

Sluttrapport 1998 –2002 og Årsrapport for 2002

Prosjekt nummer: P98089

Prosjekt navn: *Dairy bacteriocins*

Del 1: Sammanfattning

Part 1: Summary

A: Most important results

- Approximately 130 isolates of propionibacteria (PAB) has been isolated and taxonomically characterized (16S RNA DNA sequences , metabolic and phenotypic properties) and a PAB culture collection has been established that is available to all participants. All the strains is also distributed among the four participants for functional studies. The PAB culture collection has been established as a resource for the participants to be used in the project and this collection can also be used by the participants in future collaborator work and application. The eventual commercial use of these cultures is managed by a contract that has been signed by all partners.
- The antimicrobial potency of the PAB strains has been evaluated and is finished. Arla has investigated the antifungal activity. Danisco has investigated the inhibitory potency of PAB against Gram negative bacteria. Tine/NLH have tested the antagonistic activity against Gram-positive bacteria. The results clearly demonstrate that a large number of the PAB isolates are able to kill /inhibit growth of many microorganisms.
- It has been shown that many PAB are able to produce CLA (conjugated linoleic acid) some at high concentration.
- Important industrial properties such as tolerance to salt, growth and growth media, temperature etc. have been investigated
- Two PAB bacteriocins have been purified and characterized on the amino acid level and also the bacteriocin genes have been sequenced (see references). This is the first PAB bacteriocins that has been characterized on such detailed level. In addition two more antimicrobial compounds have been isolated and partly characterized .In order to apply the culture in food an extensive study of functional growth characteristics of selected PAB have been performed. The effect of temperature, pH and salt on growth kinetics has been elucidated.
- 4-7 potential interesting PAB for commercial use have been identified and testing of these are under way.
- The cheese trials have been finalized at Tine and Arlafoods with some of the new isolated PAB. Results have been obtained that will lead to additional trials in future cheese trials in order to establish new combination of starter cultures. In particular the new strains T5 and T114 showed promising results to be implemented in future cheese products.
- Some PAB isolates in combination with certain lactobacilli have been shown produce strong antifungal / antimould activity relevant for problems related to cheese.
- Inhibition of moulds has also been found with PAB strain LMG2868 and with a combination of PAB DC2053 in combination with Lb. Rhamnosus Lc705 in yogurt simulated conditions.
- One PhD student (Dag A. Brede) is associated to the project and NI funds will contribute significant to his PhD thesis. One person (Inger Ødegård at TINE) has finished her cand. Scient. thesis within the NI project

During the time period of the project two projects meeting per year have been organized at the site of the individual participants.

The project has been delayed due to maternal leave and reorganization of the R&D division at two of the participant` organizations. But by extending the project by one year (2002) all the

experiments that were planned have been finished. In particular, the combinations of certain PAB and certain lactobacilli have shown to exert strong antimould activity.

B: The technology in food fermentation is very much the same and by sharing the R&D cost in a NI project, one may achieve more information, competence and innovation for lower cost. In addition different expertise among the various participants leads to higher quality in R&D and more results for less cost. We are also transferring knowledge between the participants. Such synergistic effect cannot be achieved within national borders.

Fermented food in general and dairy products in particular has long and strong tradition in the Nordic countries and some of the products are competitive on the international market. For some of the Nordic produced cheeses and increase international demand is seen by increase export and licensed production of Nordic cheeses abroad. In order to comply with the increasing demand of increased safety and improved quality it is of crucial importance to have a strong R&D activity going. The present project, though very basic, is an important contribution to keep up with such demands from the consumers both nationally and internationally.

The project has also improved cooperation between the involved participants and at the final meeting in January 2003 it was agreed by some of the industrial partners to continue the investigation of making new starter cultures commercially available

Comments:

The use of starter cultures in preparing fermented food and to assure food safety also related to the concept "protective culture" is an important concept that has been used for a long time but is also in continuous improvement and the project is an important contribution for developing new bacteria to be used within this area. The present project is also developing alternative uses of starter cultures/protective cultures. The Nordic food fermentation industries were among the very first to develop and apply starter cultures. Today they are producing high quality fermented cheeses and food products intended for the home market, which have, also, been successfully exported to other countries. Also establishment of companies producing starter cultures commercially was developed early in Scandinavia and the present project is dedicated to further development of the starter culture concept. In order for the Nordic countries to be among the best and being competitive with respect to new developments of fermented foods, a joint effort is needed.

Del 2: Informations och resultatspridning

The information work carried out is according to our plans, by publishing and presentation of information within accepted agreements with the delay stated in this report. Internal in the group we have an open communication and feed-back on our work. In addition we have promoted our concepts/work to various public channels as indicated in the table below. It should also be emphasized that some information is confidential and cannot be externally communicated due to industrial interests of the participants. The partners have signed a written agreement about confidentiality and about use of the bacterial collection of approximately 130 PAB that has been established in the project. Several international research and review publications have been published. In addition we have presented our findings at various national and international conferences.

