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Abstract: Fermented foods are of importance worldwide. Most are prepared under nonsterile conditions using mixed cultures, either deliberately or unavoidably. Fungal mixed cultures show interactive relations at various levels. In this paper, inhibitory effects among fungi owing to competition, formation of organic acids, toxic proteins, and mycotoxins are discussed. In addition, fungi show inhibitory effects towards bacteria and vice versa, through pH changes, and excretion of organic acids, antibiotics, peptides, etc. Stimulatory interactions among fungi and between fungi and bacteria relate mainly to carbon and nitrogen metabolism, and they play an important role in the inherent stability of mixed-culture systems maintained by enrichment techniques. Better understanding of natural mixed-culture fermentations has evolved into the development of the concept of cocultivation employing compatible microbial strains of complementary metabolic ability. Especially in the area of direct conversion of complex carbohydrates (e.g., starch, inulin, or lignocellulosic matter into ethanol), cocultivation has much to offer. Genetic modification of starter organisms offers opportunities to improve, for example, their ability to degrade substrate with a minimum of catabolite repression, and produce final products of superior quality. This is illustrated by recent recombinant DNA constructs for alcoholic fermentations.

Key words: food, fungi, interaction, inhibition, stimulation, cocultivation.

Résumé : Les aliments fermentés sont importants partout dans le monde. La plupart sont préparés sous des conditions non-stériles, en utilisant des cultures mixtes, soit de façon délibérée soit de façon inévitable. Les cultures fongiques mixtes montrent des relations interactives à différents niveaux. Dans sa revue, l'auteur discute les effets inhibiteurs chez les champignons dus à la compétition, à la formation d'acides organiques, d'antibiotiques et de mycotoxines. De plus, les champignons montrent des effets inhibiteurs envers les bactéries et vice versa, par l'intermédiaire de changement du pH et l'excrétion d'acides organiques, d'antibiotiques, de peptides etc. Les interactions de stimulation entre champignons et entre champignons et bactéries sont principalement reliées au métabolisme du carbone et de l'azote, et elles jouent un rôle important dans la stabilité inhérente des systèmes de cultures mixtes maintenues par des techniques d'enrichissement. Une meilleure compréhension des fermentations naturelles mixtes s'est développée avec le développement du concept de co-culture impliquant des souches microbiennes compatibles possédant des dispositions métaboliques complémentaires. Surtout dans les systèmes impliquant la conversion directe des glucides (p. ex., l'amidon, l'inuline ou la matière ligno-cellulosique en éthanol), la co-culture a beaucoup à offrir. La modification génétique des organismes de départ offre des occasions d'améliorer par exemple, leur capacité à dégrader le substrat avec un minimum de répression catabolique, et des produits finis de qualité supérieure. Ce propos est illustré par la construction récente d'ADN recombinants pour les fermentations éthanoliques.

Mots clés : interaction, inhibition, stimulation, co-culture.
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Fermented foods with functional fungi

Of the wide range of fungal species, only a few are exploited for the fermentative production of foods and related substances (1). But considering the scale at which these processes are applied worldwide, they are of essential importance to mankind. Owing to economic and process technology factors, most food fermentations are not carried out under aseptic

conditions with pure cultures. Rather, mixed-culture inocula are deliberately used or, unavoidably, mixed-culture systems develop by enrichment (2). Selected examples of mixed-culture fermented foods with functional fungal flora are listed in Table 1. In principle, the phenomenon of mixed-culture food fermentation has positive and negative aspects. Advantages include complex flavor development, as well as stability for shelf life and consumer safety. Disadvantages stem from the risk of losing the balance between the functional component microorganisms, enabling for example, growth and metabolic activity of undesirable spoilage-causing microorganisms, reduced fermentation efficiency, or even food-borne disease.

Balance in mixed cultures is influenced by a complex range of factors (2) that can be categorized as follows:

Table 1. A selection of fungal mixed-culture fermented foods.

