Functional Genomics for Food Fermentation Processes

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Abstract

This review describes recent scientific and technological drivers of food fermentation research. In addition, a number of practical implications of the results of this development will be highlighted. The first part of the manuscript elaborates on the message that genome sequence information gives us an unprecedented view on the biodiversity of microbes in food fermentation. This information can be made applicable for tailoring relevant characteristics of food products through fermentation. The second part deals with the integration of genome sequence data into metabolic models and the use of these models for a number of topics that are relevant for food fermentation processes. The final part will be about metagenomics approaches to reveal the complexity and understand the functionality of undefined complex microbial consortia used in a diverse range of food fermentation processes.

INTRODUCTION

Fermentation of food raw materials is an activity that can be found all over the world and is part of almost all human cultures. As such, fermentation can be regarded as one of oldest food processing technologies. A diverse range of fermented food products can be found only in specific regions, and these products are tightly linked to local traditions in food preparation. A number of fermented food products have a global distribution and are not limited to specific human cultures. These are for instance bread, wine, beer, and fermented dairy products. The original driver for processing food raw materials by means of fermentation was the extension of shelf life. The conversion of simple and complex carbohydrates of the food raw materials into organic acids and alcohols contributes to the preservative effect. Fermentation also adds variety and nutritional fortification to starchy, bland diets. The significance of fermented food products is best exemplified in fermented cassava products such as gari and fufu. These fermented products are a major component of the diet of more than 800 million African people (Battcock & Azam-Ali 1998). In some regions in Africa, these fermented cassava products constitute more than 50% of the diet.

For centuries, the practical knowledge of (traditional) fermentation processes has been transferred from parents to their children. Consequently, fermented products have changed continuously in composition, taste, nutritional value, and appearance, naturally leading to product diversification. It was not only human hands that changed product characteristics over time (for instance by changing processing conditions) but also the composition and therefore the properties of the consortium of fermenting microbes has adapted over generations to changing substrate composition and processing conditions. The advent of microbiology in the late nineteenth century has rationalized our influence on the development of traditional fermentation processes and also has sparked the rational design of new fermentation processes. Knowledge of the actual workhorses of fermentation (yeast, molds, and bacteria) created the boundary conditions for industrialization and commercialization of food fermentation processes. This development started in the early twentieth century and resulted for instance in the development of starter cultures allowing more control, predictability, and robustness of food fermentation processes.

Traditional fermentation research in microbiology focused on isolation and phenotypic characterization of microbes originating from natural sources, such as spontaneous fermentations. This activity delivered defined single and mixed strain cultures that were suitable for industrial application. Current large-scale cheese and beer production are based on the use of such cultures. The drawback of rationalizing fermentation processes, for instance by applying defined starter cultures in an industrial production setting, is the loss of product diversity in terms of taste, flavor, nutritional content, and appearance. Modern consumers are more and more aware of the loss of variation in product characteristics and demand more diverse fermented products in which artisanal characteristics are combined with the established (microbial and industrial) safety and shelf life specifications. Food technologists and innovative food companies are therefore investing time and effort in connecting the right functional benefits of fermented food products to the emotional benefits (Juriaanse 2006). These developments are currently playing a key role in branding strategies of food companies.

Food fermentation, and in particular strain selection, offers numerous opportunities for industry to comply with the current consumer trends such as (*a*) wellness & health foods, (*b*) ethnicity & new world foods, (*c*) specialty foods, and (*d*) foods representing traditional values. The recent progress made in microbial genomics makes it possible to start exploration and exploitation of the enormous species- and strain diversity found among food fermenting microbes. Starting with the biological (i.e., physiological, metabolic, and genetic) knowledge of a limited number of laboratory strains of microbial species used on food fermentation, we are now

on the brink of uncovering the wealth of genetic and phenotypic variation found in wild-type food fermenting microbes. The notion that laboratory model strains of *Saccharomyces cerevisiae* differ significantly from the strains used in wine making is explicitly demonstrated in functional genomics studies (Dunn et al. 2005, Daran-Lapujade et al. 2003, Hughes et al. 2000, Pizarro et al. 2007). An in depth diversity analysis of dairy and nondairy *Lactococcus lactis* isolates by Rademaker et al. (2007) demonstrated the limited genetic diversity of the dairy strains within the different groups of genomic lineages of strains originating from other ecological niches. The genetic diversity found in wild strains comprises a true treasure chamber of traits that are relevant for starter culture improvement. This diversity is selectively propagated as a result of adaptation to dynamic natural environments. Genetic mechanisms such as horizontal gene transfer and the presence and activity of mobile genetic elements (i.e., plasmids, bacteriophages, and transposons) are the main drivers for changing the level of diversity (de Vos 2001).

Genome sequencing programs of food fermenting microbes and strains with probiotic properties took off in the first years of the 21st century with the selection of a limited number of known laboratory strains (Klaenhammer et al. 2002). Full genome sequencing is now rapidly progressing toward bacterial strains with relevance for specific applications such as food fermentation (Schroeter & Klaenhammer 2009). The rapid extension of genome sequencing programs is driven by the fast development in DNA sequence technology (MacLean et al. 2009).

The first part of this review addresses the use of genome sequence information of fermenting microbes for tailoring relevant characteristics of food products made through fermentation. Comparative genome hybridization (CGH) using a single reference genome (Molenaar et al. 2005) or multiple reference genomes (Rasmussen et al. 2008) has proven to be a powerful method for high resolution analysis of genetic diversity of natural isolates of lactic acid bacteria (LAB). Also, the potential of LAB to form specific flavors from amino acids was compared in all sequenced LAB species by searching their genomes for enzymes involved in proteolysis and amino acid conversions (Liu et al. 2008). The second part of this review deals with the integration of genome sequence data into genome scale stoichiometric metabolic models and the use of these models for a number of topics of high relevance for food fermentation processes. With the flood of new genomics data (sequence information, transcriptome profiles, metabolite data, etc.), streamlined data-processing pipelines and versatile database systems are crucial for data integration and analysis. The focus on well-defined biological research questions rather than unbiased data gathering is crucial for successful application of functional genomics approaches for improving fermentation processes. Finally, we will discuss the recent developments in metagenome analysis and the application to complex microbial consortia. With metagenomics approaches, it is now possible to reveal the complexity and understand the functionality of undefined complex microbial consortia used in a diverse range of food fermentation processes.

