Growth Inhibition of Various Enterobacteriaceae Species by the Yeast Hansenula anomala during Storage of Moist Cereal Grain

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Growth Inhibition of Various *Enterobacteriaceae* Species by the Yeast *Hansenula anomala* during Storage of Moist Cereal Grain

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Eleven of 13 *Enterobacteriaceae* species tested grew in moist stored wheat, highlighting a potential risk of this energy-saving airtight storage method. When *Hansenula anomala* was coinoculated, all *Enterobacteriaceae* species were significantly inhibited after 2 months of storage, six of them to below the detection limit.

Enterobacteriaceae species can be associated with severe human health problems, as demonstrated during the outbreak of a life-threatening hemolytic syndrome in May-June 2011 in Europe. During this outbreak, an enterohemorrhagic *Escherichia coli* (EHEC) strain grown on products of organic farming caused an epidemic with over 2,000 registered cases and more than 30 deaths (www.rki.de). Thus, there is pressure to minimize the number of *Enterobacteriaceae* organisms in the food chain; reducing the number of these bacteria in feed production has been shown to reduce their frequency in the food chain (1). Treatment of the above-mentioned EHEC strain with antibiotics increased toxin production, also indicating a demand for alternative antibacterial agents.

Cereal grain is a major resource for human nutrition, animal feed, and biofuel production. Diminishing postharvest losses and ensuring the hygienic quality of cereal grain are thus central issues in generating sustainable food, feed, and bioenergy production. The most efficient method for conserving cereal grain is drying. However, this method consumes much energy—in regions with a temperate climate, such as Sweden, it is responsible for up to 60% of the total energy consumption during grain production. Moreover, even dried material retains certain risks for contamination if drying is uneven or if the material absorbs moisture from humidity in the air. Airtight storage of moist grain is an energy-saving alternative that utilizes approximately 2% of the energy of high-temperature drying (16, 22). Airtight storage of moist grain is appropriate mainly for storage of animal feed, and it has been shown that the nutritional characteristics of the grain improve during storage (14). Furthermore, bioethanol production from moist stored grain was also improved, due to better accessibility of the starch for enzymatic pretreatment (18). Members of the *Enterobacteriaceae* have usually not been regarded as a problem in cereal grain. However, our results recently demonstrated that at least two identified *Enterobacteriaceae* species, *E. coli* and *Pantoea agglomerans*, can be present and grow on stored cereal grain, even at a moisture content of only 14% (14, 17). The question remains as to whether other species can also grow under these conditions.

It has been shown that the biopreservation yeast *Hansenula anomala* can prevent mold growth in moist cereal grain (19). (There is currently a debate about the correct designation of this yeast species. The name *Pichia anomala*, used in recent years, does not reflect the phylogeny of this species. A new species name, *Wickerhamomyces anomalus*, has been proposed [9, 10], but some argue to reinstate the former name, *H. anomala* [20].) Interestingly, we recently found that *H. anomala* strongly inhibited *E. coli* and *P. agglomerans* in cereal grain (14).

This study investigated the ability of a broad range of species belonging to the *Enterobacteriaceae* family to grow in moist cereal grain and tested the efficacy of *H. anomala* as a biocontrol agent against these bacteria. We also investigated whether inhibition of *Enterobacteriaceae* is a general feature of the species or restricted to a few strains.

The yeast strains *H. anomala* CBS 113, CBS 247, CBS 248, CBS 249, CBS 250, CBS 251, CBS 256, CBS 257, CBS 261, CBS 1947, CBS 5759, CBS 100487, DBVPG 3511, DBVPG 3650, DBVPG 3862, DBVPG 3863, DBVPG 3864, DBVPG 3873, NRRL Y-366, IMV Y-2037, J281, J316, J317, J348, J475, J536, YMO1, YMO2, YMO3, and YMO4 and the bacterial strains *Citrobacter freundii* ATCC 8090, *Enterobacter aerogenes* ATCC 13048, *Enterobacter cloacae* ATCC 13047, *Enterobacter sakazakii* ATCC 29544, *Erwinia carotovora* SCC3193, *E. coli* ATCC 11775, *Hafnia alvei* ATCC 13337, *Klebsiella oxytoca* ATCC 13182, *Klebsiella pneumoniae* (no. 14), *Morganella morganii* ATCC 25830, *P. agglomerans* ATCC 27155, *Proteus mirabilis* ATCC 29906, and *Serratia marcescens* ATCC 13880 used during this study were stored in glycerol stocks at −70°C in the culture collection of the Department of Microbiology, Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden.