Molds[a]	Yeasts[a]	Bacteria[a]	Substrate	Product name	Use
Amyl. rouxii	Endomyces spp. Hyp. burtonii	Ped. pentosaceus Streptococcus faecalis	Uncooked rice	Ragi	Amylolyic starter
Amyl. rouxii	Hyp. burtonii E. fibuliger	Ped. pentosaceus Ent. faecalis	Cassava	Peuyeum	Semisolid snack
Aspergillus oryzae A. sojae	Z. rouxii Candida [Torulopsis] versatilis	Ped. halophilus Ent. faecalis	Soya bean + rice/barley	Miso	Flavoring paste
A. oryzae A. sojae group	Z. rouxii Z. rouxii [soya] Z. rouxii [major] Pichia [Hansenula] spp. C. [Tlp.] spp. C. etchellsii C. versatilis	L. delbrueckii Ped. halophilus Ped. damnosus	Soya bean + wheat + salt	Soya sauce	Flavoring liquid
A. oryzae	Pi. [H.] anomala S. cerevisiae	Leuc. mesenteroides var. saké Lb. saké	Rice	Saké	Rice wine
P. roqueforti	Y. lipolytica	Leuconostoc spp. Lc. lactis Lc. lactis biovar diacetylactis Lc. lactis ssp. cremoris	Milk curd	Roquefort-type blue-veined cheese	Protein food flavoring
P. camembertii	Candida spp. Kluyveromyces spp. Saccharomyces spp. Candida [Tlp.] spp.	Brevibacterium linens Lc. lactis ssp. cremoris Lc. lactis	Milk curd	Camembert-type surface-ripened cheese	Protein food flavoring
P. nalgiovense P. chrysogenum		Micrococcus spp. Staphylococcus spp. Pediococcus spp. Lactobacillus spp.	Meat (sausage)	Salami	Protein food
R. oligosporus R. chinensis R. oryzae Mucor indicus	Trichosporon beigelii Clavispora lusitaniae C. maltosa C. intermedia Y. lipolytica	Klebsiella pneumoniae Enterobacter cloacae Lactobacillus spp.	Mostly soya beans	Tempe	Protein food
	C. [Tlp.] holmii S. cerevisiae Pi. saitoi C. krusei	Lb. plantarum Lb. fructivorans Lb. brevis var. lindneri Lb. sanfrancisco	Rye or wheat flour	Sourdough	Bread leaven
	S. cerevisiae Hsp. guilliermondii K. apiculata	Leuc. oenos Ped. acidilactici Lb. casei	Grape juice	Wine	Liquor
	S. cerevisiae Dekkera [Brettanomyces] spp.	Lactobacillus spp. Pediococcus spp.	Barley, wheat	Lambic-Gueuze	Beer

NOTE: Adapted from Ref. 2.

[a]A., Aspergillus; Amyl., Amylomyces; C., Candida; E., Endomyces; Ent., Enterococcus; H., Hansenula; Hsp., Hanseniaspora; Hyp., Hyphopichia; K., Kloeckera; Lb., Lactobacillus; Lc., Lactococcus; Leuc., Leuconostoc; P., Penicillium; Ped., Pediococcus; Pi., Pichia; R., Rhizopus; S., Saccharomyces; Tlp., Torulopsis; Y., Yarrowia; Z., Zygosaccharomyces.

Intrinsic (food properties)
   Native: water activity, nutrient composition, pH, endogenous
      enzyme activities, and antimicrobial components
   Added or generated: organic acids, alcohols, NaCl, and
      microbial primary and secondary metabolites
Extrinsic (environmental factors)
   Storage temperature, atmospheric relative humidity, oxygen
      supply, and process operations (mixing, size reduction,
      mechanical separations, and heat treatments)

Natural mixed-culture fermentations usually have an element of homeostasis, which is supported by stimulatory and inhibitory effects amongst microorganisms. A few examples of such relatively stable mixed cultures in which yeasts and lactic acid bacteria interact are found in kefir (3), sourdoughs, pickles (4), and wine. In addition, molds and yeasts interact in the presence of bacteria in amylolytic starters (ragi), tempe, and soya sauce (2). Table 2 summarizes the major factors responsible for such stimulations and inhibitions.

Fungal interactions of an inhibitory nature

Among molds
In mold-fermented foods, a certain risk exists of contamination with mycotoxin-producing mold strains. Several investigations were made into the aflatoxin formation by *Aspergillus flavus* and *Aspergillus parasiticus*. In the presence of rapidly growing molds, e.g., *Rhizopus microsporus* var. *oligosporus* (in tempe) and *Neurospora sitophila* (in oncom), the latter appear to deplete available carbon sources (5), reducing aspergilli growth. Moreover, unidentified growth inhibiting factors were formed by *R. microsporus* var. *oligosporus* and *N. sitophila* (5). Volatile metabolites of *Rhizopus oryzae* (*R. arrhizus*), e.g., ethanol, isobutanol, and 3-methyl butanol were shown to double the lag time of *A. flavus* when they were present at 3−6% (v/v) levels in the head space of stored bread (6). In the presence of other aspergilli, inhibition of growth, and (or) aflatoxin formation by *Aspergillus niger* (7) and *A. oryzae* (8) was observed. This was associated with accumulation of oxalic acid by *A. niger* (9) and gluconic acid by *A. niger* and *Aspergillus tamarii* (10). *Aspergillus niger* strains also produce antafumicins A and B (11), which are active against several phytopathogenic fungi of relevance to food quality, e.g., *Botrytis cinerea* and *Fusarium oxysporum*. While selecting *Penicillium nalgiovense* and *Penicillium camembertii* strains for use as commercial starters, it was observed that 29% of the tested strains significantly inhibited test strains of *A. flavus*, *Penicillium commune*, *Penicillium verrucosum*, and *Cladosporium* sp. (12).

