Isolation, Identification, and Characterisation of Beer-Spoilage Lactic Acid Bacteria from Microbrewed Beer from Victoria, Australia

Garry Menz[1], Christian Andrighetto[2], Angiolella Lombardi[2], Viviana Corich[3], Peter Aldred[1] and Frank Vriesekoop[1,*]

ABSTRACT

J. Inst. Brew. 116(1), 14–22, 2010

Lactic acid bacteria are the most frequently encountered beer-spoilage bacteria, and they may render beer undrinkable due to the production of lactic acid, diacetyl, and turbidity. Microbrewed beer is typically sold unpasteurised, leaving it more susceptible to spoilage by lactic acid bacteria. In this study, the incidence of lactic acid bacteria in bottled microbrewed beer from Victoria, Australia was investigated. A total of 80 beers from 19 breweries were screened for lactic acid bacteria. Almost 30% contained culturable lactic acid bacteria, and many had lactic acid levels well above the flavour threshold. Ethanol, hops, and the pH levels of the beers were not predictors for spoilage in the beers examined, and contamination appeared to be more closely linked to the source brewery. The 45 lactic acid strains isolated from these beers were identified by RAPD-PCR, with *Lactobacillus brevis* being the most frequently isolated species. All isolates were capable of spoiling beer and contained putative hop resistance genes. At typical beer levels, pH and ethanol had no effect on the growth of the particular spoilage bacteria isolated in this study.

INTRODUCTION

Beer is an inhospitable environment for many microorganisms due to a number of intrinsic and extrinsic antimicrobial hurdles[26]. These include low pH and the presence of ethanol, hop iso-α-acids and carbon dioxide. Beer is further protected by extrinsic (processing) hurdles such as the wort boil and pasteurisation (or sterile-filtration)[26]. Despite these hurdles, there is a limited range of bacteria that are capable of spoiling beer. These bacteria include the Gram-positive lactic acid bacteria and the Gram-negative acetic acid bacteria, *Pectinatus* and *Megasphaera*. Beer-spoilage organisms are of serious concern to the brewing industry, as spoilage incidents can damage brand equity and the cost of product retrieval can be high[34]. Microbrewed beer is typically more susceptible to microbial spoilage, as it is typically not pasteurised or sterile-filtered. Further, microbrewers usually lack the benefits of a well resourced quality control laboratory, and cold storage is not guaranteed throughout the distribution and retail chain.

Of the beer-spoilage bacteria, lactic acid bacteria are known to be the most prevalent, and it has been estimated that they account for 60–70% of all spoilage incidents[4]. Although selected strains of lactic acid bacteria may be employed with beneficial effects in the brewing industry[24]; for example as starter cultures in malting, for mash and wort bioacidification, or for the production of "acid" beers (Lambic, Berliner Weiss, etc.), they are typically regarded as beer-spoilage bacteria. Unlike most Gram-positive bacteria, beer-spoilage lactic acid bacteria are resistant to hop compounds and thus can spoil beer[34]. It is thought that the lactic acid bacteria undergo a multi-factorial hop adaptation process involving changes in metabolism, cell wall composition, and morphology[3,7,8], in addition to the more energy-dependent multidrug transporter, hop-efflux mechanisms (such as *horA* and *horC*)[32,34].

Spoilage by lactic acid bacteria typically results in the production of off-flavours and aromas, predominantly due to lactic acid and diacetyl. In addition, the appearance of the beer is degraded due to turbidity. The most frequently isolated beer-spoilage lactic acid bacteria include *Lactobacillus brevis, Lactobacillus lindneri* and *Pediococcus damnosus*[5,33].

The aim of this study was to investigate the frequency of contaminating lactic acid bacteria in microbrewed beer from Victoria Australia, and to identify and characterise the contaminating bacteria. In addition, two newly proposed methods of rapidly determining the ability of lactic acid bacteria to spoil beer were evaluated.

MATERIALS AND METHODS

Strain isolation

A total of 80 bottled craft beers were obtained from 19 breweries and analysed for the presence of culturable lactic acid bacteria. The majority of these beers (n = 73)

[1] Institute of Food & Crop Science, School of Science & Engineering, University of Ballarat, Mount Helen, Australia
[2] Biotechnology Group, Institute of Food Quality and Technologies, Veneto Agricoltura, Thiene, Italy
[3] Microbiology Group, Faculty of Agriculture, University of Padova, Legnaro, Italy
[*] Corresponding author. E-mail: f.vriesekoop@ballarat.edu.au
Parts of this paper were presented at the 2010, 31st IBD conference (Asia Pacific section) at the Gold Coast, Australia.

Publication no. G-2010-0401-1077

were non-pasteurised and non sterile-filtered products, and all were from the state of Victoria, Australia. The pH of each degassed beer was measured using a TPS-LC 80A pH meter (Springwood, Australia), and samples were centrifuged to remove yeast and other particulates and frozen for chemical analysis at a later date. The beers were attemperated to 30°C[18] and 100 mL of beer was membrane filtered (0.45 µm, cellulose nitrate) with vacuum assistance. Some beers required multiple filters to achieve a sample volume of 100 mL due to yeast fouling the membrane. Each membrane was placed onto MRS+ agar and the plates were incubated under microaerophilic conditions (candle jars) at 25°C for up to three weeks. MRS+ agar is based on MRS agar (Oxoid, Thebarton, Australia), modified by the addition of 5 g/L maltose[22], with the pH was adjusted to 5.5 using 2M HCl. In order to enhance the isolation of lactic acid bacteria from beer, MRS+ was made more selective by post-autoclave addition of filter-sterilised phenyethyl alcohol (3 mL/L) to inhibit Gram-negative bacteria, and cycloheximide (100 mg/L) to inhibit yeasts and moulds (both from Sigma-Aldrich, Castle Hill, Australia).

From the MRS+ plates of each beer, all colonies of different morphological appearance were Gram stained. The Gram-positive isolates (presumptive lactic acid bacteria) were subcultured several times on MRS+ agar to obtain pure cultures. Note that individual *Lb. brevis* strains can exhibit two distinct colony morphologies[21].

