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Influence of feeding supplementation and lactation cycle on milk cholesterol, fatty acid profile and milk fat globule size

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CONTENTS

Abstract

Milk and its products are a very important part in human nutrition and culture. They contain numerous components with a major role in diet like fat, protein and lactose. Especially fat, and its content on different fatty acids and cholesterol, is constantly at the focus of research because of their impact on human health and the need of an adequate healthy diet. Cholesterol is a very controversial discussed molecule: as a part of every animal and human cell, as a component of plasma membranes and a precursor in the biosynthesis of steroidal hormones, cholesterol is a crucial molecule in body homeostasis. Too high levels of blood cholesterol might have negative effects in cardiovascular health. Milk products have a relatively low level of cholesterol compared to other foods, but due to their high consumption, they are one of the most important sources of this metabolite in human diet. Modern diet should be aimed at lower contents of cholesterol, saturated and trans fatty acids and higher contents of polyunsaturated fatty acids. Therefore, milk and milk products with an altered fatty acid profile and cholesterol content may play an important role in a health-conscious nutrition.

However, to change the cholesterol and fatty acid composition of bovine milk, the fat metabolism of cows requires better understanding. For this reason, a feeding experiment was designed to investigate the impact of the dietary supplementation of unsaturated fat on blood composition, liver physiology and finally the change in milk composition. During this feeding experiment, blood and liver samples were taken at the beginning and at the end of the four week feeding periods where either a control ration or a ration supplemented with soybean oil, rapeseed oil or extruded linseed was fed. Milk samples were obtained over the whole experimental period.

As expected, the oil supplementation resulted in a tremendous reduction of fat yield and saturated fatty acids in milk, and an increase of mono- and polyunsaturated fatty acids. This confirms the enormous impact of dietary unsaturated fatty acids in diet on milk composition reported earlier in the literature. Cholesterol content in milk could be reduced with soybean and rapeseed oil supplementation, though blood cholesterol increased. It was also possible to show that cholesterol and fat yield do not always correlate. Analyzing gene expression of key factors and regulators of fat and cholesterol metabolism in liver cells it was evident, that there is almost no change in gene expression during early phase of lactation and in contrast, numerous genes, though just a small part of all analyzed genes, show a significant alteration in gene expression towards the end of lactation. 13 gene patterns taking part in fatty acid activation, transport and oxidation (ACSL-1, FABP-1, CPT1A), lipid droplet formation (PLIN-4) and gluconeogenesis (PC, PCK-1) to name a few, were used in a dynamic PCA to show the difference of early and late lactating cows. This implies that milk composition is not mainly regulated directly by genes located in liver, but may have other regulatory mechanisms in the mammary gland.

To investigate the mechanisms of gene regulation in the mammary gland and its influence on cholesterol and fat yield, as well as milk fat globule size, purified bovine milk epithelial cells were extracted from milk and investigated. In parallel, milk samples of multiparous cows were taken at different stages of lactation and milk fat globule diameter, fat and cholesterol content were analyzed. It was possible to show, that genes which are acting in cholesterol and fatty acid biosynthesis pathways have a lower expression rate in late lactating cows than in early lactating cows. This observation is accompanied by a bigger milk fat globule diameter at the beginning of

lactation and a decreasing diameter towards the end, as well as lower fat and cholesterol milk levels.

Additionally, a technical optimization experiment was performed: to expose the impact of storage of milk samples on analysis of milk parameters an experiment investigating effects of long term freezing on cholesterol, progesterone and lactoferrin was conducted. After thawing at 1, 6 and 12 months of storage at -20 °C it could be shown that different conservation agents can alter analysis in milk: a significant effect of storage time on progesterone and lactoferrin content could be observed whereas cholesterol content remained almost stable. The previously known effect of a lowered fat content after freezing and storage could also be shown in an impressive way, undermining a possible mechanic destruction of milk fat globules via ice crystal forming during the freezing process. This effect is also supported by the fact that the lower fat content appears already after a short period of freezing and is not based on a long storage time.

In summary it could be proven, that the additional feeding of plant oils can lead to a dramatic change in milk composition. The supplemented unsaturated fats resulted in a drastic increase in mono- and polyunsaturated fatty acids (inclusive CLAs) in milk paralleled with a decrease of saturated fatty acids. Fat and cholesterol content in milk could also be decreased, though cholesterol content in blood increased at the same time, proving that the mammary gland is an independently working organ. It could be shown that fat and cholesterol content do not strictly correlate but are defined via the changed milk fat globule diameter, because cholesterol is mainly located in the Milk fat globule membrane. Against all expectations it was evident that the liver does not maintain the major role in the regulation of milk composition, finding no significant changes in expression of key enzymes in fat and cholesterol metabolism. A more

important role seems to play the mammary gland itself, though further investigations have to be made to proof changed mechanisms of gene expression in mammary gland when plant oils are added to the diet of dairy cows.

Zusammenfassung

Milch und dessen Produkte stellen einen wichtigen Bestandteil der menschlichen Ernährung und Kultur dar. Sie beinhalten eine Vielzahl von Inhaltsstoffen, wie Fett, Eiweiß und Laktose, die eine wichtige Rolle im menschlichen Körper spielen. Besonders das „Fett“ rückte in den letzten Jahren zunehmend in den Fokus der Forschung wegen seinen verschiedenen Bestandteilen wie Fettsäuren und Cholesterin und deren Einfluss auf die menschliche Gesundheit. Ebenso ist das gesteigerte Verlangen nach einer gesunden Ernährung ein wichtiger Motor für dieses Forschungsgebiet. Cholesterin ist dabei der am kontroversesten diskutierte Bestandteil. Cholesterin kommt in jeder tierischen und menschlichen Zelle vor und ist ein sehr bedeutender Bestandteil von Plasmamembranen. Außerdem ist es eine Vorstufe von Steroidhormonen und ist deswegen sehr essentiell für die Homöostase im Körper. Es wird hauptsächlich in der Leber produziert, wo auch zugleich bei zu hohen Konzentrationen an Cholesterin dieses zu Galle umgewandelt wird und durch den Gastrointestinaltrakt ausgeschieden oder bei Bedarf wieder reabsorbiert wird. Milchprodukte haben im Vergleich zu anderen Lebensmitteln einen sehr geringen Anteil an Cholesterin, aber durch den hohen Konsum stellen sie eine der bedeutendsten Cholesterinquellen in unserer Ernährung dar. Der hohe Bestandteil an Fett und Cholesterin in unserer Ernährung beeinflusst sehr stark den Blutcholesterinspiegel, welcher, wenn er zu hoch ist, kombiniert mit einigen Transfettsäuren, möglicherweise fördernd auf Herzkreislaufkrankheiten wirkt. Das führt wiederum zu einem Umdenken in unserem Lebensstil, wo nun geringere Cholesteringehalte und Transfettsäuren und höhere Gehalte an ungesättigten Fettsäuren gewünscht werden. Um aber den Cholesteringehalt und die

Fettsäurezusammensetzung in der Kuhmilch zu beeinflussen muss zuerst der Fettmetabolismus der Kuh voll verstanden werden. Zu diesem Zweck wurde ein Fütterungsexperiment geplant um den Einfluss von ungesättigten Fetten, die dem Kuhfutter zugemischt werden, auf die Blutzusammensetzung, auf die Leberphysiologie und schlussendlich auf die Milchzusammensetzung zu untersuchen. Während dieses Fütterungsexperimentes wurden jeweils am Anfang und am Ende von vierwöchigen Fütterungsperioden, in denen entweder eine Kontrollration oder eine Ration mit Rapsöl, Sojaöl oder extrudiertem Leinsamen als Zusatz gefüttert wurde, Blutproben und Leberbiopsien genommen. Milchproben wurden während des gesamten Experimentes in gleichmäßigen Abständen genommen. Wie erwartet kam es zu einer enormen Reduzierung des Fettgehalts und der gesättigten Fettsäuren bei gleichzeitiger Erhöhung der einfach und mehrfach ungesättigten Fettsäuren. Dieser Effekt wurde bereits früher in der Literatur beschrieben und bestätigt den großen Einfluss von ungesättigten Fettsäuren im Futter auf die Milchzusammensetzung. Der Cholesteringehalt in der Milch konnte mit der Soja und Rapszusatz gesenkt werden, obwohl sich der Blutcholesteringehalt erhöhte. Ebenso war es möglich zu zeigen, dass der Cholesteringehalt nicht mit dem Fettgehalt in der Milch korreliert. Bei der Analyse der Expression von Genen die am Fett und Cholesterinmetabolismus beteiligt sind, war auffällig, dass beinahe keine Genexpression während der frühen Phase der Laktation zu messen war und nur ein kleiner Anteil an Genen am Ende der Laktation eine signifikante Änderung zeigte. So wurde das Genmuster von 13 Genen, die an der Fettsäure-aktivierung, -transport, und –oxidation (ACSL-1, FABP-1, CPT1A), Lipid Droplet Formation (PLIN-4) und Glukoneogenese (PC, PCK-1) teilnehmen, um einige zu nennen, in einer dynamischen PCA analysiert um den Unterschied zwischen früh und spätlaktierenden Kühen zu zeigen. Das deutet darauf hin, dass die

Milchzusammensetzung nicht hauptsächlich von Genen in der Leber reguliert wird sondern die Milchdrüse möglicherweise eine eigene interne Regulation besitzt.

Um die Mechanismen der Genregulation in der Milchdrüse und deren Einfluss auf den Cholesterin und Fettgehalt, sowie den Milchfettkügelchendurchmesser (MFD) zu erforschen, wurden aufgereinigte Milchepithelzellen (purified bovine milk epithelial cells – pbMECs) aus der Milch extrahiert und analysiert. Zusätzlich wurden Milchproben von mehrkalbigen Kühen in verschiedenen Laktationsstadien genommen und der MFD, Fett und Cholesteringehalt analysiert. Es konnte gezeigt werden, dass Gene, die eine Rolle in der Cholesterinsynthese und Fettsäuresynthese spielen, am Ende der Laktation eine niedrigere Expressionsrate aufweisen wo auch die Fett und Cholesterinmenge am geringsten ist. Dieser Effekt wird begleitet von einem größeren MFD am Anfang der Laktation und einen geringeren am Ende.

Um den Einfluss der Lagerung von Milchproben auf die Analyse verschiedener Parameter in der Milch wie Cholesterin, Progesteron und Laktoferrin aufzuzeigen, wurde ein Experiment mit Langzeitgefrierlagerung durchgeführt. Nach dem Auftauen nach 1, 6 beziehungsweise 12 monatiger Lagerung bei -20 °C konnte gezeigt werden, dass einzelne Konservierungsmittel, die den Milchproben beigefügt wurden, die Analyse beeinflussen können. Auch die Lagerungsdauer hat einen Effekt auf Progesteron und Laktoferrin in der Milch wohingegen Cholesterin relativ stabil war. Der bekannte Effekt eines verringerten Fettgehaltes nach dem einfrieren konnte in beeindruckender Weise gezeigt werden. Dies bestätigt eine mögliche mechanische Zerstörung der Fettkügelchen durch Eiskristalle die sich während des Einfrierprozesses bilden. Diese Beobachtung wird gestützt durch die Tatsache, dass

sich schon während einer kurzen Gefrierlagerung ein niedriger Fettgehalt messbar ist und dieser Effekt nicht durch eine lange Gefrierdauer verursacht wird.

Zusammenfassend ist zu sagen, dass mit der Zufütterung von pflanzlichen Ölen eine dramatische Änderung der Milchzusammensetzung erreicht werden konnte. Die supplementierten, ungesättigten Fettsäuren bewirkten eine Erhöhung des Anteils an einfach- und mehrfach-ungesättigten Fettsäuren (inklusive CLAs) in der Milch bei gleichzeitiger Senkung von gesättigten Fettsäuren. Der Fett und Cholesteringehalt in der Milch konnte ebenfalls gesenkt werden, obwohl der Cholesteringehalt im Blut gleichzeitig anstieg und somit das Euter als eigenständiges Organ bestätigt. Es konnte gezeigt werden, dass Fett und Cholesterin nicht direkt voneinander abhängig sind, sondern durch den Milchfettkugeldurchmesser definiert werden, da Cholesterin Teil der Kügelchenmembran ist. Weiters wurde in den Ergebnissen deutlich, dass entgegen den Erwartungen, die Leber nicht die Hauptrolle in der Regulation der Milchzusammensetzung einnimmt, da beinahe keine Expression von Enzymen, die am Fett und Cholesterinmetabolismus teilnehmen, gefunden wurde. Eine wichtigere Rolle scheint das Euter selbst zu haben, obwohl noch weitere Untersuchungen notwendig sind um zu beweisen, dass sich die Expression von Genen die am Fett und Cholesterinstoffwechsel beteiligt sind ändert wenn Öle zugefüttert werden.

1 Introduction

In modern dairy farming, nutrition obtains a major part in producing agricultural wares economically by providing adequate diets for high yielding cows. Especially in early lactation, at the onset of milk production, high energetic diets are needed to equalize the metabolic imbalance after calving (Reynolds et al., 2003). Attempts of reducing the metabolic imbalance at the beginning of lactation period by altering milking frequency or by continuous milking during dry period have been made (Schlamberger et al., 2010). Oil supplements are often used to increase the energy level of diets. By adding oil into diet of dairy cows, not only the energy level can be increased but also various effects on milk composition can be observed. A reduced milk fat content can be the result of an increased level of unsaturated fats in diet (Loor and Herbein, 2003b, Peterson et al., 2003). Also an altered fatty acid profile of milk and a changed milk composition as a consequence of oil supplemented rations inducing a changed ruminal biohydrogenation of unsaturated fats has been extensively reported (Glasser et al., 2008, Rego et al., 2009, Benchaar et al., 2012).

Milk is a very complex food containing fat, protein and lactose, as the main components, dispersed in water. Milk and milk products are deeply implemented into human diet and play a very important role in nutrition especially in western culture. Because of the high level of saturated fats and an increasing frequency of consume, milk got into the focus of scientific investigations around the globe (Menotti et al., 1999, Malpuech-Brugere et al., 2010). Milk fat, that is synthesised in the mammary gland or exchanged from circulating blood, is secreted into milk in form of milk fat globules ranging from 0.1 to 20 µm in diameter with an average diameter between 3.3 and 5.2 µm (Ménard et al., 2010, Lopez, 2011). 98 % of milk lipids are located in

the core of milk fat globules as triacylglycerol molecules (Lopez et al., 2010) surrounded by the milk fat globule membrane (MFGM). This membrane consists of an inner monolayer originating from the endoplasmatic reticulum and a bilayer from the cell membrane coating the lipid droplet during the secretion process. The globule membrane is a stabilizing element of the milk fat globule and contains a mixture of proteins, glycoproteins, glycolipids, phospholipids, cholesterol and sphingomyelin (Fong et al., 2007, Dewettinck et al., 2008). These parts are not distributed homogeneously in the membrane but form liquid-ordered lipid rafts of cholesterol and sphingomyelin surrounded by a liquid-disordered phase of phospholipids (Lopez et al., 2010).

As a part of the milk fat globule membrane, cholesterol is secreted into milk by the mammary gland. Cholesterol is a component of eukaryotic cell membrane and serves as a precursor of steroid hormones, oxysterols, bile acids and an intermediate of cholesterol synthesis is a pro-vitamin of vitamin D (Espenshade, 2013), but if consumed in high doses, cholesterol may also be a mediator of atherosclerosis and cardiovascular diseases (Ren et al., 2010, Weingartner et al., 2010, Weingartner et al., 2011). Milk is supposed to have positive or neutral effects on cardiovascular diseases due to other components, but milk consumption contributes extensively to overpassing the recommended 300 mg diet intake of cholesterol per day (Royo-Bordonada et al., 2003, Viturro et al., 2006a). Some efforts have been made to understand cholesterol metabolism and its cellular mechanisms of transport (Farke et al., 2006, Viturro et al., 2006b), but methods for specifically lowering cholesterol content in milk seem to be needed. Together with breeding, an altered feeding management might be a suitable attempt to reach this goal. In the past, many experiments about supplementation of the ration with different oils, high in

unsaturated fatty acids, have been conducted (Huang et al., 2008, Rego et al., 2009, Benchaar et al., 2012), but little is known about the impact of such approaches on cholesterol level in milk. Some efforts have already been made to understand the effects of oils on cholesterol level (Reklewska et al., 2002) but still more research is needed with other plant oils.

By lowering cholesterol levels in milk through feeding of plant oils, also fatty acids may favorably change, resulting in an increased level of mono and polyunsaturated fatty acids and a lower part of saturated fatty acids (Jacobs et al., 2011). Special interest was given to unsaturated fatty acids in the past. While trans fatty acids are considered to have neutral effects when consumed at moderate levels, they may increase the risk of cardiovascular diseases as they have adverse effects on blood lipids when consumed at high doses (Sun et al., 2007, Motard-Bélanger et al., 2008). Possible positive effects on diseases because of their antiatherogenic and anticancerogenic impacts are discussed to origin from conjugated linoleic acids (Lock and Bauman, 2004).

The goal of this thesis was to illuminate the effects of dietary unsaturated fatty acids on milk fat composition. Special interest was given to the fatty acid composition and the cholesterol metabolism. An important point therefore was to find correlations between feed composition, blood composition, milk composition and milk fat globule size. To prove that the gained results are comparable among each other, an experiment on the effects of time and different conservation agents on milk composition was conducted.

2 Material and Methods

2.1 Animal trial of oil supplementation and its impact on milk

This experiment was performed at the experimental farm “Hirschau” (TUM, Marzling, Germany) and 39 multiparous HF (Holstein-Friesian) cows were examined. Cows were kept in a free stall barn and were milked two times per day at 5 am and 4 pm in a double six herringbone milking parlor (DeLaval GmbH, 21509 Glinde, Germany). Cows were dried off 42 days before expected calving with an antibiotic drying supplement containing 1 g Cloxacillin (Orbenin, Pfizer, 10785 Berlin, Germany) and a non-antibiotic intramammary seal (OrbeSeal, Pfizer, 10785 Berlin, Germany). Non lactating cows were kept in a separate stable with deep littering. Cows were fed a partial mixed ration calculated on an average milk yield of 22 kg with an extra concentrate feeding according to individual milk yield. Concentrate was given as a maximum of 8 kg per day at the beginning of lactation period through an automatic concentrate dispenser with an integrated body weighing system. All cows had continuous access to fresh water. PMR was given daily at 9 am with continuous access to food and permanent recording of feed intake of every cow via an automatic feed weighing system integrated into scale pans. Non lactating cows were fed control ration with an extra part of straw to increase dry matter content and to reduce energy level. One week before expected calving cows were fed the control ration for a better adaption of ruminal microbes to the ration.

Cows used in this experiment were assigned to a fixed feeding plan of 16 months. The feeding plan consisted of repetitive four week feeding periods where either a control ration or an oil supplemented ration was fed. To the oil supplemented rations rapeseed-oil, soybean-oil or extruded linseed was added. Feeding periods were

separated by two weeks washout periods where PMR was identical to control ration. Cows were assigned to the feeding plan according to their stage of lactation. Therefore lactation was divided into early (0-100 DiM), mid (101-200 DiM) and late lactation (201-300 DiM) (DiM days in milk). If a cow was for instance in mid lactation at the beginning and at the end of the four week feeding period the cow was used for sampling. This design made it possible to assign one cow to several groups but not necessarily in succession. If a cow was selected, blood and liver samples were taken at the beginning and at the end of the feeding period representing the before and after treatment situation. During the feeding period, milk samples were collected two days per week. Milk yield as well as feed intake was measured automatically during the whole lactation. Body weight was measured by the weighing system integrated into one of four concentrate feeders.

The department responsible for animal welfare affairs (“Referat 54 der Regierung von Oberbayern”) approved all experiments.

Table 1. Ingredients and chemical composition of control diet (C) or treatment diets with supplemented extruded linseed (L) soybean oil (SO) or rapeseed oil (RO) for a 20 kg milk yield

Item	C	L	SO	RO
Cows assigned to study, N	37	39	39	34
Early (0-100 DiM)	12	13	14	10
Mid (101-200 DiM)	12	12	15	16
Late (201-300 DiM)	13	14	10	8
Parity	3.39	3.11	3.05	3.18
Mean days in milk†	134.2±14.2	133.7±13.7	126.0±12.9	125.7±13.2
	Ingredient, kg DM (dry matter)			
Grass silage	6.12	6.12	6.12	6.12
Corn silage	5.96	5.96	5.96	5.96
Barley straw	0.26	0.26	0.26	0.26
Hay	0.86	0.86	0.86	0.86
Salt	0.04	0.04	0.04	0.04
Soy meal	1.58	1.14	1.76	1.76
Wheat meal	2.29			
Extruded linseed		1.76		
Soybean oil			0.83	
Rapeseed oil				0.83
Feed lime	0.07	0.06	0.07	0.07
MILKINAL‡	0.10	0.10	0.13	0.13
SINCROPAC‡	0.10	0.15	0.15	0.15
NEL MJ/kg	6.7	6.9	7.0	7.0

†Mean ± SEM

‡ Product of Trouw Nutrition Deutschland GmbH (Burgheim, Germany)

2.2 Animal trial of lactation cycle impact on milk fat globule diameter, cholesterol and gene expression

Cows were kept at the experimental farm “Veitshof” (Physiology, TUM, Freising, Germany). Lactation was again divided into early (0-100 DiM), mid (101-200 DiM) and late lactation (201-300 DiM). Cows were kept in a free stall barn with littered resting areas and were milked in a 2 x 2 tandem milking parlour (GEA, WesfaliaSurge GmbH, Boenen, Germany). PMR was fed daily at 7 am to all cows during the whole lactation cycle and was calculated on a daily milk yield of 22 kg with additional individual concentrate feeding according performance with a maximum of 9 kg per day. Cows had continuous access to food and fresh water. For analysis of MFGD and for separating purified bovine milk epithelial cells, milk was collected only at morning milking and analyzed directly after to ensure that milk globules are not degraded and that epithelial cells are still intact for extracting RNA. Milk samples were taken randomly from cows at the different stages of lactation so that for analysis of MFGD, cholesterol content and fat percentage 27 samples of early lactating, 19 samples of mid lactating and 21 samples of late lactating cows were collected. For gene expression analysis of pbMECs (purified bovine milk epithelial cells) milk samples from 15 early, 15 mid and 12 late lactating cows were taken.

2.3 Trial of effects of milk conservation on analysis of cholesterol, progesterone and lactoferrin

Milk samples of 10 cows from “Veitshof” farm (Physiology, TUM, Freising, Germany) and experimental farm “Hirschau” (TUM, Marzling, Germany) were taken at a single day at morning milking. Samples were aliquoted and stored in 10 ml plastic vials with 10 vials per thawing date. Thawing dates were fixed after 1, 6 and 12 months of storage at -20 °C. Immediately after sampling cholesterol, progesterone and lactoferrin contents were analyzed and in parallel milk samples were sent to MPR (Milchprüfring) Bayern e.V. (Wolnzach, Germany) for analysis of fat protein and lactose content and pH measurement. Samples aliquoted for storage were divided into 6 treatment groups with two groups representing pure milk and four groups processed by adding conservation agents. One sample was frozen as pure milk (PM) serving as control, one as pure milk quickly frozen with liquid nitrogen and then stored at -20 °C (NM), one as milk treated with Thiomersal (TH) 1 µl/ml, one with Kathon CG 1 µl/ml (KA) added, one with Acidiol (AZ) 0.1 ml/30ml and one sample per thawing date was frozen after adding Sodium Hydroxide (SH) with a concentration of 1 µl/ml. Four vials per thawing date were frozen as PM for a later analysis of fat, protein, lactose and pH at MPR Bayern e.V. (Wolnzach, Germany).

