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Physico-chemical, sensory and oxidative quality of butter from cows fed 3-nitrooxypropanol

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ABSTRACT

This study aims to investigate the effects of supplementing 3-nitrooxypropanol (3-NOP) to the diet of dairy cows on physico-chemical and sensory properties, as well as oxidative stability of butter. Forty-eight Danish Holstein cows were randomly assigned to control diets or 3-NOP supplemented diets at 60 mg 3-NOP kg^{-1} feed DM. Compared to control butter, 3-NOP butter had higher proportions of short- and medium-chain fatty acids (FAs) and lower solid fat content, onset crystallization and offset melting temperatures. Sensory analysis revealed minor differences between 3-NOP and control butter, while peroxide values of 3-NOP butter was lower than control butter during storage of 12 weeks. In conclusion, the alterations in FA composition by 3-NOP led to minor changes in physical properties but improved oxidative stability of butter without major changes in sensory characteristics.
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1. Introduction

Butter is a valued commercial commodity produced solely from milk fat and referred to as an emulsion of water-in-oil. Global market review from FAO expects production of butter to increase in the next decade by an annual growth rate of 1.9% due to increasing demand (FAO, 2022). At the same time, the dairy companies have to meet demands from consumers and governments to reduce methane (CH_4) emission from their production. A recently innovated feed additive, 3-nitrooxypropanol (3-NOP), reduces enteric CH_4 emissions from dairy cows in indoor dairy systems by approximately 30% through the inhibition of an enzyme (methyl coenzyme M reductase) involved in the methanogenesis (Duin et al., 2016; Kebreab et al., 2022). According to previous studies, supplementation of 3-NOP in most studies had no significant effects on milk yield, while the effects on milk compositional aspects are inconsistent. Some studies reported no difference in milk fat content (Schilde et al., 2021; Van Gastelen et al., 2022; Van Wesemael et al., 2019), while others reported an increase in milk fat content upon 3-NOP supplementation in the diet (Lopes et al., 2016; Melgar et al., 2020b; Melgar et al., 2021). According to the changes in rumen volatile fatty acids (VFAs) composition and milk fatty acids (FAs) composition, it has been suggested that a shift in ruminal fermentation and increased availability of energy saved from inhibited methanogenesis led to increased production of *de novo* synthesized short-chain FAs (SCFAs) causing an increase in milk fat content by 3-NOP (Hristov et al., 2015; Melgar et al., 2020b; Melgar et al., 2021). Moreover, 3-NOP has been reported to decrease the concentration of long-chain FAs (LCFAs) such as C18:1 *trans* FA and *cis*-9 *trans*-11 CLA (conjugated linoleic acid) in milk, indicating that 3-NOP may affect ruminal biohydrogenation (Melgar et al., 2020b; Melgar et al., 2021).

Milk FAs are esterified to glycerol and present as triacylglycerols (TAGs). Thus, differences in degree of saturation, chain length, configuration of FAs, and distribution of FAs within TAGs can affect the physical properties of fat-based dairy products, and consequently their sensory characteristics (Chen et al., 2004; Lopez, 2020; Staniewski, Ogrodowska, Staniewska, & Kowalik, 2021). The butter melts over a wide range of temperatures due to the broad differences in melting points of FAs and their position on glycerol backbone of TAGs (Rønholdt et al., 2014). As the temperature increases, more of the solid fats in butter will transition into a liquid state, which leads to a decrease in the overall solid fat content (SFC). The amount of solid fat at serving and mouth temperature is important for firmness, spreadability, and mouthfeel of butter (Couvreur, Hurtaud, Lopez, Delaby, & Peyraud, 2006; Hurtaud, Faucon, Couvreur, & Peyraud, 2010; O'Callaghan et al., 2016). Butter made from milk with more unsaturated FAs (UFAs)

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resulted in more spreadable, softer, and less adhesive butter as a result of the increased proportion of low melting TAGs (Bobe, Hammond, Freeman, Lindberg, & Beitz, 2003; Smet et al., 2010). Moreover, the microstructure of the crystalline fat network, such as crystal size and volume of solid fat outside the milk fat globules also highly determines the firmness of butter (Buldo & Wiking, 2016) and these microstructural aspects can be manipulated during the production of butter.

Oxidative deterioration of butter during the storage can impair butter flavor, decrease storage stability and lower the nutritional value (Mortensen, 2012). During oxidation, oxygen reacts with UFAs and forms lipid peroxides which are further oxidized to several volatile compounds with off-flavors and rancid smell. The amount of lipid peroxides can be measured to determine the extent of fat oxidation and used as important qualitative criteria of fat-based products (Gordon, 2004). Color, which is essential in appetizing appearance, comes from fat soluble pigments such as carotene and vitamin A, and may potentially be affected by changes in cows' nutritional stage as well.

Due to the reported possible differences in fat content and FA composition upon 3-NOP supplementation, it is relevant to investigate possible effects on especially high-fat dairy products such as butter. The objective of this study was to investigate the effects of supplementing 3-NOP to lactating dairy cows on physical properties, sensory attributes, and oxidative stability of butter. We hypothesized that supplementing 3-NOP to diets of dairy cows would increase SCFAs and decrease polyunsaturated FAs (PUFAs) in milk fat and subsequently lowers SFC and melting point of butter, gives a softer texture, and improves oxidative stability during the storage of the butter.

2. Materials and methods

2.1. Experimental design, milk sampling and analyses

The feeding trial was conducted at the Department of Animal and Veterinary Sciences at Aarhus University, Denmark. The indoor feeding trial was designed to investigate the effect 3-NOP (Bovaer®; DSM nutritional products, Kaiseraugst, Switzerland) dose and interaction to different forage types on enteric CH_4 emission and production parameters. For this present study on butter characteristics, bulk milk from Danish Holstein cows, that were previously blocked according to parity (primiparous and multiparous) and lactation stage, were collected to represent 2 treatment groups: control (24 cows, 0 mg 3-NOP kg^{-1} DM) and 3-NOP (24 cows, 60 mg 3-NOP kg^{-1} DM). The 3-NOP was mixed to PMR with varying composition of the forage (high share of grass clover silage i.e. 60:40, grass-clover silage: corn silage, on a dry matter basis, and high share of corn silage i.e. 40:60, grass-clover silage: corn silage). The bulk milk for each 3-NOP level (control vs 3-NOP) was composed of milk from 12 cows on grass-clover based diet and from 12 cows on corn based diet in order to obtain sufficient amounts of milk. The 2 basic PMR (grass-clover silage based and corn silage based) were formulated according to NorFor (Volden, 2011) to meet 50–55% forage share of the total daily ration and the concentrate part included spring barley, rapeseed cake, rapeseed meal, sugar beet pulp, and mineral supplements. Cows were offered additional concentrate (605 ± 16.8 g; mean ± SD) as bait in GreenFeed units for measuring gas emissions. The PMR were isoenergetic and isonitrogenous and fed *ad libitum*. Cows were housed in a loose housing system and milked twice daily at 0530 and 1630 h in a 2 × 12 parallel milking parlor (SAC A/S, S. A. Christensen & Co, Kolding, Denmark). The experiment was a continuous experiment in a randomized block design with a two-week covariate period followed by a 12 week experimental period, where cows were exposed to experimental treatments. Milk from 5 consecutive milkings (3 morning and 2 evening) from each treatment group (control and 3-NOP) were collected in bulk milk tank at the end of the experiment. At this time, average DMI and milk yield were 23.2 ± 3.35 kg d^{-1} and 35.5 ± 6.83 kg d^{-1}, respectively. Three replicates of full milk from each treatment were collected for the analysis of overall milk composition by Milkoscan (FT2, FOSS Analytical, Hillerød, Denmark), pH by pH meter (PHM 220 RadioMeter, Copenhagen, Denmark) and milk fat globule size (MFGS) by static laser light scattering (Mastersizer 2000, Malvern Instruments, Malvern, UK). Approximately 600–700 L of bulk milk from each treatment group were transported to Arla Innovation Center (AIC), Aarhus, Denmark, to produce butter and only one butter trial was performed per treatment.

