The influence of thiamine and riboflavin on various spoilage microorganisms commonly found in beer

by Hucker, B., Christophersen, M. and Vriesekoop, F.

DOI: http://dx.doi.org/10. 10.1002/jib.385

The Influence of Thiamine and Riboflavin on Various Spoilage Micromicroorganisms Commonly Found in Beer

Barry Hucker[1], Melinda Christophersen[2], Frank Vriesekoop[1,3,4]

[1] Faculty of Science and Technology, Federation University Australia, Ballarat, Victoria, 3353, Australia

[2] Analytical Services Department, Carlton and United Breweries, Southbank, Victoria, 3006, Australia

[3] Department of Food Science and Agri-Food Supply Chain Management, Harper Adams University, Newport, TF10 8NB, England

[4] Corresponding author. E-mail: fvriesekoop@harper-adams.ac.uk

Abstract

Beer is generally considered a stable product due to its intrinsic "unfavourable" conditions (hops, alcohol, low oxygen etc.) that inhibit the growth of pathogenic microorganisms. However spoilage microorganism such as *Lactobacillus brevis, Pediococcus damnosus*, *Acetobacter aceti*, *Zymomonas mobilis,* and various wild yeasts (e.g. *Brettanomcyes* spp.) can have significant detrimental effects to the organoleptic properties of the final product. The presence of essential vitamins such as thiamine and riboflavin can help to enhance the growth of these microorganisms accelerating the rate of spoilage. The presence of thiamine had a noticeable effect on the lactic acid productivity of *L. brevis* and *P. damnosus*; acetaldehyde productivity in *Z. mobilis*; and acetic acid production in *Brettanomyces spp.* While riboflavin enhanced the 2,3-pentadione production by *P. damnosus* and *Brettanomyces spp*.

Key Words

Spoilage, beer, vitamins

Running Title

Effect of thiamine and riboflavin on spoilage microorganisms in beer

Introduction

Beer is typically considered a safe product to consume since no known pathogens can survive in a typical full-strength beer [1, 2]. This appears to be due predominantly to a series of intrinsic antimicrobial factors such as the inclusion of hops, a relatively low pH, and elevated ethanol and carbon dioxide content [1]. However, it is possible for spoilage bacteria such as *Lactobacillus brevis, Pediococcus damnosus, Acetobacter aceti, Zymomonas mobilis,* and wild yeasts (e.g. *Brettanomcyes* spp.) to still spoil beer through compromised hygiene practices. These microorganisms may be non-pathogenic, but can be detrimental to the quality of some styles of beer [2, 3]. They produce a range of unwanted flavours, aromas and in some instances cause turbidity. Specific spoilage characteristics include the accumulation of lactic acid (*L. brevis, P. damnosus*), vicinal diketones (diacetyl and 2,3- pentanedione; *P. damnosus*), acetaldehyde (*Z. mobilis*), acetic acid (*Brettanomcyes spp, A. aceti*), cresols, ethylphenol and eugenol (*Brettanomyces spp*). In some styles of beer, such as Lambic, gueuze, abbey style beers and sour ales, these same characteristics can be considered as desirable. In general, though, in most lager and ale styles of beer these characteristics would be considered a fault and would be rejected by the consumer.

The influence of the presence of thiamine and riboflavin on the growth of potential spoilage microorganisms in beer is currently unknown. Since these vitamins are utilised in a number of principle metabolic processes, it is likely that their presence or absence may aid or hinder the growth of beer spoilage microorganisms. Spoilage microorganisms such as *L. brevis, P. damnosus, Z. mobilis, A. aceti, Brettanomcyes lambicus* and *Brettanomyces bruxellensis* have been noted to have thiamine and/or riboflavin requirements [4-11]. This research investigated the thiamine and riboflavin requirements of a variety of known beer-spoilage microorganisms and determine whether the in the presence these vitamins aids in spoilage.

Methods and Materials

Microorganisms used in this research

Micromicroorganisms used in this research were sourced from various locations. *Lactobacillus brevis* and *Pediococcus damnosus* were isolated by Menz *et al*. [12]. *Zymomonas mobilis* (ZM4 strain – ATCC 31821) was sourced from the University of Melbourne (Melbourne, Victoria, Australia). *Acetobacter aceti* B450 was sourced from the Australian Wine Research Institute (Waite, South Australia, Australia). *Brettanomyces*

bruxellensis (5112) and *Brettanomyces lambicus* (5526) were sourced from Wyeast Laboratories Inc (Odell, OR). *L. brevis* and *P. damnosus* cultures were stored on MRS agar (Oxoid, Basingstoke, UK) containing 85% v/v full strength beer and 2 g/L maltose [(12)]. *Z. mobilis* was stored as per Vriesekoop *et* al., [(13)]. *A. aceti* was stored on an artificial medium containing 10 g/L calcium carbonate (Sigma-Aldrich, St Louis, MO), 3g/L glucose (Sigma-Aldrich, St Louis, MO), 15 g/L agar (Oxoid, Basingstoke, UK), 10 g/L yeast extract (Oxoid, Basingstoke, UK), 25 g/L mannitol (Sigma-Aldrich, St Louis, MO), 2 % v/v ethanol (Sigma-Aldrich, St Louis, MO). *Brettanomyces spp.* strains were stored on Malt Extract Agar (Oxoid, Basingstoke, UK).

All cultures were inoculated (10^3 cell/ml), and incubated at 24°C for five days. All tubes were monitored for an increase in growth (increased turbidity).

