trade-offs may explain why cross-feeding evolves and is maintained. For example, Doebeli (2002) used adaptive dynamics theory to demonstrate that cross-feeding evolution from a single ancestral strain is possible if there is a trade-off between the uptake efficiencies on the primary and secondary resources and this trade-off has a positive curvature. (Pfeiffer and Bonhoeffer 2004) suggested that the evolution of cross-feeding is favoured when the rate of ATP production is maximized and when the concentration of enzymes and intermediates of the pathway are minimized. However, these theoretical attempts to understand the emergence and maintenance of cross-feeding have failed to consider the mechanism of the cross-feeding interactions, as highlighted in (Porcher et al. 2001). Here, we seek to bridge this gap between theory and experiments and aim to give new insights into the physiological mechanisms that underlie the stable coexistence of cross-feeding polymorphisms. To that end, we developed a mathematical model that connects ecological population dynamics with Michaelis-Menten kinetics.

Our model is based on a well-known long-term experimental evolution study initiated by (Helling et al. 1987) in the mid-1980s. The authors observed the stable coexistence of three different phenotypic strains that had evolved from a unique ancestral *Escherichia coli* population grown in a glucose-limited chemostat for hundreds of generations. A posterior reconstruction of the experiment showed that this stable polymorphism was maintained by a cross-feeding interaction (Rosenzweig et al 1994). This study revealed that the evolved *E. coli* polymorphism was mainly constituted by three cross-feeding genotypes: a glucose specialist, that exhibited increased rate of glucose uptake as well as increased rate of acetate excretion; an acetate specialist and cross-feeder, characterized by an enhanced ability to use acetate; and a glycerol generalist that had an enhanced ability to assimilate glycerol when compared with the ancestral and the other two evolved clones. In addition, genetic analysis revealed that the increased uptake of acetate by the acetate specialist was due to a cis-regulatory mutation on the acetyl-CoA synthetase (Treves et al. 1998). This enzyme plays a key role in the uptake of extracellular acetate, and that mutation resulted in its overexpression. Furthermore, a structural mutation in the glycerol-3-phosphate regulon repressor (glpR) of the ancestral genotype might have set the stage for the adaptive evolution of a species with enhanced rate of glycerol uptake (Kinnersley et al. 2009). This regulon is important in the metabolism of glycerol and this mutation has been linked to the constitutive expression of genes involved in glycerol utilization. However, this observation is insufficient to explain the reason why the glycerol generalist uses glycerol more efficiently than the other strains.

Evolutionary scenario

These observations seem to suggest that the evolution of this cross-feeding polymorphism has been driven by adaptation to selective pressures for the optimization of resource utilization. The following evolutionary scenario has been proposed to explain the evolutionary adaptations underlying the evolution of this cross-feeding polymorphism (Rosenzweig et al. 1994). First, a limited-glucose

continuous environment leads to a selective pressure for the evolution of a genotype with an enhanced ability to uptake glucose. Thus, a mutant genotype such as the glucose specialist will be favoured and might arise first. However, this increased ability to scavenge glucose comes at a cost on its ability to use acetate and glycerol. Moreover, an increased rate of glucose consumption is linked to a lower efficiency of ATP production. This trade-off between rate and yield of ATP production arises from thermodynamic principles (Pfeiffer and Bonhoeffer 2002) and also because of increasing limitations in the TCA cycle or in respiratory NADH turnover (Kayser et al. 2005). One explanation for such limitation may be that an excess of glucose uptake results in a large amount of NADH produced through glycolysis. The NADH produced needs to be oxidized to NAD, however, the rate at which this reaction can happen reaches a threshold at high glucose uptake rates, and as a consequence, NADH accumulates (Wolfe 2005; Eiteman and Altman 2006). One way that *E. coli* has to overcome this energetic limitation is by excreting acetate as an alternative pathway to produce energy. This leads to the accumulation of a large amount of extracellular acetate into the environment, and thus a strain, which is able to use acetate efficiently such as the acetate specialist, would have an adaptive advantage and might emerge. Also, glycerol is excreted as a product of glucose metabolism and this creates a third resource available. This might have enabled the persistence of a strain able to use both glucose and glycerol, such as the glycerol generalist.

In this work, we first develop a simple mathematical model that captures the key biochemical pathways involved in this specific cross-feeding interaction, namely the glucose, acetate and glycerol metabolism. Second, we aim to reproduce the cross-feeding coexistence based on empirical data from this long-term evolution experiment (Helling et al. 1987; Rosenzweig et al. 1994; Treves et al. 1998) and on the biochemistry of bacteria (Table S1, appendix). With this, we hope to gain a better understanding of the physiological trade-offs that underlie such behaviour.

3.3. Methods

Numerical simulations were performed using MATLAB. When not otherwise specified in the figure legends, the parameter values used for each illustration are in Table S1. Initial concentrations of $X_{in,1}$, $X_{in,2}$ and X_{ex} were 0 and So was 3.5 μM.

Model

To investigate the role of glucose, acetate, and glycerol metabolism in the stable coexistence of the cross-feeding polymorphism, we developed a model of competition of three strains, a glucose specialist, an acetate cross-feeder (acetate specialist) and a glycerol generalist for a limiting nutrient, glucose, in chemostat. The catabolism of the limiting nutrient and its intermediates is based on previous models (Pfeiffer and Bonhoeffer 2004; MacLean and Gudelj 2006), in which for simplicity the authors assumed only a two-reaction process, glycolysis and the tricarboxylic acid (TCA) cycle. Moreover, we also include the ATP yield consumed and produced in the acetate and glycerol uptake and excretion. We assumed that the catabolic reactions are based on Michaelis-Menten kinetics for saturating enzymes, that the rate of cellular growth is proportional to the rate of ATP production (Bauchop and Elsden 1960), defined here by the proportionality constant G, and that the metabolic intermediate acetate reduces the growth rates of the organisms by a logarithmic cost function (See Table S1). The model of competition is represented below:

$$\frac{dS}{dt} = D \cdot (S_0 - S) - \left[v_{glu\max,1}(S) + c_1(S) \right] \cdot N_1 - \left[v_{glu\max,2}(S) + c_2(S) \right] \cdot N_2 - \left[v_{glu\max,3}(S) + c_3(S) \right] \cdot N_3$$

$$\frac{dX_{ex1}}{dt} = \begin{pmatrix} v_{AK\max,1}(X_{in,1} - X_{ex1}) \cdot N_1 + \left[v_{AK\max,2}(X_{in,2} - X_{ex1}) - v_{ACS\max,2}(X_{ex1}) \right] \cdot N_2 \\ + \left[v_{AK\max,3}(X_{in,3} - X_{ex1}) - v_{ACS\max,3}(X_{ex1}) \right] \cdot N_3 \end{pmatrix} - D \cdot X_{ex1}$$

$$\frac{dX_{ex2}}{dt} = \left[c_1(S) - v_{GK\max,1}(X_{ex2}) \right] \cdot N_1 + \left[c_2(S) - v_{GK\max,2}(X_{ex2}) \right] \cdot N_2 + \left[c_3(S) - v_{GK\max,3}(X_{ex3}) \right] \cdot N_3 - D \cdot X_{ex2}$$

$$\frac{dN_1}{dt} = G \cdot \left[v_{glu\max,1}(S) \cdot n_{glu,1} + v_{TCA\max,1}(X_{in,1}) \cdot n_{TCA,1} + v_{AK\max,1}(X_{in,1} - X_{ex1}) \cdot n_{AK} - v_{GK,1}(X_{ex2}) \cdot n_{GK,1} + c_1(S) \cdot n_{GK,1} \right] \cdot \text{cost}(X_{in,1}) \cdot N_1 - D \cdot N_1$$

$$\frac{dN_2}{dt} = G \cdot \begin{bmatrix} v_{glu\max,2}(S) \cdot n_{glu,2} + v_{TCA\max,2}(X_{in,2}) \cdot n_{TCA,2} - v_{ACS\max,2}(X_{in,2}) \cdot n_{ACS,2} + v_{AK\max,2}(X_{in,2} - X_{ex1}) \cdot n_{AK} \\ - v_{GK,2}(X_{ex2}) \cdot n_{GK,2} + c_2(S) \cdot n_{GK,2} \end{bmatrix} \cdot \text{cost}(X_{in,2}) \cdot N_2 - D \cdot N_2$$

$$\frac{dN_3}{dt} = G \cdot \begin{bmatrix} v_{glu\max,3}(S) \cdot n_{glu,3} + v_{TCA\max,3}(X_{in,3}) \cdot n_{TCA,3} - v_{ACS\max,3}(X_{in,3}) \cdot n_{ACS,3} + v_{AK\max,3}(X_{in,3} - X_{ex1}) \cdot n_{AK} \\ - v_{GK,3}(X_{ex2}) \cdot n_{GK,3} + c_3(S) \cdot n_{GK,3} \end{bmatrix} \cdot \text{cost}(X_{in,3}) \cdot N_3 - D \cdot N_3$$

$$\frac{dX_{in,1}}{dt} = \left[2 \cdot v_{glu\max,1}(S) - v_{TCA\max,1}(X_{in,1}) - v_{AK\max,1}(X_{in,1} - X_{ex1}) + v_{GK\max,1}(X_{ex2}) \right] \cdot N_1 - D \cdot X_{in,1}$$

$$\frac{dX_{in,2}}{dt} = \left[2 \cdot v_{glu\max,2}(S) - v_{TCA\max,2}(X_{in,2}) - v_{AK\max,2}(X_{in,2} - X_{ex1}) + v_{ACS\max,2}(X_{ex1}) + v_{GK\max,2}(X_{ex2}) \right] \cdot N_2 - D \cdot X_{in,2}$$

$$\frac{dX_{in,3}}{dt} = \left[2 \cdot v_{glu\max,3}(S) - v_{TCA\max,3}(X_{in,3}) - v_{AK\max,3}(X_{in,3} - X_{ex1}) + v_{ACS\max,3}(X_{ex1}) + v_{GK\max,3}(X_{ex3}) \right] \cdot N_3 - D \cdot X_{in,3}$$

N_1, N_2, and N_3 denote the population density of the glucose specialist, glycerol generalist, and acetate specialist, respectively. X_{in1} and X_{ex1} are the concentration of the intracellular and extracellular intermediate acetate respectively, and X_{ex2} is the concentration of the extracellular intermediate glycerol. S denotes the concentration of the resource, glucose, into the medium and S_0 the concentration of glucose that is supplied to the chemostat. D is the dilution rate. $v_{glu\max}$, $v_{TCA\max}$, $v_{ACS\max}$, $v_{AK\max}$ and $v_{GK\max}$ are the rate of glycolysis, the TCA cycle, the extracellular acetate uptake by the Acetyl-CoA synthetase (ACS), the reversible uptake and excretion of acetate by the acetate kinase (AK), and the irreversible uptake of glycerol by the glycerol kinase (AK) respectively, such as $v_{i\max} = V_{i\max} \text{X} / (K_{i\max} + \text{abs}(X))$ where I and X are the enzyme and substrate concentration, respectively. n_{glu}, n_{TCA}, n_{AK}, n_{ACS}, and n_{GK} refer to the ATP yield of the glycolysis, the TCA cycle, the uptake/excretion of acetate by the acetate kinase, the uptake of acetate by the acetyl-CoA synthetase, and the uptake of glycerol by the glycerol kinase, respectively. c represents the rate of glycerol

excretion and is defined as $c = k\ v_{glu\ max}(S)$ where k is a constant transport rate of glycerol across the membranar cell. The inhibitory effect of intracellular acetate assumes the following logarithmic function: $cost(X_{in}) = 1 / (1+ X_{in} /K_I)$ where K_I is the growth inhibitory constant (Davison and Stephanopoulos 1986), i.e. the inhibition constant of intracellular acetate. The model was parameterized using empirical data from this long-term evolution experiment (Helling et al. 1987; Rosenzweig et al. 1994; Treves et al. 1998) and data on the biochemistry of bacteria (Table S1, SI). Figure 3.1A shows the schematic representation of the acetate and glycerol cross-feeding interactions of the polymorphism.

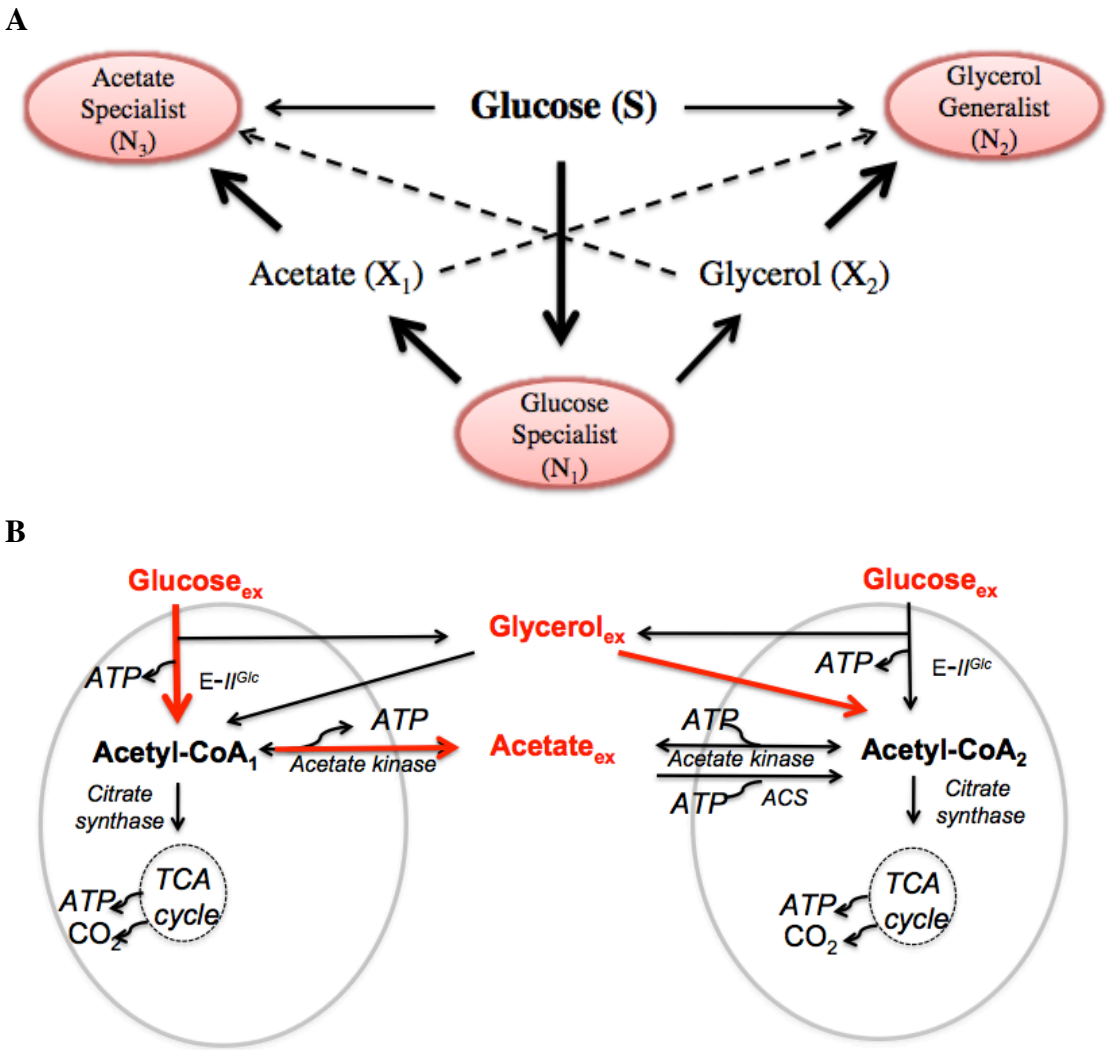

Figure 3.1. A. Schematic model illustrating the acetate and glycerol cross-feeding interactions of the polymorphism. The weight of the lines represents the

relative rates of the reactions in relation with the other phenotypes. **B. Cross-feeding interaction between a glucose specialist (left cell) and a glycerol generalist (right cell).** The red lines highlight the main reactions involved in the cross-feeding of acetate and glycerol.

Based on Helling and colleagues long-term experiments on the evolution of a cross-feeding polymorphism in *E. coli*, we assumed the following: (a) the glucose specialist has an advantage in glucose-limited chemostat due to a higher rate of glucose uptake than the acetate specialist and glycerol generalist; (b) the glucose specialist has a lower affinity for acetate than the acetate specialist. This reduced affinity enables the glucose specialist to excrete acetate faster into the environment, and the acetate can be used as energy resource posteriorly; (c) the acetate specialist exhibits an increased rate of acetate uptake because of an overexpression of the acetyl-CoA synthetase (ACS) enzyme, which is one of the main enzymes involved in the uptake of extracellular acetate; (d) by contrast, the acetyl-CoA synthetase of the glucose specialist is repressed (Treves et al. 1998). In sum, the experiments have shown that $V_{glu\,\max,1} > V_{glu\,\max,3} > V_{glu\,\max,2}$; $V_{ACS\,\max,1}$ (=0) < $V_{ACS\,\max,2}$ < $V_{ACS\,\max,3}$ and $K_{AK\,\max,2}$ < $K_{AK\,\max,1}$ < $K_{AK\,\max,3}$ (Helling et al. 1987; Rosenzweig et al. 1994; Treves et al. 1998). These metabolic traits underlie two main trade-offs. First, there is a trade-off between resource specialization and generalization, in other words, while the specialist uses preferentially and more efficiently a unique resource, the generalist is able to use different types of resources with a moderate efficiency. The second trade-off occurs between rate and efficiency of ATP producing pathways in resources oxidation. The glucose specialist benefits from an efficient utilization of glucose, however this is at a cost on the ATP yield produced. This cost is due to the partial degradation of glucose by fermentation (low ATP yield process) and which results in the production of incomplete oxidized metabolites. By contrast, both the glycerol generalist and acetate specialist have lower glucose uptake rates than the glucose specialist, but they benefit from being able to oxidize completely glucose by respiration (high ATP yield process). Also, it should be noted that our model assumes that the uptake and excretion of acetate mediated by the acetate kinase is

modeled as a unique reversible process, whereas the uptake of glucose, the first step of the TCA cycle (mediated by the citrate synthase), the acetate uptake by the acetyl-CoA synthetase, and both the glycerol uptake and glycerol excretion are irreversible processes.

3.4. Results/ Discussion

In this study, we aim to give a better insight into the physiological mechanisms underpinning the stable coexistence of the cross-feeding polymorphism observed in the evolution experiment performed by Helling and colleagues (Helling et al. 1987). To approach this question we developed a simple mathematical model that captures the key metabolic reactions of cross-feeding bacteria, and parametrized the model with experimental data of *E. coli* biology and biochemistry. Based on the trade-offs described in the previous section, our model did not recover the stable coexistence of the polymorphism. Instead, we found that the glucose specialist outcompeted the two other strains (results not shown). This suggests that our model is not taking into account either a cost on the growth of the glucose specialist or a benefit on the growths of the two other competitors (acetate specialist and glycerol generalist). Here, we proposed two separate hypotheses that might explain this stable coexistence.

Our first hypothesis (*H1*) is that acetate inhibits cell growth by a logarithmic function. The toxic effect of acetate on the bacterial growth rate when grown under aerobic conditions, also known as the bacterial Crabtree effect (Doelle et al. 1982), has been widely acknowledged in the literature (Davison and Stephanopoulos 1986; Luli and Strohl 1990; Diez-Gonzalez and Russell 1997; Eiteman and Altman 2006). Previous experiments on *E. coli* have suggested that acetate reduces *E. coli* growth rate logarithmically. This logarithmic cost has been observed in both *E.coli* batch and fed-batch cultures (Davison and Stephanopoulos 1986; Luli and Strohl 1990). The mechanism of acetate toxicity has been explained by an increase of protons from the dissociation of acetate inside the cells. This increase in intracellular protons (H^+)

results in the decrease of the proton-motive force, and this leads to the uncoupling of many metabolic processes involved in cellular growth (Davison and Stephanopoulos 1986; Luli and Strohl 1990).

Our second hypothesis (*H2*) is that the rate of the citrate synthase enzyme of the glucose specialist is reduced. The citrate synthase enzyme is the rate-limiting step to the entrance into the TCA cycle, and is inhibited by NADH, which is a specific allosteric inhibitor (Walsh and Koshland 1985; Molgat et al. 1992; Eiteman and Altman 2006). Thus, we suggest that the inhibition of the glucose specialist citrate synthase is caused by the accumulation of intracellular NADH produced through glycolysis. This increase in NADH levels has an important implication for the activity of the citrate synthase as it results on the reduction of metabolites flow through the TCA cycle, and consequently the uncoupling between glycolysis and the TCA cycle (Eiteman and Altman 2006). In the following sections, we present the results of the polymorphism competitions under each hypothesis separately.

Two phenotypes competition

H1- Growth inhibition by acetate

By competing the glucose and acetate specialists (Table S1), we observed that stable coexistence is possible if acetate has a growth inhibitory effect on the competing strains such that the toxic effect of intracellular acetate is higher in the growth of the glucose specialist than in the growth of the acetate specialist (fig. 3.2A). This result seems to suggest that the acetate specialist evolved a higher resistance to intracellular acetate than the glucose specialist. In light of this observation, we can speculate that this resistance evolved because of the selective pressure for a phenotype able to uptake acetate efficiently but at a lower cost. However, further research would need to be done to test this prediction. To evaluate if the same principle applies to the competition between the glucose specialist and the glycerol generalist, we next competed both strains under the same conditions. Our results suggest that the glucose specialist also exhibits a higher growth inhibition by intracellular acetate than the

glycerol generalist (fig. 3.2B). Interestingly, the equilibrium frequencies obtained are consistent with the equilibrium frequencies observed experimentally (Rosenzweig et al. 1994).

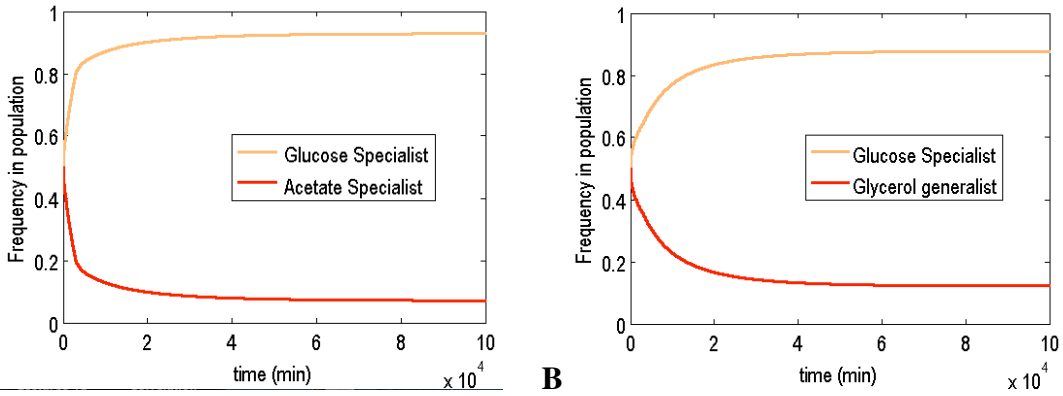

Figure 3.2. Competition in a glucose-limited chemostat when acetate has a different inhibitory effect on the competitors' growth. A. Competition between the glucose and acetate specialists. $K_{I,1}$= 4.5 and $k_{I,3}$=4.9. **B.** Competition between the glucose specialist and the glycerol generalist. $K_{I,1}$= 4.5 and $k_{I,2}$=5.3.

H2- Citrate synthase inhibition

We then sought to evaluate the possible importance of the inhibition of the glucose specialist citrate synthase (*H2*) on the glucose specialist competitive ability. To test this hypothesis we repeated the same competition experiments as the ones performed in the previous section but assumed that the rate of the glucose specialist citrate synthase ($V_{TCA\,\max,1}$) is decreased such as $V_{TCA\,\max\,1} = V_{TCA\,\max,2}$ /1.3 and $V_{TCA\,\max,1} = V_{TCA\,\max,2}$ /1.3, and also assumed an equal inhibition of acetate on the strains growth ($K_{I,1}$= $K_{I,2}$= $K_{I,3}$). Interestingly, we observed that under these conditions, coexistence of the two competitors was also allowed in both competition experiments (fig. 3.3A and 3.3B). Furthermore, the strains equilibrium frequencies are similar to the ones obtained in Figure 3.2A and 3.2B.

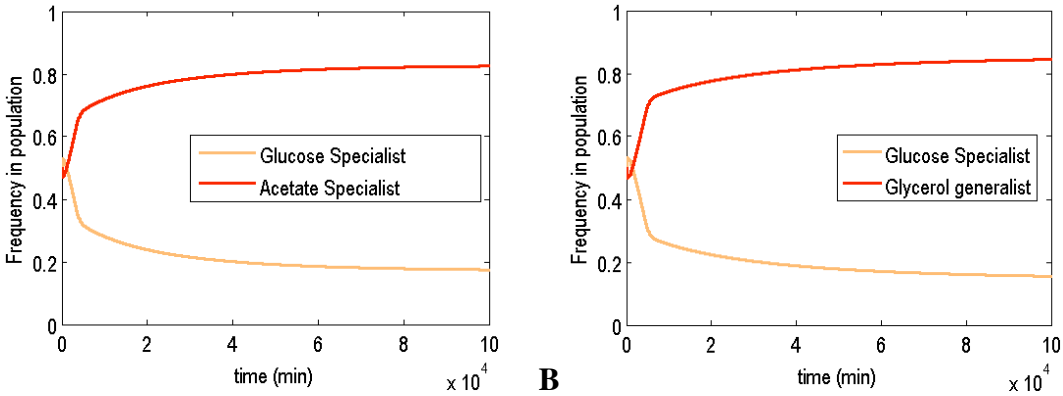

Figure 3.3. Competition in a glucose-limited chemostat when the citrate synthase activity of the glucose specialist is repressed. A. Competition between the glucose and acetate specialists. **B.** Competition between the glucose specialist and the glycerol generalist. $V_{TCA\,\max,1} = V_{TCA\,\max,2}$ /1.3.