2.4 Sampling

2.4.1 Milk samples

Milk samples for the experiment analyzing the impact of oil supplements on milk composition were taken twice a week at morning (5am) and evening (4pm) milking via an automatic separation system integrated in the milking pan. Morning samples were stored at 7 °C until evening milking and directly after mixed together according to milk yield to get a representative daily milk sample. After mixing morning and evening samples, milk was filled into 10ml plastic vials and stored prior analysis at -20°C and milk was also filled into vials provided by MPR Bayern e.V. (Wolnzach, Germany) and sent to analysis of fat, protein, lactose, urea and somatic cell count directly after. For analysis of MFGD milk samples were taken at morning milking and were directly analyzed after sampling to avoid destruction of MFG. For extraction of pbMECs, 2L of milk was used per cow directly after morning milking to ensure the extraction of living cells.

2.4.2 Blood samples

Blood samples were collected via punctuation of jugular vein. Cows were separated after morning milking, fixed and punctuated with a Vacuette blood sampling system. Blood was taken in K2EDTA tubes (Vacuette, Greiner Bio-One, Kremsmünster, Austria). Immediately after sampling, blood was centrifuged at 4500 rpm for 15 min at 4°C. Blood plasma was then separated and aliquoted in Eppendorf tubes (Eppendorf AG, Hamburg, Germany) each 1.5ml and stored at -80°C prior to analysis.

2.4.3 Liver samples

Liver samples were collected via biopsy of liver tissue with a semi-automatic Bard Magnum biopsy instrument (Bard biopsy systems, Tempe, AZ, USA) according to a method described earlier (Sigl, 2013). Before biopsy a 20x20cm field on the right side of the head-fixed cow was shaved at the crossing point of an imaginary line running from tuber coxae to the elbow joint and the 11th intercostal space. The shaved area was then washed and disinfected with 70% ethanol. Skin and muscle tissue was desensitized with a local anesthetic (10 ml procaine hydrochloride 20 mg/ml, *Isocaine ad us. vet.*, Selectavet Dr. Otto Fischer GmbH, Weyarn, Germany) and thereafter disinfected with an iodine solution (*Vet-Sept*, Albrecht GmbH, Aulendorf, Germany). At the center of the prepared site a small incision was made to admit the trocar (12 Gauge, 2.7 mm; Bard biopsy systems, Tempe, AZ, USA) for a blind percutaneous biopsy. Biopsy needle was inserted pointing to the elbow joint at the left side of the cow. Obtained liver samples, of approximately 100 mg dry weight, were immediately frozen in liquid nitrogen and stored at -80 °C until further analysis. After biopsy, the surgery area was disinfected with an iodine solution (Vet-Sept, Albrecht GmbH, Aulendorf, Germany) and cows were kept for one day in a separated area for observation of eventually occurring complications like fever or anorexia. This separation of cows helped the farm personnel to manage the waiting period for the medication used by milking treated cows at the end. The experiment was approved by the local department of animal welfare ("Referat 54 der Regierung von Oberbayern").

2.5 Analysis

2.5.1 Milk

2.5.1.1 Major Milk Components

Fat, Protein, Lactose and Urea were measured at MPR Bayern e.V. (Wolnzach, Germany) using a MilkoScan-FT-6000 spectrometer (Foss, 3400 Hillerod, Denmark). Milk samples were sent to MPR in measurement vessels provided by the laboratory prefilled with Acidiol as conservation agent. Analysis of major milk components was performed with infrared absorption measurement.

2.5.1.2 Cholesterol

Cholesterol content of milk samples was analyzed via a recently approved method (Viturro et al., 2010). Frozen milk samples (-20°C) were thawed for 30 minutes at 37 °C. Duplicates of 4 ml per milk sample were used for analysis, incubated for 15 minutes at 80 °C with 10 ml KOH. After incubation and cooling down to room temperature 3 ml Hexane (Sigma Aldrich, Taufkirchen, Germany) was added and centrifuged for 1 min at 4500 rpm. Fat dissolved in Hexane was pipetted into glass vials and put into water bath with 80 °C to evaporate Hexane. To the remaining fat 4 ml Isopropanol (99,8%) (Scharlau, Sentmenat, Spain) was added to restore the original fat concentration of the sample. After extraction of fat and cholesterol out of milk, cholesterol content was measured using a Tecan-Magellan photometer (Tecan Group Ltd., 8708 Männedorf, Switzerland). Before measurement 200 µl/well of cholesterol reagent (Cholesterol, Thermo Fisher Scientific Inc., Massachusetts, USA) was pipetted into a 96 well microtitre plate and 10 µl/well of the sample was added and incubated for 2 hours at 37 °C. Mean measured light absorption of three wells

per sample was compared with a standard curve also pipetted on the plate. Light absorption was measured with a light wavelength of 492 nm. Before measurement the plate was shaked for 10 seconds

2.5.1.3 Fatty Acids

Fatty Acid Composition was measured at the Institute of Bioanalytics (Technische Universität München, 85354 Freising, Germany) using gas chromatography. For analysis a Hewlett Packard GC Flame Ionization Detector (FID) (HP/Agilent 6890 Series, Agilent Technologies, 95051 Santa Clara California, USA), equipped with a Hewlett Packard Injector (HP 7683 series), an Agilent Auto Sampler (7683 series) and a WCOT Fused Silica capillary column (select fame Varian cp 7420) (100m x 0,25 mm ID), with hydrogen (40,0 ml/min) as carrier gas and nitrogen (30,0 ml/min) as make-up gas (Agilent Technologies, 95051 Santa Clara California, USA) was used (Altenhofer et al., 2014b, Firl et al., 2014). Gathered chromatograms were evaluated with Chromeleon 6.80 software (Dionex, Thermo Fischer Scientific Inc., Massachusetts, USA). Approximately 1.5 percent of peaks could not be identified.

2.5.1.4 Progesterone

Progesterone content was measured at every thawing date of the milk conservation experiment. Milk samples were thawed and centrifuged for 15 min at 4500 rpm. Skim milk was removed and progesterone content of skim milk was measured using an enzyme immune essay (EIA) described in Prakash et al. (1988) and Danowski (2012) with a monoclonal antibody (anti-progesterone clone 2H4 1:3500, Sigma-Aldrich, München, Germany) and a progesterone-3CMO (1:35000) label (Steraloids Inc., Rhode Island, USA). This label was coupled to horseradish peroxidase enzyme (HRP, Roche Applied Science, Mannheim, Germany) as mentioned in Danowski

(2012). For measurement of progesterone, 96 well microtitre plates (MaxiSorpNunc, Thermo Fisher Scientific, Waltham, USA) where used, coated with 1 µg/100mL affinity-cleaned goat anti-rabbit IgG (Physiology, TUM, Freising, Germany), incubated overnight and then washed twice with phosphate buffered saline added with 0.05 % Tween 80, pH 7.4 (Roth, Karlsruhe, Germany) (PBST). After washing steps 20 µL skimmed milk samples per well were added in duplicates. Progesterone-3CMO label and antibody were added, incubated in the dark and washed with PBST. Plates were measured at 450 nm by a photometer (Sunrise, Tecan Trading, Switzerland) with detectable thresholds of <0.2 ng/mL and > 3 ng/mL in skimmed milk (Danowski, 2012).

2.5.1.5 Lactoferrin

Milk lactoferrin concentration was measured at the beginning and after 1, 6 and 12 months of the conservation experiment using a competitive ELISA (enzyme linked immune assay) following the instructions given in Danowski (2012) using precoated (unspecific antibody goat anti-rabbit IgG) 96-well micro plates. After incubation and washing steps with PBST, diluted skimmed milk samples were applied onto the plate and incubated with capture antibody (produced in rabbit after immunization with bovine lactoferrin, colostrum isolate, Sigma-Aldrich, München, Germany). Concentration was measured with a Microplate Reader (Sunrise, Tecan Trading, Switzerland) at 450 nm and calculated against the optical density of the standard curve (Danowski, 2012, Danowski et al., 2013).

2.5.1.6 Milk fat globule diameter

Milk fat globule diameter was analyzed at the laboratory of the Chair for Food Process Engineering and Dairy Technology (TUM, Germany). A dispersion unit

Hydro S and a Mastersizer 2000 (Malvern Instruments, Worcestershire, UK) was used. Measurement is based on the low angle laser light scattering (LALLS) method. With this method it is possible to measure dry and wet samples with a size range between 0.02 and 2000 µm. After measurement values were analyzed using Mastersizer 2000 software (Malvern Instruments GmbH, Herrenberg, Germany) producing surface-area-related equivalent diameter D[3,2], volume-related equivalent diameter D[4,3], as well as Median value for each milk sample.

2.5.1.7 Purified bovine milk epithelial cells

For extraction of pbMECs out of milk (described in Sigl (2013)), 2 L fresh milk were aliquoted into 4 x 500 mL and centrifuged for 30 min at 4 °C at 1850 x g in a Heraeus Multifuge X3 Centrifuge (Thermo Fisher Scientific, Waltham, USA). The upper fat layer and skim milk was discarded carefully. All following steps were performed on ice to avoid RNA degradation. The remaining cell pellets were resuspended with 80 mL phosphate buffered saline (PBS) and aliquoted into two falcon tubes. After a second centrifugation step (1850 x g, 15 min, 4 °C) cells were washed with 10 mL PBS (1850 x g, 15 min, 4 °C) and resuspended with 500 µL PBS, pooled in one reaction tube and centrifuged in a 5415R Eppendorf centrifuge (Eppendorf, Hamburg, Germany) for 10 min at 400 x g at 4 °C. After this centrifugation step, 1 mL of PBS containing 1 % bovine serum albumin (BSA-PBS) was added and mixed with an antibody (Mouse Monoclonal Antibodies Cytokeratin 8, Clone-43, 1 mg/mL, EXBIO, Praha, Czech Republic). These Antibodies are specific for surface proteins on bovine epithelial cells. Unbound antibodies were removed by centrifugation at 300 x g at 4 °C for 8 min. Remaining cell-antibody complex was resuspended in 1 mL BSA-PBS and antibody-coated Dynabeads (25µL; PanMouseIgG, Invitrogen, Dynal AS, Oslo, Norway) were added. Separation was managed by fixing the

pbMEC-antibody coated Dynabeads complexes with a magnet for 2 minutes at room temperature and discarding the supernatant. Remaining pbMECs were washed with 1 mL PBS and resuspended with 700 µL QIAzol (Qiagen GmbH, Hilden, Germany) and stored at -80 °C prior analysis. RNA extraction was done following the instructions provided by the used extraction kit (miRNeasy Mini Kit, Qiagen GmbH, Hilden, Germany) eluting a final volume of 25 µL. Purity and concentration of RNA was measured with a NanoDrop spectrophotometer (NanoDrop ND-1000, Thermo Fisher Scientific, Massachusetts, USA). RNA concentration of every sample was verified against RNAse free water as blank. Integrity of RNA in each sample was measured by using an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). All steps were performed on ice to avoid RNA degradation. RNA was frozen at -80 °C until further analysis. cDNA was synthesized using the enzyme M-MLV Reverse Transcriptase, (RNAse H Minus, Point Mutant Promega, Wisconsin, USA) and random primers 0.5 µg/µL (Fermentas, St. Leon-Rot, Germany) for reversed transcription in an Eppendorf Mastercycler Gradient using the following temperature program: 21°C for 10 min, 48°C for 50 min and 90°C for 2 min. Gained cDNA was then diluted 1:1 with DNase/RNAse-free water.

Gene expression in pbMECs was measured at the Institute of Physiology using quantitative real-time polymerase chain reaction (RT-qPCR). For this reaction 1 µl of diluted cDNA, 5 µl SsoFast™EvaGreen® Supermix (BIO RAD), 0.2 µl each of 20 pmol/µl gene-specific forward and reverse primers (Table 1), and 3.6 µl DNase/RNase-free water was used. Pipetted plates (MicroAmp®Fast 96-Well Reaction Plate, Applied Biosystems) were analyzed for gene expression in a Step One Plus real-time PCR System (Applied Biosystems) under the following conditions: 30 sec. at 98°Cfor polymerase activation, 40 cycles of 5 sec. at 95°C for denaturation

and 20 sec. annealing and extension at 60°C. Melting curve analysis was carried out using incremental temperatures from 65°C to 95°C (rising by 0.5°C).

Table 2. Primer sequences of forward and reverse primers and PCR product length for the 13 genes measured in milk epithelial cells.

Gene	Function	Primer	Product length (bp)
ABCA1	Transmembrane transport	for: GGCTCTCAGACCTAGGCATC rev: GGTAAGGTGCCATCTGAGGT	118
ACACA	Fatty acid synthesis	for: ACACTCAGAGCATCGTCCAG rev: TGACCGTTCTGGAATTGTGT	124
AGPAT1	Triglyceride synthesis	for: GGAACATTCCTCAGCACCTT rev: GAGAAGAGTCCAGCCTCCAC	69
DGAT1	Triglyceride synthesis	for: AATTGGTGTGTGGTGATGCT rev: CCAGGATGCCATACTTGATG	76
FDFT1	Cholesterol synthesis	for: CCACCACAGGTCACAGTTTC rev: TGTGGGCTCCTTCTCTTTTT	89
GPAM	Triglyceride synthesis	for: AATGAGAGCCTCTGGAGCAT rev: CCGTTTTGACACATCCGTAG	73
HMGCS1	Cholesterol synthesis	for: CCTCTCTGGCCATGTGTATG rev: TGCTTTCCTCACCAGAAGTG	77
INSIG1	Regulator	for: TTCTGAACTTTGCGATGACC rev: TCGCATAGCTGATGGTTTTC	63
INSIG2	Regulator	for: TCTGCAGTGCCACAGTGTTA rev: CTTGGACCTGTTTGGGATTT	102
PPARG	Regulator	for: CATAATGCCATCAGGTTTGG rev: GTCAGCAGACTCTGGGTTCA	102
SCAP	Regulator	for: GTTTCCTTTTGGGACCTGAA rev: CTGCCAAAGTTGCAGACAAT	128
SCD	Fatty acid synthesis	for: TTGTCCACTTTCTCCTGCTG rev: GTAGCCATCACTGCCTCTGA	99
THRSP	Regulator	for: CACTTCGCTAGCCTTCATCA rev: CTGTCCCATTTTCTCCTGGT	87

2.5.2 Blood

Blood cholesterol content was measured at synlab vet GmbH (synlab vet GmbH, Augsburg, Germany) using photometry on a Beckman Coulter AU5800 (Beckman Coulter, California, US) following the instructions provided by the used cholesterol analysis kit (Beckman Coulter, California, US). For measurement of non-esterified-fatty-acids (NEFA), Blood beta-hydroxy-butyric-acid (BHBA), triglyceride and glucose levels plasma samples were sent to the Clinic for Ruminants of the Ludwig-Maximilians-University Munich (80539 Munich, Germany) where a Roche C311 auto

analyzer system (Roche Deutschland Holding GmbH, 79639 Grenzach-Wyhlen, Germany) was used. For measuring NEFAs a Randox NEFA Kit and a RANBUT D-3-Hydroxybutyrate Kit for BHBA measurement (Randox Laboratories Ltd, BT294QY Crumlin, UK), a GPO-PAP Kit (LT-SYS, Eberhard Lehmann GmbH, 14167 Berlin, Germany) for triglycerides, and a COBAS GLUH2 Kit (Roche Deutschland Holding GmbH, 79639 Grenzach-Wyhlen, Germany) for glucose level determination was used.

2.5.3 Liver

For extraction of RNA out of liver, 25 mg of liver tissue and 200 mg of MatrixGreen 1.4 mm ceramic spheres were filled into FastPrep tubes (MP Biomedicals, Santa Ana, California, USA). After adding 700 µL QIAzol Lysis reagent (Qiagen GmbH, Hilden, Germany) samples were homogenized for 40 seconds at 4.5 m/s using a MagNALyser (Roche Applied Science, Mannheim, Germany). RNA extraction was done following the instructions provided by the used extraction kit (miRNeasy Mini Kit, Qiagen GmbH, Hilden, Germany) eluting a final volume of 25 µL. Purity and concentration of RNA was measured with a NanoDrop spectrophotometer (NanoDrop ND-1000, Thermo Fisher Scientific, Massachusetts, USA). RNA concentration of every sample was verified against RNAse free water as blank. Integrity of RNA in each sample was measured by using an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). All steps were performed on ice to avoid RNA degradation. RNA was frozen at -80 °C until further analysis. cDNA was synthesized following the manufacturers protocol (miRNeasy Mini Kit, Qiagen GmbH, Hilden, Germany) as explained in chapter 2.5.1.7. *Purified bovine milk epithelial cells*.

Gene expression in liver was measured at the Institute of Biotechnology AS CR in Prague (Gene Expression Laboratoy, Biotechnology Institute of the Czech Academy of Sciences, Czech Republic) on a BioMark™ HD platform employing gene expression Dynamic Array 48.48 chips (Fluidigm). Before analysis 2 µL of cDNA sample, previously diluted to 10 ng original RNA/µL, were pre-amplified on a final volume of 10µL containing 25nM of each primer under following conditions: 10 min at 95 °C (polymerase activation), 18 cycles at 95 °C for 15 sec (denaturation) plus 4 min at 59 °C (annealing and extension) (Viturro et al., 2014). Bovine mRNA sequences of the selected target genes were gained from the National Center for Biotechnology Information (NCBI) Gene Database (NCBI, National Library of Medicine, Bethesda, MD, USA, http://www.ncbi.nlm.nih.gov/gene). A pair of forward and reverse primers was designed for each of the 48 analyzed genes (Table 3) using the software Primer3 (http://frodo.wi.mit.edu/). Because gene expression was measured under same conditions on a chip, samples were designed aiming at the following optimal properties: melting temperature 60 °C, Primer GC content 50 % and product length of 80 bp. After primer design, primers were checked virtually via data bank blast search (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi?PAGE_TYPE=BlastSearch&PROG_DEF=blastn&BLAST_PROG_DEF=megaBlast&BLAST_SPEC=OGP__9913__10708) (Viturro et al., 2014).

Table 3. Primer sequences of forward and reverse primers and PCR product length for the 48 genes measured in liver cells.

Name	Function	Sequence	Productlength (bp)
HMGCS1	Cholesterolsynthesis	for: CCTCTCTGGCCATGTGTATG rev: TGCTTTCCTCACCAGAAGTG	77
HMGCR	Cholesterolsynthesis	for: TGAGACGACTGCTTGGTTTC rev: TGCAGAGGACACAGCATGTA	112
FDFT	Cholesterolsynthesis	for: TGTGGGCTCCTTCTCTTTTT rev: CCACCACAGGTCACAGTTTC	89
ApoB	Lipoproteintransport	for: CCCTTGTCAACACTGATTGG rev: TTCTGACACGTGCTTCTTCC	75
MTTP	Lipoproteintransport	for: AAGGATGCCCTTCTCTCTCA rev: TCTTCTTTCTTTGCCGTCCT	133
ACAT	Ketone-body-synthesis	for: GGAGGAGCTGTTTCTCTTGG rev: CTTCAGTGCATGAGCCAAAT	78
HMGCS2	Ketone-body-synthesis	for: ACGTCTGCCCTCTTTCAACT rev: CAGAAGGACCCATTCAGGAT	125
CYP7A1	Bileacidsynthesis	for: TCAGACACAGCTGGACAACA rev: GAGGGAAGCACTGGAAAGTC	76
PCK1	Gluconeogenesis	for: AGGAGGGTGTGATCAAGAGG rev: GATGGGCACCGTATCTCTTT	131
PCK2	Gluconeogenesis	for: TGAACACAAAGGGAAGGTCA rev: ATGCTCAGCCAGTGTTCAAG	96
PC	Gluconeogenesis	for: GCTGTTCGTGGAGAAGTTCA rev: AGGATGTTCCCGTACTGGTC	78
PK	Glycolysis	for: GAGGAGTCTTCCCTGTGCTC rev: AAGCCACAGAGCTTTCCATT	106
ACLY	FA synthesis	for: TGGACTTCGACTACGTCTGC rev: GATCTCCTTGTGACCCCAGT	107
CPT1A	FA oxidation	for: GACGTGGATTTCCACTCCTT rev: CGGGCTGGTTTTACATTTCT	72
CPT2	FA oxidation	for: GGTCACTCAGGACAAGCAGA rev: TGGATCAGGGTTGAATGAGA	127
ACADL	FA oxidation	for: AATGCCAAAAAGGATGGAAG rev: TCACACAGCCACCCATTAGT	74
ACADVL	FA oxidation	for: TCAGAGCATCGGTTTCAAAG rev: AGGGCTCCGTTAGACAGAAA	116
ACOX1	FA oxidation	for: ATCGTACTTGCCCAGCTCTT rev: GGGCCAATGTCTCCTACAGT	122
CD36	FA trafficking	for: GGTGTGGGCAGATACCTTCT rev: ATGGCTTGGTGCTATCTGTG	62
ACSL1	FA trafficking	for: TCATCGACAGGAAGAAGCAC rev: TCGATCTTCTCGGGAGCTAT	67
ACSL4	FA trafficking	for: CTCTGACCAGTCCAGCAAAA	78

		rev: ACAGCAGCCATGAGTGTAGG	
ACSL5	FA trafficking	for: ATAGGCCAGAGTGGGTCATC	74
		rev: GGTGTCATACAAGGGCACAG	
FABP1	FA trafficking	for: CCAGAAGAGCTGTTGGATCA	127
		rev: CTTCCCCTTCTGGATGATGT	
ACBP	FA trafficking	for: TAAGACCAAGCCAGCAGATG	112
		rev: CTTGCCTTTGAAGTCCAACA	
GPAM	TG synthesis	for: AATGAGAGCCTCTGGAGCAT	73
		rev: CCGTTTTGACACATCCGTAG	
DGAT1	TG synthesis	for: AATTGGTGTGTGGTGATGCT	76
		rev: CCAGGATGCCATACTTGATG	
AGPAT1	TG synthesis	for: GGAACATTCCTCAGCACCTT	69
		rev: GAGAAGAGTCCAGCCTCCAC	
PLIN2	Lipid droplet	for: CAAGGGAGCTGTGACTGGTA	84
		rev: ATCACCCGACTTCGTAGGAC	
PLIN4	Lipid droplet	for: GGAGAACAAACCATCCCAAG	61
		rev: CTGACAGAGGTCCATGGTTG	
ASS1	Arginine synthesis	for: TGAACGTGCAGGGAGACTAC	112
		rev: GTCTATTTGGCGGTGACCTT	
CS	Citricacidcycle	for: GAATTTGCTGGTTGCAGAGA	74
		rev: GGCCTATTAAAGGGTGGACA	
IGF1	Regulator	for: AAGCAATGGGAAAAATCAGC	117
		rev: GGGCCAGATAGAAGAGATGC	
IGFBP3	Regulator	for: CGAGACAAGCCTCATCAAGA	127
		rev: GGCCCCTTGAAGATACAAAA	
GHR	Regulator	for: AGTGCGTGTGAGAACCAGAC	65
		rev: TCAGGAGCACCTCACTGAAC	
ADIPOR2	Regulator	for: GAGGCGTCTGTCCTTTCTTC	89
		rev: ATGAACCCCTCATCTTCCTG	
SREBP2	Regulator	for: TGCAAAGGTCAAAGATGAGC	99
		rev: AGAGGCAGAGGAAGGTGAG	
SCAP	Regulator	for: GTTTCCTTTTGGGACCTGAA	128
		rev: CTGCCAAAGTTGCAGACAAT	
LPIN1	Regulator	for: GAGCATAGAGGACCGAGGAG	85
		rev: CAGACACCCTTCGCAAGTTA	
INSIG2	Regulator	for: TCTGCAGTGCCACAGTGTTA	102
		rev: CTTGGACCTGTTTGGGATTT	
PPARA	Regulator	for: TTTCCACAAGTGCCTTTCAG	72
		rev: TTCTCAGATCTTGGCATTCG	
PPARG	Regulator	for: CATAATGCCATCAGGTTTGG	102
		rev: GTCAGCAGACTCTGGGTTCA	
LXRA	Regulator	for: CCGGGAAGAGATGTCTTTGT	65
		rev: CCACTGCAGAATCAGGAGAA	
THRSP	Regulator	for: CACTTCGCTAGCCTTCATCA	87
		rev: CTGTCCCATTTTCTCCTGGT	
OSBP	Regulator	for: CGAGGGACAAAGCATTACCT	120
		rev: TATTCCAGCAGTGGCTTCAG	

HNF4	Reference gene	for: GGTGGGGTAGCTGTGATTTT rev: AAAGTGGCATGGATTTCTCC	101
GADPH	Reference gene	for: TCAAGAAGGTGGTGAAGCAG rev: TTGAAGTCGCAGGAGACAAC	88
ACTB	Reference gene	for: AGATGTGGATCAGCAAGCAG	120

The reaction mix, loaded at the chip, consisted of 2.5 µL SsoFast™EvaGreen Supermix (Bio-Rad), 0.1 µL ROX (diluted 1:4, Invitrogen), 0.25 µL Binding dye loading reagent (Fluidigm), 1 µL cDNA. For the reaction, 5 µL of a premix consisting of 2 µL 10 µM forward and reverse primers, 2.5 µL loading reagent (Fluidigm) and water, was loaded. Samples and Primers were mixed followed by the qPCR cycles: 98 °C for 40 sec, 30 repetitions of 95 °C during 10 sec and 60 °C during 40 sec. At the end of the amplification, a melting curve determination step was included. An interplate calibration was included in all chips (Viturro et al., 2014).