Publications

1. Faye T, Langsrud T, Nes I F, Holo H. (2000). Biochemical and genetic characterization of propionicin T1, a new bacteriocin from *Propionibacterium thoenii*. Appl. Environ. Microbiol. **66**: 4230-6.
3. Nes, I. F. and H. Holo. (2000). Class II Antimicrobial Peptides from Lactic Acid Bacteria. Biopolymers **55**: 50-61.
4. Holo, H., Faye, T., Brede, T.A., Nilsen, T., Ødegård, I., Langsrud, T., Brendehaug, J., Nes, I.F. (2002): Bacteriocins of propionic acid bacteria. Lait **82**: 59-68
5. Faye T, Brede DA, Langsrud T, Nes IF, Holo H. An antimicrobial peptide is produced by extracellular processing of a protein from *Propionibacterium jensenii*. (2002) J Bacteriol. 3649-3656
6. Faye T, Brede DA, Langsrud T, Nes IF, Holo H. (2003) The prevalence of the genes encoding the antimicrobial peptides propionicin T1 and PAMP and their expression in the classical propionibacteria. Submitted for publication in Appl. Environm. Microbiol.

Presentations at national and international conferences/meetings

1. Holo, H. "Single hit mechanism of LAB bacteriocins." Invited speaker at the Conference on bacteriocins of lactic acid bacteria. "Advances and applications of bacteriocins". Banff, Canada, 27.4-2.5 2000.
2. Nes, I.F., Cintas, L., Nilsen, T., Hernandez, P., Holo, H.: "Novel bacteriocins from lactic acid bacteria." Invited speaker at the Conference on bacteriocins of lactic acid bacteria. "Advances and applications of bacteriocins". Banff, Canada, 27.4-2.5 2000.
3. Nes, I. F: "Bacteriocins and their regulation." Invited speaker at the 1st. International American Society of Microbiology Conference on Enterococci. 27th Feb. – 2nd March 2000, Banff, Canada.
4. Faye, T., T. Langsrud, I. F. Nes, and H. Holo. Bakteriosiner hos Propionsyrebakterier-karakterisering, produksjon og utnyttelse. Konferansen norsk mat i internasjonal konkurranse (NFR), Oslo, 5-6 september 2000 (foredrag).
5. Faye, T., T. Langsrud, I. F. Nes, and H. Holo. Isolation of bacteriocins from *Propionibacteria*. *International Dairy Federation symposium on cheese ripening and technology*, Banff, Canada, 12-16 mars 2000 (poster).
6. Holo, H: Bacteriocins of propionibacteria. 3rd International symposium on propionibacteria. Zürich, Sveits, 8.7-11.7-2001.
7. Faye, T., Langsrud, T., Nes, I.F., Holo, H.: "Isolation and partial characterization of a protease activated antimicrobial peptide from *Propionibacterium jensenii*." 3rd International symposium on propionibacteria. Zürich, Sveits, 8.7-11.7-2001.
8. Poster presentation of the project at the Nordic Industrial Fund conference "The Future for Nordic Food, Innovation in a European Context" January 28-29 2002, Stockholm.
9. Poster presentation of the project on Research Seminar at the Agricultural University of Norway, March 1st. 2002.
10. Faye T, Brede DA, Langsrud T, Nes IF, Holo H. An antimicrobial peptide is produced by extracellular processing of a protein from *propionibacterium jensenii*
Poster presented at the Seventh Symposium on Lactic Acid Bacteria Egmond aan Zee, the Netherlands, 1-5 September 2002.

Del 3: Lista över kontaktadresser

Agricultural University of Norway
Professor Ingolf F. Nes (Project leader)
Department of Chemistry and Biotechnology
Laboratory of Microbial Gene Technology
PO Box 5051, N-1432 ÅS, NORWAY
Phone: +47 64949471 Fax: +47 64941465
Email: Ingolf.Nes@IBF.NLH.NO

ARLAFoods
Dr. Ulla Svensson
Torsgata 14, ARLA
S105 46 Stockholm, SWEDEN
Tel: +46 8 677 32 27 Fax: +46 8 20 33 29
Ulla.Svensson@arlafoods.com

DANISCO CULTOR
Dr. Dieter Elsser
Danisco Cultor Niebüll GmbH
Busch-Johannsen-Strasse I
D-25899 Niebüll, GERMANY
Tlf: +49 4661 602-261
Fax: +49 4661 602-289
gsdie@danisco.com

