



Midterm Status Report 2003 and Application for Continuation in 2004

For research projects financed by grants from
The Directorate for Food, Fisheries and Agro Business
under the Danish Ministry of Food, Agriculture and Fisheries

1. Research program

Research in organic farming 2000-2005 (DARCOF II)

2. Project title and number

Production of raw milk cheese and content of phyto-estrogens from organic produced milk (II-11)

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6. Project period (month, year)

Start of project: May, 2002
End of project: July, 2004

7. Midterm description of the project, its results and progress, and application for continuation in 2004

Project summary

a)

The production of organic cheese is mainly based on the production of a number of cheese types, which are also produced from conventional milk. In order to be able to increase the market share of organically produced cheese, it is important that these cheese types are of a quality that justifies an additional price in relation to the conventionally produced cheese. It is often maintained that cheese made from raw milk can obtain a richer sensory quality than conventional products. However, documentation is needed to demonstrate that the flavour of organic cheese is different from cheeses made from pasteurized milk.

In Denmark, the production of cheese based on unpasteurized milk is subject to a very restrictive policy owing to the potential problems with microbiological safety. In order to be able to evaluate the microbiological safety of cheese manufacturing with raw milk, a number of critical control points in the production from raw material to finished product must be established, and suggestions for their monitoring must be made. This can be solved by using a Hazard Analysis Critical Control Point (HACCP) from stable to table which will provide guidelines for safe processing procedures. The specific data generated in the project will further be used in a risk simulation model which will take into account the variations in prevalence and concentration of specific pathogenic bacteria encountered in the production, as well as the variations observed in the process- and product parameters which may influence growth or inactivation.

The present project will – based on the production of red-lead putty cheese made with raw and pasteurized organic milk, respectively, from a specific dairy - compare the flavour and texture development by means of texture measurements, flavour analyses based on high vacuum distillation, peptide profiling and electronic nose measurements. It will further develop a generic HACCP model and a risk simulation model for the specific cheese which may serve as a tool for public safety managers.

b)

In organic milk production, the use of leguminous plants such as clover, lupin, horse beans and peas has increased because of nitrogen fixation into the ground and as an important energy and protein source for the cow. As an example, clover-grass, on energy basis, has been more than half the total intake in the summer period, and in the winter period, silage from clover-grass covers an average of 2/3 of the total roughage intake. Furthermore, organic dairy cows are given a large amount of cereals.

Especially leguminous plants, but also cereals, nuts, vegetables and berries contain natural high amounts of plant-estrogens, named phyto-estrogens. Phyto-estrogens are a large group of compounds with estrogen-like effects. Recent investigations on the effects of phyto-estrogens on various tissues have suggested that these compounds may improve human health, particularly by protecting against certain chronic diseases.

Phyto-estrogens can be divided in three main groups, isoflavonoids, coumestans and lignans. The concentration of isoflavonoids in clover for example, is more than 20 mg/g dry tissue. Therefore, clover is a potential source of phytoestrogens in cows milk and therefore also in humans as consumers of milk, especially if clovers are used as feed crops in organic milk production. Only a few studies have examined phytoestrogens in milk from cows, including a study from Finland and one from Australia. However, to our knowledge, there are no such studies of the content of phytoestrogens in milk from Danish herds.

The objective of the present project is to determine the concentration of phyto-estrogens in feed- and milk samples collected from different Danish organic herds in relation to the ration composition, especially the content of leguminous plants.

Table A.1: Work package list (from application)

No.	Work package title	Participants*	Budget (1.000 DKK)	Start	End	Deliverable no(s):
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1	Flavour and texture development in cheeses made from raw and pasteurised milk	DJF, ANF	966	04-02	12-03	D1-6; D10
2	Elaboration of a HACCP and a risk simulation model for a specific raw milk cheese production	KVL, MLI	1273.2	04-02	12-03	D7-11
3	Phytoestrogens in organic milk	DJF, HEF, PVF	500	06-02	12-03	D1.1

* Responsible participants are underlined

Objectives and expected achievements

Main objective (**maximum 10 lines**):

a) The overall aim is to improve the prospects of producing high quality organic cheese with acceptable microbial safety and of high sensory quality from unpasteurized milk.

