Journal of Industrial Microbiology & Biotechnology (1997) 18, 284–291

Use of virginiamycin to control the growth of lactic acid bacteria during alcohol fermentation

SH Hynes, DM Kjarsgaard, KC Thomas and WM Ingledew

Department of Applied Microbiology and Food Science, University of Saskatchewan, Agriculture Building, 51 Campus Drive, Saskatoon, SK, Canada S7N 5A8

The antibiotic virginiamycin was investigated for its effects on growth and lactic acid production by seven strains of lactobacilli during the alcoholic fermentation of wheat mash by yeast. The lowest concentration of virginiamycin tested (0.5 mg Lactrol™ kg^{-1} mash), was effective against most of the lactic acid bacteria under study, but *Lactobacillus plantarum* was not significantly inhibited at this concentration. The use of virginiamycin prevented or reduced potential yield losses of up to 11% of the produced ethanol due to the growth and metabolism of lactobacilli. However, when the same concentration of virginiamycin was added to mash not inoculated with yeast, *Lactobacillus rhamnosus* and *L. paracasei* grew after an extensive lag of 48 h and *L. plantarum* grew after a similar lag even in the presence of 2 mg virginiamycin kg^{-1} mash. Results showed a variation in sensitivity to virginiamycin between the different strains tested and also a possible reduction in effectiveness of virginiamycin over prolonged incubation in wheat mash, especially in the absence of yeast.

Keywords: fuel alcohol; virginiamycin; lactic bacteria; yield reduction

Introduction

Growth of contaminating lactic acid bacteria is a major problem in industrial alcohol fermentations. These bacteria can grow under the conditions of yeast fermentation and reduce alcohol yields by consuming glucose that could have been used by yeast for ethanol production. They also compete with yeast for other nutrients in mash, and lactic acid concentrations between 1 and 4% have been reported to inhibit yeast growth [5,8,10,17–19,21,22]. There are variations in the degree to which different lactobacilli affect yeast fermentation both between and within the homofermentative and heterofermentative groups [2,8,9,15,19].

To avoid stuck fermentations and decreased ethanol yields it is important to prevent growth of contaminating lactobacilli. Methods used in the fermentation industry to control these bacteria include stringent cleaning and sanitation, acid washing of yeast destined for reuse, and the use of antibiotics during fermentation. Penicillin has been used, but often over 1.5 mg L^{-1} is added in batch fermentations due to the possibility of induced enzymatic degradation of this antibiotic by some bacteria and the rather poor stability [17] of penicillin G below pH 5. Virginiamycin, a streptogramin antibiotic produced by *Streptomyces virginiae* [6], has great potential for the alcohol industry. It has had limited use in human medicine but extensive use as an additive in animal feeds to promote growth [6] and is now under test in the fermentation industry in both South and North America (personal communication, W Knight and J Cuomo, Pfizer Inc, Exton, PA, USA).

Virginiamycin is composed of two factors, M and S [6,7]. Factor M (a polyunsaturated cyclic peptolide) has a molecular weight of 525 ($C_{28}H_{35}N_3O_7$) while factor S (a cyclic hexadepsipeptide), has a molecular weight of 823 ($C_{43}H_{19}N_7O_{10}$) [1,6]. The two components pass through the cell membrane of Gram-positive bacteria (Gram-negative bacteria are generally impermeable to factor M) and once in the cytoplasm, one molecule of M and one of S are bound by the 50S ribosomal subunit, inhibiting protein synthesis [6]. The activity of a mixture of M and S is 10–100 times that of the individual components and depends on the ratio of the components [6]. This synergy is partly due to a 6-fold increase in the affinity of factor S for the ribosome in the presence of factor M [6]. Individually the M and S components have a bacteriostatic effect while a mixture of the two components is usually bactericidal [6,7] The minimum inhibitory concentration (MIC) of the mixture of M and S for Gram-positive bacteria is generally in the range of 0.1–5 mg L^{-1} [1,6], however there is variation in susceptibility, even within species, to virginiamycin. The MIC for *Lactobacillus fermentum* ranges from 0.5 to greater than 64 mg L^{-1}, although most lactobacilli isolated from animals were sensitive to 2 mg L^{-1} or less [10,11,20]. Yeast fermentation was not affected by a virginiamycin concentration of 2 mg L^{-1} while 20 mg L^{-1} decreased the rate of glucose uptake by yeast [14].

The antibiotic virginiamycin has potential for controlling and preventing contamination by lactic acid bacteria in the alcohol fermentation industry as it is effective against Gram-positive bacteria at concentrations below those which affect yeast. It is active in the pH range encountered during fermentation (unpublished data) and is inactivated during ethanol distillation (therefore no residues remain after distillation in alcohol or in distillers grains) [14; Pfizer Animal Health, Lactrol Technical Information Pamphlet 793-002-4/95B].

The goal of the present study was to determine the opti-

Correspondence: Dr WM Ingledew, Applied Microbiology and Food Science, University of Saskatchewan, Agriculture Building, 51 Campus Drive, Saskatoon, Saskatchewan, Canada S7N 5A8
Received 5 August 1996; accepted 18 December 1996

mal concentration of virginiamycin needed to control the growth of selected lactobacilli in wheat mash and to examine the effectiveness of this concentration in protecting a yeast fermentation artificially contaminated with industrial isolates of lactic acid bacteria.

Materials and methods

Bacterial strains

The bacteria used in this study are listed in Table 1 along with their sources and their suspected fermentative action on sugars. The ability of the lactic acid bacteria to grow in deMan, Rogosa, Sharpe (MRS) broth (Unipath, Nepean, ON, Canada) in the presence of 10% ethanol was ascertained prior to selecting strains for this study.

