Biotechnology for Biofuels

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Saccharomyces cerevisiae expressing bacteriophage endolysins reduce *Lactobacillus* contamination during fermentation

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Abstract

Background: One of the challenges facing the fuel ethanol industry is the management of bacterial contamination during fermentation. *Lactobacillus* species are the predominant contaminants that decrease the profitability of biofuel production by reducing ethanol yields and causing "stuck" fermentations, which incur additional economic losses via expensive antibiotic treatments and disinfection costs. The current use of antibiotic treatments has led to the emergence of drug-resistant bacterial strains, and antibiotic residues in distillers dried grains with solubles (DDGS) are a concern for the feed and food industries. This underscores the need for new, non-antibiotic, eco-friendly mitigation strategies for bacterial contamination. The specific objectives of this work were to (1) express genes encoding bacteriophage lytic enzymes (endolysins) in *Saccharomyces cerevisiae*, (2) assess the lytic activity of the yeast-expressed enzymes against different species of *Lactobacillus* that commonly contaminate fuel ethanol fermentations, and (3) test the ability of yeast expressing lytic enzymes to reduce *Lactobacillus fermentum* during fermentation. Implementing antibiotic-free strategies to reduce fermentation contaminants will enable more cost-effective fuel ethanol production and will impact both producers and consumers in the farm-to-fork continuum.

Results: Two genes encoding the lytic enzymes LysA and LysA2 were individually expressed in *S. cerevisiae* on multi-copy plasmids under the control of a galactose-inducible promoter. The enzymes purified from yeast were lytic against *Lactobacillus* isolates collected from fermentors at a commercial dry grind ethanol facility including *Lactobacillus fermentum*, *Lactobacillus brevis*, and *Lactobacillus mucosae*. Reductions of *L. fermentum* in experimentally infected fermentations with yeast expressing LysA or LysA2 ranged from 0.5 $\log_{10}$ colony-forming units per mL (CFU/mL) to 1.8 $\log_{10}$ (CFU/mL) over 72 h and fermentations treated with transformed yeast lysate showed reductions that ranged from 0.9 $\log_{10}$ (CFU/mL) to 3.3 $\log_{10}$ (CFU/mL). Likewise, lactic acid and acetic acid levels were reduced in all experimentally infected fermentations containing transformed yeast (harboring endolysin expressing plasmids) relative to the corresponding fermentations with untransformed yeast.

Conclusions: This study demonstrates the feasibility of using yeast expressing bacteriophage endolysins to reduce *L. fermentum* contamination during fuel ethanol fermentations.

Keywords: Bacteriophage, Lysin, Endolysin, Yeast, *Saccharomyces cerevisiae*, Fermentation, Contamination, Lactic acid, Acetic acid, *Lactobacillus*

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Background

The primary feedstock for fuel ethanol production in the United States is glucose from corn starch [1], with annual production at roughly 13 billion gallons in 2012 [2]. Strategies to meet future production demands include developing genotypes of corn that yield a higher starch content, increasing land area designated for corn production, and utilizing alternative feedstocks to supplement corn-based fuel ethanol. Lignocellulosic biomass is a promising alternative renewable carbon source; however, the technology is still evolving and is constrained by production costs [3]. Therefore, in addition to research on commercializing lignocellulose to fermentable sugars, methods to improve the efficiency of ethanol production from starch-based feedstocks must continue.

Fuel ethanol production is not performed under aseptic conditions, and the economics of fuel ethanol production are threatened by Gram-positive and Gram-negative bacteria that contaminate commercial fuel ethanol plants and reduce ethanol yields [4-7]. Lactic acid bacteria (LAB) are the primary bacterial contaminants of fuel ethanol fermentations with species of *Lactobacillus* predominating. Chronic LAB contamination reduces available glucose for conversion to ethanol and reduces essential micronutrients required for optimal yeast growth, resulting in reduced ethanol yields. Acute infections are unpredictable and are characterized by the accumulation of bacterial by-products such as lactic acid and acetic acid. These organic acids inhibit yeast growth [8-15] and may cause stuck fermentations (incomplete conversion of glucose to ethanol) that require costly shutdowns of facilities for cleaning and disinfecting [4,7,9,16]. Lactobacilli flourish in the glucose-rich fermentation environment and are well adapted for survival under the high ethanol, low pH, and low oxygen conditions present during fermentation [6,17,18]. For example, *Lactobacillus fermentum* infection of *Saccharomyces cerevisiae* fermentations can reduce ethanol yields by up to 27% [4].

Contamination may occur at different locations within the fuel ethanol production pipeline. Previous work showed that lactobacilli are found at locations after the heat exchanger (unpublished data). Samples taken from strategic points along the production line demonstrated that saccharification tanks, continuous yeast propagation systems, and biofilms may act as reservoirs of bacteria that continually reintroduce contaminants [6,19]. There are numerous strategies to control contamination, including the use of chemical treatments, natural compounds, plant-derived compounds, and antibiotics (reviewed in [20]). Each of these control strategies varies in effectiveness and burdens fuel ethanol facilities with additional costs [20-24]. Antibiotics are the most common strategy used to control contamination in fuel ethanol facilities [5,25]. Penicillin and virginiamycin [26,27] are frequently applied to fermentations to control bacterial contamination, which has resulted in the emergence of multidrug-resistant isolates [17,25], limiting their effectiveness. Thirty-eight percent of *Lactobacillus* isolates from a dry grind ethanol plant that routinely used virginiamycin were found to harbor the *vat(E)* gene, whose product inactivates virginiamycin by acetylation of its hydroxyl group [28].

Bacteriophage endolysins are lytic enzymes that offer a novel approach to reduce bacterial contamination in fuel ethanol facilities and avoid the resistance pitfalls that are often associated with antibiotics and other antimicrobials (such as hop acids [29]). Endolysins have co-evolved with their host strains to degrade the peptidoglycan (PG) cell wall [30-32]. Once the cell wall has been compromised, the high internal pressure of bacterial cells causes osmolysis (lysis from within), enabling the release of phage progeny at the end of the phage lytic replication cycle [33]. Gram-positive bacteria, which have a thick PG cell wall (up to 40 layers thick) and no outer membrane, can also lyse Gram-positive bacteria when exposed externally to purified endolysins (lysis from without) [30-32]. In contrast, the cell walls of Gram-negative bacteria harbor an outer membrane that blocks access of most endolysins to the PG located in the periplasmic space. PG is unique to bacteria and exists as a complex polymer structure [34] of alternating units of *N*-acetyl glucosamine and *N*-acetylmuramic acid, which are cross-linked by oligopeptide chains attached to muramic acid residues [35]. Endolysins have a modular structure, usually composed of N-terminal catalytic domain(s) (that is, endopeptidase, glycosidase, and amidases) and a C-terminal cell wall binding domain (CBD) [36,37]. Endolysins show near-species specificity and are highly refractory to resistance development [30-32], likely due to their co-evolution with their host, and because of the need to target highly immutable PG structures in order to ensure phage escape from the host cell and the clade survival. Further decreasing the risk of resistance development to endolysins is the localization of the Gram-positive PG layer outside the cytoplasmic membrane, since most antibiotic resistance mechanisms act on compounds that localize intracellularly (for example, efflux pumps; see [38] for a review). There have been no reports to date of extracellular inactivation of PG hydrolases (endolysins).

Applying purified endolysins may be prohibitively expensive for large-scale commercial fermentations. To address this issue in the brewing and wine-making industries, it has been proposed that genetic engineering can improve microbial catalysts to resist contamination [39,40]. Previous work has demonstrated brewer's yeast bred to contain anti-contaminant properties [41]. Schoeman *et al.* [42] constructed a bactericidal yeast strain expressing and secreting the *Pediococcus acidilactici* pediocin, an antibacterial peptide with specificity for *Listeria* and *Leuconostoc* species.

Our previous work [43] evaluated the abilities of four recombinant endolysins (LysA, LysA2, LysgaY, and λSa2) that were purified from *Escherichia coli* to lyse a variety of lactobacilli under different pH values and ethanol concentrations. In this study, we demonstrate that *S. cerevisiae* expressing either of two phage lytic enzymes (LysA or LysA2) reduces the concentration of *L. fermentum* in experimentally infected corn mash fermentations.

Methods

Strains and plasmids

Saccharomyces cerevisiae strain BY4727 (*his*Δ200 *leu*Δ*0 lys2*Δ0 *met15*Δ0 trp1Δ63 *ura3*Δ0) [44] was kindly provided by Dr. Ron Hector and used for all yeast experiments described in this work. The media used for culturing BY4727 was similar to that described previously [45]. All yeast transformations were conducted following Invitrogen's small-scale transformation protocol. Untransformed yeasts were grown in yeast extract peptone dextrose (YPD) medium (1% yeast extract, 2% peptone, and 2% dextrose), and transformed yeasts were grown on synthetic drop-out medium containing yeast nitrogen base without amino acids (0.67%), 2% glucose, and supplemented with amino acids. The drop-out medium without uracil (SD_{-ura}) contained all standard amino acids (76 mg/L final) and lacked uracil (Sigma, St. Louis, MO). The induction medium was similar to the SD_{-ura} medium except that glucose was replaced with galactose (final concentration 2%). Glucose and galactose were filter sterilized using Nalgene Rapid-Flow sterile disposable bottle top polyethersulfone (PES) membrane filters (Fisher Scientific, Pittsburgh, PA).

