Chapter 5
/Human cultures and microbial ecosystems

"For in the eighteenth century there was nothing to hinder bacteria busy at decomposition, and so there was no human activity, either constructive or destructive, no manifestation of germinating or decaying life that was not accompanied by stench."

-Patrick Süskind, *Das Parfum*

Background

Cheese is an everyday artifact of microbial artistry. Cheesemaking cultivates complex cultures of bacteria and fungi to protect milk from dangerous bacterial spoilage, a practice that developed long before bacteria had been discovered. In a biological world completely surrounded by rich communities of microorganisms, but a cultural world that often emphasizes antisepsis, cheeses and other microbe-rich foods lie at the heart of a post-Pasteurian debate over the positive impact of microbes on our health and happiness[1].

With rising antibacterial resistance and appreciation for how bacteria maintain our digestive[2] and immune[3] health, attempting to strike a balance between cultivating helpful bacteria and keeping dangerous bacterial infections at bay is more important than ever. Biotechnology and synthetic biology will likely play a role in developing healthy bacterial communities, with designer bacterial ecosystems engineered to improve human and environmental health. However, before we can have the domesticated biotechnology that scientists like Freeman Dyson predict[4], we must first re-domesticate the microbes that have evolved with us over many thousands of years.

Cheesemaking, microbial ecosystems, and biotechnology each present examples of complex mixed cultures. All bring diverse groups of lifeforms together into intricate ecologies of competition and collaboration, impacting our culture and our environment. Heather Paxson, an anthropologist who studies the microbial politics of artisanal

cheesemaking writes on the interactions between human cultures and microbes, "To speak doubly of cheese cultures—bacterial and human—is thus no idle pun."[1] What can these different cultures offer each other? Can scientists and biological engineers learn from human cultures as readily as they do from microbial cultures? Indeed, can those oft-battling "two cultures"[5] of the arts and sciences work together through something as simple as cheese to ease the friction at the interface of human and bacterial cultures?

Cheesemaking

Cheese begins when *Lactobacillus* bacteria naturally present in raw milk or added as a starter culture break down lactose into high concentrations of lactic acid. The low pH curdles the milk, separating the liquid whey from the curds made of the milk's fat and protein. Rennet, an enzyme mixture found in the stomach lining of young veal and certain types of mold, is added to further break down the milk proteins, hardening the curds so that they can be pressed, washed, aged, and processed.

Different cheeses are distinguished by the source and quality of the milk (cow, sheep, goat; grass-fed, raw, low-fat) and how they are aged and processed, but they can also be separated by the microbes involved in their production. Swiss cheese's characteristic holes come from carbon dioxide produced by the bacteria *Propionibacterium freudenreichii* rather than *L. lactis*, while different species of fungus

from the genus *Penicillium* give us not only the antibiotic penicillin, but also provide blue cheese with its stinky blueness and brie cheese its soft white rind.

Most cheese rinds don't just harbor single species, however, but complex biofilms, communities of bacteria and fungi that deposit themselves on the cheese surface and grow in dense layers as the cheese ages in dark, humid caves. Cheeses are washed, brined, and stored in different ways to cultivate unique microbial communities and thus unique cheese flavors.

Microbial communities

In these and other microbial communities we see most clearly that no microbe is an island. Bacteria and fungi in the cheese rind communicate with each other and share nutrients in intricate ways that we are only beginning to understand. Beyond cheese, every surface around us—the soil, air, and water, and even our own bodies—is host to complex ecosystems of interacting microbes. Cheese rind offers a simplified system in which to study complex microbial interactions, a model that Rachel Dutton, a Bauer Fellow at the Harvard FAS Center for Systems Biology, is using in order to uncover details of how microorganisms cooperate in nature. With only tens of species working together instead of the hundreds or thousands that could be present in more complex environments, cheese rind offers us the ability to combine different scales of

experimental understanding. Large-scale gene sequencing can be mixed with more classical microbiological experiments on one or more bacterial species working in isolated cultures, providing a deeper understanding of the biology of microbial communities.

Studying bacteria in pure cultures has allowed microbiologists to untangle thousands of the chemical reactions that make up the cell's metabolism over many decades. Despite exhaustive study, however, almost one third of the more than 4000 genes in the *E. coli* genome still have unknown functions[6]. Many of these genes seem to be entirely unnecessary to the cells growing in isolated laboratory conditions—single deletions from the *E. coli* genome has no effect on how well the cells can grow in rich media[7]. It's likely that many of these seemingly unnecessary genes are actually used by the bacteria in their more natural context, surrounded by, competing, and communicating with hundreds of other microbial strains and species[8].

