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Inhibition of *Brevibacterium linens* by Probiotics from Dairy Products

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Summary

Brevibacterium linens is an important species in dairy products rendering a specific taste and aroma to numerous smear ripened and blue veined cheeses due to proteolysis. However, the presence of the species in South African blue veined cheeses is undesirable and consumers demand the product void of the species. Accordingly, numerous methods including microbial inhibition using fungi and bacterial probiotic cultures with possible inhibitory effects were applied in an attempt to inhibit the species. None of the fungi, however, proved to be successful, whereas *Lactobacillus rhamnosus* and *Bifidobacterium lactis*, two typical probiotic species applied in dairy products, showed inhibitory effects against *B. linens* when tested using the spot-on-lawn assay.

Key words: Brevibacterium linens, Lactobacillus rhamnosus, Bifidobacterium lactis, inhibition, blue cheese

Introduction

A number of European smear cheeses are ripened by a complex bacterial flora present on the surfaces of these cheeses. Well known examples of these smear cheeses include Tilsit, Romadour, Limburger, Harzer, Münster and Weinkäse (*1*). The smear, often reddish in colour, consists of yeasts and bacteria (*2*). These aerobic microorganisms have a direct impact on the flavour, texture, appearance and development of the cheeses. *Brevibacterium linens*, an important dairy microorganism predominant in the surface flora, contributes to the final surface flavour, colour and aroma due to its strong proteolytic activity as well as the production of methanethiol (*3*). The role of the species in the flavour and aroma formation during the production and maturation of cheeses has been investigated by a number of authors (*1,4–6*), who highlight the positive contribution of this species to cheese production by accelerating the ripening process (*7*).

The presence of *B. linens* is also detected on the surfaces of blue veined cheeses, usually after the *Penicillium* species have consumed the lactic acid and deacidified the cheese. The enhanced pH level encourages the aerophilic acid-sensitive bacterial flora to become established (*8*). Although the species plays an important role in the development of typical flavours due to proteolysis in these cheeses, the South African consumer in general demands all cheeses void of *B. linens*, as they neither prefer the taste created nor the colour. Consequently, large portions of the blue cheese surfaces must be cut off prior to packaging to get rid of the surface flora, resulting in huge financial losses.

Since the application of fungi as biocontrol agents proved to be successful (*9–11*) and the antagonistic action of bacteriocins in lactic acid bacteria is well known (*12,13*), attempts to apply these organisms as natural inhibitors of *B. linens* seemed logical. Accordingly, in this study we endeavoured to find a natural means of inhibiting the growth of *B. linens* using selected yeasts from

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dairy products and lactic acid bacteria with probiotic activity. These organisms were selected because, if positive, should have no or limited effect on the final blue cheese product.

Materials and Methods

Media

All media were prepared in accordance with the manufacturer's instructions and autoclaved at 121 °C for 15 min. Aliquots of between 15 and 20 mL of sterile media were dispensed into 90-mm Petri dishes and allowed to dry overnight at room temperature. The experimental media included: plate count agar (PCA) (Biolab diagnostics, Merck) and De Man Rogosa Sharpe agar (MRS) (Biolab diagnostics, Merck) for lactic acid bacteria. In accordance with Toolens and Koning-Theune (*14*), Lab Lemco agar with 0.5 % glucose, 0.5 % $CaCO_3$, 5.5 % NaCl and 0.02 % pimafucin (LC-GS) was used for the isolation and enumeration of *B. linens*. LC-GS was incubated at 25 °C for 5 to 7 days.

Microbial interactions

The spot-on-lawn assay (*15*) was used to examine interactions between microorganisms and *B. linens* cultures. The basal medium used was either tryptone soy agar (TSA) or PCA agar for bacteria and malt extract agar for yeasts (MEA) (Oxoid). The respective basal medium was prepared and kept in a molten state at 50 °C. Freshly grown (24–48 h) cultures were seeded at 10^5–10^6 CFU/mL into the molten medium and poured into Petri dishes. After cooling and solidification, the medium was spot-inoculated on the surface with freshly grown (24–48 h) inoculum species. On each occasion, control agar plates containing the seeded species were handled similarly but without the spot inoculation. All plates were incubated at 25 °C and observed after 1, 3, 5, 7 and 9 days. Inhibition of the seeded organism by the spot-inoculated isolate was evident as a clear zone surrounding the growing spot culture.