L. fermentum 0605-B44, *Lactobacillus brevis* 0605-48, and *Lactobacillus mucosae* 0713-2 were isolated from fermentors at commercial dry grind ethanol facilities located within the Midwestern United States [4]. Lactobacilli were grown to mid-log phase ($OD_{600\ nm}$ = 0.4-0.6) at 37°C without shaking in de Man, Rogosa and Sharpe (MRS) broth. The optical densities at 600 nm ($OD_{600\ nm}$) of the overnight bacterial cultures were measured using the SmartSpec Plus spectrophotometer (Bio-Rad, Hercules, CA).

The His_6-tagged recombinant endolysin genes encoding LysA and LysA2 were yeast codon optimized, commercially synthesized, and cloned into the pUC57 vector (GenScript, Piscataway, NJ). Endolysin genes were then PCR amplified using the high-fidelity Easy-A polymerase (Stratagene, La Jolla, CA). The PCR-amplified endolysin genes were TOPO cloned into Invitrogen's high copy yeast expression vector pYES2.1 (Invitrogen, Carlsbad, CA) using standard molecular techniques. Endolysin expression was under the control of the Gal1 galactose-inducible promoter. A schematic of the vector harboring the endolysin that was transformed into BY4727 (Figure 1) was created using SeqBuilder (Lasergene version 10.0.0; DNASTAR, Madison, WI, USA).

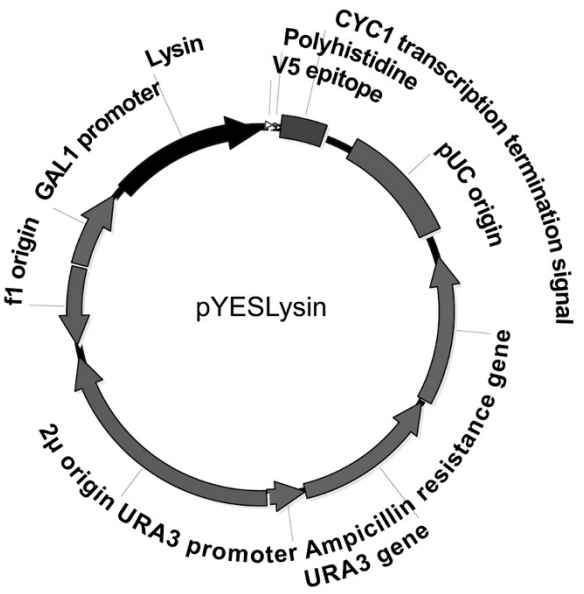

Figure 1 Schematic of yeast expression vector. High copy yeast expression vector used to transform yeast strain BY4727 for corn mash fermentation assays, containing the URA3 selectable marker and either LysA or LysA2, which were expressed under the regulation of the Gal1 promoter.

Vector-gene combinations were first transformed into TOP10 chemically competent *E. coli* cells by following Invitrogen's One Shot TOP10 chemical transformation protocol.

Yeast expression and purification of endolysins

The liquid SD_{-ura} medium was inoculated with transformed yeast cells from an SD_{-ura} medium 2% agar plate and grown at 30 °C overnight. The $OD_{600\ nm}$ of the overnight yeast culture was measured using the SmartSpec Plus spectrophotometer. Cells were isolated via centrifugation and resuspended in 1 L of induction medium (in a 2-L flask) to an $OD_{600\ nm}$ equivalent to 0.4. Induced cultures were incubated at 30°C with shaking at 200 RPM for 24 h. Cells were harvested by centrifugation for 5 min at 1,500 × g and resuspended in 10 mL of lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 0.1% polyethylenimine (PEI), 30% glycerol, pH 8.0) and homogenized via an EmulsiFlex-CF homogenizer (Avestin, Ottawa, Ontario, Canada) at 25,000 psi for 20 min. Lysate was centrifuged (15,000 RPM for 30 min) in a Thermo Scientific Sorvall RC 6+ centrifuge (Thermo Scientific, Waltham, MA), and the His_6-tagged proteins were purified from the clarified supernatant by metal ion affinity chromatography using Ni-NTA resin (Qiagen, Valencia, CA). Endolysins were purified as previously described (Roach *et al.* [43]) using 40 column volumes (CV) of lysis buffer and 15 CV of wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, 30% glycerol, pH 8.0), and proteins were eluted

with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, 30% glycerol, pH 8.0). The concentration of purified proteins was determined at $OD_{280\ nm}$ using a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE), and the purity was verified via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The SDS-PAGE gels were stained with LabSafe GEL Blue (G-Biosciences, St. Louis, MO).

Zymogram and turbidity reduction analysis

Zymogram and turbidity reduction assays were conducted as described previously with minor modifications [43]. For the zymogram experiments, *L. fermentum* 0605-B44 cells were grown to mid-log phase in 350 mL MRS media, pelleted at 12,000 RPM for 5 min, washed with 10 mL of zymogram buffer (10 mM Tris, 150 mM NaCl, pH 7.5), and pelleted. Cells were resuspended in 300 μL of zymogram buffer, which led to a final volume of approximately 600 μL cells in buffer. The purified proteins and Precision Plus Protein All Blue standard (Bio-Rad) were analyzed in parallel in two 15% SDS-PAGE gels, one that contained 600 μL of *L. fermentum* cells (zymogram) and one that contained 600 μL of buffer only (in place of cells), each of which was added prior to gel polymerization. Gels were electrophoresed at 150 V until completion (1.0-1.5 h). SDS-PAGE gels were stained with LabSafe GEL Blue, and zymograms were washed in deionized (DI) water for 1 h at room temperature. After 1 h, zymograms were incubated in 50 mM Tris-HCl, 1% Triton X-114, pH 5.5 at room temperature overnight or until visible translucent bands appeared (at approximately 18 h).

Turbidity reduction assays were performed in a Benchmark Plus microplate spectrophotometer (Bio-Rad) with yeast-expressed proteins purified as described above and *E.coli*-expressed proteins purified as described previously [43]. *L. fermentum*, *L. brevis*, and *L. mucosae* were grown to mid-log phase in 350 mL MRS broth, pelleted, washed in 10 mL buffer (phosphate buffered saline (PBS; Fisher Scientific), pH 7.4, 30% glycerol), and frozen overnight at -20°C. Cell pellets were thawed on ice and resuspended to $OD_{600\ nm} = 2.0$ in PBS, pH 5.5 to a final volume of 200 μL. Each designated experimental well of a 96-well plate contained 1 μM endolysin and 100 μL of cell suspension. Control samples containing just endolysin storage buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, 30% glycerol, pH 8.0), without endolysin, were included in every run. Immediately, absorbance milli-$OD_{600\ nm}$ ($mOD_{600\ nm}$) readings were taken every 30 s for 30 min and specific activities were determined ($\Delta mOD_{600\ nm}$/min/μM) on a sliding scale as described by Becker *et al.* [46]. To control for the effect of autolysis, the rates of the control samples (cells alone) were subtracted from the rates of the experimental samples.

Preparation of yeast cultures and *Lactobacillus fermentum* inoculum for fermentations

Both untransformed and transformed yeast strains BY4727 were grown for 24 h at 30°C with shaking (200 RPM) in 100 mL of YPD media and SD_{-ura} media, respectively. Cells of untransformed yeast and transformed yeast were then isolated by centrifugation at 1,500 × *g* for 5 min. The supernatants were discarded, and the cell pellets were resuspended in a volume of either 25% galactose or DI water to obtain an $OD_{600\ nm}$ equivalent to 160. A 1-mL aliquot was then used to inoculate the fermentations (described below) to obtain a final $OD_{600\ nm}$ equivalent to 8.0.

For the experimentally infected fermentations, stock cultures of *L. fermentum* 0605-B44 were grown in 50-mL static cultures of MRS media at 37°C to mid-log phase ($OD_{600\ nm} = 0.4$-0.6). Cells were harvested by centrifugation at 8,000 RPM for 5 min and resuspended in a volume of phosphate buffered saline, pH 7.4 (PBS; Fisher Scientific), which was necessary to obtain a concentration of 9 $\log_{10}$ (CFU/mL) ($OD_{600\ nm} = 1.0$ is approximately 8.7 $\log_{10}$ (CFU/mL)). Cells were then diluted by transferring 100 μL of cells at 9 $\log_{10}$ (CFU/mL) to 10 mL of PBS to obtain a cell density of 7 $\log_{10}$ (CFU/mL).

Preparation and fermentation of corn mash

Corn mash (about 33% solids) was obtained from a commercial dry grind ethanol facility and stored at -20°C as described previously [4]. For each 20-mL shake-flask fermentation, corn mash solids were diluted to 20% solids by addition of the following components to 25-mL Erlenmeyer flasks: 12.0 mL corn mash, 6.8 mL 25% galactose (induced fermentations) or DI water (non-induced fermentations), 1.0 mL of transformed yeast resuspended in 25% galactose or untransformed yeast resuspended in 1.0 mL DI water, 0.2 mL 12% $(NH_4)_2SO_2$, and 10 μL glucoamylase (Optidex L-400; Genencor International Inc., Rochester, NY). For induced fermentations, the final concentration of galactose was 10% as described previously [47].