DNA extraction and randomly amplified polymorphic DNA polymerase chain reaction (RAPD-PCR)

Lactic acid bacteria were cultured on MRS+ at 25°C in candle jars, and the DNA was extracted as described by Andrighetto et al.[2] Three type strains were included in the RAPD-PCR; *Lb. brevis* ATCC 14869, *Lb. lindneri* DSM 20690, and *P. damnosus* LMG 11484. Amplification reactions were performed as previously described[2]. PCR reactions were carried out in a PTC-100 Thermocycler (MJ Research, USA), with the following primers and amplification conditions: (i) M13: 5' GAGGGTGGCGGTTCT 3'[20]; 35 cycles of: 94°C for 1 min, 45°C for 20 sec, ramp to 72°C at 0.5 °C sec^{-1}, 72°C for 2 min, (ii) D11344: 5' AGTGAATTCGCGGTGAGATGCCA 3'[1]; an initial denaturation at 94°C for 2 min, then 35 cycles of: 94°C for 1 min, 42°C for 1 min, 72°C for 90 sec, and a final extension at 72°C for 10 min.

Amplification products were separated by electrophoresis on 1.5% (w/v) agarose gels in 0.5 × TBE buffer, and subsequently stained with ethidium bromide. Grouping of the RAPD-PCR profiles was obtained with the Gel Compar 4.1 software package (Applied Maths, Kortrjik, Belgium), using the Pearson product moment correlation coefficient and UPGMA cluster analysis. For comparison purposes, the type strains of *Lb. brevis, Lb. lindneri,* and *P. damnosus* were included in the RAPD-PCR experiments, and species were allocated with the assistance of the in-house library of RAPD-PCR profiles maintained by the Biotechnology Group, Institute of Food Quality and Technologies, Veneto Agricoltura, Thiene, Italy. The reproducibility of the RAPD-PCR over three independent analyses with the *Lb. brevis* type strain was 88%.

A representative number of isolates included in the different RAPD-PCR clusters and subclusters were identified by sequencing the V3–V8 region of the 16S rRNA gene. DNA was amplified using primer pair P1[28] and L1401[40], and following purification PCR products were submitted to DNA sequencing (3130 Genetic Analyzer, Applied Biosystems, Foster City, USA). The species attribution was performed after BlastN alignment (www.ncbi.nlm.nih.gov/BLAST) of the obtained sequences with the public database available from the National Centre for Biotechnology Information (NCBI).

Multiplex polymerase chain reaction (PCR) for putative beer-spoilage genes

Prior to multiplex PCR, cultures were induced to grow in the presence of hops in order to select those harbouring plasmids containing the putative beer-spoilage genes. This was achieved by successive culturing at 25°C in double strength modified MRS (MRS without Tween 80) with increasing concentrations of beer, as described by Haakensen et al.[17] A lager of 15 IBUs was used; therefore the 85% beer medium contained 13 IBUs. DNA was extracted as described above and the multiplex PCR was performed as per Haakensen et al.[15], but with the use of DyNAzyme II DNA Polymerase and 1× PCR-Buffer (Finnzymes, Espoo, Finland).

Production of metabolites by isolates

Isolates were adapted to hop compounds using the method of Haakensen et al.[17] as described above and were inoculated into an Italian lager beer (4.7% ABV, pH 4.2) in duplicate. Samples were incubated at 25°C for thirty days. Samples for chemical analysis were centrifuged to remove microorganisms and particulates, and frozen until required for chemical analysis.

Free vicinal diketones

Free vicinal diketones (VDK, the sum of 2,3-butanedione and 2,3-pentanedione) were measured by Head Space Gas Chromatography (HS-GC). To each vial 2.0 mL of degassed sample, 1 g of sodium sulphate, 0.1 mL of 0.028 M ascorbic acid, and 15 µL of a 2,3-hexanedione internal standard solution (24 µL/L in ethanol) were added. Vials were incubated at 50°C for 20 min with shaking, and injected into a Varian 3800 GC (Varian, Mulgrave, Australia) fitted with a SGE BP1 column (25 m × 0.53 mm ID, 5.0 µm film thickness) with nitrogen as the carrier gas at four psi. The injector was held at 150°C, the column at 100°C, and the Electron Capture Detector (ECD) was held at 250°C.

Organic acids

Organic acids were analysed by Reversed-Phase High Performance Liquid Chromatography (RP-HPLC). Samples were first passed through Solid Phase Extraction (SPE) cartridges for cleanup, and 50 µL of the filtered sample was injected into the HPLC system, which was composed of a Varian 9010 pump, Varian ProStar 410 autosampler, and a Varian 9050 UV-Vis detector (operated at 210 nm). Compounds were separated on a Synergi 4µ Hydro-RP 80A column (250 × 4.60 mm, 4 µm) (Phenomenex, Lane Cove, Australia) held at 30°C. The mobile

phase was 0.43% orthophosphoric acid with a flow rate of 1.0 mL/min. Organic acids were quantified by the external standard method.

International bittering units (IBUs)

Hop levels were determined spectrophotometrically[27] and expressed as International Bittering Units (IBUs).

Hop gradient agar plus ethanol (HGA + E) plates

Hop Gradient Agar plus Ethanol (HGA + E) plates were employed to assess the beer spoilage ability of isolates, and were prepared according to Haakensen et al.[16], but with longer incubation times (five days at 30°C).

The effect of pH and ethanol hurdles on growth

The ability of selected isolates to grow in a variety of conditions was determined using microtitre plates. Bacterial cultures were adapted to beer as described above. Cells were harvested by centrifugation (10 min, 4500 rcf, Sigma 3K15), and washed twice with sterile 0.85% NaCl. The washed pellets were resuspended in saline to a standard optical density with the assistance of McFarland standards. The diluted cultures (10 µL) were inoculated into 240 µL of the test beers in triplicate wells of sterile 96 well lidded flat-bottomed microtitre plates (Cellstar, Greiner Bio-One, Frickenhausen, Germany). The beers used were from commercial breweries and had been pasteurised, and no lactic acid bacteria could be detected on MRS+ agar. After being aseptically opened and degassed, ethanol levels were adjusted with ethanol over the range 0.5% ABV to 10% ABV, and 1 M NaOH and 2 M HCl were used to adjust the pH over the range 3.75 to 5.0. All additives were sterile filtered (0.45 µm). The microtitre plates were incubated statically at 30°C and read periodically at 590 nm, after linear shaking in a SPECTRAFluor (Tecan, Männedorf, Switzerland) microtitre plate reader. Absorbance readings were blanked against uninoculated wells for each test beer and MRS+ broth was used as a positive control. Results are reported as the number of days required until absorbance increased by 0.05 from time zero. The ethanol levels of the beers was determined by an enzymatic method (catalogue no. 10 176 290 035, Boehringer Mannheim/R-Biopharm, Darmstadt, Germany). The 'alcohol-free' beer contained 0.50% ethanol (v/v), and the Italian lager 4.7% (v/v). The pH values were 4.6 and 4.2 respectively, as determined by a pH meter.

