EVALUATION OF OIL-IN-WATER NANOEMULSIONS AS A POTENTIAL SUPPLEMENT IN DAIRY PRODUCTION

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SUMMARY

The following work evaluated oil-in-water nanoemulsions as a unique method for supplementing ruminant diets with various edible oils rich in polyunsaturated fatty acids. The assessment was based on three distinct and sequential analytical procedures: chromatographic analysis of fatty acid methyl esters (FAME), size and stability investigation utilizing a high-resolution electron microscope, and Zetasizer apparatus. This research used eight kinds of oil obtained from reputable sources: sunflower oil, maize oil, soybean oil, linseed oil, fish oil, olive oil, cottonseed oil, and sesame seed oil. The oil-in-water nanoemulsions were created using a 750-Watt, 20 kHz, 25 mm sonotrode tipped SONICS VCX750 ultrasonic processor with a nominal power of 750 Watts. The nanoemulsion method had little effect on raw oils fatty acid profile composition since the profiles of crude and nanoemulsified oils were almost identical. In addition, samples that were sonicated at 40% amplitude for 20 minutes resulted in a large droplet size distribution and a higher stability potential regardless of the type of oil used. However, when samples were sonicated at 80% amplitude for 20 minutes, the size distribution and zeta potential were smaller than those with 40% amplitude. Additionally, the nanoemulsion production is severely affected by high sonication temperatures (>70°C) and low surfactant levels; it was also found that storing edible oils nanoemulsion at room temperature for up to 15 days is acceptable. It was also clear that using Tween 80 to up to 11.2% of the emulsion did decrease the droplet size of the nanoemulsion; however, it did negatively affect the rumen fermentation, especially the acetate and propionate concentrations when compared to moderate Tween 80 level (5.6% of the emulsion). In conclusion, ultrasonication is ideal for producing nanoemulsions, mainly when the appropriate amplitude, surfactant level, and temperature are used, especially since nanoemulsion preparation had no appreciable effect on the FAME composition of the oil.

***Keywords**: Oils, Nanoemulsions, Fatty acid, Droplet size, Zeta potential*

INTRODUCTION

It has been demonstrated that edible oils can alter the fatty acid content of the rumen by affecting the activity of rumen microorganisms, particularly biohydrogenation bacteria. It has also been shown that biohydrogenation bacteria can change the fatty acid content of the rumen by explicitly affecting the accumulation of long-chain fatty acid and the formation of trans-vaccenic acid and conjugated linoleic acid (Ramirez *et al.,* 2016, Kliem *et al.,* 2017). Unsaturated fatty acids are abundant in certain vegetable oils (Poyato *et al.,* 2014); olive oil is rich in monounsaturated fatty acids, whereas sunflower and linseed oils are good sources of polyunsaturated fatty acids (PUFA). It is also well known that the physical and chemical characteristics of oils are affected not only by the fatty acid content of oils but also by the oil manufacturing method (Bhatnagar *et al.,* 2009, Poyato *et al.,* 2014). Some restrictions and limits exist on supplementing ruminant diets with edible oil owing to the risk of damaging rumen fermentation. In the rumen, dietary lipids alter the digestive pattern, which may alter the volatile fatty acid composition (Bionaz *et al.,* 2020). Dietary lipids change the digestion pattern during the fermentation process that occurs in the rumen. These modifications, some of which may reduce the content of volatile fatty acids, it is also well known that the rumen generates less methane when the diet contains more fat (Alvarez-Hess *et al.,* 2019, Bionaz *et al.,* 2020). This is because dietary fat decreases the quantity of hydrogen collected via fatty acid biohydrogenation, the amount of fermentable organic matter ingested, the pace at which fiber is digested, and the number of ruminal bacteria and their activity (Alvarez-Hess *et al.,* 2019).

Additionally, rumen biohydrogenation bacteria transform dietary unsaturated fatty acids (UFA) into saturated fatty acids to protect their cell structure against UFA, especially polyunsaturated fatty acids (Brzozowska and Oprzadek, 2016, Cancino-Padilla *et al.*, 2021). These procedures improve saturated fatty acid absorption post-ruminally. Thus, it is important to examine different ruminant diet-added oils that might prevent PUFAs from being digested by rumen lipolysis and biohydrogenation without substantially changing the rumen fermentation pattern or microbes (El-Sherbiny *et al.,* 2023). Nanoemulsions can be utilized in countless applications and sectors. Pharmacy and medication delivery are among the most prominent industries in which nanoemulsions are used as nanocarriers to treat different ailments. Recent nanoemulsion-solubilized pharmaceuticals include antibiotics, anticonvulsants, and antihypertensive agents (Jaiswal *et al.,* 2015). Oil-in-water nanoemulsion is a relatively recent form in dairy production, yet it is one of the most important nanotechnologies with multiple scientific and practical applications. Nanoemulsions are multiphase colloidal dispersions created by dispersing one liquid in another immiscible liquid at the nanoscale through physical-share-induced rupturing with droplet sizes smaller than 200 nm (Mason *et al.,* 2006). Based on our previous *in vitro* and *in vivo* trials (Yousef *et al.,* 2022, El-Sherbiny *et al.,* 2023), introducing a nanoemulsified form of edible oil to the rumen fermentation environment may result in a higher outflow of unsaturated fatty acids from the rumen (bypassing the rumen); as a result, higher accumulation of unsaturated fatty acids may be observed in both rumen and milk. However, despite the positive results, oil-in-water nanoemulsions are still a new supplement in livestock production and require more study to optimize their production conditions. Additionally, nanoemulsions may lose their capabilities over time as droplet size increases unless adequately prepared to manage droplet size distribution and protected against ripening (Tadros *et al.,* 2004). Consequently, this study aimed to track and evaluate the quality and potential of the produced nanoemulsion as a possible supplement in dairy animal feeding using four different criteria, a) storage; b) preparation temperature; c) Tween 80 level, and d) ultrasonication amplitude.