Each fermentation flask was inoculated with either untransformed yeast or transformed yeast (containing LysA or LysA2) (Table 1), plugged with a rubber stopper containing a 0.9 mm × 40 mm PrecisionGlide Needle (Becton Dickinson, East Rutherford, NJ) to allow for CO_2 escape, and placed in a shaking incubator set at a speed of 100 RPM and a temperature of 35°C for 3 h to acclimate the yeast. All fermentation flasks were then removed from the incubator. Fermentations that were designated to be experimentally infected were inoculated with 200 μL of 7 $\log_{10}$ (CFU/mL) *L. fermentum* cells to obtain a final density of 5 $\log_{10}$ (CFU/mL). Following infection, all fermentation flasks were returned to an incubator with shaking at 32°C and 100 RPM to begin the experiment

Table 1 Lactic acid, acetic acid, and ethanol concentrations over 72 h from experimentally infected fermentations

Treatment and yeast variant[1]		*N*	Lactic acid[2] (mg/mL ± SEM)				Acetic acid[2] (mg/mL ± SEM)				Ethanol[2] (mg/mL ± SEM)			
			0 h	24 h	48 h	72 h	0 h	24 h	48 h	72 h	0 h	24 h	48 h	72 h
Induced	LysA	4	$0.1^{a} \pm 0.0$	$0.4^{b} \pm 0.2$	$1.9^{b} \pm 0.7$	$2.7^{bc} \pm 1.0$	$0.2^{a} \pm 0.0$	$1.0^{bc} \pm 0.2$	$2.1^{cd} \pm 0.4$	$2.8^{bc} \pm 0.6$	$2.8^{a} \pm 0.8$	$22.0^{a} \pm 3.5$	$36.5^{a} \pm 2.6$	$45.4^{a} \pm 2.7$
	LysA2	4	$0.1^{a} \pm 0.0$	$0.1^{b} \pm 0.0$	$0.5^{c} \pm 0.3$	$1.7^{cd} \pm 0.6$	$0.1^{a} \pm 0.0$	$0.6^{c} \pm 0.1$	$1.4^{de} \pm 0.2$	$1.9^{cd} \pm 0.4$	$2.6^{a} \pm 0.6$	$18.0^{a} \pm 4.4$	$35.7^{a} \pm 6.6$	$41.2^{ab} \pm 7.9$
	Untransformed	4	$0.1^{a} \pm 0.0$	$0.9^{b} \pm 0.5$	$2.7^{b} \pm 0.4$	$3.4^{b} \pm 0.5$	$0.1^{a} \pm 0.0$	$1.3^{b} \pm 0.3$	$2.5^{bc} \pm 0.3$	$3.0^{b} \pm 0.3$	$3.0^{a} \pm 0.4$	$23.6^{a} \pm 3.3$	$36.9^{a} \pm 5.1$	$41.5^{ab} \pm 6.6$
Induced and yeast lysate-treated (BB)	LysA	4	$0.1^{a} \pm 0.0$	$0.1^{b} \pm 0.0$	$0.1^{c} \pm 0.0$	$0.1^{d} \pm 0.0$	$0.1^{a} \pm 0.0$	$0.8^{bc} \pm 0.2$	$1.1^{e} \pm 0.2$	$1.3^{de} \pm 0.3$	$3.3^{a} \pm 0.7$	$22.3^{a} \pm 3.8$	$36.9^{a} \pm 5.2$	$46.2^{a} \pm 7.0$
	LysA2	4	$0.1^{a} \pm 0.0$	$0.1^{b} \pm 0.0$	$0.1^{c} \pm 0.0$	$0.1^{d} \pm 0.0$	$0.1^{a} \pm 0.0$	$0.7^{bc} \pm 0.1$	$0.9^{e} \pm 0.2$	$1.0^{e} \pm 0.2$	$2.9^{a} \pm 0.8$	$20.2^{a} \pm 4.6$	$29.9^{ab} \pm 5.2$	$34.1^{ab} \pm 5.3$
	Untransformed	3	$0.1^{a} \pm 0.0$	$0.3^{b} \pm 0.2$	$2.1^{b} \pm 0.2$	$3.7^{b} \pm 0.6$	$0.1^{a} \pm 0.0$	$0.9^{bc} \pm 0.1$	$2.1^{cd} \pm 0.2$	$2.9^{bc} \pm 0.1$	$2.4^{a} \pm 0.5$	$17.1^{a} \pm 2.8$	$29.4^{ab} \pm 4.3$	$37.4^{ab} \pm 6.0$
Non-induced	LysA	3	$0.1^{a} \pm 0.0$	$3.3^{a} \pm 0.7$	$4.8^{a} \pm 0.8$	$6.5^{a} \pm 0.8$	$0.1^{a} \pm 0.0$	$2.1^{a} \pm 0.4$	$3.2^{ab} \pm 0.4$	$4.3^{a} \pm 0.4$	$2.5^{a} \pm 1.0$	$15.4^{a} \pm 2.4$	$21.0^{b} \pm 2.8$	$24.5^{b} \pm 2.7$
	LysA2	3	$0.1^{a} \pm 0.0$	$3.1^{a} \pm 0.5$	$5.5^{a} \pm 0.6$	$6.4^{a} \pm 0.8$	$0.1^{a} \pm 0.0$	$2.2^{a} \pm 0.1$	$3.7^{a} \pm 0.1$	$4.4^{a} \pm 0.2$	$2.3^{a} \pm 0.2$	$19.3^{a} \pm 2.1$	$30.2^{ab} \pm 3.5$	$33.4^{ab} \pm 4.1$
	Untransformed	4	$0.1^{a} \pm 0.0$	$2.9^{a} \pm 0.2$	$4.4^{a} \pm 0.6$	$6.1^{a} \pm 0.6$	$0.2^{a} \pm 0.1$	$2.0^{a} \pm 0.1$	$3.0^{ab} \pm 0.2$	$4.1^{a} \pm 0.3$	$2.8^{a} \pm 0.3$	$24.0^{a} \pm 1.2$	$31.5^{ab} \pm 0.7$	$35.6^{ab} \pm 2.7$

[1]Fermentations were conducted using *S. cerevisiae* strain BY4727. Induced fermentations contained 10% galactose, while non-induced fermentations were supplemented with deionized water. Experimentally infected fermentations were inoculated at time zero with 5 $\log_{10}$ (CFU/mL) *L. fermentum* 0605-B44. To prepare a yeast lysate containing LysA or LysA2, 10% of the fermentation volume was removed at 0 h and 0.5 h and was mechanically lysed (bead beating; BB) for five rounds of 30 s followed by 30 s on ice and returned to the fermentation.
[2]Concentrations (mg/mL ± SEM) of ethanol, lactic acid, and acetic acid with different superscript letters within a column are significantly different ($P < 0.05$) from each other using Student's *t*-test.

(0 h). Fermentation samples (250 μL) were removed at 0, 0.5, 1.0, 1.5, 24, 48, and 72 h and diluted 1:10 in PBS, pH 7.4 for bacterial CFU determination (serial dilution plating as described below) and quantification of glucose, galactose, lactic acid, and acetic acid using high performance liquid chromatography (HPLC) as described by Bischoff *et al.* [4]. For fermentations designated to be treated with mechanically lysed yeast (bead beating; BB), immediately following sample removal at 0 h and 0.5 h, 2 mL of the fermentation mixture was removed and subjected to five rounds of 30-s BB followed by 30 s on ice and returned to the fermentation. BB was performed at a 1:1 ratio of glass beads to corn mash using acid-washed glass beads (425-600 μm) (Sigma, St. Louis, MO) in a Mini-Beadbeater (BioSpec products, Bartlesville, OK).

Fermentation samples were titered for bacterial content by serial dilution plating on 1.5% MRS agar containing 100 μg/mL cyclohexamide (yeast inhibitor) and incubated anaerobically using the AnaeroPack System (Mitsubishi, Tokyo, Japan) at 37°C for 18 h. Plating was conducted using the Eddy Jet 2 spiral plater (IUL Instruments, Barcelona Spain) set in E mode 50, which dispenses a 50-μL sample. Colonies were counted and cell concentrations determined (CFU/mL) using the Flash & Go plate reader (IUL Instruments, Barcelona, Spain). A count of ten colonies or more was considered acceptable, and thus our minimum detection limit was determined to be 3.3 $\log_{10}$ (CFU/mL).

Western blot analysis

Protein extraction for the corn mash samples was conducted based on the method of Kushnirov [48] for western blot analysis. A fermentation sample of 250 μl was centrifuged at 1,500 × g for 5 min. The supernatant was removed, and the mash pellet was resuspended in a mixture of 250 μL DI water and 250 μL of 0.2 M NaOH, and held at room temperature for 5 min. Following incubation, the sample was centrifuged, resuspended in 250 μL of SDS sample buffer, and boiled for 3 min. The boiled sample was pelleted in an Eppendorf centrifuge 5415D (Eppendorf, Hamburg, Germany) at full speed for 2 min, and 15 μL of sample buffer was loaded onto a stain-free 12% precast polyacrylamide gel (Bio-Rad) and electrophoresed at 150 V for about 1 h. After electrophoresis, protein was transferred to a nitrocellulose membrane (Bio-Rad) in a semi-dry transfer chamber at 25 V for 20 min at room temperature. The transfer buffer was composed of 25 mM Tris, 192 mM glycine, and 20% HPLC grade methanol. The membrane was blocked in 0.5% alkali-soluble casein in Tris-buffered saline with Tween 20 (TBST; 10 mM Tris-HCl, 150 mM NaCl, 0.02% Tween 20) for 1 h at room temperature. The membrane was incubated with mouse anti-His tag (C-terminal) primary antibody (Invitrogen, Carlsbad, CA) for 1 h in 0.5% alkali-soluble casein-TBST (1:5,000). After incubation with the primary antibody, the membrane was washed three times with 0.5% alkali-soluble casein-TBST for 5 min each time. The membrane was then incubated with the secondary antibody (alkaline phosphatase conjugated anti-mouse) (Invitrogen, Carlsbad, CA) for 1 h in 0.5% alkali-soluble casein-TBST (1:5000). The membrane was washed in TBST three times for 5 min each time, and then washed in TBS (without Tween 20) once for 5 min. The membrane was incubated for 3 min with the substrate (Lumi-Phos WB; Fisher Scientific, Pittsburgh, PA) at a volume of 0.125 mL per cm^2 of membrane. LysA and LysA2 were detected and visualized through chemiluminescence by exposing the membrane in Bio-Rad's ChemiDoc XRS + imaging system.

Statistical analyses

Pairwise comparisons and correlations were performed using the statistical program JMP (version 9.0.0; SAS Institute Inc., Cary, NC). To measure significant differences, analyses of variance (ANOVA) were performed. If a significant difference ($P < 0.05$) was found with ANOVA, then Student's *t*-test was performed. The correlations were estimated by the restricted maximum likelihood (REML) method.

Results

Yeast-expressed endolysin purification and exolytic activity

His_6-tagged LysA and LysA2 endolysins purified from yeast migrated in SDS-PAGE analysis as discrete bands to their predicted molecular masses of 37.9 kDa and 41.1 kDa, respectively (Figure 2a), suggesting that they were not glycosylated during expression in yeast. A zymogram gel was run in parallel with *L. fermentum* 0605-B44 cells embedded in the polyacrylamide gel matrix (Figure 2b). A single translucent or dark band formed at the predicted molecular mass for LysA and LysA2, confirming that all lytic activity of each protein preparation resulted from the full-length endolysin and not from any minor contaminating bands (<25 kDa) that were co-purified (Figure 2b).

The relative activities of *E. coli* and yeast-purified endolysins were compared in turbidity reduction assays against different *Lactobacillus* species. Yeast and *E. coli*-expressed LysA and LysA2 effectively reduced the turbidity of log phase cells of *L. fermentum* 0605-B44, *L. brevis* 0605-48, and *L. mucosae* 0713-2 (Figure 3). In each instance, LysA showed higher activity than LysA2 and endolysins expressed from *E. coli* showed higher activity than yeast-expressed endolysins (Figure 3). Endolysins were most active against *L. fermentum* 0605-B44 and least active against *L. mucosae* 0713–2 (Figure 3). Yeast-expressed LysA lysed *L. fermentum* 0605-B44, *L. brevis* 0605-48, and

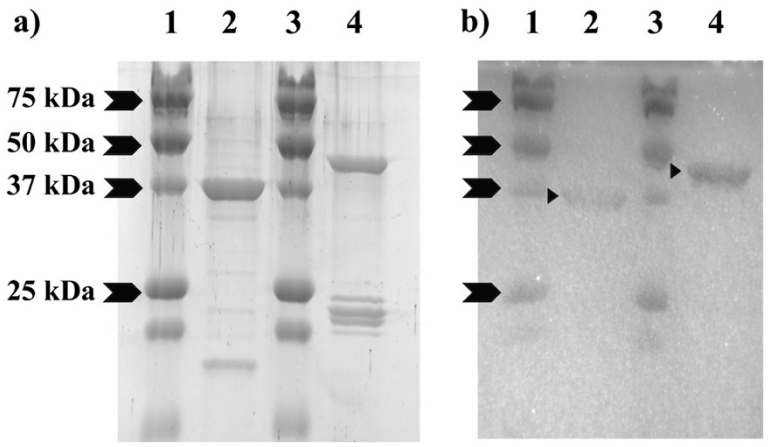

Figure 2 SDS-PAGE and zymogram analysis of purified yeast-expressed bacteriophage endolysins. (a) SDS-PAGE of yeast-expressed LysA and LysA2 purified using Ni-NTA chromatography. Lane 1, 3 Precision Plus standard; Lane 2, 2.5 μg LysA (predicted MW = 37.9 kDa); Lane 4, 2.5 μg LysA2 (predicted MW = 41.1 kDa). **(b)** Zymogram analysis with whole cells of *L. fermentum* 0605-B44 co-polymerized within the polyacrylamide gel. Endolysin exolytic activity resulted in visible clearing (dark bands indicated by black triangles) of the cell substrate at the point of protein localization, which corresponds with the predicted and observed molecular weights.

L. mucosae 0713-2 at a specific activity of 302, 241, and 194 mOD_{600}/min/μM, which was 40%, 39%, and 29% lower than the *E. coli*-expressed endolysins, respectively (Figure 3). Yeast-expressed LysA2 lysed *L. fermentum* 0605-B44, *L. brevis* 0605-48, and *L. mucosae* 0713-2 at a specific activity of 256, 119, and 116 mOD_{600}/min/μM, which was 17%, 19%, and 22% lower than the *E. coli*-expressed endolysins, respectively (Figure 3).

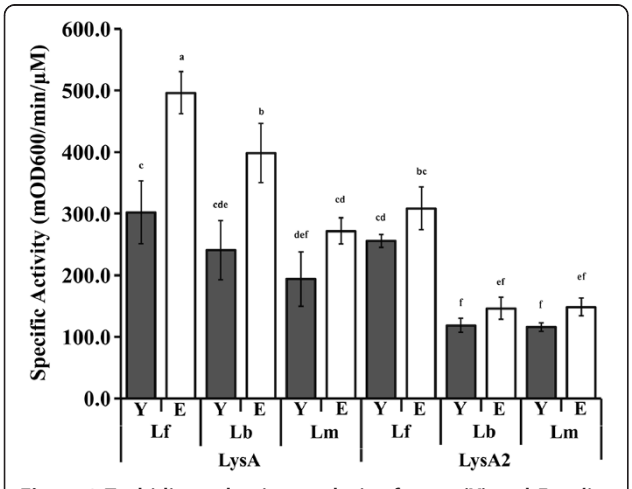

Figure 3 Turbidity reduction analysis of yeast (Y) and *E. coli* (E)-expressed LysA and LysA2. Specific activities of 1 μM endolysin were determined using mid-log phase cultures of *L. fermentum* 0605-B44 (Lf), *L. brevis* 0605-48 (Lb), and *L. mucosae* 0713-2 (Lm). To control for the effect of autolysis, rates from control samples (cells alone) were subtracted from the rates of experimental samples. Bars not connected by the same letter are significantly different ($P < 0.05$). Error bars represent the standard error of the mean (n = 3).

Expression of endolysins during fermentation reduces *Lactobacillus fermentum*

The endolysins LysA and LysA2 were evaluated for their ability to reduce the concentration of LAB in fermentations that were experimentally infected with 5 $\log_{10}$ (CFU/mL) *L. fermentum* 0605-B44 cells (Figure 4a-f). Samples were taken at early time points (0, 0.5, 1.0, and 1.5 h) (Figure 4a, c, e) and time points over three days (24, 48, and 72 h) (Figure 4b,d,f) to enumerate bacterial concentration. No bacterial colonies were observed in uninfected control fermentations (data not shown). Fermentations containing transformed yeast expressing (induced) LysA or LysA2 showed reduced bacterial concentrations at every time point relative to those containing untransformed yeast under induction conditions, transformed yeast under non-induction conditions, and untransformed yeast under non-induction conditions (Figure 4a-f). Reductions ranged from 0.5 $\log_{10}$ (CFU/mL) (1.5 h, LysA) to 1.8 $\log_{10}$ (CFU/mL) (24 h, LysA2) (Figure 4a,b).