2.2. Manufacture and chemical analysis of butter

The milk was separated into cream and skimmed milk by centrifugation at 50 °C with the flow rate of 3000 L h^{-1}. The cream fraction was standardized to approximately 30% fat and pasteurized at 85 °C for 15 s. A standard mesophilic *Lactococcus lactis* subsp (ssp). lactis biovar diacetylactis culture was used to ferment the cream at 20 °C for 20 h. The pH of the fermented cream was 5.0. The cream was churned in a batch butter churner (APV Pasilac, Silkeborg, Denmark) until it separated into butter granules and butter milk. Following the draining of buttermilk, the butter was kneaded at 12 °C until the butter was homogenous and looked dry. Then, the butter was transferred to the filling machine (Handtmann, Conserves Teknik, Ullerslev, Denmark) and filled in to rolls (of 220 g $roll^{-1}$) wrapped in black polythene. The fresh butter samples were used to analyze FA composition by GC-FID (Eurofins Steins Laboratory, Vejen, Denmark).

2.3. Color measurements

Color measurements of fresh butter were determined by Minolta Chroma meter CR-400 (Minolta, Osaka, Japan) on inner and outer samples. To take the inner measurements, the butter roll was cut into 1–2 cm thick slices. Following the calibration, L* (lightness), a* (red–green color), and b* (yellow–blue color) values were taken on 3 replicates. Whiteness index (WI), color intensity or chroma (C), and yellowness index (YI) were calculated as previously reported by Păduret (2021).

2.4. Microbial analysis and water droplet size

The safety of butter for the sensory analysis was determined by microbial counts and water droplet size distribution. Total number of contaminating microorganisms and the number of Coliforms, Enterobacteria and *Listeria monocytogenes* were assessed, following ISO standards: ISO 13559:2002(E), ISO 4832:2006 (E), ISO 21528-2:2017 (E) and ISO 11290-1, respectively.

For measurement of water droplet size, butter samples were stored at 20 °C for 3 h and then 1.5 cm of sample was filled into glass sampling tubes with an inner diameter of 10 mm. After filling the tubes, these were stored at 5 °C. The pulsed field gradient nuclear magnetic resonance (pfg-NMR) method was performed on a Bruker Minispec mq (Ettlingen, Germany), operating at 20 MHz equipped with a PH H20-10-25(33)-AVGX(Y) probe at 5 °C. The water droplet size application "g-var_mg_nf" was used to determine the water droplet size distribution in butter. A constant gradient strength γ of 0.47 T was used at a gradient pulse width δ varied automatically between 0.1 and 3.1 Tm^{-1}. For each sample, 2 technical replicates were performed with 8 gradient pulse widths δ, which were determined automatically by the system.

2.5. Sensory evaluation

For sensory evaluation, the method used was a simple version of the Difference From Control (DFC) Test described by Whelan (2017). Although there are no specific ISO or ASTM standards for this test, it is considered a useful tool for decision-making and is used as an example when experimenting with new ingredients or processing techniques. In total, 12 participants evaluated the butter of each of the two treatments, which was served in pairs at 13 °C. Samples were assessed comparing butter from treatment (3-NOP) to butter from control milk using a 6 points degree of difference scale (0 = No difference, 1 = Minor, hardly noticeable, 2 = Minor, noticeable, 3 = Considerable, 4 = Distinct and 5 = Very distinct). Additionally, for scores = 3 or above the difference is perceived to be considerable and should also be described either by selecting among the pre-defined descriptors (CATA) or describing it if it is not predefined. The samples were scored for degree of difference for odor, appearance, consistency, spreadability, mouthfeel, taste and flavor, off-flavor and the total perceived difference; besides, and the participants were asked to give a comment at the end of the evaluation. Compusense20 (Compusense Inc., Guelph, Canada) was used for the data collection.

2.6. Physical properties of butter

2.6.1. Solid fat content

The SFC was measured as a function of temperature. Glass NMR tubes (10 mm diameter) were filled to a height of 5 cm of sample. The samples were measured at increasing temperatures from 5 to 40 °C with incremental steps of 5 °C. The NMR tubes were placed in a water bath at each temperature for 30 min and SFC was measured using Bruker Minispec pNMR (Ettlingen, Germany). Two determinations on each replicate were performed.

2.6.2. Crystallization and melting

The crystallization and melting behavior of milk fat was determined by differential scanning calorimetry (DSC) (Q2000, TA Instruments, New Castle, DE, USA) in which nitrogen was used to purge the system. After 6 weeks of storage at 4 °C, 3 replicates from treatment of each weight 5–15 mg were weighted into hermetic aluminum pan and press sealed. An empty pan was used as a reference. The analysis was conducted in 3 cycles. Cycle 1: the sample was held at 5 °C for 15 min, heated at 5 °C min^{-1} to 60 °C and held for 5 min. Cycle 2: the samples was cooled from 60 °C to 5 °C at the rate of 10 °C min^{-1} and held for 25 min. Cycle 3: the sample was reheated at the rate of 5 °C min^{-1} up to 60 °C. It should be noted that cycle 1 represents the melting curve of butter while cycle 3 is melting of non-emulsified butter oil. The crystallization and melting curves were identified and integrated by Universal Analysis Software (TA Instruments, New Castle, DE, USA). Onset crystallization temperature (T-onset) from cycle 2, offset melting temperature (T-offset), peak temperature of low melting fraction (LMF) and peak temperature of high melting fraction (HMF) and enthalpy of LMF and HMF from cycle 1 and 3 were recorded, as illustrated in Fig. 1.