MATERIALS AND METHODS

Preparation of oil-in-water nanoemulsions:

To produce a droplet size distribution of 6.5 ± 0.35 μm, edible oils in water were premixed at 13,500 rpm for 2 minutes with a digital high-speed homogenizer (HG-15D Homogenizer, Daihan Scientific C., Gangwon-Do, South Korea). The oil-in-water nanoemulsion was then created utilizing the pre-homogenized solution and a Sonics VCX750 ultrasonic processor with a 750-Watt nominal power and a frequency of 20 kHz equipped with a 25 mm sonotrode tip (Sonics and Materials, Newtown, USA) (Kentish *et al.,* 2008). A 15% edible oil (sunflower oil; corn oil; soybean oil; linseed oil; fish oil; olive oil; cottonseed oil; sesame seed oil bought from local markets) was used in the oil-in-water emulsion.

Evaluation of oil-in-water nanoemulsions:

Nanoemulsions are thermodynamically unstable when compared to microemulsions. The droplet size of nanoemulsions ranges from less than 200 nm to even less than 100 nm in some circumstances (Kentish *et al.,* 2008, El-Sherbiny *et al.,* 2023). However, because there is no gravitational separation and droplet aggregation due to the reduced attractive force between the small-sized droplets, nanoemulsions have kinetic stability; therefore, tracking the nanoemulsion efficiency and stability cannot be estimated using the amount of gravitational phase separation as in Mousa et al. (2022); instead, the size distribution and Zeta potential of the produced nanoemulsion may represent a more objective approach to evaluating oil-in-water nanoemulsions. In that section, three different oil-in-water nanoemulsions (made from olive oil, corn oil, and linseed oil) were assessed based on four criteria:

1- Tracking the effect of storage time (30 days) at a room temperature of < 35°C on the average droplet size and Zeta potential. Briefly, three nanoemulsions solution (100 ml each) were prepared in different beakers for each type of oil and then stored at the room temperature range of 25-35°C. Two samples of each beaker were collected every five days (days 0, 5, 10, 15, 20, 25, and 30) and analyzed for size and potential in duplicates.
2- Studying the effect of the level of emulsifier, which in our case was Tween 80, on the nanoemulsion droplet size and potential. Three level of Tween 80 was evaluated based on the level used by Kentish et al. (2008), the first level was 2.8%, the second level was 5.6%, and the third level was 11.2% of the total emulsion volume (the rest of the emulsion formulation consisted of distilled water. For each Tween 80 level, three nanoemulsions solution (100 ml each) were prepared in different beakers for each type of oil; then, two samples of each beaker were collected and analyzed for size and potential in duplicates.

3- Investigating the effect of preparation temperature, mainly due to the heat produced during the ultrasonication. Three different temperature setup was tested, a) performing ultrasonication at a temperature of < 30°C by keeping the preparation beaker in ice during the production process, b) performing ultrasonication at a temperature between 30°C and 70°C by keeping the preparation beaker inside water during the production process, and finally, c) performing ultrasonication in a temperature that exceeds 70°C. The temperature was tracked continuously using the ultrasonic system to ensure the temperature was kept within the tested range. For each temperature range, three nanoemulsions solution (100 ml each) were prepared in different beakers for each type of oil. Two samples of each beaker were collected and analyzed for size and potential in duplicates. The samples were also screened using a transmission electron microscope.
4- Testing the effect of ultrasonication amplitude, three different amplitude level was tested, 40%, 60%, and 80%. For each amplitude level, three nanoemulsions solution (100 ml each) were prepared in different beakers for each type of oil; then, two samples of each beaker were collected and analyzed for size and potential in duplicates.

In vitro trial:

Based on the results of the Tween 80 level evaluation, it was clear that increasing the emulsifier level could result in lower droplet size distribution and high stability. That is why it was needed to test the effect of the high Tween 80 on the rumen fermentation pattern using batch fermentation cultures following the procedure of El-Sherbiny *et al.* (2023); in that study, only corn oil was used in raw and nanoemulsified form. Briefly, three cows from the slaughterhouse of El-Munib in Giza were used to provide the rumen inoculum. Slaughtered cows were fed a 50:50 dry matter (DM) diet of concentrates mixture and berseem hay and had free access to fresh water. Each cow's top, bottom, and middle rumen fluid was collected individually. The ruminal fluids from all three cows were equally blended and filtered through four layers of cheesecloth into a Schott Duran bottle (Schott North America Inc., Elmsford, New York, USA) stored at 39°C under anaerobic circumstances. The collected ruminal fluid was immediately transported to the Dairy Production, National Research Centre, Giza, Egypt laboratory, placed in a water bath pre-heated to 39°C, combined in a beaker, and diluted with the buffer solution.

The components and chemical composition of the control substrate (feed) are listed in Table 1. All elements of the control substrate were dried first, and each dry component was milled separately. A homogenous dry matter (DM) combination of the experimental substrate was formed by combining all the dry, milled materials indicated in Table 1.

Table (1): Ingredients and chemical composition of the control diet used for the *in vitro* study.

Item	Control substrate
Ingredients, g/kg of DM	
Corn grain	75.5
Cotton seed meal	116
Sunflower seed meal	85.5
Wheat bran	175
Molasse	35.5
Mineral-vitamin mixture	12.5
Berseem clover	500
Chemical composition, g/kg of DM	
Organic Matter	907.5
Ash	92.5
Crude Protein	174
Either Extract	37.2
Neutral detergent fiber	332
Acid detergent fiber	190

A portion of 400 mg of the prepared dry substrate was weighed into filter bags (ANKOM F57; Ankom Technology, Macedon, NY, USA) and put into the corresponding glass incubation bottle two hours before the experiment began; the bottles were then transported to the incubator set to 39°C (prewarming the feed). The rumen fluid was diluted 1:4 (292 mg of $K_2HPO_4.3H_2O$, 240 mg of KH_2PO_4, 480 mg of $(NH_4)_2SO_4$, 480 mg of NaCl, 100 mg of $MgSO_4.7H_2O$, 64 mg of $CaCl_2.2H_2O$, 4 mg of Na_2CO_3, and 600 mg of cysteine hydrochloride per liter). The dried substrate in a filter bag was placed in a 125-mL glass incubation bottle, and 40 mL of this combination was added. The quantities of raw maize

oil (3%) were estimated based on the DM of the substrate and added straight to the 400 mg substrate before adding the buffered rumen fluid (above the filter bags). The oil content of the produced nanoemulsified form was equal to the oil doses utilized in crude oil treatment. The amounts of supplemented corn oil nanoemulsions (3%) made with low (5.6%) and high (11.2%) Tween 80 were adjusted based on the oil content (15%) of the nanoemulsion preparation to be added at about 20% of the DM basis.