BIODIVERSITY AND GENE-TRAIT MATCHING

With the completion of a growing number of genome sequences of different, but often quite related, LAB, it has become apparent that the biodiversity within a genus and even within a species is much larger than originally anticipated. A few percent difference in the traditional molecular character of GC content (guanine-cytosine content in DNA) can translate into several hundred different genes being encoded and expressed. This major extent of biodiversity, even within one bacterial species, was clearly demonstrated in a recent publication by van Siezen et al. (2008). In this work, the genomes of two nondairy isolates of *Lactococcus lactis* were sequenced and compared with the already known genome sequences of three dairy strains. Within the five strains that were analyzed and compared, only approximately two-thirds (1300 of the more than 2000 genes) were

shared by all strains, and several hundreds of genes were identified that were not found in the dairy strains. Annotation of these newly identified genes showed the presence of some potentially very interesting functionalities such as exopolysaccharide production (important for food texture), sugar utilization (for application in a variety of nondairy food substrates), and unique activities in nitrogen metabolism (for flavor generation). Also in regulatory mechanisms, major diversity has been observed within this species using whole genome hybridization and DNA-DNA array technology for establishing gene presence (Bachmann et al. 2009). Similar strain comparisons have been performed within the species *Lactobacillus plantarum*, also identifying absence or presence of hundreds of genes in strain-to-strain comparisons (Molenaar et al. 2005, Pretzer et al. 2005b).

Below, some industrially relevant functionalities, such as industrial robustness and probiotic functionalities, will be discussed in relation to the different levels of intergenus, interspecies, and intraspecies diversity.

Industrial Robustness of Lactic Acid Bacteria

During the process of the manufacture of a typical fermented food, such as cheese, the LAB are exposed to large changes in their environment, leading to a number of different stresses. Process temperatures range from 6°C to 50°C, and osmotic value of the surrounding (cheese) environment is suddenly increased by the brining process and continues to increase during the maturation of the cheese. Furthermore, the initial clotting of the cheese occurs in large vats allowing massive exposure to oxygen for these facultative anaerobic bacteria, whereas in cheese the conditions are quite anaerobic. Finally, the initial condition of substrate excess in milk, resulting in near maximal growth, is drastically switched to starvation conditions after coagulation of the curd and separation of the whey. The LAB that are used as starters in such food fermentation are expected to perform under such hostile conditions, for at least several hours to allow rapid acidification of the food materials. Below, a number of examples are presented that lead to improved activity/survival of different LAB under these hostile conditions.

Several microorganisms, such as propionibacteria and yeasts, accumulate trehalose under different stress conditions such as aeration, low pH, and high salt concentrations. In *Propionibacterium freudenreichii*, internal trehalose concentrations of up to 6M have been reported when the cells are exposed to low pH and high salt (Cardoso et al. 2004). Within this species, varying internal concentrations of trehalose have been observed, leading to varying tolerance to stress conditions. Also, introduction of trehalose production into different microorganisms has resulted in increased stress tolerance (Padilla et al. 2004).

For some LAB, i.e., *Lactococcus lactis*, *Lactobacillus plantarum*, *Streptococcus agalactiae*, and *Enterococcus faecalis*, it has been shown in some detail that a respiratory metabolism, with active aerobic electron transport, can be reconstituted by addition of menaquinone and/or heme to the culture (Pritchard & Wimpenny 1978, Sijpesteijn 1970, Brooijmans et al. 2007, Yamamoto et al. 2005, Koebmann et al. 2008, Duwat et al. 2001, Brooijmans 2008). *L. lactis* and *S. agalactiae* show a doubling of biomass when cultivated aerobically in the presence of heme alone (Sijpesteijn 1970, Brooijmans et al. 2007, Duwat et al. 2001) or heme and menaquinone (Yamamoto et al. 2005). In fact, many species of LAB have the *cydABCD* genes present on their genome that encode the *bd*-type aerobic cytochrome. Of the 45 completely sequenced LAB genomes, these *cyd*-genes were found in approximately half the species (Brooijmans 2008). In a recent screening activity (monitoring increased biomass formation during aerobic growth on media containing heme and menaquinone), it was shown that there are potential respirators among *Lactococcus*, *Streptococcus*, *Lactobacillus*, *Carnobacterium*, *Enterococcus*, *Oenococcus*, and *Leuconostoc spp* (Brooijmans 2008).

The tolerance to different stresses is also much improved in the respiratory cell state (Rezaiki et al. 2004, Gaudu et al. 2002). The reconstituted *bd* cytochrome shows active oxygen consumption, leading to low intracellular oxygen levels under aerated conditions and protection against oxidative stress (Rezaiki et al. 2004). This protection against oxidative stress during respiration was clearly shown in a microarray study that reported down-regulation of many stress-related genes (Pedersen et al. 2008). Interestingly, a respiration-negative mutant (a *cydA* mutant) of *S. agalactiae* not only showed a lack of respiratory behavior but also a decrease in virulence (as determined in a rat model) and a decreased ability to grow in blood (Yamamoto et al. 2005, 2006).

Many LAB have components of electron transport chains (ETCs) that enable them to use various intra- and extracellular electron donors and acceptors for improved bioenergetics. The fact that these ETCs need the presence of exogenous cofactors such as heme and menaquinones to be operational does not preclude that their activity is part of the normal lifestyle of LAB. Moreover, the presence of functional ETCs allows enhanced growth and functionality on a wider variety of substrates, which may provide new applications and even new health benefits by using LAB in the food, feed, and pharmaceutical industries. The heme-sequestering by LAB should also lead to a reduced risk of colonic cancer associated with eating red meat (Sesink et al. 1999, Pierre et al. 2006).

Thiol-containing compounds such as glutathione and thioredoxin are found in most living cells. In these cells, thiol-containing compounds can play diverse roles ranging from electron carriers in electron transport processes to detoxifying agents. A general protective role of thiol-containing compounds has been reported in a range of different prokaryotic and eukaryotic microorganisms. These compounds have also been identified in several LAB, and a role in protection against oxidative stress has clearly been demonstrated (Li et al. 2003).

Lactococcus lactis is a bacterial species that is not able to synthesize glutathione. However, most strains within this species were shown to accumulate glutathione when it was supplied in the growth medium (Li et al. 2003). This uptake of glutathione resulted in improved protection against oxidative stress and also in reduced lag time when cultures were transferred in fresh growth medium. These are desired characteristics for use as starter cultures. In a metabolic engineering approach, glutathione production was introduced into *L. lactis* (Li et al. 2005). This resulted not only in improved tolerance to oxidative stress but also in better survival at low pH and high salt (Fu et al. 2006, Zhang et al. 2007).

In *L. plantarum*, thioredoxin was identified as the main thiol-containing component. Although the presence of this compound was not always essential for growth, and thioredoxin-negative mutants were still viable, the presence of thioredoxin clearly rendered increased protection against oxidative stress (Serrano 2008). By overexpression of the thioredoxin structural gene and the thioredoxin reductase gene, improved protection against oxidative stress could be achieved (Serrano et al. 2007).

All in all, a great biodiversity can be found within different genera and species of LAB with respect to industrial robustness. This is based on the presence of different genes coding for specific protection strategies against environmental stresses and/or based on different expression levels of these different genes. With this knowledge in hand, a relatively simple molecular analysis could be used to determine the presence or expression of relevant genes coding for stress response mechanisms. In this way, a molecular screening could be developed for a wide variety of LAB for the important trait of industrial robustness.

