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The Microbiology of Malting and Brewing

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SUMMARY

Brewing beer involves microbial activity at every stage, from raw material production and malting to stability in the package. Most of these activities are desirable, as beer is the result of a traditional food fermentation, but others represent threats to the quality of the final product and must be controlled actively through careful management, the daily task of maltsters and brewers globally. This review collates current knowledge relevant to the biology of brewing yeast, fermentation management, and the microbial ecology of beer and brewing.

INTRODUCTION

Beer, like any fermented food, is an immutably microbial product. Microbial activity is involved in every step of its production, defining the many sensory characteristics that contribute to final quality. While fermentation of cereal extracts by *Saccharomyces* is the most important microbial process involved in brewing, a vast array of other microbes affect the complete process (Fig. 1). Microbial interdiction at every step of the barley-to-beer continuum greatly influences the quality of beer. For an overview of the processes of malting and brewing, see the work of Bamforth (1).

BREWING YEAST

Although all strains of *Saccharomyces* will produce ethanol as a fermentation end product, in practice the strains employed in the production of beers worldwide are classified into the categories of ale and lager yeasts. The seminal text on brewing yeast is that of Boulton and Quain (2).

Ale yeasts, which are *Saccharomyces cerevisiae* strains, are the more diverse yeasts and have been isolated in innumerable locations worldwide. Such yeasts are often referred to as "top-fermenting" yeasts, insofar as in traditional open fermenters they rise to the surface of the vessel, facilitating their collection by skimming, ready for repitching into the next fermentation. The hydrostatic pressure in modern cylindroconical fermenters, many of which may contain up to 10,000 hl of fermenting beer (3), tends to overcome this tendency of ale yeast, which accordingly collects in the cone of the tank.

The nomenclature of lager yeast ("bottom-fermenting" yeast, on account of its tendency not to rise to the surface under any set of fermentation conditions) has evolved, passing through iterations of *S. carlsbergensis* and *S. cerevisiae* lager type to the currently accepted name, *S. pastorianus* (4–6). Irrespective of its name, lager yeast is a more complex organism than ale yeast, and it has been proposed that it arose in perhaps two separate steps involving the hybridization of *S. cerevisiae* with *S. bayanus* (7, 8).

It has generally come to be considered that lager yeast (unlike

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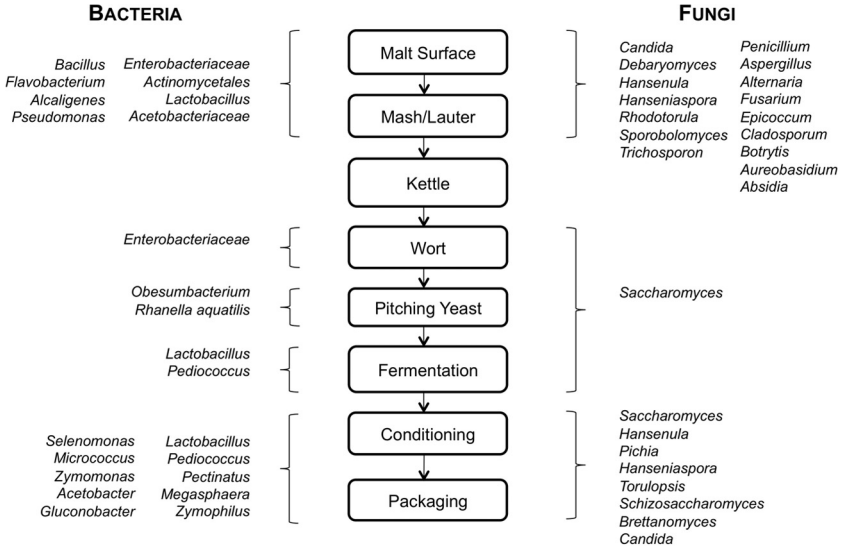

FIG 1 Microbiota of malting and brewing. The diagram shows an overview of bacterial and fungal species previously reported at all major stages of beer production. (Adapted from reference 156 with permission of the publisher.)

ale yeast) is not readily isolable from nature, though it was recently proposed that the cryotolerant strain of yeast that melded with *S. cerevisiae* in domestication circumstances to produce *S. pastorianus* originated in southern beech forests in Patagonia and represents *Saccharomyces eubayanus* sp. nov. (9).

There is far more diversity among ale strains than among lager strains (10). The latter can be divided into the Carlsberg and Tuborg types, based on chromosomal fingerprints (11), and there are comparatively minor differences between them. Casey (11) suggests that this far greater diversity of ale strains reflects their isolation in multiple locations, whereas the lager strains emerged from a very limited locality.

The genome of *S. cerevisiae* has been sequenced fully (12). Whereas the strains used for sequencing were haploid, brewing strains of yeast are polyploid or aneuploid, with 3 or 4 copies of each chromosome (13, 14). There is only limited information on the significance of this for yeast behavior, with one of the few studies being that of Galitski et al. (15), who found very few effects.

It is generally believed that the multiplicity of gene copies makes for a more stable yeast organism (10), and there may be a boost of enzyme production leading to more rapid metabolism of wort components, e.g., maltose (14). There appear to be some fundamental differences between the chromosomes in haploid and polyploid strains (10, 16). Despite the polyploid nature of brewing strains, there is evidence that there is chromosomal instability (11, 17). Repercussions include changes in flocculation and utilization of maltotriose (18). Yeast drift can also arise through the partial or complete loss of mitochondrial DNA, leading to the production of so-called "petites" (19–21). Although alcoholic fermentation is anaerobic, meaning there is no role for a respiratory function in mitochondria, the latter organelles do have other metabolic functions in brewery fermentations (22–24).

Typing of Yeast

The differentiation of brewing strains has been reviewed by Quain (25) and Casey et al. (26). Traditional approaches include examining colony morphology on plates (27), the ability of yeasts to metabolize melibiose (lager strains can do so due to their elaboration of an α-galactosidase, whereas ale strains cannot [28]), temperature tolerance (29), flocculation tests (2), behavior in small-scale fermenters (30, 31), and oxygen requirements (32, 33). Latterly, the emphasis has been on DNA-based techniques, including restriction fragment length polymorphism analysis (34), PCR (35, 36), karyotyping (11), and amplified fragment length polymorphism analysis (37). Additionally, pyrolysis mass spectroscopy (38), Fourier transform infrared spectroscopy (39), fatty acid methyl ester profiling (40), and protein fingerprinting (41) are other possibilities.

Yeast Resources and Handling

Several yeast culture collections and providers are available (Table 1). Larger brewing companies, however, tend to manage their own in-house strains, including the storage of master cultures (43, 44). Back-ups of these organisms are deposited with third parties. Storage of cultures in liquid nitrogen is deemed preferable in terms of survival, shelf life, and genetic stability compared to storage on agar, in broth, or by lyophilization (43).

While there are still brewers who simply repitch yeast from one fermentation to the next *ad infinitum* ("backslopping"), concerns about genetic drift and selection of variants mean that most brewers pitch with yeast newly propagated from the master cultures at intervals. The frequency is typically 10 to 15 "generations" (this word in a brewing context refers to successive fermentation batches), though even this may be excessive in terms of yeast deterioration (45–47). The chronological events occurring in the life cycle of yeast in brewery fermentations and the consequences for population ageing have been addressed (48).