RESULTS

Ethanol and pH values of the microbrewed beers

The pH and ethanol levels of the 80 beers analysed in this study are presented in Table I. The pH values ranged from 3.64 to 4.61, with a mean of 4.16, whilst the middle 50% of beers ranged from 4.06 to 4.27 (median ± interquartile range). Ethanol levels ranged widely from 2.9 to 11.0% (v/v), however 50% of the beers fell within the narrower range of 4.35 and 5.55% (median ± interquartile range).

Isolation of lactic acid bacteria

In this study 80 microbrewed beers were tested for the presence of lactic acid bacteria. The results were grouped into three levels based on the number of bacterial colony forming units (CFU) found on the membrane filters. Beers with 100 or less CFU/100 mL were classified as "low", beers with counts above 100 CFU/100 mL were termed "high", and when no culturable lactic acid bacteria were detected, the term "absent" was assigned. Using these groupings, Fig. 1 shows the lactic acid bacterial contamination rate for the 80 beers. Of the beers sampled, 27.5% contained culturable lactic acid bacteria (18.75% "high", 8.75% "low"), whilst 72.5% were presumed to be free from these contaminants.

The microbiological data was grouped according to the brewery from which each individual beer was produced.

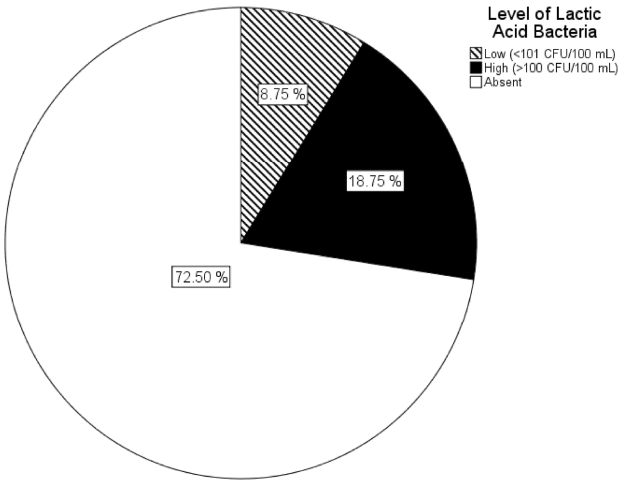

Fig. 1. Lactic acid bacterial contamination rates of the surveyed microbrewed beers (n = 80).

Table I. Statistics for the pH and ethanol levels of the beers surveyed in this study (n = 80).

	Min.	Max.	Mean	Median	Interquartile range	Standard deviation
pH[a]	3.64	4.61	4.14	4.16	0.26	0.21
% ethanol (v/v)[b]	2.90	11.0	5.20	4.95	0.90	1.20

[a] As determined with a pH meter.
[b] As stated on the beer's label.

Fig. 2 shows that lactic acid bacteria were isolated from beers sourced from 11 of the 19 breweries. All of the beers sampled from several breweries were free from culturable lactic acid bacteria, whereas all beers produced by brewery "S" contained "high" levels of lactic acid bacteria. Further, some breweries (such as "A") produced some beers with "high" levels, some with "low" levels, and some in which lactic acid bacteria were absent.

A series of ANOVAs were carried out to investigate the relationships between the hurdles (pH and alcohol by volume (ABV)) and the level of lactic acid bacteria in the beers. The ANOVAs were conducted for the data on both the absence/presence of lactic acid bacteria, and the three levels (absent/low/high). None of the ANOVAs were significant at the 0.05 level (Table II), suggesting that contamination by lactic acid bacteria was independent of pH and ABV in these samples. In addition to the ANOVAs listed in Table II, an ANOVA was used to investigate the effect of the beer's hop level (IBUs) on the contamination level (low and high). There was no significant effect observed (p-value of 0.267), indicating that the hop level was not a predictor of low or high spoilage in these samples.

The lactic acid level was measured in all of the microbrewed beers sampled. Fig. 3 groups the beers investigated according to the level of lactic acid bacteria isolated from the beer (absent, low, or high). An ANOVA showed that for those beers which contained more than 100 CFU/100 mL, the lactic acid level was significantly higher than for those that had either "low" or "absent" levels of lactic acid bacteria (p = 0.000). The majority of beers with "high" levels of lactic acid bacteria contained lactic acid levels above the flavour threshold, which suggests that these beers could be noticeably sour. All of the "low" and most of the "absent" beers were below the flavour threshold, however the high levels of lactic acid present in some of the "absent" beers suggests that these beers may have contained lactic acid bacteria, which were unculturable on the media used in this study, or that the bacteria may have previously been inactivated.

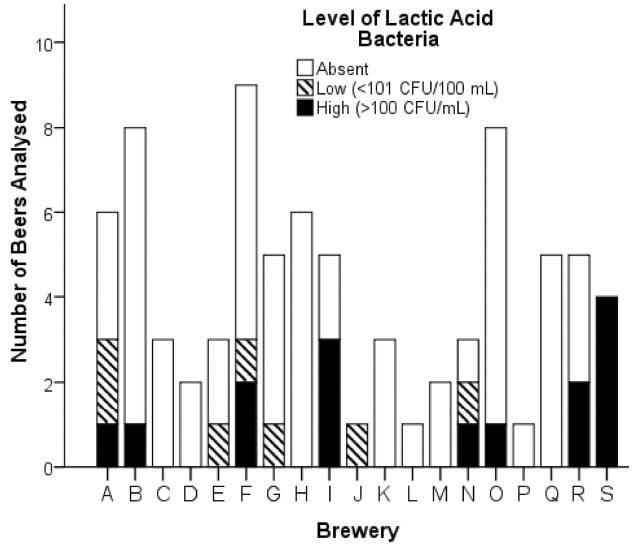

Fig. 2. Lactic acid bacterial contamination levels for each of the surveyed microbrewed beers, presented on a per brewery basis. Various numbers of beers were available in the marketplace from each brewery, which is reflected in the number of beers analysed per brewery. Each brewery was assigned a different letter at random; this coding is retained throughout this paper.

