Competitive exclusion is a major bioprotective mechanism of lactobacilli against fungal spoilage in fermented milk products

Solvej Siedlera*, Martin Holm Raua*, Susanne Bidstrupa, Justin M. Ventob,
Stina Dissing Aunsbjergc, Elleke F. Bosmaa, Laura M. McNaird, Chase L. Beiselb,e,f, Ana Rute Nevesa

a Discovery, R&D, Chr. Hansen A/S, Bøge Allé 10-12, 2970 Hørsholm, Denmark
b Department of Chemical and Biomolecular Engineering, North Carolina State University,
c Global Application, Chr. Hansen A/S, Bøge Allé 10-12, 2970 Hørsholm, Denmark
d Department of Drug Design and Pharmacology, University of Copenhagen,
e Helmholtz Institute for RNA-based Infection Research (HIRI), Helmholtz Center for Infection Research, Würzburg, Germany
f Medical Faculty, University of Würzburg, Würzburg, Germany

Running Head: Competitive exclusion by lactobacilli

#Address correspondence to S.S.
*S. S. and M. H. R. contributed equally to this work. Author order was chosen as is, because S. S. was the project leader.
ABSTRACT

A prominent feature of lactic acid bacteria (LAB) is their ability to inhibit growth of spoilage organisms in food, but hitherto research efforts to establish the mechanisms underlying bioactivity focused on the production of antimicrobial compounds by LAB. We show in this study, that competitive exclusion, i.e., competition for a limited resource by different organisms, is a major mechanism of fungal growth inhibition by lactobacilli in fermented dairy products. The depletion of the essential trace element manganese by two Lactobacillus species was uncovered as the main mechanism for growth inhibition of dairy spoilage yeast and molds. A manganese transporter (MntH1), representing one of the highest expressed gene products in both lactobacilli, facilitates the exhaustive manganese scavenging. Expression of the mntH1 gene was found to be strain-dependent, affected by species co-culturing and growth phase. Further, deletion of the mntH1 gene in one of the strains resulted in loss of bioactivity, proving this gene to be important for manganese depletion. The presence of a mntH gene displayed a distinct phylogenetic pattern within the Lactobacillus genus. Moreover, assaying the bioprotective ability in fermented milk of selected lactobacilli from ten major phylogenetic groups identified a correlation between the presence of mntH and bioprotective activity. Thus, manganese scavenging emerges as a common trait within the Lactobacillus genus, but differences in expression result in some strains showing more bioprotective effect than others.

In summary, competitive exclusion through ion depletion is herein reported a novel mechanism in LAB to delay growth of spoilage contaminants in dairy products.
IMPORTANCE
In societies that have food choices, conscious consumers demand natural solutions to keep their food healthy and fresh during storage, simultaneously reducing food waste. The use of “good bacteria” to protect food against spoilage organisms has a long successful history, even though the molecular mechanisms are not fully understood. In this study, we show that depletion of free manganese is a major bioprotective mechanism of lactobacilli in dairy products. High manganese uptake and intracellular storage provides a link to the distinct non-enzymatic manganese catalyzed oxidative stress defense mechanism, previously described for certain lactobacilli. The evaluation of representative Lactobacillus species in our study identifies multiple relevant species groups for fungal growth inhibition via manganese depletion. Hence, through the natural mechanism of nutrient depletion, the use of dedicated bioprotective lactobacilli constitutes an attractive alternative to artificial preservation.

KEYWORDS Lactic acid bacteria, bioprotection, food spoilage, manganese starvation, genome editing, Lactobacillus
Introduction

A growing demand for healthy and fresh foods without added artificial preservatives is increasing the need for natural, microbial solutions. Among microbes, lactic acid bacteria (LAB) have a long history in protecting food from spoilage and, today, bioprotective bacteria that specifically inhibit the growth of spoilage organisms have emerged as an alternative way of keeping food fresh for longer periods of time (1).

More than 200 species are covered by the exceptionally diverse genus Lactobacillus, with other genera such as Pediococcus phylogenetically intermixed (2). Believed to originally be free-living, lactobacilli today also include numerous host-adapted species, with habitats in livestock feed, plants, animals, humans and fermented foods (3). Several species have a GRAS status and are applied within the dairy industry for production of fermented milk products, and certain members are widely applied as probiotics, among these especially Lb. rhamnosus and Lb. paracasei (4, 5). These two species display a more nomadic lifestyle capable of colonizing the human and animal gastrointestinal tract, while also displaying efficient growth in milk.

In dairy products, spoilage by mold and yeast cells is one of the major problems that reduces shelf life (6). As a nutrient-rich environment, yoghurt is inherently susceptible to microbial spoilage, although certain characteristics narrow the number of potential detrimental organisms. The innate carbon source is lactose, free amino acids are scarce and instead concentrated in caseins, which require proteolysis for liberation, and the starter cultures responsible for conversion of milk to yoghurt lower the pH to around 4.5. The typically observed spoilage organisms consist of various yeast such as
Debaryomyces hansenii, Saccharomyces cerevisiae and several others, in addition to molds of especially the Penicillium genus (6).

In the past decade, the bioprotective potential of LAB has spurred considerable efforts in the scientific community to identify new strains with bioprotective properties from various food sources (7–9) as well as attempts to elucidate the mechanisms behind the observed bioactivity (10–12). Numerous metabolites produced by LAB have been identified as having antifungal and antibacterial activities (13), although in situ concentrations are typically significantly below minimal inhibitory concentrations (14). Meanwhile, other mechanisms have until now been largely unexplored.

Competitive exclusion is a widespread phenomenon in nature and includes the competition for nutrients such as a carbon source (15, 16), for a physical space, such as in the gastro-intestinal tract (17), as well as for essential ions. The concept of nutritional immunity in the human body (18) is an example of competition for essential ions, reinforced by the various intricate means of iron scavenging through the action of specific siderophores present in bacterial pathogens (19).

Manganese (Mn) is an essential trace element that is a key cofactor in all kingdoms of life, making it important for growth of bacteria, yeast and mold (20). The two major manganese uptake systems in LAB are the NRAMP-type transporter MntH and the ABC transporter SitABC. While the ABC transporter is mainly active at neutral pH, the proton driven symporter MntH is the major transport system under acidic conditions (21).