Yeast propagation, involving batches of successively increasing volumes, has been reviewed by Maule (49) and Quain (44). Yields of biomass can be limited at the high sugar concentrations employed (Crabtree effect), and some have advocated fed-batch systems analogous to those used in the production of baker's yeast (50). Gene transcription during propagation (51) and fermentation (52) has been investigated (also see reference 53). Newly

TABLE 1 List of culture collections[a]

Collection	Type of organisms	Web address
American Type Culture Collection (ATCC)	All types	www.atcc.org
CABI Bioscience	Filamentous fungi	www.cabi-bioscience.org
Centraalbureau voor Schimmelcultures	Filamentous fungi and yeasts	www.cbs.knaw.nl/
Collection Nationale de Cultures de Microorganismes	All types	http://www.pasteur.fr/recherche/unites/Cncm/index-en.html
Die Deutsche Sammlung von Mikroorganismen und Zellkulturen	All types	http://www.dsmz.de/
Herman J. Phaff Culture Collection	Yeasts and fungi	http://www.phaffcollection.org/
National Collection of Industrial and Marine Bacteria	Bacteria	www.ncimb.co.uk
National Collection of Yeast Cultures	Yeasts	www.ncyc.co.uk

[a] Derived from the work of Bamforth (42).

propagated yeast does not usually "perform" as expected in the initial commercial fermentation, in part due to a lack of synchronicity in the cell population (54).

An alternative approach to handling yeast that is attracting some attention in brewing but which is already applied widely in wineries is the use of dried yeast (55–58). Concerns include an impaired ability to handle vicinal diketones (VDKs) (59; see below), impaired flocculation of yeast, and deteriorating foam and clarity in the beer (60).

Key to successful storage and handling of brewing yeast, irrespective of whether it is handled as a slurry or as a dried product, are the storage carbohydrates that it elaborates (61). Glycogen has attracted much study as an important carbon and energy reserve in brewing yeast (62), while the importance of trehalose as a stress protectant is well studied (63).

Fermentation Control

In pursuit of a constant fermentation performance, brewers seek to achieve consistent fermentations, which demands control of the key variables of yeast quantity and health, oxygen input, wort nutritional status, temperature, and yeast-wort contact (mixing).

While traditional techniques for counting yeast, such as counts with a hemocytometer, are still widely applied, there is increasing use of instrumental approaches, often inserted in-line to achieve automated pitching control. Devices include those operating on the basis of assessing capacitance/permittivity (64, 65) and according to principles of light scatter (66).

The viability of yeast has long been assessed by staining of cells with methylene blue; however, other staining approaches have been proposed (67, 68). While these techniques inform about whether cells are alive or dead, they do not gauge the healthfulness (vitality) of the cells (69). Diverse procedures have been nominated for assessing this parameter, but none has been adopted universally. Techniques include assessments of glycogen (70), sterols (71), ATP (72), oxygen uptake rate (73), and acidification power (74, 75), as well as modifications of the methylene blue viability test (76).

While it has long been recognized that a proportion of oxygen is needed by all yeast cells to support the production of the sterols and unsaturated fatty acid components of the cell membranes (77, 78), there is a less-than-clear appreciation of why different yeast strains vary considerably in the amount that they demand (32, 79). Traditionally, the oxygen is introduced to the wort, although there have been proposals to pitch unaerated wort with yeast that has been supplied directly with oxygen (80). Ensuring contact of all yeast cells with oxygen when yeast is present at a high density is important (81). On the other hand, oxygen represents one of the stress factors encountered by yeast (82), while others include ethanol, which limits the practical alcohol concentrations that can be achieved in brewery fermentations (83). Accordingly, there is interest in the development of yeast strains with greater tolerance of high-gravity conditions (84). A review of all the stresses likely to be encountered by brewing yeast has been provided by Gibson et al. (85). There is extensive use of high-gravity brewing in commercial brewing (86), with the attendant osmotic and alcohol stresses.

One major variable that perhaps receives less detailed analysis and control than others in fermenter control is actually the wort composition (87, 88). Most brewers simply regulate the strength of the wort (degrees Plato) and pitch on that basis, assuming that the relative balance of the diverse nutrients within the feedstock is consistent and modulated by the malt selection and how that malt is processed in the brewhouse. To a first approximation, this seems to be a reasonable situation on an experiential basis, although there are two variables that many brewers do seek to regulate more closely, i.e., the clarity of the wort and the concentration of zinc ions (89, 90), although other additions to promote fermentations, particularly those with higher-strength wort, may be employed (91, 92). The presence of insoluble particles in wort (which are derived in the brewhouse and are present at a level in inverse proportion to the extent that they are removed in clarification stages prior to fermentation) promotes yeast action by their ability to nucleate carbon dioxide, thereby releasing bubbles (93). Two effects may be at play, namely, the increased resulting tendency of yeast to be moved through the fermenter and the impact that this has on lowering dissolved CO_2 levels in the wort from inhibitory concentrations (94).

The contact of yeast and wort in fermentation is not inconsequential. Often, huge fermenters are filled with several batches of wort, leading to quandaries over precisely when the yeast should be added to the fermenter and how to ensure homogeneity of yeast-wort contact throughout the vessel (95). Mechanical mixing is uncommon but advocated (96).

Fermentations may be monitored in various ways, including measuring the decrease in specific gravity of the wort (including in-process measurements) (97–99), CO_2 evolution (100, 101), the pH decrease (102), and ethanol formation (103), as well as camera-based observation of events in the fermenter (104).

At the completion of fermentation, yeast is recovered either for disposal (commonly to animal feed or production of yeast extracts [105]) or for repitching. For open fermenters, ale yeast is skimmed from the surface of the vessel, but for closed cylindroconical vessels the yeast is harvested from the cone. The population of yeast cells differs in the cone, with stratification such that older cells are located beneath the younger, more vital ones (64, 106, 107).