99% of these other strains can't be isolated and grown in culture at all: they need the dynamic microbial environment to live, making it hard to understand how they are individually contributing to the microbial ecosystem. Metagenomic sequencing of the microbes present in places as diverse as the Sargasso Sea[9] to the human elbow crease[10] has allowed us to identify much of the microbial diversity in these environments but many ecological details are missing. Projects from systems biology

like Dutton's analysis of cheese can add a valuable layer of complexity to what we can learn from sequencing alone.

Synthesizing mixed cultures

Synthetic biology too can address the complexities of how microbes work together in mixed cultures. "Synthetic" can refer to things that aren't found in nature but also can refer to how those things are made; *synthesis* brings two or more things together to make something new. Synthetic biology experiments putting cells or cell components together in a new biological context can provide us with clues about how biological systems work in nature or provide tools for new biological experiments. Wendell Lim and Michael Elowitz address this new frontier for the study of biology in "Build life to understand it" a recent commentary in *Nature*[11]:

> Conventionally, biologists have sought to understand life as it exists. Increasingly, however, from stem-cell reprogramming to microbial factories, researchers are both describing what is and exploring what could be. An analogous shift occurred in physics and chemistry, especially in the nineteenth century. Like biology, these fields once focused on explaining observed natural processes or material, such as planetary motion or 'organic' molecules. Now they study physical and chemical principles that govern what can or cannot be, in natural and artificial systems, such as semiconductors and synthetic organic molecules.

Systems and synthetic biology can thus inform each other, developing stronger models of complex biological systems as part of the design cycle[12]. When probing microbial cooperation, mathematical models from systems biology can be used to

understand how synthetic mixtures of different strains of bacteria can share metabolites in a harsh environment, providing experimental data that can strengthen the foundational models[13]. By bringing together different engineered strains and even different species we can explore how microbes communicate in nature and create new functions impossible for species working alone[14].

Moreover, as a relatively new field that combines the efforts of engineers and biologists, synthetic biology itself shows how complex mixtures of academic cultures can potentially lead to something larger than the sum of its parts. Like different bacterial species that each contribute a unique ability to the function of the community, each researcher brings their own viewpoint, their own approach to the development of the field. These different viewpoints make synthetic biology what it is, but can also lead to "culture clashes" as different groups learn to communicate and work together. Lim and Elowitz describe such clashes in their article:

> Although traditional disciplinary boundaries are dissolving, the cultural differences between scientists and engineers remain strong. For biologists, genetic modification is a tool to understand natural systems, not an end in itself. Thus, making biological systems 'engineerable' — a goal of engineers in the field of synthetic biology — can seem pointless. Many biologists wonder why engineers fail to appreciate the intricate, beautiful and sophisticated designs that occur naturally. Engineers are often equally perplexed by biologists. Why are they so obsessed about the details of one particular system? Why don't they appreciate the value of replacing a complex and idiosyncratic system with a simpler, more modular and more predictable alternative? These misunderstandings can make for fascinating conversations, but they can also prevent mutually beneficial synergies.

Here too we can learn from microbial communities, where competition plays an important role. Spirited debates on what "counts" as synthetic biology and what research will be most valuable can be useful for distinguishing the new field and developing a strong research program, only as long as we don't let such debates distract from positive work being done by members of the group. Often these debates also highlight how difficult it is to separate out individual strands from a complex community. It's not just engineers on one side and biologists on the other but rather all sorts of blurred in-betweens—in between science and technology, pure and applied research, organic and electronic. C.P. Snow warns us in *The Two Cultures and the Scientific Revolution* "The number 2 is a very dangerous number: that is why the dialectic is a dangerous process. Attempts to divide anything into two ought to be regarded with much suspicion."[5] Splitting a complex issue into just two opposing and independent factions can be as dangerous and limiting as a biological monoculture. By encouraging debate and collaboration from many sides and intermediate interests we can build stronger communities.

Indeed, engineers and biologists aren't the only people with strong and complicated interests in the future of synthetic biology. With the recent publication of the President's Bioethics Commission report on synthetic biology, the future of this new field and its technological, economic, political, social implications are being discussed from many points of view. How does synthetic biology fit into that

suspiciously binary split of science and culture? How can we incorporate other people's voices and concerns into new scientific and technological developments? How can we design a positive and just synthetic biology for the future?