2.6 Statistics

Statistical analysis was done with SPSS version 19.0 (IBM Deutschland GmbH, 71139 Ehningen, Germany) using paired t-tests and Levene's test for equality of variances. Correlations between groups were calculated using bivariate Pearson correlation analysis. Significance levels were set under a p value of < 0.05.

Data was gained after measurement of gene expression in liver via BioMark™ HD platform with BioMark Data Collection Software 2.1.1. (Fluidigm Corp., San Francisco, USA) and processed with Fluidigm Melting Curve Analysis Software 1.1.0. and Real-time PCR Analysis Software 2.1.1.. For discarding the existence and quantification of primer dimer products a melting curve analysis was performed. Reference genes were selected for their suitability with the Normfinder algorithm included in the real-time RT-qPCR data analysis software package GenEx 5.3.2 (MultiD Analyses AB, Gothenburg, Sweden, www.multid.se).

Three suitable reference genes were selected and a normalization index was calculated as the arithmetic mean of their Cq values for normalization of gene expression (Viturro et al., 2014).

$$\Delta Cq = Cq \text{ target gene} - \text{reference index}$$

$$\Delta\Delta Cq = \Delta Cq \text{ early lactation} - \Delta Cq \text{ late lactation}$$

3 First Authorship Paper Summary

3.1 Altenhofer et al. 2014 Effects of rapeseed and soybean oil dietary supplementation on bovine fat metabolism, fatty acid composition and cholesterol levels in milk.

The main goal of this scientific experiment was to study the effect of milk fat depression, induced by supplementing diet with two different plant oils, on the bovine fat metabolism, with special interest on cholesterol levels. For this purpose a feeding plan was designed with 4 week feeding periods, feeding either an oil ration or control ration divided by two weeks washout phase, where control ration was fed. 39 cows were selected according to their lactation stage and divided in three groups and fed different rations: a control group (C) without any oil supplementation and two groups with rapeseed oil (RO) or soybean oil (SO) added to the partial mixed ration (PMR). The design of the PMR was mainly done by Dr. Enrique Viturro and the Author with help of an external feeding expert Fabian Hosp. Sampling of milk four times a week, of PMR samples once a week, and of blood samples at the beginning and at the end of feeding period was completely done by the Author as well as the supervision of the experiment at the experimental farm Hirschau. Collecting of data and keeping database up to date as well as statistical analysis was also done by the Author with advisory help of Dr. Enrique Viturro. After finishing the feeding experiment and analysis of data a decrease in milk fat percentage was observed in both oil feedings with a higher decrease of -1.14 % with SO feeding than RO with -0.98 % compared to the physiological (-0.15 %) decline in the C group. There was no significant change in lactose and protein yield. The daily milk cholesterol yield, analyzed at Institute of Physiology (TUM) by the Author with help of the technical assistants, was lower in

both oil rations than in control ration. When looking at the blood cholesterol level, analyzed at synlab. Vet GmbH laboratories it showed an opposite variation than milk cholesterol content. The milk fatty acid pattern, analyzed at the Institute of Bioanalytics (TUM) by the Author with the help of Master student Melanie Spornraft and technical assistant Hermine Kienberger, showed a highly significant decrease of over 10 % in the amount of saturated fatty acids (SFA) in both oil feedings. Mono (MUFA) and poly (PUFA) unsaturated fatty acids, conjugated linoleic acids (CLA) included showed a highly significant increase. The results of this experiment suggest that the feeding of oil supplements has a high impact on milk composition, especially fat composition and its significance for human health, by decreasing fats with a potentially negative effect (SFA and cholesterol) while simultaneously increasing others with positive (MUFA, PUFA, CLA). Not published in this paper are the results of a third oil feeding where a ration with extruded linseed as the oil source was fed. These results were not included, though very similar in fatty acid composition of milk, because they were not fully comparable because of the completely different availability of fatty acids in this ration where no pure oil was added compared to the other two rations mentioned in this paper. Results of linseed feeding were presented in two non-scientific agricultural journals (Viturro and Altenhofer, 2013a, Viturro and Altenhofer, 2013b).

Journal of Dairy Research, Page 1 of 9. © *Proprietors of Journal of Dairy Research 2013*
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Effects of rapeseed and soybean oil dietary supplementation on bovine fat metabolism, fatty acid composition and cholesterol levels in milk

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The main goal of this experiment was to study the effect of milk fat depression, induced by supplementing diet with plant oils, on the bovine fat metabolism, with special interest in cholesterol levels. For this purpose 39 cows were divided in three groups and fed different rations: a control group (C) without any oil supplementation and two groups with soybean oil (SO) or rapeseed oil (RO) added to the partial mixed ration (PMR). A decrease in milk fat percentage was observed in both oil feedings with a higher decrease of − 1·14 % with SO than RO with − 0·98 % compared with the physiological (− 0·15 %) decline in the C group. There was no significant change in protein and lactose yield. The daily milk cholesterol yield was lower in both oil rations than in control ration, while the blood cholesterol level showed an opposite variation. The milk fatty acid pattern showed a highly significant decrease of over 10 % in the amount of saturated fatty acids (SFA) in both oil feedings and a highly significant increase in mono (MUFA) and poly (PUFA) unsaturated fatty acids, conjugated linoleic acids (CLA) included. The results of this experiment suggest that the feeding of oil supplements has a high impact on milk fat composition and its significance for human health, by decreasing fats with a potentially negative effect (SFA and cholesterol) while simultaneously increasing others with positive (MUFA, PUFA, CLA).

Keywords: Milk fat depression, cholesterol, fatty acid, soy oil, rapeseed oil.

Milk and milk products are main components of modern human nutrition and are deeply implanted into culture, especially in western countries. The relatively high level of saturated fats and the frequency of their consumption have attracted the attention of the scientific community in the last decades (Menotti et al. 1999; Malpuech-Brugere et al. 2010). Although milk products are considered among the aliments with a neutral or positive effect on cardiovascular health, the frequency of their consumption causes the amount of cholesterol ingested from this group of aliments to account for almost the half of the recommended amount of 300 mg cholesterol/d (milk contains a mean value of 15 mg/dl cholesterol) (Royo-Bordonada et al. 2003; Viturro et al. 2006a). Cholesterol is a central molecule in life as a substrate for steroid hormones and cellular membranes, but when present in high concentrations in blood is also a mediator in arteriosclerosis and cardiovascular diseases (Ren et al. 2010; Weingartner et al. 2011).

In the last years, significant advances have been made in understanding the bovine cholesterol metabolism and its transport into milk (Farke et al. 2006; Viturro et al. 2006b). Together with animal breeding, a suitable first attempt of alteration of milk cholesterol levels might come from feeding management. Diverse effects of different oils in the feeding ration on milk composition have already been outlined by many studies and reviews (Glasser et al. 2008; Huang et al. 2008; Rego et al. 2009), but little is known about the impact of these supplements on bovine cholesterol metabolism. Some efforts in this direction have already been made by Reklewska et al. (2002) by feeding linseed combined with a mineral mixture, but more research with other plant supplements is needed. The supplements usually employed in these experiments are rich in polyunsaturated fatty acids, molecules well known to exert cholesterol-lowering properties in other

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species (Connor, 2000; Haug et al. 2007), so their potential to positively alter milk cholesterol concentration is very high.

Simultaneously to reducing cholesterol levels, oil supplements may favourably change the fatty acid composition of milk by lowering the amount of saturated fatty acids and increasing the proportion of mono- and polyunsaturated fatty acids (Jacobs et al. 2011). Special attention is given in the last years to the unsaturated fatty acids present in milk: while various trans fatty acids (**TFA**) consumed in high doses may have adverse effects on plasma lipids and other cardiovascular risk factors (Sun et al. 2007; Motard-Bélanger et al. 2008), conjugated linoleic acids (**CLA**) have antiatherogenic and anticancerogenic impacts (Lock & Bauman, 2004).

The present work analyses the potential of soybean oil and rapeseed oil supplementation for altering bovine cholesterol metabolism and reducing the concentration of this metabolite in milk based on the hypothesis that diet-induced milk fat depression may also have an influence on the cholesterol level due to a changed blood and milk composition. Results presented in this study may serve for further research in production of 'low fat, low cholesterol milk' as an alternative to conventional milk products.

Material and methods

Animals and experimental design

The present study was performed at the research farm Hirschau of the Technische Universitaet Muenchen (TUM, 85354 Freising, Germany) from January 2011 to April 2012. 39 multiparous Holstein cows (3·39 ± 0·205, mean lactation number ± SEM) with a mean lactation yield of 30·69 ± 9·77 kg were examined. The study aimed at the comparison between three diets: soybean oil-supplemented ration (**SO**), rapeseed oil-supplemented ration (**RO**) and a control ration (**C**). The length of the feeding phases was four weeks and all rations were isoenergetic in order to avoid misinterpretation of results (Table 1). A washout period of two weeks, in which the animals received control ration, was included between all experimental feeding phases. The duration of two weeks for the washout was fixed taking the literature on the topic into account, which quotes 10 to 14 d as sufficient (Collomb et al. 2004; Zened et al. 2012). Due to practical reasons (i.e. insufficient farm personnel), a plan with alternate four-weeks feeding periods was designed in which all animals in the farm rotated simultaneously through the same feeding plan. Individuals were afterwards classified into a concrete experimental group depending on their lactation stage and the ration given at each moment. Three time periods were considered: early (days in milk (**DiM**) 1–100), mid (DiM 101–200), and late (DiM 201–300) phase (Table 1). Only the individuals which could be clearly assigned to a specific time group were considered for the analysis; those whose start and end day spanned between two different time groups were excluded to avoid misclassification of data. The result was therefore a different but comparable number of individuals for each of the nine feed-lactation stage combinations (Table 1). The department responsible for animal welfare affairs approved all experiments.

Table 1. Ingredients and chemical composition of control diet (C) or treatment diets with supplemented soybean oil (SO) or rapeseed oil (RO) for a 20 kg milk yield

Item	C	SO	RO
Cows assigned to study, *N*	37	39	34
Early (0–100 DiM)	12	14	10
Mid (101–200 DiM)	12	15	16
Late (201–300 DiM)	13	10	8
Parity	3·39	3·05	3·18
Mean days in milk†	134·2 ± 14·2	126·0 ± 12·9	125·7 ± 13·2
Ingredient, kg DM			
Grass silage	6·12	6·12	6·12
Corn silage	5·96	5·96	5·96
Barley straw	0·26	0·26	0·26
Hay	0·86	0·86	0·86
Salt	0·04	0·04	0·04
Soy meal	1·58	1·76	1·76
Wheat meal	2·29		
Soybean oil		0·83	
Rapeseed oil			0·83
Feed lime	0·07	0·07	0·07
MILKINAL‡	0·10	0·13	0·13
SINCROPAC‡	0·10	0·15	0·15
NEL MJ/kg§	6·7	7·0	7·0

† Mean ± SEM
‡ Product of Trouw Nutrition Deutschland GmbH (Burgheim, Germany)
§ 1 Mega Joule (MJ) = 238·845896627 kcal

Housing and feeding

Cows were kept in a free stall barn with rubber mat bedding and the exercise areas had a solid floor surface and a scrapper system for waste removal. Cows were milked twice daily at 5 am and 4 pm in a double six herringbone milking parlour (DeLaval GmbH, 21509 Glinde, Germany). Six weeks before expected calving, cows were dried off using an antibiotic drying supplement (Orbenin, Pfizer, 10785 Berlin, Germany) and a non-antibiotic intramammary seal (OrbeSeal, Pfizer, 10785 Berlin, Germany). Cows were fed a partial mixed ration (**PMR**) calculated on a minimal milk yield of 22 kg (Table 1) with additional concentrate feeding according to individual milk yield, with a maximum of 8 kg per d at the beginning of lactation. Concentrate was given through an automatic dispenser with an integrated body weighing system and the animals had ad libitum access to fresh water. Cows were fed daily at 9 am with continuous access to food and automatic recording of the amounts consumed at each time by an electronic weighing system integrated to the scale pans.

Sampling

PMR Samples were collected weekly for DM and composition analysis. Milk samples were collected twice a week

during the whole experiment. At the sampling days, aliquots of both morning and evening milking were obtained and mixed according to the milk yield to get a representative sample for the complete day and avoid composition differences between morning and evening milkings. Samples were sent to MPR Bayern e.V. (accredited laboratory after DIN EN ISO/IEC 17025 and DIN EN ISO 9001 certified, 85283 Wolnzach, Germany) for analysis of major milk components and several aliquots were kept at −20 °C for posterior analysis of cholesterol level and fatty acid composition.

Blood samples were taken in the morning of days 1 and 28 of each feeding period representing the 'before' and 'after' feeding situation. Samples were gained by puncturing the jugular vein, collected in K2EDTA tubes (Vacuette, Greiner Bio-One, 4550 Kremsmünster, Austria) and immediately centrifuged at 4500 rpm for 15 min. Plasma aliquots were frozen in Eppendorf tubes at −80 °C until further analysis.

Milk yield, feed intake and body weight were automatically measured and recorded daily until the end of the project for statistical evaluation. Daily milk yield data was transformed into energy corrected milk yield (**ECM**) according to the formula: ECM (kg/d) = [(0·38 × fat% + 0·21 × protein% + 1·05)/3·28] × milk yield (kg/d) (Schlamberger et al. 2010).

Analysis

Analysis of PMR composition was performed at LKS-Landwirtschaftliche Kommunikations- und Service-GmbH (accredited laboratory after DIN EN ISO/IEC 17025 and DIN ISO 9001 certified, 09577 Lichtenwalde, Germany) using Weender analysis (Table 1).

Quantification of milk fat, protein, lactose and urea by infrared spectrophotometry was performed at MPR Bayern e.V. laboratories (85283 Wolnzach, Germany) using a MilkoScan-FT-6000 spectrometer (Foss, 3400 Hillerod, Denmark). Cholesterol measurement in milk was performed after a previously approved colorimetric method (Viturro et al. 2010). Plasma cholesterol analysis was performed by synlab.vet GmbH (86156 Augsburg, Germany) using photometry. Blood beta-hydroxy-butyric-acid (**BHBA**), non-esterified-fatty-acids (**NEFA**), glucose and triglyceride levels were measured at the Clinic for Ruminants of the Ludwig-Maximilians-University Munich (80539 Munich, Germany). A Roche C311 auto analyser system (Roche Deutschland Holding GmbH, 79639 Grenzach-Wyhlen, Germany) was used with a Randox NEFA Kit for measuring NEFAs and a RANBUT D-3-Hydroxybutyrate Kit for BHBA measurement (Randox Laboratories Ltd, BT294QY Crumlin, UK), a GPO-PAP Kit (LT-SYS, Eberhard Lehmann GmbH, 14167 Berlin, Germany) for triglycerides, and a COBAS GLUH2 Kit (Roche Deutschland Holding GmbH, 79639 Grenzach-Wyhlen, Germany) for glucose level determination. Fatty acid profiling was performed by gas-chromatography (GC) at the Institute of Bioanalytics (Technische Universität München, 85354 Freising, Germany) using a Hewlett Packard GC Flame Ionisation Detector (FID) (HP/Agilent 6890 Series, Agilent Technologies, 95051 Santa Clara California, USA), equipped with a Hewlett Packard Injector (HP 7683 series), an Agilent Auto Sampler (7683 series) and a WCOT Fused Silica capillary column (select fame Varian cp 7420) (100 m × 0·25 mm ID), with hydrogen (40·0 ml/min) as carrier gas and nitrogen (30·0 ml/min) as make-up gas (Agilent Technologies, 95051 Santa Clara California, USA). Chromatograms were evaluated with Chromeleon 6.80 software with a 1.5 percentage of unidentified peaks.

Statistics

Statistical analysis was performed with SPSS 19 software (IBM Deutschland GmbH, 71139 Ehningen, Germany). Dry matter intake data is presented as the difference between the first two and the last 2 d of the feeding period. ECM, fat content, milk cholesterol, fatty acid content and blood composition is reported as difference between days 1 and 28 of the feeding period, respectively. Body weight data was pooled to weekly means (week 1 compared with week 4) before statistical analysis. Data were analysed using *t*-tests and Levene's test for equality of variances.

Results

Feeding and body weight

C, SO, and RO had comparable protein and energy levels. Starch level of C was higher compared with SO and RO due to calculated energy compensation of fat supplement in SO and RO (Table 1). The different rations offered had no significant effect on the daily feed intake and body weight during the 4 week feeding periods (Table 2).

Plasma composition

NEFA, BHBA and glucose contents are presented in Table 2. Blood plasma showed a significant increase in BHBA level in SO ($P < 0{\cdot}05$). All other components measured had no significant change between beginning and end of feeding period compared with each other.

Milk yield and milk composition

Feeding of oil supplemented rations resulted on a highly significant reduction in milk fat percentage and milk fat yield compared with C (Table 2). This difference was maintained when analysing mid and late lactating animals separately ($P < 0{\cdot}05$, Fig. 1b, c) but not for early lactating cows (Fig. 1a) with a significantly higher reduction in mid lactating cows fed SO than RO ($P < 0{\cdot}05$). There was no significant difference in ECM, protein and lactose yield of oil feedings and control feeding, except late lactating cows fed SO had a significant reduction in milk yield compared with C ($P < 0{\cdot}05$) (Table 2, Fig. 1c).

Table 2. Feed intake, body weight, milk and blood composition(mean ± SEM) changes (value at end – value at beginning of feeding period) compared among treatment groups (Control (CO), soybean oil (SO), rapeseed oil (RO)). Groups with different letter superscripts are significantly different ($P<0·05$)

Treatment group	C	SO	RO
PMR intake (kg DM)	−0·5 ± 0·87	0·75 ± 0·84	−1·29 ± 0·49
Body weight (kg)	−2·76 ± 3·62	−4·30 ± 2·15	1·90 ± 3·63
ECM (kg)	−1·71 ± 0·99	−3·06 ± 0·98	−3·06 ± 0·69
Fat (%)	−0·15 ± 0·15^{a}	−1·14 ± 0·13^{b}	−0·88 ± 0·1^{b}
Fat (g/d)	−72·32 ± 36·69^{a}	−303·75 ± 57·36^{b}	−257·35 ± 35·98^{b}
Protein (%)	−0·2 ± 0·14	−0·16 ± 0·06	−0·15 ± 0·07
Protein (g/d)	−49·36 ± 48·11	−17·61 ± 25·02	−37·74 ± 23·71
Lactose (%)	0·09 ± 0·08	−0·02 ± 0·02	0·04 ± 0·02
Milk cholesterol (mmol/l)	−0·02 ± 0·04^{a}	−0·17 ± 0·03^{b}	−0·11 ± 0·03^{ab}
Blood cholesterol (mmol/l)	0·1 ± 0·17^{a}	1·48 ± 0·2^{b}	0·68 ± 0·07^{c}
Blood NEFA	−0·04 ± 0·02	−0·03 ± 0·04	−0·06 ± 0·03
Blood BHBA	−0·07 ± 0·05^{a}	0·2 ± 0·12^{b}	−0·03 ± 0·07^{ab}
Blood glucose	0·16 ± 0·07	0·03 ± 0·06	0·16 ± 0·05
Blood triglyceride	0·00 ± 0·01	0·02 ± 0·01	0·00 ± 0·01

Milk fatty acid composition

The effects of oil supplements in diet on fatty acid composition are presented in Table 3. The content of acids of the short chain fraction (C6:0 to C17:0) decreased more in SO and RO than in C. Especially C10:0, C12:0, C14:0, and C16:0 had the greatest diminution in both SO and RO respectively with the biggest decrease of myristic and palmitic acid. Total saturated fatty acids (**SFA**) increased in C and decreased highly significantly in both SO and RO except C18:0, which showed an increase compared with C. Monounsaturated fatty acids (**MUFA**), polyunsaturated fatty acids (**PUFA**), CLAs and TFAs showed a substantial increase in SO and RO. The decrease of saturated fatty acids and the increase of unsaturated fatty acids appeared in every lactation stage for oil supplemented diets with the greatest changes in mid lactation (Fig. 1a–c). For SFA the reduction was highly significant in early, mid, and late lactation ($P<0·01$ except SO late $P=0·057$). MUFAs had the opposite effect and showed a highly significant increase in all lactation stages of oil diets ($P<0·01$ except SO late $P<0·05$). Compared with C there was also a significant higher increase in PUFA, TFA and CLA in early and mid lactating cows ($P<0·01$). Late lactating cows with SO and RO showed smaller effects compared with C than the earlier lactation periods. PUFAs, TFAs and CLAs in SO Late showed no significant difference compared with C Late ($P>0·05$). The increase in CLA including C18:2t10c12 and transC18:1 is correlated with the observed reduction of milk fat in both oil rations (Tables 2 and 3). Compared with cows fed the C diet, those with SO and RO diet had a tremendous increase in C18:1 fatty acids. In the C18:1 fraction, C18:1 cis-9 was the most prominent isoform with the biggest increase compared with C.

Cholesterol in milk and plasma

Milk and plasma cholesterol levels are presented in Fig. 2. In the mean of all lactation stages there was a highly significant decrease in milk cholesterol level in SO and a tendency to decrease in RO compared with C (Table 2). The milk cholesterol level had a bigger decrease in all lactation stages of the two oil feedings compared with C, but was significantly proved just in late lactating cows fed the SO diet ($P<0·05$). In contrast to this decrease in milk cholesterol, a significant increase in plasma cholesterol level in SO and in RO compared with C was observed. Blood cholesterol had a significant increase in all lactation stages in SO group (early $P<0·05$, mid $P<0·01$, late $P<0·01$) and a significant increase in mid and late lactation in RO group ($P<0·05$) compared with C.

Discussion

Feed intake, milk yield and major milk components

The primary objective of the study was to test the hypothesis that diet-induced milk fat depression may also have an influence on the cholesterol levels in milk. A milk fat depression (**MFD**) was successfully achieved by adding 0·83 kg of oil to the diet, as demonstrated by the drastic reduction in fat percentage and fat yield on the SO and RO groups. The altering effects of certain fatty acids on milk fat content have already been described previously (Perfield II et al. 2004; Kadegowda et al. 2008). Gama et al. (2008) reduced milk fat percentage by more than 1 % by adding 1·6 % fish oil to the diet which is similar to the reduction achieved with this experiment adding SO and RO.