Harvested yeast may either be pumped to the next fermenter

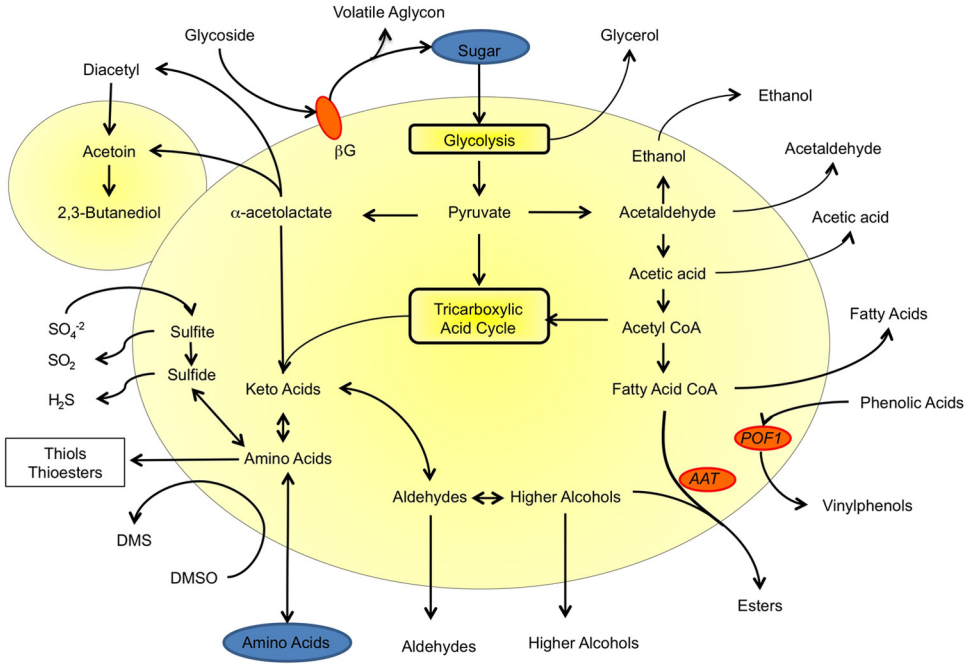

FIG 2 Overview of *Saccharomyces* metabolic activities influencing beer quality. This simplified schematic summarizes the main metabolic pathways linked to beer flavor modulation by *Saccharomyces*. βG, β-glycosidase; DMS, dimethyl sulfide; DMSO, dimethyl sulfoxide.

filling with fresh wort (cone-to-cone pitching) or stored in either a pressed or slurry form (2). It may receive acid washing to kill any bacteria that may have developed in the slurry (108). Its collection from fermenters is often through the use of centrifuges, creating damage that has implications for subsequent performance (109). The impact of serial repitching was addressed by Jenkins et al. (110), who showed that extents of deterioration vary between yeast cells.

Flocculation

A key influence on harvesting of yeast is its flocculation behavior. The flocculation of brewer's yeast was recently reviewed by Soares (111), Vidgren and Londesborough (112), and Verstrepen et al. (113). The clumping of yeast cells involves the binding of lectin-like proteins to mannoprotein receptors, promoted by calcium ions to overcome the negative zeta potential. The surface hydrophobicity of the cell is also important, and this may relate to the tendency of cell aggregates to migrate to the surface of a fermenter (top-fermenting yeast) (114). There are factors present in certain malts that lead to the premature flocculation of yeast (115, 116; see below), and meanwhile, there may be additional antiyeast materials in malt (117).

Products of Yeast Metabolism in Brewery Fermentations

During fermentation, yeast excretes a range of molecules, in addition to ethanol and CO_2, that can affect flavor (Fig. 2). While there are diverse brewing yeast strains, it has been argued that the vast majority do not differ very widely in their gene complement such that they produce unique flavor components. Strain-to-strain variation exists in the levels of some products, but there are extremely limited instances of brewing yeasts procuring flavor-active species that are not produced to at least some extent by other brewery strains.

The exception is the ale strains used for the production of traditional hefeweizen products in Germany. They have a gene coding for ferulic acid decarboxylase, which converts ferulate derived from cereal cell walls to 4-vinylguaiacol (118–121), imparting a spicy, clove-like character.

All brewing strains produce glycerol (120–122), vicinal diketones (VDKs) (123), alcohols (124, 125), esters (126, 127), short-chain fatty acids (33), organic acids (120), and diverse sulfur-containing substances (128, 129). The levels of each category that are found in beer are dependent in part upon the yeast strain, but at least as important are the precise fermentation conditions that exist, including pitching rate (130), temperature, extent of oxygen addition, C:N ratio, and duration of fermentation and maturation (2).

Of especial significance are the VDKs, diacetyl and pentanedione, which afford a buttery or honey-like character that is undesirable for most beers (123). They are produced during fermentation by the nonenzymatic degradation of acetolactate and acetohydroxybutyrate, which are metabolic intermediates in pathways of amino acid synthesis that leak out into fermenting wort. Yeast, however, will scavenge the diacetyl and pentanedione, reducing them to butanediol and pentanediol, respectively, using a range of enzymes (131–133), provided there is sufficient healthy yeast to do so. This can, however, be a relatively prolonged event, depending on the level to which the brewer seeks to lower the VDKs. Recent developments targeted toward accelerating the handling of VDKs include the addition of the enzyme acetolactate decarboxylase (e.g., derived from *Klebsiella aerogenes*), which leads to the conversion of acetolactate directly to acetoin (134). An alternative approach has been to thermally degrade newly fermented beer (denuded of yeast) to break down the precursor molecules before diverting the stream through a column of im-

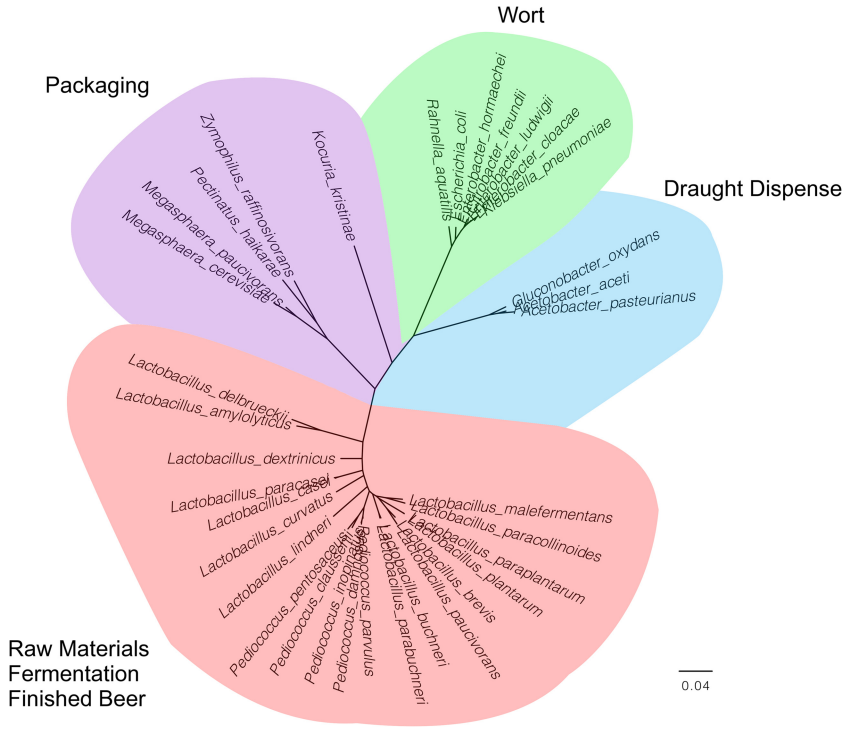

FIG 3 Phylogeny of primary beer spoilage bacteria. The maximum-likelihood tree shows the most common beer spoilage bacteria, colored by ecological niche and taxonomic group. Red, *Lactobacillales*, isolated primarily from raw materials, fermenting beer, and packaged beer; blue, acetic acid bacteria (*Rhodospirillales*), limited primarily to spoilage of draft dispensers; green, *Enterobacteriaceae*, occasional wort contaminants; purple, *Veillonellaceae*, which cause spoilage in packaged beer.

mobilized yeast (135). This represents the largest extant commercial use of immobilized yeast, although there is much interest in the potential for such yeast in continuous beer production systems (136).