Effect of thiamine and riboflavin on the growth of various beer spoilage microorganisms in a non-stressed environment

L. brevis, P. damnosus, Z. mobilis, A. aceti, B. lambicus and *B. bruxellensis* were inoculated into individual tubes containing (10 ml) of the artificial minimal media (Table 1) containing either: [1] no added thiamine and riboflavin; [2] 50 μg/L of thiamine; [3] 50 μg/L of riboflavin; or [4] 50 μg/L of thiamine and 50 μg/L riboflavin. This vitamin concentration constituted a "low vitamin" beer environment as determined previously by Hucker *et al.* [(14)]. A simple hedonic scale ("–" no growth; "+" minor growth; "++" medium growth; "+++" heavy growth) was used to measure any increase in turbidity. It must be noted that 2 % v/v ethanol was added to the *A. aceti* cultures to enable growth.

The effect of vitamins on the ability for spoilage microorganisms to proliferate in the presence of hops and ethanol

The effect of thiamine and riboflavin on the ability for spoilage microorganisms to grow in the presence of hops and ethanol were investigated. This was performed by inoculating the cultures above into an artificial minimal media (Table 1) with added hops, ethanol and vitamins. Cultures were inoculated into each flask (50 mL of media in cotton wool stoppered 100ml Erlenmeyer flasks) at 10^3 cfu/ml and were fermented for 20 days at 24 °C. An initial and final sample were taken to determine whether the presence of vitamins influenced the

spoilage-potential of these known beer spoilage microorganisms through their ability to produce off flavour indicators (lactic acid, diacetyl and acetic acid)

Sugar and organic acid analysis

Lactic and acetic acid production of the various spoilage microorganisms was analysed by using a Varian high performance liquid chromatography (HPLC) system (Varian Inc, Mulgrave, Australia) with the following conditions: a Varian 9010 pump at a flow rate of 0.6 ml/min of 0.0025 M sulphuric acid (H_2SO_4); Varian Prostar 410 autosampler (10 µL injection); Alltech 300 column heater (65 °C); Rezex ROA organic acid H+ (8%) 300 x 7.8 mm column fitted with a SecurityGuard cartridge Carbo-H 4 x 3.0 mm (Phenomenex, Torrance, CA) and a Varian 9040 Refractive Index detector (40 °C). All prepared standards were made in 0.0025 M sulphuric acid and stored at 4°C for up to six months.

Analysis of acetaldehyde

Acetaldehyde was analysed via the method described by Hucker and Vriesekoop (2008) [(15)].

Analysis of vicinal diketones

The analysis of free vicinal diketones (VDK's: 2, 3-butanedione (diacetyl) and 2, 3-pentanedione) was performed using Head Space – Gas Chromatography (HS-GC) coupled with electron capture detection and all procedures used for this analysis were based on controlled in-house testing methods, which are described briefly below.

All standards and samples were analysed using a Varian CP 3800 gas chromatograph fitted with a CTC CombiPal autosampler and equipped with head space sampling, utilising a headspace needle at 95 °C (1 ml); incubation at 50 °C for 15 minutes; injector (spilt 15:1) at 150 °C; BP1, 25 m x 0.53 mm ID, 5.0 µm film thickness, (SGE Analytical Science Pty Ltd, Ringwood, Australia); the carrier gas utilised was high purity nitrogen (4 psi); column temperature 100 °C; electron capture detector (ECD) at 240 °C.

Internal standard solution was prepared by adding 30 µL of 2,3-hexanedione (Sigma-Aldrich, St, MO) to 30 ml of ethanol in a 50 ml volumetric flask. The solution was then made to volume and 10 ml was transferred to a 250 ml volumetric flask and made to volume with

ethanol. The solution was mixed well, placed in GC vials, crimped and stored in the freezer for up to four months.

Samples (20 ml) were first degassed by swirling them for five minutes in a conical flask (100 ml). An aliquot (2 ml) of degassed sample was then transferred to a 20 ml GC vial that contained 1 g (± 0.2 g) sodium sulphate and the vials were sealed. 15 µL of internal standard solution (fresh from freezer) was quickly added to the sealed vials and the samples were analysed via HS-GC.

Results and Discussion

Effect of thiamine and riboflavin on the growth of various beer spoilage microorganisms in a non-stressed environment

All microorganisms used in this study were able to grow in the basic artificial minimal medium (Table 2). This indicates the presence of thiamine or riboflavin is not an essential requirement for growth of these microorganisms, or at least the strains used in this study. However, the addition of thiamine (50 µg/L) enhanced the growth of all beer spoilage micro-microorganisms investigated here. The addition of riboflavin (50 µ/L) did not influence the growth of *L. brevis, P. damnosus*, or *Z. mobilis*; however, *A. aceti, B. lambicus* and *B. bruxellensis* did benefit from the sole addition of riboflavin (Table 1). The combined addition of thiamine and riboflavin never produced an improved growth outcome beyond the most beneficial sole-vitamin. Despite no microbial culture having an absolute requirement for either vitamin it is apparent that the presence of particular vitamins can have a positive influence on the growth of the cultures. The presence of thiamine aided an increase in growth *L. brevis*, *P. damnosus*, and *Z. mobilis*; suggesting that beers with a higher level of this vitamin could support growth and hence increase the risk of spoilage. Similarly, an increased presence in riboflavin may increase the risk of spoilage in beer from aerobic bacteria such as *A. aceti*.