2.6.3. Firmness

The firmness was measured using a Texture Analyzer (TA.XT plus 100C, Stable Microsystems, Surrey, UK). A 60° conical probe (P/60C) was used, and the butter was penetrated for 8 mm with pre-test speed of 1 mm s^{-1}, a test speed of 0.83 mm s^{-1} and a post-test speed of 10 mm s^{-1}. Prior to the measurements the samples were subjected to temperatures of 14 °C and 5 °C. For each treatment and at each temperature, two determinations on each of two replicates were performed.

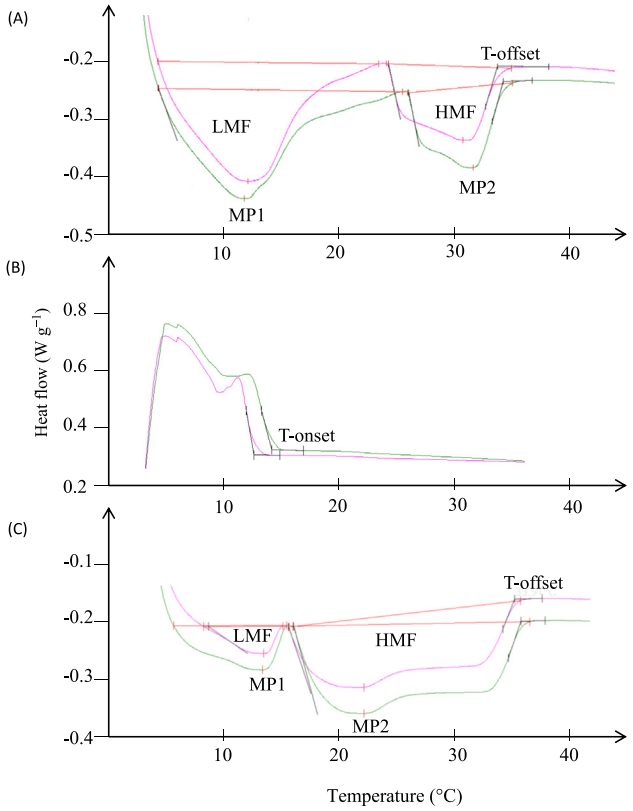

Fig. 1. (A) Melting behavior of butter (B) crystallization behavior of butter oil (C) melting behavior of butter oil from cows fed control diets (green) or diets supplemented with 3-nitrooxypropanol (3-NOP) (purple). LMF = low melting fraction, HMF = high melting fraction, MP1 = peak temperature of LMF, MP2 = peak temperature of HMF, T-offset = temperature offset of melting, T-onset = temperature onset of crystallization. Integration lines are presented in red colors.

2.7. Storage experiment and analysis of peroxide value

Twelve butter rolls from each treatment were stored at 5 and 20 °C for 10 weeks. Three representative samples from each butter roll were collected in every 2 weeks. The peroxide value (PV) of butter was determined according to IDF standard (74A:1991) with a few modifications. In short, butter was heated at 60 °C and centrifuged for 10 min at 40 °C to separate butter oil and 0.3–0.7 g of butter oil was dispersed in chloroform: methanol (2:1) mixture. The absorption of the mixture was measured by Cary 60 UV–Vis spectrophotometer (Agilent, Santa Clara, CA, USA) at 500 nm following the 5 min of interaction with iron II/thiocyanate mixture solution. Hydrogen peroxide was used as the external standard to quantify lipid peroxides.

2.8. Data analysis

Data on physico-chemical analyses were statistically analyzed using a General linear model with fixed effect of the 3-NOP treatment, using R 3.6.3 (R Core Team, 2019; https://www.r-project.org). Least squares means were computed, and multiple comparisons performed with the Tukey post hoc test. Differences were declared as significant if $P < 0.05$ as tendency if $P < 0.1$. For the sensory data, mean, standard deviation and confidence intervals were calculated and the scoring from the CATA comments were used as descriptors for the difference between treatment and control to identify systematic differences.

Table 1
Physico-chemical parameters of milk of Danish Holstein cows fed control diets or diets supplemented with 3-nitrooxypropanol (3-NOP).

Variable	Control	3-NOP	SEM[c]	*P* value
Fat (g 100 g^{-1})	4.05	3.87	0.002	<0.001
Protein (g 100 g^{-1})	3.70	3.60	0.002	<0.001
Casein (g 100 g^{-1})	2.82	2.77	0.004	<0.001
Lactose (g 100 g^{-1})	4.82	4.85	0.000	<0.001
MUN[a] (mg dL^{-1})	9.91	10.47	0.157	0.07
Citric acid (g 100 g^{-1})	0.17	0.19	0.000	<0.001
pH	6.61	6.83	0.017	<0.001
MFGS[b] (μm)	4.16	4.22	0.011	0.03

[a] Milk urea nitrogen.
[b] Milk fat globule size.
[c] Standard error of estimated marginal mean.

3. Results

3.1. Chemical composition of milk and fatty acid profile of resulting butter

Supplementation of the diet with 3-NOP affected overall milk composition to some extent (Table 1). Milk from cows fed 3-NOP diets had lower contents of fat, protein, and casein ($P < 0.001$), and higher contents of lactose and citric acid ($P < 0.001$) than cows fed control diets. The pH was higher in milk from 3-NOP fed cows than control diets ($P < 0.001$). The MFGS were larger in milk from cows fed 3-NOP diets, compared to control diets ($P = 0.03$). Table 2 shows FA composition of the butters. 3-NOP butter had a higher proportion of PUFAs than control butter ($P = 0.01$) mainly due to an increase in proportion of C18:3 ($P = 0.04$). No difference was found for monounsaturated FAs (MUFAs) proportion between treatments ($P = 0.22$), although the proportion of C18:1 *trans* 11 was lower in 3-NOP butter, than in control butter ($P < 0.01$). Furthermore, no difference was found in the proportions of saturated FAs (SFAs) and UFAs between control and 3-NOP butters. The sum of C14:0, C16:0, and C18:0 tended to be higher in 3-NOP butter than control butter ($P = 0.07$). Particularly, *de novo* synthesized FAs were affected by the treatments. Higher proportion of C4:0 ($P = 0.02$), C6:0 ($P = 0.01$), C8:0 ($P < 0.01$), C10:0 ($P < 0.01$), C12:0 ($P < 0.001$), and C15:0 ($P = 0.02$) were found in 3-NOP butter compared to control butter, whereas a lower proportion of C16:0 ($P = 0.02$) was found in 3-NOP butter compared to control butter.

Table 2
Fatty acid (FA) composition of the butter made of milk from cows fed control diets or diets supplemented with 3-nitrooxypropanol (3-NOP).