- To investigate the effect of raw material on the ripening process through the determination of sensory quality and textural parameters
- To investigate the level of contamination with specific cheese-associated foodborne pathogens from the staple to the finished cheeses in a farm dairy
- To evaluate the potential for growth or survival of these pathogens in the cheeses
- To elaborate a HACCP and a risk simulation model
- To compare the sensory quality of cheese prepared from unpasteurized milk with the analogous pasteurized cheese

b) The objective is to study the importance of phyto-estrogens in Danish organic milk production. The specific objective is to determine the concentrations of phyto-estrogens in feed- and milk samples collected from different organic herds in relation to the ration composition, especially the content of leguminous plants

C. Midterm results and progress

C.1 Description (summary) of main results and conclusions

WP1

Methods to analyze flavour components and functional properties of cheeses have been developed.

Three repetitions of Munster-type cheeses have been produced on a Danish farm having its own dairy plant. Cheeses from pasteurized and raw milk respectively were produced from the same milking. The productions were performed in the following months

- September 2002 (I)
- January 2002 (II)
- June 2003 (III)

Due to problems with the size of the cheeses and the packaging of the produced cheeses lot I was excluded from further data analysis.

Cheeses were produced from both raw and low pasteurized milk. During the production and

storage the temperature and pH development were followed. Water content in the fat free cheese has been determined in the fresh and the stored cheese. After production the cheeses have been stored at 18 °C for 9 days followed by a storage period of 6 days at 8 °C and subsequent a storage period of 30 days at 4 °C illustrating the normal period in retail. Normally the cheeses will be in retail 15 days after production.

During storage period following parameters have been measured.:

- pH
- plasmin activity,
- profile of peptides
- headspace analysis of volatiles
- texture.

pH-development in the cheeses

The results indicate that there are big deviations in the pH of the three lots of cheeses 24 hours after renneting.

Raw milk cheeses: pH 5.24-5.93

Pasteurized cheeses: pH 5.19-5.55

There was no relation between the production number and the pH, however it is seen that the differences in pH were more pronounced in the lots produced from raw milk.

Water content in the produced cheeses

As expected from the differences in pH in fresh produced cheeses the water content of the cheeses will reflect differences in the renneting and acidification moreover is the water content varying from lot to lot. Again, we see no direct relation between water content and whether the cheese was produced from raw milk or pasteurized milk (Table 1) indicating that the variation was more affected by the management of the production than by the milk type.

Table 1: Water content in the cheeses expressed in percentage

Storage (days after production)	Water content (%)	
	Raw milk cheese	Pasteurized milk cheese
Lot II (day 15)	46.8±0.8	47.3±0.9
Lot III (day 15)	49.2±0.3	42.5±0.8

pH development in the stored cheeses

As expected the pH in cheeses increased during storage.

Table 2: pH in cheeses stored at 4 °C from day 15 to day 45 comparable to retail conditions (means of pH in 5 cheeses measured in the center of the cheeses)

Storage (days after production)	pH Raw milk cheese	pH Pasteurized milk cheese
Lot II (day 15)	5.3±0.2	5.6±0.1
Lot II (day 45)	6.6±0.2	7.2±0.3
Lot III (day 15)	5.5±0.1	4.9±0.1
Lot III (day 45)	6.4±0.1	5.6±0.1

From Table 1 it is clearly seen that pH development was different from one lot to another. Especially the pH in the pasteurized cheeses from lot III was very low in pH giving rise a failed maturation.

Plasmin activity in the produced cheeses

Plasmin is one of the most important proteolytic enzymes found in milk and the activity of this enzyme was proposed to correlate to flavour development of the cheeses as well development of maturation of the cheeses. Table 3 shows the activity of plasmin extracted from the cheese 15 days after production. Seen from the results the plasmin activity in Lot II was higher in pasteurized cheeses compared with the activity in the cheeses based on unpasteurized milk while it was found to be opposite in Lot III. It is worth to notice that the differences in plasmin activity were more or less identical in the two productions of raw milk cheeses.