Table II. The p-values for ANOVAs for determining if the pH or alcohol by volume (ABV) significantly influenced the level of contamination by lactic acid bacteria (LAB). Beers with 100 or less CFU/100 mL were classified as "low", beers with counts above 100 were termed "high", and when no culturable lactic acid bacteria were detected, the term "absent" was assigned.

	LAB level: absent/present	LAB level: absent/low/high
pH	0.936	0.305
ABV	0.549	0.705

Identification of isolates

A total of 45 bacterial isolates were purified and subsequently characterized by RAPD-PCR. Fig. 4 shows the dendrogram obtained after cluster analysis of RAPD-PCR profiles. The majority (39) of the isolates clustered together with the *Lb. brevis* type strain (52% similarity), while four isolates clustered together with the *P. damnosus* type strain (80% similarity); species assignment was confirmed by 16S rDNA sequencing. Two beer isolates, namely numbers 75 and 86, did not cluster with the type strains used in RAPD-PCR analysis and after 16S rDNA sequencing they were respectively assigned to the species *Lb. paracasei* and *Leuc. pseudomesenteroides*.

The overall similarity level of the *Lb. brevis* strain profiles, obtained with the primers M13 and D11344, was relatively high (79%), suggesting the presence of similar strains in the different beer samples, probably well-

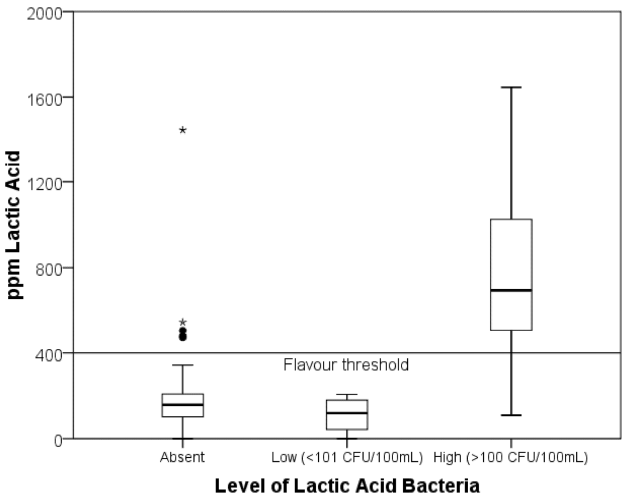

Fig. 3. Spread of lactic acid levels in the microbrewed beers. Samples for lactic acid levels were taken at the same time as those for microbiological analysis, and the results are grouped according to the level of lactic acid bacteria detected (absent, low, or high). Beers with 100 or less CFU/100 mL were classified as "low," beers with counts above 100 were termed "high," and when no culturable lactic acid bacteria were detected, the term "absent" was assigned. The solid line indicates the flavour threshold for lactic acid in beer[25]. The dots represent statistical outliers, and the stars extreme statistical outliers.

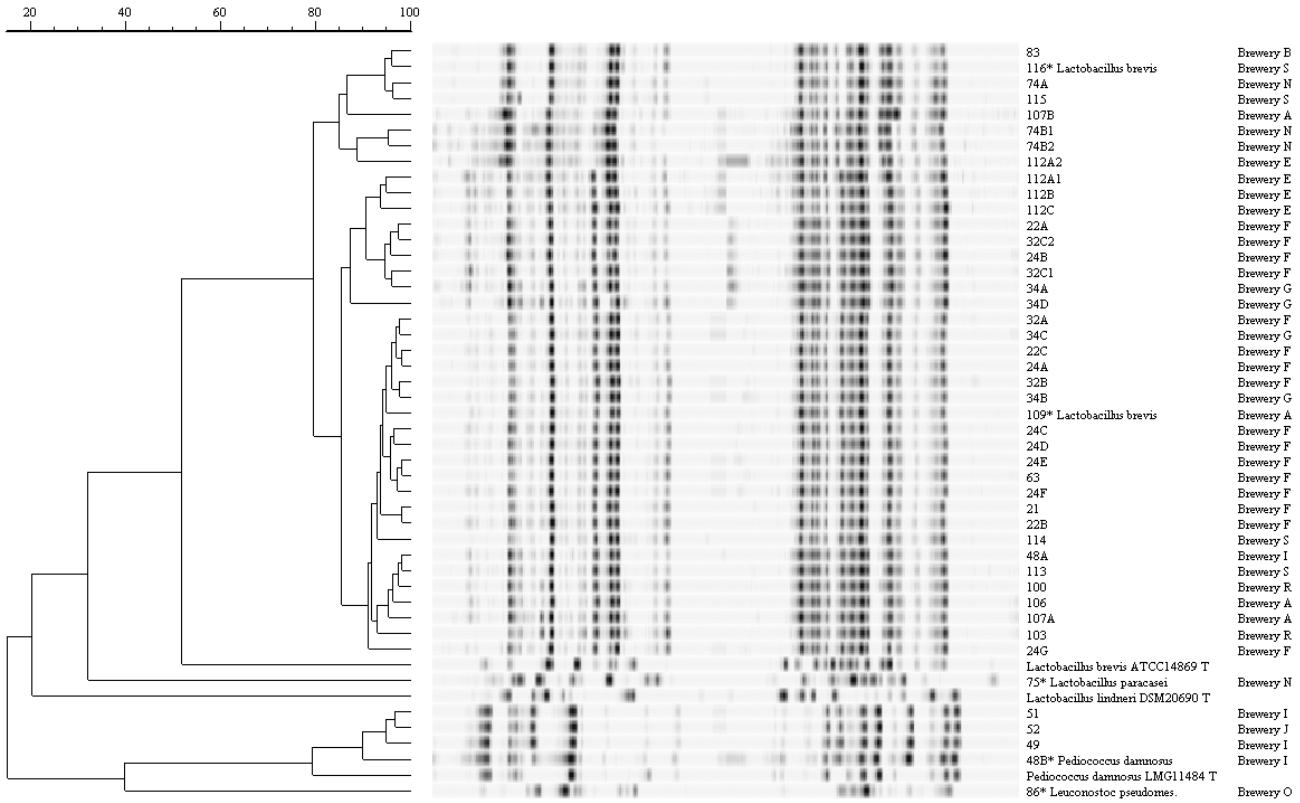

Fig. 4. Cluster analysis of RAPD-PCR patterns of beer isolates and type strains with two primer sets. 'T' indicates type strain, and '*' indicates that the strain was sequenced (16S rDNA). The final column indicates the brewery from which the contaminated beer was sourced, with each brewery being assigned a different letter at random. Coding is retained throughout this paper.