A range of esters are produced by brewing yeast, with perhaps the most important being isoamyl acetate, owing to its very low flavor threshold. Such esters are produced by the action of the enzyme alcohol acetyltransferase (AAT) on higher alcohols and acetyl-coenzyme A (acetyl-CoA) (137, 138). A major factor affecting the extent of lipid production—and, by extension, ester formation—is the amount of oxygen and unsaturated fatty acids in wort (139). AAT is also responsible for the production of thioesters (140). The mechanisms and physiological roles of ester formation in *Saccharomyces* fermentation were recently reviewed elsewhere (137, 141).

There is some interest in selecting yeast strains with elevated β-glycosidase (β-G) activity for enhancing the aroma of specialty beers. β-Glycosidases in *Saccharomyces* cleave nonvolatile glycosides derived from hops, fruit, and other plants used in brewing, cleaving a sugar moiety from the aglycon. The free aglycon may exhibit aromatic activity in this state and represents a largely untapped source of aroma in beer (142). However, there are doubts about whether the cellular location of β-G is commensurate with an ability to release such aroma compounds (143).

Production of SO_2 by yeast is significant not only with respect to a direct contribution of this material to aroma but also on account of its role in protecting against flavor deterioration, notably by scavenging the carbonyl substances that afford staling (144). Mutant yeasts capable of increased production of SO_2 have been reported (145, 146). Wort composition, for example, the level of lipid material in the wort, also has a profound effect on SO_2 production (147).

The production of SO_2 and hydrogen sulfide is linked *a priori*, through reduction of the former by sulfite reductase (148). However, it is possible to regulate their levels independently (149).

Dimethyl sulfide (DMS) is often a significant contributor to the character of lager beers, though sometimes it is reviled (150). While the bulk of the DMS originates from thermal degradation of a malt-derived precursor (3), some yeast strains are capable of reducing dimethyl sulfoxide that also originates in malt (151). Mutant forms of yeast lacking the necessary enzyme have been isolated (146), and it appears that mitochondrial function is required for the activity (24).

Genetic Modification

Saerens et al. (152) and Nevoigt (153) have reviewed genetic strategies for improving brewing yeast. As regards the application of genetic modification (154), there is sensibly no such modified strain in commercial use globally (155).

MICROBIAL ECOLOGY OF MALTING AND BREWING

While beer fermentation itself is a monocultural microbial phenomenon—with few exceptions—the complete process of beer production involves a succession of microbial constituents that dramatically influence the final product (Fig. 3). The knowledge and management of these constituents at all stages have led to dramatic increases in beer quality. Methods for interrogating the

microbial consortia of beer and brewing ingredients have been reviewed elsewhere (156, 157).

Barley

Brewing microbiology begins in the barley field, where plant-microbial interactions and the microbiological status of the grain both pre- and postharvest can have serious implications for brewhouse processing and beer quality. Although these microbes do not survive the malting and brewing processes, secretory factors may persist, affecting downstream quality.

In the field, a vast range of bacteria and fungi are present on the barley, originating from the surrounding environment, insects, and animals. Weather and other conditions will naturally affect the microbial community growing on barley, and unusually wet years in particular can encourage microbial growth and pathogenesis (158). Following harvest, barley may be stored for a time prior to malting to overcome dormancy. During this time, microbes continue to grow on and interact with the living grain, and conditions must be monitored carefully to ensure that the grain is stored in a low-moisture, low-temperature environment to minimize microbial growth (159), which can be extremely detrimental to beer quality.

A diverse set of microbes has been detected on barley (for a thorough list, see reference 158), but only a few plant-pathogenic fungi have notable relevance to beer quality. *Fusarium* spp. and several other fungal pathogens of barley and other cereals are capable of producing mycotoxins that survive the brewing process and can be detected in finished beer (160–162). A number of mycotoxins have been detected in barley, including deoxynivalenol (DON; also known as "vomitoxin"), nivalenol, T-2 toxin, HT-2 toxin, and diacetoxyscirpenol (163). DON has been implicated as the most abundantly and commonly produced mycotoxin in *Fusarium*-infected grain (164). The toxicogenic effects of DON and related mycotoxins are well established for animals and humans (for a review, see reference 165), leading to the adoption of strict quality standards for DON in malt. In addition to potentially threatening human health, high concentrations of these mycotoxins have been shown to inhibit yeast growth during beer fermentation (166, 167).

Fungal infection of barley also causes a problem with more immediate consequences to the consumer: gushing. This phenomenon is caused by hydrophobic fungal peptides (hydrophobins), which serve as nucleation sites for CO_2 bubbles in beer, resulting in the spontaneous release of gas and overfoaming once the container is opened (168). Hydrophobins are surface-active, amphipathic proteins produced by most filamentous fungi to shield the growing hyphal tip, facilitating growth across liquid-air interfaces (169). As a result of ubiquitous expression, dangerous levels are introduced into the beer process stream when excess fungal growth occurs on the grain preharvest or during storage. The link between fungal growth and gushing is well established, and suppression of *Fusarium* growth on barley by in-field application of lactic acid bacterium (LAB) starters has been used successfully to diminish gushing (170).

Fungal infection of barley may also promote gushing by eliciting a stress response in barley leading to the production of foam-active compounds, principally the plant pathogenesis-related nonspecific lipid transfer proteins (nsLTPs) (171, 172). These are normally expressed in healthy barley corns and are important foam-promoting factors when expressed at normal levels (171–173). In response to microbial infection, expression levels are increased (174), a phenomenon that has been suggested to explain gushing in beers brewed from infected grain (171, 175). In addition, nsLTPs and other pathogenesis-related proteins are toxic to yeast cells and inhibit respiration at high concentrations (176–178). However, other authors dispute the gushing potential of these proteins and instead have found that only high-molecular-weight barley proteins are positively correlated with beer gushing (179).

Different plant pathogenesis factors can also precipitate premature yeast flocculation (PYF) during fermentation. Yeast flocculation occurs when cell wall mannoproteins bind lectin-like glycoproteins on other cells, resulting in aggregation and settling (180). This normally occurs at the end of fermentation, as sugars present during early fermentation associate with the lectin surface, preventing interaction (180). In PYF, yeast aggregation and settling occur prior to full attenuation of sugar, resulting in incomplete fermentation, off-flavors, and significantly decreased beer quality (181–183). PYF can be initiated by a range of polysaccharides naturally occurring in the barley husk (183–185), released either in response to microbial infection or by degradation of the husk by microbial enzymatic activity (186).

Malt

The process of malting comprises three primary steps—steeping, germination, and kilning. The successive steeping and aeration cycles promote more than plant growth, and although kilning diminishes viable counts of microbes (159, 187), microbial activity during germination can influence beer quality downstream. After kilning, low-moisture conditions must be maintained carefully to avoid microbial spoilage, especially as malt is somewhat hygroscopic and rich in soluble nutrients at this stage.