Table 3: Plasmin activity in the cheeses

Storage (days after production)	Raw milk cheese Plasmin activity, dA 405nm/min./g cheese	Pasteurized milk cheese Plasmin activity, dA 405nm/min./g cheese
Lot II (day 15)	1073±103	2046±140
Lot III (day 15)	1284±92	498±39

Analysis of flavour components, peptides and texture in cheese

The results will first be evaluated after the last cheese production where all data will be evaluated by multi-variate analysis.

WP2

Through a literature study the factors responsible for direct or indirect contamination of raw milk with pathogenic microorganisms have been identified as outlined in Figure m1. The main contamination sources were i) the cow itself, ii) the human handler (the milker) and iii) the environment (incl. the milking area). With regard to the cow itself mastitis was found to be an important factor as pathogenic microorganisms can be excreted through the infected udder directly into the milk. Pathogenic microorganisms may also originate from the skin of the udder and teats. As illustrated in Figure m1, there are several sources from which the udder and teats are contaminated including the milker and the stable environment. On the basis of Figure m1, a list of critical points, i.e. points where contamination of the raw milk could be reduced, was made up;

- Personal hygiene of milker
- Number of disease free carriers and sick animals
- Mastitis
- Use of chemicals and fertilizer on fields used for feed or grazing
- Quality of silage
- Water quality
- Cleanliness of the cattle which is affected by stalling and type of bedding
- Pre-milking
- Teat treatment before milking
- Off-milking
- Cleaning of milking equipment and milking area

On this list mastitis and the points concerning the milking process were considered the most critical ones.

A HACCP based food safety programme has been suggested for the production of raw milk for processing of cheese. In the food safety programme *Staphylococcus aureus*, *Streptococcus agalactiae* and pathogenic *Escherichia coli* were identified as potential hazards originating from mastitic milk. *Salmonella* spp., *Listeria monocytogenes*, *E. coli*, *E. coli* O157, *Campylobacter jejuni*, *Yersinia enterocolitica*, *Mycobacterium paratuberculosis* and *Bacillus cereus* were identified as potential hazards originating from