Table III. Results from hop multiplex PCR[a].

Gene	*horA*	*hitA*	ORF5	*horC*
Proposed predictability[b]	Growth in beer	Rapid growth in beer in combination with *horA*	Inability of growth for *Pediococcus*	Rapid growth in combination with *horA*
Lactobacillus brevis	15/16	15/16	16/16	16/16
Pediococcus damnosus	3/3	3/3	3/3	3/3
Lactobacillus paracasei	0/1	0/1	1/1	0/1
Leuconostoc pseudo-mesenteroides	0/1	0/1	1/1	0/1

[a] The results are presented as number of positive strains/number of strains tested.
[b] According to Haakensen et al.[15]

adapted to the particular environment. However, in some breweries two different strains of *Lb. brevis* were detected (breweries A, E, F, G, S). In brewery I, the species *P. damnosus* was dominant, while *Lb. paracasei* was detected only in brewery N and *Leuconostoc pseudomesenteroides* only in brewery O.

A representative number of strains (21), namely isolates with a RAPD-PCR similarity level <88% or isolates with a similarity level >88%, but isolated from different breweries, were selected for further genetic characterization by means of hop multiplex PCR.

Characterisation of lactic acid bacteria

Two newly developed methods to rapidly assess the ability of lactic acid bacteria to grow in beer were evaluated in this study, namely HGA+E plates[16] and hop multiplex PCR[15]. All isolates were capable of growing in beer, and all isolates were positive for growth when tested on the HGA+E plates. The results from the hop multiplex PCR for the 21 selected strains is presented in Table III. It has been proposed that the presence of *horA* can accurately predict growth in beer[15]. With all of the *P. damnosus* isolates tested, 15 of the 16 *Lb. brevis* isolates were positive for *horA*, correctly suggesting that they could grow in beer. The *Lb. paracasei* and *Leuc. pseudomesenteroides* isolates appeared as false-negatives, although Haakensen et al.[15] did not test the predictability of the method with these species. All three *P. damnosus* isolates tested contained ORF5, and all of these isolates grew in beer; however it has been proposed that this gene predicts for the inability of growth in beer for *Pediococcus*[15]. Recently, two further genes, *bsr*A and *bsr*B have been shown to be highly correlated with beer spoilage ability for *Pediococcus*[14].

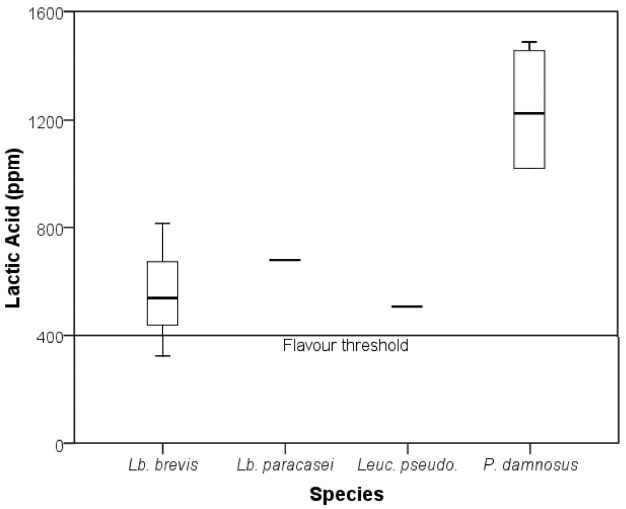

Fig. 5. Lactic acid (ppm) production for each isolate after being grown for 30 days in an Australian lager beer (5.0% ABV). *Lactobacillus brevis* n = 39, *Lb. paracasei* n = 1, *Leuconostoc pseudomesenteroides* n = 1, *Pediococcus damnosus* n = 4. The solid line indicates the flavour threshold for lactic acid in beer[25].

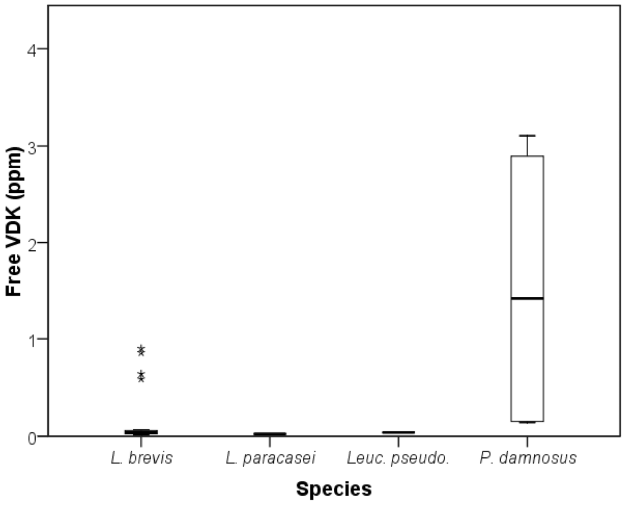

Fig. 7. Free VDK (vicinal diketone) (ppm) production for each isolate after being grown for 30 days in an Australian lager beer (5.0% ABV). *Lactobacillus brevis* n = 39, *Lb. paracasei* n = 1, *Leuconostoc pseudomesenteroides* n = 1, *Pediococcus damnosus* n = 4. The flavour threshold of diacetyl is reported to be 0.15 ppm[25]. The stars represent extreme statistical outliers.

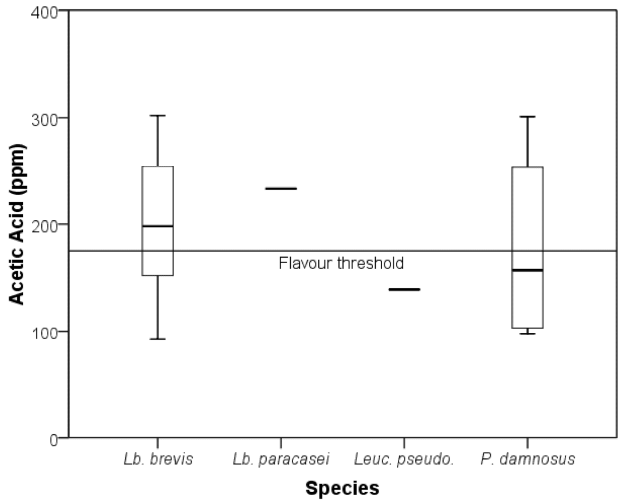

Fig. 6. Acetic acid (ppm) production for each isolate after being grown for 30 days in an Australian lager beer (5.0% ABV). *Lactobacillus brevis* n = 39, *Lb. paracasei* n = 1, *Leuconostoc pseudomesenteroides* n = 1, *Pediococcus damnosus* n = 4. The solid line indicates the flavour threshold for acetic acid in beer[25].