All yeast cultures, indicated as predominant species associated with dairy products, were selected based on previous studies (*16*). These cultures were obtained from the yeast culture collection at the University of the Free State, Bloemfontein, South Africa and maintained on yeast malt agar slants.

Lactobacillus rhamnosus and *Bifidobacterium lactis* (Wisby, Danisco Cultor Niebüll GmbH, Germany) were reactivated from the freeze-dried state by incubating in 10 mL of sterile UHT low fat milk for 6 h at 43 °C. The cultures were subsequently streaked out on MRS agar and further incubated at 43 °C under anaerobic conditions for 24 h.

Ten orange-reddish coloured cultures were isolated from blue-mould cheeses produced locally, using LC-GS media as described by Toolens and Koning-Theune (*14*) and identified as representatives of *Brevibacterium linens* according to Bergey's Manual of Determinative Bacteriology (*17*). *B. linens* (NCDO 1002) obtained from the National Collection of Dairy Organisms was used in addition to the strains isolated. The cultures were streaked out on PCA agar and incubated at 25 °C for 24–48 h until sufficient growth was observed.

Results and Discussion

Yeasts have been reported to stimulate the growth of lactic acid bacteria, *B. linens* and moulds in cheeses (*6,18–21*). Yeasts also act as antagonistic organisms, applied as natural biocontrol organisms (*9–11*) against fruit rotting and the inhibition of *Clostridium* species in dairy products (*22*). Consequently, different yeasts, moulds and probiotics associated with dairy products were examined as either the inhibitor or sensitive counterpart against *B. linens* (Table 1).

As determined by the spot-on-lawn assay, none of the yeasts inhibited or reduced the growth of *B. linens* (Table 1). However, it is interesting to note that *Zygosaccharomyces rouxii*, *Z. mellis*, *Debaryomyces anomala* and *D. bruxellensis* were almost all completely inhibited by *B. linens*. Only *Trichosporon beigelii*, *Cryptococcus albidus* and *Yarrowia lipolytica* remained unaffected by *B. linens*. Other species including *D. hansenii*, *Candida intermedia*, *C. zeylanoides*, *C. rugosa*, *Rhodotorula glutinis* and *R. mucilaginosa* were slightly affected by *B. linens.*

The *Lactobacillus rhamnosus* and *Bifidobacterium lactis* strains, both considered as probiotic organisms and frequently applied in dairy products, showed strong inhibition of *B. linens* (Table 2). However, *B. linens* showed

Table 1. The mean of results obtained from triplicate experiments carried out to determine the efficiency of the inhibition of *B. linens* growth by selected yeast species

Inhibition by *B. linens*	Rating per day				Test species	Inhibition of *B. linens*	Rating per day			
Positive or negative	3	5	7	9		Positive or negative	3	5	7	9
–	2	1	1	1	*Cryptococcus albidus*	–	1	1	1	1
+	3	2	2	2	*Candida intermedia*	–	1	1	1	1
+	2	2	1	1	*Candida rugosa*	–	1	1	1	1
+	3	3	2	2	*Candida sake*	–	1	1	1	1
+	3	2	2	2	*Candida zeylanoides*	–	1	1	1	1
+	4	4	4	4	*Debaryomyces anomala*	–	1	1	1	1
+	4	4	3	3	*Debaryomyces bruxellensis*	–	1	1	1	1