Among yeasts
Especially during winemaking, interactions among yeasts are of importance for a timely attenuation and the development of the correct flavor and acidity of the wine. The ecology of the mixed flora in wine fermentation was reviewed earlier (2). During initial fermentation stages, wild yeasts (e.g., *Hanseniaspora guilliermondii*[1] and *Kloeckera apiculata*) are

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[1] Yeast nomenclature is in accordance with Ref. 85. For the sake of recognition, some of the older names as used in the respective publications are mentioned in brackets in the text.

Table 2. Some factors involved in fungal interactions in food fermentations.

Inhibitory

Competition
   Carbon (5)
   Minerals (27)
Antibiotics
   Unidentified (2, 5, 12, 19, 22−26)
   Bacteriocins (4)
   Antafumicins (11)
   Killer toxins (16)
   Mycotoxins (18)
   Iturins (29)
   Peptides (32)
   Pyrrolnitrin (33, 34)
Metabolites
   Free fatty acids (21)
   Octanoic acid (2, 36)
   Decanoic acid (2, 36)
   Oxalic acid (9)
   Gluconic acid (10)
   Ethanol (3, 4, 6)
   Isobutyl alcohol (6)
   3-Methyl butanol (6)
   H_2O_2 (4)
   Diacetyl (4)
   Acetic acid (4, 28)
   Lactic acid (4)
   CO_2 (2, 4)

Stimulatory

Metabolites
   Pyruvate (44)
   Propionate (44)
   Succinate (44)
   Ammonia (40, 41)
   Acetic acid (44)
   Lactic acid (4)
   CO_2 (2, 37, 44)
Mutualistic degradation of carbohydrates
   Xylans (42)
   Starch (2)
   Inulin (73)
   Lactose (3)
   Maltose (48−51)
   Sucrose (47)
Lysis products
   Amino acids (50)
   Peptides (58)

NOTE: Numbers in parentheses are references.

considered essential for later flavor development. Their production of esters, glycerol, and acetoin (13) is significant. Because of their limited ethanol tolerance, wild yeasts will be inhibited and *Saccharomyces cerevisiae* will dominate when more than 5% alcohol is present. In addition to more ethanol, the latter was reported to form considerable levels of *n*-propanol (13) in the presence of wild yeasts and, thus, characteristic flavors were generated. Other wild oxidative

yeasts (e.g., *Pichia membranaefaciens*) can survive in low numbers throughout the fermentation (14). The latter are undesirable as they can lead to spoilage by film formation and acetic acid accumulation in the presence of oxygen.

Among the fermentative *S. cerevisiae* strains, the ecological importance of the killer factor and its possible causative role in sluggish or stuck fermentations (15) and in the dominance of winemaking strains is of much interest. Of the known killer types, K2 appears to be predominant in wineries. Experimental vinifications with mixed cultures of K (K2-killer) and S (sensitive) *S. cerevisiae* revealed that inoculations at a K:S mixing ratio of 1:1 or 1:10 resulted in rapid disappearance of most S cells, with a rather normal fermentation completed by the K strain. At lower K levels (K:S ratio 1:100) a majority of S cells remained alive, but apparently these were not able to lyse and to release essential nitrogen for the fermentation to be completed (15). This mechanism was hypothesized to be a basis for stuck fermentations. From a practical point of view, addition of $(NH_4)_2SO_4$ remedied this nitrogen deficiency and enabled a K-dominated completion of the fermentation. Alternatively, addition of adsorbent material (e.g., active charcoal, bentonite, or yeast cell walls) will inactivate killer toxin and allow S-completed fermentations.

Considering the effect of killer toxins on S strains it is surprising that K strains are not the dominating population in spontaneously fermented wines. Interestingly however, chromosome DNA pulsed field gel electrophoresis of 26 killer-resistant N (neutral) wine strains of *S. cerevisiae* revealed that 92% were derivatives of K2 killer toxin producing strains that had lost the ability to produce active toxin (16). Similar results were obtained in a Bordeaux winery (17) where it was observed that one karyotype of *S. cerevisiae* was stable and dominant in different wine vessels over a 2-year period. Again, almost all isolated strains were N. One could hypothesize that a minority of K cells gradually degenerates (e.g., by point mutations or deletions in the M double stranded RNA (16)) into an N population which may be protected from competing S strains by the presence of killer toxin.