Consortium competition

To evaluate the effect of these two alternative explanations for the stable coexistence of the consortium (glucose specialist, acetate specialist and glycerol generalist), we competed the three strains together. Assuming the same parameters as in the two strains competitions and a different cost of acetate inhibition (*H1*), we found that only the glucose specialist and the glycerol generalist coexist while the acetate specialist is excluded (fig. 3.4A). However, when assuming the inhibition on the glucose specialist citrate synthase (*H2*), only the glucose and acetate specialists are able to coexist while the glycerol generalist is excluded (fig. 3.5A). Assuming distinct costs than the ones assumed in the two strains competition, we were able to observe stable polymorphism coexistence (see figs. 3.4B and 3.5B). Two aspects of these results are of particular interest. First, they predict that if our hypothesis 1 is true, the three phenotypically distinct strains coexist if intracellular acetate inhibits growth in the following order: cost to glucose specialist > cost to acetate specialist > cost to glycerol generalist. Second, our results suggest that if our hypothesis 2 is true, stable coexistence is possible if $V_{TCA\,\max,1} < V_{TCA\,\max,3} < V_{TCA\,\max,2}$. One may speculate that a possible explanation for this latter observation is because of their distinct ability to use glucose and consequent effects on their citrate synthase activity.

Indeed, the glucose specialist is better at using glucose than the glycerol generalist, and the glycerol generalist,is better than the acetate specialist. As for the citrate synthase inhibition hypothesis (*H2*), our results are in agreement with bacteria biochemistry, as an increasing rate of glucose uptake results in an increasing production of NADH trough glycolysis, and NADH is a strong inhibitor of the citrate synthase. In what concerns the inhibition of intracellular acetate (*H1*), it is less clear why the acetate specialist would have a higher acetate inhibition than the glycerol generalist. Further studies would need to be done to evaluate this prediction.

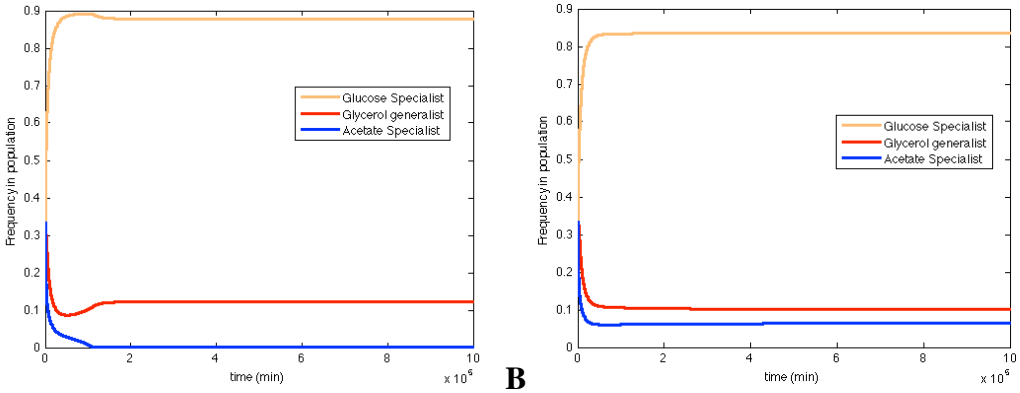

Figure 3.4. Consortium (three stains) competition in a glucose-limited chemostat when acetate has a different inhibitory effect on the competitors growth. A. $K_{I,1}$= 4.5, $K_{I,2}$=5.3 and $K_{I,3}$=4.9. **B.** $K_{I,1}$= 4.5; $K_{I,2}$= 5.4; $K_{I,3}$=5.0. Initial densities were 10^{11} cells.

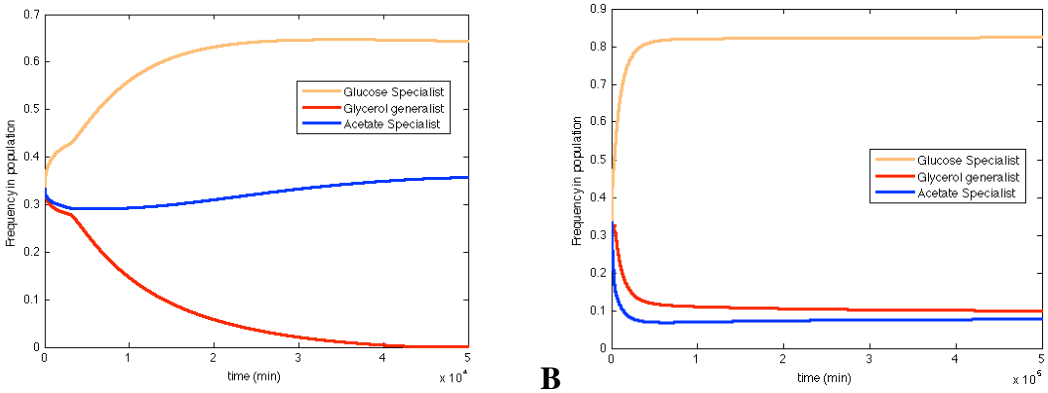

Figure 3.5. Consortium competition in a glucose-limited chemostat when the citrate synthase activity of the glucose specialist is repressed. A. $V_{TCA\ max,1} = V_{TCA\ max,2} = V_{TCA\ max,3}$ /1.3. $K_{I,1}= K_{I,2}= k_{I,3}$. **B.** $V_{TCA\ max,1} = V_{TCA\ max,2}$ /1.2 and $V_{TCA\ max,3} = V_{TCA\ max,2}$ /1.06.

In the previous section, we developed a model that considers the metabolism of acetate and glycerol cross-feeding interactions, and we were able to recover the stable *E. coli* polymorphism coexistence observed experimentally. To evaluate the biological and ecological accuracy of our model, in the next section we test whether our model is consistent with the following experimental observations:

i. Concentration of glucose at equilibrium;
ii. Importance of the increased glucose uptake of the glucose specialist;
iii. Importance of the ACS overexpression of the acetate specialist;
iv. Importance of the increased acetate excretion by the glucose specialist for the acetate specialist survival;
v. Importance of the increased glycerol uptake rate by the glycerol generalist for its coexistence;
vi. Density-independence of the equilibrium frequencies.

As shown on fig. 3.6, our equilibrium glucose concentrations are consistent with the values observed experimentally (2-8nmol/mL) (Kurlandzka et al. 1991).

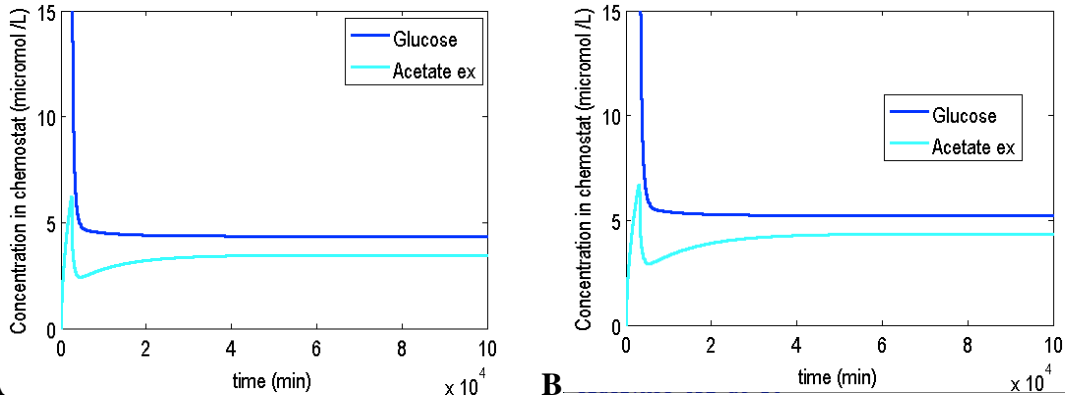

Figure 3.6. Glucose and extracellular acetate concentrations in the chemostat. **A.** $K_{I,1}$= 4.5; $K_{I,2}$= 5.4; $k_{I,3}$=5.0. $V_{TCA\ max,1} = V_{TCA\ max,2} = V_{TCA\ max,3}$. **B.** $V_{TCA\ max,1} = V_{TCA\ max,2}$ /1.2 and $V_{TCA\ max,3} = V_{TCA\ max,2}$ /1.06. $K_{I,1}= K_{I,1}= K_{I,3}$.

As expected, a "glucose specialist" mutant with no increased rate of glucose uptake dies out as it exhibits no competitive advantage in glucose (fig. 3.7A). Figure 3.7B

shows that an "acetate specialist" mutant**,** which exhibits no increased overexpression of the ACS enzyme, is excluded. These two results confirm the fundamental importance of mutations that confer specialization on different resources for the stable polymorphism coexistence. Indeed, the increased rate of glucose uptake gives an advantage in glucose while the increased overexpression of the ACS, which is the main enzyme involved in acetate utilization, gives an advantage in acetate utilization. Then, we also sought to evaluate the importance of the increased excretion of acetate by the glucose specialist to observe the stable coexistence. To that end, we assumed that a "glucose specialist" mutant, which has the same affinity for acetate (K_{AS}) than the ancestral strain and therefore a lower rate of acetate excretion than the glucose specialist, competes with the acetate specialist and the glycerol generalist. The competition results in the exclusion of the acetate specialist (fig. 3.7C). This observation suggests that the increase of acetate excretion by the glucose specialist gives an advantage to the acetate specialist and makes possible the stable coexistence. Experiments have shown that the glycerol generalist has an increased ability to scavenge glycerol and it is thought that this behaviour gives an advantage to the glycerol generalist and makes possible its coexistence with the other polymorphic strains. We tested this hypothesis in our model by competing the glucose and acetate specialists with a mutant "glycerol generalist", which has no advantage in glycerol, such as $V_{GK\ \max,\ 2} = V_{GK\ \max,\ 3}$. Interestingly, under these conditions our results suggest that the mutant glycerol generalist is excluded (fig. 3.7D).

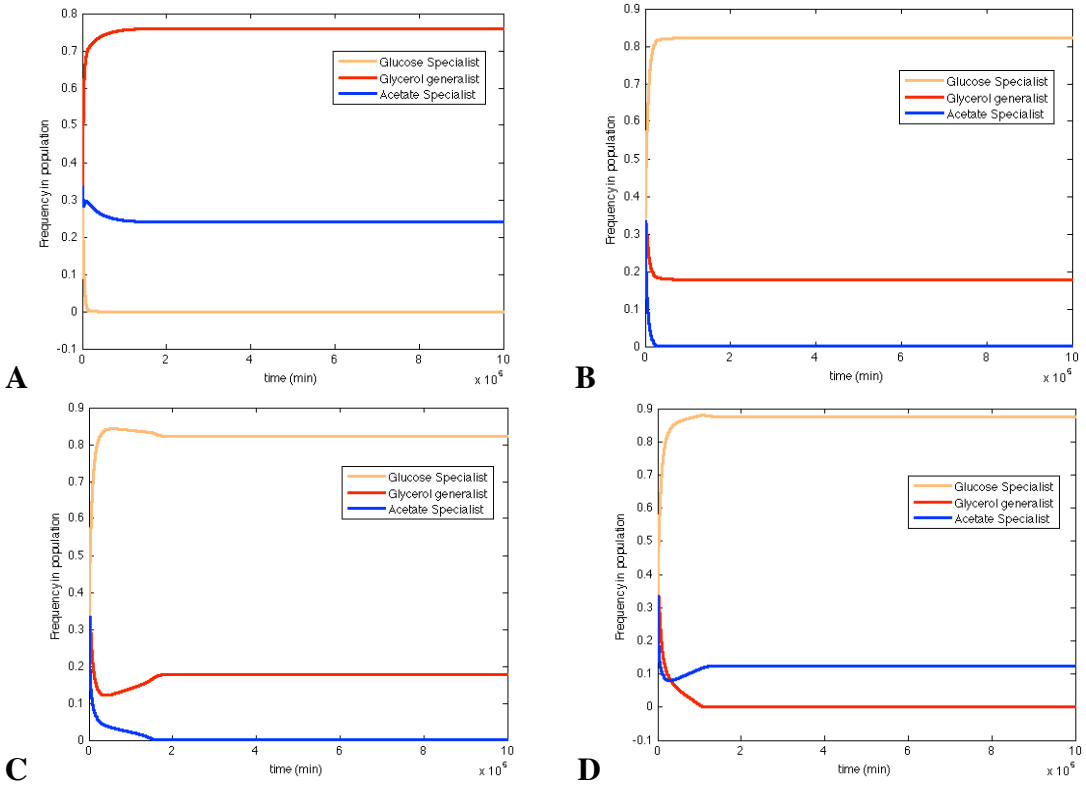

Figure 3.7. Result of the consortium competition assuming A. no increased uptake of glucose by the glucose specialist; **B.** no overexpression of the ACS enzyme of the acetate specialist; **C.** no increased excretion of acetate by the glucose specialist ($K_{AS,1} = K_{AS,3}$); **D.** no increased uptake of glycerol by the glycerol generalist ($V_{GK\ max,\ 2} = V_{GK\ max,\ 3}$);

In a following experiment, Rosenzweig et al. (1994) showed that equilibrium frequencies are density-dependent. Their results suggest that the glucose specialist is favoured by low population densities while the acetate specialist and the glycerol generalist are favoured by high population densities. The authors argue that at low densities, the concentrations of overflow metabolites (acetate and glycerol), which are determined by the population density, are too low to give a growth advantage to the cross-feeding strains. However, at high densities the metabolites concentrations are higher, and sufficient to give a growth advantage to the cross-feeders. We tested their prediction by starting the competition with different population densities. The results show that when starting with lower population densities (10^9 L^{-1}) than the ones used experimentally, both the acetate specialist and the glycerol generalist do

not persist. However, when using the population densities used in the experiment ($5.9x10^{10}$ L^{-1} and 2.4 $x10^{11}$ L^{-1}), we observed no density-dependence of the equilibrium frequencies (fig. 3.8). It should be noted that the experimental results were obtained on two strains competition and not on the consortium competition as shown in this study. Further studies should be done to understand whether our model lakes density-dependence of the equilibrium frequencies or the experiments have not been run long enough.

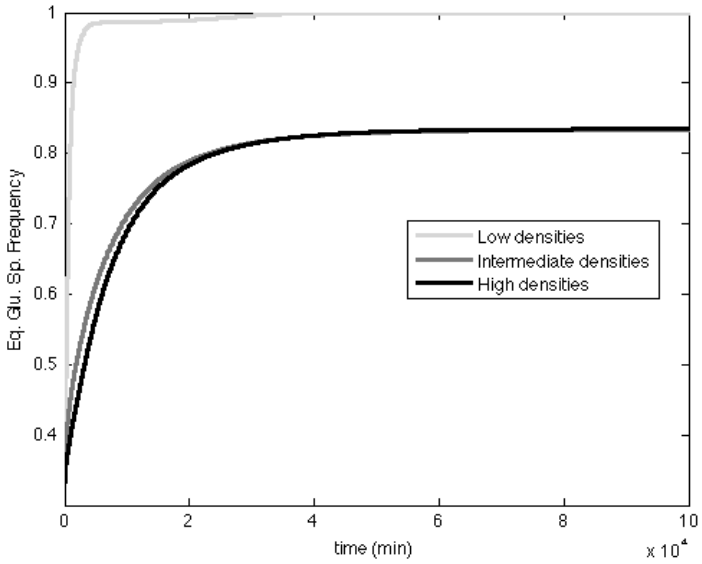

Figure 3.8. Density-dependence of the glucose specialist equilibrium frequency. Initial population densities were 10^9 L^{-1} (low densities), $5.9x10^{10}$ L^{-1} (intermediate densities) and 2.4 $x10^{11}$ L^{-1} (high densities).

3.5. Conclusion

The present work shows that our approach to model the stable coexistence of the cross-feeding polymorphism studied experimentally by Helling and colleagues (Helling et al. 1987; Kurlandzka et al. 1991; Rosenzweig et al. 1994; Kinnersley et al. 2009) is able to recover some important ecological and physiological features observed experimentally. In addition, our model suggests two further physiological

mechanisms that may allow for the stable polymorphism. Here, we propose that this is made possible because of either a distinct acetate inhibition on the phenotypes growth (cost on glucose specialist > cost on acetate specialist > cost on glycerol generalist) or a distinct cost in the citrate synthase enzyme ($V_{TCA\,\max,1} < V_{TCA\,\max,3} < V_{TCA\,\max,2}$). Although we looked at both effects separately, it would be interesting to also look at the combination of both effects.

Also, our results showed that competition between two strains and competition between three strains (consortium) need different costs. This result is of particular interest as a study on the gene expression profile of monocultures and the consortium revealed that the expression profile depends on the mixture type in which the strains are grown (Kinnersley et al. 2009). In particular, this study showed that the gene expression profile of the glucose specialist on monoculture is different than when grown in consortium. One of the main observed changes was a significant shift in the expression of two global regulators, CRP and CpxR, which are involved in stress response.

Finally we were also able to recover similar values of glucose concentration at equilibrium, the importance of the increased glucose uptake of the glucose specialist, the importance of the ACS overexpression of the acetate specialist, the importance of the increased acetate excretion by the glucose specialist for the acetate specialist survival and of the increased glycerol uptake rate by the glycerol generalist for its coexistence, but we could not observe the same density dependence observed experimentally.

3.6. Appendix

Table S1. Model parameters

Biological Parameters		
Parameter	Description	Value
$V_{glu\,max,1}$[a]	Glucose specialist maximum rate of glycolysis	2.46 μmol/min/ gm dry wt. cells[a]

$V_{glu\ max,\ 2}$[a]	Glycerol generalist maximum rate of glycolysis	1.61 μmol/min/ gm dry wt. cells[a]
$V_{glu\ max,\ 3}$[a]	Acetate specialist maximum rate of glycolysis	1.66 μmol/min/ gm dry wt. cells[a]
$K_{m\ glu}$	Half-saturation constant of glycolysis	10 μM
$V_{AK\ max,\ 1}$[b]	Glucose specialist maximum rate of acetate kinase	1185 μmol/min/ g soluble protein[a]
$V_{AK\ max,\ 2}$[b]	Glycerol generalist maximum rate of acetate kinase	1264 μmol/min/ g soluble protein[a]
$V_{AK\ max,\ 3}$[b]	Acetate specialist maximum rate of acetate kinase	1196 μmol/min/ g soluble protein[a]
$K_{m\ AK,1}$[b]	Glucose specialist half-saturation constant of acetate kinase	14 mM
$K_{m\ AK,2}$[b]	Glycerol generalist half-saturation constant of acetate kinase	12 mM
$K_{m\ AK,3}$[b]	Acetate specialist half-saturation constant of acetate kinase	32 mM
$V_{ACS\ max,\ 2}$[c]	Glycerol generalist maximum rate of ACS	3 μmol/min/g soluble protein[a]
$V_{ACS\ max,\ 2}$[c]	Acetate specialist maximum rate of ACS	122 μmol/min/g soluble protein[a]
$K_{m\ ACS}$	Half-saturation constant of ACS	200 μM
$V_{TCA\ max,}$*	Maximum rate of TCA cycle	830 μmol/min/g soluble protein[a]
$K_{m\ TCA,1}$*	Half-saturation constant of glycolysis	500 μM
n_{Glu}	ATP yield of Glycolysis	14 μmol/ μmol S
n_{TCA}	ATP yield of TCA cycle	12 μmol/ μmol X_{in}
n_{AK}	ATP yield of acetate uptake and excretion mediated by the acetate kinase	1 μmol/ μmol X_{in}
n_{ACS}	ATP yield of acetate uptake mediated by ACS	1 μmol/ μmol X_{in}
n_{GK}	ATP yield of glycerol uptake and excretion	1 μmol/ μmol S/Xin
G	Efficiency of ATP energy conversion	$G=10^9$ cell /μmol ATP (see NOTE 1)
Functions	Description	Form
$cost(X_{in})$#	Growth inhibition by acetate	$cost(X_{in})=1/(1+ X_{in}/K_I)$

Environmental parameters		
D[5]	Chemostat dilution rate	0.2 /h
α	Chemostat volume	0.17 L
S_0 [5]	Influx of nutrient in the chemostat	14 mmol/L (0.025%)

[a] Data from Table 2 of Rosenzweig et al. (1994)

[b] Data from Table 3 of Rosenzweig et al. (1994)

[c] Data from Table 4 of Rosenzweig et al. (1994)

[5] Value from Helling et al. (1987)

* Value reported in P.D.G. Weitzman, Citrate synthase from E. coli (3rd edn.), Methods in Enzymology Vol. 13, Academic Press, London (1969), pp. 22-26.

[*] Function derived from (Davison and Stephanopoulos 1986).

[a] - A single cell of *E.coli* weights $1*10^{-12}$ g (wet cell weight) that corresponds approximately to $0.2*10^{-12}$ g dry weight. It is also assumed that around 50 % of the dry weight corresponds to proteins. Here we assume that the total protein mass corresponds to 0.1% of the total soluble protein mass corresponds to 0.1% of the total soluble protein. That means that the mass of a protein per bacterium corresponds approximately to 1*10^-12 *0.5(total protein/cell mass) * 0.1 (active protein/ total protein).

Note 1. The efficiency of ATP conversion to biomass ($Y_{N/ATP}$) was determined theoretically from experimental values of the efficiency of glucose conversion to biomass ($Y_{N/Glucose}$) of *E.coli* during glucose-limited continuous culture growth (Kayser et al. 2005). For a dilution rate of 0.203 h^{-1} they obtained $Y_{N/ATP}$ =12.5 g cells.(mol ATP)$^{-1}$ that corresponds approximately to $Y_{N/ATP} = 1.25 *10^7$ cells /μmol. However, here we used $G=10^9$ cells /μmol ATP for convenience. This higher value in our model than expected could be due to an underestimation of ATP production from other metabolic pathways.

Chapter 4

FROM METABOLISM TO ECOLOGY: CROSS-FEEDING INTERACTIONS SHAPE THE BALANCE BETWEEN POLYMICROBIAL CONFLICT AND MUTUALISM

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4.1. Summary

Polymicrobial interactions are widespread in nature, and play a major role in maintaining human health and ecosystems. Whenever one organism uses metabolites produced by another organism as energy or nutrient sources, this is called cross-feeding. The ecological outcomes of cross-feeding interactions are poorly understood and potentially diverse: mutualism, competition, exploitation or commensalism. A major reason for this uncertainty is the lack of theoretical approaches linking microbial metabolism to microbial ecology. To address this issue, we explore the dynamics of a one-way interspecific cross-feeding interaction, in which food can be traded for a service (detoxification). Our results show that diverse ecological interactions (competition, mutualism, exploitation) can emerge from this simple cross-feeding interaction, and can be predicted by the metabolic, demographic and environmental parameters that govern the balance of the costs and benefits of association. In particular, our model predicts stronger mutualism for intermediate by-product toxicity because the resource-service exchange is constrained to the service being neither too vital (high toxicity impairs resource provision) nor dispensable (low toxicity reduces need for service). These results support the idea that bridging microbial ecology and metabolism is a critical step towards a better understanding of the factors governing the emergence and dynamics of polymicrobial interactions.

4.2. Introduction

Microbial communities are widespread in nature and play a major role in shaping the world we live in, ranging from maintaining human health (Backhed et al. 2005; Flint et al. 2007; Ramsey and Whiteley 2009), to shaping our ecosystems (Stams 1994; Schink 2002). Microbial cells excrete metabolites as a result of their metabolism, and such metabolic waste sets the stage for the emergence of the complex interspecific interactions observed in these communities. Whenever these metabolites can be used by other organisms as energy or nutrient resources, this is called cross-feeding. Cross-feeding is incidental when the metabolite excreted is a waste product, and therefore non costly to produce at a basal level. In some instances, cross-feeding can be cooperative, requiring an up-front investment cost to the producer, which may or may not be paid-back by the partner species utilizing the metabolite (West et al. 2006; Bull and Harcombe 2009).

From an ecological stand-point, the functional outcomes of cross-feeding interactions are potentially diverse, spanning competition, mutualism, exploitation, or commensalism. The type of ecological interaction forged depends on the net costs and benefits emerging from the association (Bronstein 1994; Connor 1995). The interaction is competitive if the net effect of the interaction is negative for all species, and is exploitative if a species benefits at the expense of the other species. In contrast, if the interaction is beneficial to both species, then they are mutualists. Costs and benefits to each partner are not constant, but depend on both biotic and abiotic factors (Bronstein 1994; Herre et al. 1999; Sachs and Simms 2006). For example, spatial structure, resource availability, the number and type of other species, and environmental perturbations, are all important factors in shaping the nature of these interspecific interactions (Brockhurst et al. 2007; Hansen et al. 2007; Bull and Harcombe 2009; Shimoyama et al. 2009; Harcombe 2010; Mitri et al. 2011).

Mutually beneficial interactions are commonly found in the complex web of metabolic exchanges among species of the human microbiota (Samuel and Gordon

2006; Mahowald et al. 2009). However, the exchange of metabolites by cross-feeding can also promote exploitation. For example, a commensal bacteria might be forced to produce a metabolite at its own expense while benefiting an opportunistic bacteria (Jagmann et al. 2010). These empirical examples raise the following question: how do fundamental ecological relationships emerge from fundamental mechanistic features of interspecific interactions?

Although it is well-acknowledged that the nature of interspecific interactions is not fixed in time or space, theoretical models have traditionally focused on a single type of ecological interaction, either competition (Case and Gilpin 1974; Frank and Amarasekare 1998), mutualism (Holland et al. 2002; West et al. 2002; Foster and Wenseleers 2006), or exploitation (Frank 1996; Hochberg and van Baalen 1998). The main motivation for such models is typically to understand the evolution of traits that underlie a specific functional form of interaction (e.g., virulence, Frank 1996). However, little attention has been directed to the role that underlying mechanistic bases of interaction play in the emergence and dynamics of these ecological interactions.

Here, we aim to address this challenge by exploring the dynamics of an incidental cross-feeding interaction. Specifically, we explore a common type of incidental cross-feeding interaction where the by-product (waste) is toxic to the producer but beneficial to the cross-feeder (Marx 2009; Shimoyama et al. 2009; Hillesland and Stahl 2010). The human microbiota, with its hundred trillion microbial cells, is one place where this type of metabolic interaction is common (Egland et al. 2004; Samuel and Gordon 2006). For example, coculture of the human gut bacterium *B. thetaiotaomicron* and methanogen *M. smithii* in gnotobiotic mice revealed that the two species are involved in a one-way cross-feeding mutualism (Samuel and Gordon 2006), while also competing for nitrogen (Samuel et al. 2007). When in coculture, *B. thetaiotaomicron* preferentially degraded fructans, resulting in the production, from fructans fermentation, of the reducing equivalents formate and hydrogen. While formate and hydrogen inhibit the metabolism of *B. thetaiotaomicron*, these waste products are a source of energy for *M. smithii*. Thus, *M. smithii* facilitates B.

thetaiotaomicron's growth by removing these reducing equivalents. In turn, the methanogen benefits from the interaction by using formate and hydrogen as source of energy for methanogenesis. A similar mechanism occurs in the novel obligate mutualism experimentally evolved between the bacterium *Desulfovibrio vulgaris* and the methanogen *Methanococcus maripaludis* (Hillesland and Stahl 2010). Lactate oxidation to acetate, carbon dioxide, and hydrogen by *D. vulgaris* is inhibited by high hydrogen levels. The presence of *M. maripaludis*, however, relieves this inhibition because *M. maripaludis* uses hydrogen for methanogenesis and therefore helps the bacterium by keeping hydrogen at low levels. In turn, the methanogen benefits by using hydrogen as an energy source. In contrast to the *B. thetaiotaomicron - M. smithii* mutualism previously described, there is no evidence of interspecific competition between *D. vulgaris* and *M. maripaludis*.

