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# 125th Anniversary Review: Microbiological Instability of Beer Caused by Spoilage Bacteria

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## ABSTRACT

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Beer has been generally recognized as a microbiologically stable beverage. However, microbiological incidents occasionally occur in the brewing industry. The microbiological instability of beer is often caused by bacteria consisting of four genera, *Lactobacillus, Pediococcus, Pectinatus* and *Megasphaera*. *Lactobacillus* and *Pediococcus* belong to the lactic acid bacteria (LAB), whereas *Pectinatus* and *Megasphaera* form a group of strict anaerobes that are known as intermediates between Gram-positive and Gram-negative bacteria. The frequencies of beer spoilage incidents caused by these four genera have been reported to exceed 90% in Europe and therefore *Lactobacillus, Pediococcus, Pectinatus* and *Megasphaera* are considered to be the principal spoilage agents in the brewing industry. Thus, this review consists of three parts involving these four genera. The first part describes spoilage LAB in alcoholic beverages with some emphasis on beer spoilage LAB. In this part, the emergence and evolution of these spoilage LAB is discussed, the insight of which is useful for developing quality control methods for these beverages. The second part is devoted to the hop resistance in beer spoilage LAB. This area of research is evolving rapidly and recent progress in this field is summarized. The third part concerns *Pectinatus* and *Megasphaera*. Although this group of beer spoilage bacteria has been described relatively recently, the incident reports in Europe increased in the early 1990s, reaching around 30% of spoilage incidents. Various aspects of *Pectinatus* and *Megasphaera*, ranging from their taxonomy and beer spoilage ability to detection and eradication methods are described.

**Key words:** beer, lactic acid bacteria, *Megasphaera, Pectinatus,* sake, spoilage, wine.

## INTRODUCTION

Beer has been recognized as a microbiologically stable beverage. This is due to the presence of ethanol (0.5–10% w/w), hop bitter compounds (ca. 17–55 ppm of iso-α-acids), high carbon dioxide content (approximately 0.5% w/v), low pH (3.8–4.7) and reduced concentration of oxygen (generally less than 0.3 ppm)[146]. Beer is also a poor medium because nutrients are almost depleted by the fermentative activities of brewing yeast. As a result, foodborne pathogens, such as *Salmonellae* and *Staphylococcus aureus,* do not grow or survive in beer[25]. Despite these rather hostile features, a number of microorganisms are able to grow in beer and are called beer spoilage microorganisms. Among the beer spoilage microorganisms, four genera, *Lactobacillus, Pediococcus, Pectinatus* and *Megasphaera,* are regarded as particularly damaging to brewers in terms of frequencies of spoilage incidents and the negative effects on the flavour profiles of beer[5,8]. Wild yeasts, such as *Saccharomyces cerevisiae* and *Dekkera* spp., are also reported as beer spoilers[5,8].

Lactic acid bacteria (LAB) contain a large group of genera and species of Gram-positive bacteria, including *Lactobacillus* and *Pediococcus*. In the period 1980–2002, approximately 60–90% of the microbiological spoilage incidents in Europe were caused by *Lactobacillus* and *Pediococcus* (Table I)[5–7]. Among these LAB, *Lactobacillus brevis, Lactobacillus lindneri* and *Pediococcus damnosus* are considered as the major beer spoilers. *L. brevis* has been reported as the most frequently detected LAB species in spoiled beer[8], and hence the most extensively studied in brewing microbiology. *L. brevis* is widespread in the food industry and natural environment, but the beer spoilage ability of *L. brevis* varies considerably, depending on the strain and the source of isolation[8,146]. Some strains spoil almost all kinds of beer, causing haze, sediment and acidification, but no diacetyl off-flavour. In contrast, *L. brevis* strains isolated from sources other than beer brewing environments generally exhibit no or very weak beer spoilage ability[104,137,139]. *L. brevis* strains are also reported to lose beer spoilage ability after repeated subcultures in broth media that do not contain hop bitter acids[146]. Due to these reasons, intra-species differentiation of beer spoilage ability in *L. brevis* is important in the brewing industry. *L. lindneri* is highly resistant to hop compounds and grows optimally at 19–23°C[8,10]. It is also reported that *L. lindneri* is unable to grow at temperatures higher than 28°C[8]. Nonetheless, this species is known to tolerate rather high thermal treatments and sometimes survives a suboptimal pasteurization process[12]. Furthermore, *L. lindneri* grows poorly in many detection media described in the brewing industry, and often causes spoilage incidents without being detected in microbiological quality control (QC) tests[140]. *L. lindneri* forms a relatively faint haze and sediment with no off-flavour formation in beer[8]. The occurrence outside beer brewing environments

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**Table I.** Percentages of beer spoilage microorganisms in incident reports during the 1980–2002 period[a].

| Genus/species[b] | 1980–1990 | 1992[c] | 1993[c] | 1997 | 1998 | 1999 | 2000 | 2001 | 2002 |
|---|---|---|---|---|---|---|---|---|---|
| *L. brevis* | 40 | 39 | 49 | 38 | 43 | 41 | 51 | 42 | 51 |
| *L. lindneri* | 25 | 12 | 15 | 5 | 4 | 10 | 6 | 13 | 11 |
| *L. plantarum* | 1 | | | 1 | 4 | 2 | 1 | 1 | 2 |
| *L. casei/paracasei* | 2 | 3 | 2 | 6 | 9 | 5 | 8 | 4 | 4 |
| *L.coryniformis* | 3 | | | 4 | 11 | 4 | 1 | 3 | 6 |
| *Ped. damnosus* | 17 | 4 | 3 | 31 | 14 | 12 | 14 | 21 | 12 |
| *Pectinatus* | 4 | 28 | 21 | 6 | 3 | 6 | 5 | 10 | 7 |
| *Megasphaera* | 2 | 7 | 3 | 2 | 2 | 4 | 4 | 4 | 2 |
| *Saccharomyces* wild yeasts | N.A.[d] | 5 | 5 | 7 | 6 | 11 | 5 | 2 | 3 |
| Non-*Saccharomyces* wild yeasts | N.A. | 0 | 0 | 0 | 3 | 4 | 5 | 0 | 2 |
| Others | N.A. | 2 | 2 | 0 | 1 | 1 | 0 | 0 | 0 |

[a] This table is adapted from the studies conducted by Back during the 1980–2002 period[5–7].
[b] *L. brevis* includes *L. brevisimilis* that exhibits phenotypical and morphological similarities to *L. brevis*. According to Back, *L. brevis* in this table consists of several types on the basis of carbohydrate fermentation profiles, arginine utilization pattern and morphological features, suggesting that this group of LAB can be further divided into separate species or subspecies.
[c] In 1992 and 1993 studies, *L. plantarum, L. casei, L. paracasei* and *L. coryniformis* were put together into one group.
[d] Not available.

has rarely been reported for this species, although it is suggested that *L. lindneri* and a closely related LAB species were isolated from wine grapes and wine making processes[9,137,139]. One striking observation is that *L. brevis* and *L. lindneri* strains grown in beer exhibit reduced cell size and more easily penetrate the membrane filters used for the removal of microorganisms in the brewing industry[2]. On the other hand, beer spoilage caused by *Ped. damnosus* is characterized by acid formation and the buttery off-flavour of diacetyl[8]. Some strains of *Ped. damnosus* produce exopolysaccharides, making the beer ropy and gelatinous[8]. *Ped. damnosus* is also known as one of the most frequent contaminants in fermentation and maturation processes, due partly to its ability to grow at a low temperature[8]. An unexpected rise in diacetyl levels during the maturation process is often caused by the presence of *Ped. damnosus*. In addition, *Ped. damnosus* is reported to adhere to brewing yeast and sometimes induces premature sedimentation of yeast cells, resulting in a retardation of the fermentation process[116]. The adherence to the brewing yeast has been observed for *L. lindneri* as well[134], suggesting that these two species tend to be latent in fermentation and maturation processes. Furthermore, *Ped. damnosus* is known as a slow grower on laboratory detection media[8,147] and has been almost exclusively isolated from beer brewing and wine making environments[8]. *L. paracollinoides* and *L. backi* have recently been proposed as a new species[21,48,141] and their frequency in beer spoilage incidents is not well known. Similar to *L. lindneri, L. paracollinoides* shows very poor culturability in many conventional media, which is especially true upon primary isolation from beer brewing environments[140]. This is probably the main reason that this species had remained uncharacterized until recently. The genetic characterization indicates that *L. paracollinoides* and *L. backi* are closely related to *L. collinoides* and *L. coryniformis,* respectively. Accordingly, some of the strains belonging to *L. paracollinoides* and *L. backi* might have been misidentified as *L. collinoides* and *L. coryniformis* in the past. *Ped. claussenii* has also been reported as a new species[41]. Some strains of *Ped. claussenii* produce exopolysaccharides. All the strains of *L. paracollinoides, L. backi* and *Ped. claussenii* characterized to date have been isolated from beer brewing environments and therefore are considered as unique LAB species to the brewing industry. In contrast, *L. casei/paracasei, L. coryniformis* and *L. plantarum* exhibit relatively weak hop resistance. Therefore these *Lactobacillus* species only spoil weakly hopped beers or those with elevated pH values[8]. Although the frequency of spoilage incidents by these lactobacilli is generally low, they are known to cause diacetyl off-flavours in beer. *Lactococcus* spp. and, to a lesser extent, *Leuconostoc* spp. are encountered in breweries, but the hop resistance of these genera is weak. Therefore spoilage incidents caused by these LAB are rare except for beers with microbiologically weak features[8].

*Pectinatus* and *Megasphaera* are strictly anaerobic bacteria and can contaminate packaged beer. The beers spoiled by *Pectinatus* not only exhibit heavy sediments, hazes, and small clots, but also exhibit an extremely unpleasant taste and odour[8]. *Pectinatus* produces hydrogen sulphide and the spoiled beer smells like a rotten egg. *Megasphaera* forms only slight hazes in beer and almost unnoticeable sediments, but causes severe off-flavours[8]. Bad smelling compounds, including butyric acid, caproic acid and hydrogen sulphide, are formed and the beer becomes undrinkable. *Megasphaera* does not tolerate ethanol very well, thus low alcohol beers are particularly at risk from this genus. The unpleasant off-flavours conferred by *Pectinatus* and *Megasphaera* are immensely damaging to the product and the corporate brand once a spoilage incident occurs. Accordingly, *Pectinatus* and *Megasphaera* are feared in the brewing industry. Combined contamination with beer spoilage LAB are not uncommon for *Pectinatus* and *Megasphaera*[8], suggesting that these beer spoilage bacteria often share habitats in the brewery.

The genus *Pectinatus* was first described by Lee et al. in 1978[88], and the first isolate of *Megasphaera* was reported by Weiss et al. in 1979[168]. Therefore the emergence of these genera as beer spoilers was relatively recent events. The number of spoilage incidents in Europe was

comparatively low in the 1980s, and approximately 6% of the spoilage incidents were caused by *Pectinatus* and *Megasphaera* during this period[5]. However, the percentage of spoilage incidents by these genera increased to 24–35% in the early 1990s and subsequently subsided somewhat in the 1997–2002 period (Table I)[6,7]. The sudden increase in incident reports in the early 1990s was most likely caused by the advances in filling technology which allowed for the production of virtually oxygen-free beer. Since *Pectinatus* and *Megasphaera* are strict anaerobes, the oxygen content in the beer is one of the deciding factors for allowing these bacteria to proliferate in beer. To date three species, *Pectinatus cerevisiiphilus, P. frisingensis* and *P. haikarae,* have been described for the genus *Pectinatus*[80,125], and three beer spoilage species, *Megasphaera cerevisiae, M. paucivorans* and *M. sueciensis,* have been reported for the genus *Megasphaera*[45,80]. Because *P. haikarae, M. paucivorans* and *M. sueciensis* have only recently been proposed as new species, most studies have been conducted with *P. cerevisiiphilus, P. frisingensis* and *M. cerevisiae*.

As described above, there have been a lot of developments in beer brewing microbiology in the past few decades. Many studies have also been carried out for spoilage microorganisms in other alcoholic beverages, such as wine, cider and sake, and a lot of insights have been gained from these industries. In this review, recent developments in the research area regarding beer spoilage LAB, *Pectinatus* and *Megasphaera* are summarized in connection with spoilage microorganisms in other alcoholic beverages. In addition, hop resistance of beer spoilage LAB is a rapidly evolving area of research and a lot of new insights have been accumulated in this field. This review also describes recent progress in hop resistance research of beer spoilage LAB.

## PART 1: BEER SPOILAGE LAB – THEIR EMERGENCE AND EVOLUTION

### I. Insights for developing detection media for spoilage LAB

**Spoilage LAB in alcoholic beverages.** LAB are generally regarded as good microorganisms and are used for a wide variety of fermented foods, such as yoghurt and pickles. Studies of LAB for beneficial applications have been extensively carried out, and LAB have been shown to enhance gut-associated immune responses and suppression of allergic reactions[1,92]. In contrast, LAB are also known as bad microorganisms that are responsible for food spoilage incidents[138]. The foods spoiled by LAB include mayonnaise, salad dressing and fermented products, all known to be protected by natural bacteriostatic agents such as organic acids and salt[138]. Hence, LAB are often the primary cause of spoilage in food products where most other microorganisms cannot grow, due to their ability to survive the inhospitable nature of these foods.

In the beer brewing industry, LAB are recognized as the principal spoilers responsible for 60–90% of spoilage incidents[5–7]. Beer spoilage LAB are shown to exhibit resistance to hop bitter acids and can grow in beer where ordinary LAB cannot grow or survive[47,146]. Hop bitter acids are reported to exert an antibacterial effect by acting as proton ionophores and can dissipate the transmembrane pH gradient required for the uptake of nutrients[127–129]. Hop resistance has been described as a distinguishing character for beer spoilage strains of LAB. In general, beer spoilage LAB are isolated from beer brewing environments and are rarely found in other sources. Therefore beer spoilage LAB can be considered as microorganisms specific to beer brewing environments[139]. But how did they emerge in the beer brewing environments and evolve to acquire beer spoilage ability? These aspects had long remained elusive in brewing microbiology. Nevertheless, several lines of evidence recently suggest that the emergence of these spoilage LAB was associated with the advent of hopped beer that presumably took place in 5th to 9th century, and since then, beer spoilage LAB have evolved to become profoundly adapted to beer brewing environments[137,139]. It has also been hypothesized that, along their long evolutionary processes, beer spoilage LAB have gradually developed complex resistance mechanisms that allow them to grow in beer brewing environments where there are very few competitors[137].

Sake and wine are also known as microbiologically stable beverages[139]. This is mainly due to their high ethanol content and low pH. Therefore most microorganisms including LAB cannot survive and grow in sake and wine. In a sense, sake and wine are similar to beer in that they are hostile environments to most microorganisms. These beverages also share a common feature in that only a limited group of LAB are recognized as the major spoilage microorganisms. Interestingly, spoilage LAB in sake and wine appear to have evolved their spoilage ability along the long history of their association with sake and wine environments[138]. In this part of the review, spoilage LAB in alcoholic beverages and their microbiological quality control methods are reviewed with some focus on beer spoilage LAB. In addition, the emergence and evolution of these LAB will be discussed.