Molds – yeasts

Secondary metabolites include mycotoxins. For example, T-2 toxin produced by *Fusarium* spp. inhibited growth and ethanol production by *S. cerevisiae* at levels from 20 ppm. It was postulated that T-2 toxin affects the energy metabolism of mitochondria (18).

Molds – bacteria

Rhizopus microsporus var. *oligosporus*, the major functional organism in tempe fermentation, was found to produce an antibiotic 5.5-kDa protein (19) with a broad pH range of activity and considerable thermal stability. Although it was active at <1 ppm against *Bacillus* spp., reported growth curves suggest that the antibiotic causes a delay of growth rather than death. This might explain why growth of bacilli in tempe may still achieve significant levels (20). In Roquefort blue-veined cheese, the presence of *Escherichia coli* and *Staphylococcus aureus* is undesirable from a hygienic and public safety point of view. It was observed that in addition to intrinsic inhibitory effects (pH, NaCl, competing lactic acid bacteria) in the cheese environment, the functional mold *Penicillium roqueforti* contributes to the inhibition of bac-

teria (21). The strongest antibacterial effect was manifested by strains having high proteolytic and lipolytic activities, and an indirect inhibition through the formation of free fatty acids was supposed.

Lactic acid bacteria are of great importance in fermented foods. Their antimicrobial effect is ascribed to competition, production of organic acids and other metabolites of low molecular mass, as well as proteinaceous bacteriocins (4). Several dairy starters, e.g., *Lactococcus lactis* ssp. *lactis* biovar. *diacetylactis* and *Lactobacillus acidophilus* reportedly (22–25), have antifungal activity against *A. fumigatus*, *R. stolonifer* and to a lesser extent against *A. parasiticus* and *Rhizopus* spp. The nature of these inhibitions remains to be investigated. Likewise, *Lactobacillus plantarum* and *Pediococcus pentosaceus* were found to inhibit *Fusarium* contamination during barley malting for brewery purposes (26). The same species of lactic acid bacteria also inhibited aflatoxin production by *A. parasiticus* in fermented meat (27). Supposedly, the latter effect included (*i*) failure of *A. parasiticus* in establishing effective competition for the nutrients necessary for aflatoxin production at the prevailing low temperature (15°C), and (*ii*) Mn deficiency caused by accumulation in lactic acid bacteria. Heterofermentative acetic acid production by lactic acid bacteria during soaking of soya beans was found to delay the germination of sporangiospores of *R. microsporus* var. *oligosporus*, the functional mold for tempe production (28).

Bacillus spp. are known to produce several antimicrobial substances. *Bacillus subtilis* is able to produce a group of broad-spectrum iturins including bacillomycin, mycosubtilin, and the peptidolipid iturin-A (29). The latter compound inhibits the growth of, for example, *Penicillium italicum*, *Penicillium viridicatum*, *Aspergillus ochraceus*, *Aspergillus versicolor*, and could be used in biorational control (i.e., using the inhibitory compound rather than the living bacterium) in foods. However, insufficient data are presently available about its safety and in vivo effectivity (29). Apparently, iturin-A does not consistently inhibit growth or aflatoxin production of *A. flavus* and *A. parasiticus* (30, 31). *Bacillus licheniformis* was observed to produce an antifungal 0.77-kDa peptide whereas *Bacillus pumilus* can be effectively used as a biological control agent against *Penicillium digitatum* on oranges when applied as a $10^{10} - 10^{12}$ colony forming units (cfu)/mL suspension (32). In the area of biological control for shelf-life extension, *Enterobacter cloacae* inhibits *Rhizopus* rot in peaches and *Pseudomonas* spp. inhibit *Penicillium expansum* on apples (32), whereas *Pseudomonas cepacia* produces the antifungal pyrrolnitrin, which is of interest in seed protection against, for example *Trichoderma viride* (33) and *Fusarium sambucinum* (34).

Yeasts – bacteria

Whereas *Pediococcus halophilus* is classified as a homofermentative lactic acid bacterium, it nevertheless forms 0.2–0.3% acetic acid from citric acid during the maturation of soya sauce. At the prevailing pH (4.6–4.8) this combination is inhibitory to *Zygosaccharomyces rouxii* (35). Conversely, bacteria can be inhibited by yeasts. For example, the malolactic fermenting *Leuconostoc oenos* are inhibited in wine by the fatty acids (e.g., undissociated decanoic acid) produced by *S. cerevisiae* (36).

Fungal interactions of a stimulatory nature

Among molds

In the area of mushroom cultivation, it is well known that the growth of the edible mushroom *Agaricus bisporus* is enhanced by thermophilic fungi, notably *Scytalidium thermophilum*, in compost. Recently it was pointed out that this stimulation was mainly due to CO_2 formed by *Scytalidium thermophilum* (37). At levels of approximately 0.5% CO_2 the effect was strongest.