For fermentations with yeast expressing LysA, the concentration of *L. fermentum* was significantly reduced at the early time points (0 h to 1.5 h) relative to the induced fermentations with untransformed yeast ($P < 0.05$) (Figure 4a). This trend was somewhat reduced from 24 h to 72 h, with fermentations containing yeast expressing LysA still showing lower concentrations of *L. fermentum*, but the differences were not significant ($P > 0.05$) (Figure 4b). At 1.5 h, the average reduction for yeast expressing LysA was 0.5 $\log_{10}$ (CFU/mL) and from 24 h to 72 h, average reductions ranged from 0.5 $\log_{10}$ (CFU/mL) (48 h) to 0.8 $\log_{10}$ (CFU/mL) (24 h) (Figure 4b). Fermentations containing yeast expressing LysA2 had significantly lower bacterial concentrations than induced fermentations

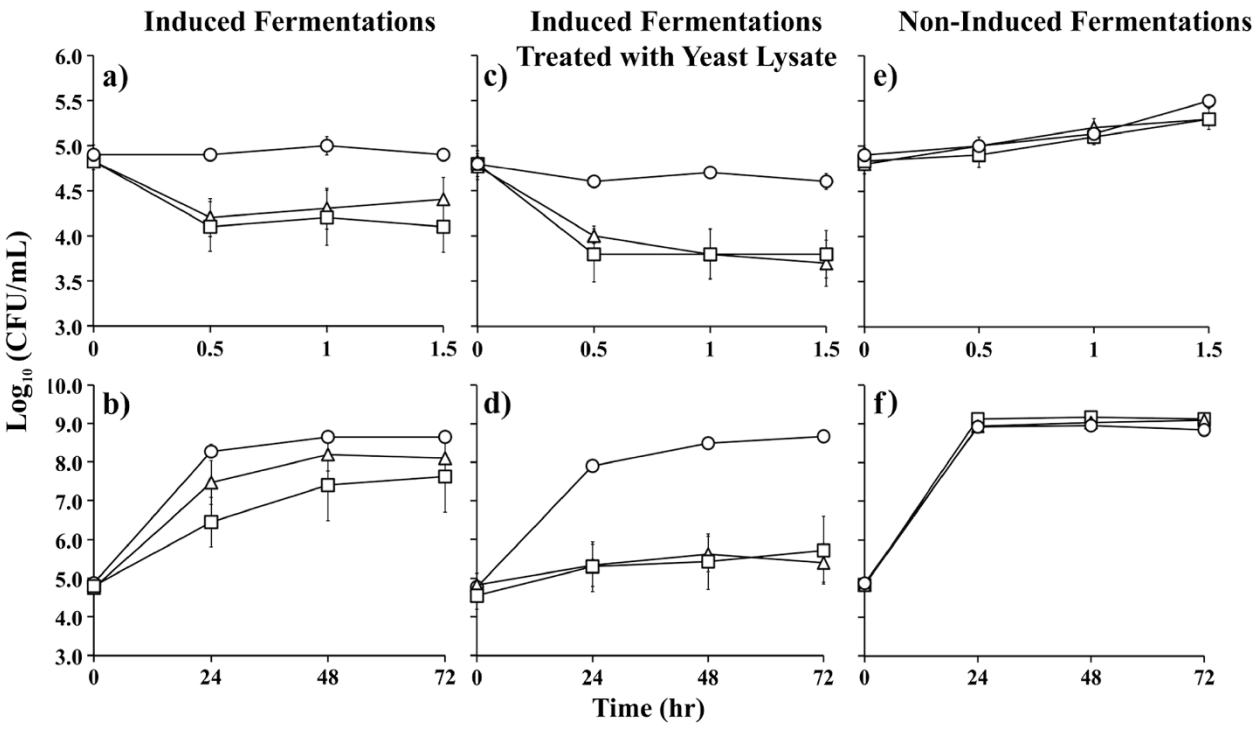

Figure 4 Reduction of *L. fermentum* during fermentation of corn mash composed of 20% solids. Fermentations were prepared with untransformed yeast (circles) or transformed yeast harboring LysA (triangles) or LysA2 (squares). The top panels **(a, c, e)** represent early time points (0 h to 1.5 h) and the bottom panels **(b, d, f)** represent long-term (72 h) fermentations. **(a-d)** Induced fermentations were induced with 6.8 mL of 25% galactose (final conc. of 10%) and **(e,f)** non-induced fermentations were treated with 6.8 mL of DI water rather than galactose. Samples of corn mash were taken at 0, 0.5, 1.0, 1.5, 24, 48, and 72 h to enumerate bacterial CFUs. **(c,d)** To prepare a yeast lysate containing LysA or LysA2, 10% of the fermentation volume (2 mL) was removed at 0 h and 0.5 h and mechanically lysed (bead beating; BB) for five rounds of 30 s followed by 30 s on ice and returned to the fermentation. Control samples included untransformed yeast under induced conditions, untransformed yeast under induced conditions and BB, and untransformed yeast under non-induced conditions.

with untransformed yeast through 24 h ($P < 0.05$) (Figure 4a,b). At 1.5 h, fermentations with yeast expressing LysA2 resulted in an average reduction of 0.8 $\log_{10}$ (CFU/mL) (Figure 4a) and from 24 h to 72 h, average reductions ranged from 0.9 $\log_{10}$ (CFU/mL) (72 h) to 1.8 $\log_{10}$ (CFU/mL) (24 h) (Figure 4b).

To augment the release of internally expressed endolysin into the fermentation medium, 10% of the fermentation volume was removed at 0 h and 0.5 h, mechanically lysed in a bead beater, and immediately returned to the culture. From 0.5 h to 72 h, the concentration of *L. fermentum* was significantly lower ($P < 0.05$) in induced fermentations containing transformed yeast expressing endolysins that were mechanically lysed than in fermentations containing untransformed yeast that were mechanically lysed (Figure 4c,d). Applying yeast lysates containing either LysA or LysA2 reduced *L. fermentum* levels in the fermentations relative to the corresponding untransformed yeast fermentations (Figure 4c,d) and demonstrated larger reductions than internal expression alone (Figure 4c, d versus 4a, b). Reductions ranged from 0.9 $\log_{10}$ (CFU/mL) (0.5 h, LysA2) to 3.3 $\log_{10}$ (CFU/mL) (72 h, LysA) (Figure 4c,d). At 1.5 h, fermentations amended with yeast lysate from transformed yeast (expressing LysA or LysA2) resulted in average reductions of 0.9 $\log_{10}$ (CFU/mL) and 0.8 $\log_{10}$ (CFU/mL), respectively (Figure 4c). From 24 h to 72 h, average reductions for LysA and LysA2 lysate-treated fermentations ranged from 2.6 $\log_{10}$ (CFU/mL) (24 h) to 3.3 $\log_{10}$ (CFU/mL) (72 h) and 2.6 $\log_{10}$ (CFU/mL) (24 h) to 3.1 $\log_{10}$ (CFU/mL) (48 h), respectively (Figure 4d).

For each time point, the effect of galactose and mechanical lysis on *L. fermentum* concentrations was evaluated with untransformed yeast fermentations. Galactose and galactose plus mechanical lysis did result in minor reductions of *L. fermentum* concentrations (Figure 4a-d) when compared to non-induced (no galactose) fermentations containing untransformed yeast and non-induced fermentations containing transformed yeast (Figure 4e-f); however, this reduction was not statistically significant ($P > 0.05$).

Western blot analysis of experimentally infected fermentations at 72 h detected the presence of LysA and LysA2 in induced fermentations, and no protein was detected in

fermentations containing transformed yeast that were not induced (Figure 5).

Expression of endolysins reduces lactic acid and acetic acid during fermentation

Experimentally infected fermentations expressing LysA or LysA2 reduced lactic acid and acetic acid levels (Table 1), and a correlation analysis revealed that the concentration of *L. fermentum* was positively correlated with the concentration of lactic acid and acetic acid ($r = 0.77$, $P < 0.0001$ and $r = 0.83$, $P < 0.0001$, respectively).

Among the infected-induced fermentations, those expressing LysA2 showed significantly reduced lactic acid at 48 h and 72 h and significantly reduced acetic acid levels at 24 h, 48 h, and 72 h ($P < 0.05$) (Table 1). Lactic acid final yields for induced fermentations with yeast expressing LysA, LysA2, and fermentations with untransformed yeast were 2.7, 1.7, and 3.4 mg/mL, respectively (Table 1). Acetic acid final yields for induced fermentations with yeast expressing LysA, LysA2, and fermentations with untransformed yeast were 2.8, 1.9, and 3.0 mg/mL, respectively (Table 1).

Induced fermentations that were treated with transformed yeast lysate contained lactic acid and acetic acid levels similar to those reported in the uninfected control fermentations (lactic acid: 0.1 mg/mL and acetic acid: ≤ 1.5 mg/mL; data not shown). The transformed yeast lysate treated fermentations demonstrated significant reductions in lactic and acetic acid at 48 h and 72 h ($P < 0.05$) (Table 1). Lactic acid final yields for lysate-treated fermentations were 0.1, 0.1, and 3.7 mg/mL, for LysA, LysA2, and untransformed yeast fermentations, respectively (Table 1). Acetic acid final yields for lysate treated fermentations were 1.3, 1.0, and 2.9 mg/mL, for LysA, LysA2, and untransformed yeast fermentations, respectively (Table 1).

Non-induced transformed yeast fermentations did not reduce lactic acid or acetic acid levels relative to the corresponding untransformed control (Table 1).

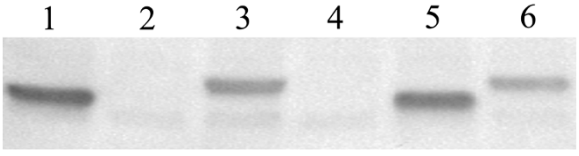

Figure 5 Representative western blot of LysA and LysA2 from experimentally infected fermentations containing transformed yeast. Fermentation samples were collected at the end of fermentation (72 h), and protein extractions were performed as described. Fermentations containing transformed yeast that were not induced served as a negative control. For analysis, 15 μL of each extract were loaded onto a 12% polyacrylamide gel. Precision Plus All Blue protein standard was used to determine relative protein size. LysA and LysA2 bands were visualized at their predicted molecular weight of 37.9 kDa and 41.1 kDa, respectively. Lane 1, fermentation containing transformed yeast expressing LysA; Lane 2, fermentation containing transformed yeast with LysA that was not induced; Lane 3, fermentation containing transformed yeast expressing LysA2; Lane 4, fermentation containing transformed yeast with LysA2 that was not induced; Lane 5, fermentation containing transformed yeast expressing LysA that was mechanically lysed; and Lane 6, fermentation containing transformed yeast expressing LysA2 that was mechanically lysed.

Ethanol concentrations in fermentations

Uninfected fermentations with untransformed yeast had ethanol concentrations that were significantly higher ($P < 0.05$) than infected non-induced fermentations with untransformed yeast at 48 h (41.6 mg/mL (data not shown) versus 31.5 mg/mL (Table 1), respectively) and 72 h (55.3 mg/mL (data not shown) versus 35.6 mg/mL (Table 1), respectively), indicating that *L. fermentum* contamination caused a stuck fermentation to occur between 24 h and 48 h. Although experimentally infected fermentations with yeast expressing LysA or LysA2 reduced the concentration of *L. fermentum* and thus lactic and acetic acid, ethanol levels were not significantly different ($P > 0.05$) from the corresponding infected-induced fermentations with untransformed yeast (Table 1). The infected-induced fermentations containing transformed yeast (expressing LysA or LysA2), including those treated with yeast lysate, had final ethanol levels that ranged from 34.1 mg/mL to 46.2 mg/mL (Table 1), and the corresponding infected-induced fermentations containing untransformed yeast had final ethanol levels of 41.5 mg/mL and 37.4 mg/mL (lysate treated) (Table 1). The uninfected fermentations containing untransformed yeast with and without galactose had ethanol levels of 55.6 mg/mL and 55.3 mg/mL, respectively (data not shown), suggesting that the ethanol yields were not altered by the addition of galactose.

Discussion

Applying enzymes (for example, alpha-amylases) to fermentations is standard procedure when preparing simultaneous saccharification and fermentation (SSF) cultures. Therefore, a potential approach to reduce bacterial contamination might be to add purified endolysins together with hydrolytic enzymes at the start of fermentation. The application of 1.0 mg purified LysA to 20 mL corn mash fermentations did not reduce bacterial contamination (results not shown), and preliminary work has suggested that purified endolysins (λSA2 and LysA) are sequestered by the corn mash solids (results not shown). Thus, the amount of enzyme needed for this approach to be successful is presently unknown and might be prohibitively expensive. We predict that endolysin expression within the fermentative yeast is much less costly than a special formulation/additive to achieve a similar result. Thus, we expressed the endolysins LysA and LysA2 in *S. cerevisiae*

during fermentation and evaluated their ability to reduce the concentration of *L. fermentum* and decrease the production of lactic acid and acetic acid in experimentally infected fermentations. Prior work by Roach *et al.* [43] demonstrated the ability of four phage encoded endolysins (LysA, LysA2, LysgaY, and λSa2), expressed and purified from *E. coli*, to lyse different species of *Lactobacillus* that were collected from fermentors at a commercial dry grind ethanol facility. The endolysins used in [43] and in this study were codon optimized for their respective host system; however, differences in protein production and processing between prokaryotic and eukaryotic organisms [49] may yield enzymes with different activities [50,51]. When expressed in yeast, LysA and LysA2 were both soluble and active (Figure 2a,b). When compared to LysA and LysA2 purified from *E. coli* in simultaneous turbidity reduction assays against *L. fermentum, L. brevis,* and *L. mucosae* (Figure 3), LysA was more active than LysA2 as previously reported [43], and the *E. coli* purified endolysins had higher specific activity than those purified from yeast (Figure 3). The small differences in the specific activities observed between yeast and *E. coli* purified proteins might be explained from the minor co-purifying contaminant proteins observed in the SDS-PAGE of the proteins purified from yeast (essentially reducing the endolysin:total protein ratio) (Figure 2), but which are absent in the *E. coli* purified proteins [43].

LysA and LysA2 were expressed in yeast without a secretion signal but nevertheless were able to reduce the *L. fermentum* concentration during fermentation (Figure 4a,b). Although the mechanism for this result is unclear, we speculate that these reductions may be a result of natural yeast cell death and subsequent release of the internally expressed endolysins. The median replicative lifespan for the average laboratory yeast strain ranges from 20 to 30 generations [52], and typically doubles after 90 min in rich media [53]. Cell death ensues via the process of apoptosis, which occurs under three different biological conditions [52]. During yeast cell lysis, alterations occur in the cell wall structure [54,55], resulting in the release of cellular components [56-58]. For example, during wine production, it is known that internal *S. cerevisiae* components are released after cell death and contribute to the sensory properties and biological stability of wine [56]. Furthermore, Hohenblum *et al.* [59] reported that at the end of fermentation with the yeast *Pichia pastoris*, 35% of the cells were dead and contamination of the supernatant with host cell proteins from lysed cells increased with increasing cell death. Additional work will need to be conducted to elucidate the exact mechanism(s) of *L. fermentum* reduction by yeast expressing endolysins (internally). However, it is known that even a single endolysin molecule (for example, one escaping from the yeast cell) should be sufficient to cleave an adequate number of bonds to lyse a bacterial cell [60].

To augment endolysin release via cell lysis, a yeast lysate was prepared from 10% of the fermentation (containing endolysin expressing yeast) at 0 h and 0.5 h during the assay and mechanically lysed. The yeast lysate was immediately returned to the fermentation and caused a significant reduction in the concentration of *L. fermentum* that was maintained throughout the remainder of the experiment (Figure 4c,d). The same effect was not observed with mechanical lysis of fermentation samples that lacked endolysin expressing yeast.

Although acetic acid and, to a lesser extent, lactic acid were present in uninfected fermentations (data not shown) [61], their levels were less than a third of those found in experimentally infected fermentations at 72 h (Table 1). When infected-induced fermentations containing untransformed yeast were compared to infected non-induced fermentations with untransformed yeast, we found that galactose-containing fermentations significantly affected lactic acid and acetic acid concentrations (Table 1). This result coincided with a reduction in *L. fermentum* concentrations in the same flasks (Figure 4). The reduced acid concentrations in these galactose-containing infected fermentations might be due to the fact that many microorganisms (such as *Lactobacillus*) prefer glucose as the primary energy and carbon source and may become stressed in the presence of uncommon sugars [62,63]. However, while an effect was seen with galactose, experimentally infected fermentations with yeast expressing LysA or LysA2 showed reduced lactic acid and acetic acid levels relative to the corresponding fermentations containing untransformed yeast under induction conditions (Table 1). Alternatives to galactose induction may include systems that require the addition of copper, methionine, and beta-estradiol, which may avoid some of the effects associated with the addition of galactose [64-66].

A measurement of ethanol levels showed that the uninfected fermentations with untransformed yeast had ethanol concentrations that were significantly higher than those of infected non-induced fermentations with untransformed yeast at 48 h and 72 h ($P < 0.05$), indicating the negative effect of *L. fermentum* on ethanol yields, and supporting the use of our shake-flask model to mimic stuck fermentation conditions experienced by industrial fermentations. Although experimentally infected fermentations with yeast expressing LysA or LysA2 reduced the concentration of *L. fermentum* and thus lactic and acetic acid, ethanol levels were not significantly different from the corresponding infected fermentations with untransformed yeast under induction conditions (Table 1). The fermentation efficiency of a given yeast strain is directly related to its stress resistance or its ability to adjust

efficiently to a dynamic environment (such as unfavorable growth conditions) and mitigate stress [62]. Our laboratory yeast strain BY4727 likely has a low stress resistance relative to industrial yeast strains, which are developed to be well adapted to fermentation conditions. For example, fermentations with the high ethanol-producing yeast strain NRRL Y-2034 had a final ethanol concentration of 112 mg/mL [4], and fermentations with the industry yeast strain Ethanol Red reached a final ethanol concentration of over 140 mg/mL [47,67]. In contrast, the concentration of ethanol produced from uninfected fermentations with our lab strain BY4727 was only about 55 mg/mL (data not shown). Thus, if endolysins were expressed in high ethanol-yielding and stress-tolerant yeast strains, differences in ethanol levels may have been observed for fermentations where bacterial contamination was reduced (fermentations with transformed yeast). We did not observe any effects of endolysin expression on ethanol production in uninfected fermentations (data not shown), but it is interesting that fermentations expressing LysA consistently had higher ethanol levels than LysA2, although not significantly, in experimentally infected fermentations (Table 1).

Employing yeast to express antimicrobial genes represents a potentially cost-effective strategy to reduce LAB contamination during batch fermentations. However, a number of issues must be addressed before this process is commercialized. First, yeast expressing endolysins internally to reduce bacterial contamination would likely require high levels of protein production that is typically observed using high copy plasmid-based expression systems. This would be suboptimal considering the high likelihood of plasmid loss under non-selective fermentation conditions. Integrating the endolysin gene into the yeast chromosome under the control of a promoter that does not require an inducing agent would be favorable for both batch and continuous fermentation systems and would ensure gene passage to all progeny yeast, but the transgene would need to be optimized for high level expression. Other strategies include secreting the endolysin out of yeast [68] or displaying the endolysin on the surface of the yeast cell wall [69]. Secretory expression of heterologous proteins in yeast is complicated with several existing technical problems, including protein misfolding, degradation by host-specific proteases, and low protein yields [68]. The secretion of heterologous proteins from yeast has been most successful with peptides, yet the secretion of large proteins has been less predictable [65]. Second, the stability of endolysins in an industrial environment and during industrial fermentation processes together with the fate of endolysins during distillers dried grains with solubles (DDGS) production will need to be examined. Initial bacterial loads have been shown to be directly correlated with decreases in final ethanol yields [9]. Thus, the stability and activity of endolysins will be most critical in the early stages of the fermentation. As for the fate of the endolysins in downstream processes, residual active enzymes would likely be denatured by the heat of drying the DDGS, which may occur at up to 232°C. Third, the use of transgenic yeast for fuel ethanol production will need to be accepted by policy makers and ethanol production facilities. The use of transgenic, commercially accepted yeast strains to produce lysates containing endolysins for treating contaminated fermentations may avoid some of the problems associated with the acceptance of using recombinant yeast during fermentation by ethanol facilities.

Conclusions

Bacteriophage endolysins are promising antimicrobial candidates for controlling LAB contamination in fuel ethanol fermentations. LysA and LysA2 purified from yeast were active against problematic lactobacilli collected from fermentors at a commercial dry grind ethanol facility including *L. fermentum*, *L. brevis*, and *L. mucosae*. When using transformed yeast expressing either LysA or LysA2 in experimentally infected small-scale corn mash fermentations, concentrations of *L. fermentum* 0605-B44 were reduced up to 1.8 $\log_{10}$ (CFU/ml), and consequently lactic acid and acetic acid levels were reduced up to 2.0 and 1.6-fold, respectively. When transformed yeast lysates were applied to experimentally infected fermentations, there were significant ($P < 0.05$) reductions in the concentration of *L. fermentum* (ranging from 0.9 $\log_{10}$ (CFU/mL) to 3.3 $\log_{10}$ (CFU/mL)), which were maintained throughout the remainder of the 72-h fermentation period. Ethanol levels achieved with our laboratory yeast strain were not comparable to ethanol levels normally achieved with industrial strains, and were not significantly different from the corresponding untransformed infected fermentations under induction conditions. Thus, stuck fermentation conditions in the mock fermentations were not mitigated. To our knowledge, this is the first report demonstrating the reduction of LAB contamination by employing yeast expressing a bacteriophage lytic enzyme during fuel ethanol production, and provides a proof of principle and basis for evaluating other antimicrobial peptides or enzymes for their potential to reduce bacterial contamination during biofuel production.

Abbreviations

ANOVA: analysis of variance; BB: bead beating; CBD: cell wall binding domain; CFU: colony-forming unit; CV: column volume; DDGS: distillers dried grains with solubles; DI: deionized water; HPLC: high performance liquid chromatography; LAB: lactic acid bacteria; OD: optical density; PBS: phosphate buffered saline; PEI: polyethylenimine; PES: polyethersulfone; PG: peptidoglycan; REML: restricted maximum likelihood; SEM: standard error of the mean; SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SSF: simultaneous saccharification and fermentation; TBS: Tris-buffered saline; TBST: Tris-buffered saline with Tween 20; YPD: yeast extract peptone dextrose.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
PAK was involved in the design of the experimental work, conducted turbidity reduction and zymogram analysis of yeast purified endolysins, performed the yeast transformation work, conducted the fermentation experiments and the analytical work involving composition of the fermentations, and was the lead writer on the manuscript. DRR was involved in the design of the experimental work, developed protocols for the purification and characterization of phage endolysins using zymogram and turbidity reduction assays, and assisted in writing and editing the manuscript. KMB, SRH, and DMD contributed to the design, coordination and assistance in all experimental work, and in the writing and editing of the manuscript. All authors read and approved the final manuscript.

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References

1. Drapcho CM, Nghiem NP, Walker TH: *Biofuels Engineering Process Technology.* New York: McGraw-Hill Companies, Inc.; 2008.
2. **U.S. ethanol production and the Renewable Fuel Standard.** http://www.eia.gov/todayinenergy/detail.cfm?id=11551.
3. Hahn-Hagerdal B, Galbe M, Gorwa-Grauslund MF, Liden G, Zacchi G: **Bio-ethanol - the fuel of tomorrow from the residues of today.** *Trends Biotechnol* 2006, **24:**549–556.
4. Bischoff KM, Liu S, Leathers TD, Worthington RE, Rich JO: **Modeling bacterial contamination of fuel ethanol fermentation.** *Biotechnol Bioeng* 2009, **103:**117–122.
5. Connolly C: **Bacterial contaminants and their effects on alcohol production.** In *The Alcohol Textbook.* 3rd edition. Edited by Jacques K, Lyons TP, Kelsall DR. Nottingham, UK: Nottingham University Press; 1997:317–334.
6. Skinner KA, Leathers TD: **Bacterial contaminants of fuel ethanol production.** *J Ind Microbiol Biotechnol* 2004, **31:**401–408.
7. Beckner M, Ivey ML, Phister TG: **Microbial contamination of fuel ethanol fermentations.** *Lett Appl Microbiol* 2011, **53:**387–394.
8. Bayrock DP, Ingledew WM: **Inhibition of yeast by lactic acid bacteria in continuous culture: nutrient depletion and/or acid toxicity?** *J Ind Microbiol Biotechnol* 2004, **31:**362–368.
9. Narendranath NV, Hynes SH, Thomas KC, Ingledew WM: **Effects of lactobacilli on yeast-catalyzed ethanol fermentations.** *Appl Environ Microbiol* 1997, **63:**4158–4163.
10. Pampulha M, Loureiro-Dias M: **Combined effect of acetic acid, pH and ethanol on intracellular pH of fermenting yeast.** *Appl Microbiol Biotechnol* 1989, **31:**547–550.
11. Narendranath NV, Thomas KC, Ingledew WM: **Effects of acetic acid and lactic acid on the growth of *Saccharomyces cerevisiae* in a minimal medium.** *J Ind Microbiol Biotechnol* 2001, **26:**171–177.
12. Narendranath NV, Thomas KC, Ingledew WM: **Acetic acid and lactic acid inhibition of growth of *Saccharomyces cerevisiae* by different mechanisms.** *Am Soc Brewing Chem* 2001, **59:**187–194.
13. Thomas KC, Hynes SH, Ingledew WM: **Influence of medium buffering capacity on inhibition of *Saccharomyces cerevisiae* growth by acetic and lactic acids.** *Appl Environ Microbiol* 2002, **68:**1616–1623.
14. Schnurer J, Magnusson J: **Antifungal lactic acid bacteria as biopreservatives.** *Trends Food Sci Tech* 2005, **16:**70–78.
15. Burtner CR, Murakami CJ, Kennedy BK, Kaeberlein M: **A molecular mechanism of chronological aging in yeast.** *Cell Cycle* 2009, **8:**1256–1270.
16. Makanjuola DB, Tymon A, Springham DG: **Some effects of lactic-acid bacteria on laboratory-scale yeast fermentations.** *Enzyme Microb Technol* 1992, **14:**350–357.
17. Bischoff KM, Skinner-Nemec KA, Leathers TD: **Antimicrobial susceptibility of *Lactobacillus* species isolated from commercial ethanol plants.** *J Ind Microbiol Biotechnol* 2007, **34:**739–744.
18. Schell DJ, Dowe N, Ibsen KN, Riley CJ, Ruth MF, Lumpkin RE: **Contaminant occurrence, identification and control in a pilot-scale corn fiber to ethanol conversion process.** *Bioresour Technol* 2007, **98:**2942–2948.
19. Skinner-Nemec KA, Nichols NN, Leathers TD: **Biofilm formation by bacterial contaminants of fuel ethanol production.** *Biotechnol Lett* 2007, **29:**379–383.
20. Muthaiyan A, Limayem A, Ricke SC: **Antimicrobial strategies for limiting bacterial contaminants in fuel bioethanol fermentations.** *Prog Energy Combust Sci* 2011, **37:**351–370.
21. Cunningham S, Stewart GG: **Effects of high-gravity brewing and acid washing on brewers' yeast.** *Am Soc Brewing Chem* 1989, **56:**12–18.
22. Bamforth CW: **pH in Brewing: an overview.** *MBAA Tech Quart* 2001, **38:**1–9.
23. Meneghin SP, Reis FC, de Almeida PG, Ceccato-Antonini SR: **Chlorine dioxide against bacteria and yeasts from the alcoholic fermentation.** *Braz J Microbiol* 2008, **39:**337–343.
24. Suzuki K, Lijima K, Sakamoto K, Sami M, Yamashita H: **A review of hop resistance in beer spoilage lactic acid bacteria.** *J Ins Brewing* 2006, **112:**173–191.
25. Lushia W, Heist P: **Antibiotic resistant bacteria in fuel ethanol fermentations.** *Ethanol Producer Mag* 2005, 80–82.
26. Bayrock DP, Thomas KC, Ingledew WM: **Control of *Lactobacillus* contaminants in continuous fuel ethanol fermentations by constant or pulsed addition of penicillin G.** *Appl Microbiol Biotechnol* 2003, **62:**498–502.
27. Stroppa CT, Andrietta MGS, Andrietta SR, Steckelberg C, Serra G: **Use of penicillin and monensin to control bacterial contamination of Brazilian alcohol fermentation.** *Int Sugar J* 2000, **102:**78–82.
28. Demeester C, Rondelet J: **Microbial acetylation of M-factor of virginiamycin.** *J Antibiot* 1976, **29:**1297–1305.
29. Sakamoto K, Margolles A, van Veen HW, Konings WN: **Hop resistance in the beer spoilage bacterium *Lactobacillus brevis* is mediated by the ATP-binding cassette multidrug transporter HorA.** *J Bacteriol* 2001, **183:**5371–5375.
30. Nelson DC, Schmelcher M, Rodriguez L, Klumpp J, Pritchard DG, Dong S, Donovan DM: **Endolysins as antimicrobials.** In *Advances in Virus Research: Bacteriophages, Part B.* Edited by Lobocka M, Szybalski WT. Waltham, MA: Elsevier, Inc; 2012:299–365.
31. Shen Y, Mitchell MS, Donovan DM, Nelson DC: **15 Phage-based enzybiotics.** In *Bacteriophages in Health and Disease.* Edited by Hyman P, Abedon ST. UK: CAB International; 2012:217–239.
32. Schmelcher M, Donovan DM, Loessner MJ: **Bacteriophage endolysins as novel antimicrobials.** *Future Microbiol* 2012, **7:**1147–1171.
33. Bernhardt TG, Wang IN, Struck DK, Young R: **Breaking free: "protein antibiotics" and phage lysis.** *Res Microbiol* 2002, **153:**493–501.
34. Schleifer KH, Kandler O: **Peptidoglycan types of bacterial cell walls and their taxonomic implications.** *Bacteriol Rev* 1972, **36:**407–477.
35. Borysowski J, Weber-Dabrowska B, Gorski A: **Bacteriophage endolysins as a novel class of antibacterial agents.** *Exp Biol Med* 2005, **231:**366–377.
36. Diaz E, Lopez R, Garcia JL: **Chimeric phage-bacterial enzymes: a clue to the modular evolution of genes.** *Proc Natl Acad Sci Unit States Am* 1990, **87:**8125–8129.
37. Sheehan MM, Garcia JL, Lopez R, Garcia P: **The lytic enzyme of the pneumococcal phage Dp-1: a chimeric lysin of intergeneric origin.** *Mol Microbiol* 1997, **25:**717–725.

38. Bischoff KM, Poole TL, Beier RC: **Antimicrobial resistance in food animals.** In *Preharvest and Postharvest Food Safety: Contemporary Issues and Future Directions.* Edited by Beier RC, Pillai SD, Phillips TD, Ziprin RL. Ames IA: Blackwell Publishing Professional; 2004:201–212.
39. Dequin S: **The potential of genetic engineering for improving brewing, wine-making and baking yeasts.** *Appl Microbiol Biotechnol* 2001, **56:**577–588.
40. Pretorius IS: **Tailoring wine yeast for the new millennium: novel approaches to the ancient art of winemaking.** *Yeast* 2000, **16:**675–729.
41. Sasaki T, Watari J, Kohgo M, Nishikiawa N, Matsui Y: **Breeding of a brewer's yeast possessing anticontaminant properties.** *J Ame Soc Brewing Chem* 1984, **42:**164–166.
42. Schoeman H, Vivier MA, Du Toit M, Dicks LMT, Pretorius IS: **The development of bactericidal yeast strains by expressing the *Pediococcus acidilactici* pediocin gene (*pedA*) in *Saccharomyces cerevisiae*.** *Yeast* 1999, **15:**647–656.
43. Roach DR, Khatibi PA, Bischoff KM, Hughes SR, Donovan DM: **Bacteriophage-encoded lytic enzymes control growth of contaminating *Lactobacillus* found in fuel ethanol fermentations.** *Biotechnol Biofuels* 2013, **6:**20.
44. Brachmann CB, Davies A, Cost GJ, Caputo E, Li J, Hieter P, Boeke JD: **Designer deletion strains derived from *Saccharomyces cerevisiae* S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications.** *Yeast* 1998, **14:**115–132.
45. Khatibi PA, Newmister SA, Rayment I, McCormick SP, Alexander NJ, Schmale DG 3rd: **Bioprospecting for trichothecene 3-O-acetyltransferases in the fungal genus *Fusarium* yields functional enzymes with different abilities to modify the mycotoxin deoxynivalenol.** *Appl Environ Microbiol* 2011, **77:**1162–1170.
46. Becker SC, Foster-Frey J, Donovan DM: **The phage K lytic enzyme LysK and lysostaphin act synergistically to kill MRSA.** *FEMS Microbiol Lett* 2008, **287:**185–191.
47. Khatibi PA, Montanti J, Nghiem NP, Hicks KB, Berger G, Brooks WS, Griffey CA, Schmale DG 3rd: **Conversion of deoxynivalenol to 3-acetyldeoxynivalenol in barley-derived fuel ethanol co-products with yeast expressing trichothecene 3-O-acetyltransferases.** *Biotechnol Biofuels* 2011, **4:**26.
48. Kushnirov VV: **Rapid and reliable protein extraction from yeast.** *Yeast* 2000, **16:**857–860.
49. Frommer WB, Ninnemann O: **Heterologous expression of genes in bacterial, fungal, animal, and plant cells.** *Annu Rev Plant Physiol Plant Mol Biol* 1995, **46:**419–444.
50. Dorr RT: **Clinical properties of yeast-derived versus *Escherichia coli*-derived granulocyte-macrophage colony-stimulating factor.** *Clin Ther* 1993, **15:**19–29. discussion 18.
51. Cohen JD, Abrams E, Eccleshall TR, Buchferer B, Marmur J: **Expression of a prokaryotic gene in yeast: isolation and characterization of mutants with increased expression.** *Mol Gen Genet* 1983, **191:**451–459.
52. Laun P, Heeren G, Rinnerthaler M, Rid R, Kossler S, Koller L, Breitenbach M: **Senescence and apoptosis in yeast mother cell-specific aging and in higher cells: a short review.** *Biochim Biophys Acta* 2008, **1783:**1328–1334.
53. Bergman LW: **Growth and maintenance of yeast.** In *Methods in Molecular Biology*, Volume 177. Edited by MacDonald PN. Totowa, NJ: Humana Press Inc; 2001:9–14.
54. Troton D, Charpentier B, Robillard B, Calvayrac R, Duteurtre B: **Evolution of the lipid contents of Champagne wine during the second fermentation of *Saccharomyces cerevisiae*.** *Am J Enol Viticult* 1989, **40:**175–182.
55. Charpentier C, Van Long TN, Bonaly R, Feuillat M: **Alteration of cell wall structure in *Saccharomyces cerevisiae* and *Saccharomyces bayanus* during autolysis.** *Appl Microbiol Biotechnol* 1986, **24:**405–413.
56. Perrot L, Charpentier M, Charpentier C, Feuillat M, Chassagne D: **Yeast adapted to wine: nitrogen compounds released during induced autolysis in a model wine.** *J Ind Microbiol Biotechnol* 2002, **29:**134–139.
57. Herker E, Jungwirth H, Lehmann KA, Maldener C, Frohlich KU, Wissing S, Buttner S, Fehr M, Sigrist S, Madeo F: **Chronological aging leads to apoptosis in yeast.** *J Cell Biol* 2004, **164:**501–507.
58. Silva MT: **Secondary necrosis: the natural outcome of the complete apoptotic program.** *FEBS Lett* 2010, **584:**4491–4499.
59. Hohenblum H, Borth N, Mattanovich D: **Assessing viability and cell-associated product of recombinant protein producing *Pichia pastoris* with flow cytometry.** *J Biotechnol* 2003, **102:**281–290.
60. Fischetti VA: **Bacteriophage endolysins: a novel anti-infective to control Gram-positive pathogens.** *Int J Med Microbiol* 2010, **300:**357–362.
61. Maiorella B, Blanch HW, Wilke CR: **By-product inhibition effects on ethanolic fermentation by *Saccharomyces cerevisiae*.** *Biotechnol Bioeng* 1983, **25:**103–121.
62. Bauer FF, Pretorius IS: **Yeast stress response and fermentation efficiency: how to survive the making of wine - a review.** *South Afr J Enol Viticult* 2000, **21:**27–51.
63. Bettenbrock K, Alpert CA: **The *gal* genes for the Leloir pathway of *Lactobacillus casei* 64H.** *Appl Environ Microbiol* 1998, **64:**2013–2019.
64. Labbe S, Thiele DJ: **Copper ion inducible and repressible promoter systems in yeast.** *Methods Enzymol* 1999, **306:**145–153.
65. Romanos MA, Scorer CA, Clare JJ: **Foreign gene expression in yeast: a review.** *Yeast* 1992, **8:**423–488.
66. McIsaac RS, Silverman SJ, McClean MN, Gibney PA, Macinskas J, Hickman MJ, Petti AA, Botstein D: **Fast-acting and nearly gratuitous induction of gene expression and protein depletion in *Saccharomyces cerevisiae*.** *Mol Biol Cell* 2011, **22:**4447–4459.
67. Nghiem N, Hicks K, Johnston D, Senske G, Kurantz M, Li M, Shetty J, Konieczny-Janda G: **Production of ethanol from winter barley by the EDGE (enhanced dry grind enzymatic) process.** *Biotechnol Biofuels* 2010, **3:**8.
68. Idiris A, Tohda H, Kumagai H, Takegawa K: **Engineering of protein secretion in yeast: strategies and impact on protein production.** *Appl Microbiol Biotechnol* 2010, **86:**403–417.
69. Ueda M, Tanaka A: **Cell surface engineering of yeast: construction of arming yeast with biocatalyst.** *J Biosci Bioeng* 2000, **90:**125–136.

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