Based on these empirical examples, we explicitly assume that the metabolic by-product (waste product) is toxic to the producer, but beneficial to the cross-feeder (non-producer). Although there is an indirect benefit of association for both producer and cross-feeder (food in exchange for detoxification), helping a competitor also comes at a cost, due to increased competition for shared resources. Our results indicate that such a resource-service cross-feeding interaction can produce diverse stable ecological outcomes: competition, exploitation (in either direction) or mutualism. We then ask: under what conditions do the reciprocal benefits of this specific mechanism of trade outweigh the interspecific competitive costs? In other words, what factors govern the occurrence of a mutually beneficial interaction? Our model emphasizes the importance of the metabolic by-product properties in governing the outcome of the ecological interaction. In particular, we show that a more toxic and more durable by-product favour mutualism, due to the service (toxic by-product removal) being more valuable as the by-product becomes a real problem to the producer. Interestingly, our model predicts that mutualism will be stronger at intermediate by-product toxicity. This occurs because of a balance between provision of a resource and need for a service. Indeed, at high by-product toxicity, the producer is highly inhibited, and thus the provision of food to the cross-feeder (resource) is reduced. However, at low by-product toxicity, the need for help (service by

detoxification) is reduced, and thus the costs of association may outweigh the benefits.

4.3. The Model

One-way by-product cross-feeding

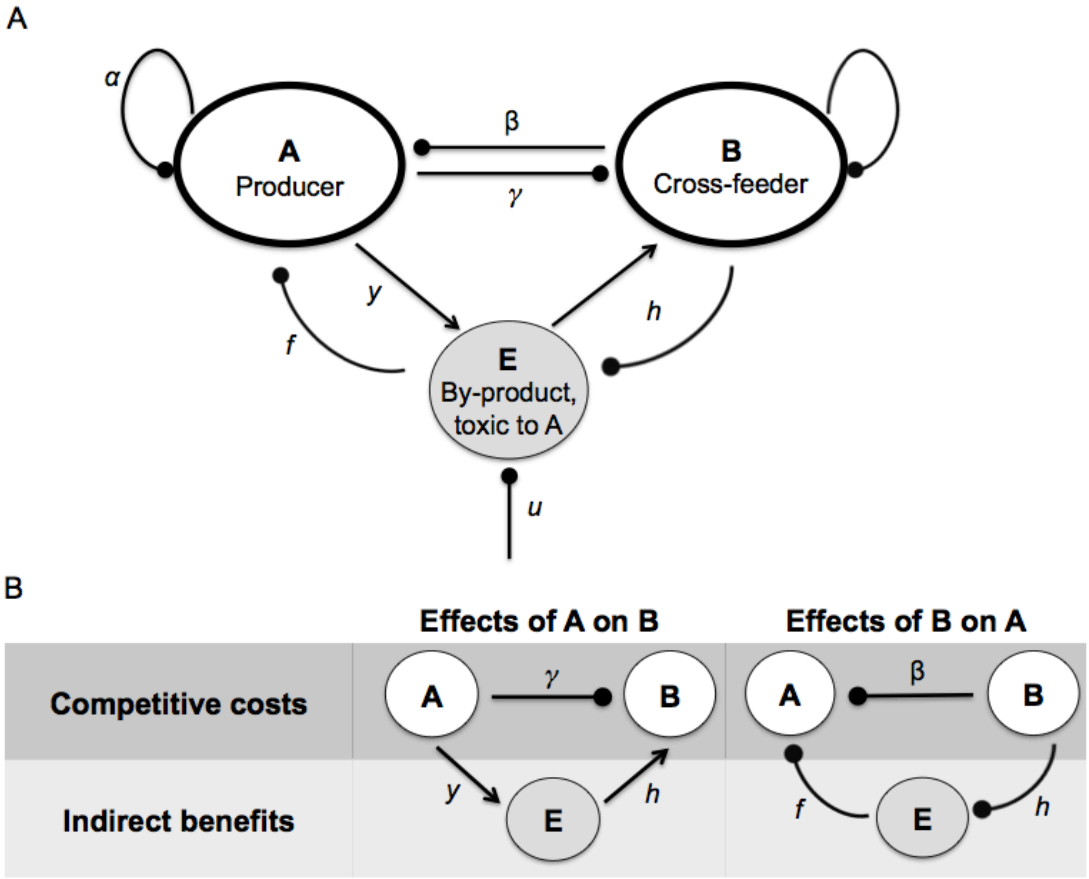

Figure 4.1. Schematic diagram of the cross-feeding model (A), and illustration of the costs and benefits of association (B). A, B and E represent producer, cross-feeder and by-product, respectively. Oval arrows represent a negative effect whereas open arrows represent a positive effect upon the population or resource they are pointing towards. Arrows are labeled with the associated rate constants (see Table 1 for definitions of other notations). The non-labeled arrow represents the cross-feeder intraspecific competition, and is normalised to 1.

Our model tracks the dynamics of a one-way cross-feeding interaction between two populations: a producer (*A*) of a by-product (*E*) and a non-producer (cross-feeder) (*B*) (see fig. 4.1 for a schematic representation of the model). Our model builds on the competitive Lotka-Volterra equations. In addition, we extend the competitive Lotka-Volterra model to include the explicit dynamics of the by-product (*E*) (Frank 1994). Specifically, we assume that the by-product (*E*) enhances the cross-feeder's growth, however it inhibits the producer's growth. Our (non-dimensional) model is defined by the following system of ordinary differential equations (see Appendix A for details on the normalization):

$$\begin{aligned} dA/dt &= (r\,(1 - \alpha A - \beta B) - fE)\,A \\ dB/dt &= ((1 - \gamma A - B) + ghE)\,B \\ dE/dt &= yA - hEB - uE \end{aligned} \qquad (1)$$

The densities of producer (*A*) and cross-feeder (*B*) are scaled to the carrying capacity of *B* (k_b), and the individual intrinsic growth rates are scaled to the intrinsic growth rate of *B* (r_b), thus *r* represents the relative intrinsic growth rate of the producer to that of the cross-feeder ($r = r_a/r_b$). *f* is the rate of the by-product toxicity on the producer, and measures the degree to which the by-product is toxic to the producer's growth. *h* represents the cross-feeder's by-product consumption rate, and *y* is the producer's by-product production rate. *g* is a conversion constant, which can be viewed as the cross-feeder's by-product uptake efficiency. *u* represents the by-product decay rate. α is the producer's intraspecific competition coefficient, and measures the degree of competition among the population of producers relative to the competition among the population of cross-feeders ($\alpha = k_b/k_a$, see Table A1). β is the cross-feeder's interspecific competition coefficient on the producer, and γ is the producer's interspecific competition coefficient on the cross-feeder, thus β and γ measure the competitive effect of *B* on *A* and *A* on *B*, respectively. It should be noted that we assume that both β and γ are strictly positive, as in the classic competitive Lotka-Volterra equations. Thus, any benefits of association will be due to the by-product dynamics (i.e. the metabolic interaction), and not imposed *a priori* to the

system. Note that when $f = g = h = y = u = 0$, we recover the classic competitive Lotka-Volterra equations.

Table 4.1. Summary of model parameters

Symbol	Definition
A	Producer
B	Cross-feeder and non-producer
E	Metabolic by-product
α	Producer intraspecific competition coefficient
β	Cross-feeder interspecific competition coefficient
γ	Producer interspecific competition coefficient
r	Relative intrinsic growth rate of the producer to that of the cross-feeder
y	By-product production rate
h	Consumption rate of by-product
u	By-product decay rate
f	By-product toxicity rate
g	Cross-feeder uptake efficiency (of by-product)

4.4. Results

Model Analysis

A stability analysis of this model (see Appendix B for detailed Model analysis) reveals that a population of pure producers ($A^* = ru/(\alpha ru + fy)$, $B^* = 0$, $E^* = ry/(\alpha ru + fy)$) is locally stable if $u > y(f + rgh)/(r(\gamma - \alpha))$ and $\alpha < \gamma$. These results reveal that any trait that increases the production and/or accumulation of the toxic by-product will compromise the stability of pure producers, and thus facilitate the invasion of cross-feeders. In turn, a population of pure cross-feeders ($A^* = 0$, $B^* = 1$, $E^* = 0$) is locally stable if $\beta > 1$, i.e. when interspecific competition of cross-feeders on producers (β) is higher than intraspecific competition within the cross-feeder population (that is normalized to 1). The model has two coexistence equilibria (see Appendix B, fig. B1). In the following analyses, we focus on parameter regimes that

allow for the stable coexistence of both species (i.e., where neither single species equilibria are stable).

Effect of association on the focal species, the producer

To understand how the focal species (producer) is affected by the biotic (cross-feeder) and abiotic (by-product) environment, we analyze the effect of changing parameter values on the densities of producers in the mixed community. We find that producers are favoured by lower by-product toxicity (f), lower interspecific competition from cross-feeders (β), and lower rate of by-product production (y) (fig. 2A-D). Interestingly, figure 4.2 reveals non-linearities in the sign of the effect of by-product consumption (h). If the by-product is weakly toxic (low f) and the cross-feeder is a moderate to strong competitor, then an increase in by-product consumption (h) decreases the density of producers in the mixed community (fig. 4.2A and 4.2C, moderate to strong competition β). In contrast, if the by-product is highly toxic (high f), and therefore a real problem to the producer, the density of producers increases with increasing by-product consumption (h) (fig. 4.2B and 4.2D). In other words, from a producer perspective, at low by-product toxicity f, the benefit of the detoxification service from the cross-feeder does not compensate for the enhanced costs of competition that result from a better-fed competitor. However, at high f the benefit from the service does compensate for the enhanced costs of competition, implying that the cross-feeder is a helper (i.e. has a net positive effect on the producer). Finally, a more durable by-product (lower u) favours the population of cross-feeders at the producers' expense (Figures 4.3A and 4.3B).

Effect of association on the cross-feeder

In turn, from a cross-feeder perspective, our results suggest that the cross-feeder is favoured by higher by-product production rate (higher y), and higher by-product consumption rate (higher h) (fig. 4.2E-H). Interestingly, figures 4.2E-H and 4.3B reveal non-linearities in the sign of the effect of increasing interspecific competition β and toxicity f. For example, if y is low, so that there is little cross-feeding potential,

increasing interspecific competition to producer (β) is beneficial to the cross-feeder (fig. 4.2G, H). In contrast, if y is high, so that the producer is a potentially valuable nutrient source, decreasing β may be beneficial to the cross-feeder if its by-product consumption rate is intermediate to high (fig. 4.2E from intermediate to high h, 4.2F) or detrimental if its by-product consumption rate is low (fig. 4.2E at low consumption h). This nonlinearity likely occurs due to a balance between the benefit from cross-feeding (higher h) and decreased interspecific competition (lower β) benefiting a potential harmer (i.e. has a net negative effect on the cross-feeder). Similarly, the effect on the cross-feeder of varying the by-product toxicity (f) depends on the nature of the interaction. Cross-feeders benefit from low f if the producer is a helper, and benefit from high f if the producer is a harmer (fig. 4.3B).

The nonlinearity in the sign of the effect of changing parameter values, as hereby described, is likely because varying the value of the parameter will affect the nature of the partner (i.e. whether the partner is a helper or a harmer). In the next sections, we describe, first, the range of ecological interactions that arise from our model and second, explore how the metabolic and environmental factors influence the nature of the resource-service interaction between producer and cross-feeder.

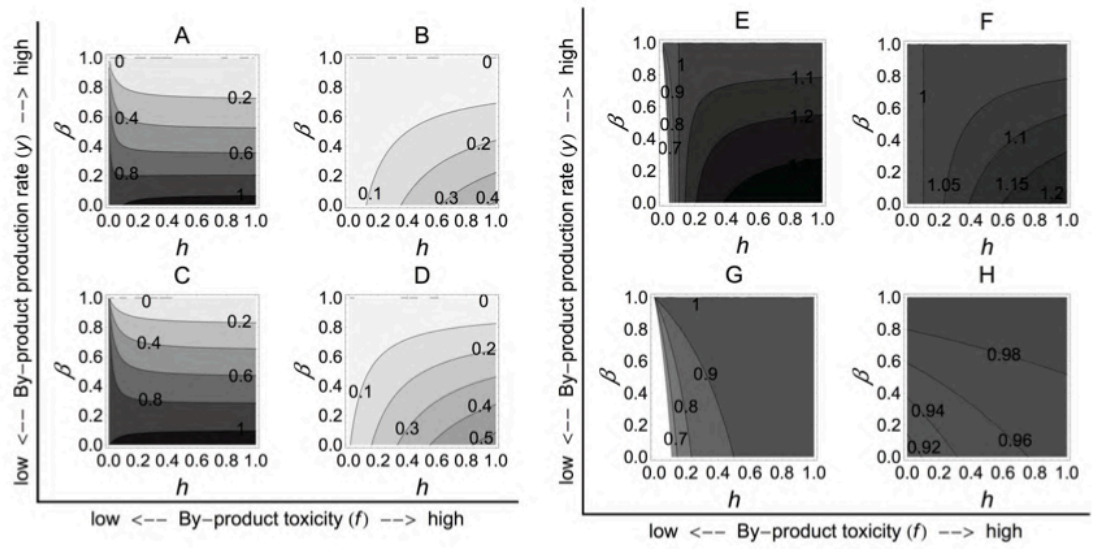

Figure 4.2. Effect of varying by-product toxicity (f) and by-product production rate (y), as well as by-product consumption rate (h) and cross-feeders' interspecific competition (β) on the stable cross-feeding community. **A-D,** Effect on the producer. Contour lines in each figure represent the density of producers at equilibrium in coculture with cross-feeders (A_B*), for values of β, h, y, and f. **E-H,** Effect on the cross-feeder. Contour lines in each figure represent the density of cross-feeders at equilibrium in coculture with producers (B_A*), for values of β, h, y, and f. Darker regions indicate higher density. The parameter values used are $r = 1$, $\alpha = 0.9$, $\gamma = 1$, $g = 1$, $u = 0.1$, $y = [1; 2]$ and $f = [0.01; 1]$ such that **A** and **E,** $y = 2$ and $f = 0.01$; **B** and **F,** $y = 2$ and $f = 1$; **C** and **G,** $y = 1$ and $f = 0.01$; **D** and **H,** $y = 1$ and $f = 1$.

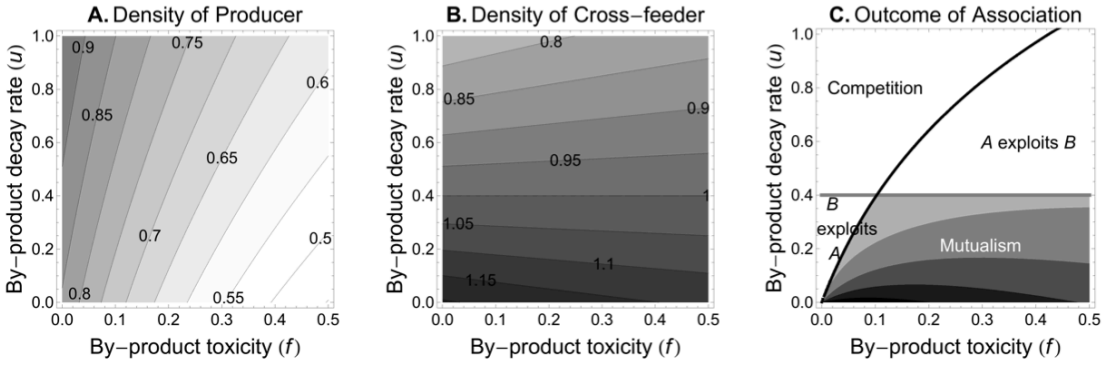

Figure 4.3. Effects of by-product durability (u) and toxicity (f) on the density of producer, density of cross-feeder, and the outcome of the association. **A-B,** darker regions indicate higher density. **C**, darker the shading stronger the mutualism. The

black line represents the threshold where $A_B^* = A_a^*$, and the grey line represents the threshold where $B_A^* = B_a^*$ (see text in Results for explanation). The parameter values used for the plots are $r = 1$, $\alpha = 0.9$, $\beta = 0.2$, $\gamma = 1$, $g = 1$, $h = 0.8$, and $y = 1.5$.

One-way cross-feeding can produce diverse ecological interactions

This one-way cross-feeding interaction where food is traded for detoxification can produce diverse ecological interactions (fig. 4.3C and 4.4). Generally, the cross-feeding interaction is mutually beneficial to both species if the density of A in coculture with B (A_B^*) is larger than the density of A alone (A_a^*), and if the density of B in coculture with A (B_A^*) is larger than the density of B alone (B_a^*) (i.e. benefits from association outweigh competitive costs endured, reciprocally). In contrast, competition occurs if both species are negatively affected by the association ($A_B^* < A_a^*$ and $B_A^* < B_a^*$, i.e. competition outweighs benefits received, reciprocally). Three other outcomes are possible. The producer might exploit the cross-feeder, and this means that the producer density is enhanced by the association at the expense of the cross-feeder density ($A_B^* > A_a^*$ and $B_A^* < B_a^*$). Here, the waste removal benefit to the producer outweighs its costs of association, but the food provision to the cross-feeder does not compensate its costs of association. Alternatively, the cross-feeder might exploit the producer, so that the density of producers is decreased by the interaction while the density of cross-feeders is increased ($A_B^* < A_a^*$ and $B_A^* > B_a^*$). Here, the food provision benefit to the cross-feeder outweighs its costs of association, but the waste removal to the producer does not compensate for its costs of association. Finally, if only one of the populations benefits while the other is not affected by the interaction, then the interaction is known as commensalism.

Interestingly, the horizontal/vertical nature of the contour lines in fig. 4.2A,B and 4.3A,B may help to explain this diversity in ecological outcomes. For example, the grey horizontal line in fig. 4.3C represents the threshold at which the cross-feeder benefits (below) or does not benefit (above) from the association (line at $B_A^* = B_a^*$), and reflects the horizontal nature of the contour lines in fig. 4.3B (effect on cross-

feeder density). Similarly, the black oblique line in fig. 4.3C defines the threshold at which the producer benefits (to the right) or does not benefit (to the left) from the association (line at $A_B^* = A_a^*$), and reflects the vertical nature of the contour lines in fig. 4.3A (effect on producer density). We now focus on the mechanisms that favour the stable mutualistic form of this association.

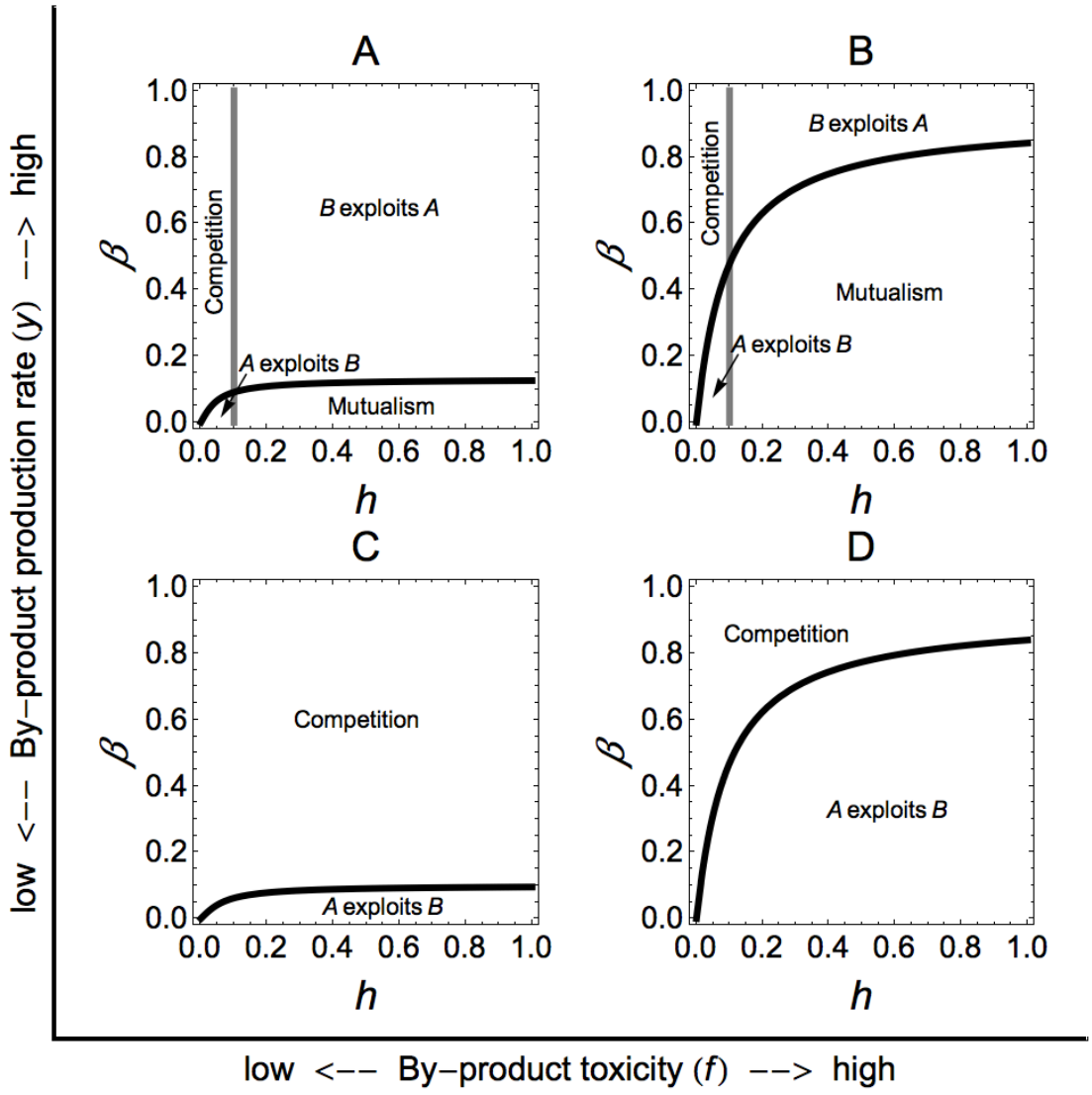

Figure 4.4. Outcome of the cross-feeding interaction for values of cross-feeder's interspecific competition (β) and by-product consumption rate (h), as well as by-product production rate (y), and by-product toxicity (f). The black line represents the threshold where $A_B^* = A_a^*$, and the grey line represents the threshold where $B_A^* = B_a^*$ (see text in Results for explanation). The parameter values used are $r = 1$, $\alpha =$

0.9, $\gamma = 1$, $u = 0.1$, $g = 1$, $y = [1; 2]$ and $f = [0.01; 1]$ such that **A,** $y = 2$ and $f = 0.01$; **B,** $y = 2$ and $f = 1$; **C,** $y = 1$ and $f = 0.01$, **D,** $y = 1$ and $f = 1$.

Mutualism (and exploitation) occurs when indirect benefits exceed competitive costs of association- Effect of interspecific competition

Mutualism can be generically explained by indirect benefits exceeding competitive costs, reciprocally (equivalent to negative interspecific competition parameters in classic Lotka-Volterra competition equations (Otto and Day 2007)). In our system, the competitive costs are defined by the interspecific competition parameters, and are separated from the indirect benefits of provision of the food resource (the effect of *A* on *B*) and the detoxification service (the effect of *B* on *A*) (fig. 4.1B).

We find analytically that the cross-feeder only benefits from the association (i.e. the indirect benefit of cross-feeding compensates for the competitive costs of interacting with the producer) if $h > u\gamma/(gy - \gamma)$. This means that mutualism cannot occur when $h < u\gamma/(gy - \gamma)$. This shows, as expected, that mutualism is favoured by lower interspecific competition γ and β (fig. 4.4).

Mutualism (and exploitation) occurs when indirect benefits exceed competitive costs of association- Effect of metabolic parameters

Given the specific nature of our cross-feeding interaction, varying the rate of by-product production (y) has opposite effects on producers and cross-feeders, as shown previously (Table 4.2). This raises the question as to what are the effects of the by-product production and consumption rates on the nature of the cross-feeding interaction?

Our results suggest that mutualism is favoured by higher by-product production rate (higher y) (fig. 4.4 and fig. C1A). As shown in figure 4, an increase in y may result in a shift from the producer exploiting the cross-feeder to a mutually beneficial

interaction. Our results also suggest that higher by-product consumption (higher h) favours mutualism (fig. 4.4 and C1), and cannot occur when $h < u\gamma/(gy - \gamma)$ (grey line in fig. 4.4*)*. Indeed, increasing the rate of by-product consumption (h) confers an indirect benefit on the producer, due to greater waste removal, but also on the cross-feeder, due to greater food consumption. However, these indirect benefits should be balanced with the competitive costs of association because increasing help to a competitor increases competitive costs, whereas helping a helper increases the feedback benefits.

Table 4.2. Summary of the main results of the cross-feeding interaction model.

	Interspecific competition of B on A (β)	**By-product production (y)**	**By-product toxicity (f)**	**Consumption of by-product (h)**	**By-product decay rate (u)**	**Relative intrinsic growth rate (r)**
Producer density (A_B*) [a]	↓	↓	↓	↑ or ↓	↑	↑
Cross-feeder density (B_A*) [a]	↑ or ↓	↑	↑ or ↓	↑	↓	↑ or ↓
Mutualism [b]	↓	↑	↑	↑	↓	↓

NOTE. **a.** ↑ means a monotonic increase with increasing parameter value, while ↓ means a monotonic decrease with increasing parameter value. **b.** Qualitative switch in ecological outcome, i.e. either outcome does not change or switches to mutualism present (↑), or outcome does not change or switches to mutualism absent (↓).