**History of spoilage LAB in alcoholic beverages.** Beer spoilage LAB were found by Pasteur in 1871 through microscopic examinations of spoiled beer[112]. Initially beer spoilage LAB were grouped into rods and cocci. Rod-shaped beer spoilage LAB strains were originally designated *Saccharobacillus pastorianus* by van Laer in 1892[159]. This species was named in honour of Pasteur and later redesignated *Lactobacillus pastorianus*. It was also reported by van Laer that *L. pastorianus* did not show culturability on ordinary nutrient media, and therefore unhopped beer solidified with gelatin was used for isolation. *L. pastorianus* was also noted to have very slow growth on beer medium. Due to its extremely low culturability on ordinary media, *L. pastorianus* had been poorly characterized, despite the fact that this species exhibits very strong beer spoilage ability[146]. However, the development of new culture techniques, which will be described later, has enabled *L. pastorianus* to be isolated from beer brewing environments[140]. Since then, the insights into this species have been accumulated and it was reported that *L. pastorianus* is a much more common beer spoiler than was previously assumed[74]. *L. pastorianus* is

now considered as a synonym of *L. pallacollinoides,* and *L. pallacollinoides* has been accepted as a formal species name[44,139]. Through the subsequent development of phylogenetic studies, the taxonomy of the rod-shaped lactobacilli has changed since the time of Pasteur and van Laer and beer spoilage lactobacilli are now divided into *L. brevis, L. lindneri, L. paracollinoides, L. backi* and other beer spoilage *Lactobacillus* species. On the other hand, coccal strains were originally named *Ped. cerevisiae* by Blacke in 1884[82]. *Ped. cerevisiae* is now designated *Ped. damnosus,* a species name proposed by Claussen[33]. *Ped. claussenii* has recently been described as a new beer spoilage LAB species[41]. *Ped. inopinatus* is also recognized as a potential beer spoiler[8,74].

Sake spoilage LAB were originally discovered by Atkinson in 1881 through microscopic examinations of spoiled sake[139,151]. In 1906, sake spoilage LAB strains were isolated by Takahashi, who found that these LAB strains were unable to grow in ordinary media, unless sake was supplemented[151,152]. One of the growth factors in sake was identified by Tamura as "hiochi acid"[150], currently a synonym of mevalonic acid. This acid is produced by *Aspergillus oryzae,* a mould used in sake mash production for the digestion of rice starch and protein. Highly ethanol-tolerant strains of sake spoilage LAB were divided into homofermenters and heterofermenters, and identified as *L. homohiochi* and *L. heterohiochi* by Kitahara[83]. *L heterohiochi* is presently regarded as a synonym of *L. fructivorans* on the basis of DNA/DNA homology[167], and *L. homohiochi* is still recognized as an independent species, although the type strain of this species has been misplaced and a search for the neo-type strain is underway[52,53]. The recent 16S rRNA gene sequence analysis indicates that some of the *L. homohiochi* strains are closely related to *L. acetotolerans,* a LAB species that exhibits a strong resistance to vinegar[139]. Current taxonomic positions of sake spoilage LAB have been described in more detail in the preceding literature[139]. On the other hand, wine spoilage LAB were discovered by Pasteur in 1866[113], and since then many studies have been conducted in this area of research. Highly ethanol-tolerant lactobacilli, including *L. fructivorans* and *L. hilgardii,* and pediococci have been known to spoil wine[8]. The type of spoilage by these LAB includes the formation of turbidity and ropiness, as well as taste alterations, caused by the bitter compounds produced from LAB[32,50]. In the case of wine making processes, LAB also play a beneficial role by conferring a desirable flavour profile on wine. The recent development of studies concerning spoilage LAB and beneficial LAB in wine will be described later in more detail.

From these backgrounds, spoilage LAB in alcoholic beverages have quite a long history of research that spans more than 100 years. It is therefore reasonable to say that they were among the first spoilage microorganisms characterized by mankind.

**Characteristic features of hard-to-culture beer spoilage LAB and development of their detection media.** Comprehensive detection of microorganisms is important to prevent spoilage incidents. However, the contamination level of microorganisms is typically very low in beer products and a few cells per 100 mL of beer should be detected in quality control (QC) tests. Detection media are generally used as a first step for routine QC tests in breweries to obtain a sufficient number of cells to determine the identity and spoilage ability of the detected microorganisms. Nevertheless, one of the most difficult aspects of QC tests in breweries is that many beer spoilage LAB are unable to grow on the culture media used for detection and isolation of LAB[8,140]. For instance, beer spoilage strains of *L. lindneri* and *L. paracollinoides* are often undetectable on QC media[140], such as MRS (de Man, Rogosa and Sharpe) agar, a medium widely used for the isolation and cultivation of LAB[40]. On the other hand,

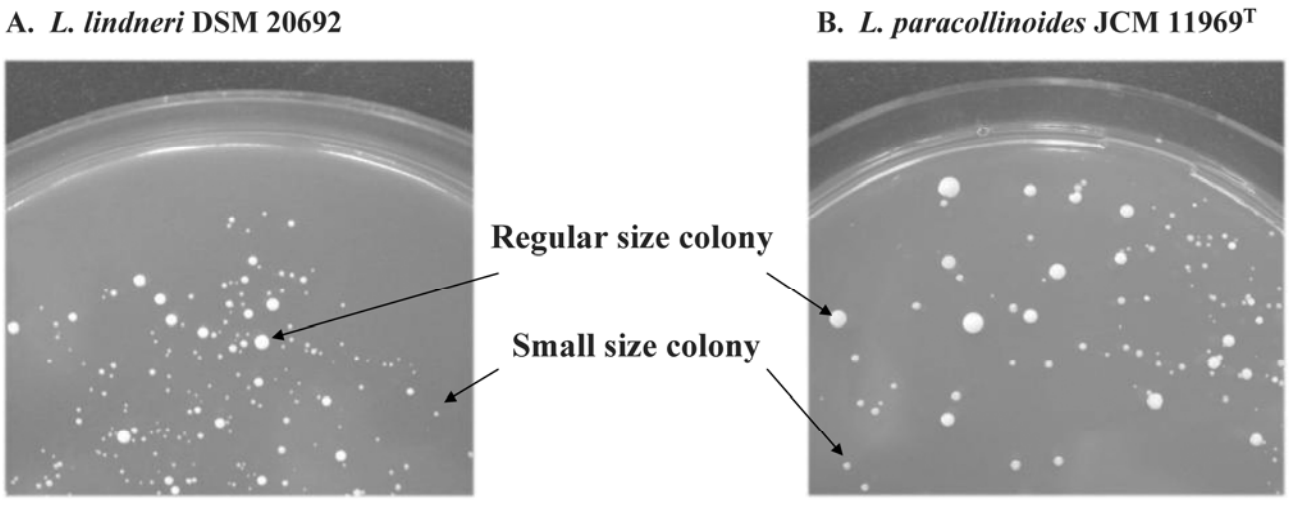


**Fig. 1.** Colonies in varying sizes after 10th subculture in beer. *Lactobacillus lindneri* DSM 20692 (A) and *L. paracollinoides* JCM 11969$^T$ (B) were repeatedly subcultured in degassed beers, and portions of the cultures were inoculated on MRS agar. After 14 days of anaerobic incubation, the colonies were photographed. The colonies that are comparable in size with those of the pre-adapted strains are indicated as regular size colonies, while unusually tiny colonies are shown as small size colonies. Similar observations were made for other beer-adapted *L. lindneri* and *L. paracollinoides* strains. In contrast, the colonies of pre-adapted strains were uniform in size, and small size colonies were not found.

some *L. lindneri* and *L. paracollinoides* strains, which initially showed excessively slow growth on MRS agar at the time of isolation, gradually acquire good culturability, when these strains were repeatedly subcultured in MRS broth[138]. In addition, *L. lindneri* and *L. paracollinoides* strains were found to grow better when media were supplemented with beer, indicating that these beer spoilage species were well adapted to beer brewing environments[138]. These observations led to the hypothesis that the culturability of *L. lindneri* and *L. paracollinoides* is affected by the status of adaptation to beer brewing environments.

To test this hypothesis, *L. lindneri* and *L. paracollinoides* strains showing good growth behaviour on MRS agar were repeatedly subcultured in degassed beer (pH 4.2) in order to adapt the strains to a beer environment. Beer-adapted *L. lindneri* and *L. paracollinoides* strains were periodically sampled to evaluate their culturability on MRS agar. As a result, all the strains tested in the study were found to grow more and more slowly as the number of subcultures in beer increased. It was also observed that the colonies formed on MRS agar progressively became smaller (Fig. 1). After 40–80 subcultures in beer, the strains were found to be unable to form colonies on MRS agar even after 14 days of incubation (Table II)[140,142]. These results indicated that long-term subculturing in beer induced a hard-to-culture state in beer spoilage *L. lindneri* and *L. paracollinoides* strains. From these observations, it was suggested that the emergence of hard-to-culture beer spoilage LAB strains in breweries was caused by their profound adaptation to beer brewing environments. Notably, among the four strains tested in the study, *L. paracollinoides* JCM 11969$^T$, *L. paracollinoides* JCM 15729 and *L. lindneri* HC92, were unable to grow or were hardly detectable on MRS agar, when they were first isolated from beer brewing environments[138]. These strains subsequently acquired good growth ability on MRS agar after gradual acclimatization to MRS medium. Therefore it can be said that these strains were brought back to their original states of primary isolation by a re-adaptation to a beer brewing environment.

To further characterize the hard-to-culture strains, these *L. lindneri* and *L. paracollinoides* strains were grown on beer solidified with agar (beer agar)[139]. As a consequence, the hard-to-culture strains were found to form colonies on beer agar. Taking advantage of these findings, the behaviour of hard-to-culture strains on MRS agar was investigated on the individual cell level. To accomplish this, the cells of hard-to-culture LAB strains were trapped on a 0.45 μm membrane filter, and microcolonies were formed on beer agar by incubating the strains for 48–96 h. After incubation, the microcolonies were double-stained with 5-(and 6-) carboxyfluorescein diacetate and propidium iodide in order to differentiate living and dead cells. As a consequence, microcolonies consisting of tens and hundreds of living cells were found on the membrane and very few cells were stained as dead (Fig. 2B). Subsequently the membranes were transferred onto MRS agar and further incubated for 18 h to evaluate their viability on MRS agar. Strikingly, most cells forming the microcolonies were stained as dead cells (Fig. 2A), indicating that highly beer-adapted *L. lindneri* and *L. paracollinoides* strains were not only unable to grow, but also tended to lose their viability on MRS agar[139].

To elucidate the underlying causes of this phenomenon, the highly beer-adapted *L. paracollinoides* strains were further investigated[138]. The pH range that supports the optimal growth of highly beer-adapted *L. paracollinoides* JCM 11969$^T$ and *L. paracollinoides* JCM 15729 was found to lie below pH 5.0 and these beer-adapted strains grew only poorly at pH 5.3[138]. This was in sharp contrast with the pre-adapted *L. paracollinoides* JCM 11969$^T$ and *L. paracollinoides* JCM 15729, which were able to grow well even at pH 5.6. The optimum growth pH found in highly beer-adapted strains can be considered extremely low compared with ordinary lactic acid bacte-

**Table II.** Effects of beer adaptation on the culturability of beer spoilage LAB on MRS agar[a].

| Strains[b] | Number of subcultures[c] | Detection time (days) | CFUs | MPN[d] |
|---|---|---|---|---|
| *L. paracollinoides* JCM 11969$^T$ | 0 | 4, 4 | 486, 445 | 460 |
| | 10 | 4, 4 | 580, 725 | 460 |
| | 30 | 7, 7 | 141, 156 | 750 |
| | 70 | N.D., N.D. | N.D., N.D. | 1100 |
| *L. paracollinoides* JCM 15729 | 0 | 4, 4 | 384, 420 | 240 |
| | 10 | 4, 4 | 214, 188 | 240 |
| | 30 | 7, 8 | 76, 70 | 240 |
| | 80 | N.D., N.D. | N.D., N.D. | 460 |
| *L. lindneri* DSM 20692 | 0 | 4, 4 | 417, 513 | 240 |
| | 10 | 4, 4 | 696, 788 | 460 |
| | 30 | 6, 6 | 181, 145 | 460 |
| | 70 | N.D., 14 | N.D., 1 | 240 |
| *L. lindneri* HC92 | 0 | 6, 6 | 516, 488 | 460 |
| | 10 | 7, 7 | 586, 612 | 1100 |
| | 20 | 8, 8 | 61, 59 | 240 |
| | 40 | N.D., 14 | N.D., 1 | 1100 |

[a] The experiments were conducted in duplicates. The detection time is shown in days and the colony forming units on MRS agar are indicated as CFUs. N.D.: Not detected.

[b] JCM: Japan Collection of Microorganisms. DSM: Deutsche Sammlung von Mikroorganismen und Zellkulturen. HC: Our culture collection principally consisting of brewery isolates.

[c] *Lactobacillus* strains were repeatedly subcultured in degassed beer (pH 4.2) for the number of times indicated in the table.

[d] The viable cell counts were calculated on the basis of the most probable number (MPN) method, using degassed beer (pH 5.0)[140].