TINE Norske Meierier
Dr. Johanne Brendehaug
Tine Meierier
Fo-Senteret avd. Voll
P.B. 50
4060 Kleppe, NORWAY
Tlf. +47 51787562 Fax: +47 51787501
Johanne.Brendehaug@tine.no

Dr. grads studerende ansatt på prosjektet:

Cand. Scient. Dag Anders Brede, Agricultural University of Norway (NLH)

Datum: February 12, 2003

Prosjektledarens underskrift:

Namnförtydligande: Ingolf F. Nes

Telefon: 64949471

Fax: 64941465

ATTACHMENTS: Annual (2002) report from the participants

Annual report for 2002

From : Agricultural University of Norway(NLH) (cand scient Dag Anders Brede, Zhian Salehian, and professor Ingolf F. Nes (project leader), and Dr. Helge Holo).

Dr.Holo is employed by TINE but is working at NLH.

Characterization of antimicrobials from propionic acid bacteria

The inhibitory compounds produced by strains T159 and T5 have been purified, but we were not able to determine their molecular structure. During 2002, the bacteriocin from strain T29 was isolated and purified. This bacteriocin is sensitive to proteolytic enzyme and heat treatment. So far, no sequence has been obtained but the work is continued in our laboratory.

Genetic approaches.

There are several reports on bacteriocin production in PAB, but only two bacteriocins have been sequenced at the protein and DNA level. The prevalence of these bacteriocins in PAB was investigated by use of PCR. The results of the study showed that

- 1:** The gene encoding the bacteriocin propionicin T1 is widely distributed in *Propionibacterium* strains.
About 67 % of strains of *P. thoenii* and 54 % of *P. jensenii* carried the gene. Whereas all the *thoenii* strains that carried the gene produced the bacteriocin, this was the case only for 20 % of the *P. jensenii* strains.
- 2:** The gene encoding PAMP, the bacteriocin activated by proteases, was found in about 60 % of strains of *P. thoenii* and 63 % of *P. jensenii*. All these strains produced the bacteriocin.
- 3:** The strain producing the hitherto uncharacterised propionicin PLG-1 produces both propionicin T1 and PAMP.
- 4:** Propionicin T1 and PAMP are not produced by any of the *P. freudenreichii* or *P. acidipropionici* strains.

Genetic approaches have also been used to study bacteriocin production and in the search for bacteriocin encoding genes. To these aims tools for genetic transformation of PAB have been developed. A propionibacterium specific cloning vector based on published shuttle vectors has been created, the bacteriocin genes have been cloned in strains of *P. freudenreichii*, and heterologous bacteriocin production has been achieved. However, the bacteriocin production was low and the clones were unstable.

Inhibition of moulds, synergistic effects with lactic acid bacteria.

A few lactic acid bacteria have been found to be inhibitory to moulds. This inhibition is, however, not always sufficient. During this project we have isolated several propionic acid bacteria with mould-inhibitory properties, but in cheese trials none were able to suppress the growth of moulds. We have studied the effects of combining lactic acid bacteria with PAB on the inhibition of growth of mould in milk. The moulds selected were *Penicillium* spp. isolated from Norwegian cheese. One

isolate, a *Penicillium palitans*, was particularly resistant to the lactic acid bacteria. By using the PAB/LAB combinations a strong inhibition was observed. The PAB having the highest antimould activity did not cause better inhibition than the other PAB tested.

References

1. Poster presentation of NI project "Dairy bacteriocins" at the Nordic Industry Fund Conference in Stockholm. 28-30 January 2002.
2. Holo, H., Faye, T., Brede, T.A., Nilsen, T., Ødegård, I., Langsrud, T., Brendehaug, J., Nes, I.F.: Bacteriocins of propionic acid bacteria. *Lait* (2002) 82: 59-68
3. Faye T, Brede DA, Langsrud T, Nes IF, Holo H. An antimicrobial peptide is produced by extracellular processing of a protein from *Propionibacterium jensenii*. *J Bacteriol.* (2002) 3649-3656
4. Faye T, Brede DA, Langsrud T, Nes IF, Holo H. An antimicrobial peptide is produced by extracellular processing of a protein from *propionibacterium jensenii*
Poster presented at the Seventh Symposium on Lactic Acid Bacteria
Egmond aan Zee, the Netherlands, 1-5 September 2002.
5. Faye T, Brede DA, Langsrud T, Nes IF, Holo H. The prevalence of the genes encoding the antimicrobial peptides propionacin T1 and PAMP and their expression in the classical propionibacteria. Submitted for publication in *Appl. Environm. Microbiol.*

Report from Arla Foods for 2002-01-01 - 2002-12-31

Participants:

Stina Björneholm Arla Foods, Sören Lillevang Arla Foods, Ulla Svensson Arla Foods,
Helena Widholm Swedish University of Agricultural Science.