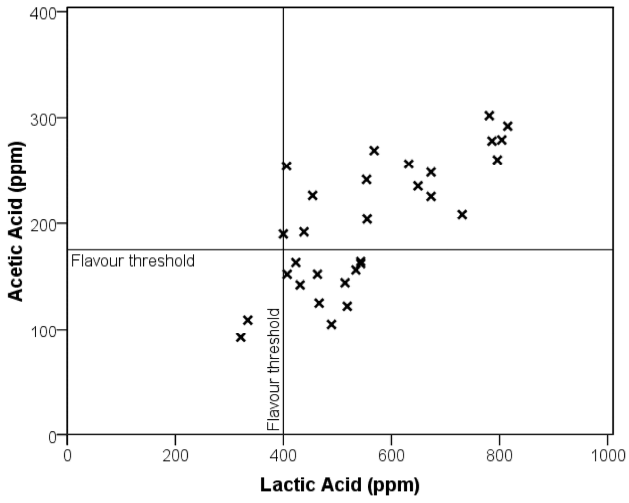

Fig. 8. Scatter plot of lactic acid and acetic acid production by the *Lactobacillus brevis* strains after 30 days growth in beer (r = 0.760, p = 0.000). The horizontal and vertical solid lines indicate the flavour threshold for acetic acid and lactic acid in beer respectively[25].

In order to understand the influence of contamination by the isolated organisms, they were inoculated into beer for 30 days, after which the levels of lactic acid, acetic acid, and vicinal diketones (diacetyl) were measured. Box plots of the data, grouped by species, are shown in Fig. 5 (lactic acid), Fig. 6 (acetic acid), and Fig. 7 (VDK). ANOVAs showed that *P. damnosus* produced significantly higher levels of both free VDK (p = 0.000) and lactic acid (p = 0.000) than *Lb. brevis*, but not acetic acid (p = 0.566). When the isolates grew in the beer, a reduction in citric acid levels was observed (data not shown), as the citric acid was utilized by the lactic acid bacteria[30].

A significant (p = 0.000) Pearson correlation was found between the lactic and acetic acid production by the *Lb. brevis* strains in beer. The Pearson correlation coefficient (r) was 0.760, indicating a strong positive linear association between the production of lactic acid and acetic acid. This relationship can be observed in the scatter plot shown as Fig. 8. This observation was in agreement with the literature, which states that *Lb. brevis* is an obligate heterofermentative organism[29]. Correlations were not investigated for the other species isolated due to the small sample size (n<5). The tolerance of selected strains, to both pH and ethanol, was evaluated using an alcohol-free beer as a basal medium in microtitre plates. MRS+ was included as a positive control, and a standard lager beer was included to confirm growth in beer. The results are presented in Table IV as days until growth in the test me-

Table IV. Days until growth[a] of selected isolates in alcohol-free beer (AFB) with modified pH and ethanol levels (v/v). Strain code is given in parentheses following the species.

	Lb.[b] *brevis* (115)	*Lb. brevis* (74A)	*Lb. brevis* (112A1)	*Lb. brevis* (34A)	*Lb. brevis* (32A)	*Lb. brevis* (24A)	*P.*[c] *damnosus* (49)	*Lb. paracasei* (75)	*Leuc.*[d] *pseudomesenteroides* (86)
MRS+ broth	<3	<3	<3	<3	<3	<3	<3	<3	<3
Lager beer	<3	<3	<3	<3	<3	<3	<3	<3	<3
AFB	<3	<3	<3	<3	<3	<3	<3	<3	<3
AFB pH 5.0	<3	<3	<3	<3	<3	<3	<3	<3	<3
AFB pH 4.5	<3	<3	<3	<3	4	<3	3	<3	<3
AFB pH 4.25	<3	<3	<3	<3	10	6	4	<3	<3
AFB pH 4.0	6	>10	6	4	>10	>10	>10	5	10
AFB pH 3.75	>10	>10	6	>10	>10	>10	>10	>10	>10
AFB 2.5% ethanol	<3	<3	<3	<3	<3	<3	<3	<3	<3
AFB 5.0% ethanol	<3	<3	<3	<3	<3	<3	<3	<3	<3
AFB 7.5% ethanol	<3	<3	<3	<3	<3	>10	<3	<3	<3
AFB 10.0% ethanol	<3	<3	<3	<3	<3	>10	<3	<3	<3

[a] Growth measured as days until absorbance at 590 nm increased by 0.05 from time zero.
[b] *Lactobacillus.*
[c] *Pediococcus.*
[d] *Leuconostoc.*

dium increased in absorbance by 0.05 at 590 nm. All isolates grew within three days in the positive control (MRS+) and the lager beer, and all except for one isolate grew within three days in 'alcohol-free' beer up to 10.0% (v/v) ethanol. Growth slowed as the pH decreased below 4.5. All strains, except for one, failed to show growth at pH 3.75, within the time frame of the experiment (10 days).

DISCUSSION

More than one in four beers sampled in this study contained culturable lactic acid bacteria, and one in five beers contained over 100 CFU/100 mL. The majority of the beers which contained >100 CFU/100 mL also had lactic acid levels well above the flavour threshold, suggesting that contamination would be noticeable when consumed. All of the isolates were capable of growth in beer, therefore the beers which were below the flavour threshold for lactic acid, yet contained lactic acid bacteria, had the potential to become spoiled over time, especially if exposed to temperatures conducive to growth and/or metabolism. The high incidence of beer-spoilage lactic acid bacteria encountered in the beers is of concern for the microbrewing industry, especially as many companies are trying to establish their brands in a competitive marketplace. A consumer is unlikely to repurchase a sour product.

High levels of contamination have been observed in other studies of unpasteurised beer. White[39] reported a contamination rate of 15% in American craft beer, whilst lactic acid bacteria were isolated from almost all of the 23 Italian microbrewed beers tested by Giusto et al.[13]. Our current study was limited to lactic acid bacteria, although it would be expected that other groups of beer-spoilage microorganisms, such as wild yeasts and acetic acid bacteria, could have been isolated, although presumably at lower frequencies.

In this study of microbrewed beers, the majority of the spoilage lactic acid bacteria were *Lb. brevis,* with four instances of *P. damnosus,* and one *Lb. paracasei* and *Leuc. pseudomesenteroides*. Various other studies have reported *Lb. brevis* and *P. damnosus* as the dominant spoilage lactic acid bacteria in beer[6,11-13,19,23,31,35-38], with other frequently isolated species including *Lb. lindneri*[5] and *Lb. plantarum*[19,36]. However, the dominance of *Lb. brevis* in this and other studies may be in part because this species grows relatively well (compared to hard-to-culture species) on the most commonly used isolation media[33]. Culture-independent techniques would be needed to provide a more accurate picture of microbial contamination.

Contamination by the lactic acid bacteria in this study resulted in the production of heightened levels of lactic acid and acetic acid, which would render the beer noticeably acidic. The *P. damnosus* isolates produced the highest levels of lactic acid, and also produced very high levels of vicinal diketones (diacetyl), leaving "buttery" off flavours and aromas in the beer.

Two recently proposed methods of assessing the beer spoilage ability of lactic acid bacteria were evaluated, both of which use hop resistance as the determining factor. Such methods are required as beer spoilage ability is not species based. The results from both the HGA+E plate and hop multiplex PCR were accurate when compared to the traditional 'growth in beer test', however since all of the isolates examined grew in beer, false-negatives and false-positives could not be tested. It is anticipated that new understandings of the multi-factorial mechanisms behind hop inhibition and resistance in lactic acid bacteria[7-10] will bring about new methods of determining beer spoilage ability.

The effect of the key antimicrobial hurdles of ethanol and pH on the growth of the isolates in beer was evaluated. All of the isolates tested were tolerant of ethanol levels typically found in beer, however pH values at the lower end provided some resistance to the growth of these strains. For example, the pH range of the middle 50% of the beers tested in this study was 4.06–4.27 and considerable inhibition of growth was observed as pH values

reached 4.0 and below. Despite this pH effect, contamination appeared to be more associated with the particular brewery, rather than to the inherent antimicrobial hurdles of the beer, as changes in the ethanol and pH hurdles at typical beer levels had little effect on growth. Further, the pH and ethanol levels of the beers did not significantly influence the contamination level, nor did hop levels influence the degree of contamination (low or high). Further evidence of brewery linked spoilage was that some breweries were free from contaminating lactic acid bacteria, whereas multiple beers from other breweries had high levels of contamination. Our findings support those of Giusto et al.[13], who found no correlations between beer type, pH, and contamination, suggesting that contamination was linked to the brewery. Our data shows that while beer design (notably pH) can assist in reducing spoilage by lactic acid bacteria, efforts to enhance microbial quality should focus on brewing practices, and in particular hygiene and sanitation regimes.

ACKNOWLEDGEMENTS

The authors wish to acknowledge the support of Melinda Christophersen and Kate Rollings (Fosters Group, Abbotsford, Australia) for completing the VDK analysis; Mr. Basel Al Maskati and Dr. Peter Varelis (University of Ballarat, Mount Helen, Australia) for assistance with HPLC method development; and the Veneto-Victoria Research Fellowship in Food Science for funding assistance.