Inhibition by *B. linens*	**Rating per day**				**Test species**	**Inhibition of *B. linens***	**Rating per day**			
Positive or negative	***3***	***5***	***7***	***9***		Positive or negative	**3**	**5**	**7**	**9**
+	2	2	2	2	*Debaryomyces hansenii*	–	1	1	1	1
+	3	3	3	3	*Kluyveromyces lactis*	–	1	1	1	1
+	3	3	3	3	*Kluyveromyces marxianus*	–	1	1	1	1
+	3	3	3	3	*Pichia haplophila*	–	1	1	1	1
+	3	2	2	2	*Rhodotorula glutinis*	–	1	1	1	1
+	3	3	3	3	*Rhodotorula minuta*	–	1	1	1	1
+	3	2	2	2	*Rodotorula mucilaginosa*	–	1	1	1	1
+	3	3	3	2	*Saccharomyces cerevisiae*	–	1	1	1	1
–	1	1	1	1	*Trichosporon beigelii**	–	1	1	1	1
+	3	3	2	2	*Torulaspora delbrueckii*	–	1	1	1	1
–	1	1	1	1	*Yarrowia lipolytica*	–	1	1	1	1
+	2	3	3	3	*Zygosaccharomyces florentinus*	–	1	1	1	1
+	4	5	5	4	*Zygosaccharomyces mellis*	–	1	1	1	1
+	5	5	5	5	*Zygosaccharomyces rouxii*	–	1	1	1	1

**Trichosporon cutaneum* var. *cutaneum*
5 – complete inhibition; 4 – notable inhibition; 3 – average inhibition; 2 – slight inhibition; 1 – no inhibition

Table 2. The mean of results obtained from triplicate experiments carried out to determine the efficiency of the inhibition of *B. linens* growth by selected probiotic and mould species

Test species/ sample	**Inhibition of *B. linens***	**Rating per day**					**Comment**
		1	**3**	**5**	**7**	**9**	
Lactobacillus rhamnosus	+	5	5	4	4	3	initial, complete inhibition
Bifidobacterium lactis	+	5	5	5	5	5	complete inhibition
Penicillium candidum	–	1	1	1	1	1	no inhibition
Penicillium camemberti	–	1	1	1	1	1	no inhibition
Penicillium caseicolum	–	1	1	1	1	1	no inhibition

5 – complete inhibition; 4 – notable inhibition; 3 – average inhibition; 2 – slight inhibition; 1 – no inhibition

reduced sensitivity to *L. rhamnosus* after approximately one week of incubation. In contrast, *B. lactis* continued to inhibit *B. linens* well for three weeks. *L. casei* var. *rhamnosus* is known to produce bacteriocins such as caseicin 80 and caseicin LHS, both heat liable bacteriocins (*23,24*), which may be responsible for the effect on *B. linens* (*25*). *Penicillium* species, added as starter cultures during the making of blue veined cheeses, have no effect on *B. linens* (Table 2).

The addition of *L. rhamnosus* and/or *B. lactis* as probiotic adjunct starters during the processing of blue-veined cheeses therefore seems a strong possibility. As indicated, both these microorganisms significantly inhibited the growth of *B. linens* on agar plates, and may also inhibit *B. linens* during the making of blue cheese. Despite these being preliminary experiments, the current data open the door for the application of probiotics in blue veined cheeses. Both species have been accepted as beneficial probiotics, which allows their incorporation into dairy products (*26–29*). Further investigation needs to focus on the application of *L. rhamnosus* and/or *B. lactis* at plant scale level into blue-mould cheese and its subsequent impact on the product.

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Inhibicija *Brevibacterium linens* probioticima u mliječnim proizvodima

Sažetak

Brevibacterium linens je zbog svoje proteolitičke aktivnosti važna bakterija koja daje specifičan okus i miris mnogim polumekim i plavo prošaranim sirevima. Unatoč tome, nepoželjna je u južnoafričkim plavo prošaranim sirevima, već se sve više traži sir bez *B. linens*. Stoga je ispitana mogućnost inhibicije rasta *B. linens* s različitim fungima i bakterijskim probiotičkim kulturama. Primjena funga nije bila uspješna za razliku od dviju tipičnih probiotičkih vrsta, *Lactobacillus rhamnosus* i *Bifidobacterium lactis*, koje su inhibirale *B. linens*, što je dokazano testom »spot-on-lawn«.