FA (g 100 g^{-1} total FA)	Control	3-NOP	SEM[e]	*P* value
C4:0	3.66	4.02	0.035	0.02
C6:0	2.34	2.58	0.018	0.01
C8:0	1.47	1.63	0.008	<0.01
C10:0	3.46	3.87	0.013	<0.01
C10:1	0.32	0.35	0.004	0.04
C12:0	3.93	4.31	0.006	<0.001
C14:0	11.66	11.75	0.023	0.10
C14:1	1.01	1.01	0.007	0.56
C15:0	1.46	1.52	0.006	0.02
C16:0	27.12	25.39	0.157	0.02
C16:1	2.05	1.96	0.022	0.10
C17:0	0.72	0.75	0.006	0.07
C17:1	0.27	0.26	0.005	0.90
C18:0	10.13	10.35	0.110	0.29
C18.1 *cis* 9	21.51	21.17	0.126	0.19
C18: 1 *trans* 9	3.13	3.13	0.011	0.82
C18:1 *trans* 11	0.83	0.81	0.001	<0.01
C18:1 *cis* 12	0.34	0.35	0.004	0.11
C18:2	3.48	3.55	0.036	0.27
Cis-9 *trans*-11 CLA	0.63	0.58	0.01	0.05
C18:3	0.89	0.94	0.008	0.04
C14:0 + C16:0 + C18:0	48.91	47.49	0.290	0.07
SFA[a]	69.21	69.40	0.20	0.57
MUFA[b]	27.70	27.27	0.19	0.25
PUFA[c]	3.10	3.33	0.02	0.01
UFA[d]	31.7	31.5	0.205	0.57

Total FA is the sum of C4:0, C6:0, C8:0, C10:0, C11:0, C12:0, C13:0, C14:0, C14:1, C15:0, C16:0, C16:1, phytanic acid, C17:0, C17:1, C18:0, C18:1 *trans*-9, C18:1 *trans*-11, C18:1 *cis*-9, C18:2 n-6, *cis*-9,*trans*-11 CLA, C18:3 n-3, C18:3 n-6, C20:0, C20:0 n-6, C20:1, C20:4 n-6, C20:5 n-3, C21:0, C22:0, C22:1 n-9, C22:2, C23:0, C24:0 and C24:1.
[a] Saturated FA.
[b] Monounsaturated FA.
[c] Polyunsaturated FA.
[d] Unsaturated FA.
[e] Standard error of estimated marginal mean.

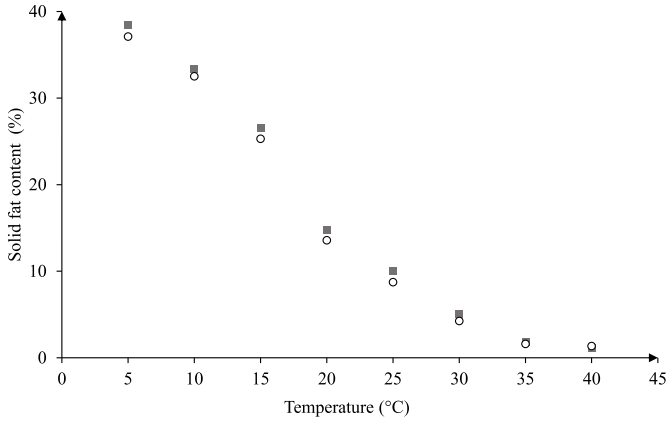

Fig. 2. Solid fat content (SFC) of butter made of milk from cows fed control diets (squares) or diets supplemented with 3-nitrooxypropanol (3-NOP) (circles).

The percentage of water droplets with more than 10 μm of volume weighted diameter of control and 3-NOP butters were 3.6 and 1.3, respectively (data not shown). Microbial analysis claimed safeness of butter for sensory analysis (data not shown).

3.2. Solid fat content, firmness and thermal behavior of butter

The SFC of butter from the treatments at different temperatures (from 5 to 40 °C) is shown in Fig. 2. The percentage of SFC was lower in 3-NOP butter than control butter from 5 to 30 °C ($P < 0.01$), whereas SFC was not differ between control and 3-NOP butters at 35 and 40 °C. The firmness of butter was determined at 5 and 14 °C and shown in Table 3 and no difference was found between control and 3-NOP butters at both temperatures ($P = 0.36$ and $P = 0.23$, respectively).

Fig. 1(B) shows crystallization curves of the butters, as determined by a cooling rate of 10 °C min^{-1}, and data on crystallization onset temperature (T-onset) are included in Table 3. Although butters behaved similarly during crystallization, the T-onset was lower for 3-NOP butter than control butter ($P < 0.001$). Melting curves of butter and butter oil are shown in Fig. 1(A) and (C), respectively. The melting curves of butter and butter oil differed in shape and size, resulting in different peak temperatures and enthalpies. Proportionally, butter has higher HMF compared to LMF and milk fat had lower HMF compared to LMF. However, butter showed a similar melting point/melting offset temperature to butter oil.

Data on melting offset temperature (T-offset), temperatures of two endothermic peaks (LMF, and HMF), enthalpies of these peaks were shown in Table 3. The melting point/melting offset temperature (T-offset) of 3-NOP butter was lower than that of control butter ($P = 0.03$). No significant difference was reported for melting

Table 3
Firmness, crystallization and melting properties of butter made of milk from cows fed control diets or diets supplemented with 3-nitrooxypropanol (3-NOP).

Variable	Control	3-NOP	SEM[e]	*P* value
Firmness (N)				
At 5 °C	19.53	21.13	1.15	0.36
At 14 °C	6.32	6.55	0.12	0.23
Melting – butter				
T-offset[a] (°C)	34.83	34.31	0.11	0.03
Peak temperature – LMF[c] (°C)	15.02	14.81	0.52	0.79
Peak temperature – HMF[d] (°C)	32.43	31.62	0.12	<0.01
Enthalpy – LMF (J g^{-1})	14.28	18.52	0.69	0.01
Enthalpy – HMF (J g^{-1})	8.76	8.91	0.46	0.83
Crystallization				
T-onset[b] (°C)	15.35	14.20	0.03	<0.001
Melting – butter oil				
T-offset (°C)	34.45	33.70	0.04	<0.001
Peak temperature – LMF (°C)	13.91	13.55	0.21	0.29
Peak temperature – HMF (°C)	22.70	21.54	0.95	0.44
Enthalpy – LMF (J g^{-1})	6.53	1.98	1.00	0.03
Enthalpy – HMF (J g^{-1})	21.36	18.61	1.08	0.15

[a] The onset crystallization.
[b] The offset melting temperature.
[c] Low melting fraction.
[d] High melting fraction.
[e] Standard error of estimated marginal mean.

peak temperature in LMF of butter between treatments ($P = 0.79$), whereas 3-NOP butter had a lower peak temperature in HMF in comparison to control butter ($P < 0.01$). For melting enthalpies in LMF and HMF, the value in LMF was greater in 3-NOP butter than control butter ($P = 0.01$), whereas value in HMF did not differ between treatments ($P = 0.83$). T-offset of 3-NOP butter oil was lower than control butter oil ($P < 0.001$). The melting peak temperatures of both LMF and HMF of butter oil did not differ between treatments ($P = 0.29$ and $P = 0.44$, respectively). However, 3-NOP butter oil had a 3-fold lower enthalpy in LMF compared to control butter oil ($P = 0.03$). In contrast, there was no significant effect of 3-NOP on enthalpy of HMF of butter oil ($P = 0.15$).