In E. coli, manganese is a critical co-factor for the superoxide dismutase (SOD) protein (22). Aerotolerant lactic acid bacteria generally lack an active SOD gene and cope with reactive oxygen species through an alternative mechanism which mainly has been studied in Lactobacillus plantarum (23). In this bacterium, intracellular manganese(II) accumulated
in excessively high concentrations of up to 25 mM manganese scavenges oxygen radicals \((O_2^-)\) as effectively as SOD (23). This defense mechanism has been shown for other lactobacilli species (e.g. \textit{Lb. casei} and \textit{Lb. fermentum}) but is not present in all members of the genus (e.g. \textit{Lb. bulgaricus} or \textit{Lb. acidophilus}) (23). The manganese(II) in \textit{Lb. plantarum} primarily associates with a large complex of nondialyzable polyphosphate-protein aggregates (24). The non-enzymatic manganese protection against superoxide \emph{in vivo} was described in the early 1980s (23), but the mechanism was shown only recently \emph{in vitro} (25). So far, reports of the mechanism in other bacteria are limited although in some organisms it could serve as auxiliary protection (26). High intracellular manganese(II) concentration has for example previously been associated with other stress defense mechanisms, such as radiation resistance (27).

In this study, we show that the competition for manganese is a major limiting factor for growth of spoilage organisms in yoghurt containing a bioprotective culture of \textit{Lb. paracasei} and \textit{Lb. rhamnosus}. Furthermore, we investigated the bioprotective potential of the \textit{Lactobacillus} genus based on this novel competition mechanism.

\section*{RESULTS}
\textbf{Manganese is the limiting factor for yeast growth in yoghurt containing a bioprotective culture}

To analyze the activity of the bioprotective culture in yoghurt in an easy and high-throughput manner, we developed an assay to determine the bioactivity based on yeast growth measured by absorbance at 600 nm. For this, we centrifuged the yoghurt and filtered the supernatant to obtain a clear aqueous phase (AQ) of the yoghurt. The AQ was
inoculated with yeast cells and the yeast growth was detected by absorbance measurement in a microplate reader after several days of incubation at 17°C. Analysis of *D. hansenii* growth by enumeration of cells was comparable in yogurt and AQ. The AQ of the reference (REF AQ) reached slightly lower cell counts possibly due to a lower amount of proteins in the AQ (supplemental Figure S1). The yeast *D. hansenii* was chosen as the reference organism, as it is a common spoilage organism and is sensitive to bioprotective cultures in yoghurt (12).

Here, the yoghurt bioprotective culture consists of an *Lb. rhamnosus* strain and an *Lb. paracasei* strain, collectively designated as BioP. A significant difference between *D. hansenii* growth in AQ of yoghurt with (BioP AQ) and without (REF AQ) bioprotective culture was detected (p<0.0001, n=6, t=9.292, df=10; Student’s t-test, two-tailed) (Fig. 1). Moreover, we noticed a difference in the bioactivity upon 10-50 % (not 80 %) dilution of BioP AQ with either tap water or MilliQ water (p<0.0001, n=3), while similar growth reduction was seen when diluting the REF AQ, i.e. no significant difference between *D. hansenii* growth levels in REF AQ when diluted 10-80 % with tap water or MilliQ water (p>0.05, n=3; df=32, 2-way ANOVA; Fig. 1A). The decreased yeast growth in MilliQ diluted samples suggests a dilution of a bioactive compound, but the results using tap water point to a lack of minerals in the BioP sample that can be replenished by tap water. To test which factor could be limiting, we added different metals to BioP AQ, which could be present in tap water, but filtered out in MilliQ water. In our assay, the added metal ion concentrations were in the same range as those present in milk (28, 29). Of all tested metals, only addition of manganese resulted in growth of *D. hansenii* that was comparable to growth in REF AQ (Fig. 1B). In particular, the growth in BioP AQ (n=5) and BioP AQ supplemented with iron, cupper, zink or magnesium ions (n=2-5) was not significantly
different (p>0.05), whereas the growth in BioP AQ and BioP AQ supplemented with manganese ions (n=4) was significantly different (p<0.0001, df=19, one-way ANOVA). This indicates availability of manganese ions as the sole limiting factor in the BioP AQ. We investigated the minimal concentration needed to promote growth of two different D. hansenii strains in BioP AQ (Supplemental Fig. S2) and in chemically defined medium (Supplemental Fig. S3), and we identified a threshold of ~0.01 mg/L under both conditions. Furthermore, we measured the manganese concentration in the AQs, which was 0.03 mg/L in REF AQ and below the quantification limit of 0.003 mg/L in BioP AQ.

Inhibition of different yeast and mold strains

After investigating the effect of manganese on growth of D. hansenii in BioP AQ, we analyzed the impact of manganese on different yeast strains (Table 1). All yeast strains comprise food isolates that are relevant for food spoilage. The growth of all tested yeast strains was inhibited in the BioP AQ compared to REF AQ (#: p<0.0001, n=3-9, df=10-23, one-way ANOVA; Fig. 2). Addition of manganese ions fully restored the growth of S. cerevisiae and Rhodoturola mucilaginosa in BioP AQ. In particular, no significant difference was detected between REF AQ and BioP AQ supplemented with manganese ions regarding the growth of R. mucilaginosa (p=0.0990) and S. cerevisiae (p=0.9860). In contrast, the growth of Cryptococcus fragi cola, Torulaspora delbrueckii, and the two D. hansenii strains was significantly different in BioP AQ supplemented with manganese ions in comparison with REF AQ (#: p<0.05 in Fig. 2). Nevertheless, significant differences were also observed between growth levels of these spoilage yeasts in BioP AQ with and without added manganese ions added (*: p<0.05 in Fig. 2), supporting the view that lack of free manganese ions in BioP AQ underlies the majority of its bioactivity. D. hansenii 1
growth was not significantly different between REF AQ and REF AQ with manganese addition (p-value 0.1112) or REF AQ and BioP AQ both supplemented with manganese (p-value 0.4442) (Supplemental Figure S4). We also tested the *D. hansenii* type strain (ATCC 36239), but the experiments with this strain were hampered due to its inability to grow in REF AQ. This exemplifies the need for relevant food isolates as many of the strains intended for academic use have lost phenotypic traits that are key for industrial applications.

After showing the effect of limited manganese availability on yeast growth, we tested the effect of manganese on the growth of three different spoilage molds from the family *Penicillium*. These strains display various levels of sensitivity to bioprotective cultures in yoghurt. Mold spores were spotted on the top of agar-solidified yoghurt with and without bioprotective culture and the plates incubated for 8 days at room temperature. The sample with the bioprotective culture showed clear inhibition of the mold growth compared to the reference. Addition of increasing manganese concentrations of up to 6 mg/L correlated positively with mold growth (Fig. 3).

**Identification of bioprotective-relevant genes by transcriptomics**

As different mechanisms of the observed manganese depletion could be hypothesized, for instance through sequestration or uptake, we performed global gene expression profiles of the two *Lactobacillus* strains with bioprotective properties to examine the underlying mechanism. Experiments were performed in milk, but the starter culture required for yoghurt production was omitted, as the relative abundance of BioP strain transcripts was comparatively marginal in yoghurt. The depicted gene count distribution (Fig. 4A) of the
co-cultured BioP strains provides an example of the large count range that is typical of RNAseq data (30), here more than five orders of magnitude. For both strains, among the most-expressed genes was an mntH manganese transporter encoding gene (mntH1), remarkably displaying fifth and seventh highest read counts of all genes in the Lb. rhamnosus and Lb. paracasei, respectively. mntH1 expression was magnitudes above the median (>100 fold) and putatively constitutes up to 1.8% of all transcripts. Such high expression, as judged from gene counts, of a manganese transporter gene is atypical. Other genes among the top-10 expressed relate to well-established highly expressed functions such as glycolysis or translation. Apart from the specified mntH1 gene, the genomes of Lb. rhamnosus and Lb. paracasei harbor one and two additional mntH homologs (mntH2, mntH3), respectively. The two specified mntH1 genes are orthologs and are in both species the most distal mntH gene to the origin of replication in the clockwise direction. For the additional mntH paralogs the expression is more than 200-fold lower; hence their products are unlikely to contribute significantly to manganese uptake. Additionally, both genomes harbor the ABC manganese transport system (sitABD) with 20- and 10-fold lower expression than the key mntH1 gene, respectively in Lb. rhamnosus and Lb. paracasei (Supplemental Table S1).

The temporal mntH1 expression (Fig. 4B) revealed a marked increase from 4 to 6 hours of milk fermentation (FDR < 10^{-37}), and with the expression remaining high during cold storage (400-500 times above median transcript levels), which indicates continued manganese uptake capacity even during product shelf life. This correlates with an increase in bioactivity during storage (Supplemental Fig. S5). Apart from temporal expression changes, mntH1 expression also seems to be a function of strain genotype and co-culturing (Fig. 4C). Culturing strains individually led to lower mntH1 expression for both
Lactobacillus strains as compared to co-culturing. Apart from co-culturing, the effect of genotype on mntH1 expression seems to be correlated to bioprotective activity. Lb. paracasei Strain C for instance was included based on its lower bioprotective activity (data not shown) and in fact displays a markedly lower mntH1 expression level (20-fold) than Strain B, the corresponding BioP Lb. paracasei (Fig. 4C). In yoghurt, two starter culture species (Streptococcus thermophilus and Lb. delbrueckii) are ordinarily present. Of the two, only S. thermophilus also harbors an mntH gene. The relative expression of this gene in a representative strain is around 25-fold lower than the BioP strains (data not shown) and thereby comparable to Lb. paracasei strain C.

MntH1 is essential for bioprotective activity

As the expression data provided strong indications of the importance of MntH1 for manganese uptake and the associated bioprotective phenotype, we sought to verify this through deletion of the mntH1 gene in the BioP Lb. paracasei Strain B using a homologous recombination strategy coupled with CRISPR-Cas9-mediated mutant selection (Supplemental Fig. S6). The bioprotective ability of the ΔmntH1 strain was evaluated by assessing yeast growth in AQ of milk fermented by individual strains (Fig. 5). While the milk fermentation profile was identical for the WT BioP Strain B and its ΔmntH1 mutant (Supplemental Fig. S7), yeast growth was only inhibited in AQ of milk fermented by the WT BioP Strain B and not by the corresponding ΔmntH1 mutant. Addition of 0.6 mg/L manganese resulted in restored yeast growth for WT BioP Strain B, comparable to the growth in AQ of milk fermented by the corresponding ΔmntH1 mutant with and without manganese addition. Complementation of the ΔmntH1 mutant with a plasmid containing the mntH1 gene under its own promoter fully restored bioactivity, while an empty plasmid
control did not (Supplemental Fig. S8). These results provide further evidence that mntH1 is responsible for the bioprotective phenotype. Culturing strains individually for a longer time in this experiment provides the strains with an increased potential for manganese uptake, compared to the co-culture with starter culture normally performed for yoghurt production. Even so, yeast inhibition by manganese uptake is insufficient in the mntH1 strain to inhibit the growth of *D. hansenii*, despite the existence of three alternative manganese transporters within the genome.

**Correlation between bioprotective activity and MntH1 phylogenetic distribution**

As the MntH1 transporter seems to be heavily involved in the increased uptake of manganese in certain lactobacilli, we investigated the presence of any mntH homologs in different species of the genus *Lactobacillus* (Supplemental Fig. S9). In total 18 strains were selected from 15 species, covering the major phylogenetic groups of the *Lactobacillus sensu lato* species tree (3). Of these, 15 strains harbored an mntH homolog while 3 strains did not. In brief, a phylogenetic basis seems to exist for the distribution of the mntH gene among the lactobacilli. For the phylogenetic group containing the dairy associated species, *Lb. delbrueckii* subsp. *bulgaricus*, *Lb. gasseri* and *Lb. helveticus*, a homolog is absent. In contrast, in representatives of the nine other studied groups, it is present. The correlation between mntH presence and yeast inhibition was subsequently investigated experimentally. We performed milk acidification with a starter culture in the presence of the different lactobacilli. The fermented milk was divided in two samples and 6 mg/L manganese was added to one sample, while the other sample was left unchanged. A bioactivity assay following *D. hansenii* growth in both conditions was performed (Fig. 6). The majority of the tested strains containing an mntH gene were bioactive, at varying levels, while those without the gene were not bioactive. The presence of the manganese
ABC transporter did not correlate better with activity, as it is only present in *Lb. rhamnosus*, *Lb. casei*, *Lb. paracasei* and *Lb. plantarum*. Addition of manganese restored the growth of *D. hansenii* completely in all samples, proving this metal to be the limiting factor.

**DISCUSSION**

In this study we found that the ability of diverse lactobacilli to scavenge manganese results in inhibited growth of yeast and mold in fermented milk. Several studies have previously investigated the ability of lactobacilli to inhibit fungi in various foods, and the usage of lactobacilli for this purpose in commercial applications is gaining considerable interest. Thus far, bacterial production of inhibitory metabolites has been the focus of most studies, but the identified compounds are mostly found in concentrations markedly below the corresponding minimum inhibitory concentration of the metabolite (14). Our study shows that competitive exclusion is a major mechanism employed by many of lactobacilli to inhibit fungal growth in fermented milk. As a mechanism to avoid unwanted fungal growth, competitive exclusion is known from the concept of “nutritional immunity” in the human body, where depletion of iron and zinc in the respective parts of the body results in inhibition of pathogenic fungal and bacterial growth (18). In the context of lactic acid bacteria, examples of competitive exclusion of nutrients are scarce. A study by Honoré et al. (16), found that *Lb. paracasei*-mediated consumption of glucose and certain amino acids in a chemically defined medium correlated with reduced mold growth on the spent culture medium.