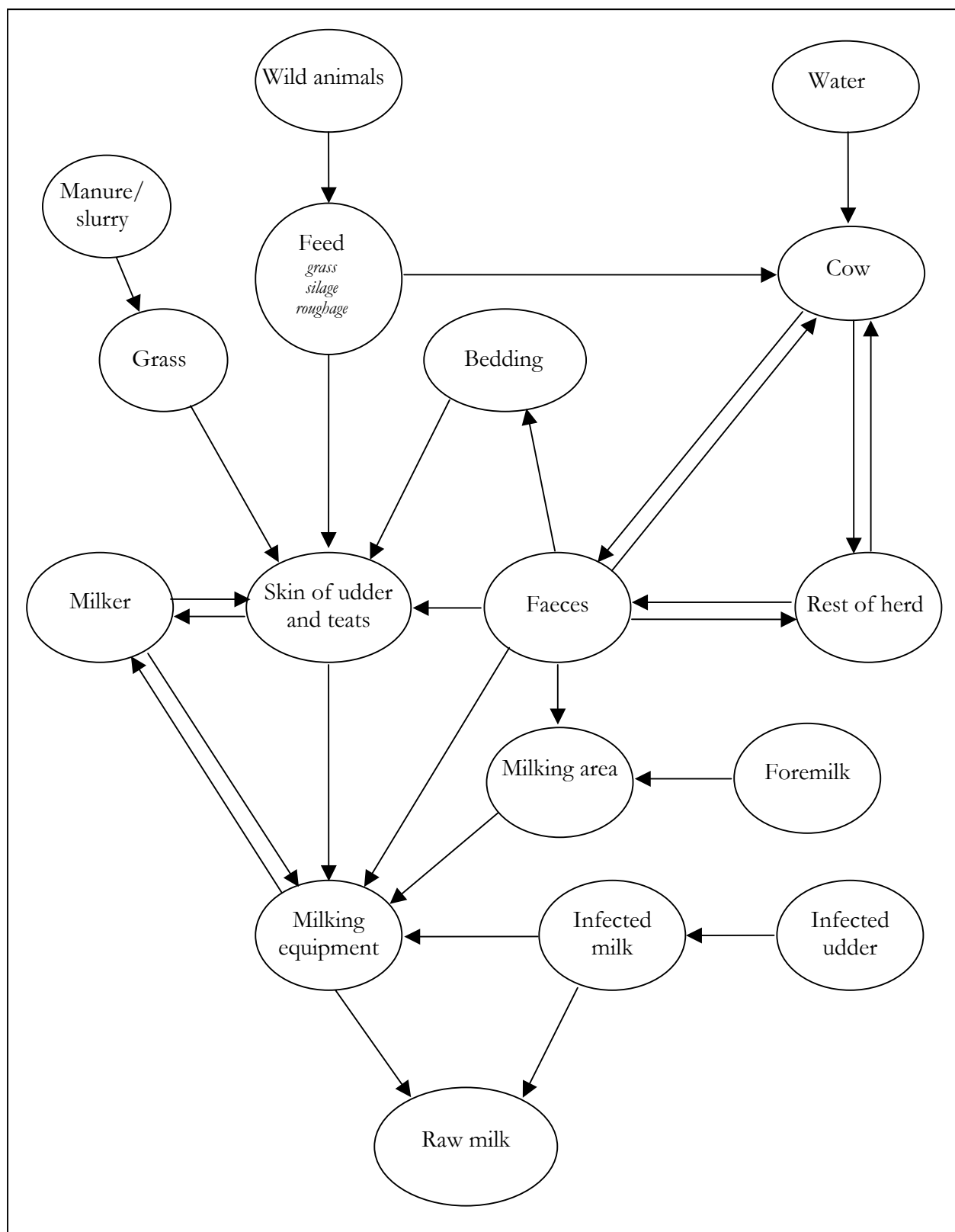


Figure m1. The routes of direct and indirect contamination of raw milk with pathogenic microorganisms.

faecal contamination of the milking equipment either directly or indirectly through contaminated skin of udder and teats. *S. aureus* may also originate from the hands of the milker as well as *Salmo-*

nella spp., *L. monocytogenes* and *B. cereus* occasionally also appears in mastitic milk. As a conclusion of the hazard analysis *Salmonella* spp., *E. coli*, *E. coli* O157, *L. monocytogenes* and *S. aureus* were considered the most important hazards as they had been implicated in several cases of illnesses from consumption of cheeses made from raw milk during the past 20 years. Critical control points (CCPs) were identified on the basis of a flow sheet of the production of raw milk where specifically the milking process was described in details. Feed (silage), water, teat treatment, pre-milking, off-milking, cleaning of milking equipment were identified as CCPs.

A single pre-milking teat treatment has been tested (Table m1). It was observed that all microbial counts on teat ends were lower after washing with wetted cotton cloths followed by air-drying before milking.

Table m1. Effect of pre-milking teat washing with wetted cotton cloths followed by air-drying on the microbial counts on teat ends (\log_{10} cfu per cow).

Sample	Mesophilic, aerobic count (TSA, 30°C)	Lactic acid bacteria (Rogosa, 30°C)	Lactobacillus count (MRS, 37°C)	Lactococcal count (M17, 30°C)	Coliform count (VRB, 37°C)
Before washing	5.5	3.1	2.9	5.4	4.2
After washing	5.4	2.9	2.7	5.2	3.9
Reduction	0.1	0.2	0.2	0.2	0.3