Effect of thiamine and riboflavin on the ability of a variety of spoilage microorganisms to grow and spoil beer in the presence of hops and ethanol

The exposure to hops (15 IBU) and ethanol (3% abv) caused no major change in the growth intensity of *L. brevis* cultivated in the absence of either thiamine or riboflavin, however

exposure to hops and ethanol caused a decrease in lactic acid production by 50 and 60% respectively (Table 3). The addition of thiamine and riboflavin to either a non-stressed or stressed *L. brevis* culture resulted in overall increases in lactic acid production. Thiamine alone provided a greater increase in lactic acid production than the sole addition of riboflavin. These results are similar to the previous findings [6, 16] which established that thiamine is required by *L. brevis* to efficiently convert pyruvate to lactic acid and ethanol.

The exposure of *P. damnosus* to hops caused a reduction of lactic acid productivity by approximately 75%; while exposure to ethanol caused a decrease in lactic acid by about 82% (Table 3). Furthermore, exposure to hops caused an increase in diacetyl productivity by 350%; while 2,3-pentadione productivity was reduced by about 25%. Exposure to ethanol caused a reduction of 20% in both diacetyl and 2,3-pentadione. This decrease in lactic acid production can be linked to a decrease in metabolism as the stresses such as hop and ethanol are inhibitory to lactic acid bacteria [12, 17-24]. The presence of thiamine resulted in a doubling of diacetyl productivity in a non-stressed culture of *P. damnosus*; while riboflavin addition in the absence of thiamine induced very little change in diacetyl productivity. The combined addition of thiamine and riboflavin to non-stressed *P. damnosus* reiterates the observations that thiamine has the greatest influence on diacetyl productivity (Table 3). The sole addition of thiamine and riboflavin enhanced the production of both lactic acid and 2,3-pentadione to non-stressed *P. damnosus*, however, there was no marked distinction between the influence of thiamine or riboflavin – neither did the combined addition of thiamine and riboflavin impose a greater productivity of either lactic acid or 2,3-pentadione. The sole and combined addition of thiamine and riboflavin to hops stressed *P. damnosus* enhanced the production of lactic acid by 22 – 29% (Table 3). This marked increase did by no means offset the large decrease in lactic acid production due to the exposure to hops. While the exposure of *P. damnosus* to hops caused a marked increase in diacetyl productivity, this increase in diacetyl biosynthesis was further enhanced by the presence of thiamine and riboflavin. The combined addition of these two vitamins caused a further synergistic increase in diacetyl productivity. The exposure to hops caused a decrease in 2,3-pentadione production; while the addition of thiamine and riboflavin to hops stressed *P. damnosus* induced an increase in 2,3-pentadione production to roughly the non-stressed level. The combined addition of thiamine and riboflavin had an additive stimulatory effect with regards to 2,3-pentadione biosynthesis (Table 3).

The observation that the presence of thiamine and riboflavin increased the lactic acid concentration implies that these vitamins can aid in increasing the risk of spoilage in a beer. The fact that the lactic acid concentration increased in conjunction with an increase in growth in the presence of thiamine (Table 3) suggests that thiamine enhances the rate of fermentation. In most LAB the pyruvic acid to lactic acid conversion facilitates the reduction of NAD^+ to NADH, which in turn is required to fuel ATP production during glycolysis in a similar manner to how yeast growth can be stimulated by influencing the intracellular redox balance through stimulation of its fermentation pathway [25, 26].

The main spoilage characteristic of *Z. mobilis* is its ability to produce copious amounts of acetaldehyde [27, 28]. The exposure of *Z. mobilis* to hops (15 IBU) and ethanol (3% abv) caused a decrease in acetaldehyde production by 7.4 and 27 % respectively. *Z. mobilis* utilises the Entner-Doudoroff pathway to produce energy [29-32] and thus relies on an active pyruvate decarboxylase. This enzyme requires thiamine diphosphate to be present to function correctly [29, 31] and therefore in the absence of this cofactor, metabolism of acetaldehyde and subsequently ethanol production, could be hindered or stopped. The presence of thiamine in a non-stressed culture of *Z. mobilis* enhanced growth and caused an increase in acetaldehyde productivity by 100% (Table 4). However, the addition of riboflavin did not enhance growth but caused a marked increase in acetaldehyde production (23%); while the combined addition of thiamine and riboflavin did not improve growth or increase acetaldehyde productivity beyond the sole influence of thiamine (Table 4). The addition of hops, in the absence of either thiamine or riboflavin, to *Z. mobilis* resulted in an observed increase in growth when compared to the non-stressed control. This might be due to the presence of both vitamins in hops [33]. The addition to either thiamine or riboflavin to a hops-exposed culture of *Z. mobilis* did not enhance growth; however as was observed with the non-hops exposed cultures, the acetaldehyde productivity increased by 86.5 % in the presence of added thiamine while riboflavin addition only caused a very minor increase in acetaldehyde. The combined addition of thiamine and riboflavin caused an increase in acetaldehyde productivity similar to the sole addition of thiamine (Table 4).

The addition of ethanol (3 % abv) to *Z. mobilis* did not decrease the observed growth, but had a negative effect (27 %) on the acetaldehyde production, which is in keeping with previous reports [27, 34]. The presence of thiamine and/or riboflavin enhanced the growth performance

of ethanol-exposed *Z. mobilis* (Table 4). The addition of thiamine to ethanol-exposed *Z. mobilis* enhanced the production of acetaldehyde by 21.3 %; while riboflavin alone had little effect on the acetaldehyde production under ethanol stressed conditions (+3.8 %). Some strains of *Z. mobilis* have been reported to have a requirement for riboflavin [(27)] but it appears that the strain used in this study does not. The combined presence of thiamine and riboflavin in ethanol-exposed *Z. mobilis* showed a synergistic enhancement of acetaldehyde accumulation.