Mutually beneficial food for detoxification cross-feeding is favoured by a more toxic by-product, but is stronger at intermediate toxicity

The level of by-product toxicity imposed on the producer (f) plays an important role in governing the balance between costs and benefits of association. Our results suggest that mutualism is favoured by higher by-product toxicity (higher f) (fig. 4.4A, 4.4B, and fig. C1B). This occurs because the more toxic is the producer's waste product, the more valuable is any help of waste removal (detoxification) provided by the cross-feeder, which means that the benefit of association to the producer is further increased by an increase in by-product toxicity. Interestingly,

however, our results also suggest that mutualism is stronger at intermediate levels of by-product toxicity, i.e. there is a greater positive net effect from association at intermediate f (fig. 4.3C). To gain more insight into this behaviour, we explore how an increase in toxicity affects the densities of producer and cross-feeder when grown alone (A_a* and B_a*, respectively), and when grown in association (A_B* and B_A*, respectively). The nonlinearity in the response of the strength of mutualism to toxicity arises because of two opposing effects. On the one hand, if by-product toxicity is low then the competitive costs of association to the producer outweigh the benefits of association (i.e. $A_B^* - A_a^* < 0$, low f in fig. C2) because of low need for detoxification (service). On the other hand, once mutualism occurs high by-product toxicity reduces the net gain of association to both partners (high f in fig. C2). This is most likely because of strong inhibition on the producer, as shown by a decrease in density of producers with increasing by-product toxicity.

Mutually beneficial food for detoxification cross-feeding is favoured by increased by-product durability

Recent studies on public goods cooperation have revealed that the costs and benefits of cooperation are shaped by the durability of public goods (Brown and Taddei 2007; Kummerli and Brown 2010). Analogously to these models, our study focuses on two lineages and an explicit molecular intermediary - so we now ask, does the durability of our toxic by-product affect the nature of the cross-feeding interaction that occurs between the producer and cross-feeder?

In monoculture, increased by-product decay rate (larger u) is beneficial for the producer. For large u, the expression of the equilibrium density of pure producers ($A^* = ru/(\alpha ru + fy)$) will approach $A^* = 1/\alpha$. Thus, the toxic effect of the by-product becomes insignificant and the population carrying capacity (equilibrium density) is only limited by its own intraspecific competition (α). However, as the by-product durability increases (lower u), the toxic by-product accumulates into the environment, and the inhibition on the producer also increases. In coculture, producers are favoured by a more fragile by-product, while cross-feeders are

favoured by a more durable by-product (fig. 3A and 3B). But what is the effect of the by-product durability on the nature of the interaction? Our results reveal that mutualism is favoured by a more durable by-product (low u) (fig. 4.3C and fig. C1D). We find analytically that mutualism cannot occur when $u > h(gy - \gamma)/\gamma$. This result suggests that when the toxic by-product is fragile (and therefore less able to accumulate) the indirect benefits of the cross-feeding association are low, because the reciprocal benefits of trading food in exchange for waste product detoxification are decreased significantly.

4.5. Discussion

Polymicrobial interactions are common in nature, and their ecological outcomes are potentially diverse. However, the link between microbial metabolism and microbial ecology is still poorly understood. In this study, we address this challenge by mapping metabolism to ecology for a simple form of polymicrobial interaction. More specifically, we explore the dynamics of a fundamental form of cross-feeding interaction where food (resource) is traded for detoxification (service). We found that a one-to-many relationship between mechanism of interaction and ecological outcome is possible. This means that diverse ecological interactions (mutualism, competition, exploitation) may emerge from a single mechanism of trade. This strongly suggests that it is not possible to predict exactly what the ecological outcome of a certain interaction is from knowledge of the metabolic interaction alone.

Our results are based on a specific mechanism of trade (detoxification for food), however, the basic principles we underline will follow for any mechanism of trade (or help to a partner) that is balanced against a direct competitive interaction (harm to a partner). This balance generates a variety of possible ecological outcomes, by allowing the net effects in both directions to switch from negative (competition outweighs help received) to positive (help outweighs competitive costs endured). It should be noted that the identification of a microbial service relationship does not

alone demonstrate net help, it is important that the service provided is set against the competitive costs, to give a proper accounting of the net effects of the interaction. To the best of our knowledge, this is the first time that the occurrence of the four diverse ecological interactions (mutualism, exploitation in either direction, and competition) from a simple mechanistic interaction between two species has been demonstrated theoretically.

In addition, we also highlight the factors that favour a mutualistic interaction between the producer and the cross-feeder (our main results are summarized in Table 2). Generally, mutualism arises when an interaction is beneficial for both partners, i.e. whenever there is an "alignment of interests" (van Baalen and Jansen 2001). Hereby, such alignment of interests arises when the resource-service exchange is fair, i.e. whenever it compensates for the competitive costs of association. Interestingly, our model suggests that mutualism (common interest) is favoured in a monotonic way (i.e. shift in a specific parameter value either results in no change in the ecological outcome or always affects mutualism qualitatively in the same direction, either switch to mutualism present or mutualism absent), however, the effect on the density of producer and/or cross-feeder presents some nonlinearities (see Table 2). For example, from a producer perspective an increase in by-product toxicity (higher f) will be detrimental no matter the type of partner, but from a cross-feeder perspective the effect of increasing toxicity depends on the nature of the producer (i.e. whether the producer is a helper or competitor).

Furthermore, our model suggests that mutualism is stronger at intermediate by-product toxicity (f). An explanation for this unintuitive result is the following. At low by-product toxicity, help from the cross-feeder to the producer (i.e. service by detoxification) is low because the by-product is less of a problem to the producer. At high by-product toxicity, the producer is strongly inhibited, and thus the help it provides to the cross-feeder (i.e. resource provision) is reduced. It should be noted that this result arises because of the nature of the mechanistic cross-feeding interaction, in which there is a trade of a waste product (which is toxic for producer while food for cross-feeder) in exchange of detoxification (service). While here we

focus on interspecific cross-feeding, we believe that this result is more general and relevant to the understanding of many kinds of interaction, in which the resource-service association is constrained to services being neither too vital nor dispensable. Here, we have focused on the metabolic and environmental parameters, however, demographic factors also play an important role in shaping the balance between costs and benefits of association, so to influence the nature of the resource-service interaction. Our results suggest that mutualism is favoured by lower relative growth of the producer to that of the cross-feeder, i.e. lower *r* (fig. C1C, and, figure C3 and Table 2 for effects on producer and cross-feeder).

Despite not having explicitly assumed a resource use trade-off, our findings suggest that a resource use trade-off in the cross-feeder (trade-off between its ability to compete for a shared energy resource - captured by the interspecific competition term β, and ability to specialize on the new resource, *h*) can foster mutualism in ecological time, as we found mutualism to be favoured by both declines in β and increases in *h*. This hypothesis will be explored in a following study. Evidence for a resource use metabolic constraint has been growing in the literature. For example, the metabolism of lactate by *Aggregatibacter actinomycetemcomitans* (an opportunistic pathogen) inhibits the uptake and metabolism of carbohydrates (e.g. glucose) (Brown and Whiteley 2007). When cocultured with commensal bacteria that produced lactate, this resource use trade-off was important for *A. actinomycetemcomitans* survival because it avoided competition with commensal bacteria for the main resource, in which *A. actinomycetemcomitans* is a poorer competitor (Ramsey et al. 2011). This result indicates that such metabolic constraints might be crucial for enhancing coculture infections featuring *A. actinomycetemcomitans*.

While our results are ecologically derived, ecological stability does not imply evolutionary stability. Now we ask, is the mutualism observed here evolutionary stable? In other words, is this by-product cooperation (Connor 1995; Sachs et al. 2004) subject to invasion by a producer cheat with reduced metabolite excretion (reduced *y*), and/or a cross-feeder cheat with reduced metabolite consumption

(reduced h)? Cross-feeding is incidental in our model as the metabolite excreted by the producer is a waste product. This implies that a producer cheat cannot reduce y because of mechanistic constraints on the production of waste. In turn, the cross-feeder is rewarded by feeding on the producer's waste product, and therefore, a cross-feeder cheat that forgoes this resource will have no selective advantage. Taken together, this suggests that this interspecific mutualism is robust to interspecific cheats because of mechanistic constraints imposed by the specific mechanism of trading food for detoxification.

However, many questions remain about the evolutionary trajectories of this mutualism. For example, it is tempting to speculate that this food for detoxification mutualism could evolve from a facultative association to an obligate association, for either one, or both species. Indeed, as long as there is enough by-product in the environment (from high production y), there may be selection on cross-feeder to reduce or lose its ability to catabolize the shared limiting resource (reduce β) to specialize on the metabolic by-product (increase h). This resource partitioning may then favour co-evolution in the producer of increased 'waste' production y (to further reduce competition).

However, this co-evolutionary scenario raises the following question: what would be the consequences of environmental perturbations? A cross-feeder with high h / low β would be relying on the producer species for food, and thus, in the absence of the latter, the cross-feeder's ability to survive could be strongly compromised. Similarly, a producer with high y would be relying on the cross-feeder for detoxification, and in the absence of the cross-feeder, it would be drowning in its own waste. Interestingly, a theory for the evolution of dependencies has been recently proposed, suggesting that functional dependencies have evolved through adaptive gene loss of dispensable functions (Morris et al. 2012). We suggest that this specific mechanism of trading food for detoxification might be a potential microbial interaction where such a mechanism could be observed - an interesting idea for future experimental research.

Previous models have made important advances in understanding the mechanisms that favour a shift along the parasitism-mutualism continuum over evolutionary time. Models based on principles of evolutionary invasion analysis have typically aimed at understanding the long-term evolution of traits that underlie a specific functional form of interaction, e.g., virulence (Yamamura 1993; Hochberg et al. 2000; Ferdy and Godelle 2005), interspecific cooperation (Doebeli and Knowlton 1998), and partner control (Johnstone and Bshary 2002). Genetic models of coevolution have also provided important insights into how temporal and spatial variability affect fitness interactions between species, and drive fluctuations between mutualism and antagonism (Gomulkiewicz et al. 2003; Nuismer et al. 2003). The novelty of our approach lies in showing a mapping from one mechanism to many functional interactions. Specifically, our model allows us to ask: given a specific context, defined by biotic and abiotic factors, can we predict where a particular mechanistic interspecific interaction will fall on a competition-exploitation-mutualism space? Overall, we suggest that a better understanding of the metabolic, demographic and environmental parameters that govern the balance between the costs and benefits of association will help us to gain new insights into how novel multispecies associations arise, and, to predict where these interactions will fall on the competition-mutualism continuum.

4.6. Appendices

Appendix A: Model Description and Non-dimensionalization

Our model tracks the dynamics of a one way cross-feeding interaction between two populations: a producer, A, of a metabolic by-product, E, and a cross-feeder (non-producer), B. Building on the competitive Lotka-Volterra model, our model explicitly captures the dynamics of the two strategists as well as the cross-feeding by-product and is defined by the following system of ordinary differential equations:

$$\frac{da}{dt} = (r_a(1 - (a + \beta b)/k_a) - fe)\, a$$
$$\frac{db}{dt} = (r_b(1 - (\gamma a + b)/k_b) + ghe)\, b \qquad \text{(A1)}$$

$de/dt = ya - heb - ue$

The above system can be rewritten in non-dimensional form (see Table A1 for a full description of the non-dimensional quantities) (Segel 1972; Murray 2002), and the non-dimensional system becomes:

$$\begin{aligned} dA/dt &= (r\,(1 - \alpha A - \beta B) - fE)\,A \\ dB/dt &= ((1 - \gamma A - B) + ghE)\,B \\ dE/dt &= yA - hEB - uE \end{aligned} \qquad \text{(A2)}$$

The densities of producer (A) and cross-feeder (B) are scaled to the carrying capacity of B (k_b), and the individual intrinsic growth rates are scaled to the intrinsic growth rate of B (r_b). We assume that the by-product enhances the cross-feeder's growth, and this (indirect) benefit is described by gh, where h represents the by-product consumption rate by the cross-feeder and g is a conversion constant, which can be viewed as the cross-feeder's uptake efficiency of the by-product. In contrast, the by-product inhibits the producer's growth at a constant rate f. u represents the by-product decay rate, and y represents the rate of the by-product production by the producer. α is the producer's intraspecific competition coefficient, and measures the degree of competition among the population of producers relative to the competition among the population of cross-feeders ($\alpha = k_b/k_a$, see Table A1). β is the cross-feeder's interspecific competition coefficients on the producer, and γ is the producer's interspecific competition coefficients on the cross-feeder, thus β and γ measure the competitive effect of B on A and A on B, respectively. Note that when $f = g = h = y = u = 0$, we recover the classic competitive Lotka-Volterra equations.

Table A1. Parameters description and respective dimensionless parameters

Symbol	Definition	Dimension	Dimensionless parameter
a	Producer	Mass	$A = a/k_b$
b	Cross-feeder and non-producer	Mass	$B = b/k_b$
e	Metabolic by-product	Mass	$E = e/k_b$
r_a, r_b	Intrinsic growth rate of A and B, respectively	$Time^{-1}$	$r = r_a/r_b$
k_a, k_b	Carrying capacity of A and B, respectively	Mass	$\alpha = k_b/k_a$
β, γ	Interspecific competition coefficients of A and B, respectively	-	$\beta = \beta\, k_b/k_a$
f	By-product toxicity rate	$mass^{-1}.\ time^{-1}$	$f = f\, k_b/r_B$
h	Consumption rate of by-product	$mass^{-1}.\ time^{-1}$	$h = h\, k_b/r_B$
y	By-product production rate	$time^{-1}$	$y = y/r_B$
g	Cross-feeder uptake efficiency (of by-product)	constant	-
u	By-product decay rate	$time^{-1}$	$u = u/r_B$
t	Time	time	$t = t\, r_B$

Appendix B: Model Equilibria and Stability Analysis

An equilibria analysis of model A2 (Otto and Day 2007) reveals that the system has six equilibria (denoted by A^*, B^*, C^*). Two equilibria are biologically invalid (note that we assume that all parameters are positive),

$A^* = 0$, $B^* = 0$, $C^* = 0$, and $A^* = 0$, $B^* = -u/h$, $C^* = -(h + u)/gh^2$.

Four equilibria are biologically valid. The producer only equilibrium is

$A^* = ru/(\alpha ru + fy)$, $E^* = ry/(\alpha ru + fy)$, $B^* = 0$.

The cross-feeder only equilibrium is

$B^* = 1, A^* = 0, E^* = 0,$

Coexistence equilibria 1 and 2 that we denote by A_1^*, B_1^*, E_1^* and A_2^*, B_2^*, E_2^* take the form:

$$A^*_1 = (A'' + z\sqrt{C}) / (2hr(\alpha - \beta\gamma)(f\gamma + \alpha rgh))$$
$$B^*_1 = (A' - \sqrt{C}) / (2hr(\alpha - \beta\gamma)) \quad \text{(B1)}$$
$$E^*_1 = (A - \sqrt{C}) / (2h(f\gamma + \alpha rgh))$$

and,

$$A^*_2 = (A'' - z\sqrt{C}) / (2hr(\alpha - \beta\gamma)(f\gamma + \alpha rgh))$$
$$B^*_2 = (A' + \sqrt{C}) / (2hr(\alpha - \beta\gamma)) \quad \text{(B2)}$$
$$E^*_2 = (A + \sqrt{C}) / (2h(f\gamma + \alpha rgh))$$

where

$$C = -4rhy(\beta - 1)(f\gamma + \alpha rgh) + [ru(\alpha - \beta\gamma) + rh(\alpha - \gamma) + (f + rgh\beta)y]^2,$$
$$z = (f + rgh\beta),$$
$$A = rh(\gamma - \alpha) + ru(\beta\gamma - \alpha) - y(f + rgh\beta),$$
$$A' = A - 2rh(\gamma - \alpha),$$
$$A'' = f^2y + ghr^2(2h\alpha - h\alpha\beta + u\alpha\beta + ghy\beta^2 - \beta(h + u\beta)\gamma) + rf(h(\alpha + 2gy\beta + \gamma - 2\beta\gamma) + u(\alpha - \beta\gamma))$$

(B3)

From the expressions for the coexistence equilibria we can note that if $\alpha = \gamma\beta$ the equilibria no longer exist rather than being inaccessible. This suggests that there is no coexistence if the product of intraspecific competition is equal to the product of interspecific competition. Also, it should be noted that there is a small parameter space where equilibrium 1 (A_1^*, B_1^*, E_1^*) and equilibrium 2 (A_2^*, B_2^*, E_2^*) are both accessible (results not shown).

The stability analysis of Model A2 reveals that the producer alone equilibrium is locally stable when $u > y(f + rgh)/(r(\gamma - \alpha))$ (i.e. u threshold for pure A^* stability) and $\alpha < \gamma$. The cross-feeder alone equilibrium is locally stable when $\beta > 1$. Thus for

sufficiently high interspecific competition of the cross-feeder on the producer, a rare population of producers cannot invade a resident population of cross-feeders. The stability analysis of the coexistence equilibria is difficult to perform analytically, so we investigated their stability using numerical simulations. In sum, our analytical and numerical analyses (fig. B1) suggest that the model is mostly defined by four main regions: i) Pure producer is locally stable, thus a rare population of cross-feeder cannot invade a resident population of producers. This region is defined by $\beta < 1$ and $u >$ threshold for pure A^* stability. None of the coexistence equilibria are accessible. (Figure B1A, B); ii) Pure cross-feeder is locally stable, thus a rare population of producer cannot invade a resident population of cross-feeder. This region is defined by $\beta > 1$ and $u <$ threshold for pure A^* stability. Both coexistence equilibria 1 and 2 may be accessible for some parameter space, but they are unstable. Any small perturbation of the initial conditions moves the system to the pure cross-feeder equilibrium. (Figure B1A, C); iii) Stable coexistence. $\beta < 1$ and $u <$ threshold for pure A^* stability, where coexistence equilibrium 2 is accessible and stable, while coexistence equilibrium 1 is non-accessible (Figure B1A, D); iv) Bistability region. This region is defined by $\beta > 1$ and $u >$ threshold for A stability. Coexistence equilibrium 1 is attainable but unstable (i.e. a repellor), and is associated with a separatrix that passes through it, and this separatrix subdivides the phase plane space into the two basins of attraction associated with the two attractors (i.e. locally stable pure A^* and locally stable pure B^* equilibria). Whether the system approaches a pure producer or pure cross-feeder equilibrium will depend on initial conditions. Any small perturbation of the initial conditions moves the system to either the pure producer or pure cross-feeder equilibrium (Figure B1A, E, F). While not discussed here, it should be noted that limit cycles (populations oscillations) are also a possible outcome. Numerical simulations suggest that limit cycles may occur when the parameters that govern the by-product dynamics are significantly lower than the effect of competition (e.g. there is a stable limit cycle under the following parameter values, $r = 1$, $\alpha = 0.8$, $\gamma = 1$, $g = 1$, $f = 0.1$, $y = 0.03$, $h = 0.01$, $\beta = 0.9$ and $u = 0.002$). However, this limit cycle is stable (results not shown), and this means that the equilibrium is also stable, therefore, our results would not be affected by the presence of this limit cycle.

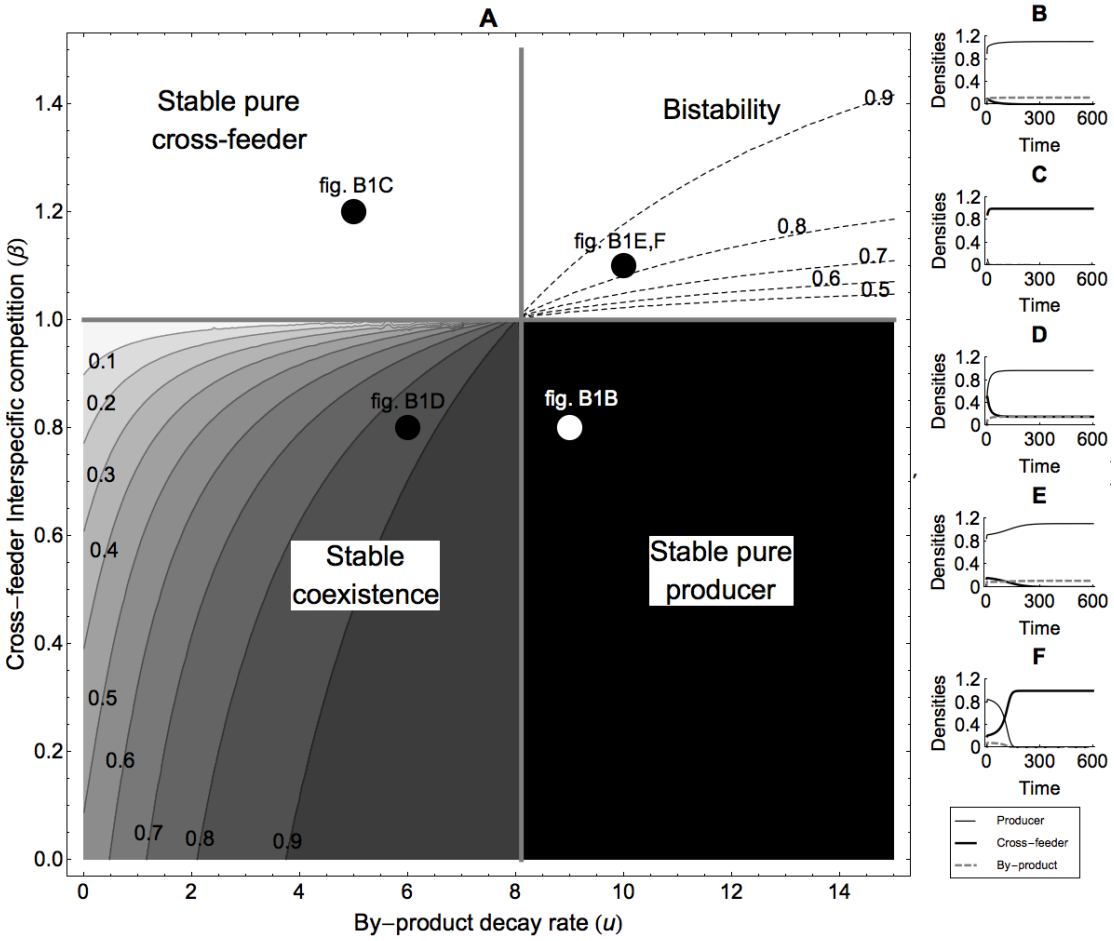

Figure B1. Illustration of the stability conditions for the equilibria of the cross-feeding model. **A,** Diagram illustrating the four main regions of equilibria stability. Black region, pure producer is locally stable. White region, pure cross-feeder is locally stable. Stable coexistence region, coexistence equilibrium 2 is stable and attainable (the contour lines represent the proportion of producer $p = A^* / (A^* + B^*)$ at coexistence equilibrium 2). Bistability region (i.e. either A or B invades), coexistence equilibrium 1 is accessible but unstable (the dashed lines represent the repellor value $p^* = A^* / (A^* + B^*)$ at coexistence equilibrium 1). The grey horizontal line represents the threshold of pure B^* stability ($\beta = 1$), and the grey vertical line represents the value of u threshold for pure A^* stability ($u = y(f + rgh)/(r(\gamma - \alpha))$, see text for more details). **B-F,** Temporal dynamics of the densities of producer and cross-feeder for parameter values falling in **B,** the stable pure producer region ($\beta = 0.8$, $u = 9$, $A_0 = 0.9$ and $B_0 = 0.1$); **C,** the stable pure cross-feeder region ($\beta = 1.2$, $u =$

6, A_0 = 0.1 and B_0 = 0.9); **D,** the stable coexistence region (β = 0.8, u = 6, A_0 = 0.5 and B_0 = 0.5); and the bistability region (β = 1.1 and u = 10) when **E,** the producer invades (A_0 = 0.85 and B_0 = 0.15), and **F,** the cross-feeder invades (A_0 = 0.80 and B_0 = 0.20). Unless stated otherwise, the parameters used are $r = 1$, $\alpha = 0.9$, $\gamma = 1$, $g = 1$, $f = 0.01$, $y = 1$, and $h = 0.8$, $E_0 = 0.01$.

Appendix C: Supplementary figures

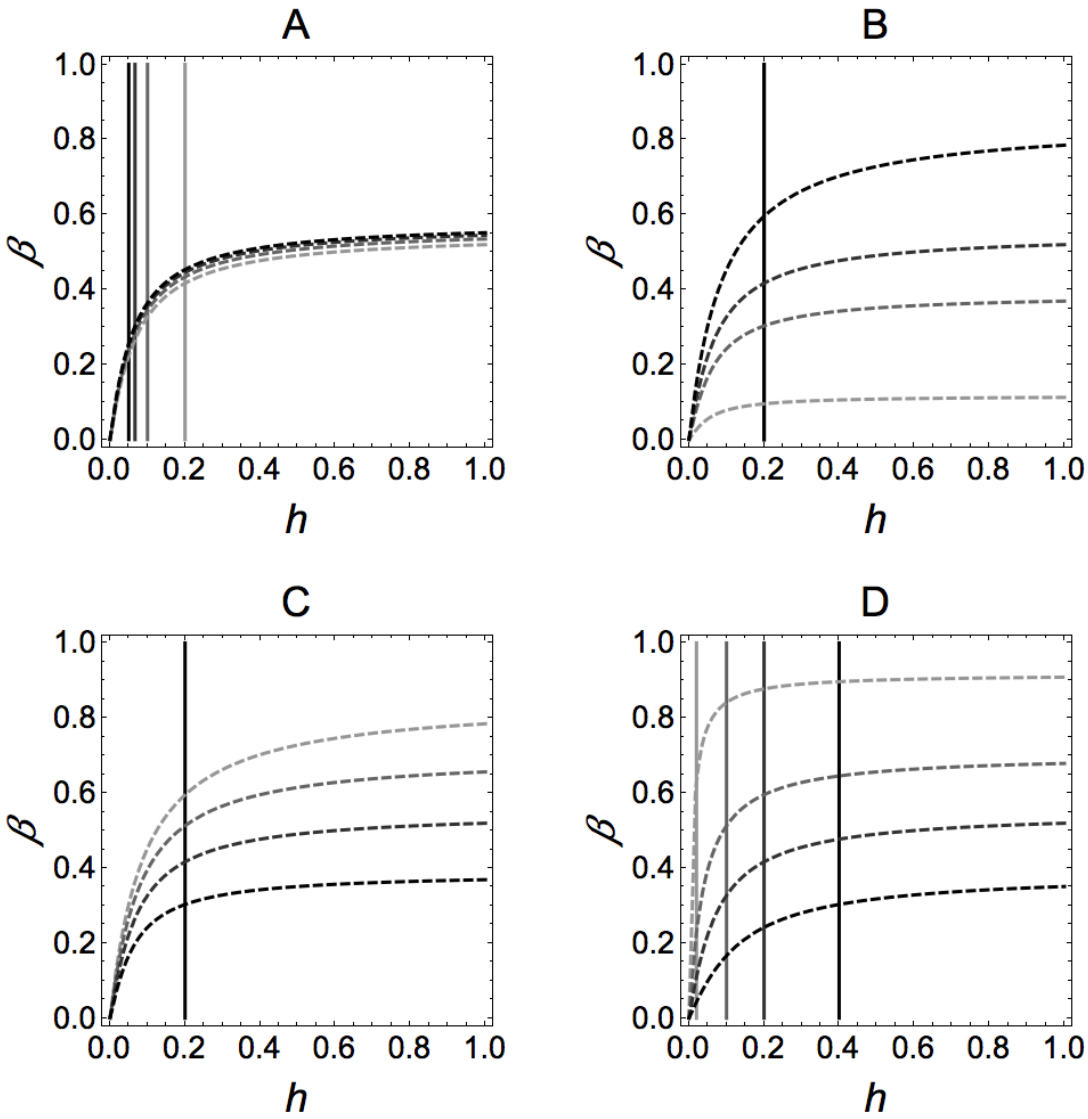

Figure C1. Effect of the demographic, environmental, and metabolic parameters on mutualism. Each dashed and full line represents the threshold where $A_B^* = A_a^*$, and $B_A^* = B_a^*$, respectively, for a given set of parameter values. Mutualism is defined by the region below the dashed line and on the right of the filled line. When not otherwise specified the parameters used for the plots are $r = 1$, $\alpha = 0.9$, $\gamma = 1$, $g = 1$, $u = 0.1$, $f = 0.1$, and $y = 1.5$. **A,** Mutualism is favoured by higher by-product production rate (higher y). Each line represents a different values of y such that y = [1.5, 2, 2.5, 3] from light to dark respectively; **B**, Mutualism is favoured by higher by-product toxicity (higher f). Each line represents a different values of f such that f = [0.01, 0.05, 0.1,0.5] from light to dark respectively. Note that $B_A^* = B_a^*$ is independent on f (see text for more details); **C**, Mutualism is favoured by lower relative growth rate (lower r). Each line represents a different values of r such that r = [0.2, 0.5, 1, 1.5] from light to dark respectively. Note that $B_A^* = B_a^*$ is independent on r (see text for more details); **D**, Mutualism is favoured by a more durable by-product (lower u). Each line represents a different values of u such that u = [0.01, 0.05, 0.1, 0.2] from light to dark respectively.