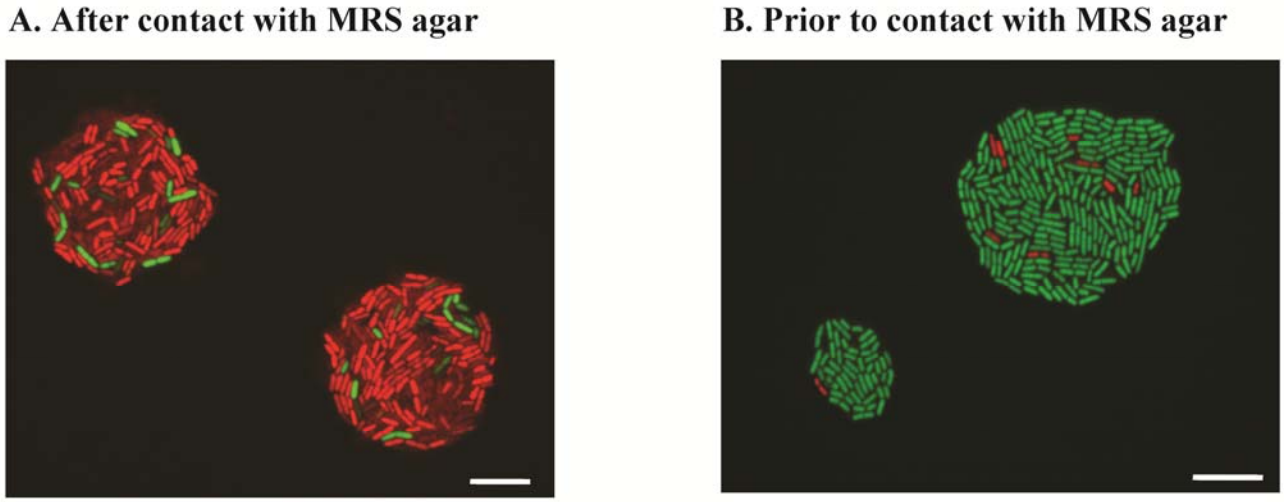


**Fig. 2.** Behaviour of hard-to-culture beer spoilage LAB on MRS agar. Hard-to-culture LAB strains were trapped on a 0.45 μm membrane filter and microcolonies were formed on beer agar by incubating the strains for 48–96 h anaerobically. The membranes were subsequently transferred onto MRS agar and the microcolonies were further incubated anaerobically at 25°C for 18 h. After the incubation, the microcolonies were double-stained with 5-(and 6-) carboxyfluorescein diacetate and propidium iodide (A). As a control, the microcolonies before transferring onto MRS agar were double-stained in the identical manner to evaluate the viability of the cells (B). In this assay, viable cells are stained green and dead cells are stained red. This figure shows an example of hard-to-culture *Lactobacillus lindneri* DSM 20692 and similar trends were observed with other hard-to-culture beer spoilage LAB. Bar, 10 μm.

ria. In fact, the pH value of MRS agar is usually adjusted to 5.7 or higher, depending on manufacturer of the medium. This is because most LAB grow well around pH 5.7. Accordingly, the downward shifts of pH range for growth were considered as one of the main reasons that beer-adapted *L. paracollinoides* strains could not grow on MRS agar. Interestingly, highly beer-adapted *L. paracollinoides* strains reacquired the ability to grow at higher pH when they were reacclimatized to MRS environments. The reacclimatization procedures were performed by repeatedly subculturing the strains in broth media mixed with beer and MRS, where the portions of beer (initially 100%) were progressively replaced with MRS broth. These results indicated that the adaptation to a certain environment significantly affected the growth pH range for beer spoilage *L. paracollinoides* strains. It was also shown that nutrients in MRS agar, such as sodium acetate, yeast extract, peptone and magnesium sulphate, inhibited the growth of highly beer-adapted *L. paracollinoides* JCM 11969$^T$ and *L. paracollinoides* JCM 15729[138]. In contrast, the pre-adapted strains and the counterparts reacclimatized to MRS environments did not show such sensitivity to any of the above compounds. Accordingly, it was suggested that nutrient status in media, in addition to pH, is an important factor for the growth of deeply beer-adapted LAB.

What are the implications for the phenomena observed in these studies? The pH range of beer typically lies between 3.8 and 4.7, and the nutrients in beer are almost exhausted after the completion of the fermentation process by the brewing yeast. Naturally LAB living in beer brewing environments are adapted to these conditions. It was therefore suggested that the adaptation to beer brewing environments substantially affects the culturability of beer spoilage LAB through alterations in the optimal growth pH range and sensitivity to nutrients. On the other hand, the pH values of detection media recommended by European Brewery Convention (EBC) and American Society of the Brewing Chemists (ASBC) often exceed 5.4[15,38,46]. Furthermore, the media ordinarily contain considerable amounts of sodium/potassium acetate, yeast extract, peptone and magnesium sulphate to enhance the growth of LAB. It was thus reasonable to say that the environments provided by the detection media were drastically different from those encountered in beer brewing environments. For other microorganisms, it has been reported that the sudden changes in living environments induce a shock state, resulting in the loss of culturability on media[154]. Accordingly, it was quite conceivable that highly beer-adapted LAB strains lapse into a shock state when they suddenly encounter an unfamiliar environment such as a conventional detection medium. Considering that many beer spoilage LAB strains are deeply adapted to beer brewing environments, brewing microbiologists should take these observations into account in formulating detection media. These new insights led to the development of ABD (advanced beer-spoiler detection) medium that adopts a pH value as low as 5.0 and contains a minimum amount of nutrients to facilitate the growth of highly beer-adapted spoilage strains (Table III)[140]. This medium has been successfully used for the detection of hitherto hard-to-culture beer-spoilage *L. lindneri, L. paracollinoides* and *Ped. damnosus* strains[140]. It was also shown that ABD medium compared favourably with the conventional media recommended by EBC and ASBC, when hard-to-culture LAB strains were tested (Fig. 3). However, some beer spoilage LAB strains grow slowly on ABD medium, due to its low nutrient status. To overcome the shortcomings, microcolony methods using carboxyfluorescein diacetate (CFDA) and species-specific fluorescence *in situ* hybridization (FISH) have been recently developed to allow a rapid detection of slowly growing

**Table III.** The compositions of various media for detection of LAB.

| Compositions (/L) | | | | | | | |
|---|---|---|---|---|---|---|---|
| **S. I. medium[a]** | | **Kunkee medium[b]** | | **MRS broth[c]** | | **ABD medium[d]** | |
| Yeast extract | 10.0 g | Tryptone | 20.0 g | Peptone | 10.0 g | MRS broth (powder) | 2.61 g |
| Polypeptone | 5.0 g | Peptone | 5.0 g | Meat extract | 8.0 g | Sodium acetate | 0.5 g |
| Glucose | 25.0 g | Yeast extract | 5.0 g | Yeast extract | 4.0 g | Cycloheximide | 10 mg |
| $MgSO_4$ $7H_2O$ | 100 mg | Glucose | 5.0 g | Glucose | 20.0 g | Agar | 15.0 g |
| $MnSO_4$ $5H_2O$ | 2.5 mg | Tween 80 | 0.5 mL | Dipotassium hydrogen | | Beer | 1,000 mL |
| $FeSO_4$ $7H_2O$ | 2.5 mg | Filtered tomato juice | 250 mL | phosphate | 2.0 g | Final pH | 5.0 |
| Sodium azide | 50 mg | Distilled water | 750 mL | Tween 80 | 1.0 g | | |
| Sodium acetate | 10.0 g | Ethanol | ca. 110 mL | Diammonium hydrogen | | | |
| Mevalonic acid | 5.0 mg | Final pH | 5.5 | citrate | 2.0 g | | |
| Agar | 0.6 g | | | Sodium acetate | 5.0 g | | |
| Ethanol | 100–150 mL | | | $MgSO_4$ | 0.2 g | | |
| Distilled water | 900–850 mL | | | $MnSO_4$ | 0.04 g | | |
| Final pH | 5.0 | | | Final pH | 5.7 | | |

[a] The composition of S. I. medium originally developed by Sugama and Iguchi[135] is shown. The reduced ethanol content may be used for accelerating the growth of sake spoilage LAB.
[b] 10% (v/v) ethanol is added after sterilization.
[c] This medium is brought to 1.0 L with distilled water. For preparation of MRS agar, ca. 1.5% (w/v) agar is added to MRS broth. For wine spoilage *L. hilgardii*, 10–15% (v/v) ethanol is added to MRS broth and the pH of the medium is adjusted to 4.5.
[d] The use of 52.2 g powder is recommended by the manufacturer (Merck, Darmstadt, Germany) for preparing 1.0 L MRS broth[140]. In the case of ABD medium, only a small portion of MRS broth is added.

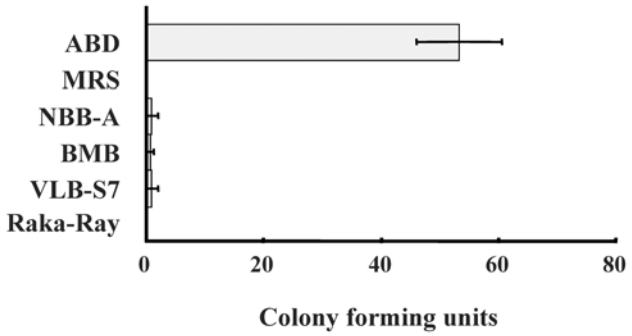


**Fig. 3.** Comparative study of various agar media for detection of hard-to-culture beer spoilage LAB. ABD medium was compared with other agar media recommended by the European Brewery Convention and the American Society of the Brewing Chemists. The hard-to-culture LAB strains were inoculated onto agar media and incubated anaerobically at 25°C. After 14 days of incubation, colony forming units were counted for each agar medium. This figure shows the example of hard-to-culture *Lactobacillus lindneri* DSM 20692 and similar trends were observed with other hard-to-culture beer spoilage LAB strains, except that NBB-A was found to be as sensitive as ABD for hard-to-culture *L. paracollinoides* strains[140].

beer spoilage LAB[3]. Using this method, the detection and species identification of slowly growing LAB are possible within 3 days of pre-enrichment on ABD medium. Notwithstanding, this approach requires a dedicated system, and QC laboratories in breweries may prefer a more traditional approach. One interesting idea might be the use of a time-dependent nutrient release system, in which nutrients are gradually released into the media, thus relieving the shock stress initially encountered by beer-adapted LAB strains and leading to the acceleration of growth in later incubation stages. A preliminary study was successfully carried out by Taskila et al.[153], and this approach may find a wider application in the brewing industry, although further studies will definitely be needed.

**Detection media for sake and wine spoilage LAB based on their characteristic features.** Sake and wine have been recognized as beverages with a high microbiological stability. Only a small number of species represent the majority of sake and wine spoilage microorganisms, and LAB are known as the predominant microorganisms in sake and wine spoilage incidents[138]. In fact, among over 300 LAB species described to date, relatively few species have been reported to spoil sake and wine. There are a number of factors contributing to the enhancement of the microbiological stability of these alcoholic beverages. For sake, the growth of LAB is primarily inhibited by its high ethanol concentration, which typically reaches 15–20% (v/v). The pH value of sake lies between 4.2–4.7 and this factor additionally contributes to the microbiological stability of sake. Therefore only highly ethanol-tolerant LAB are able to grow in sake[98,108,109]. Although wine is more diverse than sake and beer, this beverage is generally characterized as containing a relatively high amount of ethanol (typically 9–15% (v/v)) and having a low pH value (2.8–4.3)[64].

Sake spoilage LAB contain a group of microorganisms that are called hiochi bacteria[98,99]. "Hiochi" is a Japanese word that describes a phenomenon, in which sake is spoiled after the pasteurization process. Hiochi bacteria are generally composed of two groups of lactobacilli, namely hiochi-lactobacilli and true hiochi-bacilli[139]. The former group of hiochi-bacteria consists of various species of LAB, including *L. casei, L. paracasei, L. rhamnosus, L. fermentum* and *L. plantarum*. In general, hiochi-lactobacilli are less ethanol-tolerant and only able to grow at ethanol concentrations below 16.5%[139]. Hiochi-lactobacilli are also known as less heat-tolerant and cannot survive the pasteurization process in sake manufacturing. Therefore hiochi-lactobacilli generally pose less threat of significant damage to sake products. Thus this review places the main focus on the latter group of hiochi-bacteria, true hiochi-bacilli. The true hiochi-bacilli exhibit an extraordinarily high ethanol tolerance and are able to

**Table IV.** Characteristic features of spoilage LAB associated with alcoholic beverages[a].

| Species | Strains | Source | Ethanol tolerance for growth (%) | Effects of ethanol on growth | Optimal pH for growth | Requirement for mevalonic acid[b] | Growth in MRS | Culture media[c] |
|---|---|---|---|---|---|---|---|---|
| *L. fructivorans* | ATCC 8288$^T$ | Spoiled salad dressing | <10 | Inhibitory | 5.0–5.5 | Not required | Positive | MRS |
| | DSM 20607 | Spoiled wine | 18–20 | Promotive | ca 5.0 | Not required | Negative | Kunkee medium |
| | ATCC 15435, S-14, S-20 | Spoiled sake | 20–21 | Promotive | 4.5–5.0 | Essential | Negative | S. I. medium |
| *L. homohiochi* | S-24, S-40, S-48, S-57 | Spoiled sake | 21–25 | Promotive | 4.5–5.0 | Essential | Negative | S. I. medium |
| *L. hilgardii* | Strain 5 | Spoiled wine | 18–20 | Promotive | ca 4.5 | Not required | N.A. | Modified MRS |

[a] The information in the table was obtained from the prior literature[9,36,98,108,139,163,167]. N.A.: Information not available.
[b] The requirement of mevalonic acid for *L. homohiochi* is strain-dependent and other *L. homohiochi* strains may not require mevalonic acid for growth.
[c] Modified MRS contains 10–15% (v/v) ethanol and the pH is adjusted to 4.5 to facilitate the growth of wine spoilage *L. hilgardii*.

grow in media containing more than 20% (v/v) ethanol[98]. This group of hiochi-bacteria confers acidity and off-flavours, such as diacetyl, on spoiled sake products. From a taxonomic standpoint, the true hiochi-bacilli principally consist of two *Lactobacillus* species, *L. fructivorans* and *L. homohiochi*. *L. homohiochi* strains are known to grow in media containing up to 21–25% (v/v) ethanol (Table IV)[98,108,109], which makes this species among the most ethanol-tolerant LAB reported to date[77]. This level of ethanol tolerance is rarely observed with microorganisms living in ordinary environments. On the other hand, *L. fructivorans*, in addition to its high tolerance to ethanol (20–21% (v/v)), is relatively heat-tolerant and therefore survives suboptimal pasteurization processes[98,131]. As for wine, *L. fructivorans* and *L. hilgardii* have been reported as spoilage LAB[9,138]. These wine spoilage LAB show a high ethanol tolerance comparable with that of true hiochi-bacilli (Table IV) and can cause problems even in fortified wine, which is known for its high ethanol content (18–20% (v/v)) and low pH (3.5–4.0)[35].

Notably, *L. fructivorans* is recognized not only as a sake and wine spoilage LAB species, but also as a spoiler of mayonnaise and salad dressings[85,110,139,167] that contain vinegar, a bacteriostatic compound widely used as a natural preservative in the food industry. *L. fructivorans* is known to exhibit diverse features depending on the source of isolation and despite sharing the same species name they behave quite differently. Accordingly, *L. fructivorans* is a good example for illustrating an important role of the living environment. As shown in Table IV, *L. fructivorans* ATCC 8288$^T$, an isolate from salad dressing, exhibits relatively weak ethanol resistance. The growth of *L. fructivorans* ATCC 8288$^T$ is inhibited by any amount of ethanol present in medium[163] and this strain hardly grows in media containing 10% (v/v) ethanol. In contrast, *L. fructivorans* strains isolated from spoiled sake and wine show a strong ethanol resistance and are capable of growing in media containing more than 18% (v/v) ethanol. Strikingly, the growth of these sake and wine spoilage *L. fructivorans* strains are promoted by the presence of 6–10% (v/v) ethanol, a concentration that is inhibitory for *L. fructivorans* ATCC 8288$^T$ [139,163]. Accordingly, the alcoholophilicity is a characteristic feature for *L. fructivorans* strains isolated from beverages containing a high concentration of ethanol and is not ordinarily observed for *L. fructivorans* strains isolated from other sources[81,138]. In fact, most bacteria exhibit a dose-dependent inhibition of growth over the range of 1–10% (v/v) ethanol and few organisms grow above 10%[36]. Therefore the ethanol resistance and the alcoholophilicity observed with sake and wine spoilage *L. fructivorans* is extraordinary. Furthermore, the optimal pH for the growth of sake and wine spoilage *L. fructivorans* strains is rather low compared with that of ordinary *L. fructivorans* strains[139]. As described earlier in this review, beer spoilage *L. paracollinoides* strains can exhibit a considerable downward shift of optimal pH range for growth upon a deep adaptation to beer brewing environments. In fact, the tested *L. paracollinoides* strains grew optimally below pH 5.0 and hardly showed culturability at pH 5.6, whereas pre-adapted strains can grow quite well. From these analogies, a similar environmental adaptation is conceivably occurring with sake and wine spoilage *L. fructivorans* strains. This might be especially true for sake spoilage *L. fructivorans* strains that display a very poor growth ability at pH 5.5[98], the pH value known as optimal for the growth of *L. fructivorans* strains isolated from mayonnaise and salad dressing[139].