Upon steeping, microbial cells multiply rapidly on the grain and in the steep water, stimulated by dissolved nutrients, moisture, warmth, and aeration (159, 187, 188). In general, the growth of microbes during germination is deleterious to malt quality, and microbes residing on the surfaces of barley corns can compete for oxygen with the embryo, inhibiting germination (188, 189) and decreasing rootlet growth and alpha-amylase activity (190). In addition, several bacteria and fungi isolated from barley could produce significant quantities of the plant hormone indole-3-acetate *in vitro*, as well as low quantities of gibberellic acid and abscisic acid, potentially affecting germination and enzyme production (191).

The inhibitory effects of microbial growth on malt quality may be diminished by changing the steep liquor between air rests, reducing dissolved nutrients and reintroduction of suspended biomass (188), and by controlling the steep temperature, as microbial growth is limited at lower temperatures (159). Several authors have also recommended microbial inoculation of steep liquor to control the growth of detrimental microbiota during germination. *Wickerhamomyces anomalus* has been shown to inhibit *Fusarium* growth on malt when added during steeping, thereby preventing hydrophobin production and beer gushing (192). *Geotrichum candidum* (193) and *Lactobacillus plantarum* (194) have also been shown to diminish *Fusarium* growth on malt. The addition of LAB has also been shown to decrease rootlet growth, diminishing malting loss (195).

Wort

During the mashing of malt, the microbial load diminishes, but thermotolerant microbes, especially homofermentative LAB, remain active in the nutrient-rich, high-moisture environment (187). Bacterial growth during mashing can have beneficial consequences, and mash acidification by lactic acid bacteria can improve the extraction, fermentability, and nitrogen yield of wort and the foam stability, color, and flavor of beer (196). The beneficial effects of mash acidification are achieved in most breweries by direct acid addition, but microbial acidification remains the only acceptable means for mash acidification in breweries adhering to the *Reinheitsgebot* German beer purity law (196). Bacterial growth can also cause serious problems during extended mashing. For example, *Bacillus* spp. can cause excessive acidification and nitrosamine formation by reduction of nitrate to nitrite (197, 198). Growth of *Clostridium* in the mash or in wort can produce high levels of butyric acid, giving the beer a cheese-like aroma (199). Excessive bacterial growth on malt can also retard mash filtration, probably due to production of dextrans (200), and suppression of bacterial growth has been shown to improve the filterability, extraction efficiency, and nitrogen yield during mashing (192). Fungi growing on malt can produce beta-glucanases and xylanases, lowering wort viscosity and improving mash filtration (192), though this lower wort viscosity has been negatively correlated with beer foam quality (201).

Following the mash, wort is boiled for an extended period, effectively sterilizing the wort. However, wort is a nutrient-rich, high-pH (~5.5) medium, so once it leaves the kettle it is vulnerable to opportunistic spoilage agents if appropriate precautions are not taken to ensure rapid fermentation, which serves to stabilize the wort against most contaminants. The most prevalent wort spoilers are Gram-negative enterobacteria, especially species of *Klebsiella*, *Citrobacter*, *Enterobacter*, *Obesumbacterium*, and *Escherichia* (202). In wort, these bacteria produce DMS, organic acids, and 2,3-butanediol in abundance, giving beer an unpleasant fruity or vegetal aroma (202, 203). Growth of enterobacteria also inhibits the growth of *Saccharomyces* (202). Enterobacteria are aerobic and are not sensitive to hop-derived antimicrobials, so they can thrive in the oxygenated, high-sugar, high-pH environment of wort, but they are inhibited by ethanol and low pH, so they are not found in finished beer (202). However, some enterobacteria, especially *Obesumbacterium* and *Enterobacter*, are contaminants of pitching yeast, leading to serial inoculation into successive batches (202). By modern brewing convention, these bacteria are categorically considered contaminants, but it has been suggested (202) that limited activity of enterobacteria was once characteristic of certain English ales. Increased hygienic standards and updated equipment have dramatically changed this perspective in the past 40 years, and enterobacteria are now considered unwelcome (and uncommon) guests in most worts.

Beer

The cooled, oxygenated wort is pumped to fermenters, where strains of *Saccharomyces* are added to rapidly convert the wort to beer through the fermentation of maltose and other sugars to ethanol and carbon dioxide. The resulting conditions are hostile to the growth of most microorganisms: beer is high in ethanol and carbon dioxide, contains hop-derived antimicrobial compounds, and is low in pH, oxygen, and residual nutrients, though ~20% of all reducing sugars in all-malt wort consist of oligosaccharides that are not utilized by *Saccharomyces* and are felt by many brewers to contribute to the mouthfeel and flavor of the beer, as well as supporting potential microbial spoilage. The stringent conditions of beer fermentation have selected for unique groups of yeast and bacteria specialized for growth in beer—and usually not much else. All species described in this section are the most prevalent contaminants of beer from the start of fermentation through to the packaged product.

Gram-positive bacteria. LAB are prevalent in nature, associated with plant matter (including barley and malt) and humans, among other environments. Thus, their entry into the brewery is both frequent and inevitable, and their widespread dispersion in malt dust, aerosols, and equipment is unquestionable. Fortunately, most LAB are prevented from growing in beer due to the antibacterial activity of hop-derived compounds (see below). However, those that have adapted to the stringent conditions of beer (namely, developed hop tolerance) are the most prevalent beer spoilage microorganisms of the present day. These include *Pediococcus damnosus*, *Pediococcus inopinatus*, *Pediococcus dextrinicus*, *Pediococcus pentosaceous* (204), *Pediococcus parvulus* (205), *Pediococcus claussenii* (206), *Lactobacillus brevis*, *Lactobacillus casei*, *Lactobacillus paracasei*, *Lactobacillus plantarum*, *Lactobacillus buchneri*, *Lactobacillus curvatus*, *Lactobacillus coryneformis*, *Lactobacillus parabuchneri*, *Lactobacillus delbrueckii*, *Lactobacillus fermentum*, *Lactobacillus fructivorans*, (207), *Lactobacillus paucivorans* (208), *Lactobacillus paracollinoides* (209), *Lactobacillus amylolyticus* (210), *Lactobacillus lindneri* (211), *Lactobacillus paraplantarum* (212), *Lactobacillus brevisimilis* (213), and *Lactobacillus malefermentans* (214). Note that the above list includes all recognized species of LAB previously detected in beer, though not all exhibit high spoilage potential. Among these, *L. brevis* and *P. damnosus* probably represent the greatest threat to beer, being the most commonly reported contaminants of finished beers. Most species of LAB show high degrees of ethanol tolerance, but ethanol tolerance is conserved within species, and hop resistance plays a more prevalent role in conferring beer spoilage capability (215). Thus, LAB involved in other food and beverage fermentations, such as *Leuconostoc*, *Oenococcus*, *Lactococcus*, *Streptococcus*, and *Enterococcus*, have not been isolated from beer.