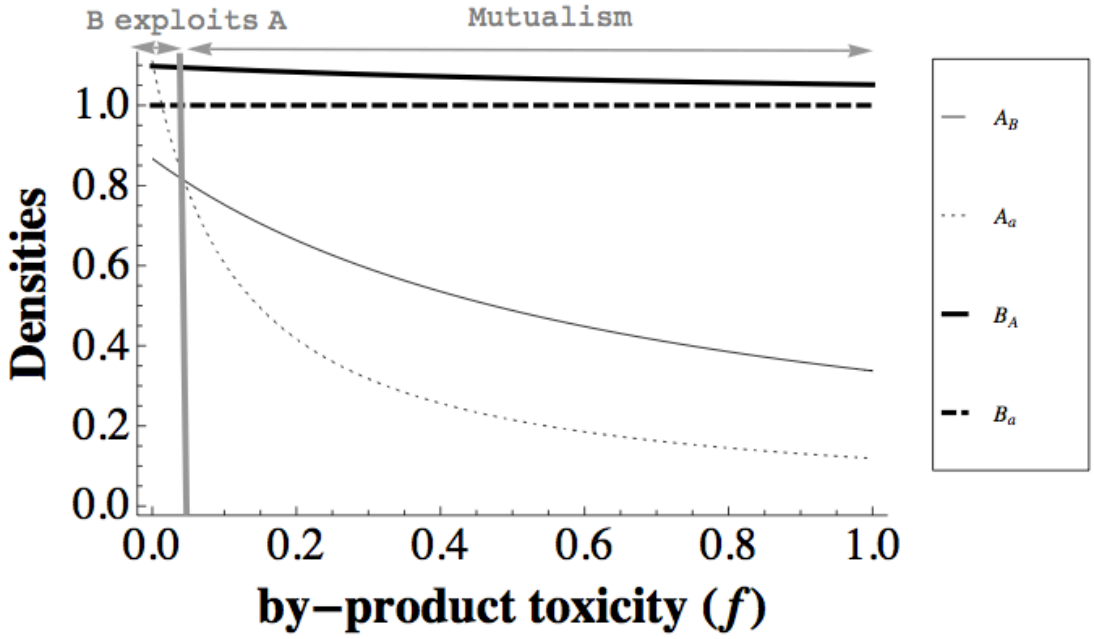

Figure C2. Effect of by-product toxicity (f) on densities of producer when grown alone and in coculture with the cross-feeder (A_a* and A_B*, respectively), and on densities of cross-feeder when grown alone and in coculture with the producer (B_a* and B_A*, respectively). The grey line indicates the frontier between cross-feeder exploits producer and mutualism. The parameter values used are $r = 1$, $\alpha = 0.9$, $\beta = 0.2$, $\gamma = 1$, $g = 1$, $h = 0.8$, $u = 0.2$, and $y = 1.5$.

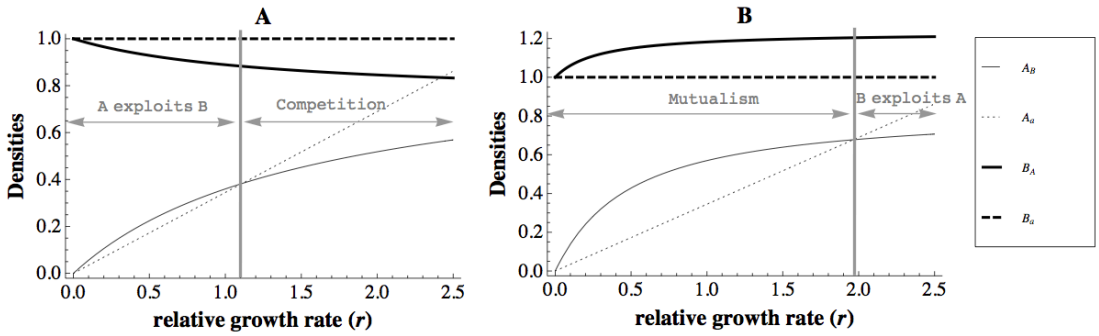

Figure C3. Effect of relative growth rate (r) on densities of producer when grown alone and in coculture with the cross-feeder (A_a* and A_B*, respectively), and on densities of cross-feeder when grown alone and coculture with the producer (B_a* and B_A*, respectively). **A,** cross-feeder does not benefit from association ($h = 0.05$). The grey line indicates the frontier between competition and A exploits B. **B**, cross-feeder benefits form association ($h = 0.3$). The grey line indicates the frontier between B

exploits A and mutualism. The other parameter values used for the plots are $\alpha = 0.9$, $\beta = 0.2, \gamma = 1, g = 1, u = 0.1, f = 0.1$ and $y = 2$.

Chapter 5

METABOLIC AND DEMOGRAPHIC FEEDBACKS SHAPE THE EMERGENT SPATIAL STRUCTURE AND FUNCTION OF MICROBIAL COMMUNITIES

This chapter is under second review in *PLOS Computational Biology* as:
Estrela S and Brown SP. Metabolic and demographic feedbacks shape the emergent spatial structure and function of microbial communities

5.1. Summary

Microbes are predominantly found in surface-attached and spatially structured polymicrobial communities. Within these communities, microbial cells excrete a wide range of metabolites, setting the stage for interspecific metabolic interactions. The links, however, between metabolic and ecological interactions (functional relationships), and species spatial organization (structural relationships) are still poorly understood. Here, we use an individual-based modelling framework to simulate the growth of a two-species surface-attached community where food (resource) is traded for detoxification (service), and investigate how metabolic constraints of individual species shape the emergent structural and functional relationships of the community. We show that strong metabolic interdependence drives the emergence of mutualism, robust interspecific mixing, and increased community productivity. Specifically, we observed a striking and highly stable emergent lineage branching pattern, generating a persistent lineage mixing that was absent when the metabolic exchange was removed. These emergent community properties are driven by demographic feedbacks, such that aid from neighbouring cells directly enhances focal cell growth, which in turn feeds back to neighbour fecundity. In contrast, weak metabolic interdependence drives conflict (exploitation

or competition), and in turn greater interspecific segregation. Together, these results support the idea that species structural and functional relationships represent the net balance of metabolic interdependencies.

5.2. Introduction

It is now widely accepted that most polymicrobial communities living in natural environments form spatially structured and surface-attached consortia (biofilms) (Costerton et al. 1995). There has recently been a great interest in investigating how spatial structure may forge and stabilize the complex web of interactions occurring within these multispecies communities, including mutualistic (Elias and Banin 2012) and competitive (Rendueles and Ghigo 2012) relationships. Empirical work in multispecies biofilms has acknowledged that species composition affects community structure and species distribution within the biofilm (Tolker-Nielsen and Molin 2000) as a result, for example, of mixing species that have distinct monoculture structures (Murga et al. 1995), or via metabolic interactions, such as cross-feeding (Nielsen et al. 2000; Christensen et al. 2002; Hansen et al. 2007; Breugelmans et al. 2008) or detoxification of exogenous waste products (Cowan et al. 2000). The type of carbon source also plays a major role in generating the diversity of spatial arrangements observed in polymicrobial communities, as varying the source of carbon likely alters the metabolic interactions between members of the community. For example, in a two-species biofilm consisting of *Burkholderia* and *Pseudomonas,* Nielsen et al. (Nielsen et al. 2000) observed that when the two species were competing for a common resource (non-cross-feeding medium), the biofilm consisted of separate microcolonies (high species segregation). In contrast, when the two species were involved in a one-way obligate cross-feeding interaction (cross-feeding medium), the microcolonies consisted of both species (greater mixing).

Evolutionary theory has suggested that spatial mixing favours the evolution of mutualism because it keeps mutualistic partners in close proximity, thereby allowing

for stronger reciprocity (Frank 1994; Doebeli and Knowlton 1998; Foster and Wenseleers 2006), which may in turn facilitate the exchange of metabolites between partners. However, it has also been proposed that, under some conditions, spatial mixing may impair mutualism because of spatial limits on exchange (Verbruggen et al. 2012), or because it hinders cooperators' clustering in within-species cooperation (Hauert and Doebeli 2004). Empirical work on the evolution of microbial cross-feeding mutualisms has also found opposite responses to environment structure. For example, Harcombe (2010) provided empirical support for the benefits of spatial structure in the evolution of mutualistic cross-feeding between *Salmonella* and auxotrophic *Escherichia coli*. However, another study on the nascent cross-feeding mutualism between *Desulfovibrio vulgaris* and *Methanococcus maripaludis* showed that mutualism was initially favoured in a well-mixed rather than static environment (Hillesland and Stahl 2010). Although the authors suggested that this different response to environmental structure is due to the lack of a direct fitness cost of cooperation in the latter cross-feeding model system (Hillesland et al. 2011), other mechanisms may be at play as well. The spatial separation (distance) between species has also been identified as a key factor for the stable coexistence of a synthetic mutualistic bacterial community (Kim et al. 2008).

While evolutionary ecology has traditionally assumed that structure is a fixed environmental property (i.e. either structured or well-mixed), there has been a recent interest in regarding structure as an emergent property of the aggregate behaviour of individuals(2008)). Individual-based simulations of microbial growth have started to shed some light on this topic. For example, Nadell et al. (Nadell et al. 2010) explored how physical and biological parameters of bacterial growth in biofilm affect lineage segregation, which in turn determines the fate of within-species cooperation. Using the same framework, it has also been proposed that within-species cooperation can be favoured due to social insulation of cooperators from non-cooperators by a second species (Mitri et al. 2011). Recently, using a mix of experiments and simulations, Momeni et al. (Momeni et al. 2013) showed that strong inter-population cooperation led to inter-population mixing in microbial communities, and specifically in a pattern of successive layering. Despite this, however, far too little attention has been given to

how specific metabolic interactions generate the emergent spatial and functional properties of microbial communities.

Our goal here is to address this question by investigating how metabolic constraints of individual species shape the emergent functional relationships and spatial structuring of a two-species community. For this, we focus on a specific type of interspecific metabolic interaction - trading food for detoxification (for empirical examples see (2006), (2010); for a theoretical approach see (Estrela et al. 2012)), and we explore how a partner's need for help (either detoxification to the producer or food to the cross-feeder) affects the ecology, spatial structure, and productivity of the two-species community.

Using an individual-based modeling (IBM) framework that models microbial population growth on a solid surface (Lardon et al. 2011), our results show that stronger metabolic interdependence generates more mutualism, more interspecific mixing, less sensitivity to initial conditions and enhanced community productivity. The emergence of this metabolism-dependent community structure and functioning is driven by demographic feedbacks, such that providing aid to a mutualistic partner generates a positive feedback on the individual's growth whereas providing aid to a competitor or exploiter generates a negative feedback on the individual's growth. In consequence, demographic feedbacks strengthen mutualistic relationships via increased lineage mixing, and weaken competitive relationships via increased segregation.

5.3. Methods

Model

Our model assumes two species, a producer (A) of a metabolic by-product (E), and a cross-feeder (B) (see fig. S1 for a schematic representation) growing on an inert surface. The producer and cross-feeder are ecological competitors for a common limiting nutrient (R, e.g. glucose) that diffuses from the bulk (above) into the

biofilm. The bulk consists of a liquid and well-mixed compartment where the concentration of nutrient (R_{bulk}) is held constant. Thus, the growths of species A, and species B, are a function of the rates of uptake of R in the local microenvironment of A and B, respectively. In addition, the cross-feeder's growth is enhanced by its ability to use the producer waste product E, while the producer's growth is decreased by E (i.e. toxic waste product). Thus the concentrations of R and E vary in space and time due to production/consumption reactions and diffusion.

The metabolic reactions and stoichiometric matrix used in the model are described in detail in Table S1. Briefly, the reaction of transformation of R into E and biomass A (X_A, cell growth of A) follows a Monod-form kinetic, and E inhibits this reaction via simple inhibition. The reaction of transformation of R and E into biomass B (X_B, cell growth of B) follows a Monod-form kinetic on R and E, respectively. Also, we assume that the producer has more affinity and is more efficient at using the main nutrient (R) than the cross-feeder, such that $K_{R,A} < K_{R,B}$ and $Y_{R,A} > Y_{R,B}$, respectively. This may represent, for example, a cost of resource generalism to the cross-feeder (Kassen 2002). We assume that the obligate cross-feeder (B_{obl}) is specialist on the producer's waste product and incapable of using the limiting nutrient. This means that the two species do not use overlapping nutrients and that the cross-feeder depends on its partner's waste product for growth. Specialization on a partner's waste product of metabolism can occur via mutations (Rosenzweig et al. 1994) or due to an exclusion mechanism in which the metabolism of the waste product inhibits the uptake of the limiting nutrient (Brown and Whiteley 2007). In addition, we assumed three facultative cross-feeders, Consistent with previous empirical work we assume that the facultative cross-feeders are able to use both the common limiting nutrient and the metabolic by-product (see e.g. (Rosenzweig et al. 1994; Turner et al. 1996; Poltak and Cooper 2011; Lawrence et al. 2012)), but differ in their degree of obligacy, varying from strongly dependent (B_{facS}) to intermediately dependent (B_{facI}) to weakly dependent (B_{facW}) on the producer's waste product for growth (see Table S2). Finally, in the producer- non-cross-feeder (B_{ncf}) association there is complete overlap of resource use. Specific parameter values used for the simulations are described in Table S2, and other simulation parameter values used

for the simulations are described in previous work (Mitri et al. 2011). Unless otherwise stated, we assume cyclic boundary conditions.

Inoculation densities are 60 cells in monoculture, and 60 cells of each species (1:1) in coculture. This means that the initial density of each individual species is held constant across culture type (i.e. monoculture and coculture), and thereby the total inoculation density of monoculture is half the total inoculation density of coculture (additive experimental design). This approach gives us a measure of how an individual species is affected by diversity only, and not by initial individual species densities.

Measuring growth rate

Growth rate is measured as $(N_f - N_i)/(t_f - t_i)$ where N_i represents the number of cells inoculated at time 0 (t_i), and N_f represents the number of cells at the end of the simulation (t_f). Unless otherwise stated, data represent growth after 96 hours, and are the mean of 3 replicates.

Segregation index

The segregation index (s) is an indicator of species segregation (or mixing) within their local neighbourood measured relative to global species frequencies, and is measured as:

$$s_A = (seg_A - \overline{p_A}) / (1 - \overline{p_A})$$

and

$$s_B = (seg_B - \overline{p_B}) / (1 - \overline{p_B})$$

where seg_A (seg_B) represents the proportion of species A (species B) in the local microenvironment (i.e. neighbourhood), and $\overline{p_A}$ ($\overline{p_B}$) is the proportion of species A (species B) in the whole population. Note that this way of measuring species segregation in an interspecific population is similar to the relatedness coefficient

used in social evolution to measure relatedness within-species (Queller and Goodnight 1989; Buttery et al. 2012). This intermixing index can also be seen as an indicator of species co-assortment, e.g., whether species A is more assorted (or segregated) with species B than if the two species were distributed randomly (i.e. when $s = 0$).

The calculation of the proportions of species A and species B in the local environment is adapted from the methodology used in Mitri et al. (Mitri et al. 2011) to measure population segregation in biofilm. In brief, for each individual cell (c_i) of a given species - i.e. either species A or species B- in a population of $N = N_A + N_B$ cells we identify all the neighbour cells (c_j) falling within a neighbourhood distance of a radius of 10 μm. The segregation of each individual cell c_i is defined as:

$$seg(c_i) = \frac{1}{N_d} \sum_{j=1}^{N_d} g(c_i, c_j)$$

where $g(c_i, c_j) = 0$ if c_i and c_j belong to different species, or, $g(c_i, c_j) = 1$ if c_i and c_j belong to the same species, and N_d is the number of cells falling within the distance of 10 μm. The segregation index seg_A (seg_B) of species A (species B) is then defined as:

$$seg_A = \frac{1}{N_A} \sum_{i=1}^{N_A} seg(c_i) \left(seg_B = \frac{1}{N_B} \sum_{i=1}^{N_B} seg(c_i) \right).$$

5.4. Results

We model the growth of a two-species microbial community on an inert surface using an individual-based modeling (IBM) framework described in detail in Lardon et al. (2011). Individual-based models have proven useful in addressing ecological and evolutionary questions in biofilms and are a powerful approach to study the

emergent properties of microbial communities (Kreft 2004; Xavier and Foster 2007; Nadell et al. 2010; Bucci et al. 2011; Merkey et al. 2011; Mitri et al. 2011; Schluter and Foster 2012). Briefly, this framework simulates the growth of bacterial cells on a surface that grow by consuming nutrients present in their local environment, and then divide. The transport of solutes into and within the biofilm occurs through diffusion, which is assumed to occur much faster than cell growth and division. Cell movement within the biofilm occurs as a result of cell growth, division, shrinking and death. Bacterial cells interact mechanically with neighbouring cells by shoving for space, a process that minimizes cells overlap. Metabolic interactions are introduced by the explicit modeling of metabolic intermediates, subject to defined stoichiometry of metabolic reactions and rates of diffusion (Table S1 and Methods, and for more details on the assumptions of the IBM framework see (Lardon et al. 2011)).

Metabolic interdependence shapes emergent functional relationships

The ecological outcome of the food for detoxification interaction depends on the balance between costs and benefits of interspecific association. The potential costs are interspecific competition for common nutrients and space, while the potential benefits are food for the cross-feeder and detoxification for the producer. To determine the type of ecological interaction forged between producer and cross-feeder, we measured the net costs and benefits from association (Bronstein 1994; Connor 1995) by comparing species growth rates when grown alone with their growth rates when grown in coculture (see Methods). If both species have an increase in growth rate relative to their growth rate in monoculture, the association is mutualistic. If both species have a decrease in growth rate when grown in coculture relative to their growth rate in monoculture, the association is competitive. If one species benefits at the expense of the other, then there is exploitation.

Analytical work under the limiting assumption of a well-mixed (planktonic) community found that diverse ecological relationships can emerge from a one-way cross-feeding interaction where nutrients are traded for detoxification (Estrela et al.

2012). Does the same result hold when the environment is spatially structured? To address this question, we first investigated how the degree of metabolic interdependency (varying along two species axes) shapes the ecological relationships between two species. Specifically, we vary by-product toxicity from non-toxic to highly toxic (variations in metabolite toxicity can occur, for instance, via changes in pH or the type of metabolite produced (Davison and Stephanopoulos 1986)) and the degree of cross-feeder obligacy from non-cross-feeder to obligate cross-feeder (see fig. S1A for a schematic representation of species interactions, and Methods). Metabolic interdependency implies that a species' chemical environment is improved in at least one specific dimension by the presence of another species (for instance, detoxification or provision of a growth substrate). However this specific chemical aid does not imply that the recipient gains a net growth advantage from association, as the two species may also compete for other limiting resources (space and/or nutrients). We found that metabolic interdependency gives rise to diverse ecological interactions, ranging from mutualism to competition (figs 5.1A, S2), thus corroborating our previous finding for well-mixed populations (Estrela et al. 2012). Specifically, mutualism only emerges when the specific help received outweighs the competitive costs endured for both partners.

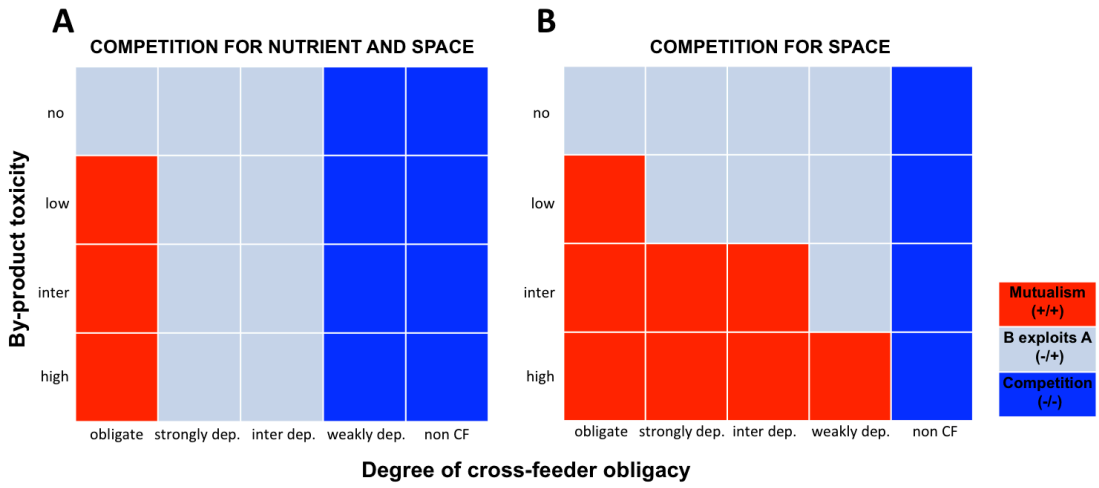

Figure 5.1. Metabolic interdependence dictates the ecological outcome of the food for detoxification interaction. Ecological outcome of interaction for varying by-product toxicity and degree of cross-feeder obligacy when the two species

compete for both nutrients and space **A,** or compete for space only **B** (see Methods and Text for further details, and fig. S1 for a schematic representation of species interactions). Red indicates mutualism, gray indicates cross-feeder (B) exploits producer (A), and blue indicates competition. CF means cross-feeding.

In figure 1A we have assumed that the two species compete for space and limited nutrients (unless cross-feeding is entirely obligate). We next ask what is the relative contribution of competition for space and nutrients to our results? Importantly, these two limiting resources are linked as winning the competition for space means getting access to nutrients, and similarly, winning the competition for nutrients means getting access to space. To disentangle their effects we relax nutrient competition (see schematic fig. S1B). As expected, we found that removing competition for nutrients leads to less negative associations, as seen by a shift from competition to exploitation, or from exploitation to mutualism (fig.1A, B). As toxicity increases, the ability of the producer to compete for a shared nutrient resource is diminished. Therefore, removal of nutrient competition has a disproportionately positive effect on mutualism as toxicity increases.

Our definition of mutualism ((Bronstein 1994; Estrela et al. 2012)) implies that the total productivity of the two species community will be greater than the summed productivities of the two species apart. However, our results also show that enhanced community productivity does not itself imply mutualism, as exploitative relationships can also lead to a community gain (figs. S3, S4). This is consistent with empirical studies that have documented that resource (niche) partitioning via cross-feeding interactions enhances community productivity (Periasamy and Kolenbrander 2009; Poltak and Cooper 2011; Ramsey et al. 2011; Lawrence et al. 2012), with the caveat that enhanced community productivity does not alone dictate a mutualistic relationship.

Metabolic interdependence drives genetic intermixing

Theoretical modelling has suggested that population segregation (high relatedness) can favour within-species cooperation because segregation keeps the benefits of cooperation close to cooperators (Nadell et al. 2010; Mitri et al. 2011), although these benefits are potentially mitigated by enhanced competition among kin (Queller 1992; Frank 1998; West et al. 2002). Furthermore, it has been suggested that population mixing favours between-species cooperation because it facilitates the exchange of the benefits of cooperation, therefore creating a tension between within-species cooperation and between-species cooperation (Mitri et al. 2011). In our food for detoxification interaction, the effect of within-species cooperation on population segregation is relaxed, therefore allowing for between-species mutualism to occur under a broader range of conditions. In a recent simulation and experimental study, it has been shown that strong inter-population cooperation leads to inter-population mixing in microbial communities, and specifically in a pattern of successive layering (Momeni et al. 2013).

Based on these observations, we next hypothesized that varying metabolic interdependence would dictate the degree of genetic intermixing within the two-species community, and in a way that reflects the net costs and benefits of interspecific association. In particular, we would expect that increasing metabolic interdependence would result in higher genetic intermixing within the biofilm to facilitate trade. We generally found that, as by-product toxicity increases, intermixing increases (figs. 5.2, S5). Similarly, increasing cross-feeder obligacy leads to higher intermixing (figs. 5.2, S5), except in the non-cross feeding medium (and intermediate to high by-product toxicity). The latter scenario likely occurs because the fast growing cross-feeder cells insulate the poorly growing producer cells in separate enclaves, thus leading to greater mixing. The segregation index (Methods) provides a global statistic of population structure, but does not reveal the developmental patterning of the two intermixing species or their resulting shared architecture. Figures S11 and S12 illustrate the resulting development and architecture of the two-species community, and highlight that strong mixing is

achieved via a striking and emergent branching pattern producing increased inter-digitation and contact surface between interdependent cell lineages. Branching-like patterns within single clonal lineages have been observed previously under conditions of low nutrient availability, due to stochastic variations in a thin active growth layer (Nadell et al. 2010). The resulting separated 'towers' (observable in fig. 5.2D) are mutually repulsive, as growth towards conspecifics increases competition for limiting resources. In contrast, as mutual interdependence increases, demographic movement towards heterospecifics becomes increasingly rewarding, resulting in branching of lineages towards heterospecifics and away from conspecifics, generating a robust and stabilising mixing pattern.