Interestingly, the acidophilic and alcoholophilic nature observed for sake and wine spoilage *L. fructivorans* is also recognized for sake spoilage *L. homohiochi* and wine spoilage *L. hilgardii*[35,36,139]. Both groups of LAB are highly ethanol tolerant and the presence of ethanol at ca. 10% (v/v) promotes their growth. The optimal pH of sake spoilage *L. homohiochi* and wine spoilage *L. hilgardii* is rather low, ranging between 4.5 and 5.0. Another intriguing example is *Oenococcus oeni,* a LAB species associated with the wine making environment and often used for the secondary fermentation of wine i.e., the malolactic fermentation[20,91,93]. *O. oeni* displays a strong ethanol tolerance and survives in an environment containing more than 13% (v/v) ethanol[49]. The optimal growth pH of *O. oeni* is reported to range from 4.3 to 4.7, and the presence of 3–7% (v/v) ethanol promotes the growth of *O. oeni*[49]. These acidophilic and alcoholophilic features are strikingly similar to those of wine spoilage *L. fructivorans* and *L. hilgardii*. Also similar to spoilage LAB in alcoholic beverages, *O. oeni* strains are exclusively isolated from wine making environments[138]. These phenomena observed in LAB associated with alcoholic beverages are most likely caused by a deep adaptation to high ethanol and low pH environments, characteristic of sake and wine. Therefore S. I. (Sugama-Iguchi) medium, which simulates the sake brewing environment, is used for the detection of sake spoilage *L. fructivorans* and *L. homohiochi*[135]. Similarly, Kunkee medium simulating the wine making environment is used for the detection of wine spoilage *L. fructi-*

*vorans*[167] and modified MRS medium, containing 10–15% (v/v) ethanol and adjusted to pH 4.5, is used for the detection of wine spoilage *L. hilgardii*[35,36]. The common features of these media are the inclusion of approximately 10% (v/v) ethanol and the adoption of a low pH value (Table III). One notable exception is that only S. I. medium contains mevalonic acid[135]. This is because sake spoilage *L. fructivorans* strains and some of *L. homohiochi* strains are reported to require mevalonic acid for growth (Table IV)[98,139].

In sake brewing, the adoption of rice as a raw material led to the use of moulds for digestion of the rice starch, an essential process for supplying sake brewing yeast with nutrients. *Aspergillus* mould, called "*koji-kin*" in Japanese, is used to supply the necessary hydrolytic enzymes (α-glucosidase, glucoamylase, transglucosidase, acid protease, carboxypeptidase) for digesting rice starch and protein in sake mash production[139]. This function corresponds to that of the malt enzymes in beer mash production. In this aspect, sake brewing is different from wine making where grapes are used as a raw material and thus no saccharification process is required. Mevalonic acid is produced by *A. oryzae,* and therefore this acid is naturally present in sake. Probably affected by these circumstances, sake spoilage *L. fructivorans* strains and some of *L. homohiochi* strains require mevalonic acid for growth and are unable to grow in media lacking mevalonic acid. In contrast, mevalonic acid is not an essential growth factor for wine spoilage *L. fructivorans* and *L. hilgardii* (Table IV)[138], presumably because *Aspergillus* mould is not used for wine making processes. These observations, taken collectively, indicate that spoilage LAB in alcoholic beverages are deeply adapted to their respective environments and suggest that a high level of environmental adaptation facilitates the formation of ecological subgroups which go beyond the species status.

**The origin of spoilage LAB in alcoholic beverages.** The most likely driving forces behind the phenomena described above are connected with the long history of beer, sake and wine. For instance, beer brewing has been documented in Babylon[23] from about 7,000 B.C. The advent of hopped beer is considered to be a much later event and the origin of the use of hops in beer brewing is suggested to be sometime between the 5th and 9th centuries[16,69]. Some argue that the origin of sake can be traced back 3,000 years, from which time sake has evolved to the present form through various transitions[111]. The documented literature indicates that the advent of what is currently recognized as sake had a probable origin in the Nara period of 710–794[14]. On the other hand, wine is considered to have an origin as old as humanity, because ethanol fermentation occurs spontaneously from grapes through their contact with *Saccharomyces* yeast. It has been reported[13] that wine making technology existed around 3,500 B.C. Therefore, from a historical point of view, it seems reasonable to say that these alcoholic beverages have quite an old origin that dates back more than 1,000 years.

Taking these historical backgrounds into account, spoilage LAB in alcoholic beverages have had ample time to become highly adapted to their own living environments. Thus the long-term environmental adaptation is presumably the underlying reason why these spoilage LAB are unable to grow on ordinary culture media for LAB, and require dedicated media simulating the environments of the respective beverages. In addition, beer, sake and wine are inhospitable environments where ordinary microorganisms cannot survive, and represent niche environments that accommodate only a few other competitors. Therefore spoilage LAB in these alcoholic beverages have been through a distinctive evolutionary process under the protection of a harsh living environment. This situation is somewhat analogous to those that occurred in the Galapagos Islands, an archipelago of volcanic islands distributed around the equator in the Pacific Ocean, 972 km west of continental Ecuador, where distinctive species have evolved and flourished in the isolated environment. This hypothesis is supported by the fact that beer spoilage *L. brevis* strains form a distinctive subgroup that can be discriminated from *L. brevis* strains isolated from other sources on the basis of *gyrB* gene sequences[105]. Moreover, a comparative study on electrophoretic mobility of D-lactate dehydrogenase (LDH) also suggested that the beer spoilage *L. brevis* is a phylogenetically distinct subgroup that can be discriminated from non-spoilage *L. brevis*[149]. From a phenotypic viewpoint, it has been shown that beer spoilage *L. brevis* strains tend to show preference for maltose over glucose as a fermentable sugar[117], suggesting that beer spoilage *L. brevis* strains are well adapted to beer brewing environments. From these findings, it is conceivable that a particular subgroup of *L. brevis* strains chose beer and related environments for their habitats and evolved along the history of beer brewing[139]. Furthermore, many beer spoilage species, including *L. lindneri* and *L. paracollinoides,* have been known to be almost exclusively isolated from beer brewing environments[133,137,139], indicating that beer spoilage LAB strains have been closely associated with beer brewing environments.

Recent studies showed that microorganisms living in the environmental extremes exhibit stress-dependence, meaning that a particular stress factor that inhibits other microorganisms facilitates their growth, and in many cases the microorganisms living in environmental extremes cannot grow without those stress factors[24,86]. *Tetragenococcus halophilus,* a LAB species used for the fermentation of soy sauce, is a good example[138]. The final product of soy sauce contains approximately 15–20% (w/v) salt and can be preserved at room temperature without microbiological problems. In a sense, soy sauce represents an environmental extreme where most microorganisms cannot survive. Nonetheless, *T. halophilus* tolerates the fermentation processes of soy sauce. *T. halophilus* is known to grow only poorly in media containing no salt and approximately a 7% (w/v) salt concentration supports the optimal growth of *T. halophilus* strains[138], indicating a dependence by *T. halophilus* on the stress factor. Similar to this case, the detection media used for beer, sake and wine closely mimic the living environments for spoilage LAB in the respective alcoholic beverages. The stress factors in these media inhibit the growth of competitive microorganisms, while selectively facilitating the growth of spoilage LAB. In a sense, the detection media in the alcoholic beverage industries are the epitome of microbial Galapagos Islands where spoilage LAB have evolved in the history of beer, sake and wine.

## II. Species-independent genetic markers for determining the spoilage ability of LAB

**General background.** It is vitally important to evaluate the spoilage ability of detected microorganisms. In other words, the data from the microbiological QC tests must determine the validity as to whether products can be distributed in the marketplace. Therefore rapid feedback is required for the determination of the spoilage ability of LAB in alcoholic beverages. However, the inoculation tests into products, one of the most accurate evaluations for spoilage ability, are very time-consuming, typically requiring several weeks and are therefore considered impractical[146]. Thus the application of rapid molecular microbiological tests is being studied. If a certain spoilage trait is associated with a particular species, species-specific methods are useful and can be developed relatively easily on the basis of gene sequences unique to the species. However, many non-spoilage strains exist among the spoilage species in alcoholic beverages, although spoilage traits observed in LAB are broadly associated with species status[146]. For example, *L. brevis* is known as one of the major beer spoilage species, but many strains belonging to *L. brevis* exhibit no spoilage ability and cannot grow in beer[104,146]. Another example is that some strains of *L. fructivorans* show a high level of ethanol tolerance and spoil sake and wine, whereas other *L. fructivorans* strains are relatively ethanol-sensitive and cannot grow in these alcoholic beverages[163]. Therefore the determination of species is not sufficient for discriminating the intra-species differences in spoilage ability. In addition to this problem, as yet uncharacterized species often emerge, causing spoilage incidents[137,146]. Species-specific approaches are only effective for already well-characterized spoilage species and cannot cope with unencountered ones. Therefore species-independent methods that can determine the spoilage ability of detected microorganisms are desired. In earlier sections of this review, a long-term association with production environments has been described for spoilage LAB in alcoholic beverages. In this section, the species-independent methods for evaluating spoilage ability will be discussed, which takes advantage of the fact that spoilage LAB have been adapted to the production environments through the long history of the respective alcoholic beverages.

**Microbiological QC methods for beer spoilage LAB.** Hop compounds in beer are reported to exert an antibacterial effect by acting as proton ionophores and dissipate the transmembrane pH gradient, which prevents LAB strains from growing in beer[127–129]. Hop stress conditions have also been suggested to reduce the level of intracellular manganese, leading to the inhibition of metabolic activities in hop-sensitive LAB[127–129]. Therefore hop resistance ability has been known as a distinguishing character for beer spoilage strains of LAB. The hop resistance mechanisms are regarded as multifactorial dynamic properties and appear to consist of various active and passive defence systems[17,143]. The active defence mechanisms involve efflux pumps, such as HorA and HorC, which have been proposed to transport hop bitter acids out of cells[75,76,121]. HorA has been shown as an ATP-binding cassette (ABC) family of multidrug transporters, while HorC was suggested to depend on proton motive force (PMF) to exclude hop bitter acids out of bacterial cells. The activities of HorA and HorC presumably result in a reduced influx of the undissociated and membrane-permeable hop bitter acids into the cytoplasm and thereby limit the antibacterial protonophoric effect of hop-derived compounds.

Hop resistance genes, *horA* and *horC,* were originally found in beer spoilage *L. brevis*[123,146], but later studies showed that these two genes were also detectable in other beer spoilage species, including *L. lindneri*, *L. paracollinoides* and *Ped. damnosus*[146]. In addition, the PCR-based methods utilizing *horA* and *horC* were reported to discriminate the intra-species differences in beer spoilage ability, which cannot be accomplished by species-specific identification methods[74,124,144]. In fact, *horA* and *horC* were found to be present in 94% and 96% of beer spoilage LAB, respectively, and all of the strains tested possessed at least one of the genetic markers (Fig. 4). These results indicate that *horA* and *horC* are excellent species-independent genetic markers for differentiating the beer spoilage ability of LAB. Furthermore, it has been shown that the *horA* and *horC* genes are detectable in newly found beer spoilage species, *L. backi* and *Ped. claussenii*[74,138], demonstrating that these genetic markers are useful for evaluating beer spoilage ability of as yet uncharacterized species. Very recently, *bsrA* and *bsrB*, presumably encoding multidrug ABC transporters, have been identified in beer spoilage *Pediococcus* isolates[56]. Although *bsrA* and *bsrB* were not found in beer spoilage *Lactobacillus,* the presence or absence of these two genetic markers was shown to be highly correlated with the beer spoilage ability of *Pediococcus* strains, whereas *horA* and *horC* showed a relatively weak correlation.

Interestingly, HorA showed a 54% identity with OmrA, an ABC family of a multidrug transporter identified in *O. oeni*[22]. *O. oeni* is used for malolactic fermentation, a process in wine making where tart-tasting malic acid, naturally present in grape must, is converted to softer-

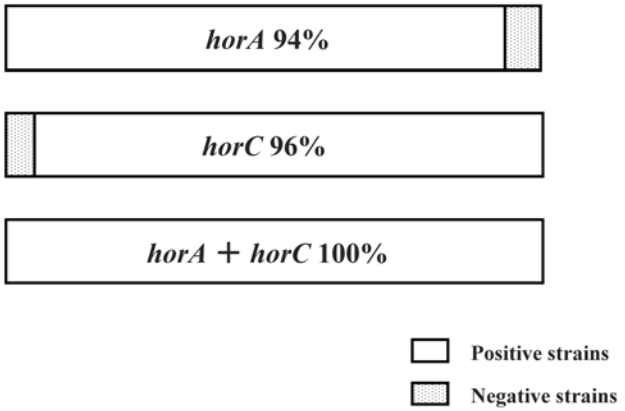


**Fig. 4.** Compensatory relationship for *horA*- and *horC*-specific determination of beer spoilage ability of LAB strains. A total of 51 strains belonging to various beer spoilage species were examined by PCR and Southern blot analysis. It was shown that beer spoilage LAB strains possess at least one of the genetic markers, indicating that *horA* and *horC* are excellent genetic markers for comprehensibly determining beer spoilage ability of LAB[144].

tasting lactic acid. Malolactic fermentation is known to create a rounder, fuller mouthfeel[91,93]. *O. oeni* tolerates the harsh environments typically encountered in wine and becomes a predominant flora in the later stage of the fermentation process. OmrA has been demonstrated to confer tolerance not only to ethanol, but also to multiple stress factors found in wine making environments[22]. Therefore it is quite conceivable that HorA confers tolerance to a variety of stress factors in beer brewing environments, as well as resistance to hop bitter acids, thereby contributing to the adaptation of spoilage LAB to beer brewing environments. On the other hand, non-spoilage LAB strains carrying *horA* homologs have been occasionally isolated from breweries and other sources[121,144]. Considering the role of OmrA as a protective agent to general stress factors, it is tempting to argue that the *horA* homologs found in non-spoilage LAB are more dedicated to environmental stress factors rather than hop bitter acids.

**Discriminatory methods for sake and wine spoilage LAB.** In wine making, LAB contamination sometimes leads to a spoilage phenomenon called ropiness[39,42,164]. Ropiness is caused by the production of exopolysaccharides (EPS) from LAB, which makes the wine gelatinous and slimy. In this type of wine spoilage, glucosyltransferase genes, *gtf*, were identified as the causative agents[42]. The presence or absence of these genetic markers was found to be highly correlated with the ropy phenotype of *Lactobacillus* and *Pediococcus* isolates[169]. Therefore, these genetic markers are useful for discriminating the intra-species differences in this type of wine spoilage ability and can be utilized in a species-independent fashion. For instance, the *gtf* gene was found in *O. oeni*, a species generally regarded as beneficial in wine making, and shown to produce EPS in the detected *O. oeni* strain[31,42]. It is widely known that some strains of *O. oeni* are suitable for malolactic fermentation, while others are not suitable[119]. Therefore the presence or absence of the *gtf* gene can be considered as one of the indicators to determine the suitability of *O. oeni* strains to wine making. This method might also find application in discriminating problematic LAB strains with a ropy phenotype from beneficial strains, which could contribute to the formation of a desirable flavour profile in wine.