REFERENCES

1. Akopyanz, N., Bukanov, N. O., Westblom, T. U., Kresovich, S. and Berg, D. E., DNA diversity among clinical isolates of *Helicobacter pylori* detected by PCR-based RAPD fingerprinting. *Nucl. Acids Res.,* 1992, **20,** 5137-5142.
2. Andrighetto, C., Zampese, L. and Lombardi, A., RAPD-PCR characterization of lactobacilli isolated from artisanal meat plants and traditional fermented sausages of Veneto region (Italy). *Lett. Appl. Microbiol.,* 2001, **33,** 26-30.
3. Asano, S., Suzuki, K., Iijima, K., Motoyama, Y., Kuriyama, H. and Kitagawa, Y., Effects of morphological changes in beer-spoilage lactic acid bacteria on membrane filtration in breweries. *J. Biosci. Bioeng.,* 2007, **104,** 334-338.
4. Back, W., Secondary contaminations in the filling area. *Brauwelt Int.,* 1994, **12,** 326-333.
5. Back, W., Brewery. In: Colour Atlas and Handbook of Beverage Biology, W. Back, Ed., Verlag Hans Carl: Nürnberg, Germany, 2005, pp. 10-112.
6. Back, W., Breu, S. and Weigand, C., Infektionsursachen im Jahre 1987. *Brauwelt,* 1988, **178,** 1358-1362.
7. Behr, J., Gänzle, M. G. and Vogel, R. F., Characterization of a highly hop-resistant *Lactobacillus brevis* strain lacking hop transport. *Appl. Environ. Microbiol.,* 2006, **72,** 6483-6492.
8. Behr, J., Israel, L., Gänzle, M. G. and Vogel, R. F., Proteomic approach for characterization of hop-inducible proteins in *Lactobacillus brevis. Appl. Environ. Microbiol.,* 2007, **73,** 3300-3306.
9. Behr, J. and Vogel, R. F., Mechanisms of hop inhibition: Hop ionophores. *J. Agric. Food Chem.,* 2009, **57,** 6074-6081.
10. Behr, J. and Vogel, R. F., Mechanisms of hop inhibition include the transmembrane redox reaction. *Appl. Environ. Microbiol.,* 2010, **76,** 142-149.
11. Eschenbecher, F., Zur kenntnis der biersäuernden laktobazillen. *Brauwissenschaft,* 1968, **21,** 424-437, 464-471.
12. Eschenbecher, F., Zur kenntnis der biersäuernden laktobazillen. *Brauwissenschaft,* 1969, **22,** 14-28.
13. Giusto, C., Manzano, M., Bartolomeoli, I., Buiatti, S. and Comi, G., Identificazione mediante PCR-DGGE dei batteri lattici alteranti la birra artigianale. *Industrie delle Bevande,* 2005, **34(197),** 207-210, 224.
14. Haakensen, M., Pittet, V., Morrow, K., Schubert, A., Ferguson, J. and Ziola, B., Ability of novel ATP-binding cassette multidrug resistance genes to predict growth of *Pediococcus* isolates in beer. *J. Am. Soc. Brew. Chem.,* 2009, **67,** 170-176.
15. Haakensen, M., Schubert, A. and Ziola, B., Multiplex PCR for putative *Lactobacillus* and *Pediococcus* beer-spoilage genes and ability of gene presence to predict growth in beer. *J. Am. Soc. Brew. Chem.,* 2008, **66,** 63-70.
16. Haakensen, M., Schubert, A. and Ziola, B., Broth and agar hop-gradient plates used to evaluate the beer-spoilage potential of *Lactobacillus* and *Pediococcus* isolates. *Int. J. Food Microbiol.,* 2009, **130,** 56-60.
17. Haakensen, M. C., Butt, L., Chaban, B., Deneer, H., Ziola, B. and Dowgiert, T., *horA*-specific real-time PCR for detection of beer-spoilage lactic acid bacteria. *J. Am. Soc. Brew. Chem.,* 2007, **65,** 157-165.
18. Hammond, J., Brennan, M. and Price, A., The control of microbial spoilage of beer. *J. Inst. Brew.,* 1999, **105,** 113-120.
19. Hollerová, I. and Kubizniaková, P., Monitoring Gram positive bacterial contamination in Czech breweries. *J. Inst. Brew.,* 2001, **107,** 355-358.
20. Huey, B. and Hall, J., Hypervariable DNA fingerprinting in *Escherichia coli*: Minisatellite probe from bacteriophage M13. *J. Bacteriol.,* 1989, **171,** 2528-2532.
21. Jakava-Viljanen, M., Åvall-Jääskeläinen, Messner, P., Sleytr, U. B. and Palva, A., Isolation of three new surface layer protein genes (*slp*) from *Lactobacillus brevis* ATCC 14869 and characterization of the change in their expression under aerated and anaerobic conditions. *J. Bacteriol.,* 2002, **184,** 6786-6795.
22. Lawrence, D. R. and Leedham, P. A., The detection of lactic acid bacteria. *J. Inst. Brew.,* 1979, **85,** 119-121.
23. Lin, J., Cao, Y., Sun, J. and Lu, J., Monitoring spoilage bacteria and wild yeasts in Eastern Chinese breweries. *J. Am. Soc. Brew. Chem.,* 2008, **66,** 43-47.
24. Lowe, D. P. and Arendt, E. K., The use and effects of lactic acid bacteria in malting and brewing with their relationships to antifungal activity, mycotoxins and gushing: A review. *J. Inst. Brew.,* 2004, **110,** 163-180.
25. Meilgaard, M. C., Flavor chemistry of Beer: Part II: Flavor and threshold of 239 aroma volatiles. *Tech. Q. Master Brew. Assoc. Am.,* 1975, **12,** 151-168.
26. Menz, G., Aldred, P. and Vriesekoop, F., Pathogens in beer. In: Beer in Health and Disease Prevention, V. R. Preedy, Ed., Academic Press: Amsterdam, 2009, pp. 403-413.
27. Menz, G., Vriesekoop, F., Zarei, M., Zhu, B. and Aldred, P., The growth and survival of food-borne pathogens in sweet and fermenting brewers' wort. *Int. J. Food Microbiol.,* 2010, doi: 10.1016/j. ijfoodmicro.2010.02.018.
28. Muyzer, G., De Waal, E. and Uitterlinden, A., Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.,* 1993, **59,** 695.
29. Priest, F. G., Gram-positive brewery bacteria. In: Brewing Microbiology, 3rd ed., F. G. Priest and I. Campbell, Eds., Kluwer Academic/Plenum Publishers: New York, 2003, pp. 181-217.
30. Qian, F., Bohak, I., Back, W., Becker, T. and Krottenthaler, M., Analysis of metabolic activities of beer-spoilage lactic acid bacteria in Chinese beer. Proceedings of the European Brewing Convention Congress, Hamburg, 2009, poster P114. Available from http://www.ebc2009hamburg.org/ (last accessed March 2010).
31. Rinck, M. and Wackerbauer, K., Detection and serological classification of lactic acid bacteria, especially *L. lindneri. Monatsschr. Brauwiss.,* 1987, **8,** 324-327.

32. Suzuki, K., Beer spoilage lactic acid bacteria. In: Beer in Health and Disease Prevention, V. R. Preedy, Ed., Academic Press: Amsterdam, 2009, pp. 150-164 (supplementary chapter).
33. Suzuki, K., Asano, S., Iijima, K. and Kitamoto, K., Sake and beer spoilage lactic acid bacteria – A review. *J. Inst. Brew.,* 2008, **114,** 209-223.
34. Suzuki, K., Iijima, K., Sakamoto, K., Sami, M. and Yamashita, H., A review of hop resistance in beer spoilage lactic acid bacteria. *J. Inst. Brew.,* 2006, **112,** 173-191.
35. Thelen, K., Beimfohr, C. and Snaidr, J., VIT-Bier: The rapid and easy detection method for beer-spoiling bacteria. *Tech. Q. Master Brew. Assoc. Am.,* 2004, **41,** 115-119.
36. Thelen, K., Beimfohr, C. and Snaidr, J., Evaluation study of the frequency of different beer-spoiling bacteria using the VIT analysis. *Tech. Q. Master Brew. Assoc. Am.,* 2006, **43,** 31-35.
37. Wackerbauer, K. and Emeis, C. C., Über die Bierschädlichen Bakterien der Gattung *Lactobacillus* (Biermilchsäurestäbchen). II. Physiologische Untersuchungen an Laktobacillen. *Monatsschr. Brauerei,* 1968, **21,** 328-333.
38. Wackerbauer, W. and Emeis, C. C., Methoden der Brauereibiologischen Betriebskontrolle. I. Nährböden zum Spurennachweis der Stäbchenförmigen Milchsäurebakterien. *Monatsschr. Brauerei,* 1967, **20,** 160-164.
39. White, C., Analysis of the first large-scale testing of craft beer. *Tech. Q. Master Brew. Assoc. Am.,* 2008, **45,** 13-16.
40. Zoetendal, E., Akkermans, A. and De Vos, W., Temperature gradient gel electrophoresis analysis of 16S rRNA from human fecal samples reveals stable and host-specific communities of active bacteria. *Appl. Environ. Microbiol.,* 1998, **64,** 3854.

(Manuscript accepted for publication March 2010)