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Short communication

Biocontrol of dairy moulds by antagonistic dairy yeast *Debaryomyces hansenii* in yoghurt and cheese at elevated temperatures ☆

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ABSTRACT

Some strains of the yeast *Debaryomyces hansenii* are known to be antagonistic toward moulds. In this study, we describe the inhibitory effects of a dairy strain of *D. hansenii* as a biocontrol agent against a number of dairy moulds in plain yoghurt and cheese under non-refrigerated conditions. This antagonistic yeast showed inhibition of growth of the following dairy moulds: *Aspergillus* sp., *Byssochlamys fulva*, *Byssochlamys nivea*, *Cladosporium* sp., *Eurotium chevalieri*, *Penicillium candidum* and *Penicillium roqueforti*. However, the inhibitory effect of this antagonistic yeast against dairy moulds is dependent upon the concentration of the moulds: the lower the concentration, the more effective the yeast. The findings of this study have implications for both cheese maturation and dairy biopreservation. Good manufacturing practice and hygiene to keep the contaminant load down is essential for the prevention of dairy spoilage.

1. Introduction

Yeasts have a long history of proven safe use in food and beverage fermentations (Jakobsen & Narvhus, 1996). There are few, known pathogenic yeasts that cause human infections (Fleet, 1990). Some yeasts exhibit antagonistic activity toward other microorganisms such as moulds and other yeasts by producing mycocins (killer toxins) (Magliani, Conti, Gerloni, Bertolotti, & Polonelli, 1997). This property has been well studied and exploited in the biological control of postharvest diseases of fruits (Fleet, 2003; Spadaro & Gullino, 2004). Some non-antagonistic (non-mycocinogenic) yeasts can inhibit growth of other microorganisms by competing for nutrients and space (Droby, Chalutz, Wilson, & Wisniewski, 1989; Spadaro & Gullino, 2004).

There have been no reports of using yeasts as biocontrol agents in the dairy systems. In contrast, antagonistic yeasts have been used in the control of undesirable yeasts in the wine and brewing industry (Magliani et al., 1997; Vaughan, O'Sullivan, & van Sinderen, 2005). There are a range of yeasts that are antagonistic toward other yeasts, moulds and even bacteria and these antagonistic yeasts are mainly concentrated in the genera of *Kluyveromyces*, *Pichia*, *Saccharomyces* and *Williopsis* (Hernandez et al., 2008; Izgu & Altinbay, 1997; Lefyedi & Taylor, 2007; Magliani et al., 1997; Michalcakova, Sulo, & Slavikova, 1993; Suzzi, Romano, Ponti, & Montuschi, 1995).

Debaryomyces hansenii is a yeast with applications in biotechnological processes such as dairy and meat fermentations, synthesis of fine chemicals and production of lytic enzymes (Breur & Harms, 2006). This yeast is an important secondary ripening culture for smear (surface)-ripened soft cheeses (Bonaiti, Leclercq-Peerlat, Latrille, & Corrieu, 2004).

Information on the antagonistic activity of dairy strains of *D. hansenii* is scarce. Only one dairy strain of *D. hansenii*, along with several non-dairy strains of the same yeast, is able to inhibit mycelial growth and sporulation of *Penicillium roqueforti* in cheese agar assays (van den Tempel & Jakobsen, 2000). A report indicates that a dairy strain of *D. hansenii* inhibits the germination of the spores of *Clostridium tyrobutyricum* and *Clostridium butyricum* (Fatichenti, Bergere, Deiana, & Farris, 1983).

The antagonistic activity of *D. hansenii* of non-dairy origins is relatively well known, especially among those of plant origin. Addis, Fleet, Cox, Kolak, and Leung (2001) report the anti-yeast activity of several strains of *D. hansenii* isolated from salted fish, whereas those dairy strains of the same yeast do not show anti-yeast activity. The antagonistic activity among strains of *D. hansenii* of plant origin appears to be common, showing inhibition of moulds on fruits, especially against *Penicillium* (Droby et al., 1989; Fleet, 2003; Wilson & Wisniewski, 1989).