Bacterial growth conditions

All lactobacilli except *Lactobacillus delbrueckii* were grown with shaking (150 rpm) at 30°C in 50 ml of MRS broth in 250-ml screw-capped, side-arm flasks. The headspaces of the flasks were flushed with filter-sterilized CO_2 on inoculation. *L. delbrueckii* was grown at 30°C without shaking in 15 ml of MRS broth in screw-capped tubes in a CO_2 incubator (National Appliance Co, Portland, OR, USA).

Growth was followed using a Klett Summerson colorimeter (Klett Mfg Co, New York, NY, USA) with a number 66 red filter (640–700 nm). All lactobacilli were grown to late exponential phase and then transferred to fresh medium and grown under the same conditions to early stationary phase. This culture was then used as the inoculum for wheat mash to give either 1×10^5 or 1×10^6 bacteria g^{-1} of mash.

In one series of experiments, *L. rhamnosus* was grown in wheat mash for varying lengths of time before the addition of yeast. In this case, each fermentor containing 500 g mash was inoculated at 0 h with 1×10^6 cells of *L. rhamnosus* g^{-1}, prepared as above. The inoculated mashes were then incubated for 0, 12, 24, or 36 h before the addition of a normal yeast inoculum of 10^6 yeast g^{-1} mash. Virginiamycin was added to fermentors immediately after yeast inoculation.

Table 1 *Lactobacillus* strains used, their suspected fermentative action on sugars and their sources

Strain	Fermentation type	Source
L. delbrueckii ATCC 9649	homofermentative	sour grain mash
L. fermentum ATCC 1493	heterofermentative	fermented beets
L. fructivorans ATCC 15435	heterofermentative	spoiled sake
L. homohiochi ATCC 15434	homofermentative	spoiled sake
L. paracasei ssp *paracasei* 2[a]	homofermentative or facultatively heterofermentative	industrial strain[b]
L. plantarum 1[a]	homofermentative or facultatively heterofermentative	industrial strain[b]
L. rhamnosus ATCC 15280	homofermentative or facultatively heterofermentative	corn steep liquor

[a]Identified by API 50 CHL kits for *Lactobacillus* (bioMerieux, Montreal, PQ, Canada).
[b]Dr Jaime Finguerut, Centro de Technologia Copersucar, Bairro Santo Antonio, Piracicaba, Brazil.

Preparation of yeast inoculum

Active dry yeast ('Allyeast Superstart', Alltech Inc, Nicholasville, KY, USA) was conditioned [5] and added to wheat mash in fermentors to obtain 1×10^6 or 1×10^7 viable yeast g^{-1} of mash.

Preparation of wheat mash for fermentation

Seven kilograms of ground (setting 5 on a S 500 Disk Mill, Glen Mills Inc, Clifton, NJ, USA) hard red spring wheat were dispersed, with continuous stirring, into 19 L of 60°C distilled water containing 1 mM calcium chloride. Gelatinization and liquefaction were carried out as described [23]. Water lost through evaporation was made up with sterile distilled water so that the final concentration of dissolved solids was 25 g per 100 ml of the liquid portion of the mash.

The liquefied mash was strained through a sterile, stainless steel food grade sieve (1.5-mm hole size), cooled and chemically sterilized with diethyl pyrocarbonate (DEPC) (Sigma Chemical Co, St Louis, MO, USA), as described by Chin and Ingledew [5]. Aliquots of 500 g mash were aseptically transferred to sterile, jacketed 500-ml (working volume) Celstir bioreactors (Wheaton Instruments, Millville, NJ, USA) to which a solution of yeast extract (AYE-2200, Gilette Foods Inc, Union, NJ, USA) had been added, before autoclaving, to give a final concentration of 0.45% w/v). Other more economical nitrogenous substances such as urea could have been used.

The fermentors were connected to a D3-G water bath circulator (Haake Inc, Saddle Brook, NJ, USA) maintained at 30°C, and stirred on magnetic stir plates (IKA-Labortechnik, Staufen, Germany). Thirty minutes prior to inoculation, 0.8 ml of membrane filter-sterilized glucoamylase (Alcoholase II, Alltech Inc), was added to each fermentor to saccharify the dextrins.

Preparation of virginiamycin

Lactrol™ (100% activity, Pfizer, Richmond, VA, USA) was the source of virginiamycin. Lactrol was dissolved in 95% ethanol (1 mg ml^{-1}) and added to mash, to give 0.5, 1.0, 2.0 or 6.0 mg Lactrol (hereafter termed virginiamycin) kg^{-1} of prepared mash (levels of ethanol solvent added with the virginiamycin were insignificant in comparison with ethanol produced).

Viable counts of bacteria and yeast

Viable counts of bacteria and yeast were determined by the membrane filtration method. Five or 10 ml of serially diluted samples were filtered through sterile membrane filters (0.45 μm). For enumeration of lactobacilli, the membranes were placed on plates of MRS agar and incubated at 30°C in a CO_2 incubator. Where necessary, cycloheximide (0.001%) (Sigma Chemical Co) was added to the MRS to inhibit yeast growth. Yeasts were enumerated by placing membranes on plates of yeast extract-peptone-dextrose

(YPD) agar (yeast extract 1%, peptone 1%, dextrose 2%, agar 1.5% (w/v)) and incubating aerobically at 27°C. Where necessary, 0.005% (w/v) gentamicin and 0.01% (w/v) oxytetracycline (Sigma Chemical Co) were added to the YPD agar to inhibit bacterial growth.

Determination of dissolved solids

The dissolved solids concentrations of mashes were determined using a DMA 45 density meter (Anton Paar KG, Graz, Austria), by measuring the specific gravity of the supernatant phase obtained by centrifugation of samples at $10\,300 \times g$ for 15 min. The readings were converted to grams of dissolved solids per 100 ml.

Determination of titratable acidity

Concentrations of lactic acid were estimated by titration of mash to pH 7.0 (Accumet pH meter 10, Fisher Scientific, Nepean, ON, Canada) with 0.1 N standard sodium hydroxide (Mallinckrodt Chemical Inc, Chesterfield, MO, USA). Ten-gram samples of mash were diluted with 50 ml distilled water prior to titration.

Ethanol assay

Ethanol concentrations were determined enzymatically using the alcohol dehydrogenase assay (Sigma Technical Bulletin No. 331 UV, Sigma Chemical Co).

HPLC analysis

Ethanol, lactic acid, and residual sugars were determined by HPLC analysis. Five microliters of diluted filtrate (0.22-μm pore size membrane filter) were injected into a FAM-PAK™ column (Waters Chromatographic Division, Milford, MA, USA) maintained at 65°C. Components were eluted from the column with 1.5 mM orthophosphoric acid at a flow rate of 1 ml min^{-1} and detected with a differential refractometer (Model 410, Waters Chromatographic Division). Methanol was used as the internal standard. The chromatographic data were processed by the Maxima 810 program (Waters Chromatographic Division).

Results

Minimal concentration of virginiamycin needed to control growth of lactobacilli in wheat mash in the absence of yeast

The growth and lactic acid production of *L. fermentum* in mashes not inoculated with yeast was controlled by virginiamycin at each of the concentrations studied (0.5, 2.0 and 6.0 mg kg^{-1} mash) (Figure 1). In mash containing 0.5 mg of virginiamycin kg^{-1}, the viable numbers of *L. fermentum* decreased by slightly more than 1 log unit during an incubation period of 72 h while at the higher concentrations there was a 2 log unit decrease. In the absence of virginiamycin there was an increase to over 10^9 bacteria ml^{-1}. Virginiamycin at a concentration of 0.5 mg kg^{-1} was less effective in controlling the growth of *L. rhamnosus*. The number of viable cells remained at approximately 1×10^5 g^{-1} for 36 h, after which there was a rapid increase in cell numbers to levels similar to those reached in the absence of virginiamycin. Virginiamycin at 2.0 and 6.0 mg kg^{-1} decreased the number of viable *L. rhamnosus* by 1 log unit (results not shown). When added to mash after the viable numbers of *L. rhamnosus* had reached 6×10^8 ml^{-1}, virginiamycin (0.5 mg kg^{-1}) controlled further growth (Figure 2) and lactic acid production. It is probable, there-

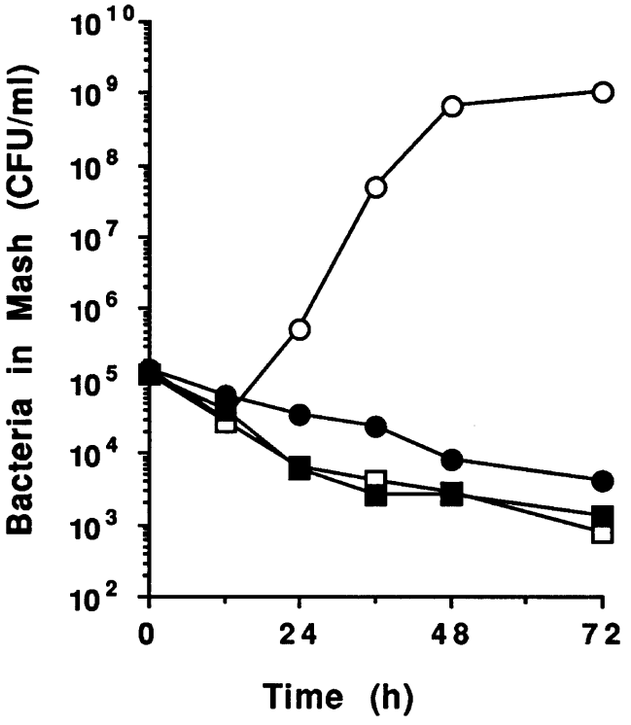

Figure 1 Viable counts of *L. fermentum* in wheat mash treated with increasing concentrations of virginiamycin (no yeast added). ○, No virginiamycin; ●, 0.5 mg kg^{-1}; ■, 2.0 mg kg^{-1}; □, 6.0 mg virginiamycin kg^{-1}.

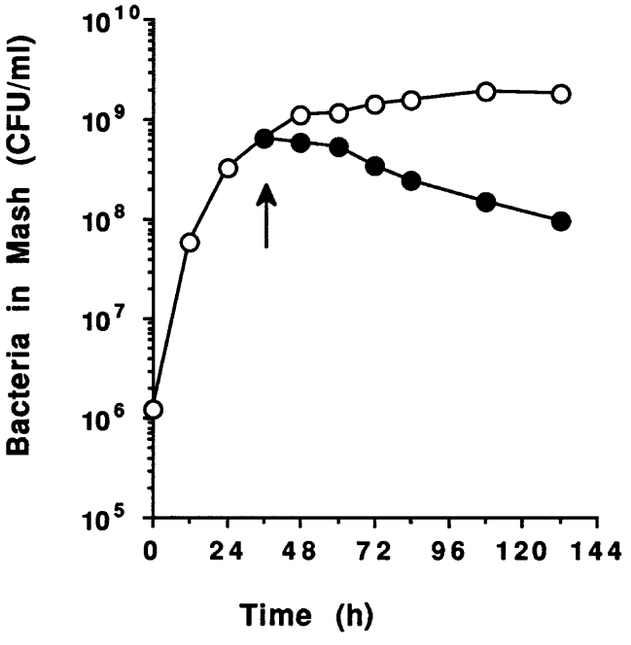

Figure 2 Viable counts of *L. rhamnosus* in wheat mash (no yeast added). Arrow indicates time of virginiamycin addition. ○, No virginiamycin; ●, 0.5 mg virginiamycin kg^{-1}.

fore, that the addition of virginiamycin led to an immediate cessation of growth.

L. paracasei showed a sensitivity to virginiamycin similar to that exhibited by *L. rhamnosus*. Virginiamycin at 0.5 mg kg^{-1} controlled growth of *L. paracasei* for 48 h but thereafter the viable numbers increased rapidly. There was no increase in viable numbers of *L. paracasei* when the virginiamycin concentration in mash was raised to 1 mg kg^{-1}.

L. plantarum appeared to be insensitive or resistant to low concentrations of virginiamycin. The growth of this organism was only slightly retarded by virginiamycin concentrations of 0.5 and 1 mg kg^{-1} and the viable numbers increased rapidly after a lag of 48 h, when 2 mg of virginiamycin kg^{-1} were present.

The effect of virginiamycin on all of the lactobacilli under study was as least partially bacteriostatic, as colonies arising from samples removed from virginiamycin-treated mash took longer to appear than those from untreated mash.

It was determined that 0.5 mg virginiamycin kg^{-1} mash was sufficient to control growth and lactic acid production in wheat mashes inoculated with *L. fermentum*, *L. rhamnosus* and *L. paracasei*. The increase in viable numbers of *L. rhamnosus* and *L. paracasei* observed after prolonged incubation in the presence of virginiamycin, took place after yeast fermentation under the same conditions would be complete. The 0.5 mg virginiamycin kg^{-1} mash concentration was therefore chosen for use in the following studies where the effect of virginiamycin during alcoholic fermentations was investigated.

Effectiveness of virginiamycin in protecting alcoholic fermentation by yeast in the presence of added lactobacilli

Virginiamycin at a concentration of 0.5 mg kg^{-1} controlled the growth of *L. fermentum* or *L. rhamnosus* during alcoholic fermentation by yeast. However, preliminary experiments showed that if the yeast inoculum in the mash was high (1×10^7 yeast g^{-1} mash), the growth and lactic acid production by the bacteria, even in the absence of virginiamycin, was not sufficient to have an effect on fermentation. The maximum number of viable yeast attained (approximately 2×10^8 ml^{-1}), the fermentation time (approximately 30 h), the titratable acidity (approximately 4 ml of 0.1 N NaOH per 10 g mash), and the final ethanol concentration (approximately 13% v/v) were similar in virginiamycin-treated and untreated mashes and in mashes inoculated with this level of yeast alone. Similar results were obtained when mash was inoculated with yeast and *L. fructivorans* or *L. homohiochi*. It is likely that the lactobacilli were not able to compete for nutrients when the yeast inoculation was 1×10^7 cells g^{-1} mash. This yeast inoculation level is typical for brewing. In subsequent experiments, the yeast inoculum was reduced to 1×10^6 g^{-1} mash (a value more representative of many industrial conditions where ADY is used in batch fermentation).

With the lower level of yeast inoculation, the addition of selected lactic acid bacteria led to losses in ethanol yield (Table 2). These losses were prevented or reduced by the addition of virginiamycin. However no significant differences in the maximum number of viable yeast or the rates of fermentation were observed between any of the treatments (results not shown). It is apparent therefore that the growth of the lactobacilli did not lead to the depletion of any nutrient essential for yeast growth. Moreover, control experiments in the absence of lactic acid bacteria showed that virginiamycin, at 0.5 mg kg^{-1}, had no significant effect on fermentation by yeast.

Virginiamycin (0.5 mg kg^{-1}) effectively controlled the growth (Figure 3) and lactic acid production of *L. rhamnosus* co-inoculated with 1×10^6 yeast cells g^{-1} mash. In the absence of virginiamycin, bacterial numbers increased from approximately 1×10^6 to 6×10^8 ml^{-1} during a 48-h fermentation period and 5.8 mg lactic acid ml^{-1} was produced (Table 2). The ethanol yield was 93% of that observed in the control (no lactobacilli) (Table 2). In the presence of virginiamycin, the viable numbers of bacteria did not increase (Figure 3), lactic acid production was limited to 1.12 mg ml^{-1} (Table 2) and the ethanol concentration, 96 mg ml^{-1}, was similar to that in the control fermentation (Table 2). Virginiamycin was also effective in controlling growth and lactic acid production by *L. paracasei* (results were similar to those shown in Figure 3). Viable numbers increased from approximately 3×10^6 to 3×10^9 ml^{-1} mash in the absence of virginiamycin while in the presence of virginiamycin the cell numbers increased to only 1×10^7 ml^{-1}. Lactic acid concentrations after 48 h of fermentation were 11.76 mg ml^{-1} and 1.60 mg ml^{-1} in the absence and presence of virginiamycin respectively (Table 2). Ethanol concentrations were only 89% of that of the control in the absence of virginiamycin but increased to 98% in its presence (Table 2).

Growth and lactic acid production by *L. plantarum* were not effectively controlled by virginiamycin (Figure 4). This may be related to the very fast growth of this bacterium in wheat mash. Over the first 24 h of fermentation, viable cell numbers increased from 2.6×10^6 to 2.5×10^9 ml^{-1} in the absence of virginiamycin, and to 7.5×10^8 ml^{-1} in the presence of virginiamycin. However, after 24 h there was a rapid loss in bacterial viability in both virginiamycin-treated and untreated fermentors (Figure 4). A similar loss of viability was seen when *L. fermentum* was co-inoculated with yeast and it is assumed that this was due to the combined toxic effects of ethanol, lactic acid and low pH.

The addition of *L. plantarum* to yeast fermentations increased lactic acid production, and reduced ethanol yield compared to the control fermentation with yeast alone (Table 2). Virginiamycin decreased the amount of lactic acid produced from 11.5 mg ml^{-1} to 7 mg ml^{-1} but increased the ethanol yield to only 93% of that of the control. Among the lactobacilli studied here, *L. plantarum* appears to be the least sensitive to virginiamycin. Virginiamycin also controlled the growth of *L. delbrueckii*, but even in the absence of virginiamycin, this organism grew slowly in wheat mash and had no significant effect on ethanol production (Table 2).

The observed losses in ethanol yield were greater than expected losses calculated from the amount of glucose diverted for the production of lactic acid (Table 2).

Table 2 Concentrations of ethanol and lactic acid produced by various lactobacilli in the presence and absence of virginiamycin (0.5 mg kg^{-1})

Bacterium used	Virginiamycin treatment (+ or –)	Ethanol produced (mg ml^{-1})	Ethanol as % of amount produced in the control	Lactic acid (mg ml^{-1})	Calculated loss of ethanol due to lactic acid (mg ml^{-1})[a]
L. rhamnosus	–	90.24	92.84	5.76	2.94
L. rhamnosus	+	96.16	98.93	1.12	0.57
L. paracasei	–	86.20	88.68	11.76	6.01
L. paracasei	+	95.36	98.11	1.60	0.82
L. plantarum	–	87.40	89.92	11.52	5.89
L. plantarum	+	90.76	93.37	7.04	3.60
L. delbrueckii	–	97.68	100.49	0.80	0.41
L. delbrueckii	+	95.68	98.44	0.52	0.27
Control, yeast alone	–	97.20		0.60	0.31

Amounts produced by control fermentations of yeast alone are given for comparison. (Values were determined by HPLC analyses of samples taken after 48 h of fermentation.)

[a]Assumes homofermentative production of lactic acid. Therefore 1 mole of lactic acid made from pyruvate is the loss of 1 mole of ethanol.

$$\text{Lactic acid (mg ml}^{-1}) \times \frac{46 \text{ (molecular weight of ethanol)}}{90 \text{ (molecular weight of lactic acid)}} = \text{calculated ethanol loss (mg ml}^{-1}).$$

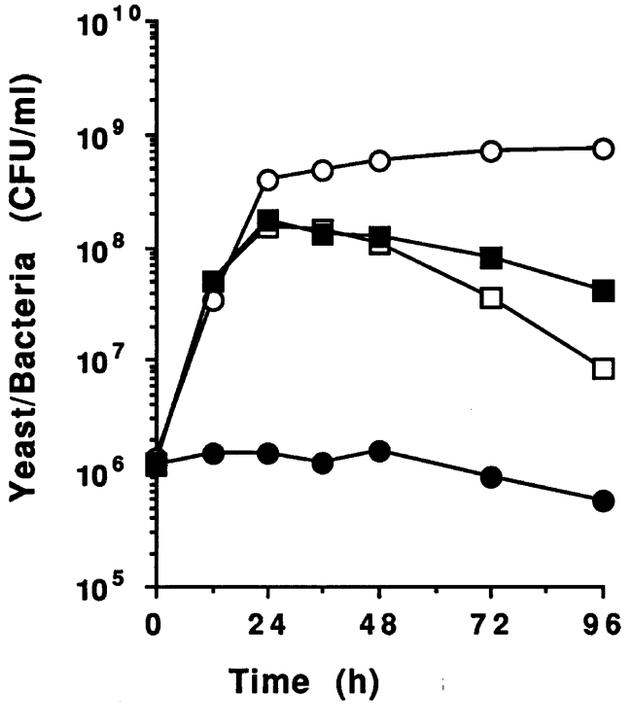

Figure 3 Viable yeast (enumeration on YPD agar plus gentamicin and oxytetracycline to inhibit bacteria) and bacteria (enumeration on MRS agar with cycloheximide to inhibit yeast), in wheat mash inoculated with yeast and *L. rhamnosus* (both at 1×10^6 g^{-1}). Yeast counts: □, no virginiamycin; ■, 0.5 mg virginiamycin kg^{-1}. Bacterial counts: ○, no virginiamycin; ●, 0.5 mg virginiamycin kg^{-1}.

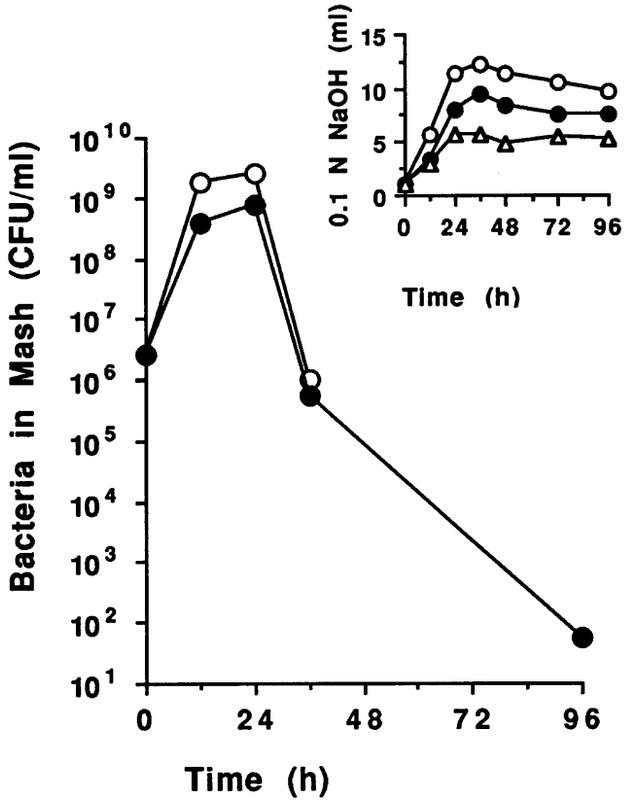

Figure 4 Viable bacteria (enumerated on MRS agar with cycloheximide to inhibit yeast) in wheat mash inoculated with yeast and *L. plantarum* (both at 1×10^6 g^{-1}), and (inset) titration of above mashes, and a control mash inoculated with yeast alone (1×10^6 g^{-1}). ○, No virginiamycin; ●, 0.5 mg virginiamycin kg^{-1}; △, yeast alone (no bacteria or virginiamycin).