3.3. Butter color and sensory attributes

The color parameters on both the outside and inside of butter are shown in Table 4. No significant difference was found for L* between control and 3-NOP butters neither on outside nor inside samples ($P = 0.24$ and $P = 0.82$, respectively). All butters reported negative values for a* (greenish direction) and positive values for b* (yellowish direction). Outside a* was higher in 3-NOP butter than control butter ($P = 0.02$), whereas inside a* was not affected by the treatments ($P = 0.94$). No significant difference was found for the outside b* of butter ($P = 0.10$). However, inside b*was lower in 3-NOP butter than control butter ($P = 0.03$). Outside h0 was higher in 3-NOP ($P < 0.01$) butter while inside h0 was not affected by treatments ($P = 0.20$). Treatments had no effect on YI and WI on both outside and inside of the butter.

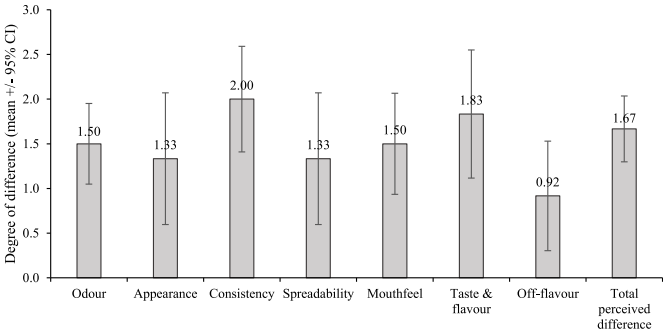

Fig. 3. The degree of difference (mean ± 95% CI) in the sensory perception between butter made of milk from cows fed control diets or diets supplemented with 3-nitrooxypropanol (3-NOP).

The reported differences in sensory attributes between 3-NOP and control butters had mean gap scores ranging from 0.92 to 2.00 (Fig. 3). These differences were considered minor, varying from hardly noticeable (1) to minor noticeable (2). The lowest score was for off-flavor, while the highest was for consistency, and taste while values for flavor and odor scores were in between. With the low level of difference, most of the comments were given at the end. The comments differed from 'No real noticeable difference' to 'Considerable taste difference' and, in general, the comments supported the mean gap scores showing minor differences between butters. Some of the comments pointed out that 3-NOP butter was slightly more yellow, slightly harder and with slightly more farmy/aromatic/lactic flavor notes. In a direct comparison of treatment to control, the perceived sensory difference was at a minor level between hardly noticeable to noticeable.

3.4. Oxidative stability of butter

As shown in Fig. 4, initial PV of 3-NOP butter was similar to control butter ($P = 0.79$). However, PV increased during the storage and was numerically lower in 3-NOP butter all the time points compared to control butter at both 5 and 20 °C. Significantly lower PV was observed in 3-NOP butter compared to control butter at 5 °C after 4 and 10 weeks of storage (0.31 vs 0.51 meq O_2 kg^{-1} fat, ($P < 0.01$) and 0.33 vs 0.52 meq O_2 kg^{-1} fat, ($P < 0.01$) respectively) and after 10 weeks of storage at 20 °C (0.40 vs 0.62 meq O_2 kg^{-1} fat ($P = 0.03$)).

4. Discussion

In the present study, the butter produced of milk from cows fed control and 3-NOP diets fulfilled EU regulations for butter (no 1272/2009) regarding fat, water and solid nonfat (SNF) contents. In addition, water droplet size and microbial counts were in accordance with national and EU regulations.

Table 4
Inner and outer color parameters of butter made of milk from cows fed control diets or diets supplemented with 3-nitrooxypropanol (3-NOP).

Variable	Outside				Inside			
	Control	3-NOP	SEM[a]	*P* value	Control	3-NOP	SEM[a]	*P* value
Brightness (L*)	90.50	89.60	0.47	0.24	90.65	90.95	0.90	0.82
Redness (a*)	−3.33	−3.71	0.07	0.02	−3.66	−3.67	0.06	0.94
Yellowness (b*)	34.47	33.04	0.48	0.10	32.68	30.97	0.35	0.03
Chroma ©	34.63	33.25	0.48	0.11	32.89	31.18	0.34	0.02
Yellowness index (YI)	54.42	52.68	0.97	0.27	51.54	48.64	1.03	0.12
Whiteness index (WI)	64.09	65.16	0.56	0.24	65.78	67.53	0.56	0.09

[a] Standard error of estimated marginal mean.

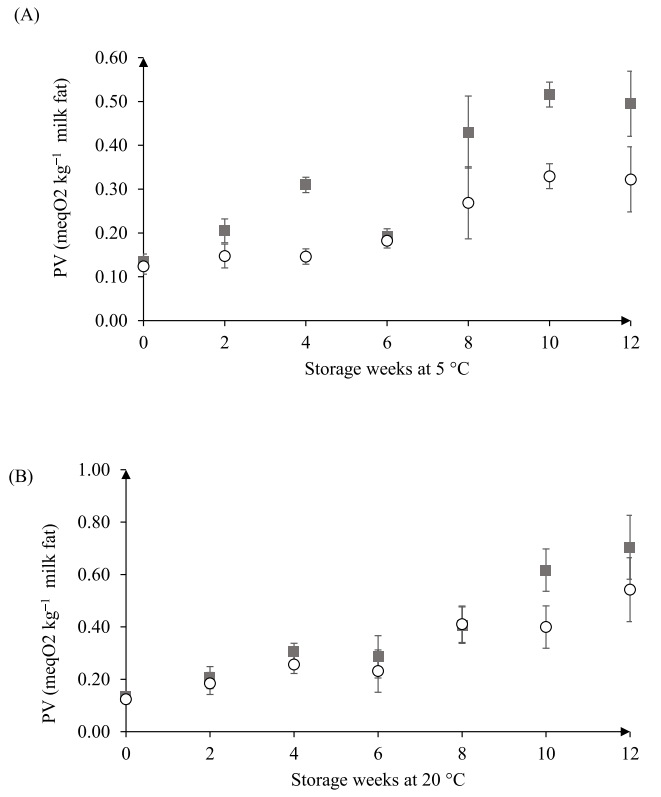

Fig. 4. Changes in peroxide value (PV) of butter made of milk from cows fed control diets (squares) or diets supplemented with 3-nitrooxypropanol (3-NOP) (circles), stored at (A) 5 °C and (B) 20 °C over a period of 12 weeks. Bars indicate standard error of means of triplicates.