What is the physiological significance for wine spoilage LAB to acquire the glucosyltransferase genes? In general, microorganisms adopt unique survival strategies under harsh environments. A biofilm is an aggregate of microorganisms in which cells adhere to each other and/or to a surface. These adherent cells are frequently embedded within a self-produced matrix of EPS and a community of microorganisms. In biofilms, microorganisms are protected from various environmental stress factors[7,8,134]. Therefore the glucosyltransferase genes have been conceivably acquired and shared by various species of wine spoilage LAB to survive in wine making environments, and the ropiness is caused as a part of a survival strategy for spoilage LAB living under harsh environments. Interestingly, the *gtf* gene was detected in some of the beer spoilage *Ped. claussenii* strains that exhibit ropy phenotypes[114]. The presence of this gene may play a role in the survival of *Ped. claussenii* strains in beer brewing environments. It is also reported that *L. brevis* subsp. *frigidus* produces EPS and renders spoiled beer ropy and viscous[8,12]. This particular group of beer spoilage *L. brevis* forms the encapsulation around the cells, which functions as a protective barrier. Accordingly, *L. brevis* subsp. *frigidus* is known as one of the most resistant beer spoilage microorganisms to sanitizing agents[8].

In wine and cider, bitterness is one type of spoilage, in which a metabolite of LAB designated acrolein combines with polyphenols in these beverages leading to the formation of bitter compounds[50]. LAB belonging to the genus *Lactobacillus* have been described as responsible for this flavour alteration by a particular metabolic pathway of glycerol[32,50]. In this pathway, glycerol is dehydrated to 3-hydroxypropionaldehyde (3-HPA), which can be transformed into acrolein by chemical dehydration under acidic and/or heat conditions. In addition to ethanol, glycerol is a main product of fermentation by yeast during wine and cider production[50]. It contributes to smoothness and roundness on the palate and hence the degradation of glycerol, in addition to the formation of acrolein, has a negative influence on the sensorial quality of wine and cider. *L. collinoides* and *L. hilgardii* are reported to be responsible for bitterness in these beverages[32]. But not all the strains of these species can degrade glycerol and thereby cause this type of spoilage in wine and cider. The presence or absence of the glycerol dehydratase gene is highly correlated with this type of spoilage ability in wine and cider spoilage LAB[32]. Therefore the glycerol dehydratase gene can be used as a species-independent genetic marker to identify spoilage LAB that cause bitterness.

So what is the physiological significance for wine and cider spoilage LAB to acquire the glycerol dehydratase genes? The metabolic pathway mediated by glycerol dehydratase is reported to enhance ATP production in glycolysis, through the generation of $NAD^+$, thereby contributing to the acquirement of energy in spoilage LAB[50]. In addition, 3-HPA, one of the metabolites in this pathway, is also known as reuterin[50]. Reuterin inhibits the growth of Gram-negative and Gram-positive bacteria, along with yeasts and fungi[138]. Therefore acquiring glycerol dehydratase genes in wine and cider presumably provides a competitive advantage both in energy production and in the exclusion of other competing microorganisms and thus helps the survival of spoilage LAB in the harsh environments encountered in wine and cider.

In contrast, species-independent genetic markers have not been well studied for sake spoilage LAB. Nonetheless, it has been reported that sake spoilage *L. fructivorans* and *L. homohiochi* present a common antigen on the cell wall[170]. This antigen was not found in non-spoilage LAB strains and therefore was considered as a specific antigen to sake spoilage *L. fructivorans* and *L. homohiochi*[170]. It is tempting to argue that this antigen is one of the properties acquired and shared in the long history of cohabitation of these spoilage species in sake brewing environments. In addition, an insertion sequence, designated ISLfr1, was found in sake spoilage *L. fructivorans*, which was not observed in non-spoilage *L. fructivorans* isolated from other sources such as salad dressing[163]. Accordingly, this genetic marker was proposed as a method to discriminate sake spoilage *L. fructivorans* strains from non-spoilage ones. Furthermore, immunological and PCR-based meth-

ods have been proposed for the comprehensive detection of sake spoilage bacteria, including *L. fructivorans, L. homohiochi* and less ethanol-tolerant hiochi-lactobacilli[106,107]. The targets of these methods were identified as a bacterial elongation factor Tu (EF-Tu) and the gene encoding this protein. EF-Tu is an envelope-associated protein and is released from the cell by osmotic shock. EF-Tu is a highly conserved protein and thus can be considered as a phylogenetic marker, rather than a marker targeting a specific spoilage trait.

**Horizontal gene acquisition of spoilage LAB in alcoholic beverages.** Interestingly, it is considered that the species-independent genetic markers, so far described in beer, wine and cider spoilage LAB, have been acquired through horizontal gene transfer. These hypotheses are supported by unusually high nucleotide sequence identities of the genetic markers found in different species and genera[32,42,144,146]. For instance, the *horA* and *horC* genes, as well as their flanking open reading frame (ORF) regions, were found to be well-conserved in various beer spoilage LAB species, including *L. brevis, L. paracollinoides, L. lindneri, L. backi* and *Ped. damnosus*[74,144,146]. The interspecies nucleotide sequence identities of these genetic markers and surrounding regions are approximately 99%[144,146]. Notably, in the instance of *L. backi* and *Ped. inopinatus* strains isolated from the same brewery site, ca. 5.6 kb regions containing *horA* were found to be 100% identical between these two species[74], indicating that hop resistance genes are actually spreading in beer brewing environments. In addition, the species-independent genetic markers identified in spoilage LAB of alcoholic beverages were shown to be carried by mobile DNA units, such as plasmids and putative transposons. Accordingly, these mobile DNA units, carrying the genetic markers, presumably become widespread in a variety of spoilage LAB species living in the respective production environments of alcoholic beverages[138]. The postulated horizontal transfer models of hop resistance genes are shown in Fig. 5 as one example. Similarly, the horizontal acquisitions of the genes responsible for spoilage, such as the *gtf* and glycerol dehydratase genes, have been envisaged for spoilage LAB in wine and cider[32,42,169]. Therefore it has been anticipated that these genetic markers can prevent spoilage incidents caused by as yet uncharacterized species that are unidentifiable with conventional species-specific approaches.

Furthermore, it has been hypothesized that spoilage LAB have emerged through horizontal gene transfer as

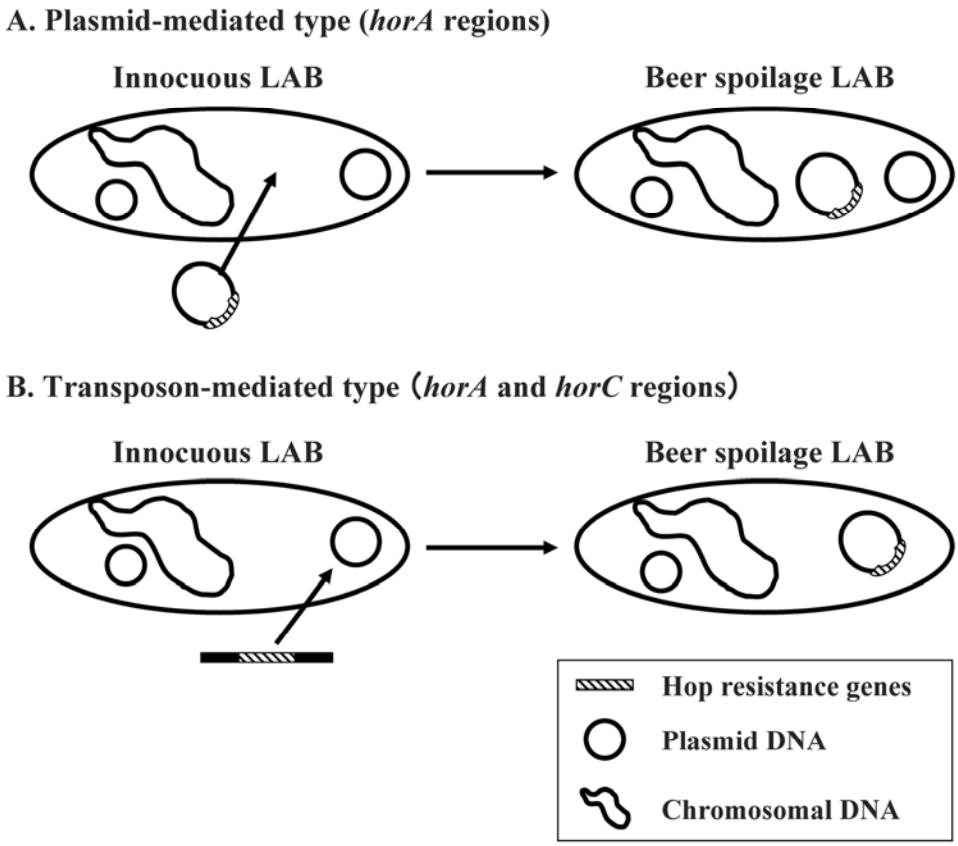


**Fig. 5.** Hypothetical horizontal transfer of *horA* and *horC*. Two modes of horizontal transfer of hop resistance genes, plasmid-mediated (A) and transposon-mediated (B) types, have been postulated on the basis of the nucleotide sequence identities and ORF analysis of *horA*- and *horC*-containing DNA regions identified in *Lactobacillus brevis, L. lindneri, L. paracollinoides* and *Pediococcus damnosus*. The exact mechanisms underlying the horizontal gene transfer of *horA* and *horC* are currently unknown, but several mechanisms, including conjugative transmission of hop resistance genes, are postulated.

part of their complex adaptation and evolutionary processes[137]. In a sense, the existence of these species-independent genetic markers is a vindication that through progressively acquiring and sharing useful genetic properties, spoilage LAB have become closely associated with their own living environments in the long history of alcoholic beverages [138]. Therefore microbiological QC tests considering this aspect will provide useful information in evaluating the spoilage ability of LAB.

Intriguingly, other genes involved in energy generation are also implicated in horizontal gene transfer. In acidic media such as wine, decarboxylation of amino acids to their corresponding amines is considered to provide energy through electrogenic transport, as well as to assist in maintaining an optimal internal pH for the LAB[94]. For example, the histamine-producing pathway was found to be encoded on an unstable plasmid in *L. hilgardii*. In this pathway, the coupled reactions of histidine decarboxylation and histidine/histamine exchange generate a transmembrane pH gradient and electrical potential, resulting in an enhanced proton motive force (secondary energy generation). The proteins involved in histidine decarboxylation system in *L. hilgardii* were found to be 99–100% identical with those of *O. oeni,* suggesting a horizontal acquisition of these systems between the two wine-associated species, *L. hilgardii* and *O. oeni*[94]. In addition to this energy generation system, the mediation of horizontal gene transfer has been suggested in the acquisition of putrescine production system in *O. oeni*[95]. The pathway from arginine and ornithine to putrescine is considered to contribute to energy production in LAB, in a manner similar to that of the histidine/histamine pathway. Generally, the acquisitions of energy through amino acid metabolism, in combination with organic acid metabolism such as malate, pyruvate and citrate, are considered to play a crucial role in LAB surviving in acidic environments[37,84,96,97]. In other words, the acquisition of these metabolic systems, directly or indirectly, contribute to energy production and PMF generation in conditions where nutrients are otherwise scarce. Therefore these studies further indicate that horizontal gene transfer plays an important role in the survival of spoilage LAB living in harsh environments. It is only natural that spoilage LAB have evolved in the long history of alcoholic beverages by sharing multiple genes to improve their chances of survival. This hypothesis also suggests that more species-independent genetic markers will be discovered in spoilage LAB of alcoholic beverages, which will make QC tests more accurate.

## PART 2: HOP RESISTANCE IN BEER SPOILAGE LAB

The hop resistance of beer spoilage LAB is a progressively evolving area of research and many studies have been conducted to elucidate the inhibitory effects of hops and the resistance to these inhibitory effects. In this review, recent progress in this area of research is briefly summarized.

**Hop resistance mechanisms associated with the cytoplasmic membrane.** Because hop bitter acids are assumed to intrude into the cells as proton ionophores, it is important for beer spoilage LAB to ameliorate the intrusion of hop compounds into the cell. The *horA* and *horC* genes, originally identified in *L. brevis,* have been shown to confer hop resistance on LAB[146]. HorA, a product of the *horA* gene, was demonstrated to act as an ABC transporter and efflux hop bitter acids out of the cell (Fig. 6A)[121]. It was also shown that HorA confers resistance to multiple drugs that are structurally unrelated to hop bitter acids, making this protein the second member of the multidrug ABC transporters discovered in bacteria[121]. On the other hand, the presumed secondary structure of HorC is similar to those of PMF-dependent multidrug transporters belonging to the resistance-nodulation-cell division (RND) superfamily (Fig. 6B)[137]. The functional expression of HorC in *L. brevis* demonstrated that this protein confers resistance to hop bitter acids, as well as other structurally unrelated drugs. Therefore HorC was postulated to function as a PMF-dependent multidrug efflux pump and a defence mechanism similar to that of HorA was hypothesized[75,76]. Accordingly, the activities of HorA and HorC presumably result in a reduced net influx of the undissociated and membrane-permeable hop bitter acids into the cytoplasm, and thereby limit the antibacterial protonophoric effect of hop-derived compounds. Since beer spoilage LAB strains develop resistance against rather high concentrations of hop bitter acids, the question arises whether functional expression of HorA and HorC is sufficient to confer hop resistance or whether other activities could contribute to defence mechanisms against hop bitter acids.

Hop compounds are weak acids, which can cross the cytoplasmic membrane in the undissociated form[127–129]. Due to the higher intracellular pH, hop bitter acids dissociate internally, thereby dissipating the transmembrane pH gradient. As a result of this protonophoric action of hop bitter acids, the viability of the exposed bacteria decreases. On the other hand, microorganisms have been found to increase PMF-generating activities in their cytoplasmic membranes when they are confronted with a high influx of protons[161]. Therefore it is conceivable that to defend against the antibacterial effects of hop bitter acids, beer spoilage LAB strains respond by increasing the rate at which protons are expelled out of the cell. In fact, the hop resistant LAB strains were found to maintain a larger transmembrane pH gradient than hop sensitive strains[127,128] and *L. brevis* ABBC45 was demonstrated to increase its activity of proton translocating ATPase upon acclimatization to hop bitter acids[122]. These findings suggest that the extrusion of protons by proton translocating ATPase counteracts the ionophoric effects of hop compounds and helps beer spoilage LAB strains maintain the transmembrane pH gradient. Furthermore, Western blot analysis of membrane proteins with antisera raised against the α- and β-subunits of $F_oF_1$-ATPase from *Enterococcus hirae* showed that there was increased expression of the proton translocating ATPase after the hop adaptation of *L. brevis* ABBC45[122]. The expression levels, as well as the ATPase activity, decreased to the initial non-adapted levels when the hop-adapted cells were cultured further without hop bitter acids. These observations strongly indicate that proton pumping by the membrane-bound ATPase contributes considerably to the resistance of LAB to hop bitter acids.