Other major milk parameters, such as milk yield and protein yield were not affected by oil supplementation

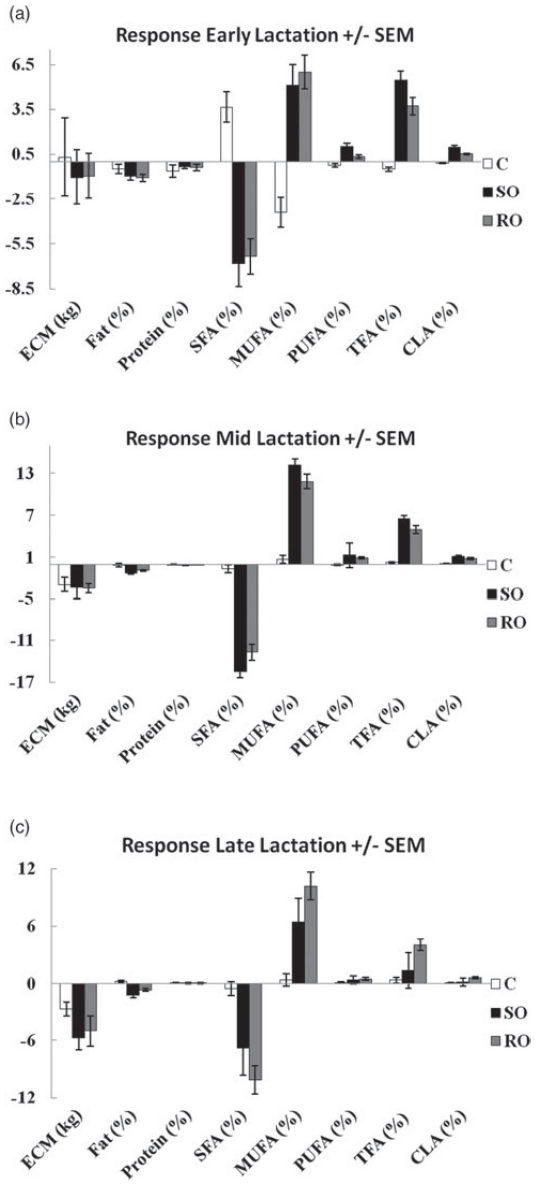

Fig. 1. (a) Percent change in milk composition parameters after the 28 d feed supplementation in individuals at early lactation stage (DiM 0 to 100). (b) Percent change in milk composition parameters after the 28 d feed supplementation in individuals at mid lactation stage (DiM 101 to 200). (c) Milk composition response (value at end – value at beginning of feeding period) of early (a) mid (b) and late (c) lactating cows fed control PMR (C) or soybean oil supplement (SO) or rapeseed oil supplement (RO) on energy corrected milk (ECM kg), fat %, protein %, saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), trans fatty acids (TFA), conjugated linoleic acid (CLA).

compared with the control diet. The influence of MFD on protein yield is not clear in the available literature and the results from similar feeding experiments are contradictory. For example, in the experiments by He et al. (2012) cows were fed diets with up to 4 % supplemental fat, observing no effect in milk yield and protein yield, but resulting on an increased milk protein concentration. However, previous research also reports unaffected milk yield, protein yield and lactose yield but a decrease in milk protein concentration by 2·5 % oil supplementation (Abdelqader et al. 2009).

Body weight and dry matter intake (**DMI**) were not affected in our study, probably due to the short feeding periods of four weeks. AlZahal et al. (2008), Huang et al. (2008) and Jacobs et al. (2011) also failed to observe changes in DMI by feeding SO or RO. He et al. (2012) described a slightly reduced DMI when cows were fed an oil diet rich in C18:1 or C18:2 whereas body weight remained unchanged.

Blood plasma

The unaffected blood plasma parameters glucose, triglycerides, NEFA and BHBA in RO (except the increase in BHBA in SO) are in good agreement with previous studies which also reported that oil feedings did not change these plasma metabolites (Abdelqader et al. 2009; Dai et al. 2011). In the study of Abdelqader et al. (2009) though a corn oil diet was fed and the plasma levels showed no significant difference compared with a control ration without oil supplement. In Dai et al. (2011) NEFAs showed a numerical increase but did not reach significance, whereas all other plasma metabolites stayed unchanged when fed RO.

Fatty acid composition

Feeding vegetable oils, rich in C18:1 and C18:2 fatty acids, comes naturally associated with a lowering of the amount of milk saturated fat and an increase of unsaturated fat, as widely described before (Dai et al. 2011; Jacobs et al. 2011; He et al. 2012). Stearic acid (C18:0) is the only SFA increasing in the oil feeding groups, due to the abundance of C18 unsaturated fatty acids in SO and RO diet. This effect is in agreement with Loor & Herbein (2003a) and Huang et al. (2008) who presented an increase of C18:0 in a SO diet compared with a diet with no supplemented oil and is also present in other ruminant species such as goats (Chilliard & Ferlay, 2004).

The decrease in C4:0 to C17:0 fraction, originating from de novo lipogenesis, is strongly correlated to the CLA content and follows the theory formulated by Bauman and co-workers (Bauman et al. 2008). Interestingly, the SO diet showed a bigger potential to reduce this group of fats than the RO diet, consistent with previous studies which reported a decrease in FA shorter than 16 carbons with a bigger decrease in C18:2 – rich soybean oil supplement than in C18:1-rich rapeseed oil supplemented diet because of an altered ruminal biohydrogenation and its intermediates

Table 3. Milk fatty acid composition of treatment groups (control (C), soybean oil (SO), rapeseed oil (RO)), comparing secretion response (value at end – value at beginning of feeding period) (mean ± SEM). Groups with different letter superscripts are significantly different ($P<0·05$)

Treatment group			
Fatty acid. %†	C	SO	RO
4:0	−0·014 ± 0·07^{a}	−0·351 ± 0·13^{b}	−0·179 ± 0·07^{ab}
6:0	0·063 ± 0·05^{a}	−0·498 ± 0·09^{b}	−0·389 ± 0·06^{b}
8:0	0·052 ± 0·03^{a}	−0·373 ± 0·06^{b}	−0·297 ± 0·04^{b}
10:0	0·125 ± 0·08^{a}	−0·993 ± 0·14^{b}	−0·853 ± 0·09b
10:1	0·011 ± 0·08^{a}	−0·086 ± 0·02^{b}	−0·081 ± 0·01^{b}
11:0	0·004 ± 0·00^{a}	−0·025 ± 0·00^{b}	−0·015 ± 0·00^{c}
12:0	0·142 ± 0·10^{a}	−1·045 ± 0·15^{b}	−0·998 ± 0·10^{b}
12:1 *cis*-9	0·003 ± 0·00^{a}	−0·015 ± 0·00^{b}	−0·019 ± 0·00^{b}
13:0	0·011 ± 0·01^{a}	−0·036 ± 0·01^{b}	−0·019 ± 0·00^{c}
Anteiso 13:0	0·003 ± 0·00^{a}	−0·020 ± 0·00^{b}	−0·022 ± 0·00^{b}
14:0	0·336 ± 0·19^{a}	−1·903 ± 0·28^{b}	−1·523 ± 0·21^{b}
Iso-14:0	0·005 ± 0·00^{a}	−0·013 ± 0·00^{b}	−0·011 ± 0·00^{b}
14:1 *cis*-9	0·013 ± 0·03	0·113 ± 0·05	0·035 ± 0·04
15:0	0·089 ± 0·04^{a}	−0·231 ± 0·04^{b}	−0·131 ± 0·03^{c}
Iso-15:0	0·019 ± 0·01^{a}	−0·019 ± 0·01^{b}	−0·029 ± 0·00^{b}
Anteiso-15:0	0·036 ± 0·01^{a}	−0·001 ± 0·01^{b}	−0·020 ± 0·01^{b}
16:0	−0·045 ± 0·52^{a}	−6·427 ± 0·72^{b}	−8·007 ± 0·60^{b}
Iso-16:0	0·010 ± 0·01^{a}	0·000 ± 0·01^{ab}	−0·019 ± 0·01^{b}
16:1 *trans*-9	−0·000 ± 0·00^{a}	0·112 ± 0·01^{b}	0·061 ± 0·01^{c}
16:1 *cis*-9	−0·115 ± 0·04	−0·020 ± 0·11	−0·214 ± 0·07
17:0	0·002 ± 0·01^{a}	−0·099 ± 0·01^{b}	−0·075 ± 0·01^{b}
Iso-17:0	0·013 ± 0·00^{a}	0·036 ± 0·01^{b}	0·006 ± 0·00^{a}
Anteiso-17:0	0·010 ± 0·01	0·007 ± 0·01	0·004 ± 0·01
17:1 *cis*-9	−0·011 ± 0·01	−0·037 ± 0·01	−0·028 ± 0·01
18:0	−0·083 ± 0·33^{a}	1·827 ± 0·25^{b}	2·146 ± 0·41^{b}
Iso-18:0	−0·002 ± 0·00^{a}	−0·011 ± 0·00^{b}	−0·006 ± 0·00^{ab}
18:1 ∑*trans*-6/*trans*-9/*trans*-10	0·012 ± 0·02	2·277 ± 0·31	2·241 ± 0·27
18:1 *trans*-11	0·016 ± 0·08	1·604 ± 0·37	1·390 ± 0·17
18:1 *cis*-9	−0·622 ± 0·47	5·083 ± 0·69	6·006 ± 0·63
18:1 *cis*-11	−0·040 ± 0·02^{a}	−0·003 ± 0·03^{a}	0·184 ± 0·03^{b}
18:1 *cis*-12	0·005 ± 0·01^{a}	0·139 ± 0·05^{b}	0·058 ± 0·02^{b}
18:2 *cis*-9, *cis*-12	−0·066 ± 0·04^{a}	0·214 ± 0·05^{b}	0·004 ± 0·04^{a}
18:2 *cis*-9, *trans*-11 (CLA)	0·023 ± 0·03^{a}	0·797 ± 0·15^{b}	0·653 ± 0·06^{b}
18:2 *tans*-10, *cis*-12 (CLA)	0·000 ± 0·00^{a}	0·013 ± 0·00^{b}	0·018 ± 0·01^{b}
18:3 *cis*-9, *cis*-12, *cis*-15	−0·005 ± 0·01	0·020 ± 0·03	0·006 ± 0·02
20:0	0·004 ± 0·00^{a}	0·030 ± 0·03^{ab}	0·086 ± 0·03^{b}
20:1 *cis*-11	−0·002 ± 0·00^{a}	0·010 ± 0·00^{b}	0·079 ± 0·01^{c}
20:2 *cis*-11, *cis*-14	0·000 ± 0·00	0·000 ± 0·00	0·001 ± 0·00
20:3 *cis*-8, *cis*-11, *cis*-14	−0·001 ± 0·01^{a}	−0·017 ± 0·00^{b}	−0·005 ± 0·00^{a}
20:4 *cis*-5, *cis*-8, *cis*-11, *cis*-14	−0·005 ± 0·01^{a}	−0·036 ± 0·00^{b}	−0·017 ± 0·01^{a}
20:5 *cis*-5, *cis*-8, *cis*-11, *cis*-14, *cis*-17	−0·004 ± 0·00^{a}	−0·010 ± 0·00^{b}	−0·015 ± 0·00^{b}
21:0	0·002 ± 0·00^{a}	−0·001 ± 0·00^{b}	−0·001 ± 0·00^{b}
22:0	0·004 ± 0·00	0·002 ± 0·00	0·001 ± 0·00
22:1 *cis*-13	−0·001 ± 0·00^{a}	−0·002 ± 0·00^{a}	0·004 ± 0·00^{b}
22:5 *cis*-7, *cis*-10, *cis*-13, *cis*-16, *cis*-19	−0·003 ± 0·01^{a}	−0·020 ± 0·00^{b}	−0·014 ± 0·00^{b}
22:6 *cis*-4, *cis*-7, *cis*-10, *cis*-13, *cis*-16, *cis*-19	0·002 ± 0·00	0·005 ± 0·00	0·002 ± 0·00
23:0	0·002 ± 0·00	0·002 ± 0·00	0·003 ± 0·00
24:0	0·004 ± 0·00^{a}	0·003 ± 0·00^{a}	−0·003 ± 0·00^{b}
Summation			
SFA‡	0·791 ± 0·55^{a}	−10·139 ± 1·17^{b}	−10·350 ± 0·85^{b}
MUFA§	−0·733 ± 0·53^{a}	9·174 ± 1·06^{b}	9·717 ± 0·8^{b}
PUFA¶	−0·058 ± 0·06^{a}	0·965 ± 0·15^{b}	0·633 ± 0·08^{b}
CLA††	0·023 ± 0·03^{a}	0·809 ± 0·15^{b}	0·671 ± 0·07^{b}
TFA‡‡	0·051 ± 0·13^{a}	4·801 ± 0·64^{b}	4·363 ± 0·35^{b}

† Results are shown as delta values day 2 vs. day 26 of feeding period ± SEM
‡ Saturated fatty acids: ∑ (C4:0, C6:0, C8:0, C10:0, C11:0, C12:0, C13:0, anteiso-C13:0, C14:0, iso-C14:0, C15:0, iso-C15:0, anteiso-C15:0, C16:0, iso-C16:0, C17:0, iso-C17:0, anteiso-C17:0, C18:0, iso-C18:0, C20:0, C21:0, C22:0, C23:0, C24:0). Iso and anteiso Fatty Acids are not shown in the table
§ Monounsaturated fatty acids: ∑ (C10:1, C12:1 c9, C14:1 c9, C16:1 t9, C16:1 c9, C17:1 c9, C18:1 t6+t9+t10, C18:1 t11, C18:1 c9, C18:1 c11, C18:1 c12, C20:1 c11, C22:1 c13)
¶ Polyunsaturated fatty acids: ∑ (C18:2 c9c12, C18:2 c9t11 CLA, C18:2 t10c12 CLA, C18:3 c9c12c15, C20:2 c11c14, C20:3 n-6, C20:4 n-6, C20:5 n-3, C22:5 n-3, C22:6 n-3)
†† Conjugated linoleic acid: ∑ (C18:2 c9t11, C18:2 t10c12)
‡‡ Trans fatty acid: ∑ (C16:1 t9, C18:1 t6+t9+t10, C18:1 t11, C18:2 c9t11 CLA, C18:2 t10c12 CLA)

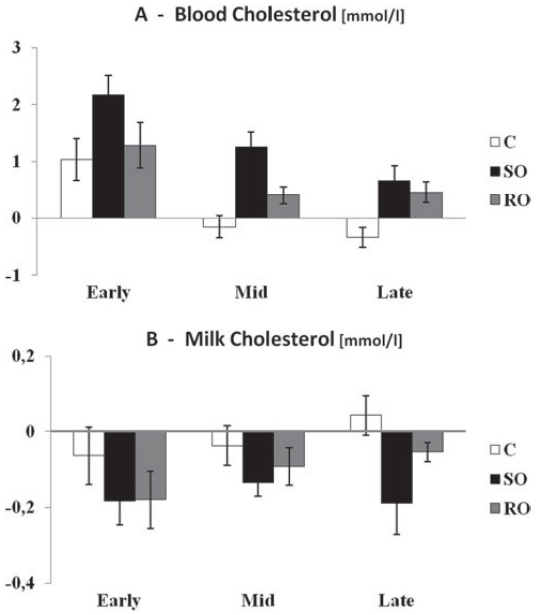

Fig. 2. Response (value at end – value at beginning of feeding period) after feeding soybean oil supplement (SO) or rapeseed oil supplement (RO) compared with control (C) on blood cholesterol [mmol/l] (panel A) and milk cholesterol [mmol/l](panel B).

(He et al. 2012). Especially the drastic reduction of the myristic acid amount is of great interest due to its potential benefits for human health (Müller et al. 2001; Dabadie et al. 2005).

As observed in our study, AlZahal et al. (2008) associated milk fat depression with an increase in total trans C18:1 and total CLA. The negative correlation of milk fat content and CLA C18:2 t10c12 was also well described by Peterson et al. (2003), Bauman et al. (2008), and Glasser et al. (2010). Zened et al. (2012) suggested that a shift from the trans – 11 to trans – 10 pathway of ruminal biohydrogenation associated with milk fat depression was due to oil supplementation of a high-wheat diet. Loor & Herbein (2003b) induced a reduced milk fat production by performing a CLA C18:2t10c12 infusion and proposed that its uptake by the mammary gland may decrease the de novo fatty acid synthesis and desaturation of long chain fatty acids. The decrease of desaturated long chain fatty acids is apparent in this experiment for the fatty acids C20:3n-6, C20:4n-6 and C22:5 for SO and C20:5n-3 in SO and RO. In our experiments, an increase in other unsaturated long chain fatty acids (C22:1 in RO or C20:1n-9 in SO and RO) was also observed, in agreement with Rego et al. (2009) who detected an increase in C20:1 and a decrease in C20:3, C20:4, C20:5 FAs by feeding SO or RO. An increased milk TFA level was also observed after oil supplementation, but the role of this group of fats in human diet is still a topic of discussion and might need a differentiation between fats of ruminant and industrial origin. The publications reporting a potential negative effects of trans fatty acids produced by ruminants refer to very high intake doses (Sun et al. 2007; Motard-Bélanger et al. 2008), whereas Jakobsen et al. (2008) and Gebauer et al. (2011) suggests that ruminal trans fatty acids are not associated with a higher risk of coronary diseases.

Milk and plasma cholesterol

Diet induced milk fat depression can be also considered as a powerful method for studying milk fat synthesis and general bovine fat metabolism. Many studies on this extreme metabolic situation have previously reported its effects on milk composition (Gama et al. 2008; Glasser et al. 2010; He et al. 2012), but to our knowledge none of them focused on milk cholesterol level and its variation in comparison with other fat components. An increase in milk cholesterol level and a decrease in the amount of this metabolite in blood during the lactating cycle is a natural process well known in the existing literature (Maynard et al. 1931; Puppione et al. 1980; Strzałkowska et al. 2009). In the case of fat supplementation of diet, the inverse relationship of these parameters is maintained and a decreased milk cholesterol level effect is always accompanied by an increased plasma cholesterol concentration. The increased plasma cholesterol levels observed in SO and RO are similar to effects described by Bremmer et al. (1998), Khorasani & Kennelly (1998), and Abdelqader et al. (2009). Previously, Nestel et al. (1978) also suggested that this hypercholesteremia might be due to an increased requirement of cholesterol for the transport of long chain fatty acids as chylomicrons from the intestine into blood, which leads to an enhanced cholesterol biosynthesis in the intestine and a decreased faecal excretion of bile acids. The high amount of fatty acids available from vegetable oils in our experimental feedings may therefore be the reason for the increased blood cholesterol levels observed in the SO and RO groups and confirm this theory. In addition, the induced milk fat depression results in a lower need for milk cholesterol as part of the milk fat and the resulting reduced transport and synthesis of this metabolite to and in the mammary gland. A possible explanation of this effect is the reduction of the expression of the cholesterol transport regulatory genes in the mammary epithelial cells and milk fat globules described by Mani et al. (2011). On the other hand, Singh et al. (2012) observed increased milk cholesterol content accompanied by a non-affected total plasma cholesterol level after feeding buffaloes with *Asparagus racemosus*. However, recent results from our group (unpublished data) suggest that physiological cholesterol levels highly differ between this species and *Bos taurus*.

Conclusions

In summary, a diet induced milk fat depression after supplementation with RO and SO positively affects milk fat

composition by not only reducing the proportion of saturated fatty acids and increasing mono- and polyunsaturated fatty acids and CLAs, but also significantly reducing the milk cholesterol level. Whether this is a secondary effect due to the mechanisms of milk fat globule formation or the result of a specific gene expression regulation mechanism should be elucidated in future works.

The present work is dedicated to the memory of Prof. Heinrich H. D. Meyer, who led this project on its early stages. Financial Support from Bayerisches Staatsministerium für Ernährung, Landwirtschaft und Forsten (Munich, Germany).

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3.2 Altenhofer et al. 2014 Effects of 1 year long-term freezing with different preservatives on milk cholesterol, progesterone and lactoferrin determination.

This study was performed as a side-project of the Thesis in order to determine effects of long term freezing on milk composition and to evaluate the possible effects on analysis of four conservation substances (Acidiol, Kathon CG, NaOH and Thiomersal) on the determination of milk progesterone, lactoferrin, and cholesterol concentration. This research project was planned by Prof. H.H.D. Meyer, Dr. Enrique Viturro, Prof. Michael Pfaffl and the Author as well as the technical assistants Christine Fochtmann and Waltraud Schmid. Analysis of milk samples was done by the Author with support of the technical assistants. Therefore milk was collected at the research farm Veitshof (Technical University Munich, Freising, Germany), separated in aliquots and stored at - 20°C in 10ml plastic vials prior analysis. Stored samples were thawed and directly after analyzed after 1 month, 6 months and 12 months. It could be shown that the preservatives used in this experiment are not equally appropriate for all chosen analyzing methods used in this experiment. Preservatives and storage conditions for milk samples have to be carefully selected during the study design depending on the parameters to be measured.

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ORIGINAL RESEARCH

Effects of 1 year long-term freezing with different preservatives on milk cholesterol, progesterone and lactoferrin determination

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This study was performed to determine the effects of long-term freezing on milk composition and to evaluate the possible effects on analysis of four preservative substances (Azidiol, Kathon CG, NaOH and Thiomersal) on the determination of milk cholesterol, progesterone and lactoferrin concentration. Collected milk was separated in aliquots, stored at 20 °C in 10mL plastic vials and analysed after 1, 6 and 12 months. It could be shown that the preservatives are not equally appropriate for all analysing methods used in this experiment. Preservatives and storage conditions for milk samples have to be carefully selected during the study design depending on the parameters to be measured.

Keywords Milk, Storage, Freezing, Analysis, Azidiol, Thiomersal.

INTRODUCTION

Freezing of milk obtained during a study is normally performed to extend the storage time of samples until they are analysed. The aim of this study was to determine a possible effect of freezing and adding preservatives on the analysis of cholesterol, lactoferrin and progesterone in bovine milk. Sampling, storage and analyses of milk samples have to be performed in a standardised manner to minimise their possible effects on the biological results obtained. There have already been some efforts made to determine the effects of preservatives on major milk components (Bertrand 1996; Sánchez *et al.* 2005; Barbano *et al.* 2010). The addition of preservatives does not always act in a beneficial way for analyses in laboratories and may cause a significant variation in the results (Bertrand 1996) and have to be carefully verified for their suitability for usage in studies. Azidiol (sodium azide/chloramphenicol) is a very commonly used liquid preservative for milk and elongates the life of milk even at room temperature (Barcina *et al.* 1987). Kathon CG, a broad-spectrum biozide, is known as a cosmetics preservative (Groot and Weyland 1988) and a milk preservative (Herzog *et al.* 1988). The other preservative used in this study, Thiomersal, is an organic mercury compound, widely used as a bacteriostatic conservation agent. Sodium hydroxide was used for the treatment of coagulated milk (Rushton 1929) and may therefore be a good preservation substance for milk samples. The effects of freezing on milk's composition without any preservative addition were also described earlier (Voutsinas *et al.* 1995; Zhang *et al.* 2006). However, to our knowledge, there are few experiments published describing the effects of freezing and added preservatives on cholesterol, lactoferrin and progesterone in bovine milk up to now, even though these parameters are measured quite commonly in milk science studies.

MATERIALS AND METHODS

Milk collection and storage

Samples were obtained from 10 individual cows immediately after evening milking. Milk was aliquoted in 10-mL plastic vials (Sarstedt 51582;

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Nümbrecht, Germany) and frozen at −20 °C. Samples were parted in six treatment groups: two groups without a preservative – pure milk (PM) as the control group and PM immediately frozen with liquid nitrogen and then stored at −20 °C (NM); four groups containing preservatives – one with Azidiol 0.1 mL/30 mL added (AZ), one with Kathon CG 10 µL/10 mL (KA), one with Thiomersal 10 µL/10 mL (TH) and a group with sodium hydroxide NaOH 1 mol 10 µL/10 mL (SH). Additional PM vials were stored for analysis of fat, protein, lactose and urea at MPR Bayern e.V. (accredited laboratory after DIN EN ISO/IEC 17025 and DIN EN ISO 9001 certified, Wolnzach, Germany). The milk was thawed after 1, 6 and 12 months. The parameters before freezing are named Time0.

