Microbial communities from spontaneous fermented foods as model system for experimental evolution

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Abstract

Evolutionary forces are widely recognised as the key drivers of patterns of biodiversity. This has resulted in a large body of theory, some of which has been tested experimentally by mimicking evolutionary processes in the laboratory. In this paper we first explain what model systems are used for experimental testing of evolutionary theory, ranging from simple microbial communities in the laboratory and, more recently, to complex (natural) communities. We conclude that microbial communities of spontaneous fermented foods are a very interesting model system to study evolutionary questions on complex communities. It is a model system that combines the complexity of a natural community with the ease of analyses of a synthetic defined community. It therefore gives the researchers the ability to investigate the behaviour of specific species in a natural community without becoming too complex. Due to developing sequencing techniques, the complexity in these communities can be analysed with relative ease while hypotheses developed in less complex systems can be tested.

In the second part of the paper we explain which research questions with an evolutionary background can be addressed using these microbial communities from fermented foods. We discuss species frequency in space and time, the diversity-stability relationship, niche space, fluctuating environment and community coalescence. Hypotheses of the influence of these factors on community evolution are given as well as a short indication of the experimental set-up of such studies when microbial communities of spontaneous fermented food are used.

Introduction and scope

Evolutionary forces are an important factor in shaping biodiversity in ecosystems. Research into understanding how these evolutionary forces shape and maintain this diversity has been mainly comparative and retroactive, trying to reconstruct evolution by observing and interpreting current (evolved) states of communities. Relatively recently manipulative experimental approaches have been developed making use of techniques of experimental evolution and high throughput DNA amplicon sequencing. Since experimental tracking of communities is challenging, thus far, most experimental evolutionary research is performed using highly simplified systems. Often, one or only a few organisms or genotypes are used for long-term propagation experiments without considering possible interactions with other organisms and with the natural environment. Experiments using

complete communities from a natural environment could provide insights in interactions and evolution occurring in nature. In many fields of biology, the concept that "everything is connected" is extensively discussed and studied, resulting in models of metabolic networks, genetic regulatory networks and trophic structures. Currently, evolutionary research rarely includes experiments addressing the evolutionary impact of this connectivity, mainly due to technical difficulties. Understanding the influence of evolution on co-existing organisms could deepen our ecological understanding of community performance. Organisms sharing the same ecosystem live in close proximity to each other for a considerable amount of time. Due to this co-existence, evolutionary processes will modulate the interactions such as the exchange of metabolites, communication, predation and competition for resources.

Evolutionary research on a complex natural community can be challenging, due to a large diversity of organisms in different trophic levels and their interactions. Therefore, a natural model system is required with a limited number of species and interactions. Also, the generation time of this community should be short enough to observe evolution in action.

In this paper, we propose the use of bacterial communities from spontaneous fermented food products for evolution experiments. Our paper has four elements. First we will show how community evolution has been studied so far and what experimental model systems were used. Second, we will explain the added value of using microbial communities from nature in general and in particular the communities from spontaneous fermented products. Third, we will show how these model systems can add to our understanding of community development and performance by showing what evolutionary theories can be tested with the model. Finally, we finish with a short outlook on possibilities for future research.

1. Studies of evolution of single species within natural communities

For many years the study of evolution was based on the sampling of variation that can be found in nature and by looking back to reconstruct how that variation arose. Darwin's finches are a well-known example of how existing variation can give us insight in evolutionary processes such as adaptive radiation (Grant, Grant, Smith, Abbott, & Abbott, 1976; Lack, 1947). This way of comparing variations in nature is still applied successfully. Examples are the studies of the interaction between population dynamics and selection of Soay sheep in Scotland, UK (Clutton-Brock & Pemberton, 2004) or cichlid fish radiations in the African Great Lakes (Seehausen, 2015). Structurally mapping or monitoring of individuals in a natural environment addresses some of the questions related to evolutionary processes. However, such natural systems can only be used to

study the effect of a changes in environment or interactions when they occur naturally, initiated by chance and without much replication.

The field of ecology deals with networks of interactions in very complex natural systems. Evolutionary research adds the influence of time on these systems, which makes the study more complex. Most factors affecting the direction of evolution (like environmental conditions, interactions with other organisms and availability of genes and mutations) are difficult to disentangle in experimental setups. Not the least because the activity of one organism can change the environment of co-occurring organisms. The ecology and evolution of one particular organism is often studied on ecological islands (like pieces of woodland fragments surrounded by agricultural land, lakes or actual islands in seas or rivers) because they can offer a confined semi-closed system and simplified context (Whittaker & Fernández-Palacios, 2007). Still, taking all interactions into account in an experimental setup, even a confined environment, delivers too many variables and unknowns to obtain valuable research outcomes. Another challenge is that multicellular eukaryotic organisms in general have reproduction times of weeks to years, making prospective laboratory evolutionary studies unfeasible. As a result most research is based on fossils or would take several generations of researchers to have a small glimpse of the whole picture of evolution.

To be able to analyse complete community structures, including all players and interactions, we require a model system that is simpler than these natural systems while it still contains the dynamics of a complex system.

2. Microbial model systems

In this section we will explain various model systems that are used for evolution experiments.

Single strain evolution experiments

In contrast to mapping and monitoring of eukaryotes, already in the 19th century Dallinger started controlled evolution experiments with bacteria. He studied adaptation of bacteria by culturing bacterial communities while slowly changing environmental conditions (Dallinger, 1887). A large scale follow up of this new way of studying evolution with controlled experiments came only much later (Atwood, Schneider, & Ryan, 1951; Dykhuizen & Hartl, 1983; Richard E. Lenski, Rose, Simpson, & Tadler, 1991; Richard E Lenski, 2017).

Microorganisms are of interest to evolutionary biologists because they are small, have short reproduction times and can easily be stored and preserved for long periods of time (Elena & Lenski, 2003). The short generation time of microorganisms allows us to see evolution in action and even try to find ways to predict evolution (de Visser & Krug, 2014). The ability to store bacterial cultures by freezing and later thawing them without loss of viability allows for direct competition experiments between evolved and ancestral types. Most experiments focus on the evolution of a single bacterial strain in a defined laboratory environment (Richard E. Lenski, 2017; Richard E. Lenski, Ofria, Collier, & Adami, 1999). More information about how the study of evolution developed and how evolution experiments are generally set up can be found in Box 1.

Due to the small size, short generation time and ease of storage, the use of microorganisms already mitigated a lot of challenges of evolutionary research. As most natural occurring microorganisms live in close proximity to hundreds or even thousands of other bacterial species and organisms from other taxa, the approach of experimental evolution using microbes could be expanded to the community level. However, structuring the complexity of most natural environments would mean analysing large quantities of data and could have too higher levels of complexity to allow the formulation on predictions for evolutionary experiments. As a solution and to simplify these microbial communities, they can be shaped into synthetic communities with only a limited number of focal (micro-) organisms.

Synthetic communities

Naturally co-occurring bacteria can be isolated from their environment and brought together in the lab in pre-determined concentrations. These so-called synthetic communities can be used for studying evolutionary processes under strictly defined conditions (Großkopf & Soyer 2014; De Roy, Marzorati, Van den Abbeele, et al. 2013; Fredrickson 2015)(De Roy et al., 2013; Wittebolle et al., 2009).

Due to previous interactions and co-evolution these bacteria are more likely to resemble or at least represent essential parts a natural community compared to combinations of lab strains which have no historical connection (De Roy et al., 2014; Røder, Sørensen, & Burmølle, 2016). In this way, synthetic communities are assumed to represent nature more accurately than most artificial communities while keeping the simplicity that is needed for experiments.

Using synthetic communities also poses two challenges. First of all, researchers face the difficulty of isolating the bacteria that are the key players in the community. Some community members might not grow on culture media in the laboratory and will

therefore be excluded from the community of isolates. Other bacteria that were isolated might not have been a member of the natural community but were incidentally present. The second challenge is to achieve the relevant or representative degree of complexity. A very simple model will not represent nature accurately. An illustration is the experiment on the influence of phages on culture diversity of Spus and his colleagues (2015). They found that the simple bacterial blends used in their experiments did not represent the diversity of the original complex starter enough to evaluate and grade the role of phage predation (M Spus et al., 2015). Later Spus repeated the experiment with more complex blends of bacterial strains which showed the influence of phage predation on community diversity (Maciej Spus, 2016).

Although the approach of extracting strains from natural communities into synthetic is very valuable, these two challenges might make the translation into "real life" complexity unrealistic (Yu, Krause, Beck, & Chistoserdova, 2016). This motivated the search for a better model system. What we need to find is small confined 'islands' of microorganisms in which the number of players and their interactions is limited and therefore manageable. In these 'islands' no selection or extraction of species into synthetic communities is required for communities to be experimentally tractable.

Bell and colleagues found these "islands" in the form of small pools formed by the roots of beech trees (e.g. Bell, Newman, Silverman, Turner, & Lilley, 2005; Fiegna, Moreno-Letelier, Bell, & Barraclough, 2015). As the number of players in the pools on beech tree roots is already quite limited, it is not necessary to extract some players and put them together in a set frequency. The natural communities can directly be used for experiments. Also the communities can be rebuild by using isolated strains for experiments with an even lower complexity. Consequently, all the steps between single strain behaviour towards the behaviour in the complexity of nature can be compared. The risk of losing vital interactions will be low and observations in the lab should represent nature best.

Fermented foods as model systems

Traditionally fermented products can form another 'island-group' of interest (Bessmeltseva, Viiard, Simm, Paalme, & Sarand, 2014; Erkus et al., 2013; Schoustra, Kasase, Toarta, Kassen, & Poulain, 2013). Many traditional fermented products rely on spontaneous fermentation, which means that they have little human interference as they are not produced using defined starter cultures, but are fermented by a naturally available microbial community. These natural communities are usually diverse but not too complex (e.g. up to 13 main players in three traditional fermented products from Zambia (Schoustra et al., 2013)).

In order to improve organoleptic properties of these products, producers often re-use a finished fermented product for the production of a next batch of the same product (Smid et al., 2014). In the food science domain this process is referred to as back-slopping (Nout, 1992). Back-slopping can also occur accidentally, by the re-use of non-sterilised fermentation equipment, like previously used vessels. These vessels will become the natural habitat of the fermenting microorganisms. In other production methods this is done purposely, like for the production of Illa-type Mabisi, a fermented milk product from Zambia, as well as for parmesan cheese production (Gatti, Bottari, Lazzi, Neviani, & Mucchetti, 2014; Moonga, Schoustra, Linnemann, Shindano, & Smid, 2017). The so called natural whey starters which are used for the production of Parmigiano Reggiano (parmesan cheese) consist of bacteria which have been living together for long periods of time with enough nutrients available to go though many generations. This method is intended for the production of a stable quality product, but can be compared with a standard evolution experiment (as explained in box 2). Also the diverse microbial interactions are therefore assumed to be more those of an evolved community.

Many traditional fermented products are dominated by communities of lactic acid bacteria (Franz et al., 2014; Gadaga, Mutukumira, Narvhus, & Feresu, 1999; Ravyts, Vuyst, & Leroy, 2012; Tamang, Watanabe, & Holzapfel, 2016). The physiology, metabolism and genetics of lactic acid bacteria have been studied in great detail because of their dominant role (Gänzle, 2015; Teusink et al., 2006; Teusink & Smid, 2006). The extended knowledge of metabolite production and growth profiles of these bacteria, can help in understanding the observed evolutionary pathways. Due to an ongoing development in sequencing techniques the methods of analysing these complex communities are becoming more available and affordable. This now makes it feasible to characterize large numbers of communities required for analyses of evolutionary outcomes of replicated experimental evolution experiments. Using food products as a model system stimulates collaboration between fundamental research groups focussing on evolution and research groups working in the field of food sciences and applied microbiology. This multidisciplinary approach is expected to lead to fermented food with improved properties. An example of this type of research can be found in box 3.

In summary, the microbial communities present in spontaneous fermented products make a very useful and interesting model system for evolutionary research. Three aspects contribute to this; 1) their limited complexity that still represents nature, 2) the production methods allows for the communities to adapt to their environment, and 3) the available knowledge about the individual players in the community. In the next section we will explain some concepts concerning community evolution that can be addressed using these communities.

3. Evolutionary theory

The suitability of model system depends on the experimental design for testing hypotheses concerning community evolution. Here we will highlight some theories that could be addressed in experiments using microbial communities from natural systems such as fermented products. For these theories we will also indicate how the experimental setup could look like. Figure 1 indicates how much the different model systems mentioned in section 2 would represent nature and how easy it is to study individual species and community structure over time. The figure also shows how well the different evolutionary questions and theories mentioned in this section can be addressed using these model systems. Table 1 lists (dis)advantages of these different model systems for answering the different evolutionary questions.

Evolution of community structure

In natural communities the frequency of various species varies in space and time. In these communities, variations in the patterns of species abundance in space and time can be measured and differences in these patterns can be linked to potential causal factors. This can be done by sampling microbial communities in the same food product or type of product, but derived from in different geographical regions and over time. The different environments and slight differences in production methods of the fermented foods are variations in selection pressures that shape the microbial community structure. The differences and similarities found can be mapped and analysed. Linked with environmental data, patterns could be found that are potentially caused by environmental factors. Taken together, observations of different patterns will allow to generate hypotheses and predictions that are testable using experimental communities.

Testing of predictions on what factors have the biggest influence on community diversification, can be done by challenging the microbial communities in the laboratory in a selection experiment. These factors can be related to the degree of diversity of the community, the number of niches that are available in the environment as well as the evolving interactions within the community.

Diversity stability hypothesis

The biodiversity-stability hypothesis poses that a more diverse system has a higher stability in terms of functionality (McCann, 2000). The functionality of microbial communities in fermented food products is based on their ability to convert the available nutrients in the food matrix into metabolites to obtain the required product

characteristics. The clear definition of functionality allows for an easy assessment of the loss or change of functionality, e.g. unsuccessful acidification, reduced breakdown of proteins or off-flavour production. A higher diversity of the microbial community can result a stable functionality of the community due to a back-up function (Awasthi, Singh, Soni, Singh, & Kalra, 2014; Bell et al., 2005; Wittebolle et al., 2009). If for various reasons certain members of the community are not present anymore or for instance due to bacteriophage attack unable to perform their function, a diverse community might contain members that can take over the lost function. A higher diversity also causes a lower number of unoccupied niches, due to for example unused nutrients (Mallon, Van Elsas, Salles, Elsas, & Salles, 2015). In that case those niches are not available for any invader, which makes the whole system more likely to keep its functionality and not be destabilised by a non-co-operator, like a food spoiling or pathogenic microorganism (De Roy et al., 2013).

Whether indeed a natural community is more stable when it is more diverse can be tested by manipulating these natural communities to become less diverse. During propagation in an evolution experiment, a fraction of the communities is periodically transferred to fresh medium. By using sequential propagation with an extremely high dilution factor of the inoculum, only those bacteria present in the highest numbers will remain, which strongly decreases the diversity in the community. Whether the diversity which is lost was crucial can be tested by studying the change in fermented end-product characteristics and testing the resilience of the microbial community against stress or invaders.

Sometimes predators can cause stability. These non-co-operators can stabilise a diverse community when growth rates of different members greatly differ. The member with the highest growth rate is the preferred victim of a predator according to the kill the winner principle (Maciej Spus, 2016; Thingstad & Lignell, 1997). The fastest grower in a community could potentially provide most nutrients for a predator and will therefore be the preferred pray, keeping the community stable. This phenomenon is closely related to "negative frequency dependent selection" of the focal strains, where an increase in frequency of an organism has a negative effect on the fitness of that organism (Feldgarden, Stoebel, Brisson, & Dykhuizen, 2003; Kawecki et al., 2012; Koskella & Lively, 2009). Apart from predation, this negative frequency dependent selection can also be caused by various other limiting forces, like food resources, cross-feeding or physical space. The magnitude of the influence of these forces can be studied by reconstructing natural bacterial communities using frequencies that differ from the frequency found in nature. The speed in which these communities will return to their original frequencies, if they do, can give indication of the strength of these forces.

Niche space

Over the years various hypotheses have been developed concerning niches in established communities. One of the oldest hypotheses concerning niches is the niche exclusion principle which states that one niche can only be occupied by one organism (Gause 1934; Hardin 1960). If two species occupy the exact same niche, descendants of the most fit organism will gradually take over from the descendants of the less fit organism.

In natural environments, however, the niche is defined by various depletable resources, like nutrients and space, and non-depletable resources, like temperature and pH, which together form a multidimensional niche space (Hutchinson 1957). Because of all these different dimensions, in theory organisms can live together in a community as long as one dimension in the niche space is not overlapping between the two organisms (Ashby et al., 2017; May, 1974; Pacala & Roughgarden, 1982). The magnitude of the overlap of niches, determines the level of competition between the species. This allows for many different organisms in a natural community as there are many different niches available.