Growth of propionibacterium together with lactic acid bacteria in milk

During year 2002 the metabolism of propionic acid bacteria and lactic acid bacteria when grown together in milk has been studied.

The aim of the project was to find out more about the interactions between the two major bacterial groups participating in the cheese making process. By cultivation of 29 strains of propionibacteria together with the lactic acid bacteria of two different starter cultures in milk, we were able to investigate the influence on growth and fermentation products. Since the fermentation substances of propionibacteria are deeply involved in the characteristic of the final product, this knowledge can be used as guidelines in the choice of a good combination for cheese making.

Abstract

This project was performed in order to receive knowledge about the interaction between different propionic acid bacterial strains and commonly used commercially available mesophilic cultures. Twenty nine strains of propionibacteria were co cultured with the two starter cultures CH N11 and Probat 505 in milk. An inoculation of 10^6 bacteria/ml of each kind was added to heat treated milk and incubated at 30°C for 72 ± 1 h. The fermentation products were analysed by pH measurement, viable counts on sodium lactate (SL) agar (propionibacteria) and calcium citrate (KCA) agar (lactic acid bacteria) and organic acids by HPLC analysis. Interpretation of the viable counts of propionibacteria was difficult since the lactic acid bacteria dominated the SL plates. Comparing the two series of samples with either CH N11 or Probat 505, the pH of the supernatants from cultures with Probat 505 was generally lower. The fermentation products varied also between mixed cultures with CH N11 and Probat 505. Together with CH N11 14 cultures produced acetate but very low amounts of propionate. On the other hand, with Probat 505, no acetate was produced and higher levels of propionate could be seen. In none of the supernatants any glucose, ethanol or butyrate was visible. The results suggests that the choice of mesophilic culture during cheese-making can have a great influence on the development of the propionic acid bacteria and flavour from propionic acid.

NIF project P 98089
“DAIRY BACTERIOCINS”



Scientific Report

4th project-year / 01.11.2001 – 31.10.2002

Main task for DANISCO in the 4th project year was to test the use of the isolated propionic acid bacteria (PAB) as protective cultures in yoghurt. These strains have been chosen on basis of previously done screening tests by all project partners. Objective target of these experiments was mainly, to extend shelf life of yoghurt products by an inhibition of yeasts and moulds.

1. Methods – test design in general

For all experiments, yoghurt samples have been prepared under practical conditions and by an application of common used yoghurt starter cultures (Yoghurt 709 Pellet DIP®50u). Pasteurised and homogenized full-cream milk (3,5% fat) was inoculated by the yoghurt culture (0,1 g in 1000 ml) together with the test strains (PAB, inoculation rate: 5×10^6 cfu/ml). Batches of 2000 ml have been fermented at 42°C and the fermentation was stopped at a final pH-value of 4.5. After that, samples have been contaminated with yeasts and in further trials with different moulds. During storage tests over 4 weeks at 6°C and over 3 weeks at 10°C, pH-values have been measured and the cell densities of test strains have been detected. Furthermore, organoleptic properties such as optical appearance, consistency, mouth feeling and smell/taste of reference samples (prepared with PAB) have been tested at beginning of the storage period and after 28 days at a storage temperature of 10°C.

2. Inhibition tests with yeasts

In all, 66 trials with different combinations of test- and indicator-strains have been carried out. As indicator strains (yeasts), *Kluyveromyces marxianus* CBS1555, *Rhodotorula mucilaginosa* CFSQE 63 and *Candida pulcherrima* 1-50/13 have been added to yoghurt samples of 100 g with inoculation rates of approximately 100 cfu/ml. 7 different PAB-strains alone and in combination with two different lactic acid bacteria strains (*Lb. rhamnosus* Lc705 and *Lb. paracasei ssp. paracasei* DC20) have been added to the vat milk together with the starter cultures.

As first detectable influence of the added test strains, a shortening of fermentation time caused by some of the added PAB-strains have been measured. The addition of *Propionibacterium jensenii* 2868 (LMG) reduced the fermentation time from 5,6h to 4,7h while *Propionibacterium freudenreichii* W9 (Danisco) showed some inhibitory effect on the starter cultures (**Fig 1.**)