The main spoilage characteristic of *A. aceti* is through its ability to oxidize ethanol to acetic acid, producing vinegary off-flavours and aromas [(2, 35)]. High levels of acetic acid will cause a drop in the pH, which has been shown to be detrimental towards yeast during the secondary fermentation [(35)]. The presence of thiamine and/or riboflavin had no marked influence on the growth performance *of A. aceti* (Table 4). Similarly, the presence of thiamine to a non-stressed *A. aceti* culture had no significant impact on the accumulation of acetic acid; while the presence of riboflavin to a non-stressed culture caused a minor but noticeable rise in acetic acid production The exposure of *A. aceti* to hops did not affect growth, but caused a decrease in acetic acid production by 25%; while the exposure to a higher level of ethanol caused an increase in growth, and a ten-fold increase in acetic acid production (Table 4). The increase in growth and acetic acid production is highly likely due to the fact that ethanol is the preferred growth substrate and main metabolic by-product related to energy production for *A. aceti* [(36-38)].

The presence of thiamine to either hops or ethanol exposed *A. aceti* induces a small increase in acetic acid produced when compared to the control treatment; while the presence of riboflavin to the *A. aceti* cultures provided a slightly greater improvement in acetic acid production. In all treatments 3 % v/v ethanol was added to all cultures to allow for growth. Therefore the ethanol stressed treatments had a further 3 % v/v ethanol applied to stress the cultures in a similar fashion to the other cultures. This increase in acetic acid in the ethanol stressed cultures is most likely due to this increase in available substrate, as the other treatments recorded similar concentrations, and the fact that all ethanol treatments recorded an increase of almost two times that of the hop and non-stressed treatments. So despite the presence of the different applied stresses, the overall effect of the addition of thiamine and riboflavin is similar for *A. aceti*. The increase in the growth of the ethanol stressed cultures when compared to the other treatments, reiterates that this is most likely due to the increased availability of the substrate.

Apart from spoilage bacteria in beer, there are also a number of yeasts that can cause significant spoilage issues in beers [39]. *Brettanomyces* spp. are most commonly associated with beer production as a cause of spoilage [35, 40] but can be beneficial in case of Belgium Lambic beers and the modern cool ship ales [41, 42]. In this study the presence thiamine or riboflavin to both non-exposed *Brettanomyces* cultures stimulated growth from medium to heavy growth (Table 5). Thiamine alone caused a marked increase in acetic acid accumulation of 43.5 and 78.2 % in *B. lambicus* and *B. bruxellensis* respectively; while riboflavin alone caused less pronounced increase in acetic acid production of 19.3 and 11.7 % by *B. lambicus* and *B. bruxellensis* respectively. However, the combined presence of thiamine and riboflavin in non-exposed *B. bruxellensis* resulted in an increase in acetic acid similar to that of thiamine alone (+74.4 %); whereas the combined presence of the vitamins in non-exposed *B. lambicus* caused an increase in acetic acid production markedly lower than the sole presence of thiamine and similar to that of the sole presence of riboflavin (Table 5). The exposure to hops and ethanol did not impose a noticeable effect with regard to growth; however, the exposure caused a decrease in acetic acid production by 5-8% and 17-31% respectively. The presence of thiamine and/or riboflavin caused an improvement in growth to heavy growth in all instances for both *Brettanomyces* spp. (Table 5), which indicates that *Brettanomyces* nascent vitamin synthesis is sufficient for reasonable growth but benefits from exogenous supplied vitamins. When exposed to hops (15 IBU) both *B. lambicus* and *B. bruxellensis* increase acetic acid production the most when thiamine is present; while the presence of riboflavin causes only a minor increase in acetic acid accumulation in *B. bruxellensis* with *B. lambicus* benefiting more with regards to acetic acid production from the presence of riboflavin when exposed to hops. Interestingly, both *Brettanomyces* spp. produce higher levels of acetic acid when exposed to ethanol in the presence of riboflavin compared to the presence of thiamine. The combined presence of thiamine and riboflavin provides an acetic acid productivity similar to the sole presence of thiamine, undoing the additional benefit from the sole addition of riboflavin.

Conclusion

The presence of either thiamine or riboflavin is capable of enhancing the spoilage potential of all spoilage micro-microorganisms investigated here. In many instances this was regardless of

the presence of hops or ethanol. Very noticeable, the presence of thiamine has the ability to markedly enhance the productivity of: lactic acid in ethanol-exposed *L. brevis* and *P. damnosus*; diacetyl in hops and ethanol exposed *P. damnosus*; acetaldehyde in hops and ethanol exposed *Z. mobilis*; and acetic acid in hops and ethanol exposed *Brettanomyces* spp. While the addition of riboflavin always enhanced the spoilage potential of all micro-microorganisms investigated here; the most noticeable enhancement by riboflavin was observed in hops and ethanol exposed *P. damnosus* with regards to 2,3-pentadione production, and ethanol exposed *Brettanomyces spp*.

While this study did not investigate the influence of thiamine and riboflavin in actual beer; it does show that the thiamine and riboflavin present in beer has the potential to provide many spoilage micro-microorganisms with spoilage-stimulants that are intrinsic to beer itself.