Synthetic Aesthetics

I had the tremendous opportunity to work explicitly in-between the two cultures of art and science in order to address some of these questions about future of synthetic biology. As a Synthetic Aesthetics resident I spent a month learning and working with artists, designers, and social scientists, trying to find a common ground from which to build a better synthetic biology.

Synthetic Aesthetics residencies pair synthetic biologists with artists for a month of work in the lab and the studio, and I had the pleasure of working with Sissel Tolaas, someone who describes herself not as an artist, but as a "professional in-betweener." Her work on smell, how we communicate about and through odors is as much chemistry as it is art. She combines a powerful ability to identify smells with specialized knowledge of what combination of molecules will exactly replicate that particular complex scent. Together in our individual lab spaces we explored the ways that we both isolate and recreate the natural world through biological or chemical means, our methods, our goals, our intentions.

In cheese we found a perfect "model organism." Stinky and full of bacteria, cheese has a lot to offer someone who studies difficult smells and someone who studies bacteria. Cheesemaking is itself a culture/science hybrid, an art form forged out of biological materials, creating a cultural object treasured by culinary cultures through millennia.

As a scientific "model organism," not only does cheese provide a simplified microbial community in which to study bacterial interactions, but cheeses closely resemble a simplified human microbiome. Milk curdling lactic acid bacteria are common on the insides of humans, in the mammalian gut and in raw milk, while the rinds of many stinky cheeses are washed with salt water during aging to cultivate microbes suited to the salty and moist environment of human skin. Descriptions of human body odors often overlap with those of cheese[15]; *Propionibacterium* used to make Swiss cheese is a major contributor to the smell of the human armpit[16] and Limburger cheese offers a remarkably close substitute for the smell of human feet, an attractant for certain species of mosquito[17].

We were fascinated by the similarities between cheese and human microbiodiversity and curious about the historic origin of cheese microflora. Given the physicality of cheesemaking (figure 5.3), we speculated on the human origins of many of the unique cheese flavors. To explore this hypothesis and to foreground the microbiology of our food and bodies, we sought out to make cheeses with starter

cultures isolated from the human body. Swabs from hands, feet, noses, and armpits were inoculated into fresh, pasteurized, organic whole milk (figure 5.4) and incubated overnight at 37° Celsius. The milk curds were then strained and pressed, yelding unique smelling fresh cheeses (figure 5.5). Eight cheeses were produced in total for further study, with bacterial origins from the bodies of the Synthetic Aesthetics team.

The odor and flavor of different cheeses emerges from a complex interaction of the proteins and enzymes in milk, the metabolism of the starter culture bacteria, and the population of bacteria and fungi involved in the ripening[18]. In modern industrial cheese production these species are often added intentionally as carefully balanced starter cultures to pasteurized milk, but historical and artisanal cheese production allow(ed) for a wide range of species, milk quality, and flavors[19]. Variety in cheese production emerged as a result of geographic distinction, with different regions producing their own "authentic" cheese[20], linking unique combinations of the *terroir* of the milk, the craft of the cheese production, and bacterial populations that remains powerful even in the face of industrialized food production[1].

Our cheeses are no different, and though they were made from the same milk using the same processes, varied widely in texture, color, and odor due to their different microbial sources. The cheeses were at once artistic and scientific objects, challenging the observer to confront the microbiological aspects of their food and their body, while offering a unique medium in which to study the interactions of microbes and the

volatile compounds that they produce. We thus analyzed the cheeses from both artistic and scientific points of view, breaking down the microbial populations and the odor profiles of each cheese. Volunteer human noses gave detailed descriptions of the cheeses' smells (table 5.1) and the smells of bacterial cultures isolated from each cheese (table 5.2). To achieve a wide descriptive range of odors we solicited opinions from profession odor artists, cheese store employees, and colleagues from both art and science (n=15).