In contrast to the raw oil form, the nanoemulsified oil was added directly to the bottle containing the buffered rumen fluid and bags containing the substrate to imitate the drinking procedure used in the lactating goat experiment (Yousef *et al.,* 2022). For each of the four *in vitro* experiments, three duplicates of batch culture fermentation trials were carried out (three bottles were used for each treatment). Three bottles contained only the dry control substrate, while three others had only culture fluid and no substrate (blank). Each *in vitro* experiment was repeated twice, and each run (repetition) began with a fresh collection of rumen fluid. The bottles were filled with carbon dioxide, tightened with an aluminum cap, and sealed with a rubber stopper. The bottles were then incubated for 24 hours in an anaerobic atmosphere with a pH of 6.5, a temperature of 39°C, and a shaking incubator set at 100 rpm.

Used substrate samples were collected at the beginning of each experiment run and stored at - 20°C until undergoing chemical analysis in triplicate. After 24 hours of incubation, the total gas production for each trial repeat was calculated by subtracting the volume of gas produced in flasks containing substrate and buffered rumen fluid from the volume of gas produced in flasks having no substrate or rumen fluid. The fermentation process was stopped by placing the bottles in a refrigerator at 5 ºC. The filter bags were removed from the bottles and dried in a forced-air oven at 50°C for 48 hours. Three glass tubes containing 5 mL of fermentation fluid samples from each bottle were examined for ammonia nitrogen (NH_3-N), volatile fatty acid (VFA), and fatty acid methyl esters (FAME).

Sampling and chemical analysis:

All samples collected from the evaluation stage were screened for size and potential. Nanoemulsions' particle size and zeta potential were measured using a dynamic light scattering (DLS) device (Zetasizer Nano-ZSP, Malvern Instruments Ltd., Worcestershire, UK). The nanoemulsified materials were decanted into polystyrene cuvettes, and measurements were taken at wavelengths of 633 nm and 10 mW, with measurement angles of 13° and 173°. Measurements were taken twice. Samples of temperature setup were also screened using a transmission electron microscope. Initially, samples were added to 3520C-MB SPI supplies carbon coated 200 mesh copper grids with an additional staining agent, 1% phosphotungstic acid, and then screened with a JEOL JEM-2100 high-resolution and analytical electron microscope equipped with STEM unit (bright- and dark-field detectors) and EDXS detector.

Concentrate and roughage samples collected throughout the *in vitro* study were dried at 55°C for 48 hours before being milled to pass a 1-mm screen (FZ102, Shanghai-Hong Ji Instrument Co., Shanghai, China) and composite. On collected samples, analytical DM (method no. 934.01), ash (method no. 942.05), crude protein (method no. 954.01), and ether extract (method no. 920.39) were measured (AOAC, 2005). Neutral detergent fiber (NDF; Van Soest *et al.* (1991)) and acid detergent fiber (ADF; AOAC (2005); method 973.18) analyses were performed using an ANKOM200 Fiber Analyzer equipment (ANKOM Technology Corporation, Macedon, NY, USA). Before NDF analysis, samples were processed with alpha-amylase and sodium sulfite. Organic matter (OM) was estimated using the difference between NDF and ADF without ash residue.

As for the *in vitro* fermentation parameters, before transferring the bottle to the refrigerator, the pH was measured immediately using a pH meter in the batch fermentation culture trials (Orion star pH meter, Thermo Fisher Scientific Inc., Germany). The total gas production (TGP) was determined for each trial repetition by subtracting the volume of gas produced in flasks containing substrate and buffered rumen fluid from the volume of gas produced in flasks containing neither substrate nor rumen fluid. By subtracting the original (substrates) and final (residues) substrate DM weights, the *in vitro* dry matter degradations (IVDMD) were calculated. According to El-Sherbiny *et al.* (2016), the colorimetric Nessler method determined the ammonia nitrogen content (NH_3-N). The volatile fatty acids were evaluated with few adjustments, according to El-Sherbiny *et al.* (2016). Briefly, 0.8 mL of fermentation liquid was blended with 0.2 mL of a solution containing 250 g of metaphosphoric acid/L. At the Central Laboratories Network, National Research Centre in Cairo, Egypt, the concentration and molar proportions of VFAs were assessed using a gas chromatograph (GC) equipped with an automated sampler (Model 7890B; Agilent Technologies, Palo Alto, CA, USA). The GC-MS was fitted with an HP-FFAPv capillary column (19091F-112; 0.320 mm outer diameter, 0.50 m inner diameter, and 25 m length; J & W Agilent Technologies Inc., Palo Alto, California, United States). The integrator was

calibrated using a mixture of known amounts of individual short-chain fatty acids (acetate, propionate, and butyrate) as an external reference (Sigma Chemie GmbH, Stein, Germany). The VFA peaks were found qualitatively and statistically by combining Fluka-purchased individual VFAs with external standards (Sigma Aldrich, MO, USA). For data processing, Microsoft Workstation 5.0 was employed.