LAB spoil beer through acidification, haze formation, and/or diacetyl production, which gives the beer an intense aroma of artificial butter. Many strains can also produce exopolysaccharides (EPS) in beer, lending an oily consistency or, in extreme cases, the formation of slime (215, 216). *Pediococcus* spp., in particular, are known for diacetyl and EPS production, and because they exhibit strong growth at low temperatures, they are common contaminants of both lager and ale breweries (204).

Aside from LAB, very few Gram-positive organisms have been reported in beer. *Kocuria kristinae* (previously *Micrococcus kristinae*) has been reported as a beer spoiler, but with low potential due to its sensitivity to hops, ethanol, and pH (217). The *Bacillaceae* have not traditionally been considered capable of beer spoilage, but four species containing the hop resistance *horA* gene (see below)—*Bacillus cereus*, *Bacillus licheniformis*, *Staphylococcus epidermidis*, and *Paenibacillus humicus*—have been isolated from spoiled, home-brewed beer and exhibited growth when reinoculated into beer (218).

A major factor limiting which organisms can spoil beer (particularly ethanol- and pH-tolerant Gram-positive bacteria) is the

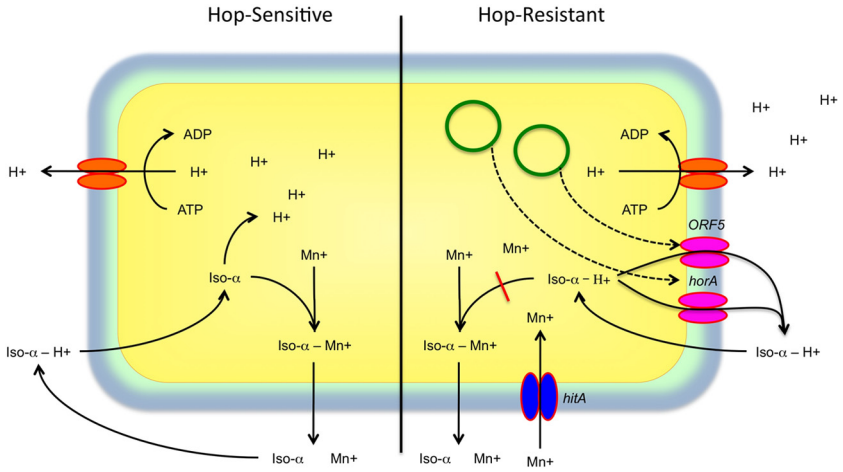

FIG 4 Schematic overview of main mechanisms of hop toxicity and resistance in Gram-positive bacteria. Iso-α, iso-alpha-acids. Green loops indicate plasmids carrying the hop resistance genes *horA* and *ORF5*.

presence of hop-derived bittering compounds. Hops contain a range of compounds that inhibit the growth of Gram-positive bacteria. Principal among these are the iso-alpha-acids, which are produced from the hop alpha-acids during wort boiling (1). The iso-alpha-acids function as proton ionophores, dissipating the transmembrane proton gradient, decreasing cytoplasmic pH, and squelching proton motive force (219). This impairs enzymatic activity and nutrient transport, halting growth and ultimately killing the cell (220, 221). In addition, iso-alpha-acids participate in transmembrane redox reactions in association with manganese, causing oxidative stress to the bacterial cell (222), which explains the manganese-dependent enhancement of transmembrane potential observed previously (219, 221) (Fig. 4).

Hop challenge involves multiple mechanisms for bacteriostasis, and thus hop resistance involves a complex cellular response. A key factor in hop resistance is the plasmid-encoded, ATP-dependent transporter protein HorA, which purges hop compounds from the cell (223). Another plasmid-encoded multidrug transporter, ORF5, has been shown to confer hop resistance across multiple species of LAB (209). In addition, resistant cells upregulate expression of the hop-inducible cation transporter HitA, which may facilitate manganese transport into hop-stressed cells despite proton gradient dissipation (224). Hop stress in *L. brevis* also induces expression of a broad range of proteins involved in redox homeostasis, DNA repair, and protein repair, facilitating a shift toward energy balance and metabolic regulation to cope with low-pH conditions and oxidative stress (225). The multiple modes of inhibition exerted by hop challenge and the complex response elicited in resistant bacteria indicate that hop-resistant LAB are specialized for growth in beer through resistance to oxidative and acid stress (219, 222). Early work demonstrated that iso-alpha-acids have no impact on the growth of Gram-negative bacteria (226), but no further work has been done to determine the mechanism for resistance. Iso-alpha-acids also display little or no inhibition of yeasts. In *Saccharomyces cerevisiae*, this is due to relegation of iso-alpha-acids within the vacuole, their active expulsion across the cell membrane, and modification of the cell wall structure in response to hop stress (227), but the mechanism has not been studied in other yeasts.

Gram-negative bacteria. The aerobic, Gram-negative acetic acid bacteria (AAB) were once a serious threat to beer production, but their activity in modern beer production is negligible, as oxygen exposure can be avoided (204). In a bygone age, when beer was aged in barrels without the luxuries at the disposal of the modern brewer (e.g., conical steel fermenters and controlled headspace), AAB were a more prevalent threat, and they are still commonly found in barrel-aged beers (228). These AAB include *Acetobacter aceti*, *Acetobacter pasteurianus*, and *Gluconobacter oxydans*. These bacteria spoil beer through the oxidation of ethanol to acetate, effectively transforming beer into vinegar.

As dissolved oxygen concentrations declined in beers with the introduction of modern techniques, a new threat replaced the enemies of old. These new contaminants were the obligate anaerobic *Veillonellaceae* organisms, including *Pectinatus*, *Megasphaera*, *Selenomonas*, and *Zymophilus*. Members of this family belong to the Gram-positive phylum *Firmicutes* but stain Gram negative and possess a lipid bilayer. Most *Veillonellaceae* organisms are found in aquatic sediment or mammalian intestines, but those mentioned above have been reported only for beer, where they cause spoilage through haze formation, overwhelming production of propionic acid, acetic acid, hydrogen sulfide, and mercaptans, and inhibition of yeast growth and alcohol production (229). *Veillonellaceae* organisms have been reported to grow in beer at a pH of ≥4.3 and with ≤5% (wt/vol) ethanol (204). Similar to enterobacteria, some of these bacteria can be introduced to beer through their association with pitching yeast (230), causing product spoilage before ethanol and pH reach inhibitory levels and contaminating future batches through repitching. Spoilage cases from these organisms have surfaced only in recent years, concurrent with the growth of nonpasteurized beers and with improved bottling equipment leading to lower dissolved oxygen in the packaged beer (204).

Zymomonas mobilis is a problem in beers containing adjunct sugars. This bacterium can grow under conditions of extreme pH (>3.4) and ethanol content (<10% [wt/vol]), is iso-alpha-acid resistant, and spoils beer through production of acetaldehyde and hydrogen sulfide, giving the beer an aroma of rotten eggs (204). However, this bacterium cannot ferment maltose or maltotriose, the primary carbohydrates in wort and beer, so it is not a common contaminant of beer (204). Spoilage is limited to beers supple-

mented with other sugars, e.g., sucrose added to carbonated English cask ales (231).