Effects of preincubation of mash with L. rhamnosus before inoculating mash with yeast

In order to simulate a heavier bacterial contamination, the mash was inoculated with *L. rhamnosus* and incubated for various lengths of time prior to inoculation with yeast and addition of virginiamycin. The numbers of viable *L. rhamnosus* cells attained following preincubation times of 0, 12, 24 and 36 h were 1.2×10^6, 5.8×10^7, 3.2×10^8 and 6.4×10^8 ml^{-1} respectively.

In fermentors with no preincubation, virginiamycin had a bacteriostatic effect on *L. rhamnosus* (Figure 5a). In fermentors preincubated for 12 h, the effect of virginiamycin was more bactericidal and viable numbers decreased by two log units over the next 72 h (Figure 5b). In the fermentors preincubated for 24 or 36 h, growth was partially controlled by virginiamycin (Figure 5c, d). However, 36 h after the introduction of the yeast, the viability of bacteria decreased

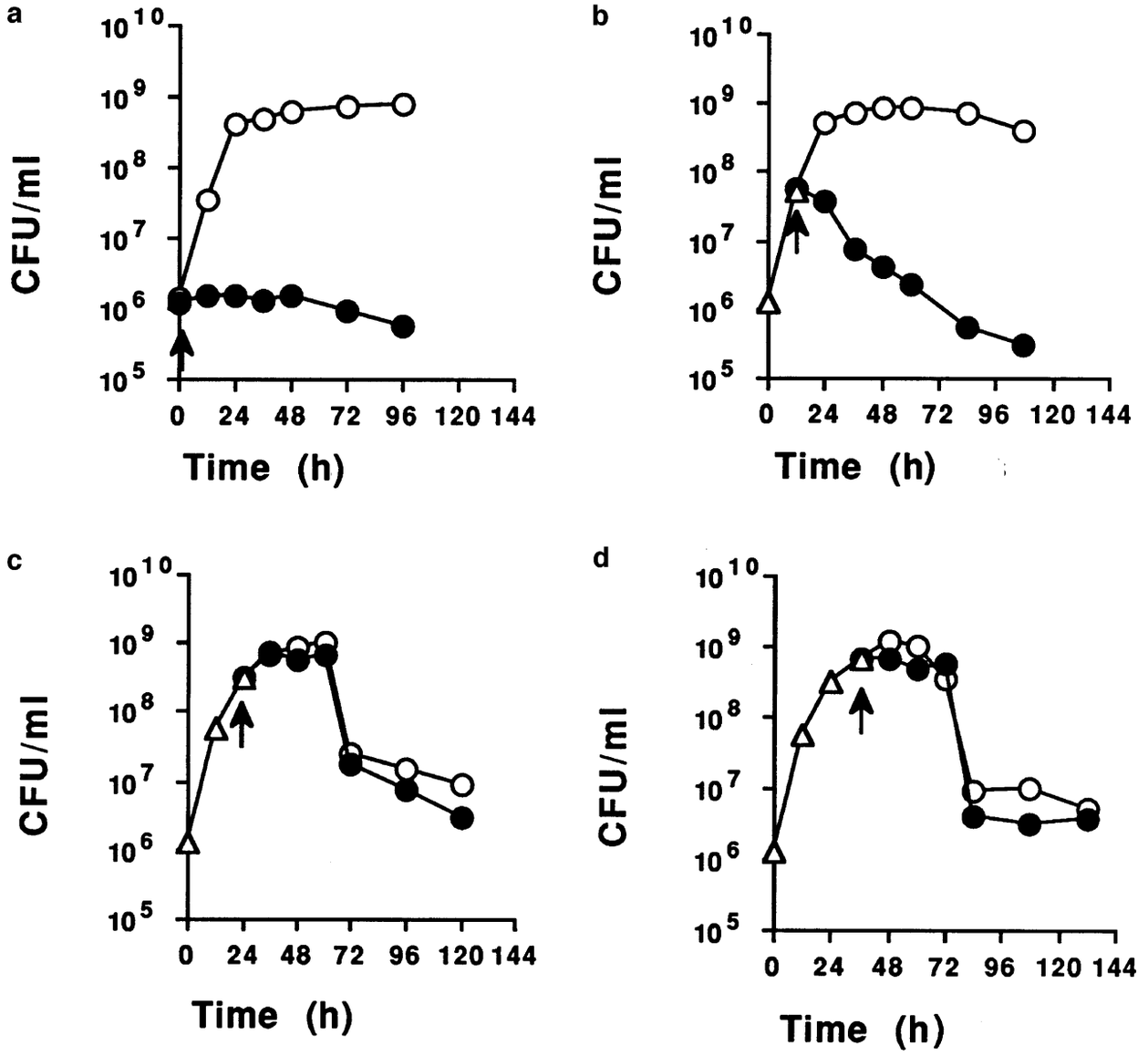

Figure 5 Viable bacteria (enumeration on MRS agar with cycloheximide to inhibit yeast) in mash preincubated with *L. rhamnosus* for: (a) 0 h, (b) 12 h, (c) 24 h and (d) 36 h before inoculation with yeast (1×10^6 g^{-1}) and addition of virginiamycin. The arrow denotes the time of addition of yeast/virginiamycin. △, Bacterial counts during preincubation time; ○, bacterial counts after yeast inoculation, no virginiamycin; ●, bacterial counts after yeast inoculation, 0.5 mg virginiamycin kg^{-1}.

rapidly in both the virginiamycin treated and untreated fermentors. Again these losses in viability are most likely due to a combination of high lactic acid and ethanol concentrations and lower pH.