Here, the reduction of available manganese was proven as an effective inhibition mechanism against all the tested yeasts and molds. Yet, two yeast strains, *T. delbrueckii*
and *C. fragicola*, only displayed partially restored growth upon manganese addition (Fig. 2), indicating that other mechanisms such as higher acid production or other antimicrobial compounds, could be involved as well (31). In milk, manganese is one of the essential trace metals with the lowest concentration (28, 29) and, consequently, the bacterial consumption required to reach a growth inhibitory concentration is low. Unlike the addition of other ions, the addition of 0.03 mg/L manganese restored *D. hansenii*, growth. This correlates well with the determined minimal manganese concentration threshold for *D. hansenii* growth of ~0.01 mg/L. In contrast, addition of higher concentration was needed when the assay was performed in yoghurt (6 mg/L) compared to its aqueous phase (Fig. 3, Supplemental Fig. S1). This can be attributed to viable bacteria present in the yoghurt capable of taking up additional manganese during storage. Transcriptional profiling of BioP strains also identified high *mntH1* gene expression during storage (Fig. 4B), indicating continued transport activity in this period and thereby a potential for further bacterial manganese uptake during storage.

In the BioP strains, the expression level of the *mntH1* gene is exceptionally high, even surpassing most glycolytic genes. In comparison, an *S. thermophilus* starter culture strain and the less yeast growth inhibiting *Lb. paracasei* strain C display a 25-fold lower *mntH1* expression level. Consequently, an excessively high *mntH* expression level seems to be crucial for high bioprotective activity. The *mntH1* expression level of the BioP strains depends however on the stage of fermentation (Fig. 4B). A significant temporal increase in expression is observed, which could be an effect of a lowered manganese concentration caused by increases in cell quantity. This finding is in line with the manganese concentration-dependent *mntH* regulation mediated by MntR, previously observed in *Bacillus subtilis* (32). For the stationary phase samples taken after 18 hours of growth at
7°C, strain divergence in expression is observed, with expression remaining high in the *Lb. paracasei* strain B and less so for the *Lb. rhamnosus* strain A, possibly signifying species-dependent activity during cold storage. The level of *mntH1* expression was observed to increase during co-culture of BioP strains, as compared to individual growth, possibly revealing a mutualistic or commensalistic relationship between the two organisms.

Deleting the highly transcribed *mntH1* gene in *Lb. paracasei* strain B proved this particular MntH1 transporter to be essential for the bioprotective phenotype. As the mechanism of bioprotection occurs by manganese scavenging, it follows that MntH1 is also the transporter responsible for manganese depletion. Although two additional MntH transporters and an ABC transport system (*sitABC*) exist in *Lb. paracasei*, there is no apparent sufficient regulatory response towards sustaining manganese uptake levels in the ∆*mntH1* strain. Either the transport kinetics or the underlying transcriptional regulation of the alternative transport systems, are inadequate to achieve the required manganese uptake level for inhibition of yeast growth. The relevance of the other transport systems could however be a function of manganese concentration and pH. The ABC transporter type has for instance been shown to display improved activity at neutral or alkaline pH, while MntH displayed improved activity in acidic medium (21, 33). Previous studies of MntH in lactobacilli have provided ambiguous findings. In *L. casei*, deletion of two *mntH* transporters were required for reducing intracellular concentrations of manganese (34), while in another *L. casei* study, despite deletions in both *mntH* and *sitABC*, growth rate was unaltered (35). The low manganese concentration tested in the former and latter study were however set at 0.1 mg/L and 0.08 mg/L, respectively, thus around 3 times higher than the concentration in milk. Possibly, these are too high concentrations for a significant effect of *mntH1* deletion to materialize. Alternatively, significant strain variation in MntH1
activity exists, as already identified among the *Lactobacillus* strains A, B and C examined in this study.

Variation in MntH activity could also explain our observation that not all selected lactobacilli (Fig. 6) harboring an *mntH* gene were equally proficient at inhibiting growth of *D. hansenii*. Therefore, the presence of an *mntH* gene *per se* does not ensure good inhibition potential of spoilage organisms, rather the expression level could be decisive. In the current experimental setting, the ability to take up manganese also requires the ability to grow in milk.

The main purpose of excessive manganese uptake appears to be the requirement for high intracellular manganese concentrations towards superoxide stress protection. As a simple and more efficient alternative exists in the manganese superoxide dismutase protein, additional reasons for high intracellular manganese concentrations may exist. It could be speculated whether excessive manganese scavenging as a competitive exclusion mechanism in itself could provide a fitness advantage in natural settings, and thereby partially explain this characteristic among many lactobacilli.

**Conclusion**

A bacterial mechanism, originally identified as providing protection against oxidative stress can from this study be coupled to facilitate another general bacterial mechanism, that of competitive exclusion. To our knowledge, such coupling in bacteria is without prior example. Moreover, it was shown that the principle of competitive exclusion, through the action of bioprotective strains, can be harnessed towards inhibiting the growth of unwanted spoilage organisms in a food source. The bioprotective activity of a strain in the present context, yoghurt, can be defined as the sum of its ability to grow in milk, grow at the given
fermentation temperature and not least the expression of an *mntH* manganese transporter gene. Without the production of an antibiotic or other small molecule, competitive exclusion constitutes a mechanism that should be difficult for spoilage organisms to overcome by spontaneous mutation, making it an ideal mechanism for protection of food, from a consumer, commercial, and regulatory perspective.

**MATERIALS AND METHODS**

**Strains and growth conditions**

Strains used in this study are shown in Table 1. The yeast strains were streaked on YGC plates (1 g/L yeast extract (Merck), 20 g/L D-glucose (Merck) and 0.1 g/L chloramphenicol (Sigma-Aldrich)) and grown for 48 hours at 25°C. Single colonies were inoculated to 25 ml YG media (1 g/L yeast extract (Merck), 20 g/L D-glucose (Merck)) and grown over night at 25°C with shaking. Afterwards, glycerol was added to a final concentration of 15% (w/v) and the yeast cells were stored at -80°C until further use.

The bacterial strains were streaked on MRS (Sigma-Aldrich) plates and incubated anaerobically at 37°C for 48 hours. Single colonies were inoculated into 3 ml MRS medium and grown over night at 37°C. 10 µl of the overnight culture was added to 2 ml heat-treated milk containing 0.02% (v/w) commercial starter culture and a pH indicator. The acidification was performed at 43°C and stopped after ~6 hours when a pH of 4.5 was reached. The fermented milk was kept in the fridge upon further use.

**Yoghurt production**
Reduced-fat (1.5% w/v) homogenized milk was heat-treated at 90±1°C for 20 min and cooled immediately. A commercial starter culture (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*) was inoculated at 0.02% (v/w) in 3 L buckets. One bucket was inoculated with bioprotective culture (+BioP) in total concentration of 100 U/T and one bucket was used as a reference (REF) and only inoculated with the starter culture. All buckets were incubated in a water bath at 43°C and fermented at these conditions until pH of 4.60 was reached. At this time point, the fermented milk products were divided into 200 mL bottles and cooled down.

**Aqueous phase (AQ) production and manganese concentration determination**

The fermented milk product was centrifuged (10 min at 5000 rpm) and the supernatant (AQ) was sterile filtered. The manganese concentration of the AQ was determined by inductively coupled plasma mass spectrometry at Eurofins Steins Laboratorium A/S.

**Assay to detect bioactivity in AQ against yeasts**

The AQ of reference and BioP were transferred to a sterile 96-well plate (150 µl in each well) and dilutions were performed or different concentrations of metals were added (manganese: 6 mg/L– 6 ng/L, iron: 6 – 0.09 mg/L, copper: 0.1 mg/L, zinc: 4.2 mg/L, magnesium: 60 mg/L).

Washed *Lb. paracasei* (wild type strain B and ∆mntH1) cultures were added to 2 mL milk and grown for ~18 hours at 37°C until pH 4.5 was reached. The fermented milk was centrifuged (10 min at 5000 rpm) and the supernatant (AQ) was transferred to a sterile 96-well plate (150 µl in each well).
The different yeasts (see Table 1) were taken from the glycerol stock and diluted in MilliQ H₂O to inoculate with ~20 cells per well. The plates were incubated at 17°C for several days and the yeast growth was determined by measuring the absorbance at 600 nm.

Assay to detect bioactivity of lactobacilli against yeast in fermented milk

Lactobacilli were selected from the Chr. Hansen A/S strain collection. If available, species type strains were selected, while strains from the remaining species were selected based on the availability of the corresponding genome sequence, in an otherwise unbiased selection process. 150 µl of the fermented milk was transferred to individual wells in a 96 well plate. Manganese (6 mg/L) was added to half of the samples and the wells were inoculated with about 20 cells of the respective yeast. After 4 days of incubation at 17°C a dilution row was spotted on selective YGC agar plates to analyze the yeast growth. In some cases, the growth was enumerated by optical inspection, where a value of 0 was given for no growth, a value of 1 for 1-2 colonies, a value of 2 for 2-10 colonies, a value of 3 for 10-30, a value of 4 for 30-70 and a value of 5 for confluent growth.

Assay to detect bioactivity in yoghurt against molds

The fermented milk products were divided into 200 mL bottles and cooled down. Different manganese concentrations (6 ng/L - 6 mg/L) were added to fermented milk products with BioP. All the fermented milk samples were heated to a temperature of 40°C and supplemented with 40 ml of a 5% sterile agar solution that had been melted and cooled down to 60°C. This solution of fermented milk and agar was then poured into sterile Petri dishes and the plates were dried in a LAF bench for 30 min. Three target contaminants, *P. brevirepactum, P. crustosum* and *P. solitum*, were added in concentrations of 500 spores/spot. The plates were incubated at 22±1°C for 8 days before assessment of mold growth.
Isolation and processing of RNA

Initially, three independent precultures of each strain were cultivated anaerobically overnight at 37°C. Cultures were washed twice in 0.9 % saline solution and inoculated in milk, here skim milk powder reconstituted (9.5 %) in distilled water. Strain A was inoculated to an OD$_{600}$ corresponding to 0.1, Strain B to 0.5, Strain C to 0.3 and the combined BioP culture to 0.1. The differences in inoculation level were chosen based on preliminary acidification rates, a growth rate proxy, with an inverse relationship between acidification rate and selected inoculation level. Prior to inoculation, lactic acid was added to reduce pH to 5.5 to simulate the acidification normally provided by the starter culture.

Milk fermentation was performed at 37°C in 2 mL deep-well plates covered by foil and proceeded either for 4 hours and 6 hours before harvest, or for 6 hours at 37 °C and followed by 18 hours at 7°C. Growth was performed in triplicate samples for each strain and not all strains were subjected to all conditions. Cell harvest was performed by mixing a 1:2 volume of milk fermentate (2 mL) to RNAProtect Bacteria Reagent (Qiagen) (4 mL) followed by a procedure for separation of cells from milk fermentate, adapted from Derzelle et al. (36). A 2 mL volume of 1 M sodium citrate solution and 0.78 mL saline solution (0.145 M sodium chloride, 0.016 M Sodium β-glycerophosphate, 0.1 % Tween 80, pH 7) were added and after 5 minutes centrifuged at 10,000g for 2 min. The resulting cell pellet was washed with cold phosphate buffer (5 mM sodium phosphate, 1 mM EDTA, pH 7), centrifuged as before, supernatant discarded and the cell pellet frozen at -80°C. Cell pellet was dissolved in Tris-EDTA buffer containing lysozyme (15 mg/mL), proteinase K (1.3 mg/mL) and mutanolysin (50U) and was shaken at 1400 rpm for 10 min at 37°C. The subsequent RNA extraction procedure was performed with the Rneasy Protect Bacteria Mini Kit (Qiagen) as per the manufacturer’s instructions, including removal of DNA with
DNAse I. The quality of total RNA was evaluated using a Bioanalyzer 2100 (Agilent).

Depletion of ribosomal RNA, library preparation and sequencing (Illumina 50 bp single-end) was performed at BGI Europe A/S, Denmark.

**RNAseq data analysis**

Obtained raw reads, 15-45 million per sample, were trimmed with Trimmomatic (37) using default parameters and mapping was performed using CLC Genomics Workbench v10 (Qiagen) with the following parameters, mismatch cost 2, insertion cost 3, deletion cost 3, length fraction 0.8, similarity fraction 0.9, strand specific, local alignment and maximum number of hits for a read 10. For reads with 2-10 hits, reads were randomly assigned a gene. For BioP culture samples, mapping was performed towards both genomes simultaneously, and to the relevant individual genome for strain A/B/C samples. Strain C reads were mapped to the strain B genome, as they are identical species. Following mapping, total gene counts were extracted. Within the SARTools (38) framework, the DESeq2 R package (39) was applied for normalization of sample gene counts using the median ratio method and subsequent estimation of differential expression. For comparison of mntH gene counts it was necessary to compare gene counts between samples of differing species, that harbor distinct genes and therefore cannot immediately be normalized together using DESeq2. Therefore, an additional normalization between sample types containing different species compositions (BioP, Strain A, Strain B or Strain C) was performed on within-species normalized gene counts applying Transcripts Per kilobase Million (TPM) normalization. For BioP culture samples, additional TPM normalization was performed for the sample as a whole (sample-normalized), but also individually for Strain A and Strain B (strain-normalized). This gives different gene count
distributions as more counts were overall associated with Strain B over Strain A for the 6-509 hour samples.

**Generation of ΔmntH1 mutant**

Clean deletion of the entire ΔmntH1 gene in *Lb. paracasei* Strain B was performed by a combination of homologous recombination and CRISPR-Cas9 counter selection (Text S1), as previously described (40). Briefly, 500 bp homology arms flanking *mntH1* were employed for homologous recombination while a targeting CRISPR-Cas9 nuclease was utilized for selection of ΔmntH1 deletion mutants. Transformation of *Lb. paracasei* was adapted from Song *et al.* (41) and to improve transformation efficiency, a procedure for obtaining non-methylated plasmids was employed (42). Once a correct mutant was isolated and sequence verified, the plasmids were cured through non-selective growth cycles.

**Statistical analysis**

Statistical analysis were conducting using Graph Pad Prism 8.2.0 (GraphPad Software, Inc., San Diego, Canada). When applying one- or two-way ANOVA analysis, multiple comparisons were corrected for using Tukey’s statistical hypothesis testing. All relevant statistical information is reported along with p- and n-values.

**Data availability statement**

Transcriptomic data are available from the Sequence Read Archive (SRA) repository of the National Center for Biotechnology Information under BioProject PRJNA395470. Strains will be available for research purposes upon request.

**Acknowledgements**
We acknowledge our colleagues in the Discovery function of the R&D organization as well as the Dairy Bioprotection and Production Development departments in Chr. Hansen A/S for valuable discussions and sharing of results. We acknowledge Sonali Sirdesai from the University of Groningen and Morten Adler Hedegaard from the University of Copenhagen for assisting during the initial stages of this project. This work was supported in part by the National Science Foundation [MCB-1413044 to C.L.B.].

Conflict of interests

Certain authors are employed at Chr. Hansen A/S, a company that develops and commercializes bioprotective cultures.

REFERENCES

1. Leyva Salas M, Mounier J, Valence F, Coton M, Thierry A, Coton E. 2017. Antifungal Microbial Agents for Food Biopreservation—A Review. Microorganisms 5:37.

2. Salvetti E, Harris HMB, Felis GE, O’Toole PW. 2018. Comparative Genomics of the Genus Lactobacillus Reveals Robust Phylogroups That Provide the Basis for Reclassification. Appl Environ Microbiol 84:e00993-18.

3. Duar RM, Lin XB, Zheng J, Martino ME, Grenier T, Pérez-Muñoz ME, Leulier F, Gänzle M, Walter J. 2017. Lifestyles in transition: evolution and natural history of the genus Lactobacillus. FEMS Microbiol Rev 41:S27–S48.

4. Smokvina T, Wels M, Polka J, Cherva C, Brisse S, Boekhorst J, Vlieg JET van H, Siezen RJ. 2013. Lactobacillus paracasei Comparative Genomics: Towards Species Pan-Genome Definition and Exploitation of Diversity. PLoS One 8:e68731.

5. Douillard FP, Ribbera A, Kant R, Pietilä TE, Järvinen HM, Messing M, Randazzo CL, Paulin L, Laine F, Ritari J, Caggia C, Lähteinen T, Brouns SJJ, Satokari R, von Ossowski I, Reunanen J, Palva A, de Vos WM. 2013. Comparative Genomic and Functional Analysis of 100 Lactobacillus rhamnosus Strains and Their Comparison with Strain GG. PLoS Genet 9:e1003683.

6. Garnier L, Valence F, Mounier J, Garnier L, Valence F, Mounier J. 2017. Diversity and Control of Spoilage Fungi in Dairy Products: An Update. Microorganisms 5:42.

7. Magnusson J, Ström K, Roos S, Sjögren J, Schnürer J. 2003. Broad and complex antifungal activity among environmental isolates of lactic acid bacteria. FEMS Microbiol Lett 219:129–135.

8. Bulgasem BY, Lani MN, Hassan Z, Yusoff WMW, Fnaish SG. 2016. Antifungal Activity of Lactic Acid Bacteria Strains Isolated from Natural Honey against Pathogenic Candida Species. Mycobiology 44:302–309.

9. Wakil, SM, Laba, SA, Fasiku, SA. 2014. Isolation and identification of antimicrobial-producing lactic acid bacteria from fermented cucumber. African J Biotechnol 13:2556–2564.
10. Sjögren J, Magnusson J, Broberg A, Schnürer J, Kenne L. 2003. Antifungal 3-hydroxy fatty acids from *Lactobacillus plantarum* MiLAB 14. *Appl Environ Microbiol* 69:7554–7.

11. Schnürer J, Magnusson J. 2005. Antifungal lactic acid bacteria as biopreservatives. *Trends Food Sci Technol* 16:70–78.

12. McNair LKF, Siedler S, Vinther JMO, Hansen AM, Neves AR, Garrigues C, Jäger AK, Franzky H, Staerk D. 2018. Identification and characterization of a new antifungal peptide in fermented milk product containing bioprotective *Lactobacillus* cultures. *FEMS Yeast Res* 18.

13. Crowley S, Mahony J, van Sinderen D. 2013. Current perspectives on antifungal lactic acid bacteria as natural bio-preservatives. *Trends Food Sci Technol* 33:93–109.

14. Siedler S, Balti R, Neves AR. 2019. Bioprotective mechanisms of lactic acid bacteria against fungal spoilage of food. *Curr Opin Biotechnol* 56:138–146.

15. Haidar R, Fermaud M, Calvo-Garrido C, Roudet J, Deschamps A. 2016. Modes of action for biological control of *Botrytis cinerea* by antagonistic bacteria. *Phytopathol Mediterr* 55:301–322.

16. Honoré AH, Aunshjerg SD, Ebrahimi P, Thorsen M, Benfeldt C, Knøchel S, Skov T. 2016. Metabolic footprinting for investigation of antifungal properties of *Lactobacillus paracasei*. *Anal Bioanal Chem* 408:83–96.

17. Golowczyc MA, Mobili P, Garrote GL, Abraham AG, De Antoni GL. 2007. Protective action of *Lactobacillus kefir* carrying S-layer protein against *Salmonella enterica* serovar *Enteritidis*. *Int J Food Microbiol* 118:264–273.

18. Gerwien F, Skrahina V, Kasper L, Hube B, Brunke S. 2018. Metals in fungal virulence. *FEMS Microbiol Rev* 42.

19. Wandersman C, Delepelaire P. 2004. Bacterial Iron Sources: From Siderophores to Hemophores. *Annu Rev Microbiol* 58:611–647.