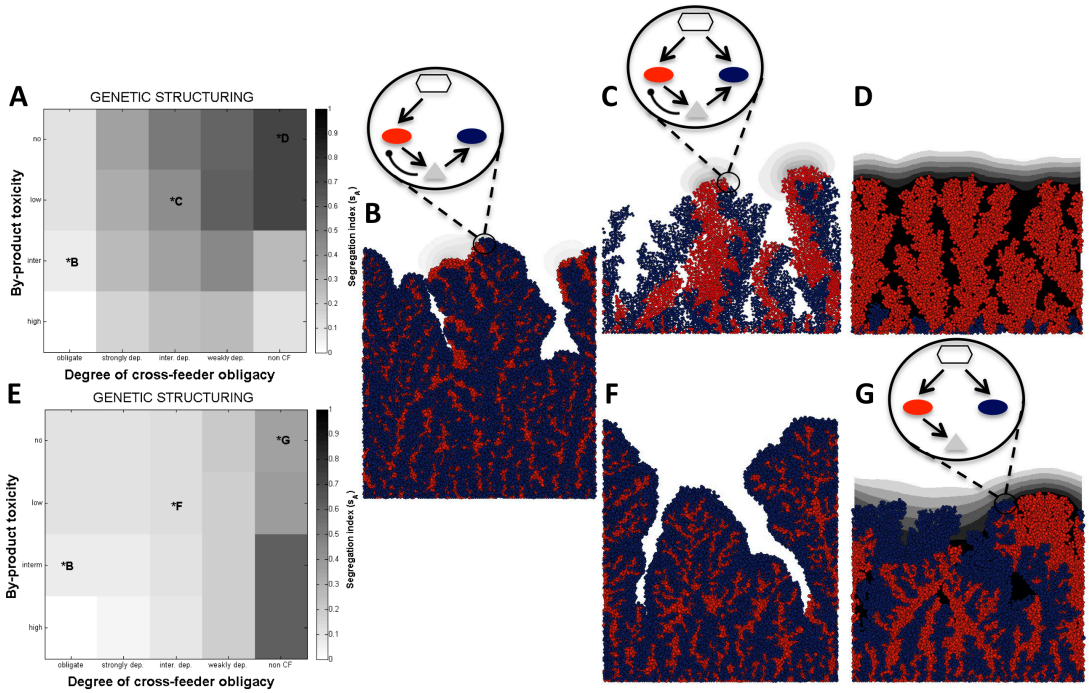

Figure 5.2. Metabolic interdependence drives genetic mixing. Producer segregation index (s_A, see Methods) for varying by-product toxicity and degree of cross-feeder obligacy when the two species compete for both nutrients and space **A,** or compete for space only **E.** Lighter regions indicate greater mixing (see Methods for further details and fig. S5 for cross-feeder segregation index). Data are the mean of 3 replicates. **B-D, F, G.** Biofilm images of community growth from one of the associations represented in **A or E**. Producer is represented in red, and facultative cross-feeder, obligate cross-feeder, and non-cross-feeder are represented in blue. By-product is in gray. The schematics illustrate the metabolic interaction scenarios.

Oval, hexagon, and triangle, represent bacteria, main nutrient, and by-product, respectively. Open arrows represent a positive effect, whereas oval arrows represent a negative effect upon the population or resource they are pointing toward. See fig. S1 for a complete schematic representation of all metabolic interaction scenarios.

Strong interdependence generates more robust mixing

It has been recently documented that population intermixing of a yeast obligate cooperative community is robust to a broad range of initial conditions, including initial ratio and densities (Momeni et al. 2013). In this study, however, the authors assumed that cells were randomly seeded. Given this, we hypothesized that the degree of intermixing at inoculation may influence the ecological and structural relationship of the two species trading food for detoxification, by modulating the establishment of key metabolic and demographic feedbacks. Indeed, increasing segregation at inoculation might have two opposite effects: on the one hand, we would expect the costs of interspecific competition to be delayed, but on the other hand, the benefits of trade would be reduced.

To examine this, we repeated the simulations of monoculture, facultative cross-feeding coculture, and obligate cross-feeding coculture, but now the cells were inoculated in two microcolonies of size 30 μm and separated by a distance of 70 μm from each other (coculture) or in a single microcolony of size 30 μm (monoculture). The degree of initial intermixing was changed by varying the proportions of producer and cross-feeder in each microcolony but keeping the total number of inoculated cells constant and 1:1. This means that, for example, when both microcolonies were inoculated with equal number of cells of producer and cross-feeder type, then they were completely intermixed (i.e. segregation index, s, equal to 0, see Methods). When one microcolony was inoculated with cells of producer type only and the other microcolony with cells of cross-feeder type only, then they were fully segregated (i.e. segregation index, s, equal to 1). Note that monoculture simulations were repeated using the same seeding rule to prevent any bias from inoculation crowding effects when we are comparing monoculture and coculture growth.

In the absence of metabolic interaction, the two species (here, differing only in colour) tend to segregate, independently of initial intermixing (fig. 3B). This agrees with modelling (Nadell et al. 2010) and empirical work on the social amoeba *Dictyostelium discoideum* (Buttery et al. 2012) showing that spatially structured growth is a passive mechanism that increases relatedness (or lineage segregation). But what happens when the lineages experience metabolic interactions? Our results suggest that the emergent patterns of lineage mixing (fig. 5.2A) are highly robust against variation in initial inoculum mixing, except when the two species are completely segregated in two separate microcolonies at inoculation (fig. 5.3A, fig. S6A-C). Indeed, if the two species are strongly interdependent, they are conditioned to mix to grow. Thus, when initially segregated, such strong initial segregation may delay (fig. S7A, B) or even prevent (e.g. when interdependency is too high; fig. S7C) the structural relationship to be forged. This result also supports the idea that spatial distance between species plays a critical role for the stable coexistence of obligate mutualistic bacterial communities (Kim et al. 2008). We also found that the strongly interdependent community shows a strong signature of negative frequency dependent selection (the rare lineage is favoured), ensuring a stable coexistence frequency of around 34% producers, regardless of their initial frequency (fig. 5.3C). In contrast, the control community is sensitive to the proportion of producers at inoculation (fig. 5.3D), due to the absence of stabilising mechanisms of interaction.

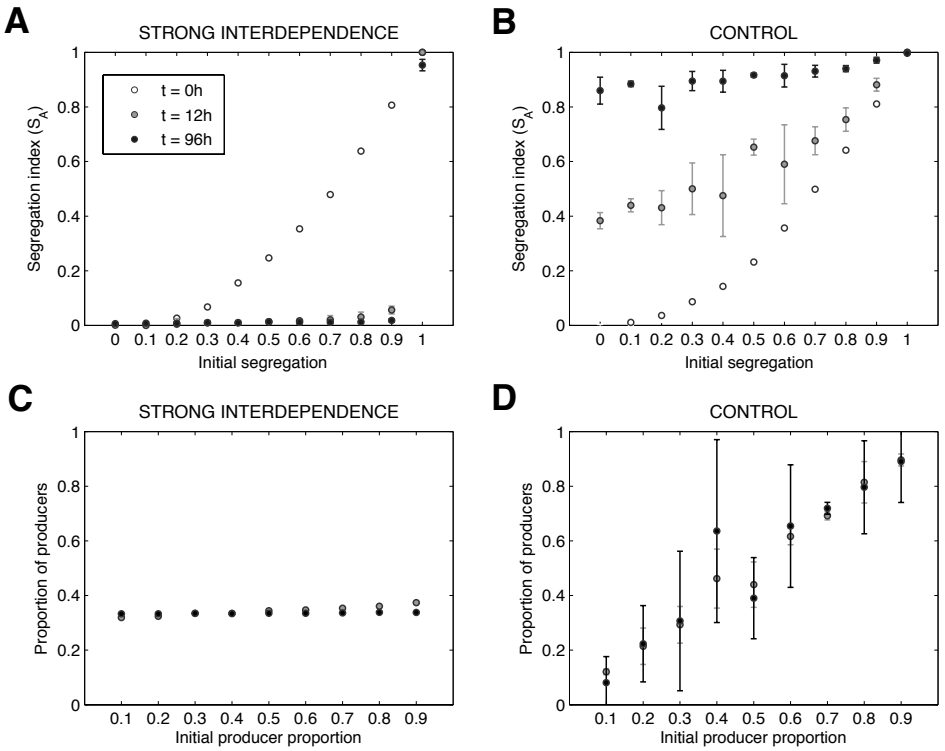

Figure 5.3. Strong interdependence generates communities that are robust to variation in initial conditions. A, B, Emergent population structure (segregation index, s_A,) as a function of initial intermixing, for two scenarios. A, strong interdependence (i.e. obligate cross-feeding and high by-product toxicity) and B, no interdependence (control scenario). Population structure is recorded at inoculation (open circles), and after 12 (grey dots) and 96 (black dots) hours. Initial population structure was varied by varying the proportions of producer (species A) and cross-feeder cells (species B) in two adjacent micro-colonies (of size 30 μm separated by a distance of 70 μm) while maintaining a constant total inoculation density and ratio (1:1). An initial segregation 0 means that each microcolony received equal numbers of A and B, whereas initial segregation of 1 means that one microcolony was pure A and the other pure B. An increment in initial segregation of 0.1 means a 5% increase (or decrease) in the number of cells of species A (or species B) inoculated in each microcolony. **C, D.** Proportion of producers as a function of initial producer proportion for strong interdependence (i.e. obligate cross-feeding and high by-product toxicity) and control scenario, respectively, and after 12 (grey dots) and 96

(black dots) hours growth (initial segregation = 0). Data are the mean of 3 replicates and error bars are the SD of the mean.

Demographic drivers of intermixing

To further understand the demographic drivers of intermixing, we break the demographic feedbacks by modifying both initial segregation conditions and the mass-transfer regime (by-product diffusion). First, we simulated the growth of an initially segregated two-species community and separately tracked the growth rates of cells situated nearer towards or further apart from the heterospecific lineage. We found that when metabolic interdependence is high, the cells that are closer to interspecific cells grow better than the cells that are further away from interspecific cells (fig. S8A). As shown in figure S13, obligate cross-feeder cells closer to producer cells grow towards the producer cells, i.e. towards the by-product. In turn, this reduces the concentration of toxic by-product in the microenvironment of producer cells that are closer to the obligate cross-feeder, thus favouring the growth of those neighbouring producer cells. This result highlights the importance of demographic feedbacks that follow from growth benefits of trading resources for detoxification in shaping community function and genetic structure. At a more macroscale, the results of demographic feedbacks among mutualists are clear in fig. 5.3C, where we see the signature of negative frequency-dependence driving the two partners to a stable coexistence point irrespective of initial frequency, and in fig. 5.4AB where we see an accelerating growth of mutualists with increasing heterospecific proximity and mixing.

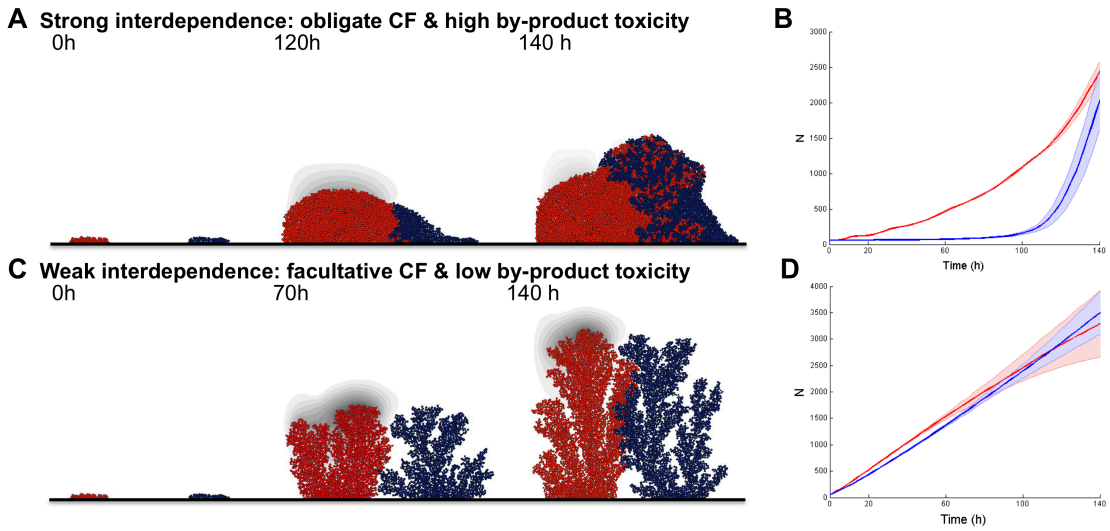

Figure 5.4. Demographic signatures of functional relationships given initial species segregation. A, B. The two species are strongly interdependent. **C, D.** The two species are weakly interdependent. Producer is represented in red and cross-feeder is represented in blue. By-product is in gray. Simulations were initiated with two segregated microcolonies (1:1). Boundaries on the sides are permeable to the by-product and non-cyclic. **B, D**. Time series of species biomass (N). The thick lines represent the mean (n=9) and shaded areas represent the standard deviation.

Furthermore, as observed earlier for intermixed inocula (fig. 5.2B and fig. S11), the community branching structure emerges as the community grows, but the branching pattern is now- with separated inocula- more pronounced, probably because of reduced space constraints (fig. 5.4A, fig. S13). The emergence of similar architectures and intermixing statistics between Figures S13 and S11 (i.e. separate and intermixed inocula, respectively) highlights the robust community developmental programme that results from strong metabolic interdependencies, which in turn deliver a high-functioning community.

Given facultative cross-feeding, the cross-feeder can grow using the shared limiting nutrient (e.g. glucose) as well as the producer by-product. When the by-product is weakly toxic both producer and cross-feeder cells that grow closer to interspecific cells grow better than the cells that are further away (fig. S8B, fig. S14), but the

disadvantage of cells growing further away is now smaller and mixing is reduced. As by-product toxicity increases, producer cells growing closer to the cross-feeder can even grow more slowly than those further away, despite receiving greater detoxification benefits. The producer cells adjacent to cross-feeding cells suffer due to the increased competition for the shared limiting nutrient (fig. S8B). At a more macroscale, the results of demographic feedbacks among weakly interdependent partners (fig. 5.4CD) can be seen by a negative correlation between the densities of producer and cross-feeder across replicates following lineage contact (fig. S9B), as a stochastic advantage to one lineage spells a cost to the competitor lineage (generating the increased variance around the mean in fig. 5.4D). In contrast, strong interdependence generates a positive correlation between producer and cross-feeder across replicates following contact (fig. S9A), as a stochastic advantage to one lineage drives further advantages to its partner lineage.

The ability to effectively carry out a food-for-detoxification exchange depends ultimately on an effective process of molecular transport from producer to consumer cell. In our final manipulation, we vary the rate of diffusion to explore the importance of mass-transfer processes on the establishment and maintenance of metabolic and demographic feedbacks. We found that when the two species are initially spatially segregated, increasing diffusion improves the performance of both species, due to an enhanced metabolic flux kick-starting the exchange (figs. 5.5A, S10A). In contrast, when the two species are initially mixed, performances (lineage growth rates) are scarcely touched by changes in diffusion over two orders of magnitude, as the initial proximity of the partner lineages assures effective inter-cellular transport even at very low rates of diffusion (figs. 5.5B, S10B). The effect of diffusion is however very pronounced on the resulting strength of mutualism. When diffusion is very low, mutualism is far stronger simply because the producers are in much more trouble when alone (fig. 5.5B). In contrast, as diffusion increases, solitary producer colonies suffer less from their byproduct toxicity due to a rapid abiotic removal process, making the net benefit of partnership much weaker (fig. 5.5B). Together, these results illustrate the important and interacting roles played by

initial segregation and diffusion in establishing an effective metabolic exchange, and consequently the emergent function and genetic structure of communities.

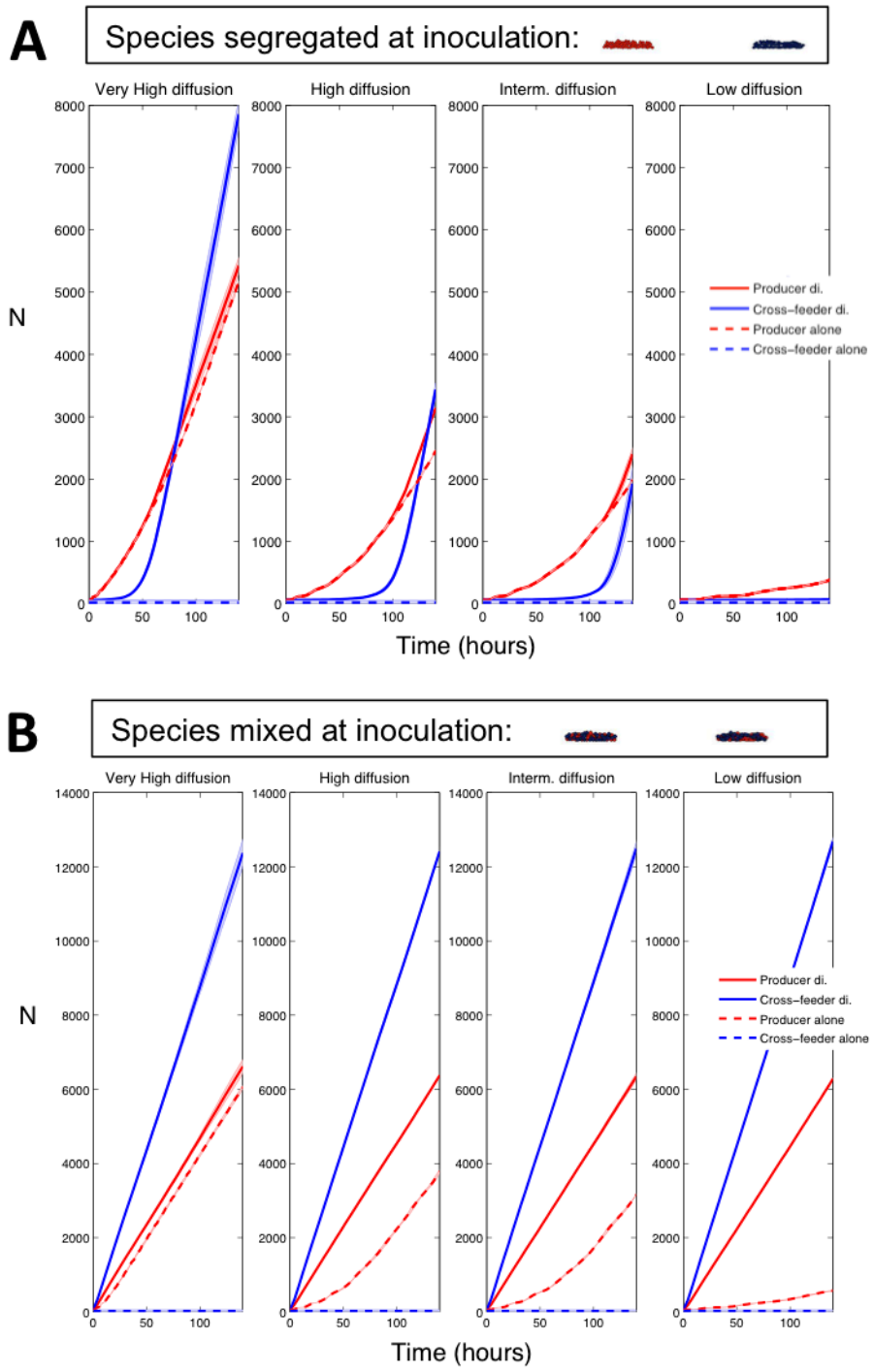

Figure 5.5. Effect of varying diffusion and initial segregation on the emergent properties of strongly interdependent communities. A, The two species are

initially segregated. **B,** The two species are initially mixed. Time series of species biomass (N) when grown in diculture (solid line) or alone (dashed line). The thick lines represent the mean (n=3) and shaded areas represent the standard deviation. See fig. 3 legend for further details on seeding conditions. By-product diffusion rates are [$10D_E$; $1.4D_E$; D_E; $0.14D_E$] from very high to low, respectively (see Table S2).

5.5. Discussion

While it is well acknowledged that spatial structure plays a critical role in shaping the ecological outcome of species interactions, our understanding of how community structure and function emerge from the mechanistic bases of species interactions is still poorly understood. Here, we addressed this question by investigating how metabolic constraints of individual species shape the emergent functional and structural relationships of a two-species microbial community that trades food for detoxification.

Specifically, our main findings reveal that mutual interdependence generates a robust and highly stabilising mixing pattern. This happens because demographic movement towards heterospecifics becomes increasingly rewarding, resulting in branching of lineages towards heterospecifics and away from conspecifics. These demographic feedbacks strengthen mutualistic relationships via increased lineage mixing, and weaken competitive relationships via increased segregation. Furthermore, we show that initial mixing and diffusion play a critical role in establishing effective metabolic exchange, and therefore in defining the emergent functional and structural relationships among species.

Strong metabolic interdependence is commonly found in syntrophic (cross-feeding) relationships (Schink 2002), and empirical evidence for the importance of spatial distribution in the functioning of metabolically interdependent syntrophic consortia is growing in the literature (Pernthaler et al. 2008; Haroon et al. 2013). But, what if mutualism is based on bidirectional cross-feeding rather than a food for detoxification mutualism? Recent work has suggested that strong inter-population

cooperation, in which each strain depends on the provision of an essential metabolite by the other strain, leads to population mixing in a pattern of successive layering (for yeast see (Momeni et al. 2013), for *E. coli* see (Brenner and Arnold 2011)). This discrepancy in spatial pattern between their findings and ours suggests that the nature of the mechanistic interaction (e.g. bidirectional cross-feeding *vs* food for detoxification cross-feeding) may play a critical role in defining the type of emergent spatial pattern occurring between members of the community.

A striking result in our simulations is the emergent two-species branching structure of communities that exhibit strong interdependence (figs S11, S13). Branching patterns are commonly found in nature (e.g. neurons, blood vessels, trees). In bacteria, branching has been observed in swarming colonies, including *Bacillus subtilis* (Julkowska et al. 2004) and *Pseudomonas aeruginosa* (Kohler et al. 2000; Xavier et al. 2011; van Ditmarsch et al. 2013), but what may explain such community architecture here? Branching seems to emerge because of lineage growth with demographic movement away from conspecifics and towards interspecifics (helpers), thereby maximizing the surface contact area with interspecifics. Figure S13 suggests that the first mover is the obligate cross-feeder which branches into regions of high by-product concentrations (high toxicity for producer). This relieves inhibition on the producer, which can now grow until toxicity returns.

Here, we have assumed that the facultative cross-feeder is able to use both the common resource and the by-product independently of their concentrations in the environment. This means that the trade-off between the cross-feeder's ability to use both nutrients is fixed, and not under regulatory control. Regulatory control, however, plays a critical role in bacterial metabolism and social dynamics (Kummerli and Brown 2010; Xavier et al. 2011). One could relax this assumption and allow for regulatory control in our cross-feeding model. For example, common resource vs by-product consumption could be a plastic trait that depends on the local concentration of the by-product. Specifically, one could assume a scenario where the metabolism of the by-product inhibits the uptake of the common resource (Brown and Whiteley 2007). While outside the scope of this study, we believe that

investigating how metabolic plasticity in resource use affects the structure-function dynamics of interspecific interactions would add to our understanding of mapping metabolism to ecology and structure in polymicrobial communities.

Our work looks at interspecific mutualisms that arise due to by-product mutualisms, as the benefit provided to the other species occurs as a result of a trait carrying no immediate, direct cost to the actor (Connor 1995; Sachs et al. 2004). Additionally, our model assumes that cell movement is purely due to demographic processes of cell growth. This means that there is no behavioural mechanism that preferentially directs help towards a mutualistic partner (such as in partner choice, (Sachs et al. 2004; Foster and Wenseleers 2006)) or makes an individual preferentially move towards a mutualistic partner. While it is unclear whether partner choice exists in bacteria, motility (An et al. 2006) and chemotaxis are behavioural mechanisms that allow bacterial cells to move towards favourable environments (e.g. food gradient) and therefore influence species functional and structural relationships. It would be interesting to see how these mechanisms would affect the functioning and structuring of our food for detoxification association. One would nevertheless expect a similar general structural pattern even when behavioural processes are at play, i.e. mix when the benefits of association outweigh the costs, but segregate when the costs of association outweigh the benefits.

Another explicit assumption of our model is that cells are growing on an inert surface and that the nutrient diffuses from the bulk (above) into the biofilm. This implies that only the cells that are at the surface of the biofilm are able to access the nutrient and grow. This is a common assumption when using this individual-based framework, but this may not always be the case as in, for example, the gut environment (see (Schluter and Foster 2012) for an individual-based model of host-microbiota interactions where the authors assume bidirectional nutrient gradient). Under these conditions, and assuming sloughing of microbial cells, different emergent structures and branching patterns may arise.

Our study illustrates how community structural and functional relationships emerge from metabolic signatures of interspecific interaction. Although we focused on a specific mechanism of trade - food for detoxification of a metabolic by-product - we believe that our approach of mapping metabolism into function and spatial organization can be extended to other types of microbial associations. It would be interesting, for instance, to investigate what are the emergent functional and structural relationships of a two-species community trading food for detoxification of an exogenous toxic metabolite (e.g. antibiotic). Also, trading food for detoxification implies that when mutualism emerges, it is intrinsically resistant to interspecific cheating strategies. This conclusion lends greater relevance to our ecological results, however it still leaves open a number of questions on the potential for coevolutionary dynamics within this mutualistic space, for instance towards greater rates of waste production (Estrela et al. 2012).

Finally, we suggest that further research into the interplay between the molecular mechanisms of species interactions and the ensuing population and community dynamics is needed to foster our understanding of how natural microbial communities emerge and are maintained in the first place, as well as predict how they may be affected by environmental perturbations on both ecological and evolutionary timescales (O'Brien et al. 2013).