Source	*Bacteria Isolated*	*Odors*
Hand-1	*Providencia vermicola* *Morganella morganii* *Proteus mirabilis*	yeast, ocean salt, sour old cheese, feet
Foot-1	*Providencia vermicola* *Morganella morganii* *Proteus mirabilis*	sweat, big toe nail, cat feet, sweet, milky, orange juice in the fridge too long, fungus, buttery cheese, soapy, light perfume
Armpit-1	*Providencia vermicola* *Morganella morganii* *Proteus mirabilis*	Feta cheese, turkish shop, nutty, fruity, fishy
Nose-2	*Providencia vermicola* *Morganella morganii* *Proteus mirabilis*	cheesy feet, cow, cheese factory, old subway station, toilet cleaner
Armpit-2	*Enterococcus faecalis* *Hafnia alvei*	neutral, perfumed, industrial, synthetic, fermentation, car pollution, burning, sharp, chemical
Armpit-3	*Micobacterium lactium* *Enterococcus faecalis* *Bacillus pumilus* *Bacillus clausii*	neutral, sour, floral, smooth, yogurt
Foot-5	*Providencia vermicola* *Proteus mirabilis*	yeast, jam, feet, putrid, sour, rotten
Armpit-4	*Enterococcus faecalis*	yogurt, sour, fresh cream, butter, whey

Cheese smellomics

We analyzed the bacterial community thriving in the cheeses with 16S ribosomal RNA sequencing (table 5.1). Samples of each of the eight final cheeses were streaked onto LB plates and different species were colony purified. Genomic DNA was isolated using the Qiagen DNEasy kit and 16S RNA was PCR amplified with universal primers (forward: 5' GGT TAC CTT GTT ACG ACT T 3', reverse: 5'-AGA GTT TGA TCC TGG CTC AG-3'). Approximately 1.3 kilobase fragments were gel purified and sequenced to identify species origin. We identified several species of bacteria: *Providencia vermicola*, *Morganella morganii*, *Proteus mirabilis*, *Enterococcus faecalis* *Hafnia alvei*, *Micobacterium lactium*, *Bacillus pumilus*, and *Bacillus clausii*. Many of the identified species have been found in metagenomic sequencing of isolates from the human body, as well as in standard cheeses (table 5.2). In particular, *Proteus vulgaris*, closely related to *P. mirabilis*, is found on cheeses and noted for its strong aroma, *E. faecalis* is a lactic acid bacteria commonly found in raw milk and cheese, and *H. alvei* is an enterobacteria common in cheese and added as a secondary culture in certain artisanal cheeses for a cauliflower-like flavor. *B. pumilus* has been identified in cheese spoilage, as has *M. lactium*, an actinobacterium that is also commonly found on unspoiled cheese and closely related to bacteria commonly found on washed rind cheeses (Rachel Dutton, personal communication). The cross-over between bacteria found offering a tantalizing

hint at how our bacterial symbionts have come to be part of our culinary cultures, how bacterial and human cultures co-evolve.

Of the bacteria isolated from the cheeses, *P. mirabilis* had the most powerful unpleasant odor (table 5.2). Cheeses that did not contain *P. mirabilis* were identified as the nicest and most neutral smelling, all of which were of armpit origin. The diversity we observed in isolated colonies, however, was not sufficient to explain the difference in the smells between cheeses. Several of the cheeses had identical bacterial populations but profoundly different odors. Many species that are present on the human body will not be able to grow in the milk and cheese, and furthermore, many of the species that can grow together in the milk will not grow in isolated colonies on LB agar, creating a bottleneck in the identification of the microbial diversity. Deeper sequencing from whole genomic DNA isolated from the cheeses themselves will likely identify much of the missing diversity from these preliminary experiments.

Before the advent of facile gene sequencing technology, identification of microbial species was challenging, and impossible for the vast majority of unculturable microbes. In the 1960s there was significant effort made to identify bacterial species by the identify of the volatile compounds they produced, in particular as a diagnostic of bacterial infection[21].While many of the volatile compounds identified in GC/MS headspace analyses are common to multiple species, there are often unique traces that allow for the identification of individual species based on scent[22,23]. While the clinical

value has diminished, the importance of bacterial volatile in flavor production in wine and cheese production, there has been significant work in the identification [24] and cataloguing[25] of bacterial odors[26].

GC/MS headspace analysis was performed on four of our cheeses by collaborators at International Flavors and Fragrances to identify the chemical composition of the cheese volatiles (Figure 5.6, GC traces provided as Appendix A figure S5 and identified compounds are indexed in table S2). This "electronic nose" technology is practiced by perfumers, flavorists, and food scientists to decompose, identify, and recreate naturally occurring odors. Tolaas's work in recreating smells of cities, spaces, and bodies, employs a mixture of this technical identification of odorants and biological identification and subjective descriptions by people with trained and untrained noses. As in other synthetic disciplines, the recreation of complex odors and comparison between the real odorant and the synthetic model[27] will verify the accuracy and strength of the modeling technology. Synthetic chemical models of the odor of Swiss cheese verify the identification of a handful of compounds that make up the primary odor and flavor of Swiss cheese[28].