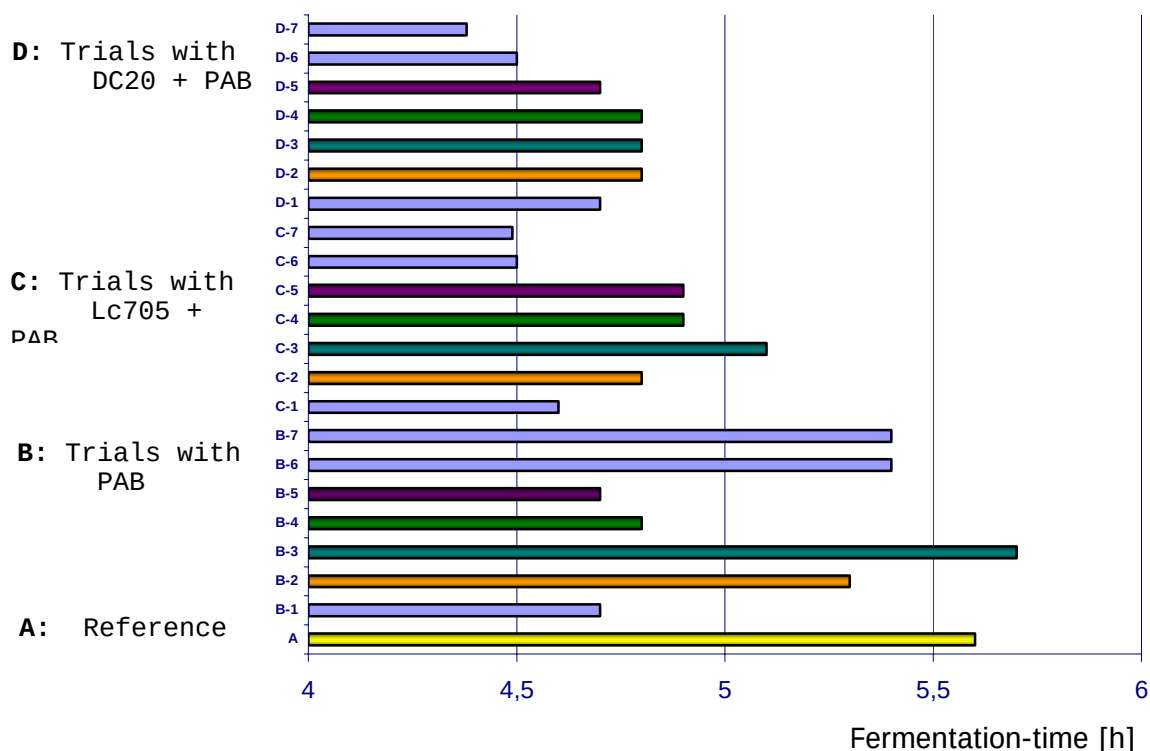


Fig. 1: Influence of test strains (PAB and combinations of PAB- with LAB-strains) on the fermentation-time of yoghurt

The organoleptic tests showed, that all as protective cultures added strains didn't influence the properties of yoghurt samples. No significant differences between the references and all other samples have been detected at the end of the storage period. Also no significant post-acidification, caused by the added test strains, was detectable.

Growth or inhibition effects of the added PAB-strains and combinations (PAB + LAB) have been detected by cell counting of yeasts in samples which have been stored at 10°C after 7 and 21 days and in samples, stored at 6°C, after 14 and 28 days. Generally can be summarized that all combinations had only slight effects on the growth of yeasts in yoghurt.

Indicator strain *Kluyveromyces marxianus* CBS 155 grew very well at 10°C in reference samples, after 7 days cell densities of $5,8 \times 10^5$ cfu/g have been detected. Only PAB 7 (*Propionibacterium freudenreichii* DC 2053 and *Propionibacterium freudenreichii* DC 1178 suppressed the growth of yeasts on a small scale (approximately 0,5 log), whereas combinations with LAB-strains showed an inhibition of approximately 1 log at 6°C after 14 days (**Fig. 2**)

All used combinations and single PAB strains didn't influence the growth of *Rhodotorula mucilaginosa* CFSQE in a significant way. No differences of cell counts have been detected at the end of storage.

Candida pulcherrima 1-50, which has been isolated from a yoghurt product, was slightly inhibited by combinations of *Lb. paracasei* ssp. *paracasei* and *Propionibacterium freudenreichii* DC 2053 and by *Propionibacterium freudenreichii* DC 1178 at a storage temperature of 6°C. Differences of 1 log have been detected after 14 days, whereas cell densities after 28 days were approximately on a high level of 2×10^6 cfu/g. No inhibition effects have been measured at 10°C.