TSA: Tryptic soy agar

MRS: De Mann, Rogosa and Sharp agar

VRB: Violet, red bile agar

The mesophilic aerobic count (TSA, 30°C) on washed teats averaged 6.2 \log_{10} cfu per cow in the winter (9th December) where the cattle stayed inside the deep bedding stable. This count was 0.8 \log_{10} cfu lower per cow in the spring (19th May) as well as in the summer (4th September) where the cattle were grazing. Also the average faecal coli counts (EMBA, 44°C) on washed teats were lower in the grazing period ($\leq 2.7 \log_{10}$ cfu per cow) compared to the period where the cattle stayed in the deep bedding stable (3.1 \log_{10} cfu per cow). Swabs from the teat cups used for milking showed that all of the 16 sets tested harboured faecal coli (selective enrichment, 44°C) on the outside. However, on average the counts were higher in the winter (3.4 \log_{10} cfu per set of cups) than in the summer ($\leq 2.7 \log_{10}$ cfu per set of cups). It was also observed that pressure spraying of the floor in the milking area during milking increased the faecal contamination on the outside of the teat cups considerably (Table m2).

Table m2. Effect of pressure spraying of the floor in the milking area during milking on the microbial counts on teat cups (\log_{10} cfu per set of cups).

Sample	Mesophilic, aerobic count (TSA, 30°C)	Staphylococcal count (BP, 35°C)	Faecal coli count (EMBA, 44°C)	Non-coliform enteric count (XLD, 35°C)
Before spraying	6.1	6.0	2.9	3.8
After spraying	6.2	6.0	3.8	4.3
Increase	0.1	0	0.9	0.5

TSA: Tryptone soy agar

BP: Baird-Parker agar

EMBA: Eosin methylene blue agar

XLD: Xylose Lysine Desoxycholate medium

Swabs from the inside of the teat cups showed that bacteria originating both from the teat skin flora (*Staphylococcus* spp.) as well as from faecal contamination (*Enterobacteriaceae*) were transferred to the inside of the milking equipment. Typical *S. aureus* colonies were found in four out of nine

samples and in one out of nine samples *E. coli* colonies were found. However, none of the 16 samples tested for faecal coli with a selective enrichment procedure were positive.

Sampling of the raw milk used for production of soft cheeses has so far shown that the mesophilic microbial counts were 0.5 – 1 log₁₀ cfu/ml lower in the grazing period than in the stable period. This difference was even more pronounced for the coliform counts (VRB, 37°C) which were 1.0, 1.7 and 4.0 log₁₀ cfu/ml for samples taken in the beginning of the grazing period, in the middle of the grazing period and in the stable period, respectively.

So far microbiological analyses on cheeses from three productions have been conducted. Preliminary data analysis of these experiments shows that there have been no obvious systematic differences between the microbial counts of cheeses made from raw or pasteurized milk after storage for 3 weeks. In the milk and the fresh cheeses some differences have been observed but final conclusions cannot be made yet, as the last cheese experiment will be conducted in November 2003.

WP3.

Milk samples have been collected from a large number of individual cows to account for the variation in content of phyto-estrogens related to season and different feeding regimes. Related feed samples of silage and red clover pasture was also collected. Blood samples from selected cows was obtained for preparation of serum samples.

The content of the isoflavonoid genistein and the lignan enterolactone was analysed in serum from 11 cows from an organic herd when housed and on pasture. Serum was analysed by Time Resolved Fluorometric Immuno Assay (TR-FIA) using commercially available kits. Preliminary results showed concentrations of enterolactone between 300 and 1500 nmol/l and concentrations of genistein between 5 and 50 nmol/l. Concentrations of enterolactone were highest in cows on on pasture while concentrations of genistein were lowest on pasture. Further studies will be performed on blood samples from other cows.

An HPLC-method for the quantification of isoflavonoids in silage from clover-grass has been developed. Isoflavonoids occurs primarily as glycosides and to a much lesser extent as free aglycones in silage from clover-grass and in cows milk. Normally the isoflavonoids are hydrolysed to their respective aglycones and then quantified. The developed HPLC-method can be used both to quantify isoflavonglycosides as well as the free aglycones. Quantification of isoflavonoids in non-hydrolysed and hydrolysed samples of silage from clover-grass is ongoing. Regarding the quantification of isoflavonoids in cows milk this will be performed according to the method of Antignac *et al.* (2003) in which the isoflavonoids are hydrolysed enzymatically to the free aglycones and then identified and quantified by LC-MS/MS. Currently, the method is tested and quantification of isoflavonoids in cows milk will be performed in the beginning of 2004.