There exists a need to develop natural alternatives such as biocontrol agents to chemical preservation and protective cultures appear to be promising for the biological control of spoilage microorganisms. To the best of our knowledge, the deliberate use of antagonistic yeasts to control spoilage moulds in dairy products has not been explored. In this paper, we report on using the

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antagonistic dairy yeast *D. hansenii* as a biocontrol agent against a range of dairy moulds in plain yoghurt and cheese under non-refrigerated conditions.

2. Materials and methods

2.1. Microorganisms, media and culturing conditions

The microorganisms used in this research were from Fonterra Research Centre. These included the yeast *D. hansenii* B9010 and the moulds, *Aspergillus* sp., *Byssochlamys fulva*, *Byssochlamys nivea*, *Eurotium chevalieri*, *Cladosporium* sp., *Penicillium candidum* and *P. roqueforti*. *D. hansenii* B9010 was identified as an antagonistic yeast in a preliminary screening study (data not shown). The mixed yoghurt culture MY-900 was from Danisco.

Yeasts were grown in YEPD broth comprised of the following components (% w/v): Bacto-yeast extract (Difco), 1.0; Bacto-peptone (Difco), 1.0; dextrose (Merck), 2.0. The ingredients were dissolved in deionised water and pH was adjusted to 5.0, followed by autoclaving at 121 °C for 15 min. Yeast cultures were incubated at 30 °C for 24 h with aeration (150 rpm). Yoghurt cultures were grown in reconstituted skim milk (RSM), which was pre-autoclaved at 115 °C for 15 min and were incubated at 37 °C for 24 h.

2.2. Inhibition by D. hansenii against moulds in plain yoghurt

Six bottles of pre-sterilised RSM (100 mL each) were inoculated with 1% v/v of yoghurt culture MY-900 (a blend of *Streptococcus thermophilus* and *Lactobacillus bulgaricus*). This was followed by adding moulds to each bottle of RSM at concentrations of 10^2, 10^4 or 10^6 spores/mL. One of the RSM bottles at each spore level was then inoculated with 1% v/v of the antagonistic yeast *D. hansenii* B9010 (treatment) and the other bottle without the yeast B9010 (control). Inoculated RSMs were incubated at 30 °C for up to 14 days. Growth of moulds was indicated by the formation of mould colonies on the yoghurt surface.

2.3. Inhibition by D. hansenii against moulds on cheese

This experiment was conducted based on the procedure of Droby et al. (1989) with modifications. A 10-mm diameter well cutter was used to cut 36 wells on a block of cheese (500 g size) with 5-mm depth. Three hundred microliter of the antagonistic yeast broth culture (*D. hansenii* B9010) was inoculated into 18 wells on one half of the cheese block. The cheese block was loosely covered with tinfoil and was air-dried for 2 h. Two hundred microliter of the yeast culture was then drawn off from each well. Mould spore suspension was diluted with sterile deionised water to give spore concentrations of 10^2, 10^5 and 10^8 spores/mL. One hundred microliter of mould spore suspension was inoculated into the 36 wells on the cheese block with six wells for each concentration. The cheese block was then covered with ethanol-soaked tissues and tinfoil to prevent contamination and drying during incubation. The inoculated cheese block was incubated at 20 °C for up to 14 days. Growth of moulds was indicated by the formation of mould colonies in the wells on the cheese.

3. Results and discussion

In this research, we report on the use of an antagonistic dairy yeast *D. hansenii* B9010 to inhibit growth of dairy spoilage moulds in yoghurt and cheese stored under non-chilled conditions. This antagonistic yeast is non-fermentative, and therefore, is not expected to grow in the plain yoghurt and cheese but may grow on cheese surface.

3.1. Inhibition of spoilage moulds by D. hansenii B9010 in yoghurt

As shown in Table 1, the antagonistic yeast B9010 significantly inhibited or retarded growth of the target moulds: *Aspergillus*, *Byssochlamys*, *Eurotium*. The inhibitory effect was dependent on mould species and mould spore concentrations: the lower the concentration, the more effective the yeast. Filtrate of the yeast B9010 was also effective in inhibiting *Aspergillus*, suggesting that mycocin might be involved. Surprisingly, there was no growth of the target mould *B. nivea* at 10^4 spores/mL without yeast B9010, whereas growth occurred in the presence of the yeast. This could be due to insufficient mixing of spores with the milk so that there were no spores present near or on the surface.