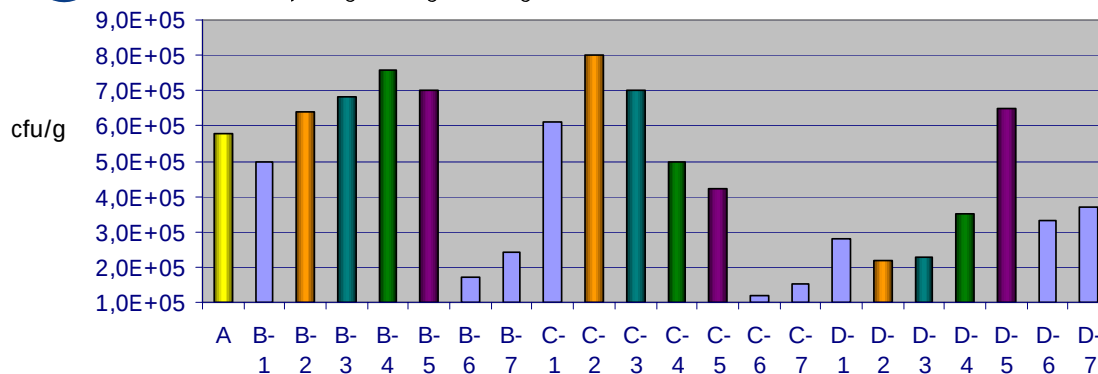


Fig. 2: Inhibition effects on *Kluyveromyces marxianus* CBS 1555 in yoghurt

Colony-
diameter
[mm]

3. Inhibition of moulds

For a detection of the inhibitory effects on moulds, caused by an application of PAB-strains, yoghurt have been fermented under above described conditions. From fermented yoghurt-samples, agar plates with yoghurt as a mixture with a heated agar-solution have been prepared. Spore suspensions (20 µl) of different moulds have been spotted on the surface of the “yoghurt-medium” and by this way prepared plates have been incubated at 25°C for 5 days. After incubation, colony diameters on plates with a reference-medium (prepared with yoghurt, fermented without test strains) have been compared with colony diameters on plates, prepared with yoghurt which was fermented with test strains too (PAB and combinations of PaB with LAB-strains). (Fig. 3)

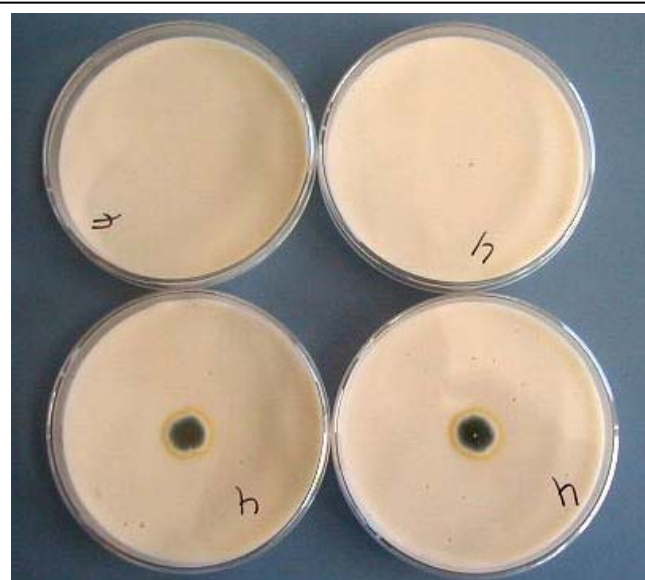


Fig. 3: “Yoghurt-spot-test”: Inhibition of moulds

Four different test strains (N159, W9, P27 and LMG 2868) have been chosen for the before described “yoghurt-spot-test”. As references, a combination of DC 2053 (PAB) and *Lb. rhamnosus* Lc705 (BIO PROFIT) have been used in the same way as the four selected single PAB-strains. As indicator-strains, spore-suspensions of 10 different moulds have been applied. 5 of them have been isolated from commercial available yoghurts from Poland, the five others were from a Swiss yoghurt product, what means, that this range represented a typical spoilage flora in this product category.

Analysing of colony diameters after 5 days resulted as following:

All four as single strains used PAB isolates (N159, W9, P27 and LMG2868) influenced the growth of the moulds very slightly (Fig. 4). We measured from case to case slightly reduced diameters by the application of these PAB-strains but it can be reasoned that the detected effects wouldn't extend shelf life of such products significantly. On the other hand, long inhibitory effects have been detected by an application of the combinations (PAB + LAB). These results confirmed again, that synergistic effects between PAB and LAB-strain support antagonistic effects also in practice.