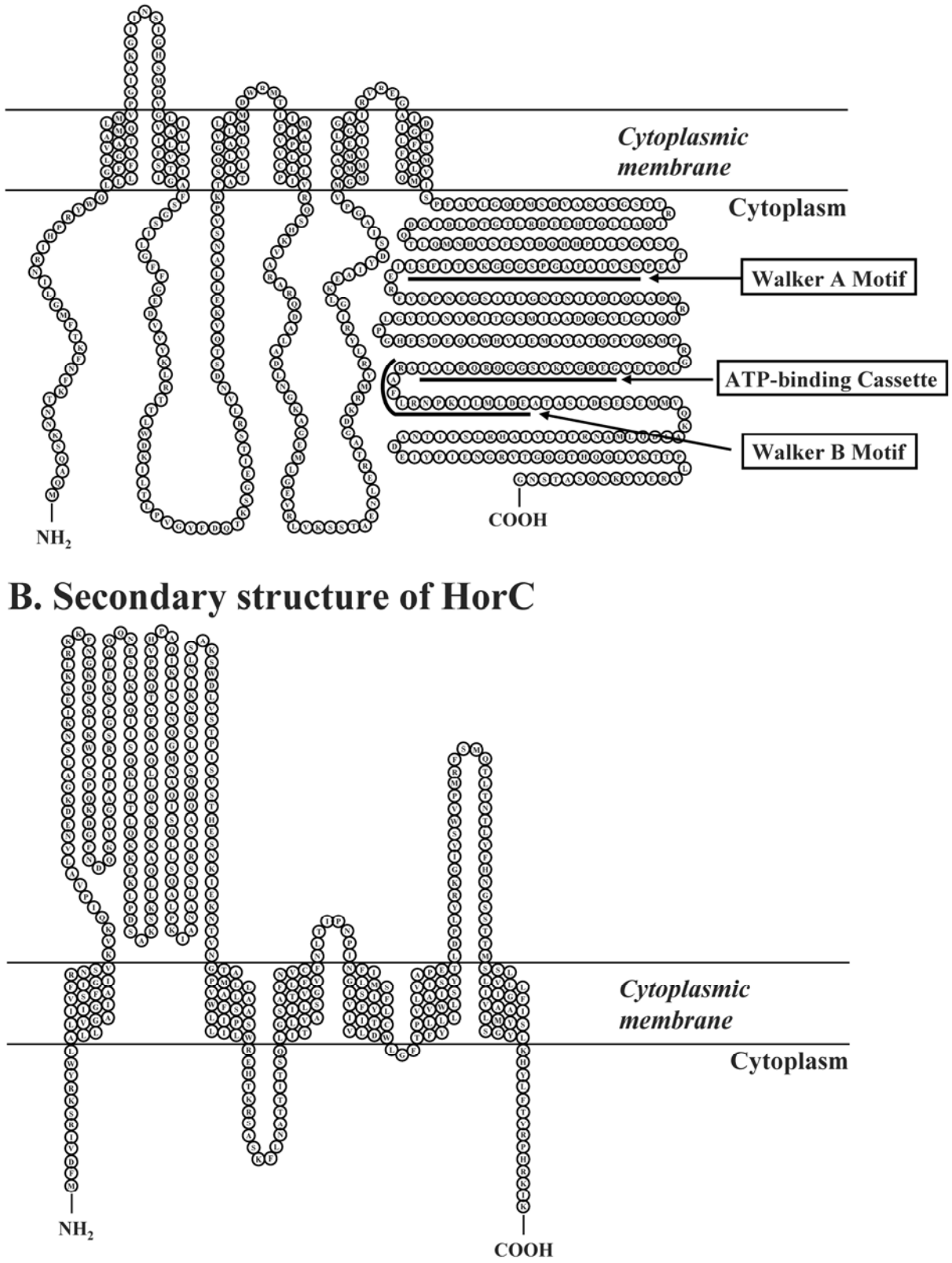


**Fig. 6.** Secondary structure models of HorA (A) and HorC (B). (A) The secondary structure of HorA is cited from the prior literature[137] with some modifications. The conserved residues of ABC transporters, corresponding to the putative ATP-binding cassette, Walker A motif and Walker B motif, are underlined. (B) The secondary structure of HorC was constructed by the SOSUI program[137]. This structure is similar to those of PMF-dependent multidrug transporters belonging to the resistance-nodulation-cell division (RND) superfamily[75,144]. In this figure, the alphabets in circles represent corresponding amino acids in abbreviated forms.

Given that these defence mechanisms are energy-consuming in nature, beer spoilage LAB strains require substantial energy sources to grow in beer. Nevertheless beer is generally considered as a poor medium to support the growth of bacteria, because most of the nutrients have been depleted by the brewing yeast. Furthermore, it has been reported that the protonophoric action of hop compounds inhibits the uptake of nutrients by bacteria[127,128]. Despite these disadvantages, beer spoilage LAB strains are still capable of growth in beer. Indeed three beer spoilage LAB strains, *L. brevis* ABBC45, *L. lindneri* DSM 20690$^T$ and *L. paracollinoides* JCM 11969$^T$, were found to exhibit strong ATP-yielding ability in beer[145]. In addition, the ATP pool in the hop-resistant LAB strains was found to be larger than in the hop-sensitive strains[129]. To investigate energy sources, *L. brevis* ABBC45, *L. lindneri* DSM 20690$^T$ and *L. paracollinoides* JCM 11969$^T$ were inoculated into beer. It was shown that citrate, pyruvate, malate and arginine were consumed to support the growth of the beer spoilage LAB strains[145]. The four components induced considerable ATP production, even in the presence of hop compounds, accounting for the ATP-yielding ability of the beer spoilage LAB strains observed in beer. As discussed earlier, the metabolisms of organic acids and amino acids in LAB are known to directly or indirectly enhance the energy production and PMF generation in conditions where nutrients are otherwise scarce. The putative metabolic pathways of these substrates have been discussed in previous literature[145,146].

In contrast to these active hop resistance mechanisms described above, passive defence mechanisms are also important, in which energy sources are not required once they are established. In *L. brevis,* the membrane composition was reported to change towards the incorporation of more saturated fatty acids, such as C16:0, rendering the membrane less fluid and protecting the cell against the intruding hop bitter acids[17]. This phenomenon is reminiscent of sake spoilage *L. fructivorans* that possesses long-chain fatty acids, exceeding 24 carbons in length, which are not observed in ordinary LAB[77,157,158]. The proportion of these long-chain fatty acids reaches 30–40% in the entire fatty acid composition of the membrane, when sake spoilage *L. fructivorans* is grown in an environment containing a high concentration of ethanol[77]. It is presumed that these unusually long-chain fatty acids prevent the intrusion of ethanol into the cytoplasmic membrane. In wine-associated *O. oeni,* the changes in membrane fluidity coupled with the upregulation of heat-shock proteins led to a reduction in the permeability of the membrane and reinforcement of membrane structures, thereby protecting the cell from the bactericidal effects of ethanol[34,54,55]. From these observations, defence mechanisms associated with the cytoplasmic membrane are generally important for LAB living in harsh environments.

**Hop resistance mechanisms associated with the cell wall.** In beer spoilage *L. brevis,* it has been shown that higher molecular weight lipoteichoic acids (LTAs) in the cell wall increase in response to the presence of hop bitter acids[17,172]. These changes in the compositions of LTAs are suggested to reduce the intrusion of hop bitter acids into cells by increasing the barrier functions of the cell wall against hop bitter acids. LTAs are also hypothesized to act as reservoirs of divalent cations, such as $Mn^{2+}$, which are otherwise scarce as a result of complexation with hop compounds[17,162]. The altered LTAs have an increased potential to bind divalent cations and to compete for them with hop bitter acids, thus reducing the detrimental effects of hops towards the cell. This type of resistance can also be considered as a passive defence mechanism that requires little energy once established. In relation to the reservoir function of LTAs for $Mn^{2+}$, Hayashi et al.[65] proposed HitA as one of the mediators of hop resistance in *L. brevis* and suggested that HitA plays a role in the uptake of divalent cations, such as $Mn^{2+}$, while hop bitter acids reduce the intracellular divalent cations. In fact, many of the proteins involved in energy generation and redox homeostasis are dependent on $Mn^{2+}$, therefore intracellular $Mn^{2+}$ plays an important role in LAB[18]. Accordingly, these mechanisms may function in concert for beer spoilage LAB to counteract the loss of intracellular $Mn^{2+}$.

In sake spoilage *L. fructivorans* and *L. homohiochi*, the presence of ethanol has been reported to induce an increase in cell wall thickness[70,72]. It was suggested that the increase in cell wall thickness is involved in the ethanol tolerance observed in sake spoilage LAB. Furthermore, the *gtf* gene that encodes glucosyltransferase is known to exist in some strains of wine-associated *O. oeni*[42]. The presence of this gene induces the formation of a cell envelope mainly consisting of β-glucans and elevates the ethanol tolerance of the *O. oeni* strains that possess the *gtf* gene[42]. Accordingly, the defence mechanisms associated with the cell wall appear to play a vital role as well for the spoilage LAB in alcoholic beverages.

**Other hop resistance mechanisms.** It has been reported that $Mn^{2+}$-dependent enzymes are induced by hop bitter acids in *L. brevis*[18]. These hop-inducible enzymes are suggested to be involved in energy generation and redox homeostasis. One explanation for this phenomenon is that the cells respond to $Mn^{2+}$ limitation by upregulating these enzymes, thus compensating for the reduced levels of intracellular manganese and the lost activities of $Mn^{2+}$-dependent enzymes[18]. Very recently, the antibacterial mechanisms of hop compounds were suggested to involve proton ionophoric actions and redox-reactive uncoupler activities occurring in parallel[19,162]. Accordingly, it is plausible that beer spoilage LAB have to cope with oxidative stress induced by hop compounds, in addition to PMF depletion. Thus, the observed upregulation of $Mn^{2+}$-dependent enzymes responsible for redox homeostasis is most likely part of a defensive response to the oxidative stress caused by hop bitter acids[19,162]. On the other hand, a morphological shift into smaller rods was observed in beer-adapted *L. brevis* and *L. lindneri* cells (Fig. 7)[2]. The diminished cell size is presumably due to the efforts by the beer spoilage LAB to reduce the surface area that is in contact with the beer. This is conceivable as beer contains many bactericidal factors, including hop compounds. In addition to reducing the defence perimeters, the minimized cell surface area presumably helps beer spoilage LAB to deploy membrane-bound resistance mechanisms more efficiently[143]. In similar cases, it has been observed that sake spoilage LAB remain morphologically compact in the presence of a high ethanol content, while ethanol-sensitive LAB tend to exhibit elongated cell forms[71]. From

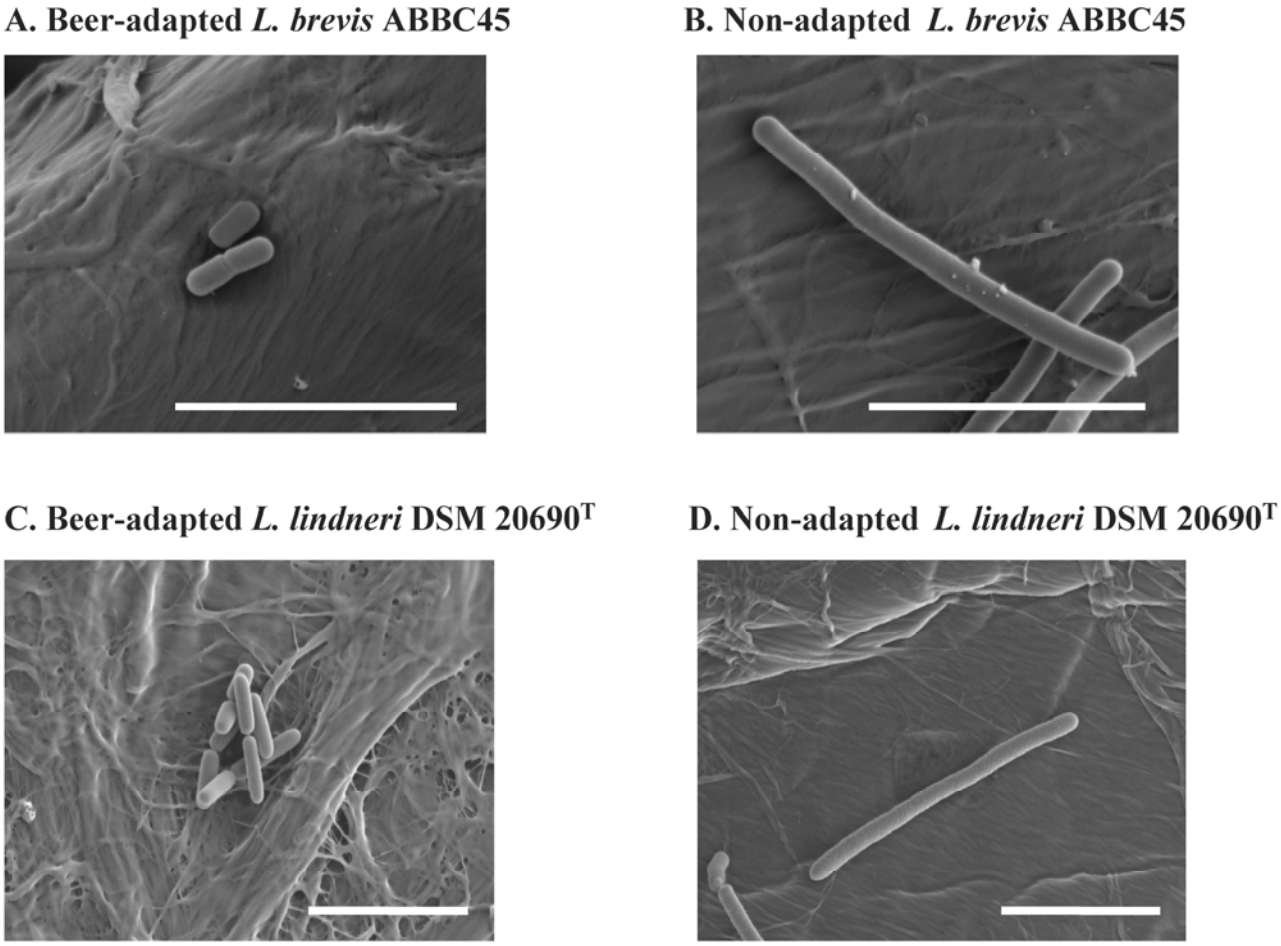


**Fig. 7.** Effects of beer adaptation on morphological features of beer spoilage LAB. Beer spoilage LAB strains were grown in degassed beer for (A) and (C), and in MRS broth for (B) and (D). Cells were trapped on a membrane filter and the morphological features of beer-adapted and non-adapted strains were compared, using scanning electron microscopy[2]. Bar, 5 μm.

these observations, the reduced surface area that is in contact with external environments is probably advantageous for spoilage LAB that must survive in a hostile milieu.

The hop resistance mechanisms described above are summarized in Fig. 8. As discussed in this part of the review, hop resistance mechanisms are more complex than previously assumed. Presumably these multiple layers of defence systems in beer spoilage LAB have been acquired progressively through centuries of beer brewing history. Undoubtedly these are only part of the whole resistance mechanisms of beer spoilage LAB and novel defence mechanisms will be found in the future. In addition, the inhibitory actions of hop compounds have recently been shown to involve oxidative stress. This newly found inhibitory mechanism goes beyond the proton ionophore activities that have been traditionally accepted[162]. It is hoped that a more comprehensive picture will emerge concerning hop resistance of beer spoilage LAB that will eventually lead to more accurate QC tests in breweries.

## PART 3: *PECTINATUS* AND *MEGASPHAERA*

**Current taxonomy of *Pectinatus* and *Megasphaera*.** *Pectinatus* is an anaerobic contaminant of unpasteurized beer that has been isolated since the 1970s[88,89]. This strictly anaerobic bacterium that stains Gram-negative is affiliated to the *Sporomusa* sub-branch of the class *Clostridia* in the phylum *Firmicutes*[66]. The genus *Pectinatus* produces unpleasant off-flavours, such as hydrogen sulphide, which makes beer smell like a rotten egg[8]. *Pectinatus* is also noted for the production of a large amount of propionic acid, often exceeding 1,000 mg/litre in spoiled beer[60,148]. The cells are usually motile and the active young cells form an "X" shape during movement, whereas old cells have a characteristic slow snake-like movement[125]. A striking feature of the genus *Pectinatus* is the comb-like arrangement of flagella on the concave side of the cells, which is the origin of the genus name *Pectinatus,* meaning "comb-like shape" in Latin[125]. The genus *Pectinatus,* first described in 1978[88], was isolated from beer that had been stored at 30°C. The type species of this genus is *P. cerevisiiphilus,* a name that means a beer-loving comb-shaped bacterium in Latin[125]. Another species, *P. frisingensis,* although already isolated in 1978 and identified as *P. cerevisiiphilus* in 1981[63], was finally established as a distinct species in 1990[125]. New species, *P. portalensis* and *P. haikarae,* were proposed in 2004[51] and 2006[80], respectively. However, on the basis of 16S rRNA gene sequence analysis and several key phenotypic fea-

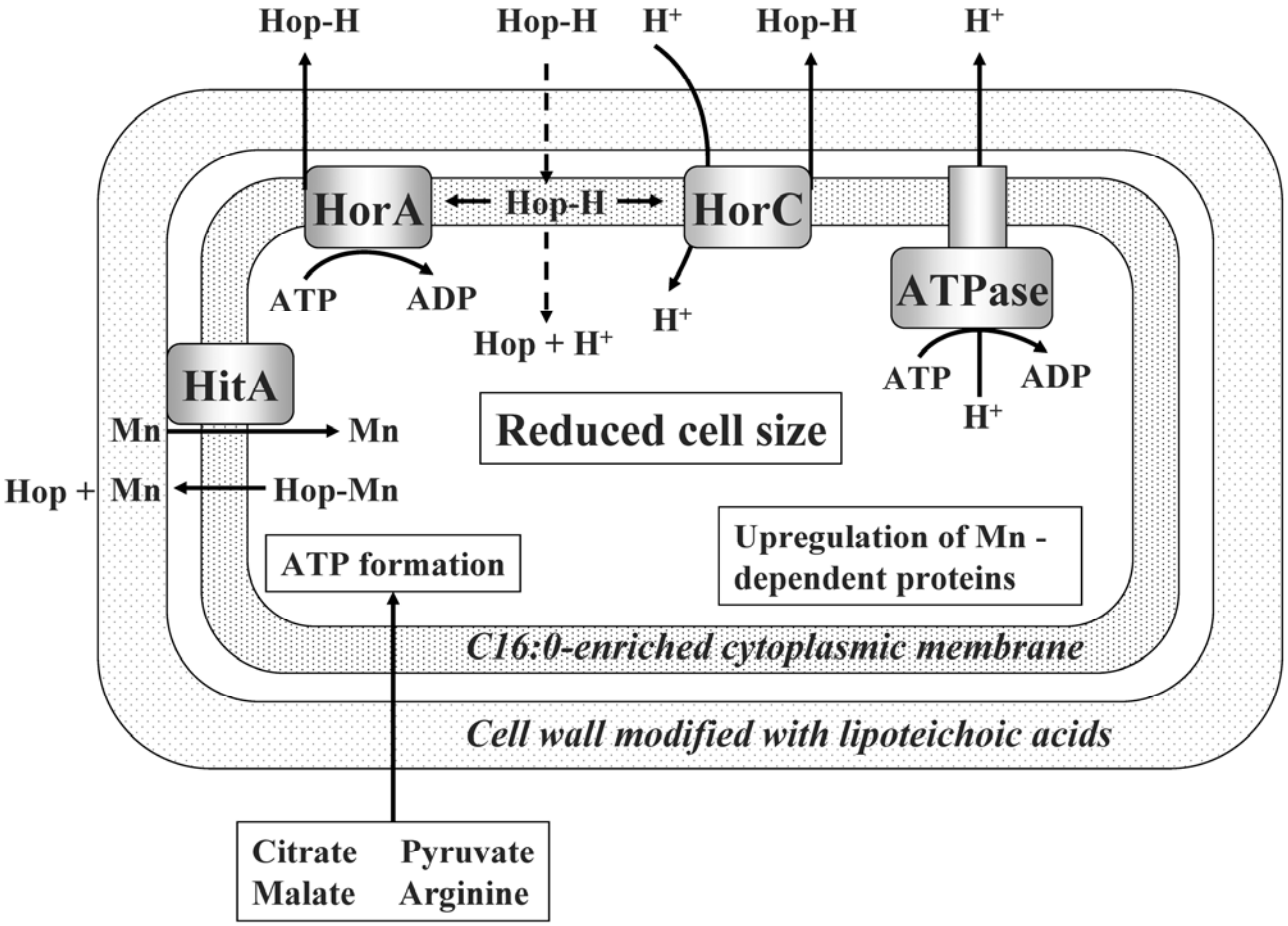


**Fig. 8.** Complex hop resistance mechanisms in beer spoilage *Lactobacillus brevis*. Hop resistance mechanisms recently reported are comprised of the following defence systems. (1) Mechanisms for prevention of hop incursions involve HorA and HorC as efflux transporters, cytoplasmic membrane modifications and cell wall modifications. These systems presumably function together to reduce the incursion of undissociated and membrane-permeable hop compounds (Hop-H). Proton-translocating ATPase also counteracts the proton ionophoric actions of hop compounds by pumping out intruding protons. (2) Intracellular $Mn^{2+}$ levels are maintained by the actions of putative $Mn^{2+}$ transporter, HitA. In addition, the modified cell walls function as $Mn^{2+}$ reservoirs and presumably counteract the loss of intracellular $Mn^{2+}$. Furthermore, $Mn^{2+}$-dependent proteins are upregulated in response to hop compounds. The upregulation of these proteins presumably compensates for the loss of their activities caused by reduced intracellular $Mn^{2+}$ levels. It is also hypothesized that the upregulated $Mn^{2+}$-dependent proteins that are involved in redox homeostasis counteract the oxidative stress conferred by hop compounds. (3) Metabolisms with citrate, pyruvate, malate and arginine supply ATP and proton motive force (PMF) for active defence mechanisms such as HorA, HorC and proton-translocating ATPase. (4) Morphological shifts into smaller rods reduce the contact areas against hostile external milieu and help beer spoilage LAB to deploy more efficiently the membrane-bound defence mechanisms, such as HorA, HorC and proton-translocating ATPase.

tures, it was ascertained that the cultures cited as the type strain of the species *P. portalensis,* CECT 5841$^T$ and LMG 22865$^T$, do not conform to the original description[160]. It has therefore been proposed that the name *P. portalensis* should be rejected[160]. Therefore currently *P. cerevisiiphilus, P. frisingensis* and *P. haikarae* can be considered as validly described species of *Pectinatus*. Other morphologically similar bacteria, principally isolated from pitching yeast, have been described and designated as *Selenomonas lacticifex, Zymophilus raffinosivorans* and *Z. paucivorans*[125]. These strictly anaerobic bacteria are phylogenetically related to *Pectinatus* based on 16S rRNA gene sequence comparisons.

*Pectinatus* species are also of academic interest insofar as they are intermediates between Gram-negative and Gram-positive eubacteria[26]. *Pectinatus* isolates stain Gram-negative to Gram-variable, and possess an outer membrane that is typical of Gram-negative bacteria[66]. On the other hand, *Pectinatus* has very thick peptidoglycan layer and a cytoplasmic membrane characteristic of Gram-positive bacteria[66]. In addition, *Pectinatus* groups with Gram-positive eubacteria based on the 16S rRNA gene phylogenetic analysis[63,125].

*Megasphaera,* like *Pectinatus, Selenomonas* and *Zymophilus,* belongs to the group of bacterial species with a Gram-negative cell envelope within the phylum of Gram-positive bacteria[125,132]. The first isolate of *Megasphaera* was made from German beer by Weiss et al. in 1979[168] and a new species *Megasphaera cerevisiae* was proposed by Engelmann and Weiss in 1985[45]. At present, the beer spoilage group of the genus *Megasphaera* includes three species, *M. cerevisiae, M. paucivorans* and *M. suecien-*

*sis*[80]. *Megasphaera* spoils mainly low-alcohol beers by producing turbidity, hydrogen sulphide and short-chain fatty acids[8]. *Megasphaera* is a strictly anaerobic, Gram-negative coccus, mainly arranged in pairs and occasionally in short chains. Mean cell size is 1.2 × 1.0 μm. *Megasphaera* is a mesophilic bacterium and growth occurs at 15–37°C with an optimum around 30°C, but not at 10 or 45°C[80].

**Ecological aspects of *Pectinatus* and *Megasphaera.*** *Pectinatus* and *Megasphaera* constitute an important group of spoilage bacteria of unpasteurized packaged beer. The natural environment of these anaerobic beer spoilage bacteria and the source of contamination are not well known. Most of the strains described and characterized to date have been almost exclusively isolated from beer. However, *Pectinatus* has sporadically been found in lubrication oil mixed with beer, drainage and water pipe systems, air of the filling hall and the filling machine, as well as on the floor of the filling hall, in condensed water on the ceiling, in chain lubricants and in the steeping water of malt before milling[11,43,61,89,130]. Other studies further showed that *Pectinatus* is found in conveyors, sewers, supporting structures of filler, and loose tiles or cracks of damaged floors and walls in breweries[8,100]. Therefore, the *Pectinatus* findings have been concentrated in the filling hall and other hidden spots in breweries. It has thus been considered that *Pectinatus* is a permanent inhabitant in breweries where it finds niches for prolonged survival. These findings also indicate water and other aqueous environments are a likely source of contamination[8,59]. Moreover, despite its anaerobic nature, *Pectinatus* can survive in aerosols and can possibly be transferred via the air into beer[43]. The reported presence of *Pectinatus* in air near unclean bottles and close to the bottling machine in the filling area indicates the possibility that air or aerosols may be another contamination source[61]. The ecological aspects of *Megasphaera* are not well known, but the simultaneous findings of *Pectinatus* and *Megasphaera* are reported[8]. Hence it is conceivable that *Megasphaera* also shares ecological niches with *Pectinatus*.

*Pectinatus* species grow at temperatures between 15 and 40°C with the optimum being 30 to 32°C[8,125]. The growth of *Pectinatus* slows down considerably at low temperatures and therefore *Pectinatus* species are rarely found in the fermentation and maturation processes in bottom-fermented beer, but may occur in these processes in top-fermented beers[61], where the fermentation temperature is comparatively higher. Organic acids, such as propionic acid produced by *Pectinatus,* are reported as a main reason for the inhibition of growth and ethanol production of *S. cerevisiae* in fermentations above 15°C[30]. On the other hand, the survival of *Pectinatus* was better at lower temperatures (8–15°C) than 32°C[29]. Therefore *Pectinatus* tends to persist in environments with a lower temperature. Furthermore, temperatures also inversely influence the oxygen resistance of *Pectinatus*. The time of decimal reduction (an inactivation kinetic parameter used to indicate rate of microbial reduction) decreased by 6.7 fold when the temperature decreased from 32°C to 8°C[29]. Therefore *Pectinatus* is better able to survive at lower temperatures. In some breweries, the temperature of the filling hall is maintained low to suppress the proliferation of microorganisms. Accordingly, these filling halls are at risk by *Pectinatus* contamination, unless the filling environments are stringently cleaned and disinfected. It has also been reported that the oxygen resistance of *P. frisingensis* is higher than that of *P. cerevisiiphilus*[58], which partially explains the predominant findings of this species in the filling area and in spoiled beer.

According to Back[6], *Pectinatus* and *Megasphaera* are inhabitants of biofilms where mixed populations of microorganisms are present. In the hypothesized biofilms, yeast and aerobic bacteria consume dissolved oxygen thereby creating anaerobic environments, and subsequently lactic acid bacteria produce lactate that can be consumed by *Pectinatus* and *Megasphaera*[87,90]. Furthermore, these biofilm communities are typically protected by a slime covering produced by coexisting microorganisms such as yeast and aerobic bacteria[6]. Since *Pectinatus* is sometimes found with lactic acid bacteria in the filling hall[8], this suggests that the biofilm communities consisting of these bacteria are actually formed in the filling area. Accordingly, the biofilms involving the mixed populations of microorganisms could be part of the reason why *Pectinatus* and possibly *Megasphaera* persist in breweries.

**Beer spoilage ability of *Pectinatus* and *Megasphaera.*** There are a number of factors affecting the beer spoilage ability of *Pectinatus* and *Megasphaera*. For instance, it has been observed that beer with a low ethanol content is more prone to *Pectinatus* and *Megasphaera*[8]. So the rate of spoilage caused by these bacteria is inversely dependent on the ethanol content of the beer. The growth of *Megasphaera* is already restricted in commercial beer with an ethanol content of 3.5% (w/v)[61]. *Pectinatus* is more ethanol tolerant, thus growing rather well in beer with a higher ethanol content (3.7–4.4% (w/v))[61], although its growth rate becomes slower as the ethanol content in the beer increases. It has also been reported that *Pectinatus* does not grow in beer with an ethanol content exceeding 5.2% (w/v)[63].

The pH of beer is another important characteristic affecting the growth of microorganisms in beer. As the pH of beer becomes higher, it is more susceptible to *Pectinatus* and *Megasphaera* contamination[8]. Of the two genera, *Pectinatus* is more tolerant to a low pH environment and a pH value of 4.1 is required for retardation of growth[57], although the presence of ethanol reduces the resistance of *Pectinatus* to low pH in a dose-dependent manner. The maximum biomass production was observed at pH 6.2 for *P. cerevisiiphilus* and between pH 4.5 and 4.9 for *P. frisingensis*[155,156], indicating that *P. frisingensis* is more acidophilic. On the other hand, the retardation in the growth of *Megasphaera* was observed in beer with pH value of 4.5[62], but the growth was reported to still occur in beer with the pH value of 4.38 but not pH 4.0[59]. However, similar to the case with *Pectinatus,* the pH tolerance of *Megasphaera* can differ, depending on the ethanol content and other factors in the beer.

The oxygen content of the beer is also one of the most decisive factors that affects the growth of *Pectinatus* and *Megasphaera*[61]. The dissolved oxygen content in beer has decreased considerably in recent years due to advances in filling technologies. At present, an air volume of 1 mL or less in the headspace is easily achievable, as is an oxygen

content of as little as 0.3 mg/litre[61]. Back reported that *Pectinatus* and *Megasphaera* strains are able to grow in beer with less than 0.3 mg/litre of dissolved oxygen[8], so contamination caused by these bacteria is mainly encountered in large modern breweries with sophisticated filling technologies. However, it has been shown that *Pectinatus* grows in wort with 0.96 mg/litre dissolved oxygen[29]. Therefore, depending on the type of beer or contamination level, spoilage can occur at higher dissolved oxygen levels than 0.3 mg/litre.

The beer spoilage ability of *Selenomonas* and *Zymophilus* is poorly studied, but the laboratory inoculation tests indicate that *S. lacticifex* is able to grow in beer with pH value of 4.3–4.6, suggesting that this species is categorized as an obligate or potential beer spoiler[78,126]. In contrast, *Z. raffinosivorans* and *Z. paucivorans* cannot grow in beer with the pH value of 4.6 and are only able to grow in beer with pH value of 5.0 and 6.0, respectively[126]. These observations indicate that *Z. raffinosivorans* is a potential beer spoiler, while *Z. paucivorans* is considered as an indicator microorganism of contamination in beer brewing environments. *Selenomonas* and *Zymophilus* are mainly isolated from pitching yeast and to the best knowledge of this author have not been implicated as the causative agents in beer spoilage incidents.

**Detection and identification of *Pectinatus* and *Megasphaera.*** Many agar media and broths have been reported for the isolation and cultivation of *Pectinatus* strains. MRS, NBB-A and Raka-Ray are recommended by European Brewery Convention (EBC) for detection of *Pectinatus*[46]. These media are also recommended for the detection of *Megasphaera,* but due to the inability of this species to utilize glucose, supplementation with fructose is recommended by the EBC[46]. When selecting agar media, caution should be exercised with membrane filtration methods, because the culturability of the cells may be lost on the membrane filter, resulting in drastically reduced colony forming units. The culturability of *Pectinatus* and *Megasphaera* on agar media can also be affected by the incubation system and the membrane filter (materials and manufacturers etc.) used in the QC tests. Thus one must evaluate the recovery rate of *Pectinatus* and *Megasphaera* before adopting a particular agar medium and other related parameters, including medium formulations, should be optimized. In addition to agar media, a selective medium for the isolation and differentiation of *Pectinatus* strains and *Megasphaera cerevisiae* was developed by Lee and designated "Selective Medium for *Megasphaera* and *Pectinatus*" (SMMP)[87]. The SMMP medium is beer-based and supplemented with reducing agents, 1% lactate as the sole carbon source, 20 ppm cycloheximide to inhibit yeasts and 5 ppm crystal violet with 25 ppm sodium fusidate to inhibit Gram-positive bacteria. Enterobacteria are suppressed by the ethanol present in the beer and the low pH of the SMMP medium.

Equally important with the media used for cultivation of *Pectinatus* and *Megasphaera* is the maintenance of strictly anaerobic culture conditions. The anaerobic chamber and the Gas Pak system provide adequate growth conditions[61]. The use of pre-reduced media and special reducing agents can enhance growth[61], although depending on media used, growth retardation may be observed with *P. cerevisiiphilus* when cysteine hydrochloride is supplemented as the reducing agent.

In terms of *Pectinatus* and *Megasphaera,* the species-specific approach seems to be adequate for the rough determination of beer spoilage ability, because the intra-species differences in beer spoilage ability are relatively smaller than those observed in beer spoilage LAB. For species-specific identification of *Pectinatus* and *Megasphaera*, various molecular biological methods have been proposed and PCR-based methods, using 16S rRNA genes or the spacer regions between 16S rRNA gene and 23S rRNA gene, have been developed[79,101,102,120]. More recently, multiplex PCR methods have been developed to identify all the six *Pectinatus* and *Megasphaera* species currently known. In this method, multiple primer sets specific to a number of microorganisms are mixed in one single reaction tube, making the PCR procedure less laborious and time consuming[73]. Additionally, group-specific real-time PCR has been proposed in combination with an end-point melting curve analysis of PCR products[78]. In this melting curve analysis, species-specific discrimination of the amplified products is performed based on the melting behaviour, which is a function of their GC/AT ratio, length and sequence. Accordingly, the method enables the identification and differentiation of the nine species of *Pectinatus, Megasphaera, Selenomonas* and *Zymophilus* in one single reaction. However, most of these methods described above require a culturing step for pre-enrichment to enhance the sensitivity of the PCR methods for the detection of a trace level of *Pectinatus* and *Megasphaera* present in beer. To overcome the problem, a fluorescent *in situ* hybridization (FISH) technique, based on the binding of a fluorescein-labelled oligonucleotide probe to rRNA, was evaluated as the detection and identification method of *Pectinatus*[171]. As a result, it was demonstrated that this FISH technique was directly applicable to the beer samples without a culturing step and was able to detect *Pectinatus* in a species-specific manner within 5 h.

Strain-specific identification is also important to determine the contamination route of microorganisms. Motoyama et al.[103] used three different restriction enzymes (*Eco*R I, *Hin*d III and *Bam*H I) for the ribotyping of *Pectinatus* strains. When all of these enzymes were combined, 34 *P. frisingensis* strains were grouped into 17 distinct ribotypes and 5 *P. cerevisiiphilus* strains were grouped into 3 ribotypes. Ribotyping has also been used for characterization of *Megasphaera* strains using three restriction enzymes (*Eco*R I, *Pst* I and *Pvu* II)[136]. As a consequence, the strains tested in this study were divided into seven ribotypes. The levels of intra-species discrimination within *Pectinatus* and *Megasphaera* achieved by these approaches are considered as useful for more accurate investigations of contamination routes in breweries.

**Eradication of *Pectinatus* and *Megasphaera*.** In general, beer spoilage microorganisms are sensitive to heat treatment, and it has been suggested that all beer spoilage microorganisms are killed off at 30 pasteurization units (PU)[12]. The typical beer spoilage bacteria are already killed below 15 PU and many of these perish at 5–8 PU. However, some beer spoilage lactobacilli are known to exhibit a moderate level of heat tolerance. For example, *L.*

*lindneri* can tolerate up to 17 PU and *L. brevis* subsp. *frigidus*, because of its mucus encapsulation, may tolerate even up to 27 PU[12]. In contrast, the heat resistance of *Pectinatus* is relatively low and the D-60 value (a measurement of heat resistance at 60°C, in which the time required for a decimal reduction of tested microorganism is shown) for this species was suggested to be close to 0.4 min[166]. Other researchers[61] also reported that treatment at 58–60°C for one min, which is less than normal pasteurization treatment, is sufficient to kill *Pectinatus*. As for *Megasphaera,* the D-60 value of 0.55 min was reported in wort and beer[165]. Therefore the ordinary thermal treatment appears to be sufficient to control *Pectinatus* and *Megasphaera*.

*Pectinatus* strains are also susceptible to most disinfectants used in breweries. These include iodine, chlorine, peracetic acid and formaldehyde[61]. *Megasphaera* is killed easily by oxidizing agents and quaternary ammonium compounds[59], but iodine is suggested to be less effective for *Megasphaera*[59].

Accordingly, *Pectinatus* and *Megasphaera* are relatively easy to control with thermal treatment and sanitizing agents. Despite these facts, *Pectinatus* and *Megasphaera* tend to persist in breweries. This is presumably because *Pectinatus* and *Megasphaera* are latent in hard-to-access corners or biofilms, which are difficult to clean and disinfect. Therefore it is vitally important to determine the hiding spots of *Pectinatus* and *Megasphaera* in breweries and to eradicate them.

**Other physiological properties to be noted.** Hop bitter acids generally restrict the growth of Gram-positive bacteria in beer. Nevertheless, *Pectinatus* and *Megasphaera* are rather tolerant to hop bitter compounds, as evidenced by the fact that the growth occurs in beer with the range of 33–38 bitterness units (BU)[4,8]. Gram-negative bacteria are known to be resistant to bactericidal lipophilic compounds including hop bitter acids, the resistance of which is conferred by the outer membrane barrier and multiple efflux systems[66]. *Pectinatus* and *Megasphaera* have been recently assigned to the *Sporomusa* sub-branch in the family *Acidaminococcaceae* of the class *Clostridia* of the phylum *Firmicutes*[26]. Despite the assignment to the class *Clostridia* of Gram-positive bacteria, cells of *Pectinatus* spp. stain Gram-negative and possess an outer membrane. It has also been shown that they contain a lipopolysaccharide (LPS) typical of Gram-negative bacteria[66]. Electron microscopic examinations have revealed that the outer membrane of *Pectinatus* spp. exhibits an unusual wrinkled appearance with numerous bulges[60,63]. Notably *P. cerevisiiphilus* has been shown to be susceptible to vancomycin and bacitracin[67], which are large molecules that are normally unable to penetrate the outer membrane of Gram-negative bacteria. It has also been demonstrated that *P. frisingensis* is sensitive to nisin, a large antibacterial peptide that normally does not affect Gram-negative bacteria[27]. These results suggest that the outer membrane of *Pectinatus,* in the conditions tested, does not act as an effective permeability barrier. Therefore the outer membrane of *Pectinatus* may not be responsible for its relatively strong tolerance to hop bitter acids. Or alternatively, the permeability properties of the *Pectinatus* outer membrane could be different between hop bitter acids and those compounds tested in the above studies. It is also conceivable that *Pectinatus* and possibly *Megasphaera* are protected by complex resistance systems including effective efflux systems of hop bitter acids.

*Pectinatus* and *Megasphaera* also grow in acidic and ethanol-containing environments under anaerobic conditions where the usual Gram-negative bacteria, such as *Escherichia coli* and *Salmonella,* cannot grow. Coupled with a rather high tolerance to hop bitter acids, these are indeed the principal factors that allow *Pectinatus* and *Megasphaera* to spoil beer. *P. frisingensis,* in particular, is reported to tolerate well the environments encountered in beer. For example, this species is able to maintain a higher intracellular pH than the external pH even at pH 4.5[28]. In fact, *P. frisingensis* was found to grow optimally at pH values in the range of 4.1 and 5.1 and was shown to grow, although poorly, at an ethanol content up to 1.2 mol/L (7.2% (w/v))[156]. In comparison with *P. frisingensis, P. cerevisiiphilus* and *M. cerevisiae* exhibited somewhat lower levels of ethanol tolerance and grew less well in a low pH environment[61,156]. These observations indicate that *P. frisingensis* is potentially the most frequent species associated with beer spoilage incidents among these species.

**Future perspectives for *Pectinatus* and *Megasphaera*.** Bacteria in the genus *Pectinatus* and *Megasphaera* are unique from both applied and academic view points[26,61,66]. From an academic standpoint, they are known as intermediates between Gram-negative and Gram-positive bacteria. *Pectinatus* and *Megasphaera* isolates stain Gram-negative, and possess an outer membrane and LPS typical of Gram-negative bacteria. Conversely, they have a very thick peptidoglycan layer and cytoplasmic membrane, characteristics that are typical of Gram-positive bacteria. The 16S rRNA phylogenetic analysis also indicates that these genera are assigned to the class *"Clostridia"* of the phylum *Firmicutes,* a group of Gram-positive bacteria. In fact, the relatively high hop resistance of *Pectinatus* and *Megasphaera* is similar to that of Gram-negative bacteria, such as Enterobacteria, whereas the relatively strong tolerance to ethanol and acidic conditions is reminiscent of Gram-positive bacteria, such as LAB. Some species belonging to *Clostridia*, such as *Clostridium acetobutylicum*, can grow at a somewhat low pH value (up to 4.2) and can occasionally affect low alcohol or alcohol-free beers[12]. *Pectinatus* and *Megasphaera,* especially *P. frisingensis,* show more strong resistance to ethanol and low pH environments than ordinary *Clostridium* strains. It would be very interesting to explore the mechanisms underlying the resistance of *Pectinatus* and *Megasphaera* to ethanol and acidic conditions. The rather high hop resistance of *Pectinatus* and *Megasphaera* will be also an intriguing avenue for future research.

The origins of *Pectinatus* and *Megasphaera* are also unknown. Several studies indicate that *Pectinatus* and *Megasphaera* are secondary contaminants that most likely enter beer products during the filling operation[61] and the findings of *Pectinatus* spp. appear to be concentrated in the filling area and other places in breweries[8,11,43,61,89,130]. Therefore *Pectinatus* spp. are considered as permanent inhabitants, rather than occasional invaders of the brewery, and they presumably find niches in breweries where

they can survive. On the other hand, one study analyzing lipopolysaccharides in *Pectinatus* suggests that this genus may be originally associated with plants[66]. This is because O-chains rich in deoxysugars, which were observed in some of the *Pectinatus* strains, are often found in plant-associated bacteria including saprophytic or pathogenic bacteria of plants. So the authors hypothesized that *Pectinatus* spp. were carried to breweries with plant materials such as cereals, rice and hops. However, the occurrences of *Pectinatus* and *Megasphaera* are largely unknown outside the beer brewing environment and further ecological studies will be needed to elucidate the origins of *Pectinatus* and *Megasphaera*. One intriguing observation is that *Pectinatus,* and to a lesser extent *Megasphaera,* are sometimes found simultaneously with LAB in beer brewing environments[6,8]. On the other hand, a close association between *Saccharomyces* yeast and beer spoilage LAB has been reported in prior literature[137,139]. Accordingly, it is interesting to imagine that these three groups of microorganisms form a community where *Saccharomyces* yeast provides the anaerobic conditions, while the LAB supply lactate as a nutrient for *Pectinatus* and *Megasphaera*. In this hypothetical community, *Saccharomyces* yeast and beer spoilage LAB create an acidic environment that contains a moderate amount of ethanol, which together with hop bitter acids in beer give *Pectinatus* and *Megasphaera* a competitive advantage over other microorganisms. So it is definitely a possibility that *Pectinatus* and *Megasphaera* have emerged and persisted as a minor constituent in brewing environments since the beginning of beer brewing.

The incident reports of *Pectinatus* and *Megasphaera* in Europe culminated in the early 1990s reaching ca. 30% of the entire spoilage incidents, but this number subsided somewhat in the late 1990s and early 2000s[5–7]. This is presumably because the technologies for detecting and eradicating *Pectinatus* and *Megasphaera* had advanced in modern breweries. However, the spoilage incidents caused by these anaerobic bacteria are particularly damaging as the affected products have very strong sulphuric notes reminiscent of rotten eggs. It is also notable that strong turbidity occurs in beers spoiled by *Pectinatus* strains, making the beer completely undrinkable[8]. As filling technologies in breweries advance globally, the need for more rapid detection and identification methods will be more and more desired to prevent spoilage incidents by these bacteria. Indeed, further studies from academic and applied standpoints are currently in progress for both *Pectinatus* and *Megasphaera*.

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