5.6. Appendix

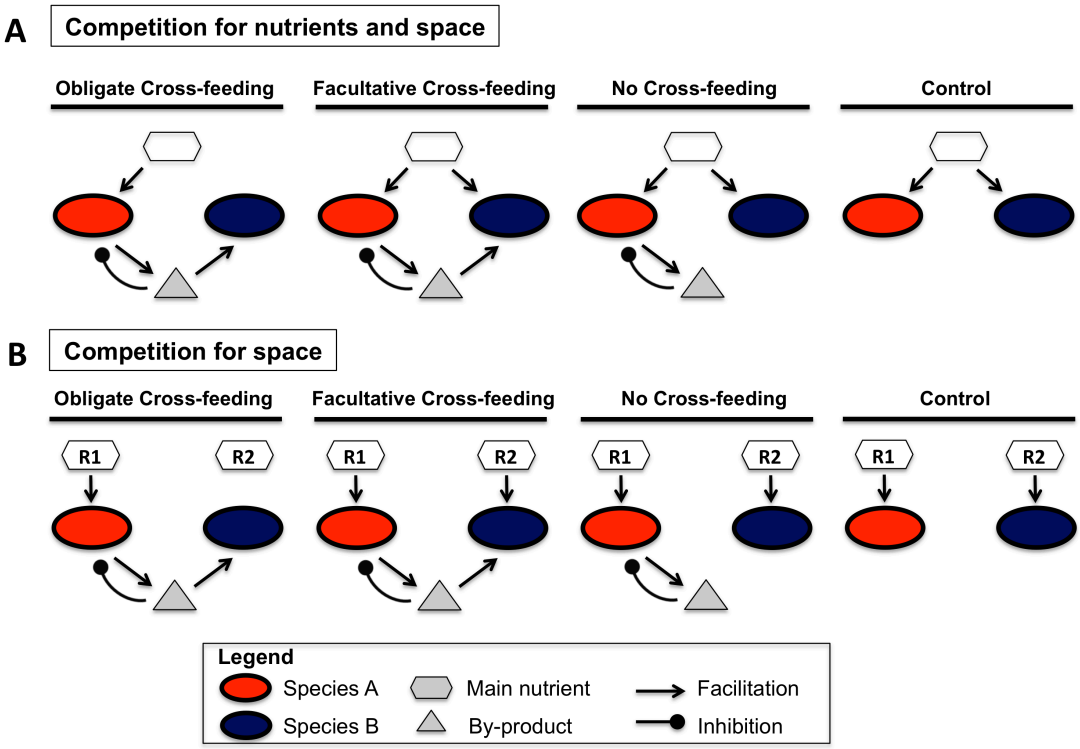

Figure S1. Schematic representation of species interactions.

A, The two species compete for a common nutrient and space. From left to right: Obligate food for detoxification, i.e. no competition for the shared nutrient; Facultative food for detoxification, i.e. the cross-feeder is able to use both by-product and common nutrient; Non cross-feeding medium, i.e. complete overlap in resource use and no cross-feeding; and, control community where both species are identical except for their color (see text for more details). **B,** The two species compete only for space. Oval, hexagon, and triangle, represent bacteria, main nutrient, and by-product, respectively. Open arrows represent a positive effect, whereas oval arrows represent a negative effect upon the population or resource they are pointing toward.

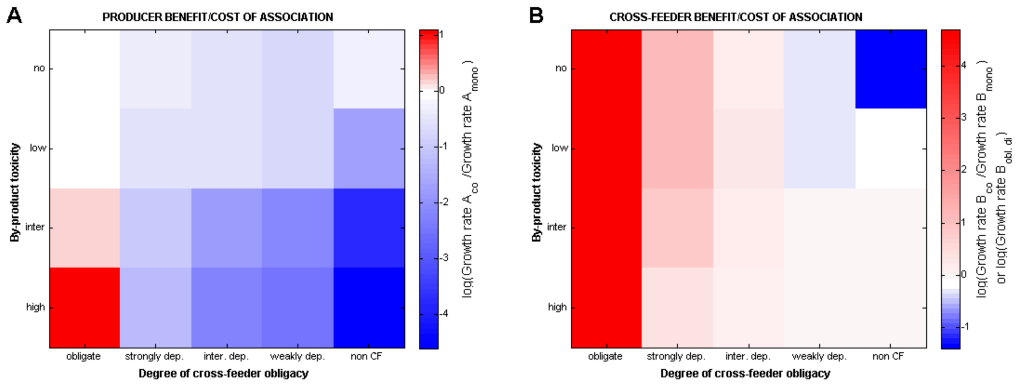

Figure S2. Benefits of association increase with need for help (need for detoxification, and need for food). A, B. Data represent log growth rate of producer (cross-feeder) in coculture relative to producer (cross-feeder) in monoculture for varying by-product toxicity and cross-feeder degree of obligacy. Measured as $\log(X_{co}/X_{mono})$ where X_{co} and X_{mono} represent growth rate in coculture and monoculture, respectively (for growth rate calculation see Methods). To note that obligate cross-feeder growth rate is measured as $\log(B_{co})$ because the obligate cross-feeder cannot grow in monoculture. Positive and negative values indicate a net gain and loss from association, respectively.

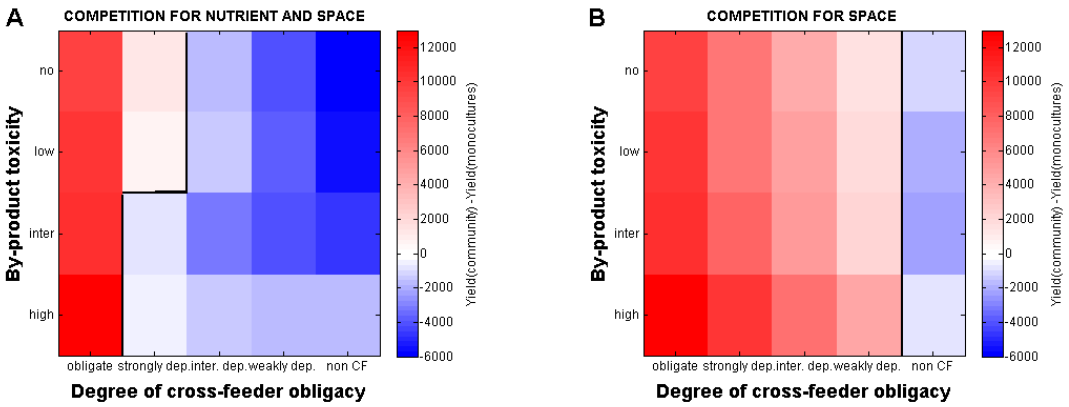

Figure S3. Enhanced community productivity does not itself imply mutualism. **A,** The two species compete for a common nutrient and space. **B,** The two species compete only for space. Indeed, exploitative relationships can also lead to a community gain (see fig. 1). Data represent $(A_{co} + B_{co}) - (A_{mono} + B_{mono})$ and are the mean of 3 replicates. The black line separates the gain (+) and loss (-) regions.

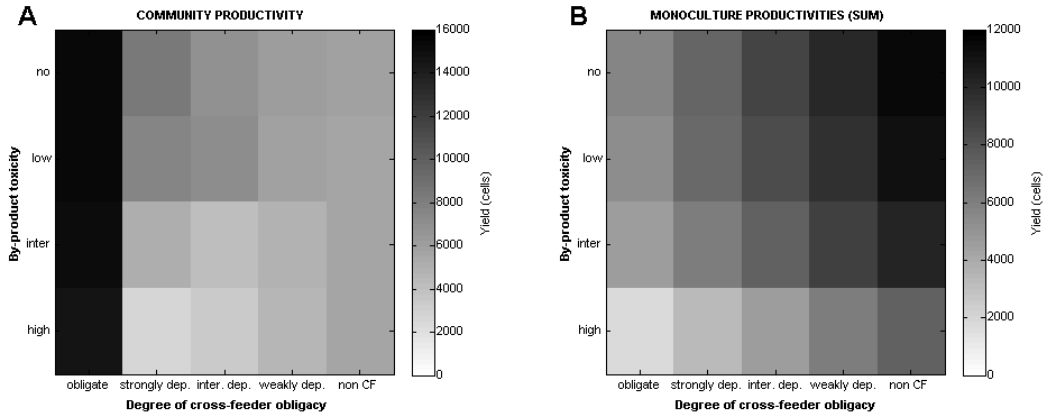

Figure S4. Metabolic interdependence drives community functioning (productivity). A. Productivity of the community (A_{co} + B_{co}), and **B.** sum of monocultures (A_{mono} + B_{mono}) for varying by-product toxicity and degree of cross-feeder obligacy (see Methods for further details). Data are the mean of 3 replicates.

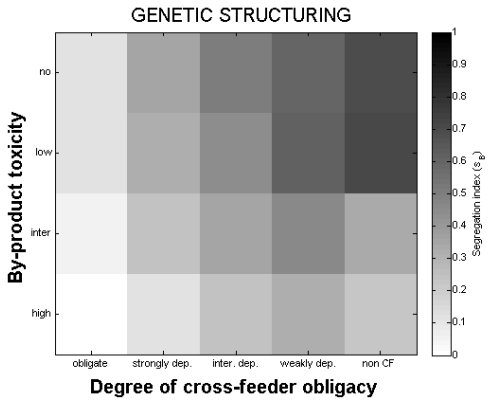

Figure S5. Genetic mixing increases with need for help (need for detoxification, and need for food). Cross-feeder segregation index (s_B) for varying by-product toxicity and degree of cross-feeder obligacy (see Methods section for further details). Lighter regions indicate greater mixing. Data are the mean of 3 replicates.

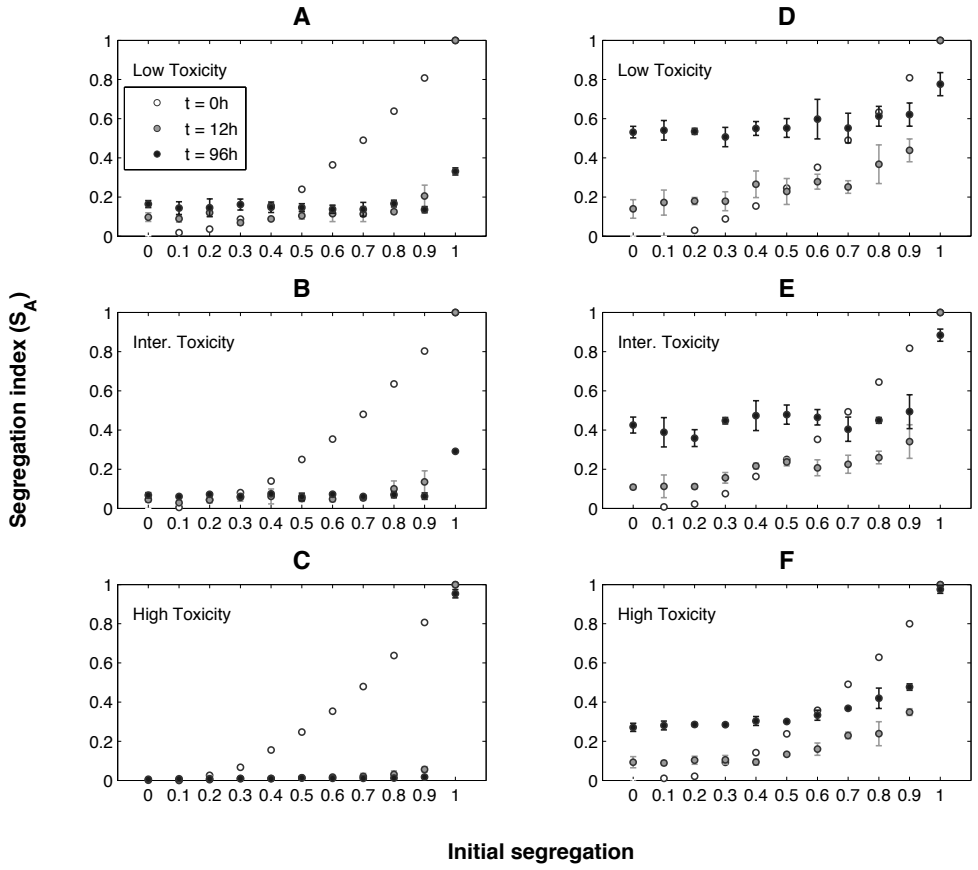

Figure S6. Stronger interdependence generates more robust community intermixing to intermixing at inoculation. A-C. Obligate cross-feeding (A-B_{obl}). **D-F.** Facultative cross-feeding (A-B_{facI} scenario)**.** Two microcolonies of size 30 μm separated by a distance of 70 μm were inoculated with varying proportions of producer and cross-feeder cells but constant inoculation density (1:1). In the x-axis, 0 means that the two microcolonies were inoculated with equal number of cells of species A and B and represents $s \sim 0$, whereas 1 means clonal microcolonies at inoculation, and therefore $s = 1$. An increment of 0.1 means a 5% increase (or decrease) in the number of cells of species A (or species B) inoculated in each microcolony. Data represent producer segregation index at inoculation (white circles), and after 12 and 96 hours growth (grey and black dots, respectively). Data are the mean of 3 replicates and error bars are the SD of the mean. **A, D,** low by-product toxicity**; B, E,** intermediate by-product toxicity; **C, F,** high by-product toxicity.

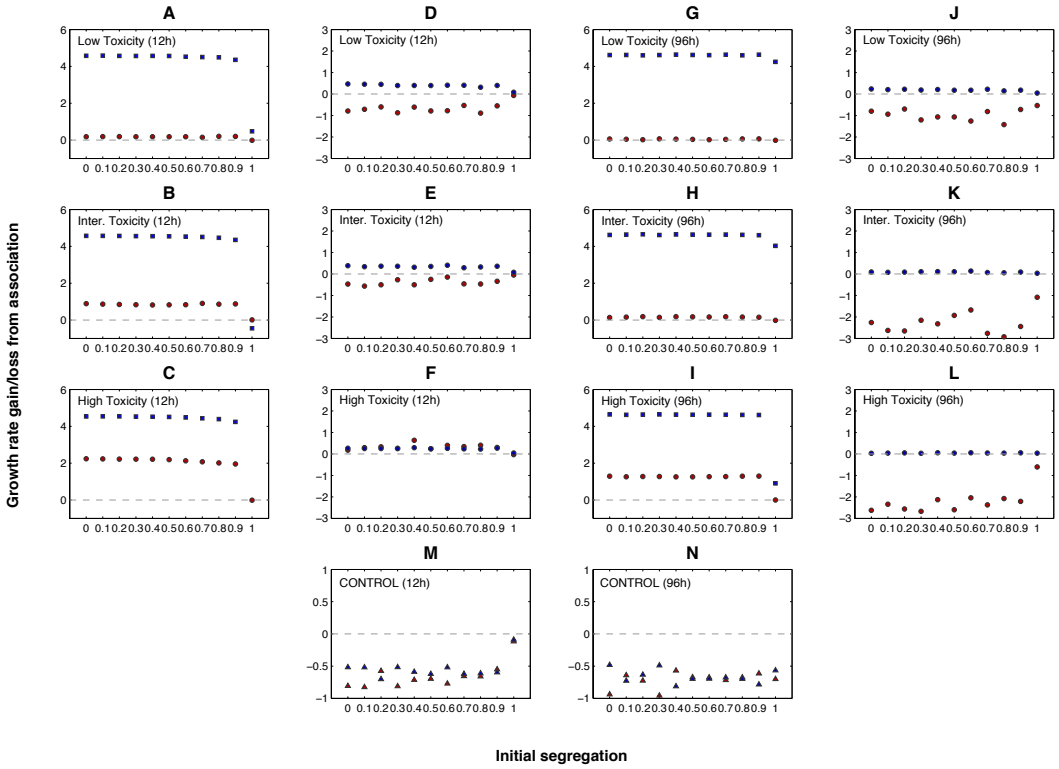

Figure S7. Costs and benefits of association for varying degree of intermixing at inoculation. A-C, G-I. Obligate cross-feeding, after 12h and 96h growth, respectively. **D-F, J-L.** Facultative cross-feeding (A-B_{facI}) after 12h and 96h growth, respectively. **M-N.** Control, after 12h and 96h growth, respectively. Measured as $\log(X_{co} / X_{mono})$ where X_{co} and X_{mono} represent growth rate in coculture and monoculture, respectively (for growth rate calculation see Methods). To note that obligate cross-feeder growth rate is measured as $\log(B_{co})$ because the obligate cross-feeder cannot grow in monoculture. Positive and negative values indicate a net gain and loss from association, respectively. Red dots represent producer, blue squares represent obligate cross-feeder, and blue dots represent facultative cross-feeder. In the control scenario, the two types are identical except for their color, i.e. red-tagged cells or blue-tagged cells. See legend figure 3 for details on inoculation conditions.

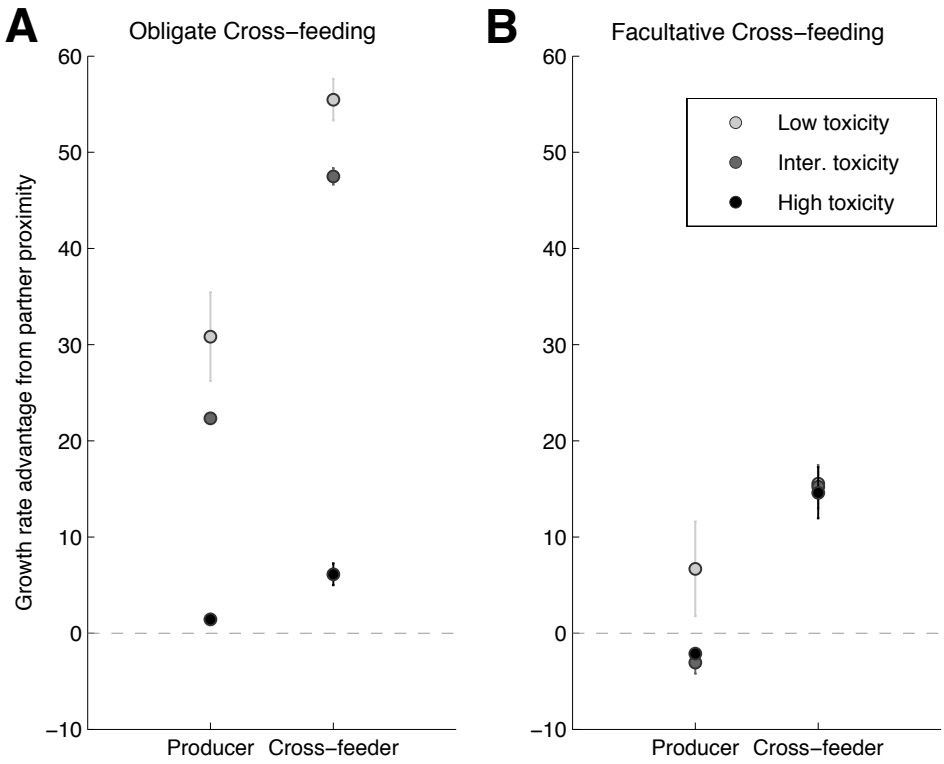

Figure S8. Effect of interspecific partner proximity at seeding. A. Obligate cross-feeding. **B,** Facultative cross-feeding (A-B_{facI}). Growth rate advantage is measured as the difference between the growth rate of a producer (cross-feeder) growing close to a cross-feeder (producer) and the growth rate of a producer (cross-feeder) growing far from a cross-feeder (producer). Thus, positive values mean a growth rate advantage from interspecific partner proximity whereas negative values mean a growth rate disadvantage from interspecific partner proximity. Boundaries on the sides of the domain are permeable to the by-product and non cyclic. Data represent 120 hours growth, are the mean of 3 replicates and error bars are the SD of the mean.

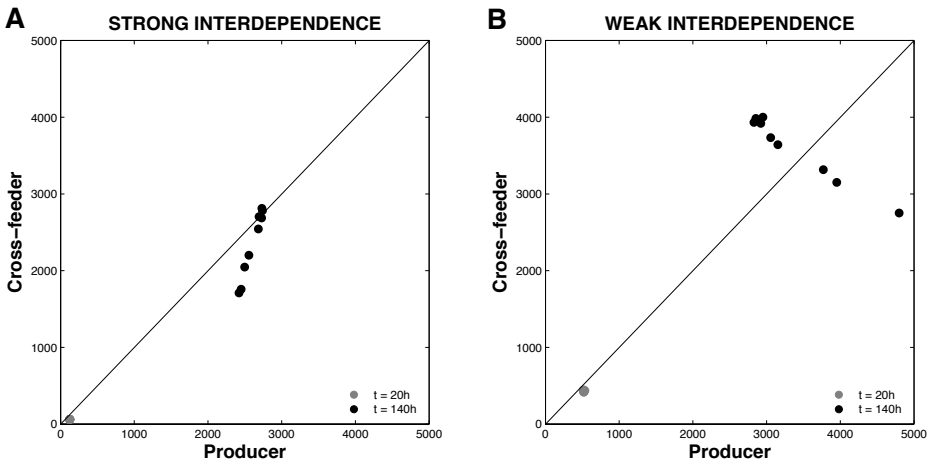

Figure S9. Results of demographic feedbacks on functional relationships. **A.** The results of demographic feedbacks among strongly interdependent partners can be seen by a positive correlation between the densities of producer and cross-feeder across replicates following lineage contact (fig. 4AB). **B**. The results of demographic feedbacks among weakly interdependent partners can be seen by a negative correlation between the densities of producer and cross-feeder across replicates following lineage contact (fig. 4CD). See legend figure 4 for further details.

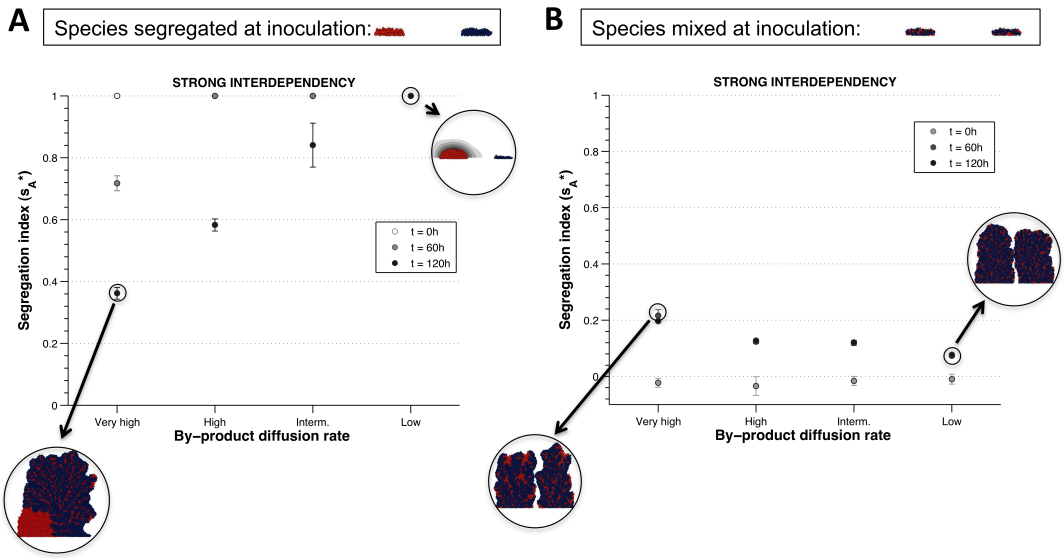

Figure S10. Effect of by-product diffusion rate on strongly interdependent communities given initial segregation A, and, initial mixing B. Producer segregation index (s_A*) was measured for a neighbourhood of 5um (see legend fig.3

and Methods section for further details). Given the strong mixing pattern of strongly interdependent communities, here we decreased the size of the neighbourhood to measure spatial structuring even more locally. By-product diffusion rates are [$10D_E$; $1.4D_E$; D_E; $0.14D_E$] from very high to low, respectively (see Table S2). Data are the mean of 3 replicates.

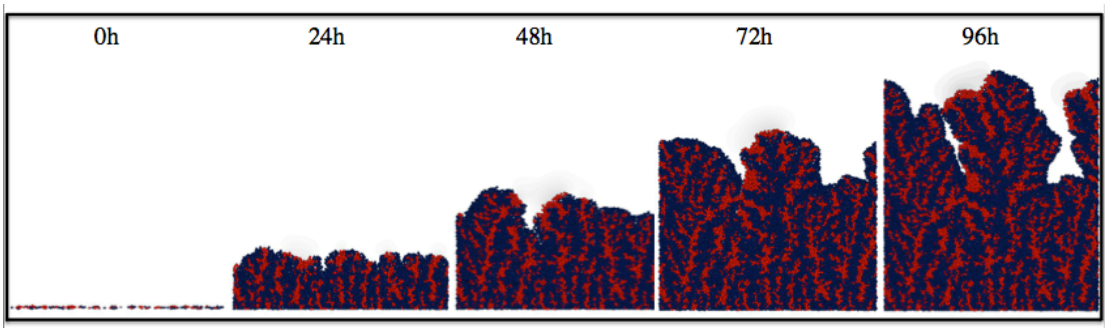

Figure S11. Images from a simulation of the producer-obligate cross-feeder community growth represented in figure 2B. This simulation shows that stronger interdependency leads to greater mixing and illustrates the emergent branching pattern.

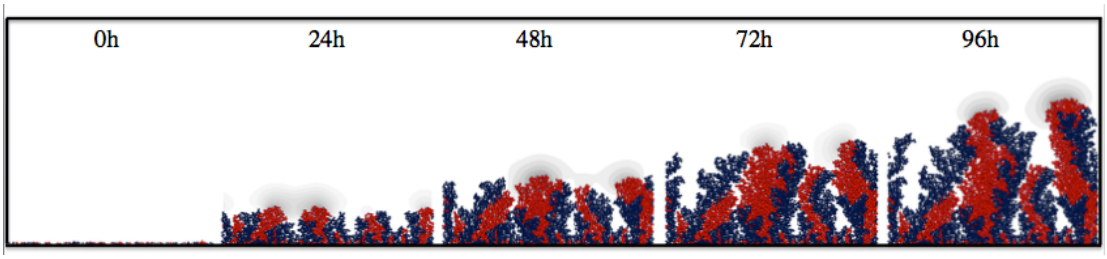

Figure S12. Images from a simulation of the producer-facultative cross-feeder community growth represented in figure 2C. This simulation illustrates that weaker interdependency leads to lower mixing. Some degree of community branching is observed.