Samples of raw oils, nanoemulsions, and rumen fluid were analyzed for fatty acid methyl esters (FAME) using GC. The approach for FAME analysis was outlined by El-Sherbiny *et al.* (2023). In brief, 3 mL of 2 M NaOH was used to hydrolyze the samples in a closed system utilizing 15 mL Pyrex tubes with Teflon stoppers. The hydrolyzed samples were incubated for 40 minutes at 90 °C in a block heater. The extracted materials were then esterified with 0.5 M NaOH in methanol and transformed to FAME in boron trifluoride (1.3 M; Fluka-Sigma Aldrich, St. Louis, MO, USA). Using a gas GC-MS system (7890B, Agilent, Santa Clara, CA, USA) with a mass spectrometer detector and a 100 m fused silica capillary column (0.25 mm i.d., coated with 0.25 m Agilent HP; Chrompack CP7420; Agilent Technologies, Santa Clara) (5977A). Hydrogen flowed at 1.3 mL/min as the carrier gas throughout the FAME chromatographic analysis. The injector and detector temperatures were 200 °C and 250 °C, respectively. The oven temperature was adjusted to begin at 120 °C for 7 minutes before increasing by 7 °C per minute to 140 °C, where it remained for 10 minutes before rising by 4 °C per minute to 240 °C. Open Lab CDS version 2.6 was used to identify the peaks by comparing their retention periods to those of the applicable FAME standards (37 FAME Mix, Sigma Aldrich, PA, USA) (Agilent, Santa Clara, CA, USA). In addition, the retention times of a reference standard and conjugated linoleic acid peaks were compared to identify them (a mixture of cis- and trans-9,11 and 10,12- octadecadienoic acid methyl esters; Sigma Aldrich, PA, USA), and the FA compositions were expressed as grams per one hundred grams of total FA. Chromatographic FA studies were conducted by the Central Service Unit of the National Research Centre in Egypt.

Statistical analysis:

Data were analyzed using a one-way ANOVA with the treatment as a constant factor. All analyses were conducted using SAS software (SAS® OnDemand for Academics, 2022 SAS Institute Inc., Cary, NC, USA). At $p \leq 0.05$ and $0.05 < p \leq 0.10$, respectively, treatment effects were deemed significant or trending toward significance.

RESULTS AND DISCUSSION

Fatty acid composition of the used edible oils:

The fatty acid composition of selected edible oils and used surfactant (Tween 80) are present in Table 2. Samples of sunflower oil, corn oil, soybean oil, linseed oil, fish oil, olive oil, cottonseed oil, and sesame seed oil were purchased from local suppliers in the local market to study their fatty acid (FA) composition and to track the changes occurred on their FA as a result of oil-in-water nanoemulsions preparation. The targeted oils were chosen based on availability from trusted suppliers to ensure the purity of each type of oil. Based on the fatty acid results, linseed and fish oil had the most moderate content of fatty acid; a high linoleic acid profile with a moderate level of oleic acid characterized corn oil, soybean, and cottonseed oil. The sunflower oil was higher in linoleic acid than olive oil, which was high in oleic acid profile. As for the sesame seed oil, it was characterized by an almost similar profile of oleic and linoleic acid. The nanoemulsified oils' fatty acid profile presented in Table 3 showed an almost identical profile compared to the crude oil; a slight increase in oleic and linoleic acid profile was stated, which could be explained by the high content of Tween 80 in oleic and linoleic acids profile. Generally, the nanoemulsion procedure didn’t affect crude oil's fatty acid profile composition, as both profiles of crude oils and nanoemulsified oils were almost comparable.

Evaluation of oil-in-water nanoemulsions: effect of storage at room temperature:

One of the most decisive factors of using nanoemulsions in the dairy farm is their ability to preserve their properties for long periods, which is why it was necessary to track the effect of storage time (30 days) on the oil-in-water droplet size (nm) and Zeta potential (mV). Looking at Table 4, it is clear that the oil-in-water nanoemulsion is sensitive to the time it is stored. This sensitivity is shown by a positive correlation beginning on day 10 of storage, also when a significant shift in droplet size and Zeta potential was detected. Even after 15 days of storage, any of the three oil nanoemulsions can still be considered in the nano-size range.

Table (2): Fatty acid composition (g/100g FA) of Tween 80 and the selected edible oils purchased from the local market.

Item	Tested Oils[1]								
	SFO	**CO**	**SBO**	**LSO**	**FO**	**OO**	**CSO**	**SSO**	**Tween80**
C14:0	0.09	0.1	0.11	0.1	0.41	0.02	0.8	0.11	4.12
C16:0	10.5	12.2	11.9	5.2	7.03	16.6	24.4	9.05	15.3
C16:1	0.09	0.2	0.12	0.1	6.33	1.86	0.4	0.15	7.92
C18:0	4.43	2.1	4.74	3.8	1.39	2.7	2.2	6.16	6.32
C18:1	18.6	28.3	21.6	18.7	5.46	61.2	17.2	40.9	50.7
C18:2	60.7	55.1	52.9	16.2	2.48	16.5	54.6	42.2	11.7
C18:3	4.76	0.9	7.65	55.2	1.95	0.66	0.3	0.33	3.98
C20:0	Nd	0.5	0.25	0.2	0.59	0.46	0.1	0.76	Nd
C20:1	0.83	0.3	0.19	0.2	Nd	Nd	Nd	0.21	Nd
C20:2n6	Nd	Nd	Nd	0.1	0.11	Nd	Nd	Nd	Nd
C20:5n3	Nd	Nd	Nd	Nd	35.8	Nd	Nd	Nd	Nd
C21:0	Nd	Nd	Nd	Nd	7.36	Nd	Nd	Nd	Nd
C22:0	Nd	0.1	0.36	0.2	0.24	Nd	Nd	0.13	Nd
C22:1	Nd	Nd	0.11	Nd	2.36	Nd	Nd	Nd	Nd
C22:6n3	Nd	Nd	Nd	Nd	28.4	Nd	Nd	Nd	Nd
C24:0	Nd	0.2	0.07	Nd	0.09	Nd	Nd	Nd	Nd
SFA[2]	15.02	15.2	17.43	9.5	17.11	19.78	27.5	16.21	25.7
UFA[3]	84.98	84.8	82.57	90.5	82.89	80.22	72.5	83.79	74.3
MUFA[4]	19.52	28.8	22.02	19	14.15	63.06	17.6	41.26	58.6
PUFA[5]	65.46	56	60.55	71.5	68.74	17.16	54.9	42.53	15.7