References

1. Menz, G., Aldred, P., and Vriesekoop, F. (2009) Pathogens in Beer, In *Beer in Health and Disease Prevention* (Preedy, V., Ed.), pp 403-413, Academic Press, USA.
2. Vriesekoop, F., Krahl, M., Hucker, B., and Menz, G. (2012) 125th Anniversary Review: Bacteria in brewing: The good, the bad and the ugly., *Journal of the Institute of Brewing 118*, 335-345.
3. Garofalo, C., Osimani, A., Milanović, V., Taccari, M., Aquilanti, L., and Clementi, F. (2015) The Occurrence of Beer Spoilage Lactic Acid Bacteria in Craft Beer Production, *Journal of Food Science 80*, M2845–M2852.
4. Holzapfel, W. H., Franz, C. M. A. P., Ludwig, W., and Dicks, L. M. T. (2009) Genus III. Pediococcus, In *Bergey's Manual of Systematic Bacteriology* (De Vos, P., Garrity, G., Jones, D., Krieg, N., Ludwig, W., Rainey, F., Schleifer, K.-H., and Whitman, W., Eds.) 2nd ed., pp 513-520, Springer Science, USA.
5. Russell, C., Bhandari, R., and Walker, T. (1955) Studies of lactic acid bacteria associated with brewery products. IV. Interrelationships in requirements for purines, folic acid and *p*-aminobenzoic acid, *Journal of Agricultural and Food Chemistry 6*, 633-636.
6. Russell, C., Bhandari, R. R., and Walker, T. K. (1954) Vitamin requirements of thirty-four lactic acid bacteria associated with brewery products, *Journal of General Microbiology 10*, 371-376.
7. Hammes, W., and Hertel, C. (2009) Genus I. Lactobacillus, In *Bergey's Manual of Systematic Bacteriology* (De Vos, P., Garrity, G., Jones, D., Krieg, N., Ludwig, W., Rainey, F., Schleifer, K.-H., and Whitman, W., Eds.) 2nd ed., pp 465-494, Springer Science, USA.
8. Kandler, O., and Weiss, N. (1986) Section 14 - Regular, Nonsporing Gram-Positive Rods, In *Bergey's Manual of Systematic Bacteriology* (Sneath, P. H. A., Mair, N. S., Sharpe, M. E., and Holt, J. G., Eds.), pp 1208-1234, Lippincott Williams & Wilkins, USA.

9. Sievers, M., and Swings, J. (2005) Genus I. Acetobacter, In *Bergey's Manual of Systematic Bacteriology* (Brenner, D., Krieg, N., and Staley, J., Eds.) 2nd ed., pp 51-52, Springer Science, USA.
10. Walsh, J. (2009) Evaluating egg white fining as a means to inhibit *Brettanomyces bruxellensis* within merlot wine, pp 1-63, University of California, Davis, USA.
11. Romano, A., Perello, M. C., Revel, G. d., and Lonvaud-Funel, A. (2008) Growth and volatile compound production by Brettanomyces/Dekkera bruxellensis in red wine, *Journal of Applied Microbiology 104*, 1577-1585.
12. Menz, G., Andrighetto, C., Lombardi, A., Corich, V., Aldred, P., and Vriesekoop, F. (2010) Isolation, identification and characterisation of beer-spoilage lactic acid bacteria form microbrewed beer from Victoria, Australia, *Journal of the Institute of Brewing 116*, 14-22.
13. Vriesekoop, F., Rasmusson, M., and Pamment, N. (2002) Respective effects of sodium and chloride ions on filament formation and growth and ethanol production in Zymomonas mobilis fermentations, *Letters in Applied Microbiology 35*, 27-31.
14. Hucker, B., Wakeling, L., and Vriesekoop, F. (2011) The quantitative analysis of thiamine and riboflavin and their respective vitamers in fermented alcoholic beverages, *Journal of Agricultural and Food Chemistry 59*, 12278-12285.
15. Hucker, B., and Vriesekoop, F. (2008) Acetaldehyde stimulates ethanol-stressed *Saccharomyces cerevisiae*, grown on various carbon sources, *Folia Microbiolica 53*, 505-508.
16. Walker, J. R. L. (1959) Pyruvate metabolism in *Lactobacillus brevis*, *Biochemical Journal 72*, 188-192.
17. Asano, S., Iijima, K., Suzuki, K., Motoyama, Y., Ogata, T., and Kitagawa, Y. (2009) Rapid detection and identification of beer-spoilage lactic acid bacteria by microcolony method, *Journal of Bioscience and Bioengineering 108*, 124-129.
18. Asano, S., Suzuki, K., Iijima, K., Motoyama, Y., Kuriyama, H., and Kitagawa, Y. (2007) Effects of morphological changes in beer-spoilage lactic acid bacteria on membrane filtration in breweries, *Journal of Bioscience and Bioengineering 104*, 334-338.
19. Bhandari, R. R., Russell, C., and Walker, T. (1954) Studies of lactic acid bacteria associated with brewery products. I. Identification of types isolated from beer and from yeast, *Journal of Agricultural and Food Chemistry 5*, 27-31.
20. Booysen, C., Dicks, L. M. T., Meijering, I., and Ackermann, A. (2002) Isolation, identification and changes in the composition of lactic acid bacteria during the malting of two different barley cultivars, *International Journal of Food Microbiology 76*, 63-73.
21. Fernandez, J., and Simpson, W. (1993) Aspects of the resistance of lactic acid bacteria to hop bitter acids, *Journal of Applied Microbiology 75*, 315-319.
22. Fernandez, J., and Simpson, W. (1995) Measurement and prediction of the susceptibility of larger beer to spoilage by lactic acid bacteria, *Journal of Applied Bacteriology 78*, 419-425.
23. Simpson, W., and Fernandez, J. (1994) Mechanism of resistance of lactic acid bacteria to *trans*-isohumulone, *Journal of the American Society of Brewing Chemists 52*, 9-11.
24. Simpson, W. J., and Fernandez, J. L. (1992) Selection of beer-spoilage lactic acid bacteria and induction of their ability to grow in beer, *Letters in Applied Microbiology 14*, 13-16.