20. Jensen AN, Jensen LT. 2014. Chapter 1. Manganese Transport, Trafficking and Function in Invertebrates, p. 1–33. In *Manganese in health and disease*.

21. Porcheron G, Garénaux A, Proulx J, Sabri M, Dozois CM. 2013. Iron, copper, zinc, and manganese transport and regulation in pathogenic *Enterobacteria*: correlations between strains, site of infection and the relative importance of the different metal transport systems for virulence. *Front Cell Infect Microbiol* 3:90.

22. Takeda Y, Avila H. 1986. Structure and gene expression of the *E. coli* Mn-superoxide dismutase gene. *Nucleic Acids Res* 14:4577–89.

23. Archibald FS, Fridovich I. 1981. Manganese, Superoxide Dismutase, and Oxygen Tolerance in Some Lactic Acid Bacteria. *J Bacteriol* 146:928–936.

24. Archibald FS, Fridovich I. 1982. Investigations of the state of the manganese in *Lactobacillus plantarum*. *Arch Biochem Biophys* 215:589–596.

25. Barness K, Gralla EB, Valentine JS, Cabelli DE. 2012. Biologically relevant mechanism for catalytic superoxide removal by simple manganese compounds. *Proc Natl Acad Sci* 109:6892–6897.

26. Culotta VC, Daly MJ. 2013. Manganese complexes: diverse metabolic routes to oxidative stress resistance in prokaryotes and yeast. *Antioxid Redox Signal* 19:933–44.

27. Sun H, Xu G, Zhan H, Chen H, Sun Z, Tian B, Hua Y. 2010. Identification and evaluation of the role of the manganese efflux protein in *Deinococcus radiodurans*. *BMC Microbiol* 10:319.

28. Nantapo CTW, Muchenje V. 2013. Winter and spring variation in daily milk yield and mineral composition of Jersey, Friesian cows and their crosses under a pasture-based dairy system. *S Afr J Anim Sci* 43.

29. Miller GD, Jarvis JK, McBean LD, National Dairy Council. 2007. *Handbook of dairy foods*.
and nutrition. CRC Press.

30. Dillies M-A, Rau A, Aubert J, Hennequet-Antier C, Jeanmougin M, Servant N, Keime C, Marot G, Castel D, Estelle J, Guerne C, Jagla B, Jouneau L, Laloe D, Le Gall C, Schaeffer B, Le Crom S, Guedj M, Jaffrezic F. French StatOmique Consortium. 2013. A comprehensive evaluation of normalization methods for Illumina high-throughput RNA sequencing data analysis. Brief Bioinform 14:671–683.

31. Lindgren SE, Dobrogosz WJ. 1990. Antagonistic activities of lactic acid bacteria in food and feed fermentations. FEMS Microbiol Rev 7:149–63.

32. Que Q, Helmann JD. 2002. Manganese homeostasis in *Bacillus subtilis* is regulated by MntR, a bifunctional regulator related to the diphtheria toxin repressor family of proteins. Mol Microbiol 35:1454–1468.

33. Kehres DG, Janakiraman A, Slauchoch JM, Maguire ME. 2002. Regulation of *Salmonella enterica* serovar Typhimurium mntH transcription by H(2)O(2), Fe(2+), and Mn(2+). J Bacteriol 184:3151–8.

34. Serata M, Yasuda E, Sako T. 2018. Effect of superoxide dismutase and manganese on superoxide tolerance in *Lactobacillus casei* strain Shirota and analysis of multiple manganese transporters. Biosci microbiota, food Heal 37:31–38.

35. Groot MNN, Klaassens E, Vos WM de, Delcour J, Hols P, Kleerebezem M. 2005. Genome-based in silico detection of putative manganese transport systems in *Lactobacillus plantarum* and their genetic analysis. Microbiology 151:1229–1238.

36. Derzelle S, Bolotin A, Mistou M-Y, Rul F. 2005. Proteome analysis of *Streptococcus thermophilus* grown in milk reveals pyruvate formate-lyase as the major upregulated protein. Appl Environ Microbiol 71:8597–605.

37. Bolger AM, Lohse M, Usadel B. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30:2114–20.

38. Varet H, Brillet-Guéguen L, Coppée J-Y, Dillies M-A. 2016. SARTools: A DESeq2- and EdgeR-Based R Pipeline for Comprehensive Differential Analysis of RNA-Seq Data. PLoS One 11:e0157022.

39. Love MI, Huber W, Anders S. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol 15:550.

40. Leenay RT, Vento JM, Shah M, Martino ME, Leulier F, Beisel CL. 2019. Genome Editing with CRISPR-Cas9 in *Lactobacillus plantarum* Revealed That Editing Outcomes Can Vary Across Strains and Between Methods. Biotechnol J 14:1700583.

41. Song X, Huang H, Xiong Z, Ai L, Yang S. 2017. CRISPR-Cas9D10A Nickase-Assisted Genome Editing in *Lactobacillus casei*. Appl Environ Microbiol 83:e01259-17.

42. Zhang G, Wang W, Deng A, Sun Z, Zhang Y, Liang Y, Che Y, Wen T. 2012. A Mimicking-of-DNA-Methylation-Patterns Pipeline for Overcoming the Restriction Barrier of Bacteria. PLoS Genet 8:e1002987.

### Table 1: Strains used in this study

| Strains | Comments/Accession no. | Source |
|---------|------------------------|--------|
| Yeast   |                        |        |
Torulaspora delbrueckii  Chr. Hansen A/S
Cryptococcus fragicola  Chr. Hansen A/S
Saccharomyces cerevisiae  Chr. Hansen A/S
Debaryomyces hansenii Strain 1  Chr. Hansen A/S
Debaryomyces hansenii Strain 2  Chr. Hansen A/S
Rhodoturola mucilaginosa  Chr. Hansen A/S

Molds
Penicillium brevicompactum  Chr. Hansen A/S
Penicillium crustosum  Chr. Hansen A/S
Penicillium solitum  Chr. Hansen A/S

Lactobacilli
Lb. rhamnosus Strain A  Part of BioP culture  Chr. Hansen A/S
Lb. paracasei Strain B  Part of BioP culture  Chr. Hansen A/S
Lb. paracasei Strain C  Chr. Hansen A/S
Lb. rhamnosus (strain 1)  Chr. Hansen A/S
Lb. rhamnosus (strain 2)  Chr. Hansen A/S
Lb. rhamnosus (strain 3)  Chr. Hansen A/S
Lb. rhamnosus (strain 4) AZCQ00000000.1  LMG 6400  Chr. Hansen A/S
Lb. salivarius  Chr. Hansen A/S
Lb. casei  Chr. Hansen A/S
Lb. paracasei  Chr. Hansen A/S
Lb. fermentum  Chr. Hansen A/S
Lb. sakei AZDN00000000  LMG 9468  Chr. Hansen A/S
Lb. reuteri AZDD00000000  LMG 9213  Chr. Hansen A/S
Lb. plantarum  Chr. Hansen A/S
Lb. brevis  Chr. Hansen A/S
Lb. kefiri AYYV00000000  DSM20587  Chr. Hansen A/S
Lb. alimentarius  Chr. Hansen A/S
Pediococcus acidilactici AEEG00000000  NCFB2767  Chr. Hansen A/S
Lb. delbrueckii subsp. bulgaricus mntH not present  Chr. Hansen A/S
Lb. helveticus mntH not present  Chr. Hansen A/S
Lb. gasseri mntH not present  DSM20243
Lb. plantarum

ΔmntH1 Lb. paracasei Strain B ΔmntH1 This study

Table 2: Oligonucleotides used in this study

| Name   | Sequence                                                                 |
|--------|--------------------------------------------------------------------------|
| oJV2   | GGCCGCATGGTTTGCCACCTAAAAACGGCATGCTCATAACCTTGTTCTGATCGTAGCTATCCTCGAT     |
Table 3: Plasmids used in this study

| Plasmid  | Description                                                                 | Resistance | Source                                |
|----------|-----------------------------------------------------------------------------|------------|---------------------------------------|
| pCB578   | E. coli-lactobacilli shuttle vector containing SpCas9, tracrRNA, and a repeat-spacer-repeat array targeting mntH1 | Erm        | Leenay et al. 2019                   |
| pJV114   | Shuttle vector with pCB578 base containing a spacer targeting mntH1           | Erm        | This work                             |
| pCB591   | E. coli-lactobacilli shuttle vector for homologous recombination template cloning | Amp (E. coli) Cm (Lactobacillus) | Leenay et al. 2019                   |
| pJV88    | Shuttle vector with pCB591 base containing the mntH1 gene and 500-bp homology arms on either side of mntH1 that serves as a recombination template to generate a clean deletion | Amp (E. coli) Cm (Lactobacillus) | This work                             |
| pJV91    | Shuttle vector with pCB591 base containing 500-bp homology arms on either side of mntH1 that serves as a recombination template to generate a clean deletion | Amp (E. coli) Cm (Lactobacillus) | This work                             |
| pNZ8148  | Broad-host range LAB expression vector containing NisA promoter and pepN terminator | Cm         | Mobitech                             |
| pEFB021  | pNZ8148 in which NisA promoter has been replaced by the 207 bp native mntH1 promoter followed by mntH1 | Cm         | This work                             |

Figure captions

Figure 1: Growth of *D. hansenii* in aqueous phase (AQ) of reference (REF) yoghurt (red bars) and bioprotective strains (BioP) containing yoghurt (blue bars). (A) The REF AQ and BioP AQ were diluted with tap water (clear bars) or MilliQ water (striped bars). Growth of *D. hansenii* was measured at 600 nm after 4 days of incubation at 17°C. (B) Effect of complementation BioP AQ with metals found in milk on the growth of *D. hansenii* (light blue bars). Growth was measured after 5 days of incubation at 17°C and compared to
REF AQ without any addition of metal ions. Metal concentrations were used as found in milk (0.3 mg/L Fe $^{2+}$, 0.1 mg/L Cu $^{2+}$, 4.2 mg/L Zn $^{2+}$, 60 mg/L Mg $^{2+}$, 0.03 mg/L Mn $^{2+}$). Mean and standard deviation of three replicates are indicated by the bars and error bars. Hash tags (#) indicate data being statistically significantly different (p<0.0001) from REF AQ (panel A: Student’s t-test, panel B: one-way ANOVA), and asterisks (*) indicate data being different from BioP AQ diluted with MilliQ water (panel A; two-way ANOVA) or undiluted/non-supplemented BioP AQ (panel B; one-way ANOVA).

**Figure 2:** Yeast growth in BioP AQ without (dark blue) and with (light blue) the addition of 0.6 mg/L manganese and in REF AQ (red) after 5 days of incubation at 17°C. The following yeasts were tested: *D. hansenii* strain 1 (A), *D. hansenii* strain 2 (B), *S. cerevisiae* (C), *R. mucilaginosa* (D), *C. fragicola* (E) and *T. delbrueckii* (F). Individual data point for the 6-9 replicates are shown along with indications of mean ± standard deviation. Hash tags (#) indicate data being statistically significantly different (p<0.05) from REF AQ, and asterisks (*) indicate data being different from non-supplemented BioP AQ (one-way ANOVA).

**Figure 3:** Growth of 3 different molds, (A) *Penicillium brevcompactum*, (B) *Penicillium crustosum* and (C) *Penicillium solitum* on plates prepared from milk fermented with starter culture alone (REF) or both starter and bioprotective culture (BioP). Different manganese concentrations were added as indicated. The spoilage molds were added in concentrations of 500 spores/spot. The plates were incubated at 22°C for 8 days.

**Figure 4:** Overall and mntH1-specific gene count distribution A: Sample-normalized gene count distribution of BioP *Lb. rhamnosus* and *Lb. paracasei* during co-culture after 6 hours milk fermentation. A white circle denotes the mntH1 count level. B: Temporal, sample-normalized mntH1 count levels of BioP strains during 37°C milk fermentation (4 hours, 6 hours), and after 6 hours of 37°C milk fermentation and 18 hours of 7°C storage (6 + 18 hours). C: Normalized mntH1 count levels after 6 hours of co-culture (Strains A+B) and individual culture (Strain A, Strain B, Strain C) in milk. Normalization is here performed individually for each strain, also for co-culture. Included are the BioP strains (Strains A, B) and an *Lb. paracasei* with a lower level of bioprotective activity (Strain C).

**Figure 5:** Effect of mntH1 deletion on yeast growth inhibition. Yeast growth in AQ of milk fermented by wildtype *Lb. paracasei* Strain B (WT), ΔmntH1 *Lb. paracasei* Strain B (ΔmntH1), without (full bars) and with 0.6 mg/L manganese addition (dashed bars). Two yeast, *D. hansenii* 1 (A) and *D. hansenii* 2 (B) were included and the growth was measured after 5 days of incubation at 17°C. Mean and standard deviation of
two biological replicates are indicated by bars and error bars. Hash tags (#) indicate data being statistically significantly different (p<0.0001) from Wt with manganese (Student’s t-test).

**Figure 6**: Growth scores of *D. hansenii* after 4 days of incubation at 17°C in fermented milk with a strain containing (blue) and not containing (red) an *mntH* gene. Addition of 6 mg/L manganese restored the yeast growth in all cases (black). The average and standard deviation of two biological independent experiments is shown (n=2).