Sample analyses

Samples were thawed in a 37 °C water bath and refilled into bar-coded vials for their transport to MPR Bayern e.V. laboratories where they were analysed for fat, protein, lactose, urea and pH via infrared absorption measurement using a MilkoScan–FT–6000 system (Foss, Hillerod, Denmark). Additionally, PM, AZ, KA, TH and NA samples were examined in our laboratories on their cholesterol level by a recently approved colorimetric method (Viturro *et al.* 2010) where fat is extracted from milk by adding Hexane as the main extraction step. The extracted fat is then analysed by measuring the colorimetric reaction after adding a cholesterol reagent (Cholesterol; Thermo Fisher Scientific, Waltham, MA, USA) binding on cholesterol molecules. Lactoferrin was analysed by a competitive enzyme-linked immunosorbent assay (ELISA) well described by Danowski *et al.* (2012), and progesterone levels of skimmed milk were determined with an ELISA described in Meyer *et al.* (1986) at our laboratory at the Institute of Physiology (Freising, Germany).

Statistical analyses

Values were analysed comparing the contents of cholesterol, progesterone and lactoferrin of every thawing date (1, 6 and 12 months) among each other and with Time0. The results were considered significantly varied at a thawing date when $P \leq 0.05$. Fat, protein, lactose, urea and pH were analysed comparing values of thawing date 1, 6 and 12 months to Time0 (significant when $P \leq 0.05$). Statistical analyses were performed using SPSS 19 (IBM Deutschland GmbH, Ehningen, Germany). *P*-values comparing means were calculated using *t*-test and Levine's test for equality of variances.

RESULTS AND DISCUSSION

Milk composition

Changes in major milk components and pH are shown in Table 1. There was no significant change in protein, lactose, urea and pH comparing different storage times with Time0. The visual inspection after each thawing also showed no change in the optical appearance of milk. This is partly in agreement with Zhang *et al.* (2006) who described a protein flocculation when milk was stored at −15 °C for 3 months and no change when milk was stored at −30 °C. Wendorff (2001) reported a destabilisation of protein after 6 months and a loss of 20% of protein after 9 months of storage at −15 °C, and in contrast, milk samples frozen at −27 °C showed good protein stability. It seems that the temperature of −20 °C chosen for these experiments also provides sufficient stability to milk protein, apparent in the stable protein values even after 12 months of freezing. The unaffected pII due to freezing time is compatible with the results presented in Voutsinas *et al.* (1995). The urea content rises slightly after freezing, although not significantly. An increase in urea was also reported previously, although with milk samples preserved with bronopol frozen for up to 7 days (Godden *et al.* 2000). The fat content shows a significant decrease at every thawing date $P \leq 0.01$. This effect is in agreement with (Lepri *et al.* 1997) who reported a decrease of 5.7% in human milk after a storage time of 90 days and Zhang *et al.* (2006) who also described a decrease in fat percentage after freezing. However, the decrease in this experiment amounted to a reduction of over 1%, whereas Zhang *et al.* (2006) reported a significant decrease of 0.1% with a bigger decrease at −15 °C than at −25 °C. The authors suggested that the decrease in the fat percentage may be due to the damage of fat globules via ice crystals

Table 1 Cow milk composition after different storage times at −20 °C and without the addition of preservatives

	Freezing time (months) at −20 °C			
	0	*1*	*6*	*12*
Fat (% w/v)	3.88 ± 0.29	2.38 ± 0.11 ***	2.31 ± 0.18 ***	2.87 ± 0.20 **
Protein (% w/v)	3.20 ± 0.16	3.25 ± 0.17	3.30 ± 0.16	3.23 ± 0.16
Lactose (% w/v)	4.79 ± 0.03	4.86 ± 0.04	4.88 ± 0.04	4.85 ± 0.04
Urea mg/L	230.30 ± 20.78	248.60 ± 16.202	240.20 ± 18.04	240.70 ± 15.47
pH	6.59 ± 0.01	6.60 ± 0.02	6.60 ± 0.02	6.61 ± 0.02

Values are presented as mean ± SEM and differ when $^{**}P \leq 0.01$, $^{***}P \leq 0.001$.

Table 2 Cow milk composition after different storage times at −20 °C

	Freezing time (months) at −20 °C			
	0	*1*	*6*	*12*
Cholesterol mg/dL				
PM	18.97 ± 0.94	22.28 ± 2.26	23.32 ± 2.56	25.55 ± 3.59
NM		16.70 ± 1.83	16.78 ± 1.32	18.93 ± 2.15
TH		21.58 ± 2.18	21.76 ± 2.64	23.53 ± 3.14
KA		20.71 ± 2.29	21.03 ± 2.59	25.05 ± 3.53
AZ		19.46 ± 2.41	19.78 ± 2.76	20.87 ± 3.32
SH		21.74 ± 2.26	24.07 ± 2.74	23.96 ± 3.23
Lactoferrin μg/mL				
PM	85.3 ± 17.25	110.0 ± 24.91	109.6 ± 49.41	58.9 ± 14.88
NM		107.8 ± 23.07	108.0 ± 49.66	58.3 ± 15.04
TH		108.7 ± 23.89	61.6 ± 10.44	60.7 ± 11.47
KA		107.3 ± 24.15	59.3 ± 8.79	54.2 ± 12.99
AZ		110.1 ± 20.91	60.8 ± 11.61	54.2 ± 13.11
SH		118.0 ± 26.43	56.9 ± 9.89	50.1 ± 11.37
Progesterone ng/mL				
PM	1.59 ± 0.33	1.15 ± 0.26	1.27 ± 0.29	1.58 ± 0.36
NM		1.16 ± 0.25	1.23 ± 0.26	1.58 ± 0.38
TH		1.35 ± 0.26	1.52 ± 0.30	2.03 ± 0.37
KA		1.26 ± 0.27	1.38 ± 0.28	1.76 ± 0.37
AZ		Over maximal detection limit		
SH		1.16 ± 0.25	1.19 ± 0.24	1.62 ± 0.33

Values are presented as mean ± SEM and differ when $P \leq 0.05$. PM, pure milk; NM, pure milk immediately frozen with liquid nitrogen, stored at −20 °C; TH, milk with Thiomersal (10 μL/10 mL); KA, milk with Kathon CG (10 μL/10 mL); AZ, milk with Azidiol (0.1 mL/30 mL); SH, milk with sodium hydroxide NaOH 1 mol (10 μL/10 mL).

formed during the freezing process with bigger crystals at −15 °C, and therefore, the reduced fat content may be an artefact of the infrared measurement method used in this experiment. Also Wendorff (2001) published an increase in the acid degree value of fat with a bigger increase at −15 °C than at −27 °C. Voutsinas *et al.* (1995), in contrast, reported that the fat content of bovine milk concentrates do not differ significantly after freezing at −20 °C for 6 months.

Cholesterol

As shown in Table 2, there is a big variation in the relative change of cholesterol during freezing time and different preservatives to Time0. There is also a significant difference between the relative change of PM and NM at 1 and 6 months of storage compared to Time0. This is maybe due to the effect of the building up of ice crystals during the freezing process (Wendorff 2001) that leads to a possible destruction of milk fat globules and a higher availability of intramembranous cholesterol (Huang and Kuksis 1967; Lopez *et al.* 2010) for the enzymes used in the detection method. This crystal formation might be reduced by fast freezing with liquid nitrogen. The correlation between the change of fat and cholesterol compared with Time0 in PM is presented in Figure 1. At every thawing date, especially after 1 month of storage time ($R^2 = 0.747$), there is a negative correlation between these two values visible – the lower the fat percentage gets, the higher the cholesterol content becomes. This effect could be because of the damaged milk fat globule membranes during the freezing process and the increased availability of intramembranous cholesterol thereafter and may be not the result of the changed fat content because the fat content presented in this study might be an artefact of the infrared measurement method used. TH, KA, AZ and SH samples showed no significant difference in cholesterol values compared with PM at each thawing date, although interestingly, the mean of the AZ remained the most closely to milk samples at Time0, probably because of the well-known stabilising effect of Azidiol. When absolute cholesterol values are compared between Time0 and between each thawing date, there are no significant differences ($P > 0.05$) due to high standard deviations (Table 2), but strong tendencies are visible: the cholesterol values of samples preserved with AZ and samples frozen with liquid nitrogen stay closer throughout 12 months of freezing to Time0 than PM and samples preserved with TH, KA and SH.

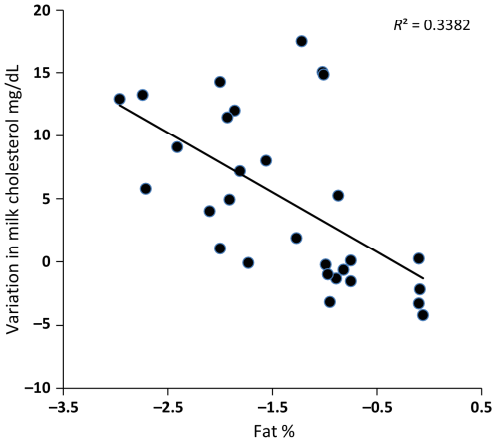

Figure 1 Correlation between change of fat % and change of cholesterol content in PM after 1, 6 and 12 months of storage at −20 °C. PM, pure milk.

Lactoferrin

Lactoferrin shows no significant change in milk content when Time0 is compared with the three thawing dates (Table 2). Although mean values are rising after 1 month of storage and thereafter decrease again, changes remain insignificant due to high standard deviation. This observation is in agreement with Evans *et al.* (1978) who reported a non-significant small increase in lactoferrin in human milk after 3 months of storage at −20 °C. Interestingly, the values of TH, KA, AZ and SH samples are lower after 6 months storage time than after 1 month, and the means of PM and NM stay at approximately the same level with increased standard deviation and are decreased after 12 months (Table 2). This is maybe due to accelerated degradation of lactoferrin when preservatives are added to the milk sample or a possible interaction between chemical conservation substances and the analysing method used.

Progesterone

The results for progesterone are also shown in Table 2, presenting a decrease in progesterone content after 3 and 6 months of storage compared with Time0. This is in agreement with Lamont *et al.* (2007) who reported a decline in progesterone after 56 days of storage at −20 °C and suggests that this effect is partially because of the storage in plastic vials and a possible adsorption of progesterone to plastic, whereas refreezing and thawing did not affect the progesterone content. At 1 and 6 months, the means of KA and TH stayed closer to the fresh milk samples at Time0 with a significant difference ($P < 0.05$) of TH compared with PM, NM and SH at 1 month of storage. Azidiol had a negative impact on progesterone analysis by interfering with the ELISA and producing a progesterone level over the maximum detection limit of the assay and was therefore excluded from statistical evaluation. When relative and absolute progesterone levels after storage are compared to Time0, there are no significant differences except that after 12 months storage, the relative change of progesterone level (128.69%) is significantly higher ($P < 0.05$) compared with Time0 (100%).

CONCLUSION

Chemical milk preservatives may have positive effects on the elongation of storage time of frozen milk, but may also have negative effects on certain analysing methods or render analysing impossible. For this reason, the preservation method has to be adapted to the design of a study and the desired parameters to be measured during it. Azidiol seems to be a good additive for cholesterol measurement as well as fast freezing with liquid nitrogen but seems to be unsuitable for progesterone analysis where Thiomersal and Kathon CG seem to be preferable against other preservatives. These results have to be taken into account in future study designs.

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3.3 Altenhofer et al. 2015 Temporal variation of milk fat globule diameter, fat and cholesterol content and milk epithelial cell gene expression in dairy cows.

The goal of this experiment was to find of regulatory mechanisms of the mammary gland and milk secretion because this is crucial to implement strategies for changing specific components of milk or to adapt its physical properties for an optimal industrial use, i.e. by modifying milk fat composition or milk fat globule diameter (MFGD). For this purpose an experiment was conducted on the experimental farm “Veitshof” (Technical University Munich, Freising, Germany) where milk samples of cows at different stages of lactation were analyzed for their changes in fat and cholesterol content as well as for the variation and possible correlation in their MFGD distribution. This part of the experiment was partly done by a bachelor student making her bachelor thesis supervised by the Author himself, Dipl.-Ing. Wolfgang Holzmüller and Dr. Enrique Viturro. In parallel, purified bovine milk epithelial cells (pbMECs) were extracted as a part of another bachelor thesis supervised again by the Author and Dr. Enrique Viturro to investigate gene expression of cells in the lactating mammary gland using a non-invasive extraction technique as an alternative to the common used biopsy technique. As a result of this experiment the amount of milk fat per milking constantly decreased (significant) from early to late lactation as well as cholesterol content per g fat and the mean MFGD, as the fat particles became less loaded. Simultaneously, a down-regulation of key genes, well known from literature and selected as key regulation genes in fat and cholesterol metabolism in liver cells by the participating bachelor student, Dr. Enrique Viturro and the Author himself, (ACACA, SCD, ABCA, GPAM, AGPAT, INSIG1, FDFT, DGAT and THRSP) was observed in pbMECs in the course of lactation. The present results confirm the

hypothesis of a direct correlation between gene expression regulation in mammary gland epithelial cells and its final consequence, the milk fat globule chemical composition and physical properties.

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ORIGINAL RESEARCH

Temporal variation of milk fat globule diameter, fat and cholesterol content and milk epithelial cell gene expression in dairy cows

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It was possible to show a connection between the temporal variation of milk fat globule diameter, fat and cholesterol content in milk and the expression of candidate genes in the mammary gland epithelial cells in milk. The beginning of lactation corresponded with higher levels of fat and cholesterol in the milk as a result of a higher expression of key enzymes in the purified bovine milk epithelial cells, paralleled with an increase in milk fat globule mean size.

Keywords Milk fat globule, Cholesterol, Gene expression, Milk epithelial cells.

INTRODUCTION

Milk is a water-based solution, which contains fat, protein, lactose, vitamins and minerals, in emulsified, colloidal or dissolved form depending on the fat or water solubility. Milk fat, either obtained from the circulating fat or synthesised in the mammary gland, is secreted as a mixture of milk fat globules (MFGs) with different sizes, with a mean diameter between 3.3 and 5.2 μm, but ranging from 0.1 to 20 μm (Ménard *et al.* 2010; Lopez 2011). Studies during the last decade showed that the MFG size distribution is not arbitrary and might be strongly regulated, as reproducible variations can be observed when comparing species or after breeding and feeding interventions (Couvreur *et al.* 2007; Lopez 2011). The interest in deeply understanding the chemical structure of the MFG arose not only because of the big potential to modify processing characteristics of milk products, but also because of the potential health benefits of some of its components, including roles as bactericidal agents, possible suppressors of multiple sclerosis, agents against colon cancer, Alzheimer's disease, depression and stress, as well as anticholesterolaemic agents (Spitsberg 2005; Dewettinck *et al.* 2008). The structure of the MFG is rather similar to other fat-in-water solutions in nature (i.e. blood lipoproteins or digestive chylomicrons). The triacyl-glycerol molecules, representing 98% of all milk lipids, are exclusively located in the centre of the structure, avoiding contact with water (Lopez *et al.* 2010). This hydrophobic core is surrounded by two membranes, an inner monolayer and an outer bilayer summarised as the milk fat globule membrane (MFGM), whose origin is the endoplasmatic reticulum for the monolayer and milk epithelial cell membrane for the bilayer and is therefore composed of a mixture of proteins, glycoproteins, glycolipids, phospholipids, cholesterol and sphingomyelin (Mather 2000; Fong *et al.* 2007; Dewettinck *et al.* 2008). Interestingly, these membrane components are not distributed as homogeneously as in the original cell membrane, and they rather contain liquid-ordered lipid rafts of sphingomyelin and cholesterol surrounded by a liquid-disordered phase of phospholipids (Lopez *et al.* 2010), supporting the idea that milk fat secretion is a fine regulated process, a subject of specific transport mechanisms.

Fat synthesis rate changes throughout lactation cycle, with the highest fat secretion at the beginning of lactation (Pollott 2004), but the different fat classes vary on a singular, characteristic

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trend, which depends on many factors (i.e. fat mobilisation from body reserves or diet (Altenhofer *et al.* 2014b)). For a better understanding of these changes, the expression of genes related to milk fat synthesis, fat secretion, fatty acid (FA) composition and MFGM-forming has been investigated, most commonly by the analysis of mammary biopsy samples or after slaughtering (Peterson *et al.* 2003; Rudolph *et al.* 2007; Bionaz and Loor 2008b; Mach *et al.* 2013). During the last years, we have optimised a technique for isolating purified bovine milk epithelial cells (pbMECs) out of fresh milk samples and postulated it as an optimal alternative to the above-mentioned traditional methods (Sorg *et al.* 2012). This technique allows a live picture of processes in the mammary gland, and it presents three important advantages: it is non-invasive, very economical and easy to perform, and samples can be taken as often as needed without causing any damage to the sensitive gland tissue, which is often the case with biopsies of the mammary gland. Also, as shown by Sorg *et al.* (2012), the obtained gene measurement results are fully comparable with the other techniques, as only living and fully intact mammary epithelial cells are extracted.

The aim of this study was to detect changes in milk fat globule diameter (MFGD) throughout lactation and a possible correlation with milk fat globule size and, especially, milk cholesterol amounts, as this crucial molecule is often not included in the analysis. A second objective was to correlate the obtained results with gene expression data obtained by employment of the noninvasive pbMEC isolation technique.

MATERIAL AND METHODS

Animals and experimental design

For data analysis, the lactation cycle was divided into early lactation (0–100 days in milk DiM), mid-lactation (101–200 DiM) and late lactation (201–300 DiM). Only multiparous cows (parity 3.5 ± 1.6, annual milk yield 8826 ± 377 kg/305 days of lactation), fitting into one of these three groups, were selected for sampling, which was performed within 2 weeks to guarantee similar conditions of housing, feeding and analysis. Only cows with no clinical signs of a mastitis were selected for sampling. Analysis of MFGD, cholesterol content and fat percentage was performed in milk samples of 27 early lactating, 19 mid-lactating and 21 late-lactating Brown Swiss cows. For gene expression analysis, pbMECs were extracted out of fresh milk samples from 15 early, 15 mid and 12 late-lactating Brown Swiss cows.

Housing and feeding

Cows were kept at the experimental farm *Veitshof* (Physiology, Technical University Munich TUM, Germany) in a free stall barn with littered resting areas combined with rubber mat bedding. Animals were milked twice a day in a 2×2 parallel milking parlour. Cows were fed a standard partial mixed ration containing 20 kg grass silage, 16 kg maize silage, 2.6 kg wheat meal and 1.8 kg soy meal as the major components and given permanent access to fresh water. Partial mixed ration was given *ad libitum* throughout the whole lactation with an extra concentrate feed, fed by an automatic dispenser, according to milk yield with a maximum of 8 kg concentrate per day. No animal presented a metabolic disease during the whole lactation.

Sampling and analysis

Milk samples were taken at the morning milking and aliquoted. One of the aliquots was sent to an external laboratory (MPR Bayern e.V., accredited laboratory after DIN EN ISO/IEC 17025 and DIN EN ISO 9001 certified, Wolnzach, Germany) for analysis of fat percentage, while MFGD was analysed at the laboratory of the Chair for Food Process Engineering and Dairy Technology (TUM, Germany) using a Mastersizer 2000 and a dispersion unit Hydro S (Malvern Instruments, Worcestershire, UK). Measurement is based on the low angle laser light scattering (LALLS) method where dry and wet samples with a size range between 0.02 and 2000 μm can be measured. Gained values were analysed using Mastersizer 2000 software (Malvern Instruments GmbH, Herrenberg, Germany) producing volume-related equivalent diameter D[4,3], surface area-related equivalent diameter D[3,2] as well as median value for each sample.

Cholesterol content was measured with a method described by Viturro *et al.* (2010) using an enzymatic reaction with a specific colorimetric kit (Cholesterol; Thermo Fisher Scientific, Waltham, MA, USA).

PbMECs were extracted via antibody-coated Dynabeads (Dynabeads Pan Mouse IgG, Invitrogen, Carlsbad, CA, USA). The separation of pbMEC-anti-cytokeratin-8-Dynabeads complexes was performed using a immune magnetic bead-based separation technique described by Sigl *et al.* (2012). Separated cells were resuspended with QIAzol (Qiagen GmbH, Hilden, Germany) and stored at −80 °C prior to RNA extraction following the extraction protocol provided by the miRNeasy Mini Kit (Qiagen GmbH). RNA concentration and purity of each sample were measured with a NanoDrop spectrophotometer (NanoDrop ND-1000, Thermo Fisher Scientific) and was verified against RNase-free water as blank. Integrity of RNA was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). cDNA was synthesised by reverse transcription using 100 ng RNA, M-MLV (H-) point mutant enzyme 200 u/μl (Promega, Madison, WI, USA) and random primers 0.5 μg/μl (Fermentas, St. Leon-Rot, Germany). The reaction was performed in an Eppendorf Mastercycler Gradient (Eppendorf AG, Hamburg, Germany) using the following temperature program: 21 °C for 10 min, 48 °C for 50 min and 90 °C for 2 min. Quantitative real-time

PCR was performed in a Step One Plus real-time PCR System (Applied Biosystems, Böblingen, Germany) using SsoFastEvaGreen Supermix (Bio-Rad, Munich, Germany) and gene-specific forward and reverse primers (Table 1) using the following conditions: 30 s at 98 °C, 40 cycles of 5 s at 95 °C and 20 s at 60 °C. Quantitative cycle (Cq) values lower than 11 were not considered for analysis due to Step One Plus real-time PCR System Software (Applied Biosystems) limitations. The ΔCq values were calculated using the formula: $\Delta Cq = Cq_{targetgene} - meanCq_{reference\ gene}$ (Pfaffl 2001). Earlier considered universal reference genes (i.e. GAPDH or beta-actin) are not suitable for mammary epithelial cells during lactation because of their high variation (Kadegowda *et al.* 2009; Yadav *et al.* 2012). For this reason, a model using the mean expression value of all measured RNAs (Mestdagh *et al.* 2009) was the method of choice.

All analysis were performed with fresh milk samples to prevent changes of milk composition caused by freezing and storage time (Altenhofer *et al.* 2014a).

Statistics

Obtained results were analysed with SPSS 19 (IBM, Armonk, NY, USA) using paired t-tests and Levene's test for equity of variances. Intergroup correlation was calculated using bivariate Pearson correlation analysis. Significance level for all samples was set under a *P*-value of 0.05. MFGD, fat content and cholesterol level of every sample were analysed, and means of every lactation stage were compared among each other. ΔCq values of genes in different lactation stages were compared. The fold change in expression in different lactation stages was calculated with the $2^{-\Delta\Delta Cq}$ method (Livak and Schmittgen 2001) comparing the mean of ΔCq values of samples in a lactation group with that of the late-lactating group as reference.

RESULTS AND DISCUSSION

Milk yield, fat and cholesterol content

Milk yield, fat and cholesterol content of milk samples analysed in this study are presented in Figure 1. Milk yield decreased constantly during the 300 days of lactation ($P < 0.01$) starting with mean 21.3 L in early lactation and 17.9 L in mid-lactation and ending with mean 13.2 L in late lactation. Milk fat content decreased from 3.56% in early lactation to 3.37% in mid-lactation and increased significantly ($P < 0.05$) in late lactation to 3.84%. Both effects are widely known (Pollott 2004; Silvestre *et al.* 2009; Strzałkowska *et al.* 2009b; Altenhofer *et al.* 2014b). In this experiment, a significant decrease of milk cholesterol concentration during the first half of the lactation cycle occurred (early lactation 20.26 ± 1.57 mg/dL vs mid-lactation 15.61 ± 0.81 mg/dL). However, this traditional way of presenting milk cholesterol data (Herdt and Smith 1996;

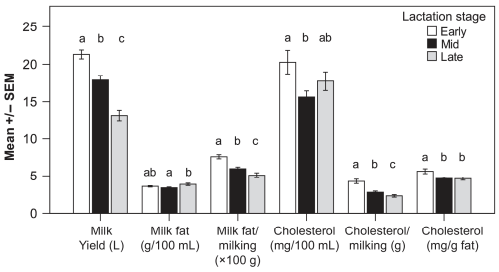

Figure 1 Fat (g/100 mL) and cholesterol content (mg/100 mL), milk yield (L) and at different stages of lactation (early 0–100 days in milk DiM, mid 101–200 DiM, and late 201–300 DiM). Values that differ significantly ($P < 0.05$) are marked with different letter superscripts (a, b, and c).

Table 1 List of primers used for RT-qPCR

Gene	*Forward primer*	*Reverse primer*	*Product length (bp)*
ABCA1	GGCTCTCAGACCTAGGCATC	GGTAAGGTGCCATCTGAGGT	118
ACACA	ACACTCAGAGCATCGTCCAG	TGACCGTTCTGGAATTGTGT	124
AGPAT1	GGAACATTCCTCAGCACCTT	GAGAAGAGTCCAGCCTCCAC	69
DGAT1	AATTGGTGTGTGGTGATGCT	CCAGGATGCCATACTTGATG	76
FDFT1	CCACCACAGGTCACAGTTTC	TGTGGGCTCCTTCTCTTTTT	89
GPAM	AATGAGAGCCTCTGGAGCAT	CCGTTTTGACACATCCGTAG	73
HMGCS1	CCTCTCTGGCCATGTGTATG	TGCTTTCCTCACCAGAAGTG	77
INSIG1	TTCTGAACTTTGCGATGACC	TCGCATAGCTGATGGTTTTC	63
INSIG2	TCTGCAGTGCCACAGTGTTA	CTTGGACCTGTTTGGGATTT	102
PPARG	CATAATGCCATCAGGTTTGG	GTCAGCAGACTCTGGGTTCA	102
SCAP	GTTTCCTTTTGGGACCTGAA	CTGCCAAAGTTGCAGACAAT	128
SCD	TTGTCCACTTTCTCCTGCTG	GTAGCCATCACTGCCTCTGA	99
THRSP	CACTTCGCTAGCCTTCATCA	CTGTCCCATTTTCTCCTGGT	87

Strzałkowska *et al.* 2009a,b) is not very effective and should rather be corrected by milk fat amount and milk yield. After correcting the data, it is clearly visible that the amount of this metabolite per milking decreases significantly ($P < 0.05$) from early to mid and late stages (Figure 1), as well as the amount of cholesterol per secreted gram of fat (5.62 ± 0.34 mg/g vs 4.63 ± 0.16 and 4.60 ± 0.21 mg/g, respectively). Cholesterol amount in milk is therefore not only regulated by its fat content and might have other regulatory factors, discarding the old hypothesis of cholesterol being passively secreted into milk among other fats. As it happens in other organs (i.e. digestive tract), this molecule might rather be the subject of a specific transport system (Farke *et al.* 2006; Viturro *et al.* 2006). This hypothesis of a specific transport system is also supported by Altenhofer *et al.* (2014b) reporting an increased cholesterol content in blood of cows fed a ration supplemented with rapeseed or soya bean oil and at the same time detecting a lower cholesterol content in milk compared to a control ration with no oil added, suggesting that cholesterol content of milk is not mainly regulated by liver or being passively transported from blood into milk but being independently regulated by mammary gland itself.

Milk fat globule diameter

Milk fat globule diameter distribution at different lactation stages is presented in Table 2. D[3,2] and D[4,3] as well as the median diameter showed a very significant higher value ($P < 0.01$) in early lactation than mid-lactation and late lactation. Comparison of mid-lactation and late lactation showed no significant differences in MFGD. This reduction in globule diameter is consistent with the data for other species from Martini *et al.* (2012), who described a significant reduction in mean globule diameter in ewes' milk from 3.15 µm at day 30 to 2.68 µm at day 120. MFGD data were co-analysed with cholesterol content, fat and milk yield revealing a very significant ($P < 0.01$) correlation with all these parameters. The positive correlation between diurnal fat yield and MFGD was already outlined by Wiking *et al.* (2003, 2004), suggesting a limitation in milk fat globule membrane production of the mammary gland when fat synthesis increases. This hypothesis was later confirmed by Wiking *et al.* (2006) after increasing milk yield through a higher milking frequency, resulting in bigger rather than in more abundant small MFGs. Availability of plasma membrane material might therefore be a rate-limiting factor in milk fat secretion in periods of intense increase of milk production, a hypothesis supported by the present data. In our opinion, the higher cholesterol content in milk at early lactation reported is the logical result of extremely increased cell membrane usage for MFG envelopment during phases of high milk production, as milk cholesterol occurs almost exclusively as an integral part of the MFG membrane (Dewettinck *et al.* 2008). This theory of a correlation between cholesterol content and milk fat globule diameter is shown in Figure 2 where surface area-related equivalent diameter is connected with cholesterol yield per milking.

Table 2 Milk fat globule diameter at different stages of lactation

Globule Diameter[1]	*Early*[2]	*Mid*[2]	*Late*[2]
Median	4.80 ± 0.15[a]	3.78 ± 0.12[b]	3.83 ± 0.10[b]
D[3,2]	5.67 ± 0.16[a]	4.49 ± 0.08[b]	4.40 ± 0.07[b]
D[4,3]	6.60 ± 0.19[a]	5.21 ± 0.10[b]	5.09 ± 0.09[b]

[1]Globule diameter (µm) is presented as median diameter, surface area-related equivalent diameter D[3,2] and volume-related equivalent diameter D[4,3]. Values differ signicantly when $P < 0.05$, marked as a, b.

[2]Lactation stage: early (0–100 days in milk DiM), mid (101–200 DiM), late (201–300 DiM).

Gene expression

Gene expression of key lipid metabolism actors is presented as ΔCT values at different stages of lactation (Table 3) and as fold changes (Figure 3a) with the late lactation (lowest overall gene expression) as a reference value. Genes involved in FA synthesis and FA desaturation showed a significant expression decrease ($P < 0.05$) towards the end of lactation when compared to both early and mid-lactation phases. Expression of acetyl-coenzyme A carboxylase alpha (ACACA), a key enzyme in FA biosynthesis (Badaoui *et al.* 2007; Bernard *et al.* 2008), is extremely reduced from early to late lactation, in accordance with Bionaz and Loor (2008b) who also described a down-regulation of ACACA between day 60 and day 240 of lactation in mammary gland biopsy samples. Stearoyl-CoA desaturase (SCD), a

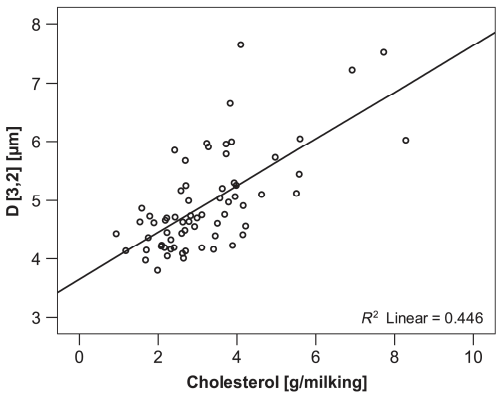

Figure 2 Cholesterol content [g/milking] related with surface area-related equivalent diameter D(3,2) [µm] of all analysed samples.

Table 3 Gene expression in mammary epithelial cells at different stages of lactation[1]

Gene	*Early*[2]	*Mid*[2]	*Late*[2]
ACACA	1.89 ± 0.39[a]	1.71 ± 0.39[a]	3.22 ± 0.39[b]
SCD	−0.94 ± 0.27[a]	−1.08 ± 0.27[a]	−0.17 ± 0.19[b]
GPAM	4.72 ± 0.34[a]	5.20 ± 0.33[ab]	5.80 ± 0.19[b]
AGPAT	−3.29 ± 0.21	−2.81 ± 0.14	−3.03 ± 0.23
HMGCS	−0.75 ± 0.36	−1.05 ± 0.42	−0.57 ± 0.16
FDFT	−2.54 ± 0.45[a]	−1.67 ± 0.29[ab]	−1.15 ± 0.36[b]
ABCA1	1.19 ± 0.47	1.12 ± 0.39	1.83 ± 0.38
INSIG1	−0.35 ± 0.69[a]	1.63 ± 0.63[b]	1.92 ± 0.80[b]
SCAP	1.28 ± 0.47	0.89 ± 0.31	1.04 ± 0.23
THRSP	−0.61 ± 0.55	−0.07 ± 0.99	−0.30 ± 0.50
PPARG	−0.09 ± 0.26	0.09 ± 0.16	−0.21 ± 0.22

[1]Gene expression is presented as delta CT values ± system error of mean (SEM). Values differ significantly when $P < 0.05$ marked as a, b.

[2]Lactation stage: early (0–100 days in milk DiM), mid (101–200 DiM), late (201–300 DiM).

prominent enzyme in the process of desaturation of FA, was also significantly down-regulated. Both variations are consistent with the observed physiological result: even when fat percentage rises slowly during lactation, the total amount of fat secreted extremely decreases. As a reinforcement of this hypothesis, Peterson *et al.* (2003) and Kadegowda *et al.* (2010) postulated a positive correlation of SCD with fat yield. Glycerol-3-phosphate acyl transferase (GPAM), 1-acylglycerol-3-phosphate O-acyl transferase 1 (AGPAT) and diacyl glycerol O-acyl transferase 1 (DGAT) are genes participating in triglyceride synthesis and are higher expressed in mammary gland than in liver (Rudolph *et al.* 2007). In our experiments, GPAM is down-regulated between early and late lactation ($P < 0.05$) (Figure 3b). A previous study, in which lower milk synthesis was induced by reducing milking frequency, a down-regulation in the expression of GPAM was also detected (Littlejohn *et al.* 2010). The expression of AGPAT and DGAT showed a tendency to down-regulation (−0.26 ΔCq and −0.40 ΔCq, respectively). This trend, though not significant, is in agreement with Peterson *et al.* (2003) and Bionaz and Loor (2008a), who described a down-regulation of these genes between early and late lactation with the onset of lactation or after inducing a decreased fat synthesis.

Concerning cholesterol output into milk, previous studies of our group revealed a down-regulation of key synthesising enzymes in liver during late lactation (Viturro *et al.* 2009). A significant down-regulation of farnesyl diphosphate farnesyl transferase (FDFT) (Figure 3c) could be also found in milk-synthesising cells in the present study. This enzyme is of an extreme informative value when studying cholesterol synthesis, as it catalyses the first step of the long synthesis pathway in which the resulting product devotes exclusively to cholesterol synthesis and is not derived to other cell pathways. The fact that FDFT is regulated in parallel for both liver and mammary gland reinforces the idea that cholesterol synthesis in bovine organisms is a finely controlled process and that this metabolite can be produced at both sites. However, the extension in which each organ participates in milk cholesterol synthesis still needs to be elucidated for *Bostaurus*, but previous studies in other species suggest a predominant role for the liver (Rudolph *et al.* 2007). Concerning cholesterol transport, the ATP-binding cassette subfamily A member 1 (ABCA1) showed a down-regulation trend between early and late lactation, as previously shown (Farke *et al.* 2006; Mani *et al.* 2011). It is important to remark that this expression suppression of both cholesterol-synthesising and cholesterol-transporting genes is paralleled with the physiological observation of a lower amount of cholesterol content per g fat in the milk at late lactation stages.

A complex network of receptors, activators and suppressors overtakes the regulation of fat synthesis at the gene expression level (Bionaz and Loor 2008b). Among them, we measured the expression of insulin-induced gene 1 (INSIG1), sterol regulatory element-binding cleavage-activating protein (SCAP), peroxysome proliferator-activated receptor gamma (PPARG) and thyroid hormone responsive SPOT14 (THRSP). The expression of INSIG1 was significantly down-regulated during lactation ($P < 0.05$), whereas a nonsignificant tendency to up-regulation of SCAP (0.23 ΔCq) and PPARG (0.12 ΔCq) and down-regulation of THRSP (0.31 ΔCq) could be observed. This observations are partly consistent with Bionaz and Loor (2008b) who reported an up-regulation of SCAP and a down-regulation of INSIG1 in early lactating compared to late-lactating cows. The trend of down-regulation of THRSP observed in this study is supported by Kadegowda *et al.* (2010), who measured a suppression of this gene in lactating mice when fat yield is reduced by feeding conjugated linoleic acids.

CONCLUSIONS

Observed as a whole, the data presented in this study confirm a direct link between milk fat composition, gene expression in the synthesising cells and the milk fat globule size. It could be shown that the higher fat and cholesterol amount in milk in early phases of lactation is reflected in higher amounts of milk fat globules with bigger size and is the product of a higher mammary gland gene expression of enzymes involved in cholesterol and fat synthesis and transport. For the first time, data were obtained directly from living pbMECs and the live milk-synthesising cells, and not from liver biopsies or slaughtering samples, remarking the suitability of this noninvasive methodology for future studies.

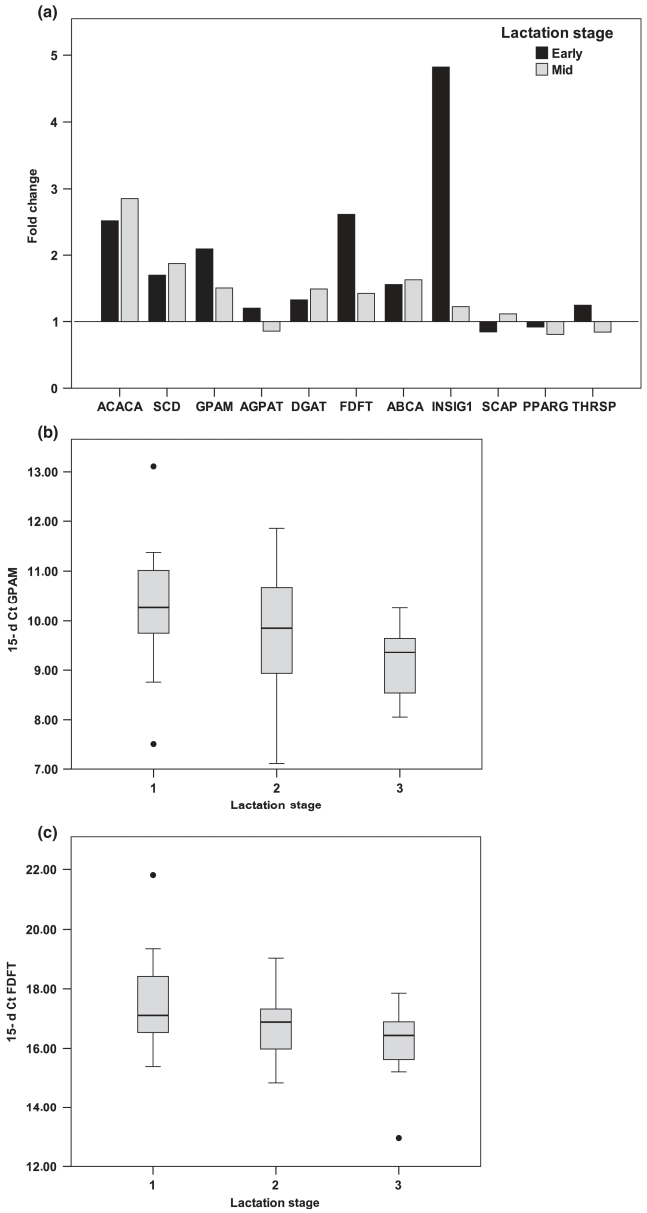

Figure 3 (a) Gene expression in purified bovine milk epithelial cells at early and mid-lactation stages calculated as fold change relative to late lactation ($2^{-\Delta\Delta Ct}$). (b) Box-plot representation of gene expression changes of GPAM and (c) FDFT at different stages of lactation (early 0–100 DiM, mid 101–200 DiM, and late 201–300 DiM) presented as 15-ΔCt values.

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4 Second Authorship Paper Summary

4.1 Viturro et al. 2014 Microfluidic high-throughput reverse-transcription quantitative PCR analysis of liver gene expression in lactating animals.

The number of publications on gene expression in animal production species has dramatically increased with the development of new genetic techniques. In the present work, we tested the suitability of a microfluidic lab-on-chip quantitative RT-qPCR methodology by measuring the gene expression of key actors of liver metabolism in cattle at different stages of lactation. Liver samples were generated via liver biopsies of cows at experimental farm Hirschau and experimental farm Veitshof. Punctuation of liver was done by Christian Altenhofer as well as RNA extraction and reverse transcription. It was chosen to compare animals in the early and late lactation phases because of the extreme adaptations in gene expression expected to occur. 48 genes were measured and 28 out of them were significantly regulated during the lactation cycle and in the same direction as previously shown with other techniques used in research. Primers had to be carefully designed because with the high throughput chip technology used Primers of selected genes had to run at the same time with the same temperature. Primer design was done by Anja Burgmaier and Christian Altenhofer with supervision of Enrique Viturro and Michael Pfaffl as well as Irmgard Riedmaier. This demonstrates that the high throughput and micro device platform used in this experiment might be a good alternative, due to its easy application, fastness, and lower economic costs. In combination with a dynamic PCA algorithm, PCA was done by Enrique Viturro and Irmgard Riedmaier, we were able to identify the set of 13 genes that allowed the clearest separation between the two

compared physiological groups with a fast, economic and reliable microfluidic RT-qPCR assay.

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ORIGINAL PAPER

Microfluidic high-throughput reverse-transcription quantitative PCR analysis of liver gene expression in lactating animals

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Abstract We have evaluated a microfluidic lab-on-chip quantitative reverse transcription (RT) quantitative PCR (qPCR) method by measuring the expression of key actors of liver metabolism in lactating cattle. Animals in the early and in the late lactation phases were chosen because of the extreme adaptations in gene expression expected to occur. During the lactation cycle, 28 out of 48 genes were significantly regulated, notably in the same direction as previously shown by other techniques. This demonstrates that this high-throughput platform represents an attractive alternative to microarrays due to its ease of application, rapidity and lower costs. A set of 13 genes was identified—in combination with a dynamic PCA algorithm—that allowed the clearest separation between the two physiologically different groups. This paves the way for classification and diagnosis of animals in different metabolic situations by a reliable microfluidic RT-qPCR assay.

Keywords Microfluidic RT-qPCR · Data analysis · PCA · Liver · Lactation phase

Introduction

Since the works in genome sequencing advance at a very fast pace, simultaneously measuring the mRNA expression of thousands of genes has become a main topic of interest for a wide spectrum of scientists working in very different species and research goals. The number of available micro device techniques such as microarrays, lab-on-chip PCR, and next generation sequencing is growing exponentially. Among the newest are, i.e., TaqMan® OpenArray® Real-Time PCR (LifeTechnologies Ltd., Paisley, UK, http://www.lifetechnologies.com) which allows to generate up to 32,000 qPCR data points from a catalog of 1.2 million assays, or the SmartChip Real-Time PCR System (Wafergen Biosystems Inc., Freemont, CA, USA, http://www.wafergen.com), which contains 5,184 nano-wells preloaded with target-specific primer pairs. However, these platforms may present some disadvantages for many scientists, since the assays available in the companies' portfolio are mostly fixed and predesigned for human or mouse. Hence the obtained results might require post-hoc reverse transcription-quantitative PCR (RT-qPCR) validation prior to publication.

For those scientific groups working with other species, i.e. in the agricultural or veterinarian field, and those whose primary research goal is not to generate huge amounts of genetic expression data, other more flexible platforms are recommended. The main goal might rather be to measure an intermediate amount of genes, previously identified as potential candidates of interest. Further, the samples should be measured in as many biological replicates as possible, with easy-to-use technologies that do not require high advanced bioinformatics knowledge. One of the available platforms links the benefits of a high throughput gene expression profiling assays with the fastness and easiness to use of conventional RT-qPCR: the Biomark HD™ real-time RT-qPCR platform (Fluidigm, San Francisco, CA, USA, http://www.fluidigm.com/biomark-hd-system.html) [1]. The system allows a reduction in the amount of material consumed of 50 to 100 times with a simultaneous throughput increase of 5 to 20 times compared to conventional single-plex RT-qPCR.

Among the fields that substantially benefit from the technical progress in animal science, dairy cattle management is

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one of those whose actual state of knowledge is unimaginable without the data generated since the first microarray studies [2], which nowadays include more than 8,000 genes measured simultaneously. The extreme metabolic adaptations of the animal after parturition, in order to face the approximately 10,000 liters of milk (and more) to be produced per lactation cycle, have almost no equivalents in nature. Since feed intake does not increase as fast as milk production during the first two months of lactation, an extreme fat mobilization from the adipose tissue occurs in order to provide the liver and the mammary gland with sufficient substrates for milk component synthesis [3]. When the rate of triglyceride synthesis in the liver does not match the slower speed of fat export via very low density lipoproteins (VLDL), a condition called fatty liver, with an incidence rate of nearly 50 % and characterized by fat accumulation inside the hepatocytes, is reached [4]. Nowadays, the main interest of the dairy research and molecular diagnostic is to be able to identify future problematic animals suffering from metabolic disorders. This can only occur when the traditional blood and milk composition analysis is completed with diagnostic gene expression profiling data.

Herein we will discuss the suitability of the high throughput Biomark platform for the measurement of gene expression changes in bovine liver, representing a physiological extremely active organ in lactating animals. For gene expression data analysis a principal components analysis (PCA) will be performed afterwards in order to see if different metabolic situations can be distinguished by regarding that gene expression data set.

Materials and methods

Animals and tissue sampling

Forty seven multiparous cows (Brown Suisse breed) were used in this experiment. Animals were housed at the research station Veitshof of the Technische Universität München (Munich, Germany) and managed on a conventional way with two milkings per day and access to feed depending on their individual milk yield. The lactation status of each animal was monitored in order to obtain two liver tissue samples: one during the early lactation phase (days 20 to 100 after parturition) and a second during the late lactation phase (days 200 to 250 after parturition).

Liver biopsy samples of approximately 100 mg dry weight were obtained by an experienced veterinarian, after local anesthesia of the zone, with a Bard Magnum biopsy instrument (Bard Biopsy Systems, Tempe, AZ, USA, http://www.bardbiopsy.com). The obtained sample was immediately frozen in liquid nitrogen and stored at −80 °C until further analysis. Samples from both early and late lactation phases could be successfully obtained for 37 of the 47 individuals that started the study. The responsible committee of animal welfare affairs approved all performed experiments.

RNA isolation and reverse transcription

Total RNA was extracted employing a peq-GOLD TriFast kit (PeqLab, Erlangen, Germany, http://www.peqlab.de) following the manufacturer's protocol. The obtained RNA was quantified by spectrophotometry and its integrity controlled with the Agilent 2100 Bioanalyzer platform (Agilent Technologies, Waldbronn, Germany, http://www.genomics.agilent.com). All extracted total RNA samples were stored at −80 °C previous to their use.

The reverse transcription was performed on a final volume of 25 μL including 500 ng RNA, 0.5 mM dNTPs, 2.5 μM random hexamer primers (Invitrogen Life Technologies, Darmstadt, Germany, www.invitrogen.com) and 100 units M-MLV H(−) reverse transcriptase (Promega, Mannheim, Germany, http://www.promega.com) together with the correspondent 5× buffer provided by the manufacturer. The temperature cycles were: annealing phase at 21 °C for 10 min, transcription at 48 °C for 50 min and degrading at 90 °C for 2 min. The obtained cDNA samples were diluted 1:1 in water prior to their utilization in the qPCR experiments and stored at −20 °C.

Primer design

Bovine mRNA sequences of the target genes were obtained from the National Center for Biotechnology Information (NCBI) Gene Database (NCBI, National Library of Medicine, Bethesda, MD, USA, http://www.ncbi.nlm.nih.gov/gene). For each of the 48 analyzed genes, a pair of forward and reverse primers was designed using the software Primer3 (http://frodo.wi.mit.edu/) aiming at the following optimal properties: melting temperature 60 °C, Primer GC content 50 % and product length of around 80 bp. All primers were checked virtually via data bank blast search (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi?PAGE_TYPE=BlastSearch&PROG_DEF=blastn&BLAST_PROG_DEF=megaBlast&BLAST_SPEC=OGP__9913__10708) and experimentally for specific product amplification by block PCR and gel electrophoresis (see ESM).

Quantitative PCR

Previous to gene expression quantification a pre-amplification step is recommended, for which the iQ Supermix and a CFX 96 cycler (both from Bio-Rad, Hercules, CA, USA, www.bio-rad.com) were employed [6]. 2 μL of cDNA sample, previously diluted to 10 ng original RNA/μL, was pre-amplified on a final volume of 10 μL containing 25 nM of each primer. The cycling conditions were: 10 min at 95 °C for polymerase activation, 18 cycles of denaturation at 95 °C for

15 sec plus 4 min annealing and extension at 59 °C. The plate was placed on ice immediately after pre-amplification and the samples diluted 1:10 previous to qPCR.