Lactic Acid Bacteria and Probiotic Biomarkers

The approach of gene-trait matching, as suggested in the previous paragraph for establishing industrial robustness, has also been used successfully to identify specific genes involved in probiotic

functionality. A large collection of strains of *L. plantarum* were compared for absence or presence of genes with DNA arrays that were designed on the basis of the genome sequence of *L. plantarum* WCFS1 (Molenaar et al. 2005). This lead to specific gene-absence/presence bar plots of each strain that were subsequently correlated with probiotic behavior. For *L. plantarum*, probiotic behavior was observed to be directly related to the ability to reside in the host, and this property was found to be directly dependent on the presence of a gene identified as coding for a mannose-specific adhesin (MSA). By performing such a gene-trait matching exercise, for the first time, a probiotic functionality could be related to the presence of a single gene (Pretzer et al. 2005a). By making a specific deletion of this single gene, leading to loss of adhesion to the host, the ultimate proof was provided that the *msa* gene was indeed a direct biomarker for probiotic functionality. In similar exercises, several of these biomarkers have now been identified in *L. plantarum* and several other probiotic LAB.

GENOME-SCALE METABOLIC MODELING

The two major challenges in industrial food fermentation are reduction of the operational costs and innovation of products and processes (Smid et al. 2005b). Cost-related topics translate into research questions on optimization of production rate or improving the safety of the process. For product and process innovation, the focus is on the development of new product functionalities with clear benefits for the customer such as improved flavor, texture, or nutritional value. Solutions for cost reduction and product innovation can be found either by implementing improved fermentation conditions or by using new (mixtures of) production strains with new or more pronounced functionality. Here, we focus on genomics-based approaches for the improvement of industrial strains that are used in fermentation processes for food and ingredient production. The knowledge of the complete genetic potential of organisms allows us to make predictions of their metabolic capabilities, which are relevant for the outcome of a fermentation process. These metabolic capabilities can be related to various different characteristics of the fermented food product, such as shelf life and microbial safety (organic acid production), flavor (amino acid and lipid degradation), texture (extracellular polysaccharide production), caloric value (sugar conversion), and nutritional value (vitamin production). One of the most comprehensive ways to translate genome sequence data of the fermenting microbes into industrially relevant knowledge of the metabolic properties of these microbes is to construct genome-scale metabolic models (Teusink & Smid 2006, Teusink et al. 2006, Smid et al. 2005a, Pastink et al. 2009, Oliveira et al. 2005).

Reconstruction of Metabolic Network and Model Development

With genome-scale metabolic models, it is possible to integrate the knowledge of all metabolic functions that are encoded and annotated in the genome (Price et al. 2004). Stoichiometric network models are a good starting point for the construction of genome-scale models given that these allow direct input of genome sequence data. Kinetic metabolic models are less suited for this purpose given that essential data, such as in vivo kinetic parameters of enzymes as well as information of in vivo conditions (concentrations of metabolites, pH, water activity, etc.), are far from complete and difficult to obtain experimentally. The stoichiometric modeling approach can therefore be regarded as a logical first step in the exploration of the basic properties of the metabolic network. The quantitative relationships between all compounds involved in metabolic conversions (stoichiometry) are described in a matrix format with all reactions (i.e., enzymes) in one dimension and all metabolites in the other. The entries in the matrix represent the stoichiometry coefficients of the reaction involved. Mass and charge balance of the reactions are essential quality criteria for drafting a good model. To construct a model, first the sequenced genome is

scanned for open reading frames that encode proteins with enzymatic activity. Approximately 20% to 40% of all annotated open reading frames found in genomes of Firmicutes encode genes with a metabolic function. The predicted metabolic enzymes are linked into putative pathways and at this stage of the reconstruction process, all relevent biochemical and physiological knowledge is used to complete the network topology (Francke et al. 2005). In short, the reconstruction process places individual proteins into a functional context that can be a protein complex or metabolic pathway. This process is not always straightforward as is exemplified by attempts to reconstruct metabolic networks from genomes which have a relatively high incidence of gene function loss. The genome of *Streptococcus thermophilus* is a good example with 10% of pseudogenes encoded in the genome (Bolotin et al. 2004, Hols et al. 2005). Reconstruction of a high quality metabolic network always requires additional physiological studies to ascertain the presence and activity of certain predicted pathways (Teusink et al. 2005). In the latter study, the vitamin and amino acid requirements of *L. plantarum* were tested experimentally and compared with the predicted presence of the biosynthesis pathways of these metabolites. In 32 out of 37 cases, the experimental results agreed with the final reconstruction (Teusink et al. 2005). By placing the encoded enzymes in the metabolic context, many refinements of the existing knowledge of the organisms can be made. A nice example is the redefinition of the use of cofactors in methionine biosynthesis in *L. plantarum* (Francke et al. 2005). The initial reconstruction of the metabolism of *L. plantarum*, based on the available genome annotation, indicated the involvement of succinyl-CoA in one of the first reactions of the methionine biosynthesis pathway. However, inspection of the entire metabolic network indicated that succinyl-CoA is not produced by the organism because it does not possess a complete tricarboxylic acid (TCA)-cycle. An in-depth phylogenetic analysis of the candidate gene assigned to this reaction (*metA*) showed that an orthologous MetA protein from *Bacillus subtilus* utilizes acetyl-CoA as reactant (Hacham et al. 2003). The latter study shows that the process of network reconstruction delivers new knowledge of the metabolic interactions in the organism of interest and leads to improved annotation of the query genome.

Sequence-based reconstructions of metabolic networks require extensive manual curation. Therefore, this process is slow and very time-consuming. Several methods have been developed to accelerate the process of network reconstruction for a query species (Notebaart et al. 2006, Karp et al. 2002). One of these methods, named AUTOGRAPH (Notebaart et al. 2006), exploits the availability of well-curated metabolic networks and uses high-resolution predictions of gene equivalency between species, allowing the transfer of gene-reaction associations from curated networks. The authors evaluated the AUTOGRAPH method by using data of a published metabolic network for *Lactococcus lactis* IL1403 (Oliveira et al. 2005). Most of the gene-reaction associations (85%) in the IL1403 network were recovered with the AUTOGRAPH algorithm. In addition, more than 200 additional genes associated to metabolic reactions were identified, underlining the relevance of the AUTOGRAPH methodology as an essential step toward the construction of metabolic networks that are suitable for mathemetical modeling. The assembled networks serve as a starting point and still require verification and refinement before they are suitable for modeling.

For the conversion of a genome-scale metabolic model into a computational model, a number of essential quality checks need to be performed. These relate to mass balance, identifying network gaps and full cofactor connectivity. Network gaps are here defined as reactions in the network that should be there based on biochemical or physiological evidence but for which there is no identifiable gene in the genome (Osterman & Overbeek 2003). For crucial steps in this process of verification and refinement, bioinformatics methods have been developed which are based on partitioning of central and intermediary metabolism into discrete, interconnected components that are shared across organisms (DeJongh et al. 2007). The method described by DeJongh et al. (2007) claims to produce substantially complete reaction networks, suitable for systems level analysis.

The next steps toward a computational model allowing predictions of flux distributions in the metabolic network have recently been outlined in great detail by Feist et al. (2009). The energy coefficients that are related to maintenance and growth and the biomass composition of the organism, preferably under the relevant physiological conditions, have to be available from literature sources or determined experimentally (Feist et al. 2009, Teusink & Smid 2006, Teusink et al. 2006). A genome-scale metabolic model of the lactic acid bacterium *L. plantarum* WCFS1 was constructed based on genomic content and experimental data (Teusink et al. 2006). In this study, the energy requirement for maintenance and growth were determined experimentally. In the traditional approach, the total ATP production for fermentative organisms is estimated on the basis of the production of lactate and acetate (Tempest & Neijssel 1984). However, under the experimental conditions, *L. plantarum* grows on a complex defined-growth medium that results in the input of multiple substrates (i.e., amino acids, citrate) that all can affect the simple relationship between ATP formation and lactate and acetate production. For instance, the model predicts the possibility that serine, which can be taken up from the growth medium, can be converted directly into lactate without contributing to the production of ATP. Teusink et al. (2006) used the genome-scale model to calculate the total amount of ATP generated by the model under the experimental constraints. Because growth is modeled as a sink flux of biomass components, in a proportion that matches the biomass composition, Teusink et al. (2006) determined the biomass composition of *L. plantarum* WCFS1 in great detail using a range of experimental growth conditions and literature sources. A comparison between three species of LAB, i.e., *L. lactis*, *S. thermophilus*, and *L. plantarum*, showed that the overall biomass composition is subject to a large species-dependent variation (Pastink et al. 2009). The resulting organism-specific biomass equations can be inserted in the stoichiometry matrix to test the functionality of the network by examining its capability to predict cellular growth and regeneration using known growth-supporting media.

Once a well-curated metabolic model is converted in a quality-controlled stoichiometry matrix, a number of analyses can be carried out with the objective to explore the metabolic capacities of the model. These techniques are known as constraint-based modeling (Price et al. 2004). The most frequently used method is flux balance analysis (FBA), a linear programming method that optimizes the flux distribution through the metabolic network to obtain the optimal value for some predefined objective, such as product yield or biomass formation (Price et al. 2003). The potential problem with applying FBA is that the objective function is not always known. Under conditions of energy limitation, optimization of biomass yield appeared to be the best objective function to predict the metabolic state of microorganisms such as *Escherichia coli* and *Saccharomyces cerevisiae* (Edwards et al. 2001, Schuetz et al. 2007, Varma & Palsson 1994, Famili et al. 2003). However, attempts to use FBA with biomass optimization as objective function failed to predict metabolic fluxes in *L. plantarum*. This lactic acid bacterium was found to produce lactate, even under glucose-limited chemostat conditions, in which FBA predicted mixed acid fermentation as an alternative pathway leading to a higher yield (Teusink et al. 2006). Given that constraint-based modeling techniques are dealing with yields (Teusink et al. 2006, 2009), extensions to the constraint-based modeling repertoire that can capture these other types of objectives would be much needed to enhance the predictive capabilities of genome-scale metabolic models. Flux variability analysis (FVA) (Mahadevan & Schilling 2003) is an example of an alternative for FBA that allows exploration of the impact of metabolic uncoupling on the metabolic capacities of the model network. FVA calculates for each reaction in the model the range of flux values that are compatible with the experimentally determined constraints on input fluxes.

With the current state-of-the-art, genome-scale metabolic models provide a metabolic context for improvement of the annotation of the gene products and for interpretation and visualization

of functional genomics data. In addition, constraint-based modeling techniques can be used for exploration and prediction of metabolic capabilities of the sequenced organism. This leads to greater understanding of the functioning of the organism of interest (Francke et al. 2005).

Application of Genome-Scale Metabolic Models

Genome-scale metabolic models have been used in a number of different ways to find answers for biological questions related to fermentation. Global metabolic models have proven their value in conjunction with high-throughput data to generate new biological knowledge. Global metabolic models are also being used to predict system behavior thereby generating new data (i.e., flux distributions) (Teusink & Smid 2006). A nice example of the first type of application is the visualization of transcriptomics data on interactive metabolic maps such as those that can be produced with the Simpheny™ modeling platform. Stevens et al. (2008) studied aerobic, nonrespiratory growth of *L. plantarum* on a complex broth medium. Under these conditions, the culture displayed growth stagnation during early growth phase. Transcriptome analysis was performed on culture samples taken before and after the phase of growth arrest. To facilitate data interpretation, transcriptome data were projected on metabolic maps created on the basis of the metabolic model for *L. plantarum* WCFS1 (Teusink et al. 2006). This exercise revealed several pyruvate-associated metabolic pathways that are expressed at a higher level after the growth arrest phase in comparison with the phase preceding the growth arrest (**Figure 1*a***). Common metabolites involved in the group of responding reactions can be uncovered by looking at metabolite connectivity in the interactive metabolic maps (**Figure 1*b***). It was found that resumption of growth after stagnation correlated with activation of CO_2-producing pathways, suggesting that a limiting CO_2 concentration induced the stagnation. This hypothesis was experimentally challenged by analyzing aerobic nonrespiratory growth of *L. plantarum* in a culture that was continuously flushed with 1% CO_2-enriched air. As predicted by the model, the latter conditions allowed noninterrupted growth of *L. plantarum*. This study illustrates the power of functional genomics approaches for the identification of growth-limiting factors in microbial fermentations. Another good example of the use of a global metabolic model in data analysis is presented by Patil & Nielsen (2005). In this study, information from a genome-scale model of *S. cerevisae* is used to analyze patterns of gene expression clustered around subnetwork structures. In the study of Marco et al. (2009), the gene-expression profile of *L. plantarum* in the mouse caecum was analyzed using the genome-scale metabolic model of *L. plantarum* WCFS1 (Teusink et al. 2005, 2006). It was found that genes involved in carbohydrate transport and metabolism form the principal functional group that is up-regulated in vivo compared with exponential phase cells grown in standard laboratory media (Marco et al. 2009).

Reconstructed metabolic networks can also help to discover new substrates for fermentation. The genome-scale metabolic model of *L. plantarum* WCFS1 (Teusink et al. 2005, 2006) predicted the possibility of the organism to grow on glycerol in the absence of fermentable sugars. However, wild-type cultures showed only marginal growth on glycerol-containing media. By applying the widely used serial dilution protocol (Lenski & Travisano 1994), mutant strains were selected that were able to grow faster on glycerol and therefore would be able to take over the population. One of the adapted strains (*L. plantarum* NZ1405) showed a growth yield on glycerol and corresponding fluxes which were similar to *in silico* predictions (Teusink et al. 2009).

Flavor Formation and Amino Acid Metabolism

Amino acids are the building blocks for proteins and the nitrogen source for other important metabolites such as nucleotides, porphyrins, coenzymes, some vitamins, and hormones, and they also serve as a source of energy.

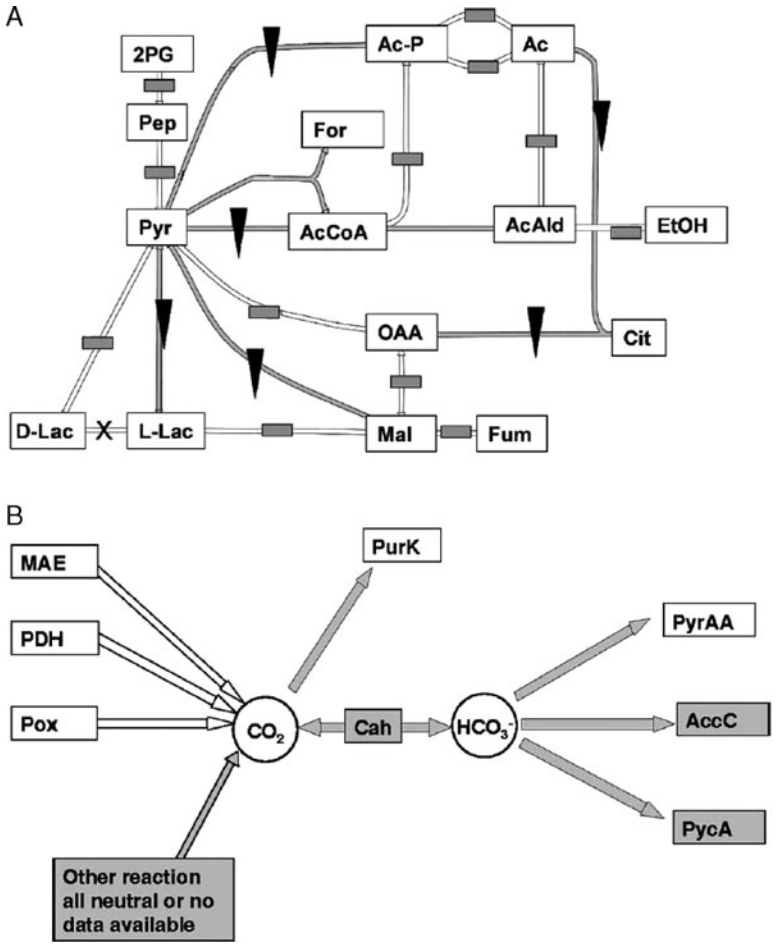

Figure 1

(*a*) Transcriptome data obtained from culture samples collected before (P1) and after (P2) growth arrest, projected on a part of the metabolic map which is zoomed in on pyruvate metabolism of *Lactobacillus plantarum* WCFS1. The filled triangles indicate reactions corresponding to up-regulated genes in P2, the gray boxes indicate no significant regulation, and a multiplication sign indicates that no data are available. All reactions were significantly regulated ($P < 0.05$), except for the pox gene reaction ($P = 0.11$). (*b*) Carbon dioxide–consuming and –producing reactions in *L. plantarum* WCFS1. Enzymes are differentially expressed at time points P1 and P2 in aerobic cultures. The open arrows indicate reactions corresponding to up-regulated genes in P2, and the gray arrows indicate no significant regulation. All reactions were significantly regulated ($P < 0.05$), except for the pox gene reaction ($P = 0.11$). Abbreviations: 2PG, 2-phosphoglycerate; Pep, phosphoenolpyruvate; Pyr, pyruvate; L-Lac, L-lactate; D-Lac, D-lactate; Mal, malate; Fum, fumarate; OAA, oxaloacetate; Cit, citrate; EtOH, ethanol; AcAld, acetaldehyde; Ac, acetate; Ac-P, acetyl phosphate; AcCoA, acetyl coenzyme A; For, formate; MAE, malic enzyme; PDH, pyruvate dehydrogenase; Pox, pyruvate oxidase; PurK, phosphoribosylamino-imidazole carboxylase; Cah, carbonate anhydrase; PyrAA, carbamoyl-phosphate synthase; AccC, acetyl-coenzyme A carboxylase; PycA, pyruvate carboxylase. Data and figure taken from Stevens et al. (2008).

Ammonia is the source of nitrogen for all amino acids; glutamine and glutamate play important roles in this regard. The amination of α-ketoglutarate by the action of glutamate dehydrogenase (GDH) is important because in most organisms this is the major pathway for the formation of α-amino groups directly from ammonia. The carbon backbones derive from the glycolytic pathway, the pentose phosphate pathway or other amino acids. Given that all amino acids except glycine are chiral, stereochemical control of amino acid biosynthesis is important.

Amino acid catabolism has important effects on the quality of fermented foods. For fermenting microbes, the amino acid catabolism also seems to play an important role in obtaining energy in a nutrient-limited environment (Christensen et al. 1999). *Lactococcus lactis* is probably the best studied LAB concerning amino acid metabolism (Van Kranenburg et al. 2002). LAB need amino acids for growth, the number and type of essential amino acids is species dependent and even strain dependent (Garault et al. 2000, Neviani et al. 1995, Van Kranenburg et al. 2002). Non-dairy (plant-associated) strains of LAB are not associated with a nutrient-rich environment such as milk, and they need fewer amino acids than adapted strains. Some nondairy *L. lactis* subsp. *cremoris* and subsp. *lactis* strains only require 1 to 3 amino acids for growth (Van Kranenburg et al. 2002). To obtain all essential amino acids, LAB are able to degrade proteins into small peptides and amino acids, which can be taken up from the environment by transporters (**Figure 2**) (Christensen et al. 1999). These peptide and amino acid transporters have been studied in much detail (Christensen et al. 1999, Kunji et al. 1996). Peptides are intracellularly degraded by peptidases into amino acids (Christensen et al. 1999). Amino acids are then converted by aminotransferases into the corresponding α-keto acids (Smit 2004). A second conversion route for amino acids is initiated by lyases (Smit 2004), such as threonine aldolase which converts threonine into acetaldehyde (Chaves

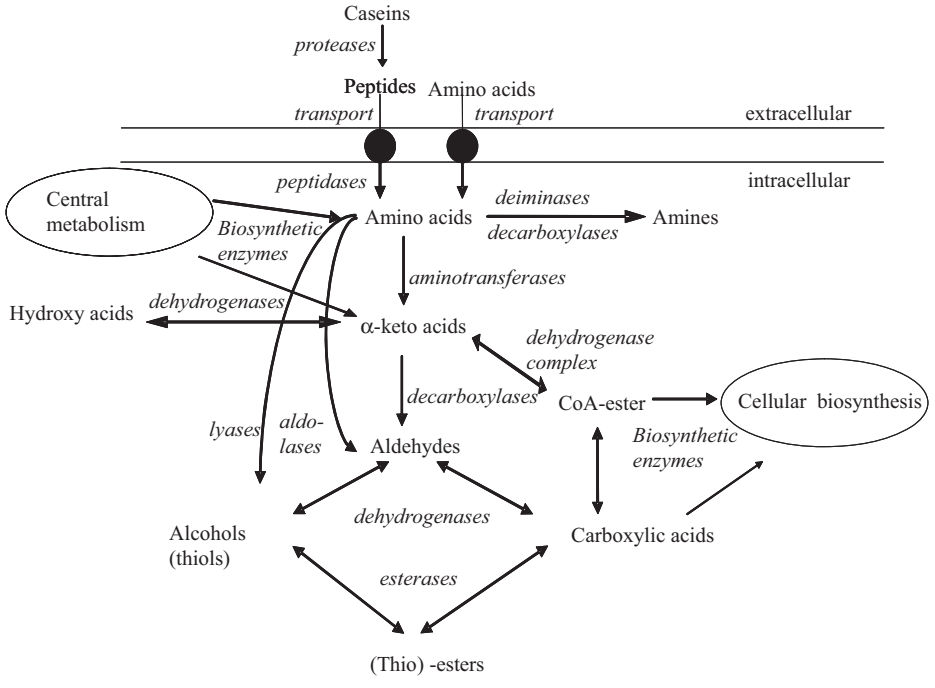

Figure 2

Summary of general protein conversion pathways present in lactic acid bacteria. Adapted from Smit (2004).

Table 1 Examples and descriptions of important amino acid–derived flavors, adapted from Smit (2004)

Flavour	Description	Metabolism
2-methylpropanal	banana, malty, chocolate-like	valine
2-methylbutanal	malty, chocolate	isoleucine
3-methylbutanal	malty, powerful, cheese	leucine
3-methylbutanol	fresh cheese, breathtaking, alcoholic	veucine
Acetaldehyde	yogurt, green, nutty, pungent	threonine
Phenylacetaldehyde	rose, floral	phenylalanine
Phenol	medicinal	tyrosine
Methional	cooked potato, meat like, sulfur	methionine
Methanethiol	"rotting" cabbage, cheese, vegetative, sulfur	methionine
Benzaldehyde	bitter almond oil, character, sweet cherry	phenylalanine

et al. 2002). A third conversion route for amino acids is the deimination/decarboxylation to amines. These amines are studied extensively because of the health risk of biogenic amines (Smit 2004).

The major flavor compounds produced during amino acid metabolism are aldehydes, alcohols, carboxylic acids, and esters. Those derived from methionine, aromatic amino acids, and branched chain amino acids are especially important for the final product flavor of fermented foods (**Table 1**). Another interesting feature of amino acid metabolism is its influence on the flavor profile of fermented products.

Most amino acids do not directly influence the production of flavor compounds, but only indirectly as precursors for aromatic compounds (Yvon et al. 1997). Flavor formation not only depends on the substrate (Guedon et al. 2001a,b) but also on the microbial species and even strains that are used. As described previously (Ayad et al. 1999, 2001; Rijnen et al. 1999; Smit et al. 2004; Williams et al. 2006; Yvon et al. 1997), there is a large variation in flavor-forming abilities among food-fermenting LAB (Pastink et al. 2008). There is also a large variation in amino acid requirements between species and strains. Experiments with single amino acid omissions have shown that the number and type of essential amino acids is strain dependent (Garault et al. 2000, Letort & Juillard 2001, Neviani et al. 1995). Of the LAB that have had their genomes sequenced, *S. thermophilus* has been reported to be auxotrophic for the fewest amino acids (Bracquart & Lorient 1979), and this was confirmed by the genome-scale model recently constructed for this species (Pastink et al. 2009). *S. thermophilus* has a relatively complete set of amino acid biosynthetic pathways, which are often also utilized for amino acid conversions. As a result, this lactic acid bacterium can produce a much larger variety of amino acid–derived flavors than other LAB (Pastink et al. 2008).

With the development of genome-scale models for several different LAB, it has become possible to do in silico comparisons on the metabolic level. In such a recent exercise by Pastink et al. (2009), the genome-scale models of *Lactococcus lactis*, *L. plantarum*, and *S. thermophilus* were compared. On the level of amino acid metabolism, *S. thermophilus* was found to be equipped with, by far, the most complete set of amino acid biosynthesis pathways, followed by *L. lactis* and, far behind, *L. plantarum*. On the level of primary (carbohydrate) metabolism, some intriguing differences were also observed. Although *L. lactis* and *L. plantarum* seemed quite similar in this respect, *S. thermophilus* showed clear differences in its inability to produce ethanol (from pyruvate) and the absence of the first enzymes (oxidative part) of the pentose phosphate pathway (PPP). This last observation is quite interesting given that the PPP is supposed to generate the reducing

equivalents (NADPH) that are utilized in biosynthetic pathways. The logical follow-up question to this finding was how does *S. thermophilus* generate its NADPH? With the support of the genome-scale metabolic model, predictions could be made on alternative options for NADPH-generation (Pastink 2009). The model predicted two reactions as most likely to be involved in NADPH-generation, namely GDH and isocitrate dehydrogenase. Given that isocitrate dehydrogenase, as part of the TCA-cycle, is involved in citrate degradation and *S. thermophilus* is not known to utilize citrate, GDH was identified as the most logical alternative for NADPH-generation. To verify the essential role of GDH for metabolism of *S. thermophilus*, the gene coding for GDH was disrupted. The resulting mutant, surprisingly, showed similar, very good growth as the wild type and similar product formation from sugar. However, amino acids were utilized in higher amounts, leading to ammonia production and different flavor compounds, and citrate utilization was induced in this mutant. These observations strongly suggest that the entire network of amino acid metabolism is used by *S. thermophilus* as an alternative route to NADPH generation. The resulting phenotype of the GDH-knockout is a rapidly growing *S. thermophilus* with an unexpected increase in amino acid utilization and flavor production (Pastink 2009).

Fermentation at Zero Growth Conditions and Ecological Implications

In many natural ecosystems, nutrient—especially carbon source—availability is extremely low and fluctuates with time. Chemoheterotrophic microorganisms in these environments live a feast-or-famine existence. When conditions are favorable, resources are primarily devoted to growth, whereas under nutrient limitation, most energy is invested in survival (Nystrom 2004). This dichotomy has been formalized by Pirt (1965) in his linear equation for substrate consumption. This model explicitly states that metabolic energy is divided between two kinds of demands: the demands of biosynthetic processes (growth) and those of nongrowth-associated processes. These latter processes are collectively termed maintenance and usually assumed to ensure cell survival. However, the exact nature of the underlying processes remains hypothetical. Maintenance energy demand is considered constant for a microorganism under given cultivation conditions. Therefore, when resources are limiting, less energy becomes available for growth-associated processes, resulting in slow or no growth. These conditions of extremely slow growth and high maintenance requirements are commonly encountered in natural environments, as well as in engineered cultivation systems such as fed-batch fermentations.

Most of the current knowledge about the physiology of slow-growing bacteria is derived from the extensively studied transition from exponential to stationary phase during batch growth, as triggered by nutrient—typically carbon—starvation. Under these conditions, metabolism is redirected to alternative substrates and global responses are induced, such as the stringent or general stress responses (Hecker et al. 2007, Srivatsan & Wang 2008). The stringent response results in the shutdown of growth-associated processes and induction of stress defense mechanisms (Srivatsan & Wang 2008). Other responses such as the apparition of subpopulations with distinct physiological states (e.g., dormant cells or spores) are also well documented (Hecker et al. 2007), and mechanisms of genetic variation have been identified (Finkel 2006, Robleto et al. 2007).

The very fast transition from a rapidly growing to a nongrowing state, as observed in batch cultures, is most likely not very relevant under natural conditions, in which exponential growth at maximum specific growth rate is very rarely encountered. In addition, as a result of the high metabolic activity in exponential phase, carbon-starved stationary phase cells are faced not only with limited nutrient resources, but also with the presence of high concentrations of end-products of their own metabolism, which often are growth inhibitory. An alternative model to study bacteria at a defined growth rate is the chemostat, in which the specific growth rate can be directly

manipulated by changing the dilution rate and which provides the additional advantage of a controlled and constant environment. However, chemostats cannot be operated at extremely low dilution rates because of homogeneity problems. This can be overcome by using biomass recycling fermentors (retentostats), i.e., chemostats with complete biomass retention. In these systems, biomass progressively increases, resulting in decreased specific substrate availability. Hence, progressively less energy is available for growth, and the specific growth rate decreases concomitantly (van Verseveld et al. 1984). Such systems have been used to study the physiological properties of various microorganisms or microbial communities at low growth rates (Chesbro et al. 1979, Muller & Babel 1996, Tappe et al. 1999). However, in contrast with carbon-starved stationary phase cells, information on retentostat cultures remains very fragmented. In particular, no genome-scale approach has been applied to the study of extremely slow-growing bacteria under such conditions.

In a recent study, *L. plantarum* was used as a model microorganism to investigate the physiology of slow growth under retentostat conditions (Goffin et al. 2010). *L. plantarum* is found in various environmental niches, including plants (Mundt & Hammer 1968) and lakes (Yanagida et al. 2007), where nutrient supply is limited (Munster 1993, Leveau & Lindow 2001). It has a relatively simple carbon metabolism mainly focused on lactate production, but the genome sequence of *L. plantarum* strain WCFS1 has revealed a high potential for metabolic flexibility and interactions with the environment (Kleerebezem et al. 2003). In order to get a complete overview of its physiology under these conditions, two genome-scale tools were used: metabolome analysis using a genome-scale metabolic model (Teusink et al. 2006) and transcriptome analysis.

Glucose-starved cells are known to switch to alternative substrates. In the study by Goffin et al. (2010), genes involved in the utilization of alternative substrates were up-regulated at low growth rates, reflecting catabolite derepression and indicating that extremely slow-growing *L. plantarum* was prepared for glucose starvation. However, the energy metabolism remained largely based on the conversion of glucose to lactate, despite the large decrease in specific growth rate (more than 100-fold), the decreased specific nutrient availability and hence the increasing proportion of energy required for maintenance. Previous studies of retentostat cultivation of LAB at higher growth rates also reported a relatively stable metabolic profile with lactate as the major end product (Major & Bull 1989). The constant metabolic behavior over a wide range of specific growth rates indicates that extreme carbon limitation and slow growth are not the trigger for a metabolic shift toward higher ATP-yielding pathways, as was reported for *L. lactis* (Koebmann et al. 2002).

Another example of energetically inefficient metabolism was revealed through the metabolic flux analysis performed with a genome-scale model: production of the amino acids Arg, Asp, Met, and Ala was observed, as a consequence of the excess catabolism of branched-chain and aromatic amino acids under conditions of limited ammonium production (Goffin et al. 2010).

A question raised by the observation of amino acid production is, why are branched chain amino acids (BCAAs) and aromatic amino acids (AAAs) catabolized in excess if they do not generate energy? A possible answer lies in the end products of their catabolism (**Figure 2**), a number of which could serve as signaling molecules for the interaction of *L. plantarum* with its environment. The production of phenylacetaldehyde and indolic compounds was observed in this study, and the production of indole-3-lactate (ILA) was suggested by the model simulations. Phenylacetaldehyde and indole-3-pyruvate (the precursor of ILA) can be converted nonenzymatically to phenylacetate and indole-3-acetate (IAA), respectively (Fernandez & Zuniga 2006, Smit et al. 2005), which are known to elicit an auxin-like response in plants (Woodward & Bartel 2005). Similarly, model simulations predicted the catabolism of methionine to α-keto-γ-methyl-thio-butyric acid (KMBA), which was further converted to methional. The observed production of methanethiol, which can be generated nonenzymatically from KMBA or methional (Smit et al. 2005), indicates

that this pathway is active in slow-growing *L. plantarum*. KMBA has been previously identified as the precursor of the plant stress hormone ethylene in several microorganisms (Arshad et al. 2004, Fukuda et al. 1989, Ince & Knowles 1986, Weingart et al. 1999), although the involved enzymes remain unknown. The metabolic analysis thus suggests that *L. plantarum* is capable of producing plant hormones—or at least plant hormone precursors—under conditions of slow, energy-limited growth. This might be a way to divert the plant metabolism toward its own interest. The production of IAA is a common strategy employed by plant pathogens to increase local substrate availability on plant leaves (Beattie & Lindow 1999).

In support of this view, several gene clusters involved in the utilization of β-glucosides, typical sugars from plant cell–wall degradation, were up-regulated under the retentostat conditions (Goffin et al. 2010). In addition, six of the nine *csc*-clusters were highly induced at extremely low growth rates. These clusters encode cell-surface protein complexes specifically found in plant-associated gram-positive bacteria and have been predicted to play a role in the degradation and utilization of oligo- or polysaccharides of plant origin (Siezen et al. 2006). In conclusion, slow-growing *L. plantarum* appears to specifically favor interactions with its environment, more specifically with plants. Interestingly, these observations were made in the absence of any plant-derived material, suggesting that it might constitute an intrinsic behavior that has been selected during evolution of the bacterium in its natural niche.

Modeling Mixed-Culture Fermentations

Interactions between microorganisms are crucial for the performance of mixed culture fermentations. A good example is the yogurt consortium consisting of *Streptococcus thermophilus* and *Lactobacillus bulgaricus*. These two species stimulate each other's growth by the exchange of growth factors associated with purine and amino acid metabolism (Courtin et al. 2002, Crittenden et al. 2003, Driessen et al. 1982). Sieuwerts et al. (2008) suggested connecting genome-scale metabolic models of the individual strains of mixed yogurt starter cultures through a limited number of interactions. Such multigenome or dual strain metabolic models could prove to be effective tools for the optimization of mixed-culture performance with respect to growth and metabolite production. Recently, Sieuwerts (2009) demonstrated the use of a dual-species, genome-scale metabolic model to identify the metabolic reactions occurring simultaneously in *S. thermophilus* and *L. bulgaricus*. Sieuwerts (2009) used the dual-strain model to predict carbon and amino acid fluxes in both bacteria. Also, metabolic reactions were identified that are predicted to be involved in the interactions between the two consortium members. The model predicted the involvement of formic acid, folic acid, and proteolysis in the nutritional exchange between the two species. In addition, the experimental observation of an increased biomass yield on lactose was confirmed in the model. These first attempts to model the interactions between bacterial species in mixed cultures demonstrate the potential value of dual strain or even mutiple strain metabolic modeling for development or improvement of mixed culture fermentations.

METAGENOMICS

Metagenomics approaches are used to reveal the complexity and understand the functionality of undefined complex microbial consortia used in a diverse range of food fermentation processes. Metagenomics refers to the study of genetic material recovered directly (i.e., in a culture-independent way) from environmental samples (Handelsman 2004). Complex fermentations can be defined as the fermentative conversion of complex food raw materials by undefined microbial consortia. In Europe, undefined starter cultures are still commonly used for the manufacturing

of particular types of cheese, such as the Dutch Gouda cheese. Although the composition of the Gouda starter cultures is known on the level of representative species, the overall quality of Gouda cheese can still not be achieved with the use of defined mixed starter cultures (Hugenholtz and & de Veer 1991). This demonstrates our lack of knowledge of the functional diversity at strain level in such starter cultures. In practice, the composition of the Gouda cheese starters is kept constant by applying well-defined procedures for starter propagation and storage. In this way, constant product quality can be assured. Many other artisanal cheeses, such as the Italian Parmigiano Reggiano, are also being produced using starter cultures with undefined composition. The latter aged Italian cheeses are produced using natural whey starters (Coppola et al. 2000). Another dairy example is the production of Kefir, which also relies on undefined starter cultures, organized in so-called Kefir grains (Garrote et al. 2001).

Despite the large economic value of complex fermentations, the basic principles governing the activity and stability of these fermentations are far from understood. These basic principles relate to the presence of microbial interaction networks that determine the industrially relevant features of the end products of fermentation such as stability in composition, acidification rate, and flavor and texture development. Knowledge of which microbes are present in the culture, their relative abundance, their specific contribution in the overall fermentation, their interactions with other microbes, and their interactions with the food matrix is an absolute requirement for obtaining better understanding and control of complex fermentations. Moreover, the food matrix may largely affect the stability and performance of mixed cultures. Hence, the general mechanisms underlying the interaction of the fermenting microbiota with the food matrix should be addressed. Metagenome analysis of undefined mixed starter cultures may shed light on the diversity and functionality of undefined starter cultures. First of all, metagenome analysis provides a parts list of all the genes, pathways, and associated functionalities present in the ecosystem (McHardy & Rigoutsos 2007). To obtain a deeper insight in the genetic diversity of the strains in the starter culture, other typing methods such as AFLP and comparative genome hybridizations will be needed to complement metagenome data.

To date, a number of different metagenome studies have been applied on microbial ecosystems with different levels of complexity. Relatively simple ecosystems such as those found in the acid mine drainage biofilm samples (Tyson et al. 2004) were analyzed by metagenomics approaches. These biofilms are dominated by two or three species of bacteria. This low degree of complexity allowed the researchers to reconstruct almost complete genomes of the two most abundant species (*Leptospirillum* group II and *Ferroplasma* type II) from the metagenome data. These data allowed the authors to construct a qualitative model describing the metabolic interactions between the two abundant species and their environment (pyrite sediment, acid mine drainage fluid, and the atmosphere). On the other hand, extremely complex microbial communities, such as the GI-tract microbiota (Zoetendal et al. 2008, Gill et al. 2006) or the microbial community found in agricultural soil, were subjected to metagenome analysis (Tringe et al. 2005). In the latter situation, 16S rRNA phylotyping indicated the presence of more than 3000 distinct phylotypes and no indication of any organism with a relatively high abundance. Moreover, it is estimated that the human collective gastrointestinal microbiome comprises more than 40,000 bacterial species (Frank & Pace 2008). As a result of this high level of complexity, only a minor proportion of the metagenome reads can be assembled, which makes it hard to analyze the community composition in phylogenetic terms. However, thanks to the fast development of sequencing techniques (Ansorge 2009, MacLean et al. 2009), one single laboratory equipped with the latest sequence machines can now produce more than 1 billion (a giga base) sequenced bases per day. This enables deep sequencing of extremely diverse microbial communities, producing sufficient coverage of less abundant species.

The diversity within undefined starter cultures for food fermentation is probably small if we consider the species level. Most likely, the genetic diversity within these microbial communities is more apparent at the strain level. Further development of effective bioinformatics methods is crucial for the analysis of metagenome data derived from simple as well as complex microbial communities.

Until now, no studies have been published on metagenome analysis of undefined starter cultures. However, the first metagenome studies on cheese starter cultures are under way in the Netherlands with the objective to explore the genetic potential of such microbial cultures and to find out how important genes (those which are associated with industrially relevant traits) are distributed among the collection of constituting strains. In this study, sequenced genomes of individual strains will provide a scaffold for interpretation of metagenome data. The expectation is that metagenome analysis will support rational reconstitution of a defined mixed starter, constituting a minimal set of strains covering the entire metagen(om)e pool.

OUTLOOK

Genomics research is currently the main driver for scientific and technological progress in the field of food fermentation research. Genome sequence information in conjunction with high-throughput analytics and the latest bioinformatics tools provides us an unprecedented view of the metabolic and physiological capabilities of microbes. Based on this detailed biological information, accurate predictions can now be made of behavior and activities of microbes in particular environments, including the well-defined industrial production conditions encountered during food fermentation processes. Genome-scale metabolic models are the first step toward understanding fermentation processes at a systems level. The construction of metabolic models of industrial microbes is rapidly becoming a routine step in tool development, facilitating product and process development related to fermented food products and food ingredients. The next steps of metagenome analysis and the development of multigenome metabolic models will supply tools for studying and eventually controlling complex fermentations involving a multitude of microbial species and strains of the same species. As a result of these developments, strain and process optimization in the future will go from labor-intensive random screening exercises and trial and error approaches toward model-based predictions.

DISCLOSURE STATEMENT

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Errata

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