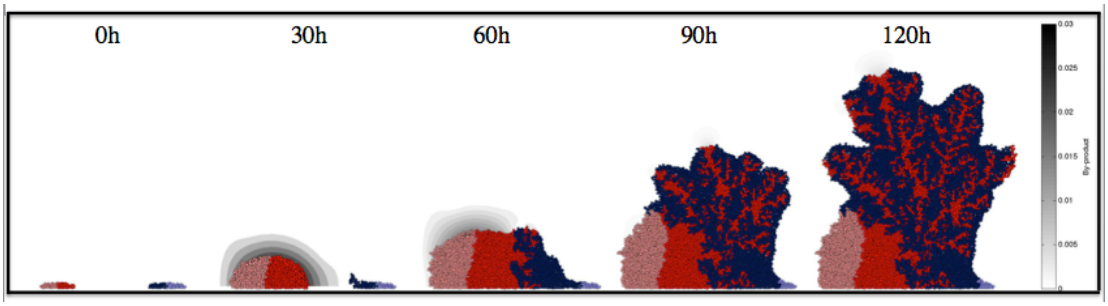

Figure S13. Images from a simulation of the producer-obligate cross-feeder community growth illustrating that when metabolic interdependence is strong the cells that are closer to interspecific cells grow better than the cells that are further away from interspecific cells. Intermediate by-product toxicity scenario. Initial conditions: two clonal microcolonies were seeded 1:1 with either producer (red) or obligate cross-feeder cells (blue). Light red and dark red cells were seeded 1:1 on the left and right side, respectively, of the producer microcolony. Dark blue and light blue cells were seeded 1:1 on the left and right side, respectively, of the cross-feeder microcolony. Boundaries on the sides of the domain are permeable to the by-product and non cyclic.

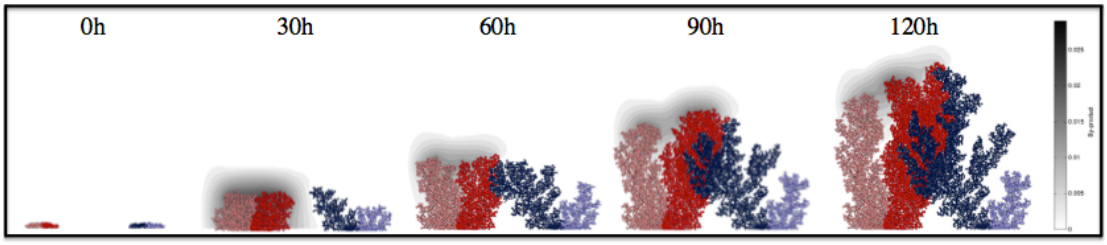

Figure S14. Images from a simulation of the producer-facultative cross-feeder community growth illustrating that the cells that are closer to interspecific cells grow better than the cells that are further away from the interspecific cells. However, given the weaker interdependence the cells growing further away from their interspecific partner grow better than when strongly interdependent. Mixing is thus reduced. Low by-product toxicity scenario. Initial conditions: two clonal microcolonies were seeded 1:1 with either producer (red) or facultative cross-feeder cells (blue). Light red and dark red cells were seeded 1:1 on the left and right side, respectively, of the producer microcolony. Dark blue and light blue cells were seeded 1:1 on the left and

right side, respectively, of the cross-feeder microcolony. Boundaries on the sides of the domain are permeable to the by-product and non cyclic.

Table S1. Reactions and respective stoichiometry of biological processes used in the models

Process	Solute		Biomass		Rate expression
	R	E	X_A	X_B	
Growth of producer, A	-1	$Y_{E,A}$	$Y_{R,A}$	-	$\mu_{\max,A}\frac{R}{K_{R,A}+R}\frac{K_{i,E}}{K_{i,E}+E}X_A$
Growth of cross-feeder, B	-1	-1	-	$Y_{R,B}+Y_{E,B}$	$\mu_{\max,B}\left(\frac{R}{K_{R,B}+R}+\frac{E}{K_E+E}\right)X_B$

Table S2. Model parameters

Symbol	Description	Value	Unit
$\mu_{max, A}$	Maximum cell growth rate of species A	1*	h^{-1}
$\mu_{max, B}$	Maximum cell growth rate of species B	1*	h^{-1}
$Y_{R, A}$	Biomass yield of species A on R	0.5*	$g . g^{-1}$
$Y_{E, A}$	Yield of E produced per R consumed	2	$g . g^{-1}$
$Y_{R, B}$	Biomass yield of species B on R	0.125 (B_{facW}) 0.25 (B_{facI}) 0.375 (B_{facS}) 0.5 (B_{ncf})	$g . g^{-1}$
$Y_{E, B}$	Biomass yield of species B on E	0.125 (B_{facS}) 0.25 (B_{facI}) 0.375 (B_{facW}) 0.5 (B_{obl})	$g . g^{-1}$
$K_{R, A}$	Species A half-saturation constant for R	3.5×10^{-5} *	$g . L^{-1}$
$K_{R, B}$	Species B half-saturation constant for R	3.5×10^{-4}	$g . L^{-1}$
K_E	Species B half-saturation constant for E	3.5×10^{-5} (B_{fac}) 3.5×10^{-6} (B_{obl})	$g . L^{-1}$
$K_{i, E}$	Half-saturation inhibition constant of E on species A	3.5×10^{-2} (low tox.) 3.5×10^{-3} (inter tox.) 3.5×10^{-4} (high tox.)	$g . L^{-1}$
R_{Bulk}	Concentration of R in the bulk	0.125*	$g . L^{-1}$
D_R	R diffusivity	9.6×10^{-7} *	$m^2 . day^{-1}$
D_E	E diffusivity	7.2×10^{-6} *	$m^2 . day^{-1}$

*Values from Mitri et al (2011)

Chapter 6

GENERAL DISCUSSION

The aim of this thesis was to gain insight into the ecological and evolutionary dynamics of microbial interactions, building from explicit metabolic mechanisms of species interactions.

Focusing specifically on cross-feeding, in this thesis I have shown that the evolutionary outcome of cooperative cross-feeding depends strongly on the shape of the trade-off curves between the costs and benefits of cooperation (ch. 2). I have derived new predictions on the physiological mechanisms that may explain the stable coexistence of a cross-feeding polymorphism that evolved from a single clone (ch. 3). I have demonstrated that diverse ecological interactions (competition, mutualism, exploitation) can emerge from a simple cross-feeding interaction (food for detoxification) and can be predicted by the metabolic, demographic, and environmental parameters that govern the balance of the costs and benefits of association (ch. 4). Finally, I showed that metabolic constraints of individual species drive the emergent functional and structural relationships among microbial species within a surface attached community (ch. 5).

These findings suggest that bridging microbial ecology and metabolism is a critical step towards a better understanding of the factors governing the emergence and dynamics of polymicrobial interactions. In the remaining sections I will discuss the potential implications of these findings for managing the health of the human microbiome, and suggest new avenues of research.

6.1. Exploring the human microbiome

Understanding the structure and functioning of polymicrobial communities is of major importance in human health, as witnessed by the key role played by the human microbiome in maintaining health and preventing diseases (Human Microbiome Project (HMP), (Turnbaugh et al. 2007)). Humans carry ten times more microbial cells than human cells. Microbes can be found almost everywhere, living both inside (gut, vagina, respiratory tract,..) and on (skin, mouth,..) the human body. While many of these microbes are beneficial to their hosts (mutualists) (Backhed et al. 2005; Ley et al. 2006; Dethlefsen et al. 2007), some can also harm their host (parasites). But this raises the question of why harm or help your host? The general theoretical framework addressing this question is virulence evolution theory, which in its most common 'trade-off' form states that parasites damage their hosts as an unavoidable side-effect of gaining transmission to the next host (Anderson and May 1982; Alizon et al. 2009). Trade-off models of virulence have met with some success in explaining the exploitation strategies of obligate, specialist parasites such as malaria (Pollitt et al. 2011) or HIV (Fraser et al. 2007). However for many bacterial pathogens their life-histories do not easily conform to the assumptions of an obligately parasitic life history and a specialism on a single host type (Brown et al. 2012), calling into question the applicability of trade-off models based on these assumptions. Here we sketch a more mechanistically grounded approach to the question of how bacterial harm (or help) to the host emerges from metabolic and demographic interactions within bacterial communities. More specifically, we ask - can we predict where host-microbe interactions will fall on the parasitism-mutualism continuum? How will they respond to perturbations (antibiotics, diet)? To what extent can we shape our microbiota and select for beneficial symbionts? These are fascinating and important questions, however, we are still far from fully satisfactory answers. Here I suggest that an integrative approach that links microbial metabolism with ecology and evolutionary theory may contribute to the understanding of the dynamics of microbiome and host-microbiome interactions, and ultimately help in managing human microbiome health.

6.2. The human microbiome: an ecological network of metabolic interactions

Although the beneficial effects of the microbiome on their host have been recognized for a long time (Pasteur 1885), these have been traditionally overshadowed by a strong emphasis on pathogens and infectious diseases. Within the past decade, however, the HMP has greatly contributed to increasing awareness of the critical role played by the human microbiome in both health and disease (Turnbaugh et al. 2007; Huttenhower et al. 2012).

Some of our current understanding of the mutualistic nature of host-microbiome relationships derives from the study of host-microbiota metabolic interactions using gnotobiotic mice models (these are germ-free mice colonized with a defined microbial community) (Backhed et al. 2005; Samuel and Gordon 2006). While the microbiota obtains food and shelter from the host, the microbiota protects the host from pathogens (Wilson 2005; Stecher and Hardt 2008; Kamada et al. 2013) by inducing the production of antimicrobial peptides that kill the pathogens (Raqib et al. 2006), modulating the host immune system (Mazmanian et al. 2005; Mazmanian et al. 2008), winning the competition for nutrients and space with pathogens (Schaible and Kaufmann 2005), or by creating an environment where pathogens cannot grow (e.g. acidic conditions by vaginal microbiome, reviewed in Hickey et al. (2012)). Moreover, another benefit to the host is the degradation of complex dietary compounds that the host is unable to digest, thereby enhancing the host's energy storage (Backhed et al. 2004; Cani and Delzenne 2007; Mahowald et al. 2009). These studies illustrate the importance of nutritional relationships for host-microbiota homeostasis, however, such metabolic exchanges are prone to exploitation by pathogens (reviewed in (Brown et al. 2008; Rohmer et al. 2011)). For example, it has been documented that some pathogens have developed clever strategies to exploit their host by stimulating the host's production of energy resources in which the pathogens are better competitors, and thus promoting a successful infection (Winter et al. 2010; Thiennimitr et al. 2011). Other manipulative parasite strategies act to clear resident commensals by engineering shifts in the host

immune state that favour the invader over the resident strains (Lysenko et al. 2005; Stecher et al. 2007; Brown et al. 2008; Brown et al. 2009; Diard et al. 2013).

Rather than exploiting their host, some pathogens can enhance the success of their colonization by getting help from the resident microbiota (Venturi and da Silva 2012). For example, a study in a murine abscess model showed that metabolic cross-feeding with a common commensal is critical for *Aggregatibacter actinomycetemcomitans* opportunistic co-culture infection (Ramsey et al. 2011).

This interplay between bacterial metabolism and virulence opens new avenues for the development of novel and more sustainable drugs and treatment strategies, but progress is challenged by the intrinsic complexity of the human microbiome.

6.3. The human microbiome in health and disease: from correlation to causation

Human microbiome sequencing projects have revealed intriguing correlations between specific patterns of microbial diversity and multiple aspects of host health, including auto-immune disorders (Round and Mazmanian 2009; Scher and Abramson 2011), diabetes (Qin et al. 2012), obesity (Turnbaugh et al. 2009), and even psychiatric conditions (Foster and McVey Neufeld 2013), but they typically fail to unravel the causal links between human microbiome and disease states (unsurprising given the enormous complexity of the human microbiome). The establishment of microbial causal roles (particularly in obesity) is gathering pace thanks to experimental manipulations of germ-free mice (Ridaura et al. 2013), however the causal mechanisms remain frequently obscure.

A major challenge to unraveling the mechanisms of microbiome functioning is the necessity to combine molecular and ecological approaches to the study of highly complex assemblies of billions of interconnected bacterial individuals. Systems biological approaches are beginning to make important headway by building and

analyzing complex computational models of metabolic interactions within microbial communities (Greenblum et al. 2013), however these approaches make strongly simplifying assumptions on the spatio-temporal dynamics of constituent species, reducing their population biology to a simple 'presence/absence' dichotomy. This simplification (shared by ecological approaches to microbial community assembly (Costello et al. 2012)) allows a mapping of potential metabolic interactions among species, but fails to predict the extent to which any interaction will be realized.

The work presented in this thesis suggests that a simple mechanism of trade can generate a diverse array of ecological relationships, spanning mutualism, competition, and exploitation; and that such diversity can arise by simply changing the properties of the metabolite that is exchanged. Furthermore, I show that it is critical to understand how the presence of one species modulates the growth of the other, and how these coupled demographies together shape the functional and spatial structuring of the community. Based on these findings, I suggest that to develop a complete mechanistic understanding of microbiome functioning, it is vital to understand how metabolic and demographic mechanisms mediate microbiome development, structure and functioning, and what are their implications for control strategies to mitigate disease states (schematic illustration, fig. 6.1).

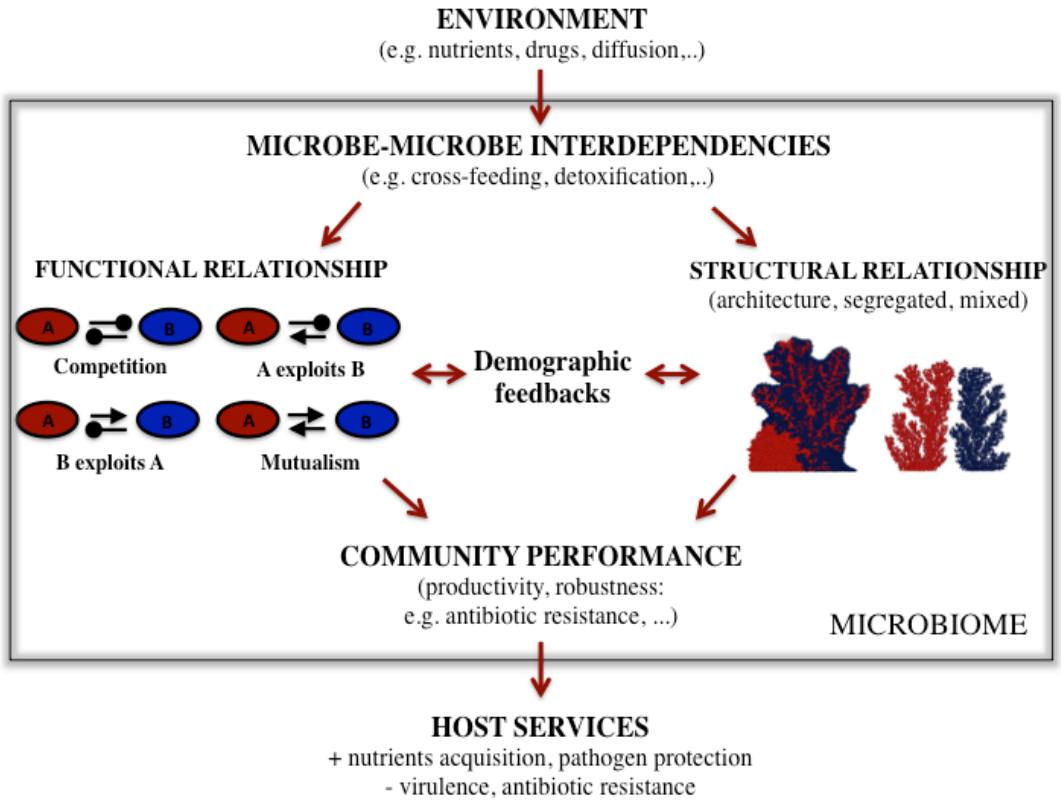

Figure 6.1. Schematic illustrating an integrative approach that aims at bridging the gap between cell and community properties to build an integrated mechanistic account of microbiome functioning.

6.4. Modulating the emergent metabolic-mediated and demographic properties of the microbiome to foster health and prevent disease

My work has focused on the functional and structural relationships among microbial species, but this raises the question of what are the implications for the functional characteristics of the community. In particular, how does this relate to the community ability to perform host services (both positive and negative)? Here I discuss how a better understanding of the emergent metabolic-mediated and demographic properties of microbial communities could be integrated into the broader theme of community-mediated resistance, and hopefully shed light into mechanisms that promote host microbiome health.

Antibiotic resistance and pathogen persistence: dynamics of species interactions

The invasion and persistence of antibiotic-resistant pathogens (e.g. *Clostridium difficile* and *Enterococcus faecium*) after antibiotic treatment is a common problem we are facing today. In a microbiome context, of particular interest is to understand the emergent resistance properties of microbial communities to antibiotic assault and pathogen persistence. The success of these pathogens is generally due to the disruption of commensal bacteria that lack resistance to the antibiotic. This competitive release opens a niche that pathogens can exploit and thus proliferate to reach high densities. A growing concern is therefore to understand how to prevent pathogen release and how to re-establish a healthy microbiome. The results presented in this thesis suggest a link between metabolic interactions and functional relationships, and whose outcome depends on the properties of their shared environment. This raises the question of what are the emergent metabolic-mediated and demographic properties of the microbiome that may promote the success of pathogens after antibiotic disruption of the normal microbiota. Although not addressed here, the framework used may be extended to explore the effect of antibiotic exposure on the dynamics of species interactions. Of particular interest would be to explore the interacting role of antibiotic concentration and initial conditions (i.e. before antibiotic exposure), including species densities, proportions, and mixing, in determining the success of pathogens.

Antibiotic resistance and pathogen persistence: species spatial arrangement

Next, one should ask whether we can manipulate species functional and spatial relationships to influence community function? Supporting evidence comes from a recent study showing the key role played by the combined action of functional and spatial relationships in conferring antibiotic resistance of a two-species microbial community constituted by *Pseudomonas aeruginosa* (*Pa*), a β-lactamase producer and antibiotic resistant, and a susceptible *Staphylococcus aureus* (*Sa*) (Connell et al. 2013). Interestingly, *Pa* protected the susceptible *Sa* from β -lactam antibiotics, and this protection was significantly enhanced due to their pre-defined spatial

arrangement (*Sa* was confined within a shell of *Pa*). Although in this study the species spatial arrangement was imposed *a priori*, this raises the interesting question of whether antibiotic protection could arise via emergent species spatial structuring.

For example, one could focus on a 'minimal' two-species community and antibiotics with a biased efficacy to one or the other species (narrow-spectrum antibiotics) or antibiotics attacking both species (broad-spectrum antibiotics), and ask what is the interplay between community function, spatial structure and community resistance.

Based on my previous findings, we would expect that mutualistic, well-mixed biofilms are more resistant to narrow-spectrum antimicrobial clearance due to partner shading. To illustrate this idea let us consider an antibiotic that preferentially targets the red strain (fig. 6.1). Under competitive interactions the two species segregate (fig. 6.1), rendering the red strain isolated and vulnerable to clearance. In contrast, under mutualistic interactions, the two species interdigitate and thus increase the average distance between the bulk fluid (maximal antimicrobial density) and target cells.

Overall, this strongly suggests that integrating structural and functional relationships into the broader theme of community-mediated resistance could shed light into the mechanisms underlying drug resistance.

Promoting microbiome health by managing polymicrobial interactions

So far I have identified mechanistically-explicit causal pathways from key environmental drivers (nutrients, drugs, flow rates) to the structure and functioning of a two-species 'minimal microbiome'. But now we should ask: how can we effectively manage human microbiome health via manipulation of their nutrient, drug, and mixing parameters? For some microbial communities, the medical priority will be to prevent the establishment of one of the species (as e.g. in the case of the commensal *Streptococcus gordonii (Sg)* and the opportunistic oral pathogen *Aggregatibacter actinomycetemcomitans (Aa)* interactions where the priority is to prevent *Aa* establishment, fig. 1.1), but for other communities, the objective may be

to encourage the growth of specific species or sets of species that are associated with human health (e.g. *Bifidobacteria* (Cani et al. 2007; Cani et al. 2009), or *Bacteroides thetaiotomicron* (Falony et al. 2009)).

Moreover, it is important to recognize the complexity that virulence is often an emergent property of multi-species interactions, in particular mutualistic relationships. In appendix A1, I demonstrated that mutualistic interactions among pathogens (e.g. HIV & malaria) are particularly dangerous due to the compounding effect of within-host demographic feedback (Eswarappa et al. 2012), highlighting the importance of identifying effective levers to reduce mutualistic interdependency involving pathogens. More generally, the existence of strong demographic interactions between species (ch. 4, 5) implies that treatment strategies cannot focus on the behaviour of target species in isolation, but rather on managing the properties of polymicrobial interactions. So this opens the door for exploring potential control strategies that will aim at maximising (when beneficial)/ minimising (when harmful) positive metabolic interactions between species. Let's illustrate this idea with the minimal *Sg-Aa* microbiome model (fig. 1.1). When under anaerobic conditions, *Sg* and *Aa* are engaged in a competitive interaction. *Sg* is a better competitor, so it outcompetes *Aa* and thus protects the host. When under aerobic conditions, *Sg* and *Aa* are engaged in a mutualistic interaction. Although *Sg* is usually a commensal to the host, the harm to the host by *Aa* reduces the host fitness. Thus, here we should aim at minimizing mutualism between *Sg* and *Aa* and therefore reduce *Aa* virulence. Figure 6.2 illustrates this idea for a diversity of potential ecological scenarios occurring between a host and a 'minimal' two-species community.

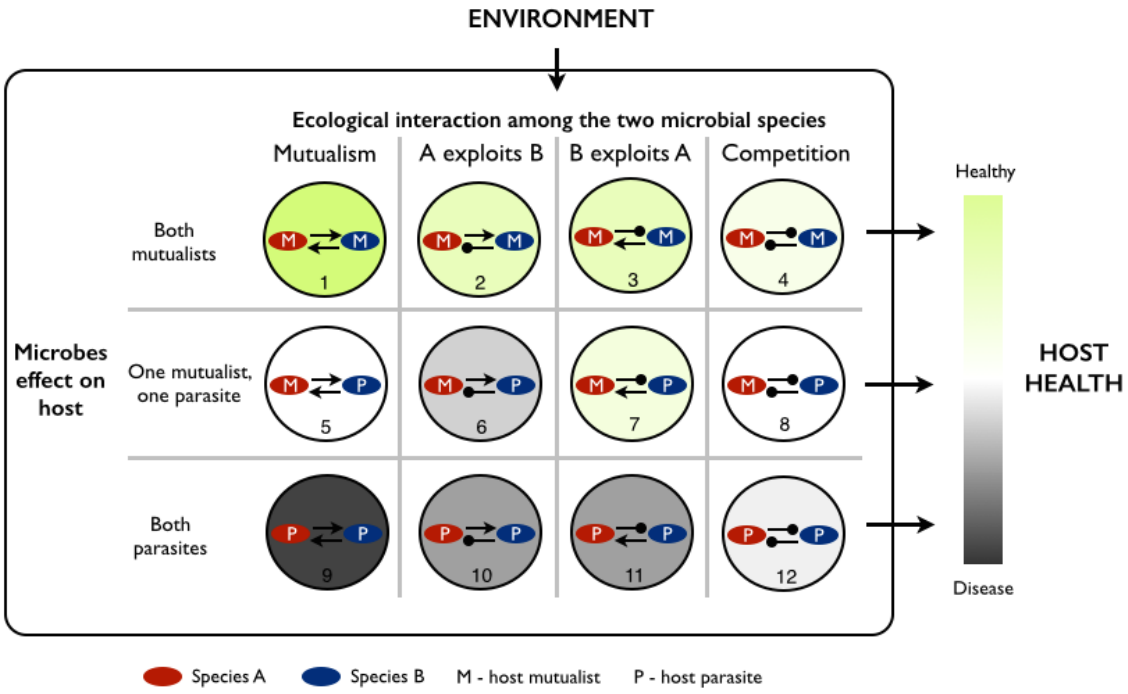

Figure 6.2. Schematic illustrating the diversity of potential ecological scenarios occurring between a host and a 'minimal' two-species community. To promote health and prevent disease, we aim at maximizing mutualism between host mutualists (1) and minimizing mutualism between host parasites (9). However, what we want to maximize crucially depends on the species that are present (i.e. mutualist or parasite) and the nature of their interaction (mutualism/exploitation/competition), which is contingent on their environment. For simplicity, the color shading illustrates potential symbionts effect on host health when assuming symmetric symbionts interaction, but the outcome will depend on the strength and symmetry of the interaction.

More generally, if one aims to develop and apply effective control strategies to promote microbiome health, it is fundamental to understand how species respond to the presence of other species in a given context, and specifically, understand the mechanisms that favor a shift along the parasitism-mutualism continuum. My findings suggest that shifts between mutualism and parasitism are likely to occur by changes in the properties of the biotic (e.g. initial densities and proportions) and abiotic environment (e.g. diffusion and decay rate). In a microbiome context, in addition to interacting with other microbial species, microbes also interact with their host. This raises therefore many interesting questions, such as what is the effect of

the host in shaping these interactions, and, how does the host manipulate its own microbiota and select for beneficial symbionts while against harmful symbionts? Despite the recent efforts in developing models that integrate interactions between a host and its microbiome (Schluter and Foster, 2012), such models are still scarce. Future work is therefore needed to develop predictive models of host-microbiome.

Finally, the work presented here has mostly focused on a minimal two-species community. While this imposes limitations on what can be understood for more diverse communities, a two-species community already gives rise to a diverse and complex network of potential ecological scenarios. Furthermore, studying the role of metabolic and demographic feedbacks in shaping the dynamics of spatially-structured microbial communities using mathematical models is challenging, in part due to the computational challenge of studying mechanistically-explicit models over space and time. But microbes are intrinsically leaky (i.e. excrete metabolic by-products) and the microbial world is inevitably explicitly spatial, so I believe that a mechanistic understanding of a two-species spatially-explicit community offers crucial insights into the dynamics of more diverse and complex communities, including the human microbiome.

6.5. Concluding remarks: challenges and opportunities for progress

A major challenge to answering these outstanding questions is the ability to link the scales from molecules to cells and communities. But this is an exciting time to work at this interface, as new technologies emerge that allow us to unravel the spatio-temporal dynamics of microbial communities (Wessel et al. 2013). For example, it is now possible to confine microbial cells in lobster traps to study their social behaviour (Connell et al. 2010), visualize single microbial cells in space and in real time (Confocal laser scanning microscopy, Fluorescence in situ hybridization), as well as spatially map the molecular environment of microbial communities (e.g. Scanning Electrochemical Microscopy (Koley et al. 2011; Liu et al. 2011), nano-scale secondary-ion mass spectrometry (Dekas et al. 2009)).

Coupling these experimental techniques with spatially explicit individual-based models of microbe-microbe interactions or host-microbe interactions is a challenging but promising research direction. But, in a time where multi-omics approaches can produce a vast amount of data at an unprecedented pace, integrating experiments with theory can be essential to effectively exploit and interpret this information.

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