When the numbers of *L. rhamnosus* were allowed to increase prior to yeast inoculation, the effect of the bacteria on fermentation became significant. Losses in ethanol yield increased with increasing bacterial preincubation time and these losses were prevented (in the case of 0-h preincubation time), reduced (in the case of 12-h preincubation) or not significantly affected (in the case of the 24-h or 36-h preincubation times) by the addition of 0.5 mg virginiamycin kg^{-1} (Table 3). In the latter cases, conditions (decreased glucose and increased lactic acid concentrations) leading to losses in potential ethanol yield already existed in the mash before the addition of virginiamycin.

In the fermentors preincubated with *L. rhamnosus* for 12 or more hours, lactic acid concentrations during fermentation reached levels sufficient to inhibit yeast (10–40 mg ml^{-1}) [5,17,18]. Inhibition by lactic acid as well as the direct diversion of glucose to lactic acid would contribute to losses in ethanol yield.

Discussion

Virginiamycin was effective in controlling growth of the majority of lactic acid bacteria. The lowest concentration tested, 0.5 mg kg^{-1}, was sufficient to control the growth of

Table 3 Concentrations of ethanol and lactic acid produced by *L. rhamnosus* in the presence and absence of virginiamycin (0.5 mg kg^{-1}) following preincubation of *L. rhamnosus* for increasing times before the addition of yeast and virginiamycin

Preincubation time (h)	Virginiamycin	Ethanol produced (mg ml^{-1})	Ethanol as % of amt produced by control	Lactic acid produced (mg ml^{-1})
0	–	90.24	92.84	5.76
0	+	96.16	98.93	1.12
12	–	83.16	85.56	12.48
12	+	88.08	90.62	3.76
24	–	83.36	85.76	17.72
24	+	82.64	85.02	15.32
36	–	79.24	81.52	16.64
36	+	81.84	84.20	15.36
Control (yeast alone)	–	97.20		

Values given are from HPLC analyses of samples 48 h after inoculation of yeast (*L. rhamnosus* had been in the mash for the preincubation times given plus 48 h, accounting for the higher concentrations of lactic acid in the preincubated samples).

L. rhamnosus, *L. fermentum*, *L. paracasei* and *L. delbrueckii*, but a higher concentration of the antibiotic may be required to control the growth of *L. plantarum*. In the absence of yeast, *L. rhamnosus* and *L. paracasei* were able to grow in the presence of 0.5 mg virginiamycin kg^{-1} after a lag of 48 h. It is not clear whether this is due to a variation in sensitivity or to a selection, mutation or degradation of virginiamycin by these lactobacilli. Resistance to virginiamycin among several genera of Gram-positive bacteria has been reported [1,3,6,10,11], as has the breakdown of virginiamycin by lactobacilli [10]. In mash co-inoculated with yeast, the effects of yeast growth and yeast fermentation products combined with the effect of virginiamycin prevented a similar increase in viable numbers of *L. rhamnosus* and *L. paracasei*.

Losses of up to 11% of produced ethanol were observed when lactobacilli were co-inoculated with yeast and these losses were reduced or prevented by the addition of virginiamycin. Losses of 6–12% of total produced alcohol (0.8–1.5% v/v ethanol concentration in fermentation) were seen when particularly aggressive lactic contaminants were present in mash in high numbers (unpublished data). Even a 1% loss of ethanol in a plant making 100 000 000 L $year^{-1}$ is a loss of revenue of over $300 000.

Increased numbers of lactobacilli present at the time of yeast inoculation led to increased losses in ethanol yield. The results also showed that the observed losses of ethanol could be only partially accounted for by direct diversion of glucose to lactic acid. Other possible causes of loss in yield are: competition for nutrients by lactobacilli resulting in decreased yeast metabolism; inhibition of alcohol fermentation by lowered pH or by lactic acid and other metabolic products of lactobacilli [13]; diversion of glucose to cellular constituents needed for growth of the lactobacilli; increased flocculation of yeast in the presence of some strains of lactobacilli [19]; or indirectly, through an inhibition of glucoamylase due to a lowering of pH by lactic acid—resulting in incomplete saccharification [16,19].

There was excessive loss of viability of co-inoculated *L. fermentum* or *L. plantarum* during the alcoholic fermentation of wheat mash by yeast—most likely due to synergistic effects of high ethanol and lactic acid concentrations and low pH. However, in the case of *L. plantarum*, in spite of this loss of viability, ethanol yields were decreased by 10%. Under different fermentation conditions, losses in ethanol yield due to these rapidly growing lactobacilli could be even greater. This emphasizes the importance of controlling these organisms by commercial pasteurization of mash, by using cleaners and sanitizers on equipment and by the addition of a suitable concentration of an antibiotic like virginiamycin.

Due to their slow growth rates, the effect of the industrial strains of *L. delbrueckii*, *L. fructivorans* and *L. homohiochi* on yeast fermentation were minimal. These organisms, at the levels tested in the present study, are not likely to be a problem in relatively rapid alcohol fermentations.

In this study, the lactobacilli which affected fermentation were those that were tolerant to ethanol, had rapid growth rates and were able to reach high numbers of viable cells prior to the completion of yeast fermentation. These organisms were isolates from the fermentation industry and appear to be adapted to compete with yeast under fermentation conditions. They grew considerably more quickly and to higher cell densities than laboratory strains of lactobacilli isolated from brewing and food sources. Bryan-Jones [4] and Barbour and Priest [2] also reported the distinctiveness of distillery lactobacilli and noted their apparent adaptation to fermentation environments.