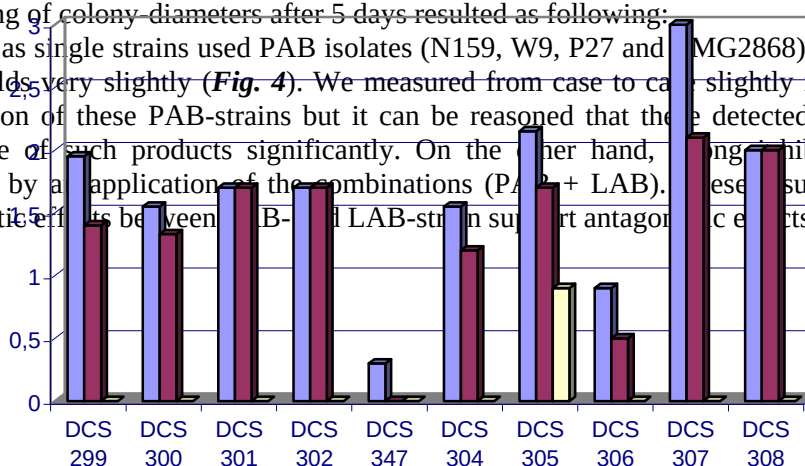


Fig. 4: Inhibition of different moulds by *Propionibacterium jensenii* LMG 2868 and by *Lb. rhamnosus* Lc705 in combination with *Propionibacterium freudenreichii* DC 2053.

Conclusion:

All achieved results and from these results extracted novel knowledge about the use of PAB-strains as protective cultures may be summarized as follows:

Propionic acid bacteria for their use in yoghurt for a prevention of spoilage caused by yeasts showed a very low potential as protective cultures, the effects on moulds can be described as very slight. In contrast to these results, combinations of lactic acid bacteria (LAB) and propionic acid bacteria (PAB) seem to have some effects. These antimicrobial activities, which have been detected mainly against moulds, make them appropriate for industrial application as biopreservatives. Metabolic activities of these cultures may contribute to the control of undesired moulds and thus a prolongation of shelf-life. It can be expected that an optimisation of these strain combinations would generate more intensive antagonistic effects. Extensive study and research on antimetabolites will be therefore necessary.

Annual report 2002 from TINE - NI project – Dairy bacteriocins

During February 2002 (week 6-8) 20 batches of cheese were produced, using the Jarlsberg cheese technology.

3 strains of Propionic Acid Bacteria (PAB) were selected on basis of their inhibition activities earlier reported in this project:

T5 *P. freudenreichii*

T114 *P. freudenreichii*

T159 *P. thoenii*

As a reference the standard (S) TINE Production strain was used in normal amount.

The test strains were used alone in the same amount, and in combination with the standard, both in normal amount. Each day one batch of cheese by the reference culture was produced.

The cheesemilk was without bactofugation (but pasteurised) and without use of nitrate. Investigation of effects against clostridium was based on the natural occurring anaerobic spores in the cheese milk. Effects against strains of Penicillium mould was not investigated due to practical problems.

Microbiological and chemical analysis was performed during the process and ripening of the cheeses. All cheeses were analysed for bacterial accounts and volatile fatty acids (GC) after 10d, 20d and 30d, and after 8 and 16 weeks, in addition to normal process controls. Sensoric evaluation was performed at the age of 8 and 16 weeks.

The results indicated that the strains T5 and T114, both used alone and together with standard (S) culture, produced normal amounts of propionic acid in cheese (figure 1A) when compared to the reference (S). T159 produced less propionic acid maybe due to lower growth in cheese.

Interesting results was observed according to the amounts of butyric acid in cheese (figure 1B). The two strains T5 and T114 seemed to keep butyric acid at a lower level during ripening of the cheese compared both to the strain T159 and to the reference S (mean value).

Sensoric evaluation showed varied results. Most cheeses had normal to high eyeformation, some had comments on cracks in texture. In taste/flavour some cheeses were good tasting, while others were quite untypical.

In conclusion, the teststrains T5 and T114 are promising alternatives for cheesemaking, while T159 may be an alternative in combination with other cultures. Further cheese trials will be of interest for us.