Many of the compounds that specifically contribute to the odor of different cheeses has been analyzed using gas chromatography headspace technology[29] (extensively reviewed in Urbach[18]). Fresh cheese odors include strong doses of diacetyl and acetaldehyde[18], while several ketones and alcohols contribute to the smell of different

types of mature cheeses[29]. Several such compounds appeared in our volatile headspace analysis, with the most pleasant and cheese-like smelling cheese, Armpit-3, containing

Bacteria	*Appearance*	*Odors*	*Also found*
Providencia vermicola	Shiny white colonies	sharp, vinegar, chlorine, swimming pool, sweet, floral, tulip	Gastrointestinal Tract
Morganella morganii	Shiny yellow colonies	*E. coli*, pungent, rotting fish, dog breath, barn, monkey house at the zoo	Skin, Airways, predatory ground beetle digestive tract, wallaby cloaca, frog skin, pea aphid, metal working fluids and aerosols, histamine production in cheese
Proteus mirabilis/ vulgaris	Fast-moving biofilm that creates bullseye appearance when spread over petri dish.	Putrid, foul,	Urogenital tract, skin, airways, swine manure, cheese volatiles
Enterococcus faecalis	Small white colonies	LB, not much of a smell, chlorine, pool bathroom	Blood, diabetic wound microbiota, raw milk, cheese
Hafnia alvei	Dense and fluffy streaks	sour, salty, corn tortillas, old leather couch, musty, gym mats	Gastrointestinal Tract, human skin microbiome, feces of the pygmy loris, yellow catfish stomach, cauliflower flavor additive for cheese production
Micobacterium lactium	bright yellow small opaque colonies	hard crumbly cheese	Skin, Irish washed-rind cheese
Bacillus pumilus	fluffy yellow	deep fried chicken, fried fat, cheedar cheese, cheese-its, brie cheese	Soil, cheese spoilage
Bacillus clausii	white colonies	stinky cheese, stinging, bleach, alcohol	Skin

the most cheese-associated ketones. Furthermore, both hand-1 and foot-5 contained isovaleric acid, a compound found in Swiss cheese[30] and human axillary odor [16].

Coupled with current metagenomic sequencing technologies, analysis of the volatile compounds produced by bacteria in isolated or mixed cultures can be used to elucidate complex metabolic and biosynthetic pathways. Currently only a small number of the biosynthetic routes for bacterial volatile compounds is known completely, in particular that of geosmin, a compound responsible for the earthy and musty smell of cellars, as well as off flavors in contaminated water and wines and the peaty flavor of whisky[31]. Metagenomic sequencing can contribute to an understanding of community metabolism, and linking this information to analysis of volatile compound metabolomics can improve our understanding of global metabolism and ability to engineer novel flavors.

Flavors and fragrances are emerging as a valuable application of synthetic biology, where enzymatic production of a wide range of chemicals and compounds can be produced through more environmentally conscious biosynthetic conversion of glucose rather than chemical conversion of petrochemicals[32]. Several iGEM teams have worked towards producing fragrant compounds in *E. coli*, including MIT's production of wintergreen and banana scent[33] and Art Science Bangalore's attempt at producing geosmin, also responsible for the romantic smell of fresh rain. Synthetic biology has also taken advantage of the power of volatiles as agents of inter- and intra-species

communication[34]. Bacterial volatiles have been shown to limit growth of other bacterial species, fungi[22,35], and plants[31]. Other volatiles have been shown to promote growth of *Arabidopsis* in some cases[36], influence cytokine production in mammalian tissues[37]. Moreover, "bacterial olfaction" was recently discovered in certain species of *Bacillus* where volatile ammonia produced by one strain affected biofilm formation in another[38]. Engineering of bacteria and yeast to produce volatile chemicals and other strains with volatile responsive promoters allows for the design of synthetic interkingdom mutualistic relationships, with great potential for the establishment and synchronization of multispecies synthetic devices[34].

Conclusions

Having broken down and analyzed the mixed cultures of our cheeses, we can begin to re-assemble the parts and re-synthesize the questions we started with. Will cheese or the way we eat it change as we learn more about microbial communities and can better engineer them? Will our relation to our food and our bodies change with an increased appreciation for the millions of non-human cells that make up our personal ecosystem? Can we design biology better with an appreciation for all the mixed cultures involved, both human and microbial?

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