Some variation in susceptibility to virginiamycin was seen among the different strains of lactobacilli used and also an apparent variation depending on the growth phase. A wide variety of strains from industrial sites needs to be identified and tested for both antibiotic sensitivity and alcohol tolerance, to fully appreciate their effect on industrial processing, and to determine which species are usually found in production plants using particular substrates.

The use of a high inoculum concentration of yeast was seen to minimize the growth of contaminating lactobacilli and reduce the losses of ethanol yield. Although this finding has not been confirmed with all strains, it may explain why lactic acid does not normally rise to high concentrations in fermentations catalysed by high numbers of yeast (for example, continuous fermentation with yeast recycle). Chin and Ingledew [5] reported no loss in ethanol when batch fermentations infected with lactobacilli were inoculated with high concentrations of yeast, and Essia Ngang *et al* [12] suggested the use of high yeast populations to help control contamination by lactic acid bacteria. This may sug-

gest a strategy for fermentation plants where lactics are seen in high numbers.

Acknowledgements

This research was partly funded by the Natural Sciences and Engineering Research Council of Canada and by the Western Grains Research Foundation. We thank Dr Jaime Finguerut, Centro de Technologia Copersucar, Brazil, for provision of cultures from Brazil.

References

1 Allignet J, V Loncle and N El Solh. 1992. Sequence of a staphylococcal plasmid gene, *vga*, encoding a putative ATP-binding protein involved in resistance to virginiamycin A-like antibiotics. Gene 117: 45–51.
2 Barbour EA and FG Priest. 1988. Some effects of *Lactobacillus* contamination in scotch whisky fermentations. J Inst Brew 94: 89–92.
3 Brady MS and SE Katz. 1992. *In vitro* effect of multiple antibiotic/antimicrobial residues on the selection for resistance in bacteria. J AOAC Int 75: 738–742.
4 Bryan-Jones G. 1975. Lactic acid bacteria in distillery fermentations. In: Lactic Acid Bacteria in Beverages and Food. Proceedings of the Fourth Long Ashton Symposium (Carr JC, CV Cutting, and CG Whiting, eds), pp 165–175, Academic Press, London.
5 Chin PM and WM Ingledew. 1994. Effect of lactic acid bacteria on wheat mash fermentations prepared with laboratory backset. Enzyme Microb Technol 16: 311–317.
6 Cocito C. 1979. Antibiotics of the virginiamycin family, inhibitors which contain synergistic components. Microbiol Rev 43: 145–198.
7 Cocito C and G Fraselle. 1973. The properties of virginiamycin-resistant mutants of *Bacillus subtilis*. J Gen Microbiol 76: 115–125.
8 Dolan TCS. 1976. Some aspects of the impact of brewing science on scotch malt whisky production. J Inst Brew 82: 177–181.
9 Dolan TCS. 1979. Bacteria in whisky production. The Brewer 65: 60–64.
10 Dutta GN and LA Devriese. 1981. Degradation of macrolide-lincosamide streptogramin antibiotics by *Lactobacillus* strains from animals. Ann Microbiol 132A: 51–57.
11 Dutta GN and LA Devriese. 1981. Sensitivity and resistance to growth promoting agents in animal lactobacilli. J Appl Bacteriol 51: 283–288.
12 Essia Ngang JJ, F Latourneau and P Villa. 1989. Alcoholic fermentation of beet molasses: effects of lactic acid on yeast fermentation parameters. Appl Microbiol Biotechnol 31: 123–128.
13 Essia Ngang JJ, F Latourneau, E Wolniewicz and P Villa. 1990. Inhibition of beet molasses alcoholic fermentation by lactobacilli. Appl Microbiol Biotechnol 33: 490–493.
14 Hamdy MK, CJ Shieh, RT Toledo, MA Pfannenstiel and R Wang. 1994. Effects of virginiamycin on alcohol fermentation by yeasts. Abstracts Am Soc Microbiol General Meeting. May 1994, Las Vegas.
15 Huang Y-C, CG Edwards, JC Peterson and KM Haag. 1996. Relationship between sluggish fermentations and the antagonism of yeast by lactic acid bacteria. Am J Enol Vitic 47: 1–10.
16 Ingledew WM. 1993. Yeasts for production of fuel ethanol. In: The Yeasts, Vol 5. Yeast Technology. 2nd edn (Rose AH and JS Harrison, eds), pp 245–291, Academic Press, New York.
17 Kelsall DR. 1995. The management of fermentations in the production of alcohol. In: The Alcohol Textbook (Lyons TP, DR Kelsall and JE Murtagh, eds), pp 89–101, Nottingham University Press, UK.
18 Maiorella B, HW Blanch and CR Wilke. 1983. By-product inhibition effects on ethanolic fermentation by *Saccharomyces cerevisiae*. Biotechnol Bioeng 25: 103–121.
19 Makanjoula DB, A Tymon and DG Springham. 1992. Some effects of lactic acid bacteria on laboratory-scale yeast fermentations. Enzyme Microb Technol 14: 350–357.
20 Nagaraja TG and MB Taylor. 1987. Susceptibility and resistance of ruminal bacteria to antimicrobial feed additives. Appl Environ Microbiol 53: 1620–1625.
21 Serra CE, MP Cereda, SZ Pinho, MA Meneguim, EA Marino, LJ Ferreira and AE Bethiol. 1980. Microbiological control of ethanol fermentation in industrial scale. Proceedings of the Fourth International Symposium on Alcohol Fuels Technology, Brazil. Vol III, pp 1051–1054.
22 Stark WH. 1954. Alcoholic fermentation of grain. In: Industrial Fermentations (Underkofler LA and RJ Hickey, eds), vol 1, pp 17–72, Chemical Publishing Co, New York.
23 Thomas KC and WM Ingledew. 1990. Fuel alcohol production: effects of free amino nitrogen on fermentation of very-high-gravity wheat mashes. Appl Environ Microbiol 56: 2046–2050.