Free niches might increase the chance of invasion by an alien species (Mallon et al., 2015; Stecher et al., 2010; Stecher, Berry, & Loy, 2013). In practice we still do not see all the possible niches, with all possible combinations of dimensions, which can be occupied. Species that might exist in a community cannot coexists with already established players in the community due to competition for non-substitutable resources. The availability of unoccupied niches may result in character displacement (Grant, 1972) and adaptive radiation (Rainey & Travisano, 1998), where organisms change to occupy other niches.

Without external fluctuations the amount on niches will remain stable and should be equal to the amount of species present in the community. By propagating a microbial community with different numbers of species or by adding or removing niches it can be tested whether these two are indeed so strongly linked.

Fluctuating environmental factors

In nature communities we cannot neglect external fluctuations. The continuously changing environmental factors in batch culturing are to a certain extent comparable to fluctuations that can be found in natural ecosystems, like seasons and tides. Under such dynamic conditions, nutrient rich periods are alternated with nutrient poor periods. These fluctuation give rise to the possibility of different organisms to flourish in different moments of time, resulting in a more diverse community. The could be a trade-off between growth rate in the exponential phase of fermentation and survival rate in the stationary phase that is following the fermentation which will result in a balance of

organisms that have a different strategy (Fitzsimmons, Schoustra, Kerr, & Kassen, 2010; Reznick, Bryant, & Bashey, 2002).

The influence of these fluctuations on community structure can be investigated by varying the time regime of the batch fermentations. In this way, the balance between fast growers and those with high survival could change. It is possibly very difficult to completely get rid of some players in the community (Spus, 2016), but it is hypothesised that when there is no stationary phase, the community will mainly consist of fast growers, while at constant low nutrient levels the community will consist of mainly slow growing survivors (van Mastrigt, Abee, Lillevang, & Smid, 2018).

Community coalescence

Another natural phenomenon that can be studied using bacterial communities from food products is the effect of coalescence of communities. The term "community coalescence" was introduces recently (Rillig et al., 2015) and describes situations were entire communities interact because their environments become translocated allowing this interaction. In nature we see this for example during soil tillage and flooding events, but also while eating and kissing. The performance of single species do not always give a good indication of how the species will behave in a community (Tikhonov, 2016). The coalesced community might be a combination of the two initial communities or be dominated by either of them, dependent on the best performing combination (Sierocinski et al., 2017). The influence of co-evolution in the outcome of community coalescence can be investigated using microbial communities from fermented products. Combining two or more co-evolved communities can provide information on how specialised the evolved interactions within the communities are. Besides this coalescence of similar communities, also the mixing of a fermented community with the community of a raw product has important implications for the formation of the fermented product. Besides studying the influence of co-evolution on the outcome of community coalescence, also the evolutionary results of regular coalescence occasions can be studied using fermented products.

4. Conclusion and way forward

Microbial communities of spontaneous fermented foods present a promising model system to experimentally test evolutionary theory. These communities bear several intrinsic advantages for executing evolution experiments that have been developed in experimental evolution starting with single strains of microorganisms: short generation times, small size and ability to be stored frozen and defrosted to perform competition

experiments (fitness tests) between evolved and ancestral lines. Moreover, these natural microbial communities have a limited number of players and form an island of microorganisms that does not have a lot of influx from the outside the confined system boundaries. These communities also have a clear function which makes it easy to check whether certain environmental changes can have an impact on this function. All these characteristics make bacterial communities from fermented food products an interesting model system to test long standing theories in community ecology and evolution and increase our understanding of evolution and its drivers.

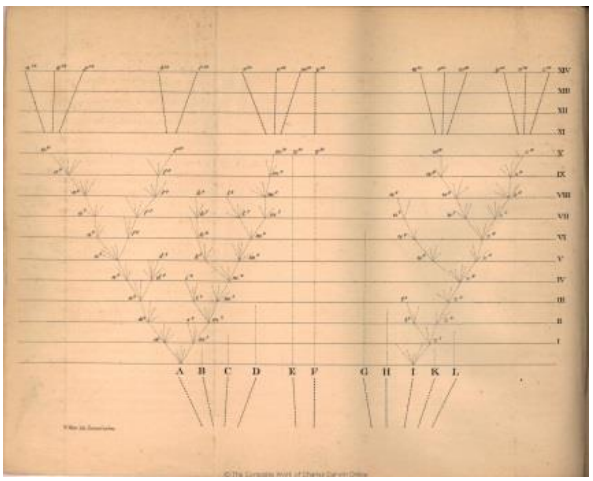
Studies using natural microbial communities will inevitably experience a high degree of natural variation in the results. To translate these findings to general concepts that are applicable to natural systems can be a challenge. Fortunately due to fast developing DNA and RNA techniques, analysing all players present in a community including their activity is becoming more and more feasible.

We have only just started to explore potential of the above described experimental systems. Apart from giving fundamental insight in microbial community dynamics and experimentally scrutinizing aspects of the evolution theory, the outcomes of research using microbial communities from fermented products will help understanding multiple-strain fermentations and how to manipulate these processes to obtain high quality fermented food products.

BOX 1 A short history of evolution experiments

Predicting evolution is something Charles Darwin already took interested in, as can be seen in the sketches he made for his book "On the origin of species" (Darwin, 1859). In this drawing lines I to X represent the phylogeny of the species found and their history. Lines XI to XIV represent the unknown future states of the species, predicting the outcomes. William Dallinger was the first to report about planned experiments with evolution (Dallinger, 1887). By slowly increasing the temperature of the environment of microorganisms, he allowed these microorganisms to adapt to a temperature at which they would normally never grow. When returning them to their old environment he concluded that this adaptation came with the costs of growing slower in their old environment. His results show the principle of adaptation and trade-offs.