Wild yeasts. Any organism that has not intentionally been introduced to a beer by the brewer is considered a spoilage organism. Thus, the principal form of wild yeast contamination in beer is from rogue strains of *Saccharomyces cerevisiae* (232). These spoil beer through ester or phenolic off-flavor production (POF), formation of haze or sediment, or superattenuation, leading to overcarbonation and diminished body. In *Saccharomyces* and other yeasts, POF is caused by decarboxylation of *p*-coumaric acid and ferulic acid to 4-vinylphenol and 4-vinylguaiacol, respectively, a property engendered by the *POF1* gene (233). These compounds give beer an unusual medicinal or spicy clove aroma and are atypical for most beers, though they are considered a marker trait of German wheat beers and some Belgian ales, as the yeasts used in these beers are POF positive.

Brettanomyces yeasts (teleomorph *Dekkera*), including *Brettanomyces bruxellensis*, *Brettanomyces custersii*, and *Brettanomyces anomalus*, are nefarious contaminants of most beers and other alcoholic beverages, though their presence is often encouraged in other types of beer (see Deviant Fermentations). These yeasts spoil beer through the production of the highly volatile phenolic compounds 4-ethylguaiacol and 4-ethylphenol, lending the aroma of bandages, sweat, and smoke. A number of other metabolites, including copious acetate production in the presence of oxygen (234), result in a wide range of off-flavors produced by these yeasts. In spite of its reputation, *Brettanomyces* is a desired component of certain beers, particularly Belgian lambic (see below) and fruit beers, in which its beta-glycosidase activity enhances fruit aroma (235). In a bygone age, *Brettanomyces* character was even considered an indispensable element of proper English stock beers, and it was first described for English beer, giving this yeast its name (236).

A large number of other non-*Saccharomyces* yeasts are capable of growth in beer, but their spoilage potential is limited under optimal storage conditions, due to the combined factors of oxygen limitation, ethanol toxicity, and competition with *Saccharomyces*. These include *Pichia anomala*, *Pichia fermentans*, *Pichia membranifaciens*, *Pichia guilliermondii*, *Candida tropicalis*, *Candida boidinii*, *Candida sake*, and *Candida parapsilosis* (all reported in reference 232); *Candida guilliermondii*, *Candida glabrata*, *Candida valida*, *Saccharomyces unisporus*, *Torulaspora delbrueckii*, and *Issatchenkia orientalis* (all reported in reference 237); and *Kluyveromyces marxianus*, *Debaryomyces hansenii*, *Zygosaccharomyces bailii*, *Zygosaccharomyces bisporus*, *Schizosaccharomyces pombe*, and *Kloeckera apiculata* (all reported in reference 207). Most of these yeasts spoil beer through the production of off-flavors (especially organic acids and POF), haze, sediment, or surface films. Like AAB, these yeasts are common throughout breweries, especially in unwashed sampling ports and on other surfaces contacting beer. They are opportunistic contaminants, causing spoilage when conditions are favorable, but are generally not an issue in modern brewing practices, due to improved oxygen control. These yeasts are more of an issue in barrel-fermented beers, where oxygen ingress stimulates their growth, hence the need to limit the headspace during barrel maturation.

Biogenic Amines

Biogenic amines (BAs) and polyamines present another serious consequence of microbial contamination of beer (238–243; for a review of BAs in beer, see reference 244). These compounds are found in a wide range of foods and beverages, including fish, meat, cheese, and wine, and are formed by microbial decarboxylation of amino acids (245, 246). BAs pose a health hazard to sensitive individuals, resulting in allergy-like reactions (247), migraine (248), and/or toxic reactions with monoamine oxidase inhibitor drugs (249, 250). BAs in beer are formed primarily during fermentation but can also be produced by microbes in barley, malt, wort, and hops (251, 252). LAB are most commonly implicated in biogenic amine formation (251), but enterobacteria and some strains of *Saccharomyces* may also play a role (251). Therefore, limitation of microbial activity during malting, wort production, and fermentation is the best strategy for minimizing BA formation (239). In mixed-culture and "spontaneous" fermentations (see Deviant Fermentations), however, many of these organisms are crucial components of the fermentation, and these beers often contain higher levels of BAs than other beers (238, 251).

Packaging and Distribution

Packaging and distributing beer represent the two greatest challenges to the microbial stability of beer. During all previous brewing processes, from wort boiling to cold conditioning, wort and beer are contained within eminently cleanable, seamless stainless steel vessels (assuming that state-of-the-art equipment and hygienic practices are employed). Upon packaging, however, the virgin product travels across complex surfaces in the filling equipment, is briefly exposed to the atmosphere, and is parsed into small vessels. Biofilms may form on the surfaces of filler heads and in filling areas, increasing the risk of microbial contamination (253). Kegs represent a particular risk, as these are reused constantly, often circulated among different breweries, and contain enclosed, complex surfaces. Kegs may see questionable conditions during return to the brewery—including prolonged exposure to warm temperatures and air—making them a potential breeding ground for colonization and biofilm formation by the microbial panoply described above.

The moment beer leaves the brewery it is out of the brewers' control and is subject to whatever conditions distributors, retailers, and consumers may impose. The package may be exposed to fluctuations in temperature, light, and/or turbulence, all of which degrade the quality of the inner product and (with the exception of light exposure) promote microbial growth. Even under optimal storage conditions, a significant volume of beer may spend several months in shipment and storage prior to consumption, increasing the probability of microbial spoilage, given the scale and distance of contemporary global beer distribution. The industry long ago addressed this issue through product stabilization via filtration, pasteurization, or some combination thereof. However, increasing demand for unpasteurized beers in recent years has increased the incidence of microbial contamination in packaged beer by microbes such as *Pectinatus* (204).

Draft systems present a particular threat to the stability of beer, as the serving mechanism itself involves introducing foreign objects into the package *in situ*, after which its stability is governed by the storage, serving, and hygienic conditions of the serving site (pub, restaurant, or private residence). The container is penetrated by the coupler, which allows gas to flow into the container and beer to flow out. Compressed carbon dioxide enters the container, maintaining the appropriate level of carbonation and driving the beer through the draft lines, through the tap faucet, and

into the drinking vessel. While the compressed gas itself should be sterile, microbes may be introduced directly into the keg by the gas lines and coupler if they have not been cleaned and sterilized properly. Dispensing equipment (coupler, lines, and tap faucet) comprises a large area of surface contact with the beer and contains a number of complex surfaces that are resistant to cleaning. Biofilms may hypothetically form along these surfaces, especially in microfissures in the draft line and crevices in the dispensing equipment. These biofilms may support the survival of microbes not typically found in beer, but the composition of draft beer biofilms has yet to be elucidated. Beer experiences a certain residence time in this unrefrigerated environment before it is dispensed to the next customer. During this time, cells may multiply in the beer trapped in the lines, causing spoilage through haze, off-flavors, and even BA production (250). A study of draft and bottled beers in Canada found that of all beers tested ($n = 98$), only the draft beers (4 of 49 beers) contained dangerous concentrations of BAs (>10 mg/liter)—implying postpackaging microbial growth—and these investigators suggested that draft beers should be avoided by BA-sensitive individuals (250). The best means of controlling draft contamination is through observation of proper hygienic practices, including cleaning and sanitization of all equipment prior to connection to a keg, replacement of all lines at regular intervals, and proper storage conditions.