Yeast was pregrown in 15 ml YPD broth (10 g liter^{-1} yeast extract, 20 g liter^{-1} bacteriological peptone [Oxoid Ltd., Basingstoke, United Kingdom], and 20 g liter^{-1} DL-glucose [Merck, KGaA, Darmstadt, Germany]) and incubated at 25°C on a rotary shaker at 150 rpm overnight. The bacterial strains were inoculated into 15 ml LB broth (Duchefa, Haarlem, The Netherlands) and incubated at 37°C overnight, apart from *E. carotovora*, which was incubated at 25°C.

Inhibition of *Enterobacteriaceae* in wheat by *H. anomala* was analyzed in test tubes ("minisilos") as previously described (21). Briefly, nonsterile dry wheat (~10% moisture; cultivar Kosack) was adjusted to a water activity (a_w) of ~0.95 with tap water and inoculated with ~10^5 CFU g^{-1} each of *H. anomala* and a bacterial

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TABLE 1 Bacterial quantification in airtight stored moist wheat inoculated with the indicated *Enterobacteriaceae* species alone (Eb) or with both the bacterium and *Hansenula anomala* (EbHa)[a]

Bacterial species	Log CFU g^{-1} grain at t_0				Log CFU g^{-1} grain t_1				Log CFU g^{-1} grain at t_2			
	Eb	EbHa	Difference	*P* value	Eb	EbHa	Difference	*P* value	Eb	EbHa	Difference	*P* value
C. freundii	4.66	4.77	−0.11	0.51	4.14	3.75	0.39	0.039	1.00	1.00	0.00	1.00
E. aerogenes	5.39	5.56	−0.17	0.07	6.05	5.55	0.50	<0.01	6.10	3.53	2.75	<0.01
E. cloacae	3.97	3.82	0.14	0.45	3.33	4.39	−1.06	<0.01	3.75	1.00	2.75	<0.01
E. sakazakii	5.43	5.80	−0.35	<0.01	2.86	3.24	−0.38	<0.01	4.93	1.00	3.93	<0.01
E. carotovora	5.42	5.54	−0.12	0.27	2.59	1.00	1.59	<0.01	1.65	1.00	0.65	<0.01
E. coli	5.91	5.82	0.09	0.51	5.60	4.95	0.65	<0.01	4.82	1.00	3.82	<0.01
H. alvei	3.11	3.49	−0.39	<0.01	1.00[b]	1.13	−0.13	0.20	1.00	1.00	0.00	1.00
K. oxytoca	5.63	5.75	−0.13	0.65	6.11	6.73	−0.62	0.04	6.13	1.52	4.61	<0.01
K. pneumoniae	3.51	5.51	−2.01	<0.01	5.73	6.01	−0.28	0.30	5.90	1.33	4.57	<0.01
M. morganii	3.62	3.58	0.04	0.89	4.63	4.86	−0.23	0.43	5.59	1.67	3.92	<0.01
P. agglomerans	5.43	5.41	0.02	0.88	6.89	6.20	0.69	<0.01	4.45	1.00	3.45	<0.01
P. mirabilis	5.40	5.63	−0.23	0.16	6.08	6.08	0.00	0.98	6.16	1.26	4.90	<0.01
S. marcescens	4.94	4.97	−0.04	0.83	5.15	4.45	0.70	<0.01	5.24	1.00	4.24	<0.01

[a] CFU values were analyzed after inoculation (t_0) and 14 days (t_1) and 60 days (t_2) later. Log CFU are mean values ($n = 3$).
[b] Below the detection limit (10 CFU g^{-1} grain).

species. Cell concentrations were enumerated with a hemocytometer. Controls contained only yeast or only bacteria. One group of treatment tubes was opened immediately (t_0), one was incubated for 14 days (t_1), and one was incubated for 60 days (t_2), at 25°C. All treatments were prepared in triplicate ($n = 3$).

After opening the minisilos, samples were suspended and homogenized as described previously (11, 15). The homogenates were serially diluted and spread on selective media to enumerate yeast (malt extract agar [Oxoid Ltd., Cambridge, United Kingdom]; incubation at 25°C for 2 to 4 days) and *Enterobacteriaceae* (violet red bile agar [Oxoid Ltd.]; incubation at 30°C for 24 h) (11). Yeast and bacterial counts were expressed as mean log CFU g^{-1} cereal grain (dry weight). The measured variable was analyzed by a two-way model, with mixtures (cocultures of *Enterobacteriaceae* strains and *Hansenula anomala* or *Enterobacteriaceae* strains only) and time (0, 14, and 60 days) as fixed factors. The numerical calculations were performed by the Proc GLM (general linear model) module of the SAS statistical package, and differences between treatments were regarded as significant for *P* values of <0.05 (26).