Molds – yeasts

In food fermentations of protein-rich raw materials usually some extent of proteolysis takes place. Mold-fermented foods, e.g., soya bean tempe (*R. microsporus* var. *oligosporus*) (20) and cheeses (*P. roqueforti*, *P. camembertii*) (38, 39), as well as some bacterial fermentations, e.g., soya bean kinema (*Bacillus* spp.) (40), have in common that up to approximately 0.2% w/w of ammonia is formed as a result of proteolytic activity. As a result, the pH of such products tends to increase by one to two pH units. It was found that in groundnuts and maize, fermentative production of ammonia could help to degrade the majority of aflatoxin B1 contamination (M.J.R. Nout, unpublished data). Another positive aspect of ammonia formation is that it is a convenient source of assimilable nitrogen for microorganisms with low proteolytic activity, e.g., yeasts (39, 41). On the other hand, excessive ammonia accumulation causes unacceptable off flavors (20).

Molds – bacteria

At a very large scale, animal feed is fermented by ruminants. The rumen microflora is a strictly anaerobic complex mixture including fungi and bacteria, which can deal quite efficiently with raw polysaccharides of vegetable origin. The finding that the rumen mold *Neocallimastix frontalis* achieves higher xylan utilization rate in the presence of the nonxylanolytic rumen bacteria *Bacteroides ruminicola*, *Succinivibrio dextrinosolvens*, and *Selenomonas ruminantium* confirms that mutualism is of importance in the rumen (42).

Yeasts – bacteria

Stable mixed cultures of yeasts and lactic acid bacteria are found in products generated and maintained using an enrichment process (e.g., kefir grains and cereal sourdoughs). Milk kefir is a refreshing dairy beverage that has undergone a mixed lactic acid – alcoholic fermentation by a mutualistic combination of yeasts and lactic acid bacteria embedded in a resilient polysaccharide (kefiran) matrix, forming grains that are used as a source of immobilized inoculum. In Spanish kefir grains the predominant yeasts (*Torulaspora delbrueckii* and *S. cerevisiae*) were unable to assimilate lactose and thus depended on other lactose assimilating microorganisms (lactic acid bacteria and a minority of Lac$^+$ yeasts) for their supply of carbon source. The lactic acid bacteria (mainly heterofermentative lactobacilli, i.e., *Lactobacillus brevis*, *Lactobacillus viridescens*, *Lactobacillus kefir*, and *Lactobacillus fermentum*) produced lactic acid that could serve partly as a source of carbon. Their antimicrobial metabolites supposedly protected the mixed culture against contaminating microorganisms (3).

Sugary kefir (43, 44) and tibicos (45) are similar mutualistic combinations of yeasts and lactic acid bacteria, embedded in polysaccharide (including dextran). They have some resemblance to "Mother of Vinegar" and tea fungus (Kombucha) (46), although the latter have a higher content of acetic acid bacteria. In contrast to milk kefir, sugary kefir and tibicos are used as an immobilized inoculum to ferment sugar solutions (i.e., molasses, sugary tea, etc.), to produce a fizzy beverage with a low (2%) alcohol and acetic acid (0.05%) and a rather high (1%) lactic acid content. In Mexican tibicos (45), *Candida valida* and *Candida famata* were found in stable combination with lactic and acetic acid bacteria. From sugary kefir, several yeasts (*Zygosaccharomyces [S.] florentinus*, *Torulaspora [S.] pretoriensis*, *S. cerevisiae*, *Candida lambica*, *C. valida*, *K. apiculata*) and lactic acid bacteria (*Lactobacillus hilgardii*, *Lactobacillus paracasei* ssp. *paracasei*, *Leuconostoc mesenteroides* ssp. *dextranicum*) were isolated, and interactive properties of *L. hilgardii*, *Z. [S.] florentinus*, and *C. lambica* were investigated (43). Stimulation of the lactobacilli by the culture supernatant of the yeasts was observed and was ascribed to CO_2 production. Subsequently, it was established that *Z. [S.] florentinus* stimulated *L. hilgardii* by several of its metabolites, i.e., CO_2, pyruvate, propionate, acetate, and succinate (44). Interestingly, no stimulatory effect on *L. hilgardii* could be detected by adding vitamins or nitrogen compounds. Conversely, *L. hilgardii* had an inhibitory effect on growth and ethanol production by *Z. [S.] florentinus*. Likewise, it was found that the growth of lactic acid bacteria (*Lactobacillus casei* ssp. *pseudoplantarum*) was stimulated during alcoholic fermentation of molasses with *S. cerevisiae* operating on a cell-recycle principle. This was explained (47) by the invertase activity caused by the high cell density of *S. cerevisiae* releasing significant levels of glucose and fructose from sucrose. At the same time, no evidence for the involvement of nitrogenous compounds was observed.