25. Vriesekoop, F., Barber, A., and Pamment, N. (2007) Acetaldehyde mediates growth stimulation of ethanol-stressed *Saccharomyces cerevisiae*: evidence of a redox-driven mechanism, *Biotechnology Letters 29*, 1099-1103.
26. Vriesekoop, F., Haas, C., and Pamment, N. (2009) The role of acetaldehyde and glycerol in the adaption to ethanol stress of *Saccharomyces cerevisiae* and other yeasts, *FEMS Yeast Research 9*, 365-371.
27. Swings, J., and De Ley, J. (1977) The biology of Zymomonas, *Microbiology and Molecular Biology Reviews 41*, 1-46.
28. Weir, P. M. (2016) The ecology of Zymomonas: a review, *Folia Microbiologica*, 1-8.
29. Bringer-Meyer, S., Schimz, K. L., and Sahm, H. (1986) Pyruvate decarboxylase from *Zymomonas mobilis*. Isolation and partial characterization, *Archives of Microbiology 146*, 105-110.
30. Dawes, E. A., Ribbons, D. W., and Rees, D. A. (1966) Sucrose utilization by *Zymomonas mobilis*: formation of a levan, *Biochemical Journal 98*, 804-812.
31. Diefenbach, R. J., and Duggleby, R. G. (1991) Pyruvate decarboxylase from Zymomonas mobilis. Structure and re-activation of apoenzyme by the cofactors thiamin diphosphate and magnesium ion., *Biochemical Journal 276*, 439-445.
32. Entner, N., and Doudoroff, M. (1952) Glucose and gluconic acid oxidation of *Pseudomonas saccharophila*, *Journal of Biological Chemistry 196*, 853-862.
33. Hucker, B., Wakeling, L., and Vriesekoop, F. (2014) Vitamins in brewing: the impact of wort production on the thiamine and riboflavin vitamer content of boiled sweet wort, *Journal of the Institute of Brewing 120*, 164-173.
34. Stanley, G. A., Hobley, T. J., and Pamment, N. B. (1997) Effect of acetaldehyde on *Saccharomyces cerevisiae* and *Zymomonas mobilis* subjected to environmental shocks, *Biotechnology and Bioengineering 53*, 71-78.
35. Gilliland, R. (1961) Brettanomyces. I. Occurance, characteristics, and effects on beer flavour, *Journal of the Institute of Brewing 67*, 257-261.
36. Bartowsky, E. J., Xia, D., Gibson, R. L., Fleet, G. H., and Henschke, P. A. (2003) Spoilage of bottled red wine by acetic acid bacteria, *Letters in Applied Microbiology 36*, 307-314.
37. Rainbow, C., and Mitson, G. W. (1953) Nutritional requirements of acetic acid bacteria, *Journal of General Microbiology 9*, 371-375.
38. Rao, M., and Stokes, J. L. (1953) Nutrition of the acetic acid bacteria, *Journal of Bacteriology 65*, 405-412.
39. Fleet, G. (1992) Spoilage yeast., *Critical Reviews in Biotechnology 12*, 1-44.
40. Schifferdecker, A. J., Dashko, S., Ishchuk, O., and Piškur, J. (2014) The wine and beer yeast Dekkera bruxellensis, *Yeast 31*, 323-332.
41. Bokulich, N., Bamforth, C. W., and Mills, D. (2012) Brewhouse-resident microbiota are responsible for multi-stage fermentation of American coolship ale, *PLoS One 7*, 35507.
42. Spitaels, F., Wieme, A. D., Janssens, M., Aerts, M., Daniel, H. M., Van Landschoot, A., De Vuyst, L., and Vandamme, P. (2014) The microbial diversity of traditional spontaneously fermented lambic beer, *PLoS One 9*, 95384.

Table 1. Artificial minimal media used in spoilage culture experiments.

Composition based on research by Moore and Rainbow (1955), Carr (1958), Shankman et al., (1947), Russell et al., (1954), Dunn et al., (1947), Liu et al., (1995), Hammes and Hertel (2009) and Sievers and Swings (2005).

Nitrogen source	**Supplier**	g/L
$(NH_4)_2SO_4$	Chem Supply[a]	5
Carbohydrate source		g/L
Maltose	Sigma-Aldrich[b]	2.5
Glucose	Sigma-Aldrich	2.5
Amino Acids	**Supplier**	mg/L
Alanine	Ajax Chemicals[c]	200
Asparagine	Sigma-Aldrich	200
Aspartic acid	Sigma-Aldrich	200
Arginine	Sigma-Aldrich	200
Cysteine	Sigma-Aldrich	200
Glutamic acid	BDH chemical Ltd[d]	200
Glutamine	Sigma-Aldrich	200
Glycine	Sigma-Aldrich	200
L-Histidine	Sigma-Aldrich	200
Isoleucine	Sigma-Aldrich	200
Leucine	Sigma-Aldrich	200
Lysine.HCl	Sigma-Aldrich	200
DL-Methionine	Sigma-Aldrich	200
Phenylalanine	Sigma-Aldrich	200
Proline	Research Organic Inc[e]	200
Serine	Sigma-Aldrich	200
Threonine	Research Organic Inc	200
LD-Tryptophan	Research Organic Inc	200
Tyrosine	Sigma-Aldrich	200
Valine	Research Organic Inc	200