DEVIANT FERMENTATIONS

For 99% of the beers on this planet, *Saccharomyces* is the sole microbial component, and any deviation is considered a flaw. However, other beers, which are gaining increased popularity worldwide, incorporate secondary, non-*Saccharomyces* starter cultures, uncharacterized "natural" starter cultures, or autochthonous, nonstarter microbiota during fermentation or maturation, leading to distinctive, unusual products.

Autochthonous Fermentations

The best-known mixed-fermentation beers are the lambics of Belgium and (to a lesser extent) their offspring, the "coolship ales" of the United States. The uniting feature of both of these beers is the lack of any inoculation whatsoever. Instead, these beers are fermented by a mixture of brewery-resident yeasts and bacteria introduced to the cooling wort during overnight exposure in a shallow, open vessel known as a coolship. The following morning, the beer is pumped into oak barrels and allowed to ferment—without racking off the lees—for up to 3 years before packaging. The brewhouse environment appears to select for similar microbiotas, as lambic and coolship ale exhibit similar successions of microbial communities. The first month is dominated by enterobacteria, including *Klebsiella*, *Enterobacter*, *Escherichia*, *Citrobacter*, *Serratia*, and *Pectobacterium* (228, 254), and non-*Saccharomyces* yeasts, primarily *Kluyveromyces* in lambic (216) and *Rhodotorula* in coolship ale (228). Enterobacteria present during this stage produce several compounds responsible for the aroma of 1- to 2-month-old lambic, including 2,3-butanediol, ethyl acetate, higher alcohols, and acetic, lactic, and succinic acids (255). After 1 month, LAB (primarily *Pediococcus*) and *Saccharomyces* spp. dominate the main, alcoholic fermentation, which lasts 3 to 4 months. *Brettanomyces bruxellensis* dominates the remainder of the fermentation and maturation (216), producing a range of characteristic aroma compounds in lambic, including caprylic and capric fatty acids and their ethyl esters (256, 257). *Brettanomyces* also hydrolyzes EPS produced by *Pediococcus* during the main fermentation, reducing the viscosity of lambic (216). Since these beers are fermented and matured in the same vessel *sur lies*, the unique flavor profile is likely influenced by microbial autolysis, contributing both substrates (lipids, proteins, and carbohydrates) and intracellular enzymes to participate in unbridled reactions. One- and 3-year-old lambic is often blended and allowed to re-ferment in the bottle to produce gueuze, which exhibits a markedly different aroma due to regrowth of *Brettanomyces* in the bottle (258).

Less familiar to Western palates are the many traditional beers enjoyed throughout Africa. These include such popular libations as *ikigage* of Rwanda (259), *bili bili* of Chad (260), *tchoukoutou* of Benin (261), *tchapalo* of Côte d'Ivoire (262), *pito* in Ghana, Togo, and Nigeria, and *dolo* in Burkina Faso (263). These beers are made from malted sorghum, and often malted millet, and otherwise involve roughly similar brewing processes consisting of a sour mash followed by alcoholic fermentation. The beers are consumed fresh in an actively fermenting state. They are opaque, sour, and mildly alcoholic and contain large amounts of suspended solids but are highly nutritious and comprise a large proportion of the local diet (261). All of these beers are fermented by backslopping flocculent yeast slurry from a previous batch, an identical process to that traditionally used for European beer inoculation. Thus, *Saccharomyces cerevisiae* dominates the fermentation of these beers, similar to other spontaneous beer fermentations. A range of other yeasts are involved, including *Meyerozyma caribbica*, *Candida tropicalis*, *Pichia kudriavzevii*, *Pichia kluyveri*, *Kodamaea ohmeri* (262), *Kluyveromyces marxianus*, *Candida melibiosica*, *Cryptococcus albidius* var. *albidius*, *Dekkera bruxellensis*, *Rhodotorula mucilaginosa*, *Debaryomyces hansenii*, *Torulaspora delbrueckii* (260), *Candida inconspicua*, *Issatchenkia orientalis*, *Candida magnolia*, *Candida humilis* (259), *Candida albicans*, *Dekkera anomala*, *Candida etchellsii*, *Candida kuwiensis*, and *Saccharomyces pastorianus* (261). LAB are the second most prominent category of microorganisms in most of these beers, and they carry out mash acidification, which is an important processing step. The most commonly observed LAB are *Lactobacillus fermentum*, *Lactobacillus buchneri* (259), *Lactobacillus delbrueckii*, *Pediococcus acidilacti*, *Leuconostoc lactis*, and *Lactococcus lactis* (263). During early fermentation, *Enterobacteriaceae* and *Staphylococcus aureus* can also be isolated, but they do not survive the fermentation and are likely killed by the low pH (259).

Mixed-Culture Fermentations

Many mixed-inoculum beer fermentations have traditionally been brewed in Belgium, with the most renowned group being the acid beers of Flanders. These beers are inoculated with a mixture of *S. cerevisiae*, *Lactobacillus* spp., and *Pediococcus* spp. and fermented in steel tanks for 7 to 8 weeks to create a fruity, refreshingly tart beer (205). Some breweries package and sell this young beer as is, while others mature the beer for 1 to 2 years in large oak casks, where *Brettanomyces* spp. and wild yeasts resident in the wood re-ferment the beer (205). The fully matured beer is then packaged straight, blended with some proportion of young, steel-fermented beer, or filtered and blended with non-sour ale prior to distribution, depending upon the preference of the brewery.

A number of other mixed-inoculum beers are produced globally and enjoy increasing popularity among niche markets. Many Belgian ales—most notably certain Trappist beers—are re-fermented in-bottle by *Brettanomyces* and occasionally other yeasts

or bacteria, providing the unique sensory character of these beers. German Berliner weisse is a low-gravity wheat beer fermented with *S. cerevisiae* and *Lactobacillus* spp. in mixed culture. Finally, there is a growing trend of American craft brews incorporating *Brettanomyces* spp. and lactic acid bacteria in the fermentation, maturation, or bottle re-fermentation process, and even a rare few purportedly conduct a fermentation entirely by *Brettanomyces*.

CONCLUSION

An enormously diverse group of microbes can contribute to the production and quality of beer. For most of these organisms, it is not possible to categorically define them as making negative contributions: it really does depend on the beer or on the role that the organism is expected to play. Thus, while lactic acid bacteria are frequently undesirable as spoilage agents, they can perform necessary functions, such as the acidification of mashes according to traditional Germanic brewing practices or as key elements in the production of sour beers. A brewing company's prized brewing strain represents a wild yeast for a competing brewer. The achievement of mastery over the diverse microbial players will differ from circumstance to circumstance and is dependent on an understanding of the organisms likely to be at play from grain to glass.

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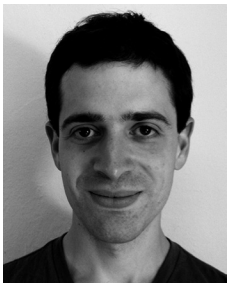

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