4.1. Effect of 3-NOP on physico-chemical properties of butter

The FA composition of butter reflected the FA composition of the raw milk used. As already observed in milk (Hristov et al., 2015; Melgar et al., 2020a, 2021; Van Gastelen et al., 2022), variations were mainly observed in *de novo* synthesized SCFAs. The 3-NOP butter had higher SCFAs and medium-chain FAs (MCFAs) contents than control butter. All *de novo* derived FAs, except C16:0, increased with 3-NOP supplementation, possibly due to (1) shift of rumen fermentation toward increased production of butyrate, which acts as main substrate for *de novo* synthesized SCFA and (2) increased availability of energy from reduced CH_4 emission (Melgar et al., 2021). However, this increase in *de novo* derived FAs did not make a difference in the sum of SFAs between the control and 3-NOP butter although some other studies reported increased proportion of SFAs upon 3-NOP supplementation (Hristov et al., 2015; Melgar et al., 2021; Van Gastelen et al., 2022).

Very limited work has been done on the effect of 3-NOP on functional properties of milk. In the present study, the lower content of C16:0 and higher content of SCFAs and C18:3 resulted in lower SFC (5–30 °C) in 3-NOP butter. The C16:0 is the major FA of butters averaging 26.25% of total FAs (Table 2) and therefore can be the major cause of the variation in SFC. However, at higher temperatures (30–40 °C), no variation in SFC levels of butter was observed. Despite the difference in SFC at 5 and 14 °C there was no difference in firmness between the control and 3-NOP butters, indicating that the microstructure also highly affects the hardness. Milk fat from cows fed 3-NOP began to crystallize at lower temperature which is likely due to lower content of C16:0 and increased content of C4:0 and total PUFAs. 3-NOP supplemented at the dose of 80 mg kg^{-1} in our previous study (unpublished data) also shifted onset-T of milk fat toward low temperature due to an increase in long-chain UFAs. The crystallization properties of milk fat are crucial in butter production and important in producing high-quality butter with desired texture, consistency, spreadability and mouthfeel. Onset-T reported in the present study was closer to the reported values of butter made of milk from cows maintained outdoors on perennial rye grass by O'Callaghan et al. (2016).

For the analysis of crystallization and melting in the present study, we first melted the butter to analyze the effects of both FA composition and butter microstructure. The latter is a result of cream and butter treatment during production. After re-crystallization in the DSC of the melted butter oil, it was melted again. The melting curves of the non-emulsified butter oil give more information about the sole effect of FA composition. Comparing Fig. 1(A) and (C), it is clear that the LMF peak is much larger than the HMF in butter but opposite in butter oil, which showed the effect of fractional crystallization during butter production helping to make butter softer and more spreadable. The increased proportions of FAs with low melting point, C4:0 and total PUFAs and decreased proportions of FAs with high melting point, C16:0, may have caused lower offset-T in butter oil from cows fed 3-NOP in the present study. T-offset of butter oil ranged within the values reported in previous studies (Buldo, Larsen, & Wiking, 2013; Larsen, Andersen, Kaufmann, & Wiking, 2014; O'Callaghan et al., 2016). Ortiz-Gonzalez et al. (2007) also reported that the melting point of butter oil is positively correlated with the concentration of C16:0.

The typical melting curve of milk fat consists of 3 distinct peaks representing LMF, medium melting fraction (MMF) and HMF. In the present study, LMF and MMF seem to have merged and appeared as one peak. Therefore, the term LMF used in this study represents both LMF and MMF. The lowered enthalpy of LMF in 3-NOP group might be due to lowered concentration of C16:0 and increased concentration of PUFAs regardless of the increased total SCFAs and MCFAs. The C16:0 was reported to positively correlate with LMF in previous studies (Buldo et al., 2013; Larsen et al., 2014). Similar to our study, previous studies found that it is mainly the LMF affected by the feeding (Buldo et al., 2013; Larsen et al., 2014). Generally, the LMF contains TAGs with a high content of long-chain UFA such as C18:1 *cis* 9 and short-chain SFA including C4:0 and C6:0 whereas MMF comprises of TAGs with one SCFAs of one UFAs (Lopez, 2018). The LMF is liquid at room temperature and therefore, the proportion or the enthalpy is important in sensory properties: spreadability and mouthfeel (Lopez, 2018; Ortiz-Gonzalez et al., 2007). The lower enthalpy in 3-NOP butter oil suggested 3-NOP butter would be less spreadable than control butter. However, this effect was not obvious in the butter from this trial.

Overall, the lower crystallization and meting values for the 3-NOP butter fits well with the lower SFC in Fig. 2. The differences in DSC parameters between butter and butter oil showed that the small effects of 3-NOP can easily be adjusted through butter production process.

4.2. Effect of 3-NOP on color and sensory attributes of butter

We assumed that no flavor compounds had been transferred or formed from the metabolism of 3-NOP to be released into milk, resulting in no difference in taste and flavor between control and 3-NOP butters. Flavor compounds in milk are either directly transferred from the feed (through lungs or the rumen) or formed during the rumen fermentation and the metabolism (Honkanen, Karvonen, & Virtanen, 1964). The previous *in vivo* and *in vitro* studies on metabolism and excretion showed that 3-NOP is

extensively metabolized in the rumen of cows and 1.9% was excreted in faeces, 3.5% in urine and 6.42% in milk while 4.98% deposited in the total edible tissues and 1.14% in the intestinal tract content and primarily exhaled as CO_2. Metabolized molecules of 3-NOP are mainly incorporated in lactose followed by fat and protein in milk and no presence of 3-NOP and 3-nitrooxypropionic acid (NOPA), which is a metabolite of 3-NOP, was reported in milk at the limit of quantification (LOQ) of 8 μg 3-NOP-equivalents kg^{-1} (Bampidis et al., 2021; Thiel, Rumbeli, Mair, Yeman, & Beilstein, 2019). The 3-NOP, however, may indirectly influence the flavor profile of dairy products by altering the chemical composition of milk. The slightly more lactic flavor noted by some of the participants may be a direct result of higher lactose content in milk from cows fed 3-NOP. The fermentation step in the butter production allowed the lactic acid bacteria to convert lactose into lactic acid and other flavor compounds. As the lactic acid concentration increases, it enhances the overall flavor profile of the butter. However, analysis of flavor profile is needed to confirm this speculation. Off-flavor of butter develops after manufacturing, during the storage by auto-oxidizing UFAs (Mortensen, 2012). There was no off-flavor detected in either butters indicating that oxidative volatiles had not been developed or below the threshold of detection by consumers after 6 weeks of storage. Having an indistinguishable off-flavor between control and 3-NOP butter could be explained by the absence of difference in UFAs content and PV at week 6 of storage.