The approach of Dallinger remains in use today by evolutionary biologists. In the last two decades a lot of evolutionary concepts have been studied using mainly single organisms in controlled laboratory environments. It shows that even very simple laboratory model systems are very useful in addressing numerous fundamental questions on the dynamics of evolution. The longest ongoing evolution experiment is the setup started by Richard Lenski. In 1988 he started transferring 12 lineages of *Escherichia coli* on a daily basis in minimal medium. He used these lines to study adaptation and diversification, trade-offs, consequences of mutators and the influence of population size on drift among various other theories in evolution (Elena & Lenski 2003; Rozen & Lenski 2000; Lenski & Travisano 1994; Lenski 2017; Deatherage et al. 2017; Sachs & Hollowell 2012 etc.).



BOX 2 Set-up of evolution experiments

The set-up of evolution experiments has a very general basis. Organisms are transferred to a set environment and are sequentially transferred to fresh medium at regular time intervals. During these transfers the organisms go through several generations, the

number of which is determined by the dilution factor of the inoculum into fresh medium. By taking samples after a particular number of transfers, changes in fitness and population composition can be monitored. A typical classic evolution experiment is performed with a single species which is allowed to evolve for a long period of time. In this way, beneficial mutations can deliver fitness advantage to variants. These advantages can be measured as increased relative abundance of the organisms carrying the beneficial mutation.

The experiments can be set-up with different variables; in starting genotypes, environment and ways of transfer. The type and number of bacteria that are transferred to the next cycle has great influence on the outcome of the results (Cremer, Melbinger, & Frey, 2012). In case of mimicking the spontaneous fermentation in a pre-used container as mentioned in this paper, it might be necessary to transfer the bacteria that attach themselves to the wall over the planktonic ones. Since mutations often occur random over the genome (with the exception of hotspots) only with a considerate number of replicates it is possible to draw conclusions to an observation.

By the production of spontaneous fermented foods using some material of an old batch to initiate a new batch ("backslopping"), the production method is a kind of evolution experiment. The microbial community that ferments the raw ingredients are transferred to a new environment which is rich in nutrients so they can undergo many generations. Stability in production practices gives a stable environment for the community causes the community to get close to an evolutionary endpoint and all individuals to reach a fitness peak. The production method of Parmesan cheese is a clear example where backslopping can cause the microbial community to stabilise and in that way stabilise product quality. Analysing these co-evolved communities can give insight in environmental factors shaping microbial communities.

BOX 3 Evolutionary applications.

Knowledge about bacterial communities in traditional fermented foods has a widespread economic and ecological application. The fermenting bacterial communities are besides a model system for fundamental evolutionary questions also a functional starter culture for food production. Knowledge obtained about these starter cultures and the their environment are a very important step in producing a safe, nutritious and tasty product.

For local African communities, the large scale production of the traditional fermented products can be of crucial economic importance. Due to current regulations, local producers are sometimes not allowed to sell non-pasteurised products on the market.

However, the pasteurisation of the raw milk will not only kill of pathogens, also the naturally present fermentation inoculum will die, leaving no starter for spontaneous fermentation. Providing a stable starter culture for these fermented products can be a great nutritional and economic importance for the local communities. So far, most bacterial starter cultures contain only one or two bacterial strains. In order to represent the original product accurately, the starter culture of the traditional fermented food might be more complex. An understanding of the complex community interactions and co-evolution is required. Results of the types of research mentioned in this article might therefore be of great importance.

Also products currently produced on industrial scale could be improved when more is known about complex starter culture dynamics. The problems industries have to deal with range from contamination by pathogens, to frequency changes, plasmid loss, phage predation and mutations. In the industrial production of the probiotic lactic acid bacterium *Lactobacillus rhamnosus*, a mutation caused this bacteria to lose its flagella, which was crucial for its probiotic activity (Sybesma, Molenaar, van IJcken, Venema, & Kort, 2013). By increasing our understanding of these problems, we might decrease their occurrence in industry.

453 Ashby, B., Watkins, E., Lourenço, J., Gupta, S., & Foster, K. R. (2017). Competing
454 species leave many potential niches unfilled. *Nature Ecology and Evolution*, *1*(10),
455 1495–1501. http://doi.org/10.1038/s41559-017-0295-3

456 Atwood, K. C., Schneider, L. K., & Ryan, F. J. (1951). Periodic Selection in Escherichia
457 Coli. *Proceedings of the National Academy of Sciences*, *37*(3), 146–155.
458 http://doi.org/10.1073/pnas.37.3.146

459 Awasthi, A., Singh, M., Soni, S. K., Singh, R., & Kalra, A. (2014). Biodiversity acts as
460 insurance of productivity of bacterial communities under abiotic perturbations. *Isme*
461 *J*, *8*(12), 2445–2452. http://doi.org/10.1038/ismej.2014.91

462 Bell, T., Newman, J. J. a, Silverman, B. B. W., Turner, S. L. S., & Lilley, A. A. K. (2005).
463 The contribution of species richness and composition to bacterial services. *Nature*,
464 *436*(7054), 1157–1160. http://doi.org/10.1038/nature03891

465 Bessmeltseva, M., Viiard, E., Simm, J., Paalme, T., & Sarand, I. (2014). Evolution of
466 bacterial consortia in spontaneously started rye sourdoughs during two months of
467 daily propagation. *PloS One*, *9*(4), e95449.
468 http://doi.org/10.1371/journal.pone.0095449

469 Clutton-Brock, T., & Pemberton, J. (2004). *Soay Sheep: Dynamics and selection in an*
470 *island population*. Cambridge: Cambridge University Press.

471 Cremer, J., Melbinger, A., & Frey, E. (2012). Growth dynamics and the evolution of
472 cooperation in microbial populations. *Scientific Reports*, *2*, 1–6.
473 http://doi.org/10.1038/srep00281

474 Dallinger, W. (1887). The President's address. *Journal of Microscopy*.

475 Darwin, C. R. (1859). *On the Origin of Species by means of natural selection, or the*
476 *preservation of favoured races in the struggle for life.* John Murray London.

477 De Roy, K., Marzorati, M., Negroni, A., Thas, O., Balloi, A., Fava, F., … Boon, N. (2013).
478 Environmental conditions and community evenness determine the outcome of
479 biological invasion. *Nature Communications*, *4*, 1383.
480 http://doi.org/10.1038/ncomms2392

481 De Roy, K., Marzorati, M., Van den Abbeele, P., Van de Wiele, T., & Boon, N. (2014).
482 Synthetic microbial ecosystems: an exciting tool to understand and apply microbial
483 communities. *Environmental Microbiology*, *16*(6), 1472–1481.
484 http://doi.org/10.1111/1462-2920.12343

485 Deatherage, D. E., Kepner, J. L., Bennett, A. F., Lenski, R. E., & Barrick, J. E. (2017).
486 Specificity of genome evolution in experimental populations of Escherichia coli
487 evolved at different temperatures. *Proceedings of the National Academy of Sciences*
488 *of the United States of America*, *114*(10), E1904–E1912.
489 http://doi.org/10.1073/pnas.1616132114

490 de Visser, J. A. G. M., & Krug, J. (2014). Empirical fitness landscapes and the
491 predictability of evolution. *Nature Reviews Genetics*, *15*(7), 480–490.
492 http://doi.org/10.1038/nrg3744

493 Dykhuizen, D. E., & Hartl, D. L. (1983). Selection in Chemostats. *Microbiology*, *47*(2),
494 150–168.

495 Elena, S. F., & Lenski, R. E. (2003). Evolution experiments with microorganisms: The
496 dynamics and genetic bases of adaptation. *Nature Reviews Genetics*, *4*(6), 457–469.
497 http://doi.org/10.1038/nrg1088

498 Erkus, O., De Jager, V. C. L., Spus, M., Van Alen-Boerrigter, I. J., Van Rijswijck, I. M. H.,
499 Hazelwood, L., … Smid, E. J. (2013). Multifactorial diversity sustains microbial
500 community stability. *ISME Journal*, *7*(11), 2126–2136.
501 http://doi.org/10.1038/ismej.2013.108

502 Feldgarden, M., Stoebel, D. M., Brisson, D., & Dykhuizen, D. E. (2003). Size doesn't
503 matter: Microbial selection experiments address ecological phenomena. *Ecology*,
504 *84*(7), 1679–1687. http://doi.org/10.1890/0012-
505 9658(2003)084[1679:SDMMSE]2.0.CO;2

506 Fiegna, F., Moreno-Letelier, A., Bell, T., & Barraclough, T. G. (2015). Evolution of species
507 interactions determines microbial community productivity in new environments. *The*
508 *ISME Journal*, *9*(5), 1235–1245. http://doi.org/10.1038/ismej.2014.215

509 Fitzsimmons, J. M., Schoustra, S. E., Kerr, J. T., & Kassen, R. (2010). Population
510 consequences of mutational events: Effects of antibiotic resistance on the r/K trade-
511 off. *Evolutionary Ecology*, *24*(1), 227–236. http://doi.org/10.1007/s10682-009-
512 9302-8

513 Franz, C. M. a. P., Huch, M., Mathara, J. M., Abriouel, H., Benomar, N., Reid, G., …
514 Holzapfel, W. H. (2014). African Fermented Foods and Probiotics. *International*
515 *Journal of Food Microbiology*, *190*, 84–96.
516 http://doi.org/10.1016/j.ijfoodmicro.2014.08.033

517 Fredrickson, J. K. (2015). Ecological communities by design. *Science*, *348*(6242), 1425–

518 1427. http://doi.org/10.1126/science.aab0946

519 Gadaga, T. H., Mutukumira, a. N., Narvhus, J. a., & Feresu, S. B. (1999). A review of
520 traditional fermented foods and beverages of Zimbabwe. *International Journal of*
521 *Food Microbiology*, *53*, 1–11. http://doi.org/10.1016/S0168-1605(99)00154-3

522 Gänzle, M. G. (2015). Lactic metabolism revisited: Metabolism of lactic acid bacteria in
523 food fermentations and food spoilage. *Current Opinion in Food Science*, *2*, 106–117.
524 http://doi.org/10.1016/j.cofs.2015.03.001

525 Gatti, M., Bottari, B., Lazzi, C., Neviani, E., & Mucchetti, G. (2014). Invited review:
526 Microbial evolution in raw-milk, long-ripened cheeses produced using undefined
527 natural whey starters. *Journal of Dairy Science*, *97*(2), 573–591.
528 http://doi.org/10.3168/jds.2013-7187

529 Gause, G. F. (1934). Experimental Analysis of Vito Volterra'S Mathematical Theory of the
530 Struggle for Existence. *Science*, *79*(2036), 16–17.
531 http://doi.org/10.1126/science.79.2036.16-a

532 Grant, P. R. (1972). Convergent and divergent character displacement. *Biological Journal*
533 *of the Linnean Society*, *4*(1), 39–68. http://doi.org/10.1111/j.1095-
534 8312.1972.tb00690.x

535 Grant, P. R., Grant, B. R., Smith, J. N., Abbott, I. J., & Abbott, L. K. (1976). Darwin's
536 finches: population variation and natural selection. *Proceedings of the National*
537 *Academy of Sciences*, *73*(1), 257–261. http://doi.org/10.1073/pnas.73.1.257

538 Großkopf, T., & Soyer, O. S. (2014). Synthetic microbial communities. *Current Opinion in*
539 *Microbiology*, *18*(1), 72–77. http://doi.org/10.1016/j.mib.2014.02.002

540 Hardin, G. (1960). The Competitive Exclusion Principle. *Science*, *131*(3409), 1292–1297.
541 http://doi.org/10.1126/science.131.3409.1292

542 Hutchinson, G. E. (1957). Concluding Remarks. *Cold Spring Harbor Symposia on*
543 *Quantitative Biology*, *22*(0), 415–427. http://doi.org/10.1101/SQB.1957.022.01.039

544 Kawecki, T. J., Lenski, R. E., Ebert, D., Hollis, B., Olivieri, I., & Whitlock, M. C. (2012).
545 Experimental evolution. *Trends in Ecology and Evolution*, *27*(10), 547–560.
546 http://doi.org/10.1016/j.tree.2012.06.001

547 Koskella, B., & Lively, C. M. (2009). Evidence for negative frequency-dependent selection
548 during experimental coevolution of a freshwater snail and a sterilizing trematode.
549 *Evolution*, *63*(9), 2213–2221. http://doi.org/10.1111/j.1558-5646.2009.00711.x

Lack, D. (1947). *Darwin's Finches*. Cambridge University Press.

Lenski, R. E. (2017). Convergence and Divergence in a Long-Term Experiment with Bacteria. *The American Naturalist*, *190*, S57–S68. http://doi.org/10.1086/691209

Lenski, R. E. (2017). Convergence and Divergence in a Long-Term Experiment with BacteriaLenski, R. E. (2017). Convergence and Divergence in a Long-Term Experiment with Bacteria. The American Naturalist, S000–S000. http://doi.org/10.1086/691209. *Tfile://wurnet.nl/homes/groen102/My Documents/Literature/Evolutionary Questions/convergene and Divergence.pdfhe American Naturalist*, S000–S000. http://doi.org/10.1086/691209

Lenski, R. E. (2017). Experimental evolution and the dynamics of adaptation and genome evolution in microbial populations. *The ISME Journal*, *11*(10), 2181–2194. http://doi.org/10.1038/ismej.2017.69

Lenski, R. E., Ofria, C., Collier, T. C., & Adami, C. (1999). Genome complexity, robustness and genetic interactions in digital organisms. *Nature*, *400*(6745), 661–664. http://doi.org/10.1038/23245

Lenski, R. E., Rose, M. R., Simpson, S. C., & Tadler, S. C. (1991). Long-Term Experimental Evolution in Escherichia coli. I. Adaptation and Divergence During 2,000 Generations. *The American Naturalist*, *138*(6), 1315–1341. http://doi.org/10.1086/285289

Lenski, R. E., & Travisano, M. (1994). Dynamics of adaptation and diversification: a 10,000-generation experiment with bacterial populations. *Proceedings of the National Academy of Sciences*, *91*(15), 6808–6814. http://doi.org/10.1073/pnas.91.15.6808

Mallon, C. A., Van Elsas, J. D., Salles, J. F., Elsas, J. D. van, & Salles, J. F. (2015). Microbial invasions: The process, patterns, and mechanisms. *Trends in Microbiology*, *23*(11), 719–729. http://doi.org/10.1016/j.tim.2015.07.013

May, R. M. (1974). On the theory of niche overlap. *Theoretical Population Biology*, *5*(3), 297–332. http://doi.org/10.1016/0040-5809(74)90055-0

McCann, K. S. (2000). The diversity–stability debate. *Nature*, *405*(6783), 228–233. http://doi.org/10.1038/35012234

Moonga, H. B., Schoustra, S., Linnemann, A., Shindano, J., & Smid, E. J. (2017). Influence of Production Methods on Communities of Lactic Acid Bacteria in Traditional Fermented Milk - Mabisi. In *Short lectures, presented at the 12the*

583 *Symposium on Lactic Acid Bacteria* (p. 1).

584 Nout, M. J. R. (1992). Accelerated natural lactic fermentation of cereal-based formulas at
585 reduced water activity. *International Journal of Food Microbiology*, *16*(4), 313–322.
586 http://doi.org/10.1016/0168-1605(92)90033-Y

587 Pacala, S. W., & Roughgarden, J. (1982). The evolution of resource partitioning in a
588 multidimensional resource space. *Theoretical Population Biology*, *22*(1), 127–145.
589 http://doi.org/10.1016/0040-5809(82)90039-9

590 Rainey, P. B., & Travisano, M. (1998). Adaptive radiation in a heterogeneous
591 environment. *Nature*, *394*, 69–72. http://doi.org/10.1038/27900

592 Ravyts, F., Vuyst, L. De, & Leroy, F. (2012). Bacterial diversity and functionalities in food
593 fermentations. *Engineering in Life Sciences*, *12*(4), 356–367.
594 http://doi.org/10.1002/elsc.201100119

595 Reznick, D. N., Bryant, M. J., & Bashey, F. (2002). r-and K-selection revisited: the role of
596 population regulation in life-history evolution. *Ecology*, *83*(6), 1509–1520.
597 http://doi.org/http://dx.doi.org/10.1890/0012-
598 9658(2002)083[1509:RAKSRT]2.0.CO;2

599 Rillig, M. C., Antonovics, J., Caruso, T., Lehmann, A., Powell, J. R., Veresoglou, S. D., &
600 Verbruggen, E. (2015). Interchange of entire communities: Microbial community
601 coalescence. *Trends in Ecology and Evolution*, *30*(8), 470–476.
602 http://doi.org/10.1016/j.tree.2015.06.004

603 Røder, H. L., Sørensen, S. J., & Burmølle, M. (2016). Studying Bacterial Multispecies
604 Biofilms: Where to Start? *Trends in Microbiology*, *24*(6), 503–13.
605 http://doi.org/10.1016/j.tim.2016.02.019

606 Rozen, D. E., & Lenski, R. E. (2000). Long- Term Experimental Evolution in *Escherichia*
607 *coli* . VIII. Dynamics of a Balanced Polymorphism. *The American Naturalist*, *155*(1),
608 24–35. http://doi.org/10.1086/303299

609 Sachs, J. L., & Hollowell, A. C. (2012). The origins of cooperative bacterial communities.
610 *mBio*, *3*(3), 1–3. http://doi.org/10.1128/mBio.00099-12

611 Schoustra, S. E., Kasase, C., Toarta, C., Kassen, R., & Poulain, A. J. (2013). Microbial
612 community structure of three traditional zambian fermented products: mabisi,
613 chibwantu and munkoyo. *PloS One*, *8*(5), e63948.
614 http://doi.org/10.1371/journal.pone.0063948

615 Seehausen, O. (2015). Process and pattern in cichlid radiations–inferences for
616 understanding unusually high rates of evolutionary diversification. *New Phytologist*,
617 *207*(2), 304–12. http://doi.org/10.1111/nph.13450

618 Sierocinski, P., Milferstedt, K., Bayer, F., Großkopf, T., Alston, M., Bastkowski, S., …
619 Buckling, A. (2017). A Single Community Dominates Structure and Function of a
620 Mixture of Multiple Methanogenic Communities. *Current Biology*, *27*(21), 3390–
621 3395. http://doi.org/10.1016/j.cub.2017.09.056

622 Smid, E. J., Erkus, O., Spus, M., Wolkers-Rooijackers, J. C. M., Alexeeva, S., &
623 Kleerebezem, M. (2014). Functional implications of the microbial community
624 structure of undefined mesophilic starter cultures. *Microbial Cell Factories*, *13*(1).
625 http://doi.org/10.1186/1475-2859-13-S1-S2

626 Spus, M. (2016). *PhD thesis: Mixed culture engeneering for steering starter functionality*.
627 Wageningen University.

628 Spus, M., Li, M., Alexeeva, S., Wolkers-Rooijackers, J. C. M., Zwietering, M. H., Abee, T.,
629 & Smid, E. J. (2015). Strain diversity and phage resistance in complex dairy starter
630 cultures. *Journal of Dairy Science*, *98*(8), 5173–82.
631 http://doi.org/10.3168/jds.2015-9535

632 Stecher, B., Berry, D., & Loy, A. (2013). Colonization resistance and microbial
633 ecophysiology: Using gnotobiotic mouse models and single-cell technology to
634 explore the intestinal jungle. *FEMS Microbiology Reviews*, *37*(5), 793–829.
635 http://doi.org/10.1111/1574-6976.12024

636 Stecher, B., Chaffron, S., Käppeli, R., Hapfelmeier, S., Freedrich, S., Weber, T. C., …
637 Hardt, W. D. (2010). Like will to like: Abundances of closely related species can
638 predict susceptibility to intestinal colonization by pathogenic and commensal
639 bacteria. *PLoS Pathogens*, *6*(1). http://doi.org/10.1371/journal.ppat.1000711

640 Sybesma, W., Molenaar, D., van IJcken, W., Venema, K., & Kort, R. (2013). Genome
641 Instability in Lactobacillus rhamnosus GG. *Applied and Environmental Microbiology*,
642 *79*(7), 2233–2239.

643 Tamang, J. P., Watanabe, K., & Holzapfel, W. H. (2016). Review: Diversity of
644 microorganisms in global fermented foods and beverages. *Frontiers in Microbiology*,
645 *7*(377), 1–28. http://doi.org/10.3389/fmicb.2016.00377

646 Teusink, B., & Smid, E. J. (2006). Modelling strategies for the industrial exploitation of
647 lactic acid bacteria. *Nature Reviews Microbiology*, *4*(1), 46–56.

http://doi.org/10.1038/nrmicro1319

Teusink, B., Wiersma, A., Molenaar, D., Francke, C., De Vos, W. M., Siezen, R. J., & Smid, E. J. (2006). Analysis of growth of Lactobacillus plantarum WCFS1 on a complex medium using a genome-scale metabolic model. *Journal of Biological Chemistry*, *281*(52), 40041–40048. http://doi.org/10.1074/jbc.M606263200

Thingstad, T. F., & Lignell, R. (1997). Theoretical models for the control of bacterial growth rate, abundance, diversity and carbon demand. *Aquatic Microbial Ecology*, *13*, 19–27. http://doi.org/10.3354/ame013019

Tikhonov, M. (2016). Community-level cohesion without cooperation. *eLife*, *5*, 1–12. http://doi.org/10.7554/eLife.15747

van Mastrigt, O., Abee, T., Lillevang, S. K., & Smid, E. J. (2018). Quantitative physiology and aroma formation of a dairy Lactococcus lactis at near-zero growth rates. *Food Microbiology*, *73*, 216–226. http://doi.org/10.1016/j.fm.2018.01.027

Whittaker, R., & Fernández-Palacios, J. (2007). *Island biogeography: ecology, evolution, and conservation*. Oxford University Press, Oxford.

Wittebolle, L., Marzorati, M., Clement, L., Balloi, A., Daffonchio, D., Heylen, K., … Boon, N. (2009). Initial community evenness favours functionality under selective stress. *Nature*, *458*(7238), 623–626. http://doi.org/10.1038/nature07840

Yu, Z., Krause, S. M. B., Beck, D. A. C., & Chistoserdova, L. (2016). A synthetic ecology perspective: How well does behavior of model organisms in the laboratory predict microbial activities in natural habitats? *Frontiers in Microbiology*, *7*(JUN). http://doi.org/10.3389/fmicb.2016.00946