Vitamins	**Supplier**	µg/L
Biotin	Sigma-Aldrich	100
Pantothenic acid	Sigma-Aldrich	100
Folic acid	Sigma-Aldrich	100
Inisitol	Sigma-Aldrich	2000
Niacin (nicotinic acid)	Sigma-Aldrich	100
p-aminobenzoic acid	Merck[f]	100
Pyridoxine hydrochloride	Sigma-Aldrich	100
Choline chloride	Sigma-Aldrich	100
Cobalamin (B12)	Sigma-Aldrich	100
Thiamine*	Sigma-Aldrich	50
Riboflavin*	Sigma-Aldrich	50
Trace Elements	**Supplier**	µg/L
H_3BO_3	Ajax Chemicals	500
$CuSO_4$	Ajax Chemicals	40
KI	Ajax Chemicals	100
$FeCl_3$	BDH Chemical Ltd	200
$MnSO_4$	Ajax Chemicals	400
Na_2MoO_4	Ajax Chemicals	200
$ZnSO_4$	Chem Supply	400
Salts	**Supplier**	g/L
KH_2PO_4	Sigma-Aldrich	1.0
K_2HPO_4	Sigma-Aldrich	1.0
$MgSO_4$	BDH Chemical Ltd	0.5
NaCl	Chem Supply	0.1
$CaCO_3$	Chem Supply	0.1
Stress Treatments*		
Hops [g] (mg/L)	Ellerslie Hop Estate Pty Ltd	750
Ethanol [h] (v/v)	CSR Distilleries	3%

[a] Beverly, Australia; [b] St Louis, USA; [c] Sydney, Australia; [d] Poole, England; [e] Clevland, USA; [f] Kilsyth, Australia; [g] Myrrhee, Australia; [h] Yarraville, Australia; * Applied where required

Table 2. Thiamine and riboflavin requirements of various beer spoilage microorganisms.

"–" no growth; "+" minor growth; "++" medium growth; "+++" heavy growth.

Spoilage microorganism	Added vitamins			
	None	**Thiamine only (50 µg/L)**	**Riboflavin only (50 µg/L)**	**Thiamine and Riboflavin (50 µg/L of each)**
Lactobacillus brevis	+	++	+	++
Pediococcus damnosus	+	++	+	++
Zymomonas mobilis	+	++	+	++
Acetobacter aceti[a]	+	++	++	++
Brettanomyces lambicus	++	+++	+++	+++
Brettanomyces bruxellensis	++	+++	+++	+++

[a] 2 % v/v ethanol added to media to improve growth

460 **Table 3.** Effect of hop and ethanol stresses on a spoilage potential of *L. brevis* and *P.*
461 *damnosus*.

462 The percentage change (Δ%) determined by comparing the vitamin treatment to the control
463 treatment where all vitamins were absent. All data is calculated from an average of
464 quadruplicate flasks analysed that has been analysed in duplicate (n = 8).

	L. brevis			***P. damnosus***						
Treatment	**Growth**	**Lactic acid (mg/L)**	**Δ %**	**Growth**	**Lactic acid (mg/L)**	**Δ %**	**Diacetyl (µg/L)**	**Δ %**	**2,3-pentanedione (µg/L)**	**Δ %**
Non Stressed Control	+[a]	108.0 ± 6.4[b]		+	112.9 ± 4.2		40.7 ± 9.8		11.0 ± 2.2	
Non Stressed Control + thiamine	++	216.0 ± 2.1	100.0	++	141.5 ± 3.4	25.3	82.4 ± 9.8	102.4	13.1 ±1.3	19.4
Non Stressed Control + riboflavin	+	137.4 ± 4.9	27.2	+	129.7 ± 3.7	14.8	43.3 ± 3.4	6.3	14.2 ± 3.3	30.1
Non Stressed Control + thiamine & riboflavin	++	222.6 ± 3.4	106.0	++	131.4 ± 5.5	16.4	91.0 ± 7.7	123.5	13.2 ± 7.6	20.7
Hops 15 IBU	**Growth**	**Lactic acid (mg/L)**	**Δ %**	**Growth**	**Lactic acid (mg/L)**	**Δ %**	**Diacetyl (µg/L)**	**Δ %**	**2,3-pentanedione (µg/L)**	**Δ %**
Hop (control)	-[c]	50.1 ± 1.4		+	29.9 ± 4.7		143.4 ± 4.9		8.1 ± 7.1	
Hops + thiamine	+	55.2 ± 1.3	10.1	+	38.0 ± 5.2	27.0	180.6 ± 9.9	25.9	11.4 ± 6.4	40.0
Hops + riboflavin	+	54.2 ± 6.7	8.2	+	36.6 ± 7.1	22.3	181.8 ± 3.8	26.8	11.4 ± 3.9	40.2
Hops + thiamine & riboflavin	+	55.7 ± 5.9	11.2	+	38.6 ± 6.1	29.1	195.5 ± 5.4	36.3	17.3 ± 8.9	113.6
Ethanol 3% abv	**Growth**	**Lactic acid (mg/L)**	**Δ %**	**Growth**	**Lactic acid (mg/L)**	**Δ %**	**Diacetyl (µg/L)**	**Δ %**	**2,3-pentanedione (µg/L)**	**Δ %**
Ethanol (control)	+	43.2 ± 7.8		+	20.2 ± 6.1		32.3 ± 8.4		9.3 ± 2.7	
Ethanol + thiamine	++	53.7 ± 7.7	24.3	++	26.7 ± 4.4	32.1	53.0 ± 6.5	64.1	12.6 ± 1.6	34.7
Ethanol + riboflavin	++	53.2 ± 9.3	23.1	++	21.6 ± 7.7	6.9	42.6 (6.3)	31.8	13.8 ± 8.6	47.3
Ethanol + thiamine & riboflavin	++	55.3 ± 8.2	28.1	++	26.1 ± 5.8	29.2	48.5 (8.4)	50.1	10.2 ± 5.2	9.5