The comment of slightly more yellow color of 3-NOP butter from some participants was not in agreement with internal color measured by colorimeter. However, sensory analysis was conducted after 6 weeks of storage while the color was measured on fresh butter samples, which may hamper a direct comparison. Moreover, the reported difference of internal yellowness (b*) is numerically smaller and may not perceived by human eye whereas external yellowness did not differ among treatments. The 3-NOP is a white granular powder and there has not been reported any effects on pigment metabolism in dairy cows, therefore no color difference was expected. The resulted L*, a* and b* parameters are within the range of previous studies of butter (Hurtaud et al., 2010; O'Callaghan et al., 2016; Păduret, 2021), and inside and outside measurements are similar. Overall, the difference in spreadability and mouthfeel between control and 3-NOP butters was not noticeable and well correlated with the result of firmness. The slightly harder texture noted by some participants might be related to numerically larger firmness value found in 3-NOP butter. So far, only one study has investigated the effect of 3-NOP supplementation on sensory characteristics of milk and dairy products, concluding that 3-NOP had no perceptible effect on milk and cheddar cheese (Melgar et al., 2020a). The current study is the first to report the effects of 3-NOP on organoleptic characteristics of the butter. The lack of difference in sensory properties between control and 3-NOP butter indicates that supplementation of 3-NOP did not change the organoleptic properties of butter.

4.3. Effect of 3-NOP on oxidative stability of butter

The initial PVs of butter were within the range of values reported by Krause, Miracle, Sanders, Dean, and Drake (2008), Viriato, Queiros, Neves, Ribeiro, and Gigante (2019), and Ullah et al. (2020) and below the maximum allowed value (0.3 meq O_2 kg^{-1} milk fat) required by European Union regulation for butter (EU regulation no 1272/2009). At all the measuring points and at both temperatures, the PV remained lower in 3-NOP butter than in control butter. The PVs are commonly used for assessment of primary oxidation products of lipids, such as hydroperoxides, and are an indicator of initial stages of oxidation. The composition of butter, including FA profiles, degree of unsaturation, i.e. number, position and configuration of double bonds in UFAs, antioxidants, pro-oxidants and salt concentration affect its oxidative stability (Gordon, 2004; Mortensen, 2012). α-Tocopherol is the most important antioxidant in butter, and previous work from our laboratories showed increased content of α-tocopherol in milk upon 3-NOP supplementation (unpublished data), which could also be linked to the improvement of oxidative stability in 3-NOP butter.

The storage conditions including time, temperature, and type of package can affect rate of butter oxidation (Krause et al., 2008; Mortensen, 2012). The PV of butter increased over the time of storage by nearly 3-fold and 5.5-fold at 5 and 20 °C, respectively. The final PV of butter after 12 weeks of storage at 20 °C almost doubled over that at 5 °C, due to an increase in the rate of oxidation. At week 6, PV of butter did not show further increase compared to PV of week 4 at both temperatures which may be a result of the formation of secondary oxidative products from peroxides. Similarly, Laikoja, Teder, and Jõudu (2017) observed an increasing and deceasing trend of PV of unsalted sweet cream butter stored at 5 and 20 °C.

5. Conclusions

Supplementation of 3-NOP to dairy cows modified the fatty acids (FAs) composition of butter, mainly by decreasing C16:0, and increasing SCFAs, MCFAs and PUFAs. Changes in FA composition lowered the solid fat content, offset temperature of melting and onset temperature of crystallization in milk fat without affecting the firmness, spreadability and mouthfeel of butter. In addition, the supplementation of 3-NOP seemed to slightly reduce the susceptibility of butter to oxidation during storage. Overall, 3-NOP supplementation at the dose of 60 mg kg^{-1} DM in Danish Holstein cows had no deleterious effects on physico-chemical properties and oxidative stability of butter and did not compromise the sensory characteristics. The results of this study suggest that 3-NOP can be used in dairy farms to reduce methane emission with no detrimental effect on milk used for butter production.

Credit author statement

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

Bampidis, V., Azimonti, G., Bastos, M.d. L., Christensen, H., Dusemund, B., Fašmon Durjava, M., et al. (2021). Safety and efficacy of a feed additive consisting of 3-nitrooxypropanol (Bovaer® 10) for ruminants for milk production and reproduction (DSM Nutritional Products Ltd). *EFSA Journal, 19*(11), Article e06905.

Bobe, G., Hammond, E., Freeman, A., Lindberg, G., & Beitz, D. (2003). Texture of butter from cows with different milk fatty acid compositions. *Journal of Dairy Science, 86*(10), 3122–3127.

Buldo, P., Larsen, M. K., & Wiking, L. (2013). Multivariate data analysis for finding the relevant fatty acids contributing to the melting fractions of cream. *Journal of the Science of Food and Agriculture, 93*(7), 1620–1625.

Buldo, P., & Wiking, L. (2016). *Butter: Properties and analysis* (pp. 535–541). Encyclopedia of Food and Health.

Chen, S., Bobe, G., Zimmerman, S., Hammond, E. G., Luhman, C. M., Boylston, T. D., et al. (2004). Physical and sensory properties of dairy products from cows with various milk fatty acid compositions. *Journal of Agricultural and Food Chemistry, 52*(11), 3422–3428.

Couvreur, S., Hurtaud, C., Lopez, C., Delaby, L., & Peyraud, J.-L. (2006). The linear relationship between the proportion of fresh grass in the cow diet, milk fatty acid composition, and butter properties. *Journal of Dairy Science, 89*(6), 1956–1969.

Duin, E. C., Wagner, T., Shima, S., Prakash, D., Cronin, B., Yáñez-Ruiz, D. R., et al. (2016). Mode of action uncovered for the specific reduction of methane emissions from ruminants by the small molecule 3-nitrooxypropanol. *Proceedings of the National Academy of Sciences, 113*(22), 6172–6177.

FAO. (2022). *OECD-FAO agricultural outlook 2022-2031*. https://www.fao.org/markets-and-trade/publications/detail/en/c/1469352. (Accessed 22 December 2022).

Gordon, M. H. (2004). Factors affecting lipid oxidation. In R. Steele (Ed.), *Understanding and measuring the shelf-life of food* (pp. 128–141). Cambridge, England: Woodhead publishing.

Hristov, A. N., Oh, J., Giallongo, F., Frederick, T. W., Harper, M. T., Weeks, H. L., et al. (2015). An inhibitor persistently decreased enteric methane emission from dairy cows with no negative effect on milk production. *Proceedings of the National Academy of Sciences, 112*(34), 10663–10668.

Honkanen, E., Karvonen, P., & Virtanen, A. I. (1964). Studies on the transfer of some flavour compounds to milk. *Acta Chemica Scandinavica, 18*(34), 295.