The production of mycocins by *D. hansenii* appears to be varied with strains within the species. Droby et al. (1989) report that mycocin is not involved in the inhibition of *Penicillium digitatum* by *D. hansenii* on grapefruit and the authors attribute the inhibitory effect to competition for nutrients and space by the antagonistic yeast. However, other strains of *D. hansenii* indeed produce mycocins that inhibit cell wall synthesis (Santos, Marquina, Barroso, &

Table 1
Inhibition by antagonistic yeast *D. hansenii* B9010 (inoculated at ~10^6 cfu/mL) against dairy moulds (inoculated at 10^2, 10^4 and 10^6 spores/mL) in plain yoghurt incubated at 30 °C[a].

	10^2 spores/mL		10^4 spores/mL		10^6 spores/mL	
Day	−B9010	+B9010	−B9010	+B9010	−B9010	+B9010
Aspergillus sp.						
1	–	–	–	–	–	–
2	–	–	–	–	+	–
3	–	–	++	+	+++	+
6	++	–	+++	+++	+++	++
7	+++	–	+++	+++	+++	+++
8	+++	–	+++	+++		
9	+++	–	+++	+++		
13	+++	–	+++	+++		
Byssochlamys fulva						
2	–	–	–	–	–	–
5	–	–	+++	–	+++	+++
6	+	–	+++	–	+++	+++
9	++	–	+++	+	+++	+++
12	+++	–	+++	++	+++	+++
14	+++	–	+++	+++	+++	+++
Eurotium chevalieri						
1	–	–	–	–	–	–
4	+	–	++	++	+++	+++
5	++	–	++	++	+++	+++
8	++	–		++		+++
11	++	–		+++		+++
13	++	–		+++		+++
14	++	–		+++		+++
Byssochlamys nivea						
1	–	–	–	–	–	–
2	–	–	–	–	–	–
3	–	–	–	–	–	–
6	+	–	–	–	+	–
7	+	–	–	+	+	+
8	++	–	–	+	+	+
9	++	–	–	+	+	+
13	+++	+	–	++	+	+
Aspergillus sp.[b]						
1	–	–	–	–	+	–
4	–	–	–	–	++	+
7	+	–	+	–	+++	++
9	+	–	+	–	+++	+++
10	+	–	+	–	+++	+++

[a] Growth was indicated by mould colony formation: –, no colony formation; +, slight colony formation; ++, moderate colony formation; +++, abundant colony formation.
[b] Filtrate of antagonistic yeast *D. hansenii* B9010 was used instead of whole cells.

Peinado, 2002). It is to be verified whether *D. hansenii* B9010 produces mycocin.

Besides mycocins and competition for space and nutrients discussed above, there may be other mechanisms for the anti-mould activity of the yeast *D. hansenii* B9010. It is possibly through the action of aroma compounds carried over from broth cultures or excreted in yoghurt and cheese, even though this yeast was not growing in the dairy system. Many aroma compounds such as aldehydes and esters have anti-fungal properties (Arroyo, Moreno, Daza, Boiaznova, & Romero, 2007; Belletti et al., 2007; Fan, Song, Beaudry, & Hildebrand, 2006; Lanciotti et al., 2004).

3.2. Inhibition of spoilage moulds by D. hansenii B9010 on cheese

As shown in Table 2, there was significant delayed or reduced growth of moulds in the cheese with the added yeast B9010, compared with the control (no added yeast B9010). This yeast was effective against growth of *Aspergillus*, *B. nivea*, *Cladosporium*, *P. candidum* and *P. roqueforti*. Concentration of mould spores was important to the effectiveness of the yeast. This inhibition of moulds could be due to a combination of competition for space and nutrients due to growth of the yeast B9010 and production of metabolites such as mycocin, because this aerobic yeast was expected to grow in the wells on the cheese surface.