Yeast counts increased to 10^7 CFU g^{-1} grain after 14 days and continued to yield the same value after 60 days; the mixtures or controls did not differ significantly (data not shown). All tested bacterial species except for *E. carotovora*, *C. freundii*, and *H. alvei* were able to survive to a considerable extent in the grain storage system (Table 1). The cell numbers of several species apparently decreased after transfer from the preculture to the grain storage system. However, six species yielded approximately 10-fold increases in CFU during incubation in pure culture, *viz*, *E. aerogenes*, *K. oxytoca*, *K. pneumoniae*, *M. morganii*, *P. agglomerans*, and *P. mirabilis*. Other species, such as *E. cloacae*, *E. sakazakii*, and *E. coli*, maintained constant cell numbers or recovered after an initial drop in cell number (Table 1). No bacterial growth was found in noninoculated controls. The fact that most strains were able to survive or grow represents a hygiene risk for storage of moist feed grain. Although most strains are probably harmless, *Enterobacteriaceae* species can be involved in pathogenic processes in humans, animals, or plants. For example, specific serovars of *E. coli* may be harmful and cause food poisoning (see above) (4), *Klebsiella* spp. cause pneumonia (23), and *E. sakazakii* may cause meningitis (5). *S. marcescens* is found on starch-rich foods, and similar to *E. cloacae*, *P. mirabilis*, *M. morganii*, and *E. aerogenes*, it may cause urinary tract infections (25, 27). *P. agglomerans* is regarded as a plant pathogen and may cause large economic losses. It is also known to cause monoarthritis (3), but in certain settings it is used as a biocontrol agent on apples (12). Our results demonstrate that there is an urgent demand for an agent to control the growth of *Enterobacteriaceae* in moist cereal feed grain.

When *H. anomala* was coinoculated on the grain, the *Enterobacteriaceae* species that were previously able to survive in the storage system were inhibited. For *E. aerogenes*, *E. carotovora*, *E. coli*, and *P. agglomerans*, a significant inhibitory effect was already observed after 14 days. After 60 days, the CFU of all tested species were reduced significantly, most of them to below the detection level; in cases where bacteria were still countable, the CFU number was <1% of the control level.

We tested an additional 29 *H. anomala* strains against *E. coli* ATCC 11775 in the test storage system. In all cases, *E. coli* CFU numbers were reduced to 1 to 10% of the initial value after 4 weeks of storage and to below the detection level after 8 weeks (results not shown), suggesting that the anti-*Enterobacteriaceae* effect is common to the species *H. anomala*.

The mechanism of enterobacterial inhibition is not yet clear. The antibacterial activity of *H. anomala* acts much more slowly than its suppression of molds, which typically is observed within a few days (7). On the other hand, its bacterial inhibition was ultimately stronger, reducing the majority of species to below the detection level. This may point to a different mode of inhibition or to different responses of bacteria and molds to the antimicrobial activity of *H. anomala*. Polonelli and Morace (24) demonstrated an antibacterial action of *H. anomala* killer proteins in agar plate assays. Such proteins could possibly be involved in the inhibition of bacteria on cereal grain; however, because *H. anomala* forms distinct colonies on cereal grains (13), the proteins would have to be soluble and spread to the whole system by diffusion in the surface moisture around the grains. With respect to the substantial inhibition of *P. agglomerans* and *E. coli* in our earlier study, where the moisture content of the grain was only 18% (14), it

seems unlikely that killer proteins are the sole mechanism, if they play any role at all. It has been suggested that the volatile ester ethyl acetate is involved in antimold activity (6, 8). An antibacterial activity of ethyl acetate has been shown (2), but to our knowledge, no further studies are available.

We have previously shown that the microbial flora in airtight stored moist grain can vary substantially between different farms. The microbial population dynamics in those systems is poorly understood (17). Our study shows that potentially pathogenic bacteria can also grow in the system. To utilize the advantages of the storage system—saving energy and improving the nutritional content of the grain—it is necessary to control the microbial flora in the system by adding an appropriate starter culture. The yeast *H. anomala* is a strong candidate for such a starter organism, as it also confers both nutritional improvement and broad antimold and antibacterial activities. Identifying the antibacterial mechanism of this yeast may result in alternative methods to combat bacterial strains without using antibiotics.

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