[1]Tested oils: SFO; Sunflower oil, CO; Corn oil, SBO; Soybean oil, LSO; Linseed oil, FO; Fish oil, OO; Olive oil, CSO; Cottonseed oil, SSO; Sesame seed oil, [2]SFA; a total of saturated fatty acids, [3]UFA; a total of unsaturated fatty acids, [4]MUFA; a total of monounsaturated fatty acids, [5]PUFA; a total of polyunsaturated fatty acids, Nd; not detected.

Table (3): Fatty acid composition (g/100g FA) of the nanoemulsified form (NE) of the selected edible oils purchased from the local market.

Item	Tested Oils[1]							
	NSFO	**NCO**	**NSBO**	**NLSO**	**NFO**	**NOO**	**NCSO**	**NSSO**
C14:0	0.07	0.21	0.92	0.09	0.32	0.02	0.52	0.14
C16:0	9.5	9.56	10.3	4.98	5.12	15.7	23.6	8.89
C16:1	0.11	0.31	0.21	0.12	5.67	1.99	0.66	0.23
C18:0	4.43	1.93	4.53	3.52	2.02	2.49	2.05	5.56
C18:1	20.7	29.9	23.1	19.3	6.11	62.9	19.1	42.9
C18:2	59.6	56.3	53.9	16.9	3.16	15.5	53.7	41.1
C18:3	5.22	0.82	5.92	53.7	1.95	0.78	0.22	0.22
C20:0	Nd	0.45	0.33	0.31	0.32	0.62	0.15	0.68
C20:1	0.37	0.22	0.36	0.38	Nd	Nd	Nd	0.18
C20:2n6	Nd	Nd	Nd	0.4	0.55	Nd	Nd	Nd
C20:5n3	Nd	Nd	Nd	Nd	35.8	Nd	Nd	Nd
C21:0	Nd	Nd	Nd	Nd	7.76	Nd	Nd	Nd
C22:0	Nd	0.1	0.27	0.3	0.24	Nd	Nd	0.1
C22:1	Nd	Nd	0.14	Nd	2.36	Nd	Nd	Nd
C22:6n3	Nd	Nd	Nd	Nd	28.5	Nd	Nd	Nd
C24:0	Nd	0.2	0.02	Nd	0.12	Nd	Nd	Nd
SFA[2]	14	12.45	16.37	9.2	15.9	18.83	26.32	15.37
UFA[3]	86	87.55	83.63	90.8	84.1	81.17	73.68	84.63
MUFA[4]	21.18	30.43	23.81	19.8	14.14	64.89	19.76	43.31
PUFA[5]	64.82	57.12	59.82	71	69.96	16.28	53.92	41.32

[1]Tested nanoemulsified (N) oils of; SFO; Sunflower oil, CO; Corn oil, SBO; Soybean oil, LSO; Linseed oil, FO; Fish oil, OO; Olive oil, CSO; Cottonseed oil, SSO; Sesame seed oil, [2]SFA; a total of saturated fatty acids, [3]UFA; a total of unsaturated fatty acids, [4]MUFA; a total of monounsaturated fatty acids, [5]PUFA; a total of polyunsaturated fatty acids, Nd; not detected.

This holds regardless of the type of oil. However, after 20 days of storage, the droplet size in all oil-in-water nanoemulsions exceeded 200 nm reaching an average size of up to 1 µm at day 30 of storage. The Zeta potential's strength indicates the degree to which nearby, similarly charged particles in dispersion are attracted to one another electrostatically. If the molecules or particles in question are small enough, having a high Zeta potential will make them more stable. Therefore, colloids that have a high zeta potential (either positive or negative) are electrically stabilized. In contrast, colloids with a low Zeta potential tend to coagulate or flocculate (Kentish *et al.,* 2008).

Table (4): Average droplet size (nm) and Zeta potential (mV) of the nanoemulsified edible oils as affected by storage (days) at room temperature (< 35°C).

Item	Day 0	Day 5	Day 10	Day 15	Day 20	Day 25	Day 30	*P*-Value
Nanoemulsified olive oil								
Average Size (nm)	39±2.6^{g}	52±5.8^{f}	123±9.3^{e}	198±13.3^{d}	235±16.3^{c}	522±6.2^{b}	1073±3.2^{a}	<0.001
Zeta potential (mV)	-48±3^{a}	-40±2^{b}	-22±1.6^{c}	-14±0.9^{d}	-11±0.8^{d}	-3±1.7^{e}	1±0.6^{f}	0.009
Nanoemulsified corn oil								
Average Size (nm)	35±2.4^{g}	46±5.3^{f}	105±8.6^{e}	176±12.3^{d}	240±15.1^{c}	553±3.3^{b}	1002±6.8^{a}	<0.001
Zeta potential (mV)	-52±4^{a}	-42±3^{b}	-26±1.9^{c}	-16±1.1^{d}	-12±0.9^{e}	-4±1.9^{f}	1±0.9^{g}	0.001
Nanoemulsified linseed oil								
Average Size (nm)	41±2.2^{g}	61±4.9^{f}	145±7.9^{e}	188±11.2^{d}	260±13.8^{c}	623±7.1^{b}	1132±5.3^{a}	0.003
Zeta potential (mV)	-44±3^{a}	-41±2^{a}	-20±1.5^{b}	-17±0.8^{b}	-11±0.6^{c}	-4±2.8^{d}	1±1.5^{e}	<0.001

$^{a–g}$ Means within a row with different superscripts differ ($p < 0.05$).