Antagonistic yeasts may impact on cheese ripening and quality (mould-ripened and smear-ripened cheeses). The inhibition of *P. candidum* and *P. roqueforti* by the antagonistic yeast *D. hansenii* B9010 is consistent with the finding by van den Tempel and Jakobsen (2000) and this is of particular concern, because the two moulds are central to the maturation of mould-ripened cheeses. If antagonistic yeasts are present, growth of moulds may be retarded or even inhibited and this can adversely affect cheese ripening and quality. It is important to select non-antagonistic yeasts as secondary cultures for mould-ripened cheeses, even smear–ripened cheeses.

The inhibition of moulds by the dairy strain B9010 of *D. hansenii* is consistent with studies on non-dairy strains of *D. hansenii* elsewhere. It has been reported that wine killer yeasts can inhibit a range of moulds (Suzzi et al., 1995). The inhibition of moulds by antagonistic yeasts appears to be a common feature and has been well studied in the biocontrol of postharvest diseases of fruits (Fleet, 2003; Spadaro & Gullino, 2004; Wilson & Wisniewski, 1989).

The use of biocontrol agents such as protective cultures alone may not be sufficient to eliminate food spoilage due to yeasts and moulds. A combination of biocontrol agents and preservatives at a reduced dose should help minimise such spoilage in conjunction with good manufacturing practice. Therefore, the use of biocontrol agents can at least help reduce the usage of chemical preservatives, if not totally replacing them. Furthermore, some antagonistic yeasts may possess probiotic properties and as such, can provide probiotic benefits when used as a biocontrol agent. This area certainly merits more research.

Table 2
Inhibition by antagonistic yeast *D. hansenii* B9010 against dairy moulds (inoculated at 10^2, 10^5 and 10^8 spores/mL) on cheese incubated at 20 °C[a].

	10^2 spores/mL		10^5 spores/mL		10^6 spores/mL	
Day	−B9010	+B9010	−B9010	+B9010	−B9010	+B9010
Aspergillus sp.						
5	0/6	0/6	0/6	0/6	0/6	0/6
7	0/6	0/6	0/6	0/6	3/6	0/6
8	0/6	0/6	1/6	0/6	3/6	0/6
9	0/6	0/6	1/6	0/6	3/6	0/6
14	1/6	0/6	1/6	0/6	3/6	0/6
Penicillium candidum						
5	0/6	0/6	0/6	0/6	0/6	0/6
7	0/6	0/6	6/6	0/6	1/6	1/6
8	1/6	0/6	6/6	0/6	3/6	1/6
9	1/6	0/6	6/6	0/6	4/6	1/6
14	1/6	1/6	6/6	1/6	4/6	3/6
Penicillium roqueforti						
5	0/6	0/6	0/6	0/6	0/6	0/6
7	0/6	0/6	2/6	0/6	2/6	0/6
8	0/6	0/6	5/6	0/6	2/6	0/6
9	1/6	0/6	5/6	0/6	3/6	0/6
14	1/6	0/6	5/6	0/6	3/6	0/6
Byssochlamys nivea						
5	0/6	0/6	0/6	0/6	0/6	0/6
7	0/6	0/6	0/6	0/6	1/6	0/6
9	0/6	0/6	0/6	0/6	3/6	1/6
12	0/6	0/6	0/6	0/6	3/6	1/6
14	0/6	0/6	0/6	0/6	3/6	1/6
Cladosporium sp.						
1	0/6	0/6	0/6	0/6	0/6	0/6
4	0/6	0/6	0/6	0/6	2/6	0/6
5	0/6	0/6	0/6	0/6	3/6	0/6
8	0/6	0/6	0/6	0/6	3/6	0/6
11	0/6	0/6	0/6	0/6	3/6	0/6
12	0/6	0/6	0/6	0/6	4/6	0/6
14	4/6	0/6	2/6	0/6	4/6	0/6

[a] The values given indicate the number of wells with mould colonies out of a total of six inoculated wells.

4. Conclusion

D. hansenii is effective as a biocontrol agent to inhibit growth of several dairy moulds in yoghurt and on cheese at non-refrigerated temperatures. However, the initial mould population is critical to the effectiveness of *D. hansenii* as a biocontrol agent. It is important to keep the load of contaminating moulds as low as possible through good manufacturing practice and hygiene.

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