Hurtaud, C., Faucon, F., Couvreur, S., & Peyraud, J. L. (2010). Linear relationship between increasing amounts of extruded linseed in dairy cow diet and milk fatty acid composition and butter properties. *Journal of Dairy Science, 93*(4), 1429–1443.

Kebreab, E., Bannink, A., Pressman, E. M., Walker, N., Karagiannis, A., van Gastelen, S., et al. (2022). A meta-analysis of effects of 3-nitrooxypropanol on methane production, yield, and intensity in dairy cattle. *Journal of Dairy Science, 106*(2), 927–936.

Krause, A. J., Miracle, R. E., Sanders, T. H., Dean, L. L., & Drake, M. A. (2008). The effect of refrigerated and frozen storage on butter flavor and texture. *Journal of Dairy Science, 91*(2), 455–465.

Laikoja, K., Teder, L., & Jõudu, I. (2017). Assessment of chemical and sensory quality of unsalted and salted sweet cream butter during storage at different temperatures and time. *Journal of Agricultural Science, 2*, 76–81.

Larsen, M., Andersen, K., Kaufmann, N., & Wiking, L. (2014). Seasonal variation in the composition and melting behavior of milk fat. *Journal of Dairy Science, 97*, 4703–4712.

Lopes, J. C., de Matos, L. F., Harper, M. T., Giallongo, F., Oh, J., Gruen, D., et al. (2016). Effect of 3-nitrooxypropanol on methane and hydrogen emissions, methane isotopic signature, and ruminal fermentation in dairy cows. *Journal of Dairy Science, 99*(8), 5335–5344.

Lopez, C. (2018). Crystallization Properties of Milk Fats. In K. Sato (Ed.), *Crystallization of Lipids: Fundamentals and Applications in Food, Cosmetics and Pharmaceuticals* (pp. 283–321). New Jersey, USA: Wiley Blackwell.

Lopez, C. (2020). Crystallization and melting properties of milk fat. In T. Truong, C. Lopez, B. Bhandari, & S. Prakash (Eds.), *Dairy fat products and functionality: Fundamental science and technology* (pp. 205–243). Cham, Switzerland: Springer International Publishing.

Melgar, A., Harper, M. T., Oh, J., Giallongo, F., Young, M. E., Ott, T. L., et al. (2020a). Effects of 3-nitrooxypropanol on rumen fermentation, lactational performance, and resumption of ovarian cyclicity in dairy cows. *Journal of Dairy Science, 103*(1), 410–432.

Melgar, A., Lage, C. F. A., Nedelkov, K., Raisanen, S. E., Stefenoni, H., Fetter, M. E., et al. (2021). Enteric methane emission, milk production, and composition of dairy cows fed 3-nitrooxypropanol. *Journal of Dairy Science, 104*(1), 357–366.

Melgar, A., Welter, K. C., Nedelkov, K., Martins, C., Harper, M. T., Oh, J., et al. (2020b). Dose-response effect of 3-nitrooxypropanol on enteric methane emissions in dairy cows. *Journal of Dairy Science, 103*(7), 6145–6156.

Mortensen, B. K. (2012). *Butter and related products: Product characteristics, production technology and quality aspects*. Odense, Denmark: International Dairy Books.

O'Callaghan, T. F., Faulkner, H., McAuliffe, S., O'Sullivan, M. G., Hennessy, D., Dillon, P., et al. (2016). Quality characteristics, chemical composition, and sensory properties of butter from cows on pasture versus indoor feeding systems. *Journal of Dairy Science, 99*(12), 9441–9460.

Ortiz-Gonzalez, G., Jimenez-Flores, R., Bremmer, D. R., Clark, J. H., DePeters, E. J., Schmidt, S. J., et al. (2007). Functional properties of butter oil made from bovine milk with experimentally altered fat composition. *Journal of Dairy Science, 90*(11), 5018–5031.

Păduret, S. (2021). The effect of fat content and fatty acids composition on color and textural properties of butter. *Molecules, 26*(15), 4565.

Rønholt, S., Buldo, P., Mortensen, K., Andersen, U., Knudsen, J. C., & Wiking, L. (2014). The effect of butter grains on physical properties of butter-like emulsions. *Journal of Dairy Science, 97*(4), 1929–1938.

R core Team (2019). https://www.r-project.org/.

Schilde, M., von Soosten, D., Huther, L., Meyer, U., Zeyner, A., & Danicke, S. (2021). Effects of 3-nitrooxypropanol and varying concentrate feed proportions in the ration on methane emission, rumen fermentation and performance of periparturient dairy cows. *Archives of Animal Nutrition, 75*(2), 79–104.

Smet, K., Coudijzer, K., Fredrick, E., De Campeneere, S., De Block, J., Wouters, J., et al. (2010). Crystallization behavior of milk fat obtained from linseed-fed cows. *Journal of Dairy Science, 93*(2), 495–505.

Staniewski, B., Ogrodowska, D., Staniewska, K., & Kowalik, J. (2021). The effect of triacylglycerol and fatty acid composition on the rheological properties of butter. *International Dairy Journal, 114*, 104913.

Thiel, A., Rumbeli, R., Mair, P., Yeman, H., & Beilstein, P. (2019). 3-NOP: ADME studies in rats and ruminating animals. *Food and Chemical Toxicology, 125*, 528–539.

Ullah, R., Nadeem, M., Imran, M., Khan, M. K., Mushtaq, Z., Asif, M., et al. (2020, Jan 16). Effect of microcapsules of chia oil on omega-3 fatty acids, antioxidant characteristics and oxidative stability of butter. *Lipids in Health and Disease, 19*, 10.

Van Gastelen, S., Dijkstra, J., Heck, J. M. L., Kindermann, M., Klop, A., de Mol, R., et al. (2022). Methane mitigation potential of 3-nitrooxypropanol in lactating cows is influenced by basal diet composition. *Journal of Dairy Science, 105*(5), 4064–4082.

Van Wesemael, D., Vandaele, L., Ampe, B., Cattrysse, H., Duval, S., Kindermann, M., et al. (2019). Reducing enteric methane emissions from dairy cattle: Two ways to supplement 3-nitrooxypropanol. *Journal of Dairy Science, 102*(2), 1780–1787.

Viriato, R. L. S., Queiros, M. S., Neves, M. I. L., Ribeiro, A. P. B., & Gigante, M. L. (2019). Improvement in the functionality of spreads based on milk fat by the addition of low melting triacylglycerols. *Food Research International, 120*, 432–440.

Volden, H. (2011). *NorFor – The Nordic feed evaluation system* (p. 130). Wageningen Academic Publishers, EAAP publication.

Whelan, V. J. (2017). Difference from control (DFC) test. In L. Rogers (Ed.), *Discrimination testing in sensory science* (pp. 209–236). Cham, Switzerland: Springer International Publishing.