Evaluation of oil-in-water nanoemulsions: effect of Tween 80 level:

The impact of surfactant level on the size distribution and zeta potential is shown in Table 5. Surfactant is fundamental for the production of oil-in-water nanoemulsions, and the droplet size and the physical properties of the produced emulsion are severely affected by the type and level of surfactant. Tween 80 is a nonionic surfactant and emulsifier frequently used in the cosmetics and food industries. This water-soluble, water-viscous synthetic chemical is essential for dispersing oil in water. Tween 80 (Sigma Aldrich, Darmstadt, Germany) is characterized by high oleic and moderate linoleic acid. According to Kentish *et al.* (2008) and El-Sherbiny et al. 2023, using Tween 80 at 5.6% of the emulsion result in a favorable droplet size distribution and high stability; however, we wanted to test the effect of including Tween 80 at the half and double of that level, 2.8%, and 11.2%, respectively.

Based on the obtained results, smaller droplet size can be observed in all types of oil-in-water nanoemulsions when using Tween 80 at 2.8%; however, the droplet size showed to decrease when the level of Tween 80 is increased, reaching a favorable droplet size distribution and higher stability when Tween 80 is supplied at 11.2% of the emulsion.

To limit the thermodynamically unfavorable contact area between non-polar groups and water, the surfactant molecules in an oil-in-water emulsion are structured so that their non-polar tails associate with one other to form a hydrophobic core (Thadros *et al.,* 2004). The surfactant molecules' hydrophilic head groups protrude into the surrounding aqueous phase. In general, surfactants reduce interfacial tension, decreasing droplet size (Thadros *et al.,* 2004, Kentish *et al.,* 2008).

Table (5): Average droplet size (nm) and Zeta potential (mV) of the nanoemulsified edible oils as affected by the level of Tween 80 in the produced emulsion (%), the preparation temperature (°C) and the ultrasonic processor amplitude (%).

Item	Nanoemulsified olive oil		Nanoemulsified corn oil		Nanoemulsified linseed oil	
	Size (nm)	Potential (mV)	Size (nm)	Potential (mV)	Size (nm)	Potential (mV)
Level of Tween 80 (%)						
2.80%	245±3.76^{a}	-6.1±0.16^{c}	213±3.03^{a}	-8.3±0.24^{c}	266±4.06^{a}	-8.1±0.25^{c}
5.60%	56±0.86^{b}	-41±1.07^{b}	49±0.69^{b}	-47±1.41^{b}	55±0.84^{b}	-43±1.35^{b}
11.2%	39±0.59^{c}	-57±1.49^{a}	35±0.49^{c}	-55±1.64^{a}	34±0.51^{c}	-49±1.54^{a}
p-Value	<0.001	0.008	<0.001	<0.001	<0.001	<0.001
Preparation temperature (°C)						
< 30°C	42±0.64^{c}	-49±1.28^{a}	35±0.49^{c}	-52±1.55^{a}	46±0.73^{c}	-43±1.35^{a}
30-70°C	66±1.01^{b}	-38±0.99^{b}	58±0.82^{b}	-41±1.22^{b}	72±1.11^{b}	-36±1.13^{b}
> 70°C	272±4.18^{a}	-11±0.28^{c}	230±3.28^{a}	-16±0.47^{c}	290±4.43^{a}	-10±0.31^{c}
p-Value	<0.001	<0.001	<0.001	0.004	<0.001	0.001
Ultrasonic processor amplitude (%)						
40%	102±1.56^{a}	-28±0.73^{c}	113±1.63^{a}	-24±0.71^{c}	134±2.05^{a}	-23±0.72^{c}
60%	89±1.36^{b}	-32±0.84^{b}	74±1.05^{b}	-36±1.07^{b}	79±1.27^{b}	-32±1.01^{b}
80%	66±1.01^{c}	-41±1.07^{a}	59±0.84^{c}	-49±1.46^{a}	63±0.96^{c}	-43±1.35^{a}
p-Value	0.007	0.001	<0.001	<0.001	<0.001	0.003

$^{a–f}$*Means within a column with different superscripts differ (p < 0.05)*

Evaluation of oil-in-water nanoemulsions: effect of sonication temperature:

According to Table 5, producing nanoemulsion under high temperatures, despite the oil type used, increasing temperature to over 70°C can result in an inconsistent emulsion due to the gathered oil droplets. The better droplet size distribution and higher zeta potential were obtained when performing ultrasonication at temperature < 30°C; however, nanoemulsion production is recommended at a temperature range of 30-70°C due to the comparable results and the low cost of production instead of supplying ice that keep production temperature under 30°C, which is not practical on farm basis. Additionally, the differences in droplet size and distribution in Fig.1 highlighted the comparison between producing nanoemulsions at a temperature range of 30-70°C and a production setup with a temperature above 70°C.

Evaluation of oil-in-water nanoemulsions: effect of sonication amplitude:

The results of applying different sonication amplitude in the production of oil-in-water nanoemulsions are presented in Table 5. Three different amplitude sets were used; 40%, 60%, and 80%. The sample sonicated at 40% amplitude for 20 min resulted in higher droplet size distribution and moderate stability potential despite the type of oil used; when samples were sonicated at 60% amplitude for 20 min, the size distribution was lower than sample sonicated under 40% amplitude by around 14%. Zeta potential was also lower, showing better stability.

The same observation was shown in samples sonicated at 80% amplitude, where lower droplet size distribution and lower zeta potential were observed compared to both 40% and 60% amplitudes. Based on the observations, sonicating nanoemulsified oils under 80% amplitude for 20 min resulted in optimal droplet size distribution and better stability potential. According to Shojaeiarani *et al.* (2020), the ultrasonic processor amplitude represents the distance the sonicator tip can longitudinally fluctuate. By increasing the amplitude, cavitation intensity within the liquid is also increased. In other words, when the sonication process's amplitude increases, the nanoemulsions' particle size substantially decreases.