Reference

Antignac, J.-P., Cariou, R., Bizec, B. L., Cravedi, J.-P. and Andre, F. (2003). Identification of phytoestrogens in bovine milk using liquid chromatography/electrospray tandem mass spectrometry. *Rapid Comm. Mass Spectrometry* **17**, 1256-1264.

C.2 Fulfilment of deliverables and milestones

(To be completed for each work package)

WP1 Flavour and texture development in cheeses made from raw and pasteurised milk	Time schedule	Deviations, if any*
Deliverables		
1 Production of cheeses	9/2002	
2 Production of cheeses	12/2002	
3 Production of cheeses	4/2003	
4 Production of cheeses	9/2003	1 month
5 International paper	12/2003	
6 End report	12/2003	
10 Discussion meeting with stakeholders	11/2003	
Milestones		

WP2 Elaboration of a HACCP and a risk simulation model for a specific raw milk cheese production	Time schedule according to application	Deviations, if any*
Deliverables		
7 Review of pathogenic bacteria in raw milk and raw milk cheeses	4/2003	8 months
8 Suggestion for a generic HACCP	7/2003	4 months
9 Elaboration of a risk simulation model	10/2003	4 months
10 Discussion meeting with stakeholders	11/2003	4 months
11 Final manuscript	12/2003	6 months
Milestones		
4 Review produced	12/2002	6 months
5 Microbiological analyses performed	6/2003	6 months
6 Generic HACCP programme produced	6/2003	6 months
7 Risk simulation model generated	12/2003	6 months

WP3 Phytoestrogens in organic milk	Time schedule according to application	Deviations, if any*
Deliverables		
1 Report	December 2003	6 months
2		
Etc.		
Milestones		
1 Sampling	June 2003	6 months
2 Laboratory analyses	October 2003	6 months
3. Evaluation and report	December 2003	6 months

* *Deviations are to be further discussed in D*

D. Description of deviations and subsequent adjustments of plans

WP2 in general has been delayed with 4 months as a result of a delay in the project start. Further 2 months' delay is expected due to a delay of the conduction of the fourth cheese experiment. WP2 at KVL will run for the time originally scheduled with adjustment for the total time delay, i.e. until medio 2004.

WP2, milestone 4 and deliverable 7: The part of the review considering the raw milk is done whereas the part on cheese will be delayed until the beginning of 2004.

WP3

We wish to expose the time schedule according to quarters mentioned in C. The analyses have been delayed since the new methods require more time than anticipated. Furthermore, the responsible person for Workpackage number 3 (Stig Purup) has been a visiting scientist at a biotechnology company in Australia from September 2002 to July 2003.

E. Project publications and other products

1. Articles in international, scientific journals with review procedures
2. Papers presented at congresses, symposiums, etc.
3. Reports, articles in agricultural journals, etc.

Majbrit Wigø Dahl (2003): Optimere egenkontrollen for primærproduktion til råmælksost (Optimization of the food safety programme applied for production of raw milk intended for cheese processing). Bachelor project. Department of Dairy and Food Science, KVL. July 2003. (In Danish. English abstract is available on Organic Eprints).

4. Oral presentations, public meetings, field days, etc.

F. Scientific education

Majbrit Wigø Dahl, Bachelor project from September 2002 to July 2003.
Kenneth Højgaard, Master project from September 2002 to February 2004.

G. National and international cooperation

Critical reflection on the project

WP1 and WP2 Raw milk cheeses:

The large variation in renneting and acidification seems to influence the results and is believed to affect the quality of the results in relation to the objective of the project.

WP3 is only technically administrated by the project leader of this project and not related to the production and studies of raw milk cheeses.

8. Budget

A. Account for any change in budgets

As a result of the delay of the project some time-related changes have been made in the budget.