Cereal sourdoughs could be described as enrichment cultures of relatively stable combinations of yeasts and (mainly) heterofermentative lactic acid bacteria. Sourdoughs based on rye, wheat, sorghum, or other cereal flours are used as a leavening agent in the production of bread. Depending on the type and composition of the flour, dough-making conditions, and incubation environment, rather characteristic mutualistic combinations develop during the long periods (months or years) that sourdoughs are maintained. Typical lactic acid bacteria encountered include *Lactobacillus sanfrancisco* (presently including *L. brevis* ssp. *lindneri*), *Lactobacillus fructivorans*, *L. fermentum*, *L. plantarum*, and *Lactobacillus reuteri*. Frequently occurring yeasts are *S. cerevisiae*, *Pichia saitoi*, *Candida krusei*, *Candida holmii*, *Candida milleri*, and *Saccharomyces exiguus*. The likely mechanism of the stability of sourdoughs has been discussed (48), and recent findings (49–51) may serve to update the understanding of this self-protecting and self-regulating system. Wild strains of sourdough lactobacilli are specialized towards assimilation of maltose, the major source of assimilable carbon in cereal dough. There is a regular supply of maltose, generated by the endogenous cereal amylases. In principle, sugar transport in lactic acid bacteria can occur via (*i*) high-affinity ATP-dependent transport or high-affinity phosphoenolpyruvate:sugar phosphotransferase systems and (*ii*) low-affinity secondary transport systems driven by (electro)chemical gradients that are effective at relatively high sugar concentra-

tions. In maltose-grown *L. sanfrancisco* maltose uptake is via maltose–H$^+$ symport. Within the cell, maltose is phosphorylated and split into glucose-1-phosphate and free glucose. About 50% of the latter leaves the cell through a glucose uniport (52). At lower pH the proton-motive force will be increased and so will be the maltose uptake. When all maltose has been taken up, the exported glucose will be assimilated in pure cultures. However, in sourdough the released glucose will be assimilated by yeasts. It was found in sourdough that glucose is exported at the same rate as maltose is imported (49) reaching glucose levels up to 13 mM. In the presence of sufficient maltose, maltosephosphorylase, produced by lactobacilli, is not glucose-repressed. This factor is considered essential for the domination of the lactobacilli over microorganisms competing for the same carbon source (i.e., maltose). As most other maltose-assimilating organisms are glucose repressed, the export of glucose would thus serve as an indirect and temporary inhibitor (49). Nevertheless, it was shown that maltose-assimilating *S. cerevisiae* compete with *L. brevis* ssp. *lindneri* resulting in reduced bacterial growth and organic acid production (53). It was also suggested (48) that lactic acid bacteria may produce antibiotics and, thus, support the stability of the sourdough population. At present, no direct evidence has been presented to confirm or contradict this hypothesis. On the other hand, most yeasts present in sourdough are quite robust (54). They tolerate acetic acid, but they do not compete with the lactic acid bacteria because they cannot assimilate maltose, only glucose and fructose. The role of yeasts as suppliers of growth factors was suggested (48). Indeed, it was found (50) that *S. cerevisiae* and *S. exiguus* excrete alanine, glutamic acid, leucine, and valine during the stationary phase of growth and that these amino acids stimulated *L. brevis* ssp. *lindneri*. It was shown earlier that lactic acid bacteria from sourdoughs required vitamins (55) and varying numbers of amino acids (56), which could be supplied either by fresh yeast extractives (57), a peptide (58), or live yeast cells (59). Also, it was observed that in wheat doughs, proline and arginine are assimilated faster by mixed yeast − lactic acid bacteria cultures than by the individual microbial components (51). However, the latter study also showed that considerable levels of free amino acids are generated in dough by proteolytic enzymes of the cereal grain and that yeasts (*S. cerevisiae*) consume, rather than release amino acids (51). It appears that the extent of acidification of lactic fermentations of cereal doughs will, inter alia, depend on the availability of micronutrients for lactic acid bacteria. If the latter are deficient in the raw material to be fermented, they will have to be supplied by yeast cells and, consequently, an equilibrium situation will be established with final pH values and acetic acid concentrations enabling adequate yeast growth (60). It still remains to be clarified to what extent yeasts play a role as growth stimulators for sourdough lactic acid bacteria.

Developments in cocultivation technology

In the preceding sections, several mechanisms were discussed whereby components of mixed cultures affect each others growth and metabolic activity. The following section deals with some mixed-culture systems with components that do not show a microbial interaction as such but which have complementary metabolic activity. For such cocultures to be

successful, the organisms must be compatible, and the medium composition and incubation conditions must be an acceptable compromise for both.