In vitro trial:

According to our earlier research findings, a higher Tween 80 improved droplet distribution and stability in the created nanoemulsion. The study's findings are summarised in Table 6, which compares the effects of creating oil-in-water nanoemulsions with low and high concentrations of Tween 80 on the fundamental parameters of the rumen, as well as the volatile fatty acid and fatty acid composition.

The treatments that utilized nanoemulsified corn oil with low Tween (NCOT1) did not affect the pH of the fermentation culture or the ammonia concentration, in contrast to the diet that served as the control (CON). However, as compared to raw corn oil (CO), high tween nanoemulsified corn (NCOT2), and the diet that served as the control, the NCOT1 had a substantially more significant influence (P<0.01) on the

amount of ammonia, IVDMD, and total volatile fatty acid content. Because of NCOT1, the molar ratios of acetate and propionate were considerably altered for the better. The incorporation of NCOT1 into the fermentation culture at a concentration of 3% of DM resulted in a reduction (P<0.01) in the amount of vaccenic acid (trans-11 C18:1), in addition to a reduction in the total number of CLA isomers. This was especially true for C18:2 cis-9 trans-11, which experienced a considerable drop in concentration compared to NCOT1.

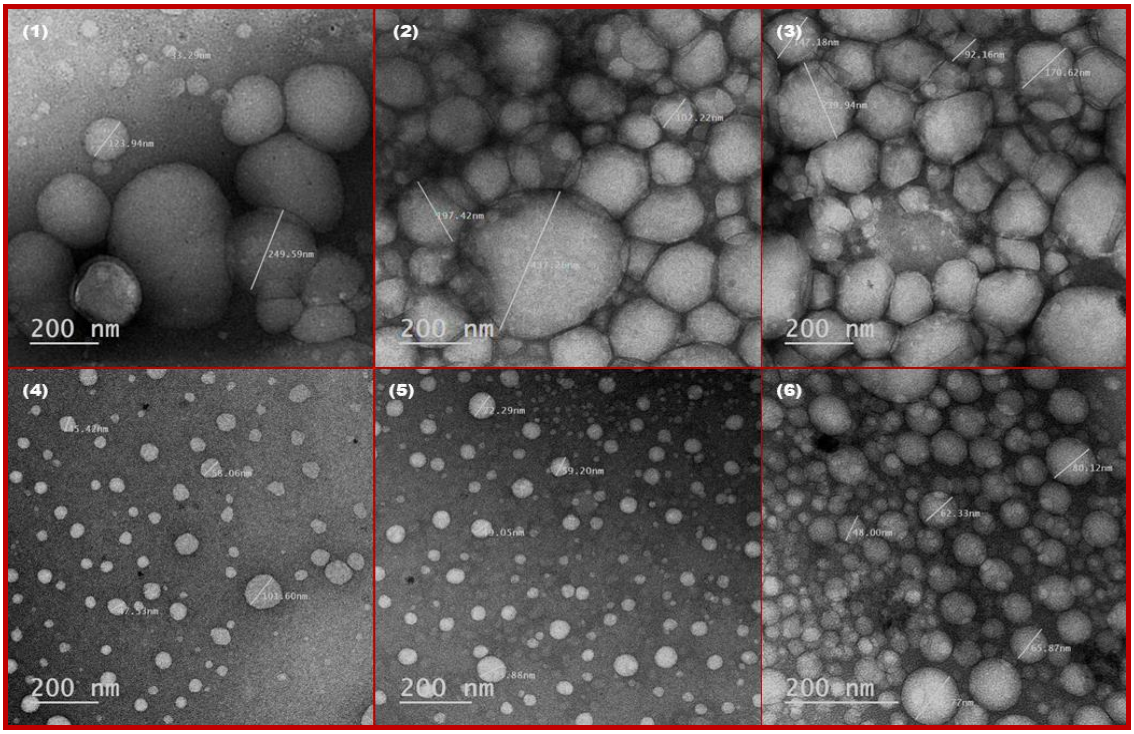

Fig 1. Transmission electron microscope micrographs showing the droplet size of oil-in-water nanoemulsions prepared from three different oils under two thermal conditions as follows: 1) NE of olive oil prepared under high temperature (> 70°C), 2) NE of corn oil prepared under high temperature (> 70°C), 3) NE of linseed oil prepared under high temperature (> 70°C), 4) NE of olive oil prepared at a temperature range of 30-70°C, 5) NE of corn oil prepared at a temperature range of 30-70°C, 6) NE of linseed oil prepared at a temperature range of 30-70°C.

In terms of C18 UFA, the supplementation of NCOT1 it has led to a significant rise (P<0.01) in the proportions of oleic acid (cis-9 C18:1), linoleic acid (cis-9 cis-12 C18:2), and linolenic acid (cis-9 cis-12 cis-15 C18:3) when compared to the raw form, NCOT2, and the control group. Based on the obtained results, it is clear that using a high Tween 80 did affect the rumen fermentation pattern in a negative, making it suitable to use Tween 80 at a moderate level (5.6%). In terms of nanoemulsion in general, it appears that this technology aids in retaining higher amounts of polyunsaturated fatty acids than raw addition. This finding could be explained by the direct prevention of ruminal lipolysis and/or biohydrogenation, which prevents a high proportion of PUFA from getting saturated under biohydrogenation conditions; however, our findings do not support this idea. Nonetheless, Bauchart *et al.* (1990) emphasize two distinct metabolic activities of biohydrogenation bacteria toward UFA, particularly linoleic acid: first, extensive biohydrogenation of UFA, and second, protection of these UFA from biohydrogenation via uptake and incorporation into cellular free fatty acids. Because of the nanodroplet size of the nanoemulsified form of used oil, the permeability or uptake of this fatty acid by the bacterial cell may be increased, preventing more considerable proportions of UFA from being bio-hydrogenated to SFA. That was corroborated by subsequent research and validated by our in vivo findings when feeding nanoemulsified corn oil to lactating dairy goats (Yousef *et al.*, 2022).