All qPCR experiments were carried out on the BioMark™ HD platform employing gene expression Dynamic Array 48.48 chips (Fluidigm). The reaction mix, loaded at the left side of the chip, consisted of: 2.5 μL SsoFast EvaGreen Supermix (Bio-Rad), 0.25 μL Binding dye loading reagent (Fluidigm), 0.1 μL ROX (diluted 1:4, Invitrogen), 1 μL pre-amplified and diluted cDNA. At the top side of the chip, 5 μL of a premix consisting of 2 μL 10 μM forward and reverse primers, 2.5 μL loading reagent (Fluidigm) and water, was loaded. At the initial step of the reaction, samples and primer mixes are combined inside the mix by a Nanoflex IFC controller (Fluidigm), followed by the qPCR cycles: 98 °C for 40 s initial denaturation and 30 repetitions of 95 °C during 10 s and 60 °C during 40 s. A melting curve determination step was included at the end of the amplification. An interplate calibration consisting of the same sample (in our case, sample nr. 31) and a genomic DNA sample were included in all chips.

Data analysis

All fluorescence data was obtained during the measurements with BioMark Data Collection Software 2.1.1. (Fluidigm) and processed with Fluidigm Melting Curve Analysis Software 1.1.0 and Real-time PCR Analysis Software 2.1.1., yielding a raw value for the cycle of quantification (Cq) for each sample-gene pair which was corrected, if necessary, for fluorescence baseline and threshold. Melting curve analysis was performed in order to discard the existence and quantification of primer dimer products. Reference genes, whose expression should remain stable among all measured experimental groups, were selected for their suitability with the Normfinder algorithm included in the real-time RT-qPCR data analysis software package GenEx 5.3.2 (MultiD Analyses AB, Gothenburg, Sweden, www.multid.se).

An index for normalization (reference gene index) was calculated as the arithmetic mean of the Cq values of three suitable reference genes and used as for normalization of gene expression calculations:

$$\textbf{reference gene index} = \textbf{arithmetic mean of three validated Cq reference genes}$$
$$\Delta\textbf{Cq} = \textbf{Cq target gene} - \textbf{reference gene index}$$
$$\Delta\Delta\textbf{Cq} = \Delta\textbf{Cq early lactation} - \Delta\textbf{Cq late lactation}$$
$$\textbf{expression change} = 2^{-\Delta\Delta\mathbf{Cq}}$$

A paired t-test with Bonferroni correction was performed with the expression change values of both lactation stages and differences were considered to be statistically significant at $p<0.05$. For a better comprehension of differences between both studied groups a fold expression value was calculated as $2^{-\Delta\Delta Cq}$ [5]. All statistics and graphics were generated with Excel 2011 (Microsoft, Redmond, WA, USA) and SPSS 12.0 Software (SPSS Inc., Chicago, IL, USA).

To visualize if the expression of the selected genes is suitable to distinguish between early and late lactation, principal components analysis (PCA) was performed using GenEx version 5.3.2 (MultiD Analyses AB). PCA is a method that enables the reduction of the multi dimensionality of gene expression data to a smaller number of dimensions, two in this case, in order to represent them in a scatter plot. The first principal component accounts for as much of the variability in the data as possible, and each succeeding component accounts for as much of the remaining variability as possible [6]. Dynamic PCA enables the selection of a pattern out of a number of genes that visualizes sub-clusters best.

Results and discussion

RNA quality

The mean total RNA concentration of the extracted liver samples was 995.16±251.54 $ng \cdot \mu L^{-1}$ and the RNA Integrity Number (RIN) 8.61±0.68. No significant differences, neither in RNA concentration nor in RIN, could be found between the two lactation phase groups. Total RNA obtained from liver with the above-explained protocol is therefore the ideal starting material for gene expression profiling studies and testing the suitability of the RT-qPCR methodology. An influence of RNA sample quality on the results can be discarded.

PCR reaction efficiency

The specificity of all designed amplifications was confirmed, first by gel electrophoresis of the obtained products (size confirmation), and second by qPCR melting curve analysis (absence of secondary PCR products and primer dimers). For the calculation of the PCR amplification efficiency value in the Biomark for each designed primer pair, a series of

dilutions (1:1, 1:4, 1:16, 1:64, 1:256) from a cDNA pool were employed in duplicates. As expected, the microfluidic qPCR platform offered a very high quantification precision over all applied assays and dilutions, as shown by a mean PCR efficiency value of 102.3±4.2 % and a mean correlation coefficient of the dilution series (r^2=0.995±0.009), confirming optimal amplification efficiencies.

Reference genes selection

The three candidates with the highest expression stability among the experimental groups were selected by Normfinder as reference gene set for this study and used for the calculation of the arithmetic mean reference gene index. The three best candidates selected, as calculated with the Normfinder tool, were: glyceraldehyde 3-phosphate dehydrogenase (GAPDH), beta-Actin, and hypoxanthine-guanine phosphoribosyl transferase (HPRT).

Analysis of gene expression by relative quantification

Out of the 48 analyzed target genes 28 showed a measured was significant alteration during the early lactation phase (Fig. 1) at key stages of the liver metabolism. For a better understanding of the regulated pathways, the 28 regulated key actors of liver metabolism in lactating cattle are resumed in a liver pathway figure (Fig. 2).

Result and discussion of the gene regulation in liver

Liver fat metabolism undergoes extreme adaptations during these first months after parturition in order to cope with the demand of fat for milk production, as clearly reflected by the significant expression change of the studied genes (all listed p values correspond to our microfluidic qPCR measurements). The applied microfluidic lab on chip qPCR system combines high-throughput with flexibility; throughput with up to 96×96 assays and flexibility in assay composition for optimal expression profiling. The obtained expression results reproduce the existing literature on the topic, demonstrating that this microfluidic platform might be an ideal alternative to the microarray and conventional single-plex RT-qPCR techniques used in the cited works:

– *Fatty activation and transport*: Acyl-CoA synthetase long-chain family member 1 (ACSL1) is responsible for the fatty acid (FA) activation by their conjugation with acetyl-CoA, which occurs directly at their entrance through the cellular membrane [7, 8]. Once activated, the FA-CoA complex is channeled through the cytosol

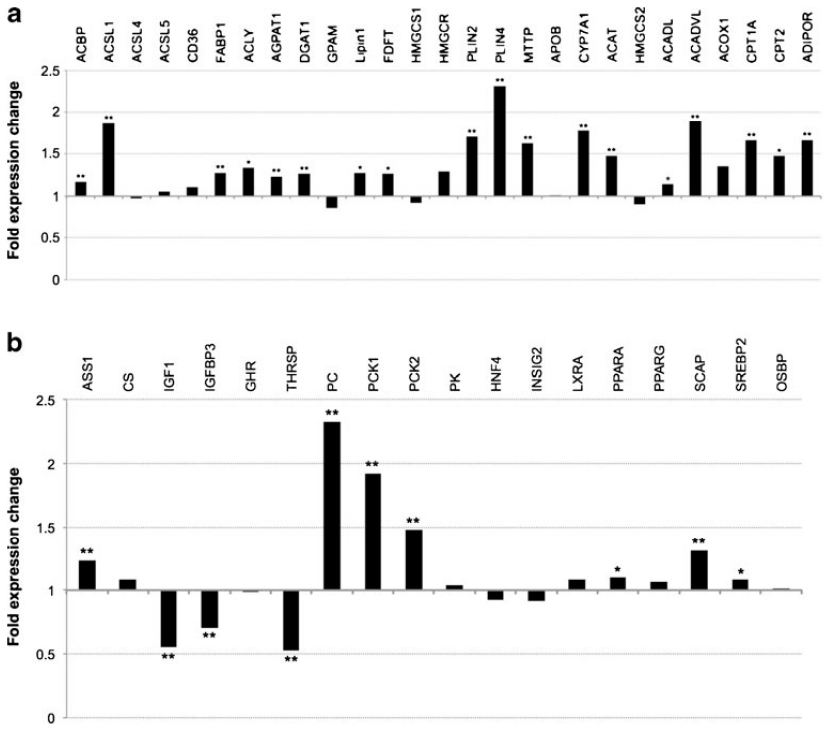

Fig. 1 Fold change in liver gene expression ($2^{-\Delta\Delta Cq}$) during the early lactation compared to late lactation (late lactation=1.00) from genes involved in **a** fat metabolism and **b** other metabolic routes and gene expression regulation. Significant differences between early and late lactation stages are marked as * (p<0.05) and ** (p<0.01)

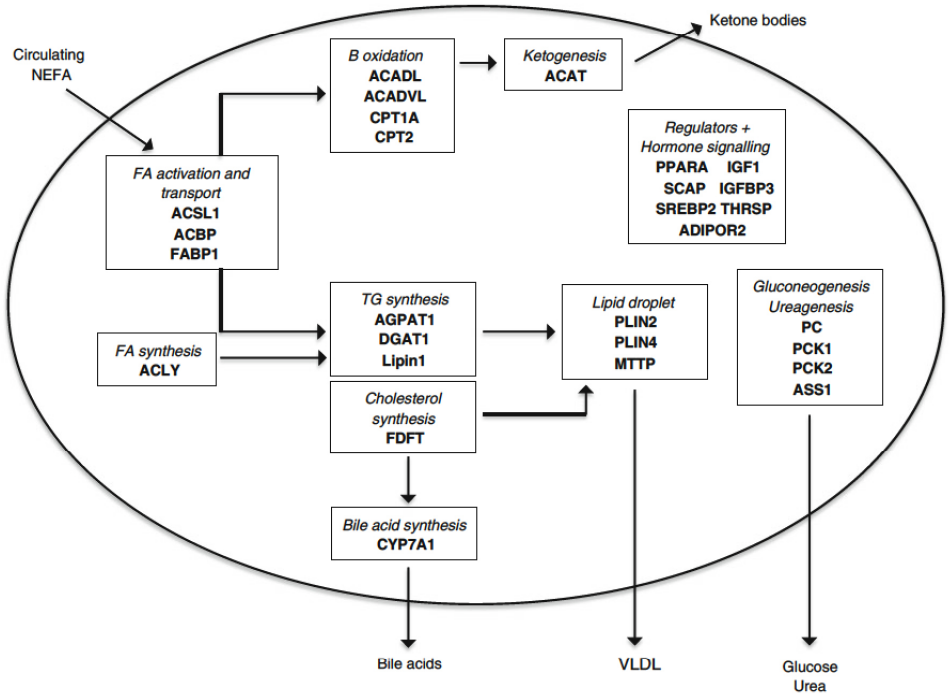

Fig. 2 Selected pathways of liver cell metabolism in which the 28 significantly regulated genes during early lactation are involved

with the help of the acyl-coA binding protein (ACBP) and different fatty acid-binding protein (FABP) isoforms [9–11]. As the import of FAs from blood needs to be increased, the expression of ACSL1, ACBP and FABP1 were significantly ($p<0.01$) increased during early lactation.

- *De novo fat synthesis* was also activated as reflected by the significant overexpression of ATP citrate lyase (ACLY) ($p<0.05$) and Farnesyl diphosphate farnesyl transferase (FDFT) ($p<0.05$) enzymes. ACLY is responsible for the ability to synthetize fatty acids out of glucose [12] while FDFT is the first enzyme of the long cholesterol synthesis pathway that catalyzes the formation of a product that leads exclusively to cholesterol synthesis. Due to the high amounts of milk cholesterol to be produced, its expression must be increased after parturition [13].
- *Triglyceride synthesis*: the triglyceride molecule is assembled out of a glycerol molecule and three, previously activated, FAs. 1-acylglycerol-3-phosphate acyltransferase (AGPAT) ($p<0.01$) catalyzes the union of the second FA molecule to generate phosphatidate, and diacylglycerol acyltransferase (DGAT) ($p<0.01$) performs the union of the third FA molecule to the complex [14]. Between these reactions, phosphatidate has to be converted into diacylglycerol by depriving it of a phosphate group, a step that avoids its conversion into phospholipids and is catalyzed by phosphatidate phosphatase, also called Lipin. The isoform 1 (Lipin1) ($p<0.05$) of this family of genes is cytosolic and relevant for the present study. The glycerol phosphate acyltransferase (GPAM), the enzyme responsible for the union of the first FA to the glycerol molecule did not show an altered expression in the present study, a fact that was previously suggested by Loor et al. [2] who showed that only AGPAT1 and DGAT1, the liver specific isoforms of these enzymes, were susceptible to gene expression changes after diet restriction. Graber et al. [8] even pointed out that GPAM should be down-regulated at the beginning of lactation in order to channel FAs preferentially towards beta-oxidation and production of energy.
- *Fatty acid oxidation*: Carnitine palmitoyltransferase isoforms 1A and 2 (CPT1A, CPT2) are mitochondrial membrane proteins that mark the rate at which FA oxidation occurs by translocating the long-chain FAs to the mitochondrial matrix where the beta-oxidation occurs. Both were significantly upregulated at early lactation (CPT1A, $p<0.01$; CPT2, $p<0.05$). Another good image of the rate of oxidation can be obtained by measuring the gene expression of the enzyme responsible for its first step, the Acetyl-CoA dehydrogenase (ACADL for long-chain fatty acids, ACADVL for very long-chain fatty acids). An overexpression of these enzymes ($p<0.05$ and $p<0.01$

respectively) during the early lactation reflects the shortage in energy during that period and the subsequent activation of FA oxidation and was well described before [2, 8, 15].

- *Lipid droplet formation*: The excessive amounts of fat in the hepatocyte are assembled into lipid droplets and continue growing until a size of 1 to 20 μm is reached and the droplet is stored in the cytosol or transformed and secreted in form of VLDL [16]. Crucial actors of the droplet growth process are the members of the gene family of perilipins (PLIN2, PLIN4 in our study), which are associated to the outer surface of the droplet with the goal of transferring lipids to its core [17]. Subsequently, they are overexpressed in this period of time ($p<0.01$) characterized by intense fat mobilization in the liver cell [18]. As shown in previous works [19–21] the formation of the lipid droplet is usually followed by its export in form of VLDL, a process in which the overexpressed ($p<0.01$) microsomal triglyceride transfer protein (MTTP) promotes the transport of lipids into the nascent VLDL.
- *Bile acid synthesis*: With the secondary aim of promoting the lipid recruitment at the liver during lactation in order to compensate the energy deficit, the bile acid synthesis enzymes are overexpressed at the initial phases of the cycle [22]. An increased bile acid synthesis is considered physiologically normal during that period, as marked by the higher levels of Cholesterol 7 alpha-hydroxylase (CYP7A1) ($p<0.01$).
- *Ketone body synthesis*: Negative energy balance, the principal physiological characteristic of the early lactation, is easily detectable with elevated levels of blood FA and ketone bodies, which are synthetized in the liver as a faster way to mobilize the energy from fat reserves. As a consequence, an overexpression of acetyl-CoA acetyltransferase (ACAT) was observed ($p<0.01$).
- *Gluconeogenesis*: The de novo synthesis of glucose out of amino acids, lactate, glycerol or propionate, increases after parturition in response to the demands for milk sugar production. Propionate is one of the final products of bacterial fermentation in the rumen and is not available in sufficient amounts at this stage due to the reduction in feed intake. Therefore, the gluconeogenic pathways with lactate or aminoacids as a starting substrate obtain priority , as reflected by the overexpression ($p<0.01$) of pyruvate carboxylase (PC) [23, 24]. Phosphoenolpyruvate carboxykinase, the key regulatory enzyme of the gluconeogenesis, was also upregulated for both its isoforms (PCK1, cytosolic; PCK2, mitochondrial; $p<0.01$) confirming the hypothesis [2, 15].
- *Urea synthesis*: The increase in protein intake has the logical effect of an increase in the need for nitrogen detoxification in the liver. Hartwell et al. [24] measured a slight increase in the expression of the urea-synthetizing enzyme arginosuccinate synthase (ASS1), which could correspond with the also slow increase in feed intake. Our results seem to confirm that hypothesis ($p<0.01$).
- *Hormone signaling*: Belonging to the adipokines, adiponectin is a protein hormone secreted by the fat tissue that regulates energy metabolism by increasing glucose uptake and FA beta-oxidation in the liver. Adiponectin receptor 2 (ADIPOR2) is overexpressed ($p<0.01$) in the cell membrane at those conditions in order to mediate adiponectin effect in the hepatocyte [25]. Not surprinsingly, a significant downregulation ($p<0.01$) of insulin growth factor 1 (IGF1) insulin growth factor binding protein 3 (IGFBP3) and thyroid hormone responsive Spot 14 (THRSP) could be observed, as correspond to the need of favoring the catabolic status that will support lactation in its early stages [26, 27].
- *Gene expression regulators*: All the above mentioned routes are subject to the regulation of a complex network of gene expression regulators that respond to FA and sterol levels and are one of the topics of more intense investigation during the last years [13, 28, 29]. In our study, we observed a significant upregulation ($p<0.05$) of peroxisome proliferator-activated receptor alpha (PPARA), sterol regulatory element binding protein 2 (SREBP2) ($p<0.05$) and SREBP cleavage-activating protein (SCAP) ($p<0.01$).

Grouping in different lactation phases

PCA was used to test if the expression differences of the candidate genes enable the differentiation between early and late lactation phase. Figure 3a shows the plot created when all quantified genes were included in the PCA. Black dots represent the samples obtained from animals in early lactation and white dots represent samples from animals in late lactation. Early lactation samples group on the upper side of the PCA plot and late lactation samples on the lower side whereas early lactation samples show a higher variance, reinforcing the idea of a different individual ability to adapt to lactation. GenEx version 5.3.2 software enables the application of a dynamic PCA in order to select the best genes for separation of groups according to the statistical difference between them. Using dynamic PCA, the gene pattern of 13 genes (IGF-1, IGFBP3, ACSL1, ADIPOR, THRSP, PCK1, CPT1A, PLIN4, ACAT, FABP1, ASS1, CYP7A1 and PC) showed the best separation between early and late lactation (Fig. 3b) whereas the variance between the samples of early lactation is not reduced. These results show that the expression of the selected genes, measured with a microfluidic lab on chip qPCR system, might enable the differentiation between individuals in different lactation stages and open future perspectives for identifying

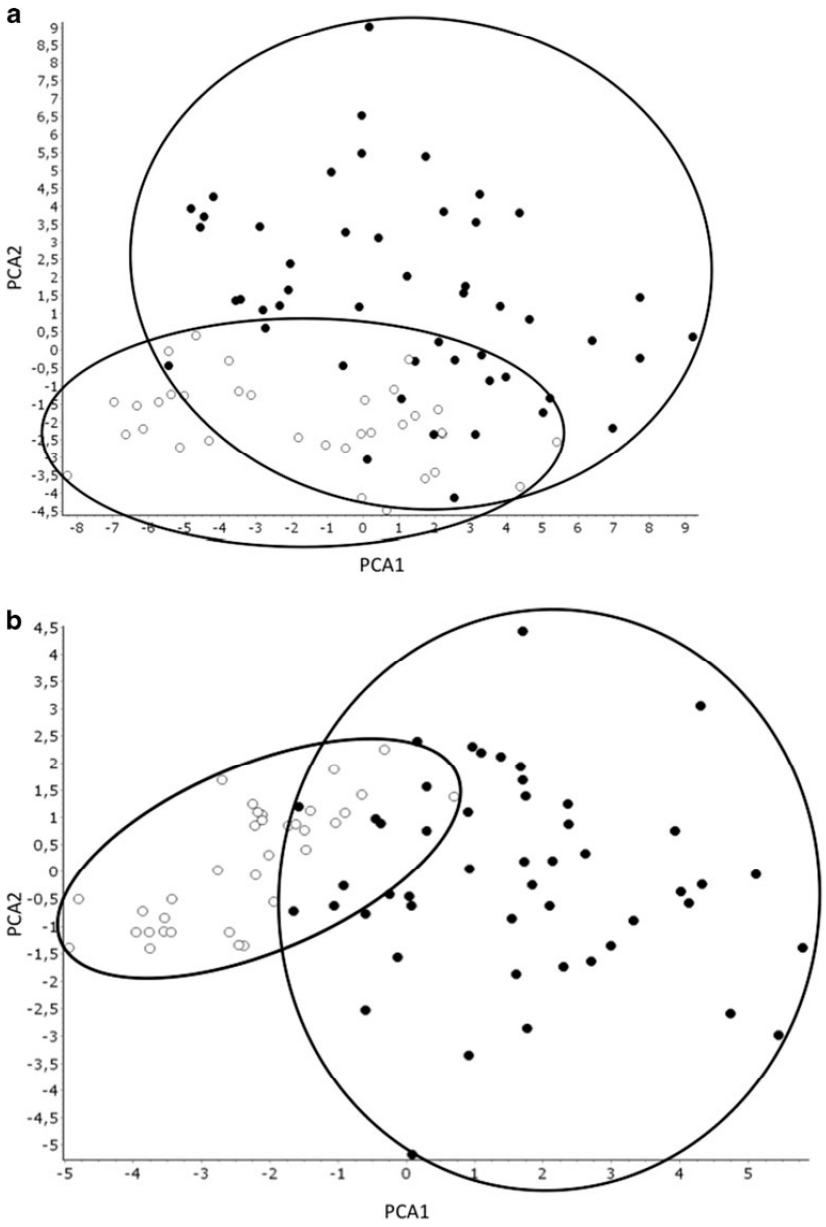

Fig. 3 **a** PCA, here calculated with the expression data of all measured genes, was employed to test if the quantified gene expression differences enable the separation of cows in early and late lactation. Black dots represent the samples obtained from animals in early lactation and white dots represent samples from animals in late lactation. **b** To determine the pattern of genes that visualizes the separation of animals in the two lactation stages best, dynamic PCA was employed. A pattern of 13 genes was identified. Black dots represent the samples obtained from animals in early lactation and white dots represent samples from animals in late lactation

which animals are better suited for a lactation cycle, helping to diminish milk yield lost, metabolic stress and diseases.

Conclusions

The tested high-throughput microfluidics platform demonstrated to be a good alternative to microarrays or conventional single-plex RT-qPCR techniques, as it reproduced the result shown in previous works with those technologies. Due to its limitations in chip space (maximal 96 samples × 96 genes), it fits especially when the purpose is to quickly analyze the expression of a limited amount of candidate genes or confirm previous hypothesis rather than performing the screening of thousand of genes. In addition, subsequently processing the obtained data with the dynamic PCA algorithm allows

identifying which of the measured genes better serve for the separation of metabolic conditions. The combination of both methods, high throughput micro device RT-qPCR and dynamic PCA algorithm, could therefore serve optimally as a screening tool and allow to quickly identifying individuals with the highest potential of developing metabolic imbalances during lactation.

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5 Discussion

5.1 Feed intake, Milk Yield and Major Milk Components

The major objective of the study was to test the hypothesis that diet-induced milk fat depression may in parallel influences cholesterol levels in milk. The successfully induced milk fat depression (**MFD**), by adding 830 g of oil to the diet in Soybean-oil (SO) Rapeseed-oil (RO), is demonstrated by the drastic reduction in fat percentage and fat yield on the SO and RO groups (Altenhofer et al., 2014b). With the adding of extruded linseed (LO) to the diet it was not possible to induce a MFD (Viturro and Altenhofer, 2013a, Viturro and Altenhofer, 2013b), maybe because of the lower availability of fatty acids in ruminal fermentation. It is described earlier in literature that certain fatty acids have an altering effect on milk fat content (Perfield II et al., 2004, Kadegowda et al., 2008) in contrast to the LO group in our experiment where fatty acids in diet can alter fatty acid composition in milk but do not significantly change fat percentage in milk (Viturro and Altenhofer, 2013a, Viturro and Altenhofer, 2013b). This effect is maybe based on the different availability of fatty acids in diet supported by Gama et al. (2008) who reduced milk fat percentage by more than 1 % by adding 1.6 % pure fish oil to the diet which is very similar to the reduction observed in this experiment adding SO and RO. Also Veira et al. (2001) reported a highly significant reduction of fat yield and fat percentage in milk of cows fed a ration with 3 % supplemented soybean oil.

Other mayor milk parameters, such as milk yield and protein yield were not affected by the three different oil supplementations except early lactating cows fed LO compared to the control diet (Viturro and Altenhofer, 2013a, Viturro and Altenhofer,

2013b, Altenhofer et al., 2014b). This effect was no surprise to the study designers because all experimental diets where expected to be iso-energetic. Yet the influence of diet induced MFD on protein yield is not clearly described in the available literature and the results from several similar feeding experiments are contradictory, as for example, in the experiments published by He et al. (2012) cows were fed diets with up to 4 % supplemental fat resulting in an increased milk protein concentration but in contrast no effect in milk yield and protein yield was visible. However, unaffected milk yield, protein yield and lactose yield but a decrease in milk protein concentration by 2.5 % oil supplementation (Abdelqader et al., 2009) was previously reported. Veira et al. (2001) reported an unchanged milk yield, protein and lactose content in milk of cows fed a diet with 3 % soybean oil added compared to a control ration with no oil supplemented.

Body weight and feed intake described as dry matter intake (**DMI**) in our experiment were not affected in all three experimental groups, probably due to the short feeding periods of four weeks or because different diets are equally valued by the tested cows (Altenhofer et al., 2014b). This observation is supported by AlZahal et al. (2008), Huang et al. (2008) and Jacobs et al. (2011), who also failed to detect changes in DMI by feeding SO or RO whereas He et al. (2012) described an unchanged body weight, but observed a slightly reduced DMI when cows were fed a diet supplemented with C18:1 or C18:2. Also Veira et al. (2001) detected a modest reduction of dry matter intake because of a slightly reduced digestibility of the ration supplemented with soybean oil.

5.2 Blood Plasma

The stable blood plasma parameters glucose, triglycerides, NEFA and BHBA in RO and SO (except the increase in BHBA in SO) (Altenhofer et al., 2014b) are in good agreement with previous studies (Abdelqader et al., 2009, Dai et al., 2011) which also reported that these plasma metabolites are unaffected with supplemental oil feedings. In the experiment of Dai et al. (2011) NEFAs appeared to show a numerical increase but detected changes did not reach significance, whereas all other plasma metabolites stayed unchanged when fed RO. In early lactating cows fed LO NEFA and BHBA show a numerical decrease but could not reach a significant level (Viturro and Altenhofer, 2013a, Viturro and Altenhofer, 2013b). This could also be because of the short feeding period. During the four week feeding of oil supplemented ration no sign of a metabolic imbalance was detected. The feeding of highly oil supplemented rations during the whole lactation cycle may result in metabolic disorders.

5.3 Fatty Acid Composition

Feeding vegetable oils, rich in C18:1, C18:2 and C18:3 fatty acids, is associated with a lowering of the amount of milk saturated fat and an increase of unsaturated fat, as widely described before (Dai et al., 2011, Jacobs et al., 2011, He et al., 2012) and also detected in this experiment with a smaller effect observed in LO group than in both groups supplemented with pure oil (Viturro and Altenhofer, 2013a, Viturro and Altenhofer, 2013b, Altenhofer et al., 2014b). Stearic acid (C18:0) is the most prominent SFA increasing in all three oil feeding groups (appendix), due to the abundance of C18 unsaturated fatty acids in the three oil supplemented groups in our experiment and the saturation of these unsaturated fatty acids because of ruminal

biohydrogenation. This effect was reported before (Loor and Herbein, 2003a, Huang et al., 2008) where authors present in their experiments an increase of C18:0 in a SO diet compared to a diet with no supplemented oil.

The decrease in C4:0 to C17:0 fraction, known to originate from de novo lipogenesis, is strongly correlated to the CLA content and follows the theory postulated by Bauman and coworkers (Bauman et al., 2008). When focusing on the diets where pure oil was supplemented, the SO diet showed a bigger potential to reduce this group of fats than the RO diet (Altenhofer et al., 2014b). This is consistent with previous studies which also reported a decrease in FA shorter than 16 carbons observing a bigger decrease in C18:2 - rich soybean oil supplemented diets than in C18:1-rich rapeseed oil supplemented diet, maybe as a consequence of an altered ruminal biohydrogenation and its intermediates (He et al., 2012) because of the high availability of fatty acids in rumen when adding pure oil. Focusing on its potential benefits for human health, especially the drastic reduction of the myristic acid amount is of high interest (Zock et al., 1994, Müller et al., 2001, Dabadie et al., 2005). Particularly the human-blood-cholesterol rising potential of myristic acid in human diets is of great interest, as dairy products are one of the major sources of myristic acid in the human diet (Zock et al., 1994) and a lower level of this fatty acid may result in a lower blood cholesterol level.

As observed in our study with feeding of pure oil (Altenhofer et al., 2014b), AlZahal et al. (2008) associated milk fat depression with an increase in total trans C18:1 and total CLA. The effect of a lower milk fat content because of a higher amount of CLA C18:2 t10c12 was earlier described by Peterson et al. (2003) and Glasser et al. (2010) maybe because of a shift from the trans - 11 to trans - 10 pathway of ruminal biohydrogenation (Zened et al., 2012). The almost unaffected milk fat content in LO

feeding was maybe due to the protected fats in the extruded linseed and therefore a smaller effect on ruminal biohydrogenation and a lower content of C18:2 t10c12 in milk as presented in appendix (Viturro and Altenhofer, 2013a, Viturro and Altenhofer, 2013b). After oil supplementation, an increased milk TFA level was observed in all three oil feeding groups, most likely because of the high amount of unsaturated C18 fatty acids and the incomplete saturation process. The role of this group of fats in human diet is still a topic of discussion. Maybe a differentiation between fats of ruminant and industrial origin is needed. The publications found in literature reporting a potential negative effects of trans fatty acids produced by ruminants refer to extremely high intake doses (Sun et al., 2007, Motard-Bélanger et al., 2008), whereas Jakobsen et al. (2008) and Gebauer et al. (2011) suggest that ruminal trans fatty acids have a neutral impact on the risk of coronary diseases.

5.4 Milk and Plasma Cholesterol and Gene expression in mammary epithelial cells

Many studies on extreme metabolic situation like milk fat depression have previously reported its effects on milk composition (Gama et al., 2008, Glasser et al., 2010, He et al., 2012), but to our knowledge none of them focused on milk cholesterol level and its variation in comparison with other fat components up to day. An increase in the amount of cholesterol in blood during and a decline of this metabolite in milk during the lactating cycle is a natural process (Maynard et al., 1931, Herdt and Smith, 1996, Strzałkowska et al., 2009). In the case of fat supplementation in form of pure oil (SO and RO) in diet, the inverse relationship of these parameters is maintained and a decreased milk cholesterol level effect is always accompanied by an increased plasma cholesterol concentration (Altenhofer et al., 2014b). The increased plasma cholesterol levels observed in SO, RO and LO, though smaller in LO than in the other

two treatment groups, are similar to effects described by Khorasani and Kennelly (1998), and Abdelqader et al. (2009). Previously, Nestel et al. (1978) postulated that this hypercholesteremiasis most probably due to an increased requirement of cholesterol for the transport of long chain fatty acids as chylomicrons from the intestine into blood, which leads to an enhanced cholesterol biosynthesis in the intestine. The high amount of fatty acids available from vegetable oils in our experimental feedings may therefore be the reason for the increased blood cholesterol levels observed in the SO, RO and LO groups and confirm this theory (Altenhofer et al., 2014b). In addition, the induced milk fat depression results in a lower need for milk cholesterol as part of the milk fat, especially part of the milk fat globule membrane, and the resulting reduced transport and synthesis of this metabolite to and in the mammary gland. A possible explanation of this effect is the reduction of the expression of the cholesterol transport regulatory genes like ATP-binding cassette subfamily A member 1 (ABCA1) and genes involved in cholesterol synthesis like farnesyl diphosphate farnesyl transferase (FDFT) or the reduction of the expression of Stearoyl-CoA desaturase (SCD), a gene involved in the desaturation of fatty acids, or Glycerol-3-phosphate acyl transferase (GPAM), a gene participating in in triglyceride synthesis, in the mammary epithelial cells and milk fat globules during lactation cycle (Mani et al., 2011, Altenhofer et al., 2015). Maybe a different regulation of these genes can be observed when plant oils are added to the diet. The unaffected gene expression of liver samples after oil supplementation (unpublished data), though regulated during lactation cycle (Viturro et al., 2014) might be the result of the short feeding periods of four weeks or the primary role of the liver of regulating the whole body metabolism masking the effects of oil in the diet on cholesterol and fat metabolism in liver cells. Another reason of the low gene expression in liver cells might be an independent regulatory network in the mammary

gland itself, preventing certain metabolites from passing from blood into mammary gland. This hypothesis is also supported by Altenhofer et al. (2015) reporting higher gene expression levels in mammary gland of genes involved in cholesterol synthesis and transport when higher contents of this metabolite are detected in milk (GPAM, SCD, FDFT and ABCA1).

To be sure that freezing of milk samples has no effect on analyzes a milk conservation experiment was conducted (Altenhofer et al., 2014a). This experiment illuminates the possible effects of freezing time and different milk conservation agents on milk composition. A high decrease in milk fat percentage was observed at every thawing date $P \leq 0.01$ (Altenhofer et al., 2014a). This effect of a reduction of fat percentage after freezing is in agreement with (Lepri et al., 1997) who reported a decrease of 5.7 percent in human milk after a short storage time of 90 days and Zhang et al. (2006) who also described a decrease in fat content after freezing. The tremendous reduction of fat percentage might be due to ice crystal forming during freezing process resulting in a destruction of milk fat globules and thereafter the measurement via infrared light absorption might be influenced because of undetectable smaller milk fat globule fragments. The possible positive effects of conservation agents on milk fat percentage were not investigated and may be the topic of another experiment. As the samples in Viturro and Altenhofer (2013a), Viturro and Altenhofer (2013b), Altenhofer et al. (2014b), Viturro et al. (2014) and Altenhofer et al. (2015) were frozen for a time period of approximately three months without conservation agents, the effect of freezing on analysis does not influence the presented statements because the effect stays the same for every analyzed sample.

6 Conclusions and Outlook

We were able to induce extreme changes in milk components by feeding supplements of soybean oil, rapeseed oil or extruded linseed. Especially fatty acid composition of milk was deeply affected, but also its total fat and cholesterol content. It was possible to reduce the amount of saturated fatty acids, especially myristic acid as an important part of saturated fats because of its cholesterol rising potential, total fat and cholesterol on the one hand and on the other hand increase the amount of mono – and polyunsaturated fatty acids, including conjugated linoleic acids. The observed effects may be an important step towards improving the nutritional quality of milk and its fatty acid composition by using oil additives in feeding rations of dairy cows. Gene expression in liver cells did not show any effect after oil feeding, although a differentiation of early and late lactating cows was possible. These results led to an experiment with milk epithelial cells in which the hypothesis that fat and cholesterol may be regulated at the mammary gland itself was tested. It was possible to show that the size of milk fat globule diameter and fat as well as cholesterol content are correlated. Higher expression of genes involved in cholesterol and fat synthesis and transport (ABCA1, FDFT for example) could also be detected in mammary gland epithelial cells when higher amounts of these metabolites are in milk. This observation points at the hypothesis that cholesterol content is not passively transported from blood to milk but has an internal regulatory mechanism. To investigate the effects of oil supplements on gene expression of mammary gland epithelial cells and its implication on milk fat globules further research is needed. Another point needed to be investigated further is the effect of oil supplements on dairy cows metabolism when oil is fed over a longer period of time. A further

experiment in which cows are fed a ration with a lower fat content, in order to reduce costs of feeding may also be conducted and results should be compared with this experiment to test its reproducibility at these conditions. To test the effects of storage time and temperature of conservation on milk analysis an experiment was conducted where it was possible to show a tremendous reduction of fat when milk was frozen underlining a possible destructive effect on milk fat globules by ice crystal forming during the freezing process as well as several impacts of conservation agents on analysis could be outlined. For future experiments, the storage time and conservation agent have to be carefully evaluated and have to be included in study design to minimize possible bias because of the impact of these treatments on analysis of specific metabolites. Also, a subsequent storage experiment can be designed to detect possible impacts of conservation agents on the destruction of milk fat globules during freezing should be performed.

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9 Scientific Communications

9.1 Peer reviewed

9.1.1 Related to Thesis

(Altenhofer et al., 2014a) Altenhofer, C., M. Pfaffl, and E. Viturro. 2014a. Effects of 1 year long-term freezing with different preservatives on milk cholesterol, progesterone and lactoferrin determination. International Journal of Dairy Technology 67(4):490-494

(Altenhofer et al., 2014b) Altenhofer C, Spornraft M, Kienberger H, Rychlik M, Herrmann J, Meyer HH & Viturro E 2014 Effects of rapeseed and soybean oil dietary supplementation on bovine fat metabolism, fatty acid composition and cholesterol levels in milk. Journal of Dairy Research 81(01) 120-128

(Altenhofer et al., 2015) Altenhofer, C., W. Holzmüller, F. Wolfertstetter, D. Wolfschoon Ribeiro, U. Kulozik, M. W. Pfaffl, and E. Viturro. submitted 2014 Temporal variation of milk fat globule diameter, fat and cholesterol content and milk epithelial cell gene expression in dairy cows. International Journal of Dairy Technology (online)

(Viturro et al., 2014) Viturro E, Altenhofer C, Zölch B, Burgmaier A, Riedmaier I & Pfaffl MW 2014 Microfluidic high-throughput reverse-transcription quantitative PCR analysis of liver gene expression in lactating animals. Microchimica Acta 1-8

9.1.2 Not related to Thesis

(Kofler et al., 2012)Kofler J, Mangweth G, Altenhofer C, Weber A, Gasser C, Schramel J, Tichy A, Peham C, Lahmheitserkennung K & Lahmheit R 2012 Messung der Bewegung lahmheitsfreier Kühe mittels Accelerometer im Schritt und Vergleich der Beschleunigungswerte nach Kleben eines Klotzes. Wiener Tierarztliche Monatsschrift 99(7) 179

(Mangweth et al., 2012)Mangweth G, Schramel J, Peham C, Gasser C, Tichy A, Altenhofer C, Weber A & Kofler J 2012 Lahmheitserkennung bei Kühen durch Messung der Bewegung im Schritt mittels Accelerometer. Lameness detection in cows by accelerometric measurement of motion at walk. Berl. Münch. Tierärztl. Wschr. 11(9-10) 386-396

9.1.3 Poster

(Viturro et al., 2012)Viturro E, Hüttinger K, Schlamberger G, Wiedemann S, Altenhofer C, Kaske M &Meyer HHD 2012 Effect of dry period absence on milk cholesterol metabolism and gene expression in dairy cows. Book of Abstracts of the 63rd Annual Meeting of the European Association for Animal Production: Bratislava, Slovakia, 27-31 August 2012

9.2 Non peer reviewed

(Viturro and Altenhofer, 2013a)Viturro E & Altenhofer C 2013a Kann Leinsaat das Energieloch stopfen? Pages 30-31 in Top Agrar Vol. 06.

(Viturro and Altenhofer, 2013b)Viturro E & Altenhofer C 2013b Reduction of the negative energy balance in early lactating dairy cows. Pages 10-12 in Feed Magazine/Kraftfutter Vol. 9-10.

10 Curriculum vitae

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11 Appendix

Table of changes of Fatty acid composition during four weeks of feeding.

	Treatment Group			
Fatty Acid %[†]	C	SO	RO	LO
4:0	-0.014±0.07[a]	-0.351±0.13[b]	-0.179±0.07[ab]	-0.046±0.05
6:0	0.063±0.05[a]	-0.498±0.09[b]	-0.389±0.06[b]	0.081±0.04
8:0	0.052±0.03[a]	-0.373±0.06[b]	-0.297±0.04[b]	0.094±0.04
10:0	0.125±0.08[a]	-0.993±0.14[b]	-0.853±0.09b	0.234±0.12
10:1	0.011±0.08[a]	-0.086±0.02[b]	-0.081±0.01[b]	0.012±0.02
11:0	0.004±0.00[a]	-0.025±0.00[b]	-0.015±0.00[c]	-0.003±0.00[d]
12:0	0.142±0.10[a]	-1.045±0.15[b]	-0.998±0.10[b]	0.198±0.16
12:1*cis*-9	0.003±0.00[a]	-0.015±0.00[b]	-0.019±0.00[b]	0.000±0.01
13:0	0.011±0.01[a]	-0.036±0.01[b]	-0.019±0.00[c]	0.001±0.01[d]
Anteiso 13:0	0.003±0.00[a]	-0.020±0.00[b]	-0.022±0.00[b]	0.001±0.01
14:0	0.336±0.19[a]	-1.903±0.28[b]	-1.523±0.21[b]	0.050±0.27[d]
Iso-14:0	0.005±0.00[a]	-0.013±0.00[b]	-0.011±0.00[b]	-0.013±0.00[d]
14:1*cis*-9	0.013±0.03	0.113±0.05	0.035±0.04	-0.052±0.04[d]
15:0	0.089±0.04[a]	-0.231±0.04[b]	-0.131±0.03[c]	-0.086±0.04[d]
Iso-15:0	0.019±0.01[a]	-0.019±0.01[b]	-0.029±0.00[b]	-0.002±0.01[d]
Anteiso-15:0	0.036±0.01[a]	-0.001±0.01[b]	-0.020±0.01[b]	0.002±0.01[d]
16:0	-0.045±0.52[a]	-6.427±0.72[b]	-8.007±0.60[b]	-3.679±0.54[d]
Iso-16:0	0.010±0.01[a]	0.000±0.01[ab]	-0.019±0.01[b]	-0.030±0.01[d]
16:1 *trans*-9	-0.000±0.00[a]	0.112±0.01[b]	0.061±0.01[c]	0.031±0.01[d]
16:1 *cis*-9	-0.115±0.04	-0.020±0.11	-0.214±0.07	-0.326±0.06[d]
17:0	0.002±0.01[a]	-0.099±0.01[b]	-0.075±0.01[b]	-0.066±0.02[d]
Iso-17:0	0.013±0.00[a]	0.036±0.01[b]	0.006±0.00[a]	-0.005±0.01[d]

Anteiso-17:0	0.010±0.01	0.007±0.01	0.004±0.01	-0.017±0.01[d]
17:1 *cis*-9	-0.011±0.01	-0.037±0.01	-0.028±0.01	-0.042±0.01
18:0	-0.083±0.33[a]	1.827±0.25[b]	2.146±0.41[b]	1.490±0.42[d]
Iso-18:0	-0.002±0.00[a]	-0.011±0.00[b]	-0.006±0.00[ab]	-0.008±0.00
18:1 ∑*trans*-6/*trans*-9/*trans*-10	0.012±0.02	2.277±0.31	2.241±0.27	0.148±0.04[d]
18:1 *trans*-11	0.016±0.08	1.604±0.37	1.390±0.17	0.642±0.09[d]
18:1 *cis*-9	-0.622±0.47	5.083±0.69	6.006±0.63	0.772±0.67
18:1 *cis*-11	-0.040±0.02[a]	-0.003±0.03[a]	0.184±0.03[b]	-0.064±0.02
18:1 *cis*-12	0.005±0.01[a]	0.139±0.05[b]	0.058±0.02[b]	0.074±0.02[d]
18:2 *cis*-9,*cis*-12	-0.066±0.04[a]	0.214±0.05[b]	0.004±0.04[a]	0.018±0.04
18:2 *cis*-9,*trans*-11 (CLA)	0.023±0.03[a]	0.797±0.15[b]	0.653±0.06[b]	0.258±0.04[d]
18:2 *tans*-10,*cis*-12 (CLA)	0.000±0.00[a]	0.013±0.00[b]	0.018±0.01[b]	0.000±0.00
18:3 *cis*-9,*cis*-12, *cis*-15	-0.005±0.01	0.020±0.03	0.006±0.02	0.292±0.03[d]
20:0	0.004±0.00[a]	0.030±0.03[ab]	0.086±0.03[b]	0.027±0.02
20:1 *cis*-11	-0.002±0.00[a]	0.010±0.00[b]	0.079±0.01[c]	-0.001±0.00
20:2 *cis*-11, *cis*-14	0.000±0.00	0.000±0.00	0.001±0.00	-0.001±0.00
20:3 *cis*-8, *cis*-11, *cis*-14	-0.001±0.01[a]	-0.017±0.00[b]	-0.005±0.00[a]	-0.012±0.00
20:4 *cis*-5, *cis*-8, *cis*-11, *cis*-14	-0.005±0.01[a]	-0.036±0.00[b]	-0.017±0.01[a]	-0.016±0.00
20:5 *cis*-5,*cis*-8,*cis*-11,*cis*-14,*cis*-17	-0.004±0.00[a]	-0.010±0.00[b]	-0.015±0.00[b]	0.022±0.00[d]
21:0	0.002±0.00[a]	-0.001±0.00[b]	-0.001±0.00[b]	-0.000±0.00
22:0	0.004±0.00	0.002±0.00	0.001±0.00	0.000±0.00
22:1 *cis*-13	-0.001±0.00[a]	-0.002±0.00[a]	0.004±0.00[b]	0.015±0.00
22:5 *cis*-7,*cis*-10,*cis*-13,*cis*-16,*cis*-19	-0.003±0.01[a]	-0.020±0.00[b]	-0.014±0.00[b]	0.012±0.00[d]

22:6 *cis*-4, *cis*-7,*cis*-10,*cis*-13,*cis*-16,*cis*-19	0.002±0.00	0.005±0.00	0.002±0.00	-0.001±0.00
23:0	0.002±0.00	0.002±0.00	0.003±0.00	0.002±0.00
24:0	0.004±0.00[a]	0.003±0.00[a]	-0.003±0.00[b]	-0.002±0.00[d]
Summation				
SFA[‡]	0.791±0.55[a]	-10.139±1.17[b]	-10.350±0.85[b]	-1.784±0.64[d]
MUFA[§]	-0.733±0.53[a]	9.174±1.06[b]	9.717±0.8[b]	1.211±0.61[d]
PUFA[¶]	-0.058±0.06[a]	0.965±0.15[b]	0.633±0.08[b]	0.573±0.07[d]
CLA[††]	0.023±0.03[a]	0.809±0.15[b]	0.671±0.07[b]	0.258±0.05[d]
TFA[‡‡]	0.051±0.13[a]	4.801±0.64[b]	4.363±0.35[b]	1.080±0.42[d]

Means are different among groups (P<0.05) when marked with X[abc]. Collumn LO is only compared to group C because of the different oil supplementation in SO and RO. LO is significantly different to C when marked with X[d].

[†]Results are shown as delta values day 2 vs. day 26 of feeding period ±SEM

[‡]Saturated fatty acids: ∑ (C4:0, C6:0, C8:0, C10:0, C11:0, C12:0, C13:0,anteiso-C13:0, C14:0, iso-C14:0, C15:0, iso-C15:0, anteiso-C15:0, C16:0, iso-C16:0, C17:0, iso-C17:0, anteiso-C17:0, C18:0, iso-C18:0, C20:0, C21:0, C22:0, C23:0, C24:0). Iso and anteiso Fatty Acids are not shown in the table.

[§]Monounsaturated fatty acids: ∑ (C10:1, C12:1 c9, C14:1 c9, C16:1 t9, C16:1 c9, C17:1 c9, C18:1 t6+t9+t10, C18:1 t11, C18:1 c9, C18:1 c11, C18:1 c12, C20:1 c11, C22:1 c13)

[¶]Polyunsaturated fatty acids: ∑ (C18:2 c9c12, C18:2 c9t11 CLA, C18:2 t10c12 CLA, C18:3 c9c12c15, C20:2 c11c14, C20:3 n-6, C20:4 n-6, C20:5 n-3, C22:5 n-3, C22:6 n-3).

[††]Conjugated linoleic acid: ∑ (C18:2 c9t11, C18:2 t10c12)

[‡‡] Trans Fatty Acid: ∑ (C16:1 t9, C18:1 t6+t9+t10, C18:1 t11, C18:2 c9t11 CLA, C18:2 t10c12 CLA