Mixed molds

In the area of mold-fermented foods, several pure culture inocula are available commercially. *Penicillium roqueforti*, *P. camembertii*, and *P. nalgiovense* are widely marketed as cheese- and meat-fermentation starters. On sausage casings, mixed inocula of *Scopulariopsis candida* and *P. nalgiovense* showed improved attachment to casings (61) because *Scopulariopsis candida* could penetrate them and *P. nalgiovense* attached into the *Scopulariopsis candida* mycelium. Also, simultaneous growth of *P. roqueforti* and *P. camembertii* is possible, as exemplified in the German cheese Bayerisch Blau (62), where *P. roqueforti* grows inside and *P. camembertii* grows on the surface of the cheese. Considering the potential mycotoxin forming properties of penicillia it would be prudent to investigate whether any stress reactions occur in such mixed cultures that could trigger mycotoxin formation. In the area of tropical mold-fermented foods it is well known that koji's for soya sauce and saké contain well-balanced mixtures of aspergilli, i.e., *A. oryzae* and *Aspergillus sojae*. The fact that such mixed cultures produce more enzymes while consuming less starch compared with single pure cultures has been associated with possible hyphal incompatibity (63). Nevertheless these individual components do not appear to either depend on or to competively exclude one another. Also, the traditional tempe inoculum (usar) contains *Rhizopus* strains (64), which grow as a mixed culture during the tempe fungal fermentation stage.

Mixed yeasts

The major objective of mixed yeast cocultivations is to convert carbohydrate sources directly into ethanol or microbial protein at maximum yield. As high-yielding and ethanol-tolerant *Saccharomyces* spp. are poor degraders of starch or pentoses, cocultures are carried out with yeasts able to degrade such substrates. During selection of compatible pairs of alcohol-producing *Saccharomyces* spp. and xylose fermenting yeasts for production of alcohol from lignocellulosic waste, it was observed that none of the *Saccharomyces* inhibited the xylose fermenters but a number of the latter inhibited *Saccharomyces*, some by killer factors (65). Using a combination of *Pichia stipitis* and a respiratory-deficient *S. cerevisiae* [*diastaticus*], mixtures of 30% xylose and 70% glucose were fermented to ethanol in a continuous culture (66). The performance of *Pichia stipitis* was a limiting factor, requiring a respiratory-deficient *S. cerevisiae* [*diastaticus*] mutant to to create a favourable O_2 profile. In addition, glucose levels had to be limited to avoid repression of the xylose assimilation. Similarly, direct conversion of soluble starch to ethanol, using a coculture of *Endomyces fibuliger* and *S. cerevisiae* was reported (67). *Endomyces fibuliger* is able to degrade starch by amylase and amyloglucosidase. In the absence of yeast extract, it was unable to assimilate the released glucose. Glucose repression of the starch degradation is avoided by its simultaneous conversion to ethanol by *S. cerevisiae*. Another combination of a starch-degrading amylase-derepressed mutant of *Schwanniomyces occidentalis*, with ethanol producing *S. cerevisiae* was used to convert grain sorghum directly

into ethanol and single cell protein (68). The derepressed *Schwanniomyces occidentalis* degraded the starch completely, but because of its low ethanol tolerance, *S. cerevisiae* was required to obtain significant ethanol yields. Per 1000 kg of sorghum, 390 L of ethanol and 185 kg crude protein were obtained (68). In combination with *C. utilis* and the derepressed *Schwanniomyces occidentalis*, protein-enriched sorghum could be obtained by conversion of $(NH_4)_2SO_4$ and starch (69). Considering the world market prices of competing vegetable proteins, it is doubtful, however, whether protein enrichment by fermentation is economically feasible.

Mixed molds and yeasts

Several molds are superior producers of carbohydrases, especially in view of the degradation of cellulose and hemicelluloses. The cellulase — hemicellulase activity of *Sclerotium rolfsii* was used to degrade alkali-treated rice straw. By simultaneous glucose transformation to ethanol using either *S. cerevisiae* or *Candida tropicalis* the glucose repression of the enzyme system could be avoided (70). In a similar way, *F. oxysporum* was incubated with *S. cerevisiae* inoculum on sorghum, resulting in ethanol yield of 68% of the theoretical value (71). Higher ethanol yields (83 – 84% of theory) were obtained in 3 days from chicory and dahlia inulin by saccharification with inulinase produced by *A. niger* and simultaneous ethanol fermentation using an ethanol tolerant *S. cerevisiae* (72).