Table (6): Effect of the supplementation of raw corn oil and nanoemulsified corn oil produced with two different levels of Tween 80 on basic rumen parameters, volatile fatty acid, and fatty acid composition in batch fermentation cultures.

Item	Treatments[1]				SEM	*P*-Value
	CON	CO	NCOT1	NCOT2		
Rumen basic parameters						
pH	6.17^{a}	6.06^{b}	6.19^{a}	6.03^{c}	0.019	0.002
TGP, mL/g DM	257^{b}	241^{d}	261^{a}	244^{c}	1.856	<0.001
Ammonia-N, mmol/L	11.2^{a}	10.1^{b}	11.4^{a}	10.3^{b}	0.108	0.005
IVDMD[2], %	54.6^{b}	51.8^{c}	55.3^{a}	52.5^{c}	0.319	0.002
Volatile fatty acids (VFA), mmol/L						
Total VFA	101^{b}	96.1^{c}	108^{a}	100^{b}	1.434	0.001
Acetate (A)	60.9^{b}	58.2^{c}	64.6^{a}	59.9^{b}	0.311	<0.001
Propionate (P)	22.5^{b}	20.8^{c}	23.1^{a}	21.5^{bc}	0.182	0.015
Butyrate	18.3^{b}	16.6^{d}	18.8^{a}	17.7^{c}	0.105	<0.001
A:P ratio	2.71^{b}	2.79^{a}	2.79^{a}	2.78^{a}	0.009	0.082
Fatty acid methyl esters, g/100g FA						
C14:0	2.68^{a}	1.75^{b}	1.47^{d}	1.68^{c}	0.094	0.001
C14:1 cis-9	1.92^{a}	1.19^{b}	0.84^{d}	0.93^{c}	0.091	0.009
C16:0	20.7^{a}	18.6^{b}	15.4^{d}	17.3^{c}	0.415	0.003
C16:1 cis-9	0.92^{b}	0.84^{c}	1.01^{a}	0.93^{b}	0.028	0.016
C18:0	28.7^{c}	31.3^{a}	25.4^{d}	29.8^{b}	0.657	0.001
C18:1 trans-10	1.69^{b}	2.21^{a}	1.17^{c}	2.24^{a}	0.116	<0.001
C18:1 trans-11	4.32^{b}	5.71^{a}	3.31^{c}	4.12^{b}	0.305	<0.001
C18:1 cis-9	6.03^{d}	11.3^{c}	19.6^{a}	12.9^{b}	1.538	<0.001
C18:2 cis-9 cis-12	3.75^{d}	3.93^{c}	9.79^{a}	4.92^{b}	0.696	<0.001
C18:2 cis-9 trans-11	0.23^{d}	0.45^{a}	0.28^{c}	0.32^{b}	0.031	<0.001
C18:2 trans-10 cis-12	0.12^{c}	0.33^{a}	0.15^{c}	0.22^{b}	0.022	0.003
C18:3 cis-9 cis-12 cis-15	0.38^{d}	0.49^{b}	0.67^{a}	0.43^{c}	0.158	0.006

$^{a–c}$ Means within a row with different superscripts differ ($p < 0.05$). [1] Treatments, control diet (CON), raw corn oil supplementation at 3% of DM (CO), nanoemulsified corn oil supplementation at 3% of DM prepared with 5.6% of tween 80 (NCOT1), and nanoemulsified corn oil supplementation at 3% of DM prepared with 11.2% of tween 80 (NCOT2). [2] In vitro dry matter degradation.

CONCLUSION

In conclusion, nanoemulsion preparation is a very sensitive process and is affected by different factors. Based on the current study, it was observed that high sonication temperature, low surfactant level, and lower sonication amplitude could result in bigger droplet size distribution and unstable nanoemulsion. It is also clear that storing produced nanoemulsion for more than 15 days could result in an increase in droplet size and a decrease in zeta potential. Consequently, it is recommended to produce edible oil-in-water nanoemulsion at 80% amplitude, 5.6% level of Tween 80, and a temperature range of 30-70°C. Extending the research to evaluate the factors that affect nanoemulsion production and storage is also needed.

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تقييم مستحلبات الزيت في الماء النانومترية كمكمل محتمل في إنتاج الألبان

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في العمل التالي، قمت بتقييم المستحلبات النانومترية كطريقة فريدة لتدعيم علف الحيوانات المجترة بالزيوت النباتية المختلفة الغنية بالأحماض الدهنية غير المشبعة. اعتمد التقييم على ثلاثة إجراءات تحليلية متتالية: تحليل الكروماتوغرافيا للأحماض الدهنية (FAME)، وفحص الحجم والثبات باستخدام ميكروسكوب إلكتروني عالي الدقة، وجهاز Zetasizer. استخدمت في هذا البحث ثمانية أنواع من الزيوت تم الحصول عليها من مصادر موثوقة: زيت عباد الشمس وزيت الذرة وزيت فول الصويا وزيت بذر الكتان وزيت السمك وزيت الزيتون وزيت بذرة القطن وزيت بذور السمسم. تم إنشاء مستحلبات نانومتريه للزيت في الماء باستخدام معالج الموجات فوق الصوتية SONICS VCX750 بقوة 750 وات و 20 كيلوهرتز و 25 مم مع قوة تبلغ 750 واط. كان لطريقة تحضير المستحلب النانومترية تأثير ضئيل على تكوين الأحماض الدهنية للزيوت الخام حيث إن خصائص الزيوت الخام والزيوت المستحلب النانومتري كانت متطابقة تقريبًا. بالإضافة إلى ذلك، أدت العينات التي تم انتاجها بسعة 40٪ لمدة 20 دقيقة إلى توزيع حجم قطيرات كبير واستقرار أعلى بغض النظر عن نوع الزيت المستخدم. ومع ذلك، عندما انتجها بسعة 80٪