B. Budget for the whole project (1.000 DKK)

Total consumption of funds from DARCOF and expected consumption this year and coming years

Year:	Consumption before 2003	Expected consumption 2003	2004	2005	Total
Man-months					
Scientific personnel	3	27	7		37
Technical personnel	1	16.5	0.5		18

Year:	Consumption before 2002	Expected consumption 2003	2004	2005	Total
Salaries					
Scientific personnel	113.6	974.4	367.7		1455.7
Technical personnel	23.8	402.5	12.5		438.8
Other operational costs	79	245.2	64		388.2
Equipment					
Others (please specify)					
Direct costs	216.4	1,622.1	444.2		2,282.7
Indirect costs (20% of direct costs)	43.3	324.2	88.8		456.3
Total	259.7	1,946.3	533		2,739

Comments:

Budget is justed in relation to the delayed project start

9. Signatures and stamps

Name	Institute	Date	Signature
Head of project Jacob Holm Nielsen	Danish Institute of Agricultural Sciences	1/10-03	

Appendix I. Detailed budget

A. Budget for each participating institute (1.000 DKr)

Name of Institute: Department of Animal Product Quality, DIAS

Year:	Consumption before 2003	Expected consumption 2003	2004	Total
Man-months				
Scientific personnel		10		10
Technical personnel		15		15

Year:	Consumption before 2003	Expected consumption 2003	2004	Total
Salaries				
Scientific personnel		360		360
Technical personnel		345		345
Other operational costs		100		100
Equipment				
Others (please specify)				
Direct costs		805		805
Indirect costs (20% of direct costs)		161		161
Total		966		966

Name of Institute: KVL

Year:	Consumption before 2003	Expected consumption 2003	2004	2005	Total
Man-months					
Scientific personnel	1	15	7		23
Technical personnel	0	1	0		1

Year:	Consumption before 2003	Expected consumption 2003	2004	2005	Total
Salaries					
Scientific personnel	34	531	326		891
Technical personnel	0	20			20
Other operational costs	1	100	49		150
Equipment					
Others (please specify)					
Direct costs	35	651	375		1061
Indirect costs (20% of direct costs)	7	130	75		212
Total	42	781	450		1273

Comments:

As the experimental work in WP2 of the project has been spread over the whole year it has been difficult to employ technical personnel. Instead Tina Beck Hansen, the scientific employee on this project, has conducted the laboratory work in collaboration with student employees. This has caused a delay in the other tasks/parts of the project. In order to be able to fulfil the deliverables of WP2 of the project we would, therefore, like to convert the rest of the salaries intended for technical personnel to salaries for scientific personnel. This change will not increase the total expenses.

B. Budget for each participating department (1.000 DKK)**Name of Institute and department: DJF – Department of Animal Nutrition & Physiology**

Year:	Consumption before 2003	Expected consumption 2003	2004	2005	Total
Man-months					
Scientific personnel	1	1	1		3
Technical personnel	1	1			2

Year:	Consumption before 2003	Expected consumption 2003	2004	2005	Total
Salaries					
Scientific personnel	39,8	41,7	41,7		123,2
Technical personnel	23,8	25			48,8
Other operational costs	47	30,2			77,2
Equipment					
Others (please specify)					
Direct costs	110,6	96,9	41,7		249,2
Indirect costs (20% of direct costs)	22,1	19,4	8,3		49,8
Total	132,7	116,3	50,0		299

C. Budget for co-financing from each participating institute (1.000 DKK)

Name of Institute: DJF-Department of Horticulture

Year:	Consumption before 2003	Expected consumption 2003	2004	2005	Total
Man-months					
Scientific personnel	1	1			2
Technical personnel		0.5	0.5		1

Year:	Consumption before 2003	Expected consumption 2003	2004	2005	Total
Salaries					
Scientific personnel	39.8	41.7			81.5
Technical personnel		12.5	12.5		25
Other operational costs	31	15	15		61
Equipment					
Others (please specify)					
Direct costs	70.8	69.2	27.5		167.5
Indirect costs (20% of direct costs)	14.2	13.8	5.5		33.5
Total	85	83	33		201

Comments: