



HAL
open science

Fluorometric determination of isocitrate dehydrogenase (EC 1.1.1.42; 1; NADP⁺ dependent) in ruminant milk

T. Larsen, R. Rupp, Nicolas N.C. Friggens, J.A.A. Pires

► To cite this version:

T. Larsen, R. Rupp, Nicolas N.C. Friggens, J.A.A. Pires. Fluorometric determination of isocitrate dehydrogenase (EC 1.1.1.42; 1; NADP⁺ dependent) in ruminant milk. *Animal*, 2022, 16 (8), 11 p. 10.1016/j.animal.2022.100593 . hal-03771699

HAL Id: hal-03771699

<https://hal.inrae.fr/hal-03771699v1>

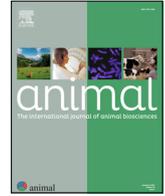
Submitted on 7 Sep 2022

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial - NoDerivatives 4.0 International License



Fluorometric determination of isocitrate dehydrogenase (EC 1.1.1.42; 1; NADP⁺ dependent) in ruminant milk

T. Larsen^{a,*}, R. Rupp^b, N.C. Friggens^c, J.A.A. Pires^d

^aDept. of Animal Science, Aarhus University, 8830 Tjele, Denmark

^bGenPhySE, Université de Toulouse, INRAE, ENVT, Castanet-Tolosan, France

^cUniversité Paris-Saclay, INRAE, AgroParisTech, Paris, France

^dINRAE, Université Clermont Auvergne, Saint-Genès-Champanelle, France



ARTICLE INFO

Article history:

Received 15 November 2021

Revised 17 June 2022

Accepted 20 June 2022

Available online 20 July 2022

Keywords:

Goat

Isocitrate

Isocitrate dehydrogenase-1

Lipogenesis

Milk enzyme

ABSTRACT

The enzyme isocitrate dehydrogenase (EC 1.1.1.42; 1; NADP⁺ dependent) located in the mammary cell cytosol mediates the synthesis of the majority of reducing equivalents for the energetically demanding milk fat and cholesterol synthesis in mammary cell cytosol. The present article presents a novel fluorometric method for quantification of the activity of this enzyme (**IDH**) in ruminant milk without pretreatment of the sample. Further, 493 goat milk samples – harvested before, during and after a nutritional restriction – were analysed for IDH activity i) with addition of extra substrate (isocitrate), and ii) with the intrinsic isocitrate solely. The IDH activity ranged from 0.22 to 15.4 units [nano moles product/(ml * min)] (un-supplemented) and from 0.22 to 45.6 units (isocitrate supplemented). The IDH activity increased considerably in milk during the nutritional restriction period concomitant with the increase in the metabolite isocitrate concentration and somatic cell count and returned to the initial level shortly after restriction period. The present 'high through-put' analytical method may be beneficial in future studies to phenotype modifications in mammary energy metabolism and milk fat synthesis, for which IDH activity may be a biomarker.

© 2022 The Author(s). Published by Elsevier B.V. on behalf of The Animal Consortium. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Implications

The present paper describes a new fluorometric method for determination of the activity of the enzyme isocitrate dehydrogenase, NADP⁺ dependent, in milk. This enzyme activity is responsible for the majority of the energy production necessary for the synthesis of fat and cholesterol in the mammary cells and thereby in the milk. The analytical method works without pretreatment of the sample and may contribute to a further insight in the energy metabolism and lipogenesis of the mammary cells.

Introduction

In ruminants, about one half of the milk fatty acids (molar percent) are derived from *de novo* synthesis by mammary epithelial cells (Bauman and Griinari, 2003). Short-chain fatty acids (4–8 carbons) and medium-chain fatty acids (10–14 carbons) arise almost exclusively from *de novo* synthesis. Long-chain fatty acids (>16 carbons) are derived from the uptake of circulating (blood) lipids and

free fatty acids. This indicates that less than half of the carbon in milk fat is incorporated by *de novo* synthesis. Lipogenesis, however, is an energy-demanding series of reductive steps dependent upon reducing equivalents in the form of NADPH, namely approx. two moles per 2C-incorporation in fatty acids. The NADPH production in ruminant mammary epithelial cells is believed to be derived from i) oxidative decarboxylation of glucose 6-phosphate, and the principal part, ii) cytosolic oxidative decarboxylation of isocitrate (Bauman et al, 1970; Gumaa et al. 1973; Chaityabutr et al, 1980). The latter process, (ii) oxidative decarboxylation of isocitrate, is mediated by two isoforms of the enzyme isocitrate dehydrogenase (**IDH**, EC 1.1.1.42; 1 and 2). Isocitrate and subsequent degradative steps are part of “The citric acid cycle” (Krebs' cycle). However, the cycle running inside the mitochondria is mainly producing NADH (reducing equivalents) mediated by the enzyme isocitrate dehydrogenase (NAD⁺ dependent; EC 1.1.1.41; isoform IDH 3). Citric acid and isocitrate are however able to cross the mitochondrial wall, the conversion of citric acid to isocitrate, and further oxidation to 2-oxoglutarate may consequently take place in the cytosol. The responsible and abundant enzyme IDH in the cytosol is NADP⁺ dependent (EC 1.1.1.42; 1) and ensures the concomitant reduction of NADP⁺ to NADPH. The cytosol is furthermore

* Corresponding author.

E-mail address: Torben.Larsen@anis.au.dk (T. Larsen).

where lipogenesis takes place and reducing equivalents are needed (Bauman et al, 1970). The oxidised metabolites from citrate may be translocated back into the mitochondria where they are recycled to regenerate citrate, the whole process known as the “isocitrate dehydrogenase cycle” – a parallel to the citric acid cycle and occurring partly outside the mitochondria. Similarly, cholesterol, a highly reduced sterol compound, also synthesised in the cytosol of mammary cells, and found in milk fat globule membranes, appears to be dependent upon NADPH production (Moore and Christie, 1979). The activity of this cytosolic isoform of IDH increases dramatically immediately after the onset of lactation (Waldschmidt and Rilling, 1973; Farrell et al 1987), when studying mammary cells. Only few studies have focused on NADP⁺ dependent IDH activity in secreted milk (Grigor and Hartmann, 1985; sow and rat).

The present study describes a fluorometric method for the quantification of IDH activity in whole milk without pretreatment of the sample. Raw milk contains varying levels of isocitrate, the substrate for IDH (Larsen, 2014). The enzyme activity was investigated based on this intrinsic isocitrate content (substrate) and with addition of extra substrate. Further, the milk IDH activity was compared to the intrinsic isocitrate content and the general energy status of the animals. It is our hope that the description of mechanisms in lactation physiology may benefit from the present, practicable, analytical procedure. Accordingly, the IDH enzyme activity might turn out to be valuable as an indicator of physiological status during lactation.

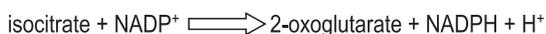
Material and methods

Analytical procedure for isocitrate dehydrogenase (IDH) in milk; EC 1.1.1.42: 1 (NADP⁺ dependent) by fluorometry.

This procedure is based on the fact that the intrinsic IDH activity in milk will oxidise isocitrate to 2-oxoglutarate while reducing NADP⁺ to NADPH₂. The reduced cofactor further reduces the (pre) fluorophore, resazurin to the fluorophore resorufin, which is detected by fluorometry.

Reaction 1:

IDH



Reaction 1 is mediated by the intrinsic isocitrate dehydrogenase (IDH) enzyme in milk.

Reaction 2:

diaphorase



Reaction 2 is mediated by added diaphorase enzyme.

Basic reagents

Tris-buffer, 100 mM, pH 7.2. Manganous sulphate, 20 mM in water. β-NADP⁺, (Roche 10 128 058 001, MW 787.4). DL-isocitrate (Acros 205010010, MW 258.07), 12.5 mM in water considering D-isocitrate.

Reagen 1 basis

Immediately before use. 18 ml Tris-buffer + 2.0 ml Mn-solution + 10 mg β-NADP⁺ [Tris = 90 mM; Mn⁺⁺ = 2.0 mM; β-NADP⁺ = 635 μM].

Reagent 1 basis is split into two equal portions, a and b (each of 10 ml). To **1a** is added 2.0 ml isocitrate-solution. To **1b** is added 2.0 ml water. [Tris = 75 mM; Mn⁺⁺ = 1.67 mM; β-NADP⁺ = 529 μM; D-isocitrate = 2.08 mM].

Reagent 2

A total of 480 units diaphorase enzyme (Toyobo DAD-301) and 8 mg resazurin (Sigma R-2127; MW 251.2) are solubilised in 40 ml water. 2.0 ml 1% Triton X-100 and six drops of Tween 80 are added [diaphorase 11.2 U/ml; resazurin 0.76 mM; Triton 0.048 %; Tween 80 0.43%].

Procedure

Raw milk was analysed “as is”. No efforts were made in order to liberate the sample from e.g. fat or cell fragments.

A black 96-well plate is used.

I) 50 μL milk is pipetted in the wells; II) 80 μL R 1a or R 1b is added; III) The plate is left for 5 min at room temperature; IV) 80 μL R 2 is added (*t* = 0 min).

The plate is placed in the fluorometer and the reaction is measured as fluorescence development (excitation 544 nm; emission 590 nm light; FluoSTAR, Galaxy Fluorometer) and compared to a standard curve. Plates were measured in intervals of 10 min during 15–135 min unless otherwise stated.

Final concentration in the incubation medium (210 μL): sample fraction 0.238; isocitrate (supplied) 792 μM; Tris-buffer 28 mM; Mn⁺⁺ 635 μM; β-NADP⁺ 202 μM; diaphorase 4.2 U/ml; resazurin 290 μM; Triton X-100: 0.018%; Tween 80: 0.16%.

One half of the plate (column 1–6) was supplied with reagent 1a (isocitrate supplementation), and the other half (column 7–12) with reagent 1b (without isocitrate supplementation, i.e. intrinsic milk isocitrate only). Standards (S₀–S₇) were placed (50 μl) on each half of the plate. Fluorometric readings were ‘calibrated’ on the highest standard (S₇) in the initial phase of incubation, and the same calibration was kept throughout the reading periods (5–135 min). Emissions of light were read at every time point (5–135 min) for both samples, and standards and calculations of enzyme activity were based on the related readings (see later).

Preparation of standards

A quantum of 3.5% fat milk was heated to 75 °C for 10 min. Twenty ml of this heat-treated milk was supplied with 16.7 mg NADPH (MW 833.4) = S₈. [NADPH = 1.0 mM]. NADPH concentrations 0; 0.05; 0.10; 0.15; 0.20; 0.25; 0.30; and 0.50 mM (S₀–S₇) were prepared from this stock by diluting with heat-treated milk.

NADPH added to the heat-treated milk with reagents (the standards) will reduce the resazurin in reagent 2, developing the fluorophore resorufin in equimolar concentrations. Fluorescence units corresponding to standard S₇ minus S₀ consequently represent the effect of 0.50 mM NADPH in 50 μl sample. In absolute terms, 0.5 mM NADPH in 50 μl sample corresponds to 0.5 μmoles/ml sample. Each reading of sample (*x*, emission of light) is compared to the interrelated reading of the standards (S₇–S₀), i.e. *x* / (S₇ – S₀); (S₇ – S₀) represents 0.5 μmoles of product per ml sample, i.e. each reading of samples during the time course is *x* / (S₇ – S₀) * 0.5 μmoles (product per ml sample). Accordingly, the enzyme activity is expressed via the slope determination (point calculations vs time of incubation), i.e. nano moles/(ml * min) (defined as units).

Enzyme activities were based on readings from 15 to 135 min’s incubation (straight-line development of product), and samples not supplied with substrate (intrinsic only) were based on 5–65 min incubation readings (linear development of product while remaining sufficient substrate). Samples showing activity beyond

the standard curve were diluted in heat-treated milk (identical sample) and re-analysed.

Validation of the assay

Relationship between enzyme concentration and activity in the sample

Eight low-medium activity samples were each divided into two portions. One portion was heat-inactivated (75 °C, 10 min), the other not. Subsamples were then created, containing 10, 20, 40, 60, and 100% un-treated milk. This approach secures the same level of substrate in subsamples. These five times eight samples were then analysed for IDH activity during 15–135 min's incubation, both supplemented with extrinsic substrate (792 µM) and also without extra supplementation of substrate (i.e. 8 samples * 5 dilutions * 2 levels of substrate). Cofactor level was constant (NADP⁺, 202 µM).

Isocitrate dehydrogenase activity response to substrate and cofactor concentrations

A series of experiments were conducted to demonstrate the influence of cofactor concentration (NADP⁺, intrinsic and added) and substrate concentration (isocitrate, intrinsic and added), taking into consideration the incubation time (reaction period). Raw goat milk from different animals were used in all test procedures, i.e. samples with varying content of intrinsic isocitrate (substrate) and varying intrinsic IDH activity.

1. Eight milk samples of low-medium enzyme activity were analysed for activity with 202 µM NADP⁺ in the incubation medium, but with varying levels of extrinsic substrate, namely, 99, 132, 198, 397, or 794 µM, i.e. a 1 by 5 factorial design.
2. 32 low-medium activity samples (mean intrinsic isocitrate 141 µM ± 36, SD) were tested in a two-by-two arrangement. All samples were tested with addition of 100 µM NADP⁺ (in the reaction medium) or without addition of NADP⁺, and with addition of 792 µM isocitrate or without.
3. High activity samples were tested in a similar design (as B2) (mean intrinsic isocitrate 273 µM ± 42, SD).
4. Sixteen milk samples of medium IDH activity were tested in a 2 by 5 factorial design. Samples were tested in ten combinations of cofactor and substrate (conc. in the reaction medium), i.e. with addition of 101 or 202 µM NADP⁺ and 99, 132, 198, 397, or 794 µM extrinsic substrate (isocitrate) (2 * 5 balanced arrangement on two plates). The intrinsic content of isocitrate ranged between 76 and 206 µM, mean of 139. In some instances, reaction velocity declined markedly due to a shortage in reaction ingredients, therefore, the speed of the reaction (slope, product/(min * ml)) was determined from 5 to 55 min after addition of reagent 2 (all samples) in order to determine reaction velocity. For all series with different NADP⁺- and isocitrate combinations, IDH activity was calculated based on its own standard curve.

Intra- and inter-assay precision

Forty milk samples of medium IDH activity were tested over 3 days. Each day 40 samples were tested in four combinations of cofactor and substrate (conc. in the reaction medium), i.e. with addition of 202 or 283 µM NADP⁺ and/or with addition of 792 or 1012 µM isocitrate (2 * 2 balanced arrangement on 2 plates). Each combination was calculated from its own standard curve. All together 40 samples in four combinations were repeated on 3 days, i.e. 480 wells with samples and 2 * 2 * 3 * 8 standards (S₀–S₇), i.e. 96 wells with standards.

Animal trial

The design of the animal trial followed precisely the protocol described in detail in Friggens et al., (2016) with the difference that 50 lactating goats were used. Briefly, early-lactation goats had ad libitum access to feed and water throughout the experiment, which lasted 17 days. The experiment consisted of a 7-day prechallenge period, followed by a 2-day challenge period, and a 5-day postchallenge period, with respectively three, two, and five milk samples taken. During the pre and postchallenge periods, the goats received a ration based on lucerne hay offered in collective troughs, complemented with concentrates that were dispensed in the milking parlour. During the challenge, the goats received chopped straw only. Proportional milk samples were taken/collected at each ordinary milking. Somatic Cell Counts (SCCs) were performed by Fossomatic (Foss, Hillerød, DK). Samples used in laboratory analyses were kept cool and frozen within 30 min from harvest.

In total, 493 morning milk samples were analysed for IDH activity, with substrate addition (792 micro moles per litre of isocitrate in incubation medium) and with intrinsic substrate only. Milk samples were further analysed for the metabolites isocitrate, triacylglycerides (TAGs), and cholesterol by enzymatic-fluorometric methods (Larsen, 2014; Larsen et al. 2011; Larsen 2012). Samples were thawed (40 °C, 10 min) from minus 18 °C and pipetted directly on the black 96-well plates without further manipulation.

Statistical analyses and calculations

Statistical analyses were performed using SAS (version 9.3; SAS Institute Inc.). Balanced data were analysed by the ANOVA procedure where class variables were cofactor (NADP⁺) and substrate (isocitrate). Covariance (considering basic, intrinsic isocitrate) was analysed by Proc. GLM and comparisons between two treatments of specific samples by Student t-tests (two-sided, paired). All simple correlations between data sets (e.g. fluorometric readings and incubation time) were analysed according to Pearson (Excel, Microsoft).

Results

Relationship between enzyme concentration and activity in the sample

Fig. 1 summarises the results of this sub-project. The enzyme activity increases markedly from 10 to 100% intact milk, both for substrate supplemented samples and for samples not supplemented with extrinsic substrate. Supplemented samples increased from 0.26 to 1.92 ($P < 0.001$), whereas un-supplemented samples increased from 0.23 to 0.37 nano moles/(min * ml) ($P < 0.001$).

Isocitrate dehydrogenase activity response to substrate and linearity in time

1) Fig. 2 shows average reaction velocity as a function of substrate concentration for eight samples. Velocity increases significantly from the low substrate concentration (99 µM) to the next level (132 µM). 198 µM substrate further increases velocity, but even higher levels of substrate do not increase enzyme activity. At these higher levels of substrate, the intrinsic enzyme seems to be saturated with substrate, the substrate is no longer limiting for the reaction velocity.

2) Low activity samples in a 2 by 2 design. Fig. 3a shows the average of all 32 samples in the four combinations. All four combinations of NADP⁺ and isocitrate revealed a linear production of fluorescence and thereby NADPH. Correlations between product and time revealed coefficients > 0.98. i.e. neither NADP⁺ nor isocitrate

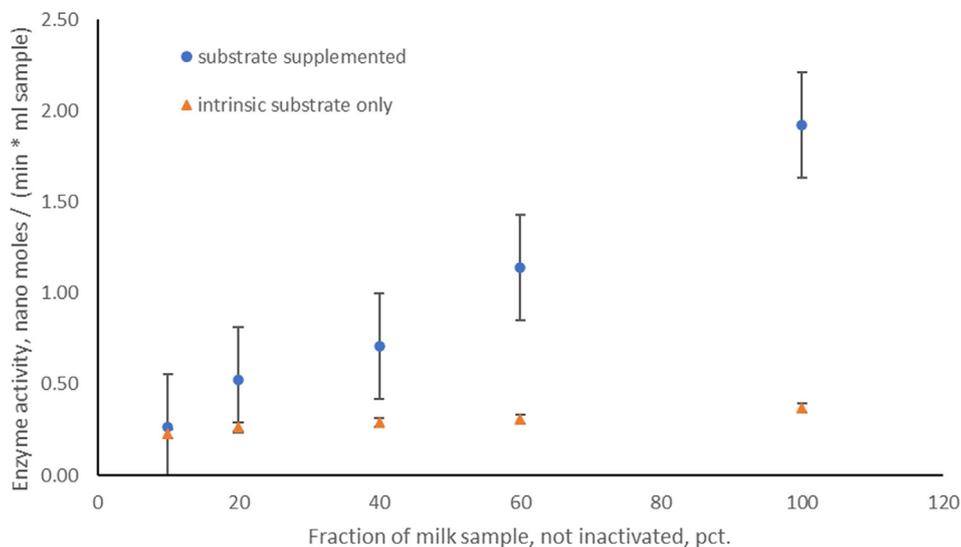


Fig. 1. Eight low-medium enzyme activity goat milk samples were diluted with a subsample of same identity that was heat-treated (75 °C, 10 min), resulting in reduced activity compared to the original sample, i.e. 100–10 pct. of initial activity. The samples (8 * 5 dilutions) were analysed with substrate addition (extrinsic, 792 µM) and without (intrinsic isocitrate only). Cofactor level (NADP⁺) was equal (202 µM). Average isocitrate dehydrogenase (IDH) activity of eight samples is shown with SE of the statistical analysis.

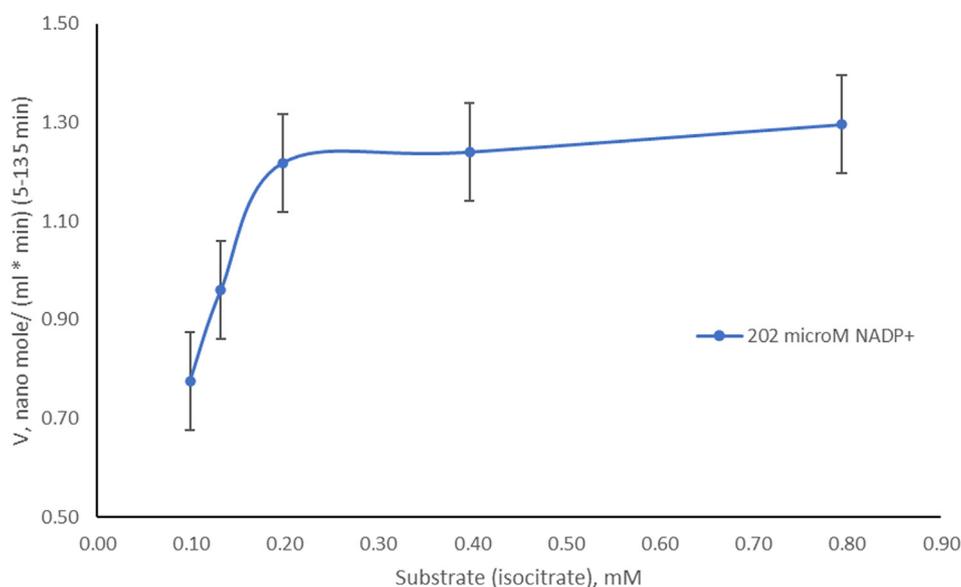


Fig. 2. Eight low-medium enzyme activity goat milk samples were analysed in a 1 by 5 factorial design; i.e. 202 µM NADP⁺ (cofactor) level * extrinsic substrate levels of 99, 132, 198, 397, or 794 µM isocitrate. Average isocitrate dehydrogenase (IDH) activity (V = enzyme velocity) of the eight determinations is shown with SE of the statistical analysis.

were exhausted from the incubation medium during 15–135 min's incubation. Mean of all 32 measurements showed that the addition of NADP⁺ and isocitrate (plus/plus) exhibited an average activity of 0.66 ± 0.32 (SD) nano mole product per min and ml. Addition of NADP⁺ without addition of isocitrate (plus/minus) exhibited 0.40 ± 0.095 SD; no NADP⁺ addition with isocitrate addition (minus/plus) exhibited 0.11 ± 0.025 SD; and no addition of NADP⁺ or isocitrate (minus/minus) showed 0.13 ± 0.020 SD nano mole product per min and ml. All combinations of cofactor and substrate were significantly different (*P* < 0.001).

3) Fig. 3b shows the average of all 24 high activity samples in the four combinations. These samples with addition of NADP⁺ exhibited an almost linear production of fluorescence, both regressions showed *r* > 0.98, whereas incubations without NADP⁺ revealed slightly curvilinear courses, *r* < 0.98, indicating exhausted

conditions by time. The IDH activity in these 2 × 24 samples was consequently calculated for 5–65 min's incubation, before exhaustion of NADP⁺ occurred. The average activity in samples supplemented with NADP⁺ and isocitrate (plus/plus) addition was 12.2 ± 1.30 nano mole product per minute and ml, and the activity of the plus/minus condition was 6.5 ± 0.10. Samples not supplemented with NADP⁺ revealed a remarkably lower activity, i.e. 1.5 and 1.5 units for plus isocitrate and minus isocitrate, respectively. All combinations of cofactor and substrate were significantly different (*P* < 0.001); the difference between the combinations minus NADP⁺ addition (i.e. minus/minus vs minus/plus) was however less pronounced (*P* = 0.027).

4) The 2 (NADP⁺ levels) by 5 (extrinsic isocitrate) experimental arrangement. Fig. 4 a and b are example of one representative milk sample only. The figures show the incubation courses including

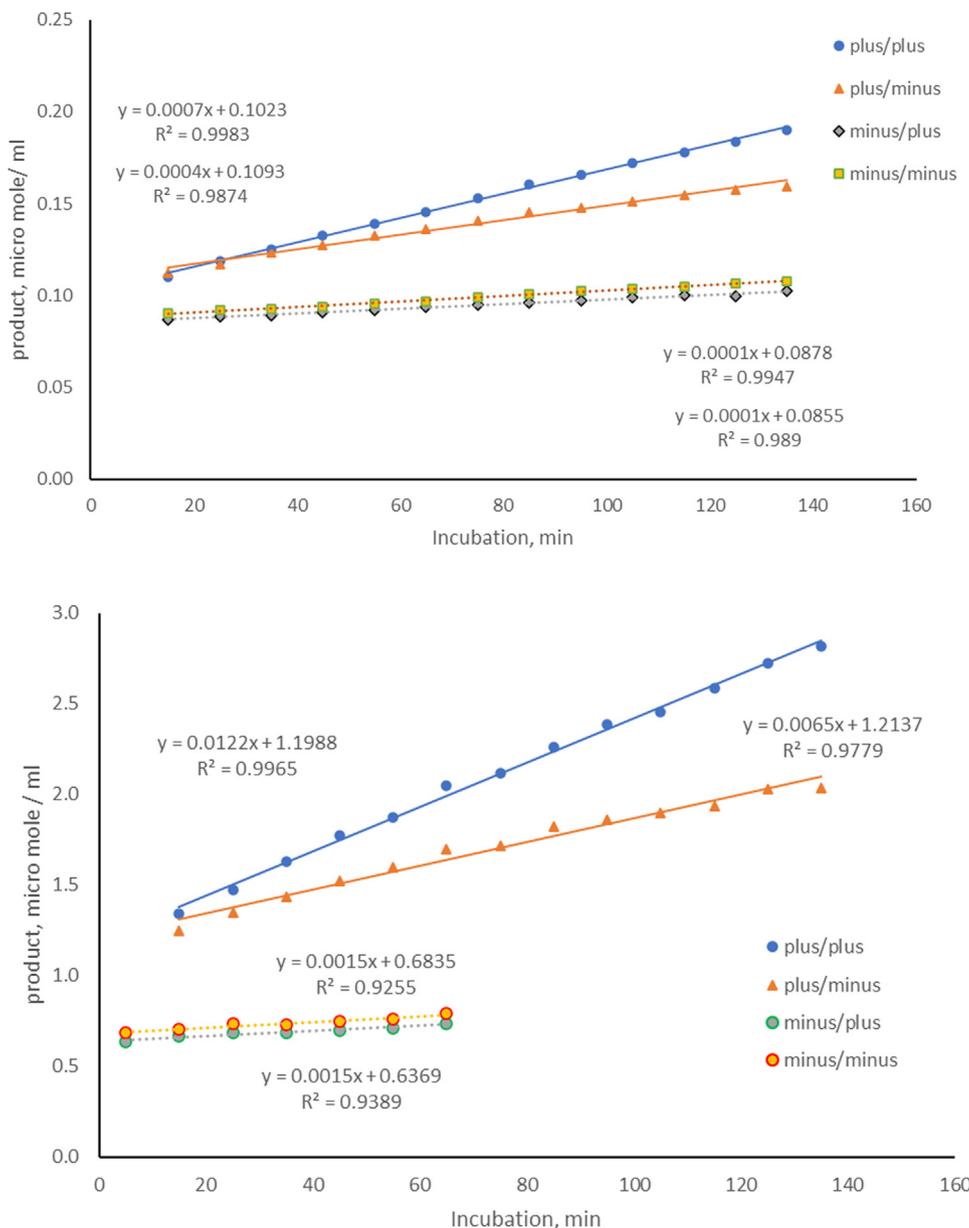


Fig. 3. Analyses of goat milk samples for isocitrate dehydrogenase activity. Samples were tested with addition of 100 μmol per litre NADP^+ (cofactor) in the reaction medium or without (plus or minus) and with the addition of 792 μmol per litre isocitrate (substrate) in the reaction medium or without (plus or minus), i.e. plus/plus designates plus NADP^+ and isocitrate, plus/minus designates plus NADP^+ , minus isocitrate, etc. a) upper diagram: Mean of 32 low to medium activity milk samples, b) lower diagram: Mean of 24 high activity milk samples. Samples without cofactor addition were estimated during 5–65 min’s incubation (only) due to expected deficiency in long-term incubations. Note the difference in ordinate values.

135 min, whereas the further calculations for enzyme activity were made for all samples during the period of 5–55 min only, in order to avoid substrate depletion among high activity samples.

Calculations of enzyme activity to time revealed correlation coefficients for all determinations ranging between 0.974 and 1.000 (r ; inter percentile_{0.02–0.98}; mean 0.995, $n = 160$), i.e. steady enzyme activity in this period. There was no effect on substrate concentration neither when the extrinsic isocitrate was considered alone (0.099–0.794 μM isocitrate), i.e. an IDH activity of 0.64–0.70 and 0.89–1.0 nano molar product/min and ml for the low and high NADP^+ levels, respectively; nor when the intrinsic sample isocitrate was included as a covariate. NADP^+ concentration, however, significantly increased turnover rate, i.e. 0.68 vs 0.94 nano mole/min and ml for 101 and 202 μM NADP^+ , respectively, $P < 0.001$. No interaction between substrate and cofactor was observed.

Precision

Isocitrate dehydrogenase activity in all samples ranged from 0.43 to 6.02 nano moles per min and ml (median 1.61) There was no overall effect of cofactor level or substrate level ($P = 0.93$); and no effect of the individual class variables (NADP^+ and isocitrate; $P = 0.90$ and $P = 0.53$, respectively). Consequently, the four combinations were considered to be the same treatment. Inter-day precision was consequently calculated from 40 samples * 2 * 2, i.e. 160 samples (N) repeated 3 days (n). Inter-day precision was on average 6.1 CV% (inter percentile_{0.05–0.95} = 1.5–10.6 CV%). Intra-day precision was consequently based on 40 samples (N) repeated four times per day (n). Intra-day precision on day 1 was 2.6 CV%, day 2: 5.7 CV%, and day 3: 3.9 CV%, on average 4.1 CV% (mean activity 1.90 units).

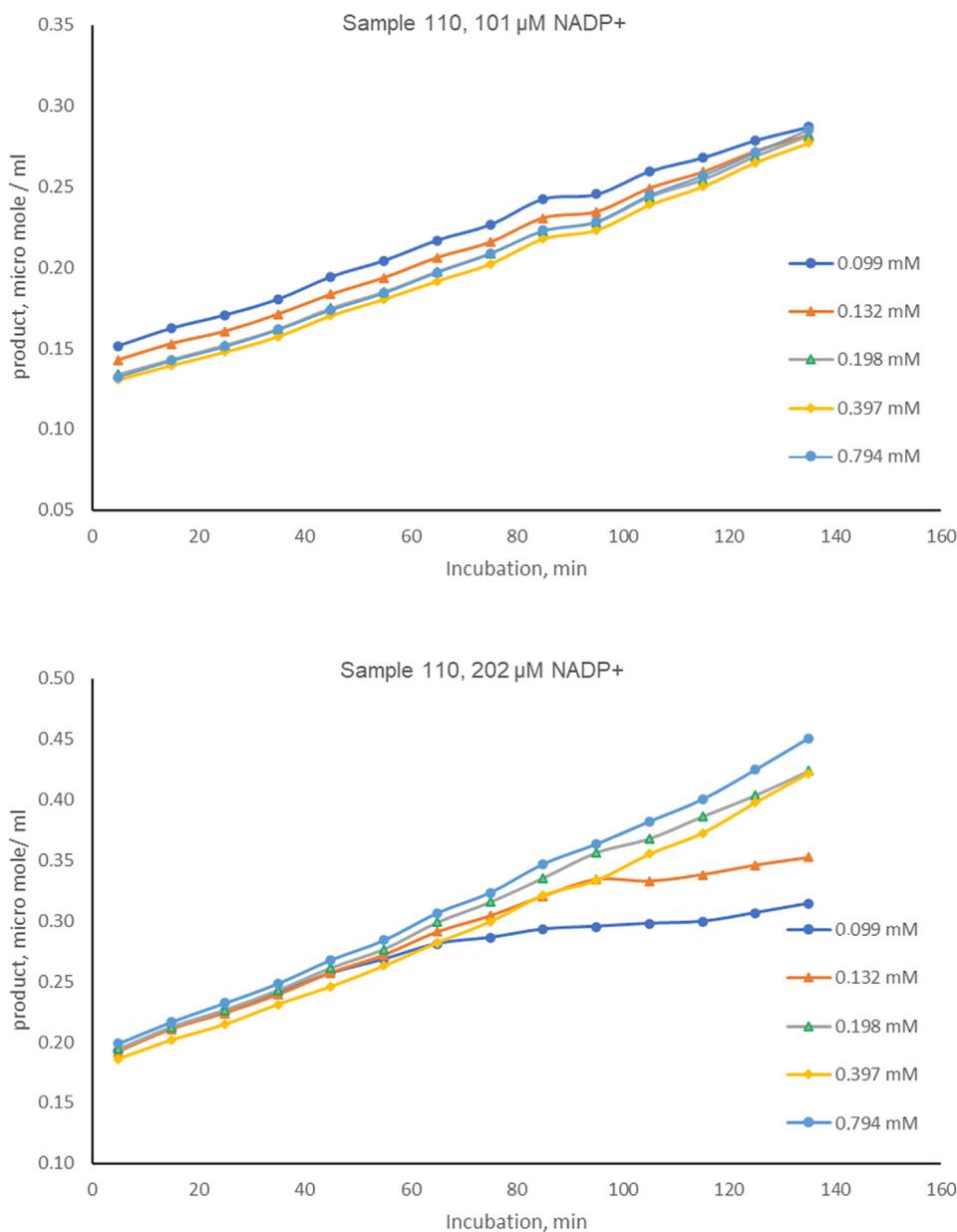


Fig. 4. Example of enzyme kinetics in a single goat milk sample incubated with five different extrinsic levels of substrate (isocitrate, 99–794 µM) and two levels of cofactor (NADP⁺), i.e. a) 101 µmol per litre and b) 202 µmol per litre in the reaction medium.

Animal trial

On all milk samples, correlations (Pearson) of the consecutive readings (15–135 min; micro moles per ml sample) with time (min) revealed a mean coefficient (*r*) of 0.996 (*n* = 493; inter percentile_{0.02–0.98} = 0.992 – 1.000) for the substrate supplied samples and 0.994 (*n* = 493; inter percentile_{0.02–0.98} = 0.991 – 1.000) for the un-supplemented samples (*n* = 493; 5–65 min). High activity samples were diluted before analyses, see Materials and Methods. Standard curves (*n* = 25) correspondingly showed a mean correlation coefficient of 0.995 (inter percentile_{0.05–0.95} = 0.992 – 0.999). Table 1 shows the distribution of the intrinsic isocitrate in the samples and the distribution of the IDH activity for substrate (isocitrate) supplemented and un-supplemented samples (intrinsic isocitrate only) and the ratio between these measurements.

The isocitrate content in the samples varied by a factor of 9 (range 34–307 µM, median 143 µM). The IDH activity in un-supplemented samples varied by a factor of 70 (0.22–15.38 units),

among supplemented samples, the activity varied more than 200 fold (0.22–45.49 units).

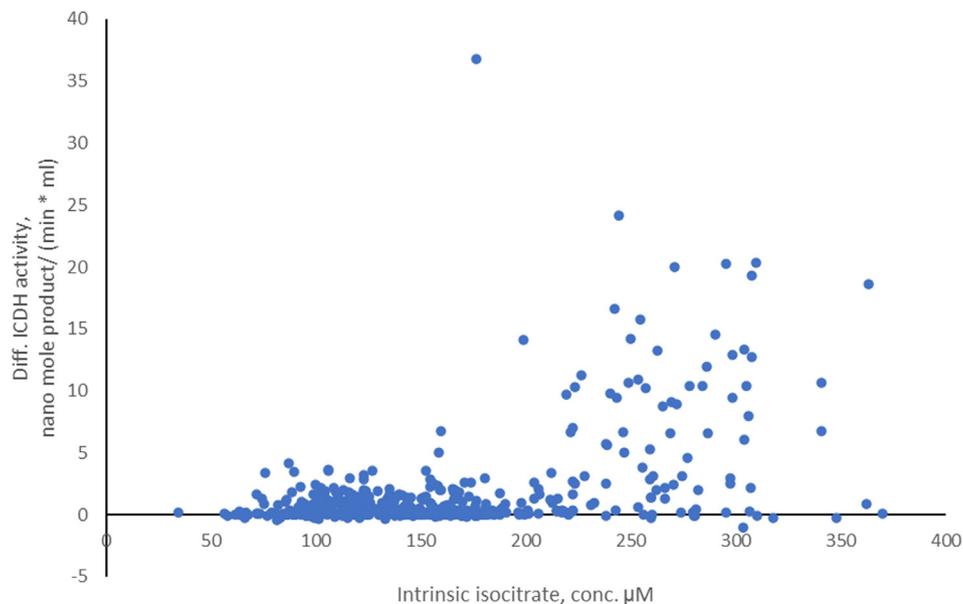
As seen from the ratios between enzyme activities (Table 1), not all samples demonstrate an increased enzyme activity when offered a higher isocitrate concentration (meaning that the substrate is not limiting under the present conditions). However, approx. 90% of the samples show higher activity when supplied with extrinsic substrate (seen by the 10-percentile). Fig. 5 shows the difference in IDH activity (plus-minus extrinsic substrate) plotted against the intrinsic isocitrate content in the same sample. Most samples with a low or moderate isocitrate content did not increase enzyme activity markedly in response to supplemental extrinsic isocitrate, whereas samples already high in intrinsic isocitrate predominantly increased IDH activity in response to supplemental extrinsic isocitrate. Fig. 6 presents intrinsic isocitrate content plotted with IDH activity throughout the experimental period and shows that the IDH activity in milk increases almost simultaneously with the increasing isocitrate concentration in

Table 1The distribution of SCC, intrinsic content of isocitrate in the milk samples (μM) and of IDH activity (units) in 493 goat milk samples.

Percentile	SCC Log_{10}	Isocitrate, μM (intrinsic)	IDH activity, supplemented samples, a	IDH activity, un-supplemented samples, b	Ratio, a/b
0	4.04	34	0.22	0.22	0.3
2	4.15	66	0.28	0.25	0.7
5	4.32	81	0.37	0.32	0.9
10	4.49	93	0.44	0.39	1.0
20	4.74	108	0.58	0.47	1.1
30	4.95	120	0.75	0.53	1.1
50	5.36	143	1.25	0.70	1.4
70	5.85	170	2.20	0.94	2.3
80	6.19	206	3.83	1.42	3.0
90	6.60	260	10.05	5.52	4.2
95	7.00	292	15.95	7.72	5.2
98	7.19	308	22.51	10.15	7.1
100	7.63	307	45.59	15.38	15.7

SCCs = Somatic Cell Counts.

IDH = isocitrate dehydrogenase (EC 1.1.1.42).

For enzyme assays: All samples were supplemented with substrate (isocitrate, 792 μM in incubation medium) or analysed with the intrinsic isocitrate only (un-supplemented). a/b is the ratio between IDH activities.**Fig. 5.** A total of 493 goat milk samples were analysed for isocitrate dehydrogenase activity a) with no addition of substrate (intrinsic isocitrate only) b) with addition of 792 μmol per litre isocitrate during incubation. Both assays were supplied with 202 μmol NADP^+ per litre (cofactor). The difference in enzyme activity (activity in samples supplemented with extra substrate minus activity in samples with intrinsic isocitrate only, b-a) is plotted against the samples' intrinsic content of isocitrate.

the milk. The concentrations of TAG and cholesterol in the milk samples are plotted in Fig. 7. TAG and cholesterol concentrations increased markedly during the nutritional challenge and returned to the initial level shortly after. The intrinsic isocitrate concentration in the milk was positively correlated with TAG (inter percentile_{0.02–0.98} = 11–78 mM; $r = 0.639$; $n = 493$) and cholesterol (inter percentile_{0.02–0.98} = 38 – 499 μM ; $r = 0.569$; $n = 493$). The ratio between cholesterol and TAG concentrations ($\mu\text{M}/\text{mM}$) increased almost by a factor of 2 during the nutritional challenge, details not shown.

SCC ranged from 4.15 to 7.20 (log_{10} ; inter percentile_{0.02–0.98}; median 5.36), Table 1. Days 1, 2 and 3 in the experiment (restriction period and 1 day after) showed significantly higher levels than the remaining days.

Discussion

Basically, all milk samples will have different 'natural', intrinsic content of IDH activity, isocitrate, NADP^+ and other potentially

influential constituents. The measured enzyme activity will be influenced by these constituents irrespective of whether they are supplied to the reaction medium or they are intrinsic in the milk sample. In simple situations, the enzyme concentration itself may be limiting (Figs. 1 and 2), i.e. saturation of the enzyme-substrate complex, in other cases, there may be an interaction between more constituents. The content of intrinsic constituents is variable (and unknown from the start) and together with the reagents added in the laboratory (including pH) will determine the reaction conditions including reaction speed (velocity). The experimental conditions chosen in the present study (concentration of substrate, cofactor, sample, pH and incubation time) revealed a wide range of enzyme activity in the chosen milk material and thereby in substrate turnover. Samples taken early in experiment and samples taken during feed challenge showed IDH activity, other dehydrogenase activity, or generally, other reducing activity – even without addition of isocitrate and cofactor (NADP^+). Interestingly, Fang et al. 1997 demonstrated that stimulation of neutrophils in blood and milk induced resazurin reduction. The intrinsic isocitrate con-

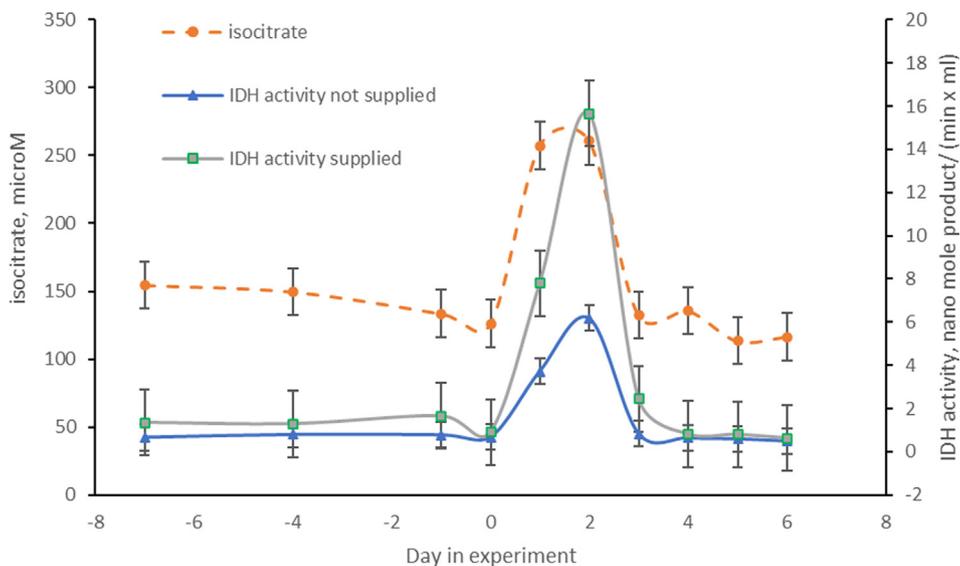


Fig. 6. The concentration of intrinsic milk isocitrate was measured in all goat milk samples throughout the entire experiment. The isocitrate dehydrogenase (IDH) activity was measured based on the intrinsic isocitrate only (substrate not supplied) and when incubations were supplemented with extra isocitrate (substrate, 792 $\mu\text{mol/l}$). SE of the statistical analysis are indicated.

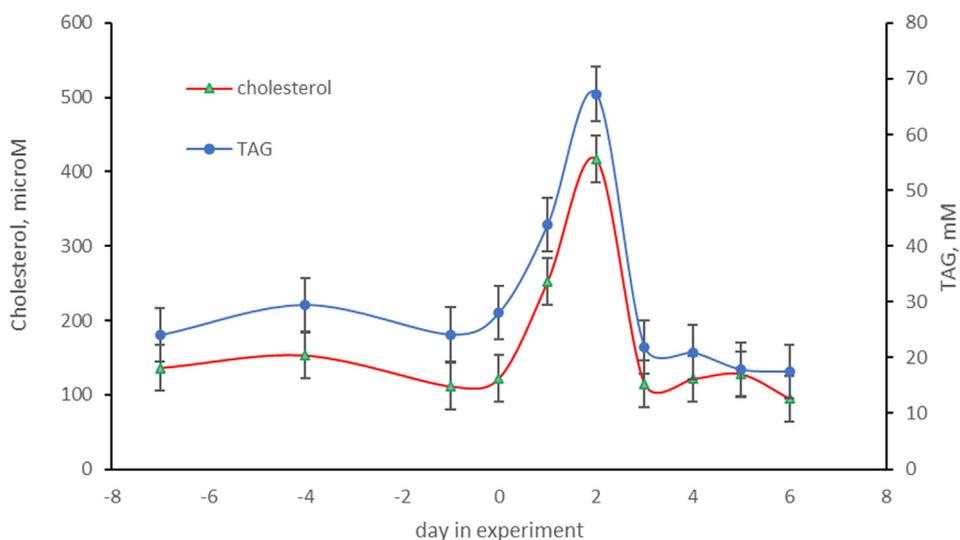


Fig. 7. Triacylglycerol (TAG) and cholesterol were determined in all goat milk samples during the experimental course (day -7 to day $+6$; $n = 493$). SE of the statistical analysis are indicated.

tent is documented whereas the intrinsic NADP^+ content is only deduced. We cannot be sure that the activity obtained in these samples does not originate from other processes producing NADH_2 or NADPH_2 that potentially will reduce the prefluophore resazurin, to resorufin (fluorescence). However, ‘high activity’ samples, not supplemented with NADP^+ (and isocitrate), markedly decrease in resazurin reduction (i.e. product, y-axis, Fig. 3b, incubation time > 65 min, not shown and used in calculations due to curvilinear course). Yet, it is interesting that all combinations of NADP^+ and isocitrate revealed an activity approximately 10 times higher in samples taken during the nutritional challenge compared to samples harvested prechallenge (Fig. 3a vs 3b). In these cases, it is obvious that the limiting intermediates are not NADP^+ or isocitrate but rather enzyme or other reducing substances. Generally, for the majority of samples, the individual samples demonstrated minor or major increased activity in response to an increase in extrinsic substrate (to a certain level under the chosen conditions), Table 1

and Figs. 1, 3a, 3b, 5. In the same manner, NADP^+ addition markedly increased IDH activity (e.g. 3a vs 3b), indicating that the cofactor in most instances was the limiting factor for the reaction of the individual samples under the present circumstances. Fig. 4a and b nicely demonstrates these situations; 4a vs 4b: at higher NADP^+ levels, substrate may become the limiting factor for enzyme activity. Fig. 4b (left part, $t = 5-65$ min): reaction velocity is equal between samples supplied with substrate, i.e. substrate is not limiting for velocity but intrinsic enzyme or cofactor may be. Fig. 4b (right part, $t = 65-135$ min): the reaction velocity is inhibited by substrate deficiency for some of the samples. NADP^+ is re-generated in the present process after the (chemically) reduced form NADPH has reduced resazurin (the prefluophore), but obviously, a certain amount of NADPH is delayed in this transfer; perhaps indicating that the second step in the reaction is slower than the first.

IDH activity plays a crucial role in ruminant anabolism. The regulation of the IDH isoforms has attracted some attention. The IDH

(NAD⁺ dependent form) of the enzyme, which is part of the citric acid cycle, is known to be allosterically regulated by ADP and ATP (Chen and Plaut, 1963), and product inhibited by NADH and 2-oxoglutarate, indicating that the enzyme plays an important regulatory role in Krebs cycle metabolism. Farrell et al. (1990) demonstrated a certain activation and inhibition of IDH (EC 1.1.1.42 NADP⁺ 1) by metal ions (Mn²⁺ and Mg²⁺), citrate and isocitrate complexes and later tested the inhibitory effect of several acyl-coenzyme (CoA) intermediates in the fatty acid synthesis (Farrell et al. 1995). The short-chain acyl CoA's which are typical intermediate *de novo* products did not have any apparent effect; however, palmitoyl-CoA (C16-CoA), one of the major end products of *de novo* mammary lipid synthesis, produced a modest effect. On the other hand, stearyl CoA (C18-CoA), which is primarily of dietary origin or from lipomobilisation for ruminants and not an end product of anabolism, completely inhibited IDH (NADP⁺ dependent); i.e. the NADP⁺ dependent form of IDH is "end product" inhibited by preformed fatty acids ready for incorporation in TAGs.

Isocitrate is a natural constituent of ruminant milk. Larsen (2014) found a mean concentration of 168 μM (inter percentile_{0.02-0.98} = 70 – 293 μM, *n* = 1760) in ordinary production animals (dairy cows) with no experimental bias. Further, milk from Danish Holstein cows contained significantly less isocitrate than milk from Danish Jersey cows (134 μM vs 211 μM, *n* = 984 vs 760).

The concentration of milk citrate increases in the perinatal period as copious milk secretion begins, passes through a maximum and then declines in the transition from colostrum to mature milk (Davies et al. 1983). Mammary epithelium is impermeable to citrate in both directions (Linzell et al. 1976); therefore, milk citrate concentration reflects mammary activity rather than general metabolism. Isocitrate is an isomer of citrate, and the two are maintained in equilibrium in the cell (Peaker and Faulkner, 1983). Chaiyabutr et al. (1981) report a positive correlation between the concentrations of citrate and isocitrate in goat milk (*r* = 0.98). Both citrate and isocitrate increased during starvation and decreased after refeeding; the ratio of isocitrate:citrate remained constant at approximately 0.022. High-fat diets can increase milk citrate concentrations (Ormrod et al. 1980; Banks et al. 1984) and isocitrate concentrations (Faulkner and Clapperton, 1981); accordingly, diet-induced milk fat depression in Holstein cows was associated with increased milk isocitrate concentrations (Bernard et al. 2020). High-fat diets may depress lipogenesis in the mammary gland and both citrate and isocitrate levels in the milk are raised whereas the concentration of 2-oxoglutarate falls. The ratio of isocitrate to 2-oxoglutarate has been suggested to reflect the cytosolic ratio of NADPH to NADP⁺ which may raise as a result of a lower requirement for reducing equivalents during the inhibition of fatty acid synthesis (Faulkner, 1980; Faulkner and Clapperton, 1981; Faulkner and Peaker, 1982). Consequently, the isocitrate to 2-oxoglutarate ratio correlated positively with the proportion of long-chain fatty acids and negatively with the proportion of medium-chain fatty acids in bulk milk throughout 1 year (Faulkner et al. 1986). Concurrent with this observation, Garnsworthy et al. (2006) found a negative association between milk citrate and the ratio between *de novo* synthesised fatty acids and preformed fatty acids. More recently, Billa et al. (2020) and Pires et al. (2021) found significant negative correlations between isocitrate and short- and medium-chain fatty acids (C10-C15 and C6-C15, respectively) and positive correlations between isocitrate and long-chain fatty acids (>C16).

A number of studies have dealt with reduced feed/nutrient intake or forced starvation of animals in order to observe the effect of energy supply on milk metabolites. In the lactating goat, starvation decreases the rate of *de novo* fatty acid synthesis in the udder, resulting in the production of milk fat consisting of predominantly long-chain fatty acids (Annison et al., 1968). During starvation, the

citrate concentration increases in milk and then falls again on refeeding (Ormrod et al. 1982) while milk 2-oxoglutarate concentrations behave in an opposite manner (Chaiyabutr et al. 1981). Billa et al. (2020) and Pires et al. (2021) observed a marked increase in milk isocitrate (and a decrease in medium-chain fatty acids) when mid- and early-lactation cows were subjected to nutritional challenges by partial feed restriction or feeding a high-straw diet (respectively) for 6 and 3 days, respectively. In contrast to this, increased energy intake in the form of propylene glycol was shown to reduce the isocitrate concentration in milk (Bjerre-Harpoth et al. 2016).

These observations strongly support the important role of citrate-isocitrate-2-oxoglutarate in the milk energy turnover by linking mammary gland lipogenesis and milk isocitrate metabolism.

From the discussion of the preceding literature, we expected to find a reduced IDH activity and thus a decrease in NADPH production during the nutritional challenge as the one performed here. This would have explained the recorded increase in milk isocitrate concentrations. However, we observed a marked increase in IDH activity during the nutritional challenge and a subsequent fast return to the initial level after cessation of the challenge. The increase in IDH activity in the present study is coincident with the marked increase in SCC. If this was cow milk, these high counts could only be due to an immune response (leucocyte transfer to milk). However, goat SCC may be different from the situation in cows, in this case also seen by the sudden decrease after refeeding, not an indication of intramammary infection. Smistad et al. (2021) concluded that goat somatic cell count, compared with dairy cows, is higher and probably more affected by physiological factors such as parity, stage of lactation, and season. The present study strongly indicates that also a nutrient challenge may affect SCC severely. The presence of indigenous enzymes in milk originating from blood or epithelial cells are well described in healthy cows, as well as cows experiencing mastitis, which is often characterised by an increase of somatic cells in the milk (e.g. Kelly et al. 2006; Larsen et al. 2010). Even more moderate feed restrictions than the present also promote exfoliation of epithelial cells in milk, and mammary epithelium permeability increases during severe restriction (Stumpf et al., 2013; Herve et al., 2019). We have formerly observed significantly elevated activities of lactate dehydrogenase (LDH, EC 1.1.1.27; 6–10 fold) in goat milk during nutritional challenges (not published), and this seems to be the situation for other enzymes including IDH in the present study also.

A relative increase in milk fat concentration and a reduced milk production are well documented during reduced energy intake (Friggens et al., 2016). In the Friggens et al. (2016) study, where the nutritional challenge of goats was similar to the present (2 days of straw-only feeding), milk production decreased from (approx.) 4000 g/d (early lactation) to 1500 g/d, and from 1800 g/d to 500 g/d (late lactation). Concurrently, milk fat concentration increased from (approx.) 38 to 89 g/kg and from 40 to 76 g/kg, i.e. a drop in fat production (weight) of 12 % in early lactation and 47 % in late lactation. In the present study, the milk TAG concentration increased by approximately 2.4 fold and the cholesterol concentration approximately 3.4 fold during the nutritional challenge, compared to the prechallenge situation (Fig. 6). The present TAG determination is based on the *molar* content. Assuming the milk during the nutritional challenge is fractionally higher in long-chain fatty acids (>C16), this means that more than 2.4 times C-atoms were incorporated in milk fat during the nutritional challenge (and more than 2.4 times by weight). Given that the total fat incorporation into milk in energy-restricted early-lactation goats is only slightly reduced, the increase in IDH activity is difficult to explain from the perspective of fatty acid synthesis. Further research in this area is needed.

Previous determinations of IDH activity in milk and tissue homogenates are based on time-consuming fractionation of the matrix (cutting with scissors, homogenisation, high-speed centrifugation, separation of infra-natants, filtration, etc.) (e.g. Bauman et al. 1970; Waldschmidt and Rilling, 1973; Grigor and Hartmann, 1985) because the used technique, spectrophotometry, requires a transparent sample. The present fluorometric determination of enzyme activity is not dependent upon transparency, but operates well in opaque matrices like crude homogenates and whole milk, not pretreated nor liberated from fat and protein. It is our hope that future research may benefit from this simple, precise and time-saving analytical procedure in order to elucidate further areas of the ruminant mammary fat synthesis and energy metabolism.

Ethics approval

Animals were cared for at the experimental INRAE research unit of Bourges (Unit approval C18-174-01) and handled in accordance with the French legislation on animal experimentation and European Convention for the Protection of Vertebrates Used for Experimental and Other Scientific Purposes (European Directive 86/609) under specific authorisation number APAFIS#8613-2017012012585646.

Data and model availability statement

None of the data were deposited in an official repository. The data/models that support the study findings are available from the authors upon request.

Author ORCIDs

R. Rupp: <https://orcid.org/0000-0003-3375-5816>.

J.A.A. Pires: <https://orcid.org/0000-0002-3773-9293>.

T. Larsen: <https://orcid.org/0000-0003-3058-5938>.

Author contributions

Torben Larsen: Conceptualization, Methodology, Formal analysis, Writing – original draft, Review and Editing, Validation

Rachel Rupp: Design of the animal experiment, Funding acquisition

Nicolas Friggens: Design of the animal experiment, Review and Editing, Funding acquisition

José Pires: Design of the animal experiment, Review and Editing, Funding acquisition

Declaration of interest

None.

Acknowledgements

We gratefully acknowledge the work of Thierry Fassier and the team at the INRAE UE0332 P3R experimental unit of Bourges (Osmoy, France) for the care of the animals and their work on the experimental protocol. Jens Clausen and Carsten Berthelsen, Aarhus University, DK, are gratefully acknowledged for the skilful laboratory analyses of milk samples.

Financial support statement

The animal trial and milk sampling were performed in the framework of the EU H2020 SMARTER project (grant n°772787), and the French APIS-GENE (Paris, France) funded ActiveGoat and Resilait projects (R.R. J.A.A.P., N.C.F.).

References

- Annisson, E.F., Linzell, J.L., West, C.E., 1968. Mammary and whole animal metabolism of glucose and fatty acids in fasting lactating goats. *Journal of Physiology* 197, 445–459. <https://doi.org/10.1113/jphysiol.1968.sp008569>.
- Banks, W., Clapperton, J.L., Girdler, A.K., Steele, A.K., 1984. Effect of inclusion of different forms of dietary fatty acid on the yield and composition of cow's milk. *Journal of Dairy Research* 51, 387–395. <https://doi.org/10.1017/S0022029900023682>.
- Bauman, D.E., Brown, R.E., Davis, C.L., 1970. Pathways of fatty acid synthesis and reducing equivalent generation in mammary gland of the rat, sow, and cow. *Archives of Biochemistry and Biophysics* 140, 237–244. [https://doi.org/10.1016/0003-9861\(70\)90028-7](https://doi.org/10.1016/0003-9861(70)90028-7).
- Bauman, D.E., Grünari, J.M., 2003. Nutritional regulation of milk fat synthesis. *Annual Review of Nutrition* 23, 203–227. <https://doi.org/10.1146/annurev.nutr.23.011702.073408>.
- Bernard, L., Fougère, H., Larsen, T., Pires, J., 2020. Short communication: Diets supplemented with starch and corn oil, marine algae, or hydrogenated palm oil differently affect selected metabolite concentrations in cow and goat milk. *Journal of Dairy Science* 103, 5647–5653. <https://doi.org/10.3168/jds.2019-18008>.
- Billa, P.A., Faulconnier, Y., Larsen, T., Leroux, C., Pires, J.A.A., 2020. Milk metabolites as noninvasive indicators of nutritional status of mid-lactation Holstein and Montbéliarde cows. *Journal of Dairy Science* 103, 3133–3146. <https://doi.org/10.3168/jds.2019-17466>.
- Bjerre-Harpoth, V., Storm, A.C., Vestergaard, M., Larsen, M., Larsen, T., 2016. Effect of postpartum propylene glycol allocation to over-conditioned Holstein cows on concentrations of milk metabolites. *Journal of Dairy Research* 83, 156–164. <https://doi.org/10.1017/S0022029916000145>.
- Chaiyabutr, N., Faulkner, A., Peaker, M., 1980. The utilization of glucose for the synthesis of milk components in the fed and starved lactating goat in vivo. *Biochemistry Journal* 186, 301–308. <https://doi.org/10.1042/bj1860301>.
- Chaiyabutr, N., Faulkner, A., Peaker, M., 1981. Changes in the concentration of minor constituents of goat's milk during starvation and on refeeding of the lactating animal and their relationship to mammary gland metabolism. *British Journal of Nutrition* 45, 149–157. <https://doi.org/10.1079/BJN19810087>.
- Chen, R.F., Plaut, G.W.E., 1963. Activation and inhibition of DPN-linked isocitrate dehydrogenase of heart by certain nucleotides. *Biochemistry* 2, 1023. <https://doi.org/10.1021/bi00905a020>.
- Davies, D.T., Holt, C., Christie, W.W., 1983. The composition of milk. In: Mepham, T. B. (Ed.), *Biochemistry of Lactation*. Elsevier, Amsterdam/New York, The Netherlands/NY, USA, pp. 71–117.
- Fang, W., Myllus, V., Sandholm, M., 1997. Resazurin reduction as a function of respiratory burst in bovine neutrophils. *American Journal of Veterinary Research* 58, 601–607.
- Farrell, H.M., Deeney, J.T., Tubbs, K., Walsh, R.A., 1987. Role of the isocitrate dehydrogenases and other Krebs cycle enzymes in lactating bovine mammary gland. *Journal of Dairy Science* 70, 781–788. [https://doi.org/10.3168/jds.S0022-0302\(87\)80074-7](https://doi.org/10.3168/jds.S0022-0302(87)80074-7).
- Farrell, H.M., Deeney, J.T., Hild, E.K., Kumosinski, T.F., 1990. Stopped flow and steady state kinetic studies of the effect of metabolites on the soluble form of NADP⁺: Isocitrate dehydrogenases. *Journal of Biological Chemistry* 265, 17637–17643.
- Farrell, H.M., Wickham, E.D., Reeves, H.C., 1995. Effects of long-chain acyl-Coenzyme A's on the activity of the soluble form of nicotinamide adenine dinucleotide phosphate-specific isocitrate dehydrogenase from lactating bovine mammary gland. *Archives of Biochemistry and Biophysics* 321, 199–208. <https://doi.org/10.1006/abbi.1995.1386>.
- Faulkner, A., 1980. The presence of cellular metabolites in milk. *Biochimica et Biophysica Acta* 630, 141–145. [https://doi.org/10.1016/0304-4165\(80\)90145-2](https://doi.org/10.1016/0304-4165(80)90145-2).
- Faulkner, A., Clapperton, J.L., 1981. Changes in the concentration of some minor constituents of milk from cows fed low- or high-fat diets. *Comparative Biochemistry and Physiology* 68A, 281–283.
- Faulkner, A., Peaker, M., 1982. Reviews of the progress of Dairy Science: Secretion of citrate into milk. *Journal of Dairy Research* 49, 159–169. <https://doi.org/10.1017/s002202990002224x>.
- Faulkner, A., Brechany, E.Y., Mabon, R.M., Pollock, H.T., 1986. Seasonal changes in the fat composition and concentration of citrate and related metabolites in cow's milk. *Journal of Dairy Research* 53, 223–227.
- Friggens, N.C., Duvaux-Ponter, C., Etienne, M.P., Mary-Huard, T., Schmidely, P., 2016. Characterizing individual differences in animal responses to a nutritional challenge: Toward improved robustness measures. *Journal of Dairy Science* 99, 2704–2718. <https://doi.org/10.3168/jds.2015-10162>.
- Garnsworthy, P.C., Masson, L.L., Lock, A.L., Mottram, T.T., 2006. Variation of milk citrate with stage of lactation and de novo fatty acid synthesis in dairy cows.

- Journal of Dairy Science 89, 1604–1612. [https://doi.org/10.3168/jds.S0022-0302\(06\)72227-5](https://doi.org/10.3168/jds.S0022-0302(06)72227-5).
- Grigor, M.R., Hartmann, P.E., 1985. NADP-linked dehydrogenases in secreted milk. *Journal of Dairy Research* 52, 501–506. <https://doi.org/10.1017/S0022029900024444>.
- Gumaa, K.A., Greenbaum, A.L., McLean, P., 1973. Adaptive changes in satellite systems related to lipogenesis in rat and sheep mammary gland and in adipose tissue. *European Journal of Biochemistry* 34, 188–198. <https://doi.org/10.1111/j.1432-1033.1973.tb02745.x>.
- Herve, L., Quesnel, H., Veron, M., Portanguen, J., Gross, J.J., Bruckmaier, R.M., Boutinaud, M., 2019. Milk yield loss in response to feed restriction is associated with mammary epithelial cell exfoliation in dairy cows. *Journal of Dairy Science* 102, 2670–2685. <https://doi.org/10.3168/jds.2018-15398>.
- Kelly, A.L., O'Flaherty, F., Fox, P.F., 2006. Review: Indigenous proteolytic enzymes in milk: A brief overview of the present state of knowledge. *International Dairy Journal* 16, 563–572. <https://doi.org/10.1016/j.idairyj.2005.10.019>.
- Larsen, T., 2012. Enzymatic-fluorometric quantification of cholesterol in bovine milk. *Food Chemistry* 135, 1261–1267. <https://doi.org/10.1016/j.foodchem.2012.05.061>.
- Larsen, T., 2014. Fluorometric determination of free and total isocitrate in bovine milk. *Journal of Dairy Science* 97, 7498–7504. <https://doi.org/10.3168/jds.2014-8018>.
- Larsen, T., Røntved, C.M., Ingvarsen, K.L., Vels, L., Bjerring, M., 2010. Enzyme activity and acute phase proteins in milk utilized as indicators of acute clinical *E. coli* LPS-induced mastitis. *Animal* 4, 1672–1679. <https://doi.org/10.1017/S1751731110000947>.
- Larsen, T., Larsen, M.K., Friggens, N.C., 2011. Enzymatic and fluorometric determination of triacylglycerols in cow milk and other opaque matrices. *Food Chemistry* 125, 1110–1115. <https://doi.org/10.1016/j.foodchem.2010.09.104>.
- Linzell, J.L., Mepham, T.B., Peaker, M., 1976. The secretion of citrate into milk. *Journal of Physiology* 190, 333–346. <https://doi.org/10.1113/jphysiol.1976.sp011541>.
- Moore, J.H., Christie, W.W., 1979. Lipid metabolism in the mammary gland of ruminant animals. *Progress in Lipid Research* 17, 347–395. [https://doi.org/10.1016/0079-6832\(79\)90012-0](https://doi.org/10.1016/0079-6832(79)90012-0).
- Ormrod, I.H.L., Thomas, P.C., Wheelock, J.V., 1980. The effect of dietary inclusions of tallow on the citric acid and soluble calcium content of cow's milk. *Proceedings from the Nutrition Society* 39, 33A.
- Ormrod, I.H.L., Holt, C., Thomas, P.C., 1982. The inorganic constituents of milk IV. Diffusible calcium and magnesium concentrations in goat's milk and the effect of starvation. *Journal of Dairy Research* 49, 179–186. <https://doi.org/10.1017/S0022029900022287>.
- Peaker, M., Faulkner, A., 1983. Soluble milk constituents. *The Proceedings of the Nutrition Society* 42, 419–425. <https://doi.org/10.1079/pns19830049>.
- Pires, J.A.A., Larsen, T., Leroux, C., 2021. Milk metabolites and fatty acids as noninvasive biomarkers of metabolic status and energy balance in early lactation cows. *Journal of Dairy Science* 105, 201–220. <https://doi.org/10.3168/jds.2021-20465>.
- Smistad, M., Sølverød, L., Inglingstad, R.A., Østerås, O., 2021. Distribution of somatic cell count and udder pathogens in Norwegian dairy goats. *Journal of Dairy Science* 104, 11878–11888. <https://doi.org/10.3168/jds.2021-20549>.
- Stumpf, M.T., Fischer, V., McManus, C.M., Kolling, G.J., Zanela, M.B., Santos, C.S., Abreu, A.S., Montagner, P., 2013. Severe feed restriction increases permeability of mammary gland cell tight junctions and reduces ethanol stability of milk. *Animal* 7, 1137–1142. <https://doi.org/10.1017/S1751731113000128>.
- Waldschmidt, M., Rilling, S., 1973. Metabolite levels and enzyme activities in the bovine mammary gland in different stages of lactation. *Journal of Dairy Research* 40, 361–370. <https://doi.org/10.1017/S0022029900014308>.