465 [a] Growth was determined on the following hedonic scale "-" no growth; "+" minor growth; "++" medium
466 growth; "+++" heavy growth
467 [b] Standard deviation of the data
468 [c] visually no growth present
469

470

471

Table 4. Effect of hop and ethanol stresses on a spoilage potential of *Z. mobilis* and *A. aceti*. The percentage change (Δ%) determined by comparing the vitamin treatment to the control treatment where all vitamins were absent. All data is calculated from an average of quadruplicate flasks analysed that has been analysed in duplicate (n = 8).

	Z. mobilis			***A. aceti***		
Treatment	**Growth**	**Acetaldehyde (mg/L)**	**Δ %**	**Growth**	**Acetic acid (g/L)**	**Δ %**
Non Stressed Control	+[a]	10.7 ± 0.5[b]		++	1.6 ± 5.7	
Non Stressed Control + thiamine	++	21.4 ± 1.1	100.0	++	1.6 ± 3.4	1.5
Non Stressed Control + riboflavin	+	13.2 ± 3.2	23.6	++	1.7 ± 5.4	6.1
Non Stressed Control + thiamine & riboflavin	++	21.6 ± 0.3	101.8	++	1.7 ± 6.2	5.3
Hops 15 IBU	**Growth**	**Acetaldehyde (mg/L)**	**Δ %**	**Growth**	**Acetic acid (g/L)**	**Δ %**
Hop (control)	++	9.9 ± 0.9		++	1.2 ± 6.9	
Hops + thiamine	++	18.5 ± 5.1	86.8	++	1.2 ± 3.4	2.5
Hops + riboflavin	++	10.5 ± 0.6	4.1	++	1.3 ± 5.1	7.2
Hops + thiamine & riboflavin	++	18.1 ± 2.6	82.8	++	1.3 ± 6.2	6.9
Ethanol 3% abv	**Growth**	**Acetaldehyde (mg/L)**	**Δ %**	**Growth**	**Acetic acid (g/L)**	**Δ %**
Ethanol (control)	+	7.8 ± 6.1		+++	17.5 ± 2.2	
Ethanol + thiamine	++	9.5 ± 2.6	21.3	+++	18.3 ± 3.2	4.5
Ethanol + riboflavin	++	8.1 ± 1.7	3.8	+++	19.7 ± 2.5	12.8
Ethanol + thiamine & riboflavin	++	10.3 ± 3.4	31.7	+++	19.4 ± 5.2	11.1

[a] Growth was determined on the following hedonic scale "-" no growth; "+" minor growth; "++" medium growth; "+++" heavy growth

[b] Standard deviation of the data

Table 5. Effect of hop and ethanol stresses on a spoilage potential of *B. lambicus* and *B. bruxellensis*.

The percentage change (Δ%) determined by comparing the vitamin treatment to the control treatment where all vitamins were absent. All data is calculated from an average of quadruplicate flasks analysed that has been analysed in duplicate (n = 8).

	B. lambicus			***B. bruxellensis***		
Treatment	**Growth**	**Acetic acid (mg/L)**	**Δ %**	**Growth**	**Acetic acid (mg/L)**	**Δ %**
Non Stressed Control	++[a]	590.1 ± 6.1[b]		++	451.4 ± 2.3	
Non Stressed Control + thiamine	+++	847.2 ± 9.8	43.57%	+++	804.2 ± 5.8	78.2%
Non Stressed Control + riboflavin	+++	706.1 ± 2.3	19.66%	+++	504.4 ± 1.9	11.7%
Non Stressed Control + thiamine & riboflavin	+++	724.3 ± 2.6	22.74%	+++	787.3 ± 9.2	74.4%
Hops 15 IBU	**Growth**	**Acetic acid (mg/L)**	**Δ %**	**Growth**	**Acetic acid (mg/L)**	**Δ %**
Hop (control)	++	561.0 ± 3.3		++	413.1 ± 5.9	
Hops + thiamine	+++	845.0 ± 8.3	50.6%	+++	751.3 ± 4.2	81.9%
Hops + riboflavin	+++	762.4 ± 8.5	35.9%	+++	423.0 ± 5.6	2.4%
Hops + thiamine & riboflavin	+++	882.3 ± 2.7	57.3%	+++	688.4 ± 6.9	66.6%
Ethanol 3% abv	**Growth**	**Acetic acid (mg/L)**	**Δ %**	**Growth**	**Acetic acid (mg/L)**	**Δ %**
Ethanol (control)	++	409.1 ± 1.7		++	376.7 ± 8.1	
Ethanol + thiamine	+++	603.4 ± 5.6	47.5%	+++	438.3 ± 7.4	16.4%
Ethanol + riboflavin	+++	629.3 ± 3.4	53.8%	+++	467.4 ± 9.9	24.1%
Ethanol + thiamine & riboflavin	+++	600.1 ± 9.5	46.7%	+++	443.2 ± 1.1	17.7%

[a] Growth was determined on the following hedonic scale "-" no growth; "+" minor growth; "++" medium growth; "+++" heavy growth

[b] Standard deviation of the data