Mixed molds and bacteria

The importance of no-cook processes for direct conversion of starch into ethanol were discussed earlier (2). Significant savings can be made on energy and sophisticated equipment if thermal gelatinization and liquefaction of starch and media sterilization are omitted. Such processes require a microbial system that efficiently degrades native starch. Cocultures for that purpose have been described (73). Another requirement is adequate control of contaminants, especially if continuous systems are employed. Interestingly, it was found that *Aspergillus awamori*, *R. oryzae [japonicus]*, and *Zymomonas mobilis* coimmobilized in alginate for direct conversion of native rice starch into ethanol, performed well in the presence of 0.1% neupectin-L, an oligogalacturonic acid with a degree of polymerization of 10 having antimicrobial activity against contaminating bacteria (73).

Mixed yeasts and bacteria

An elegant coculture system composed of the nitrogen fixing *Acetobacter diazotrophicus* and the starch degrading *Lipomyces kononenkoae* was described (74). In a basal mineral and vitamin medium without added nitrogen source, starch degradation by *L. kononenkoae* was enabled by nitrogen supply through *Acetobacter diazotrophicus*, the latter depending on assimilable carbon supplied by the yeast. It was estimated that approximately 40% of the fixed N_2 was released to the yeast, being considerably higher than the nitrogen release by other N_2-fixing organisms (e.g., *Azospirillum* spp.).

Impact of modern biotechnology

As an alternative of natural mixed cultures or compatible cocultures to ferment food-grade materials, the tools are now at hand to combine the key functions of the mixed flora into single strains of starter cultures. Several reviews deal with

the molecular-genetic aspects of foreign gene expression (75) and the application of recombinant DNA fungi in food manufacture (76). It would fall outside the scope of this paper to give a full account of all recombinant DNA constructs of interest in the food industry, but a few areas will be mentioned which appear relevant to the current issue of substitution of mixed cultures or cocultures.

The first example is the direct conversion of polysaccharides into ethanol. Limited ability to digest starch by *S. cerevisiae* can be overcome using recombined DNA constructs expressing *Schwanniomyces occidentalis* amylase and glucoamylase or *A. oryzae* α-amylase (76), and maltodextrins can be fermented by *S. cerevisiae* expressing *C. tropicalis* α-glucosidase (76) or by *S. cerevisiae* glucose derepressed mutants (77). Similarly, the glucose production of *A. oryzae* for saké brewing could be improved by increasing its ratio of glucoamylase/ α-amylase activity from 0.37 to 1.01 by expression of *Aspergillus shirousamii* glucoamylase (78). Inulin can be fermented directly into ethanol by a *S. cerevisiae* expressing *Kluyveromyces marxianus* inulinase, xylose by a *S. cerevisiae* expressing *B. pumilus* endo-1,4-β-xylanase and β-glucosidase or *Pichia stipitis* xylose reductase and xylitol dehydrogenase, and lactose by a *S. cerevisiae* expressing *A. niger* or *Kluyveromyces lactis* β-galactosidase (76). Compared with parent strains, the performance of some of these constructs is definitely superior giving earlier brewing attenuation (77) and higher alcohol yield (78) with similar or improved organoleptic characteristics (78). At present, no published data are available enabling a comparison of the performances of recombinant DNA constructs with those of equivalent cocultures.

Another area of relevance is winemaking that experiences an increasing popularity of dry yeast inoculants. Using an elegant mitochondrial DNA restriction analysis, the fate of wine *S. cerevisiae* dry inoculant during vinification at the winery level was evaluated and compared with the activity of wild yeast strains present in the must (79). Although the inoculated strain was responsible for the fermentation, the first stages of the fermentation were dominated by wild *S. cerevisiae* strains, some of which persisted throughout the process. Several recombinant DNA *S. cerevisiae* wine yeast constructs have been prepared, e.g., expressing K1 and K3 killer toxins for dominance (80) or *Trichoderma longibrachiatum* β-1,4-endoglucanase for increased frutal aroma (81). Other glycosidases (e.g., β-glucosidase, α-arabinosidase, and α-rhamnosidase) are also effective flavor enhancers by releasing volatile grape terpenols (e.g., linalol and geraniol) from their nonvolatile glycosides (82). Hybrids of cryophilic *Saccharomyces bayanus* and mesophilic *S. cerevisiae* wine strains were stable, had promising fermentation characteristics at 10°C, and released more flavor substances than parent strains (83). Presently, very little data are available to evaluate the effectiveness of these new strains. In this respect it should be taken into account that genetic modification could also lead to inferior characteristics as was found with California and Pasteur champagne parent strains, when compared with their inbred homothallic and hybrid heterothallic derivatives (84).

Conclusion

In conclusion, fungal interactions observed in natural mixed-culture fermentations may be efficiently exploited at a cocultivation level or by recombinant DNA constructs com-

bining relevant metabolic abilities. More data is becoming available to demonstrate that conversion yields of polymeric substrates can be significantly increased with these approaches. A challenge for the future is to focus ecological and biotechnological experience on the improvement of safety and organoleptic quality of foods.

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