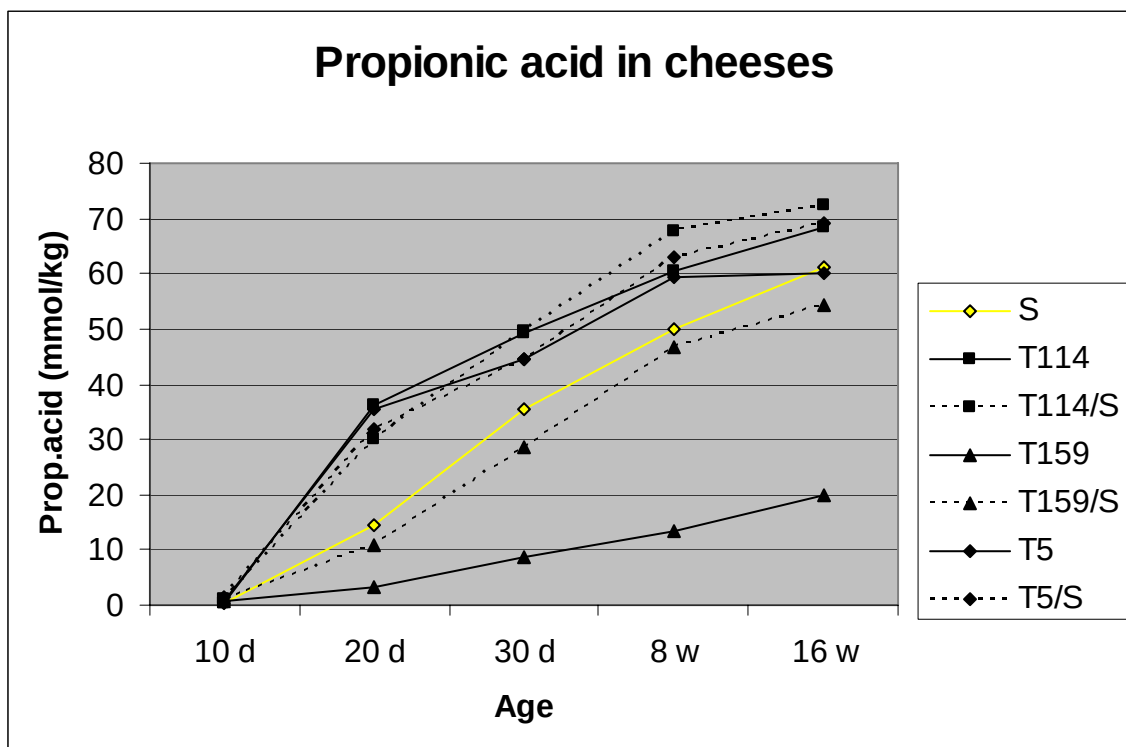


Figure 1A: Amount of Propionic acid in cheese at different age

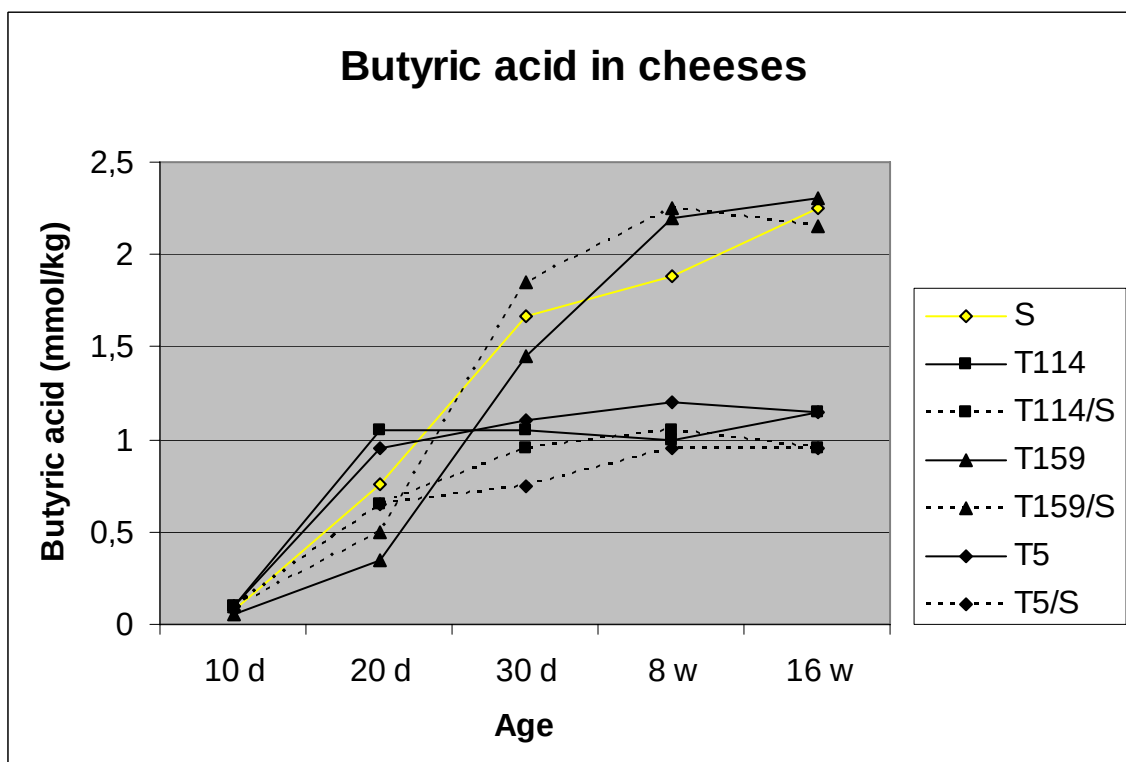


Figure 1B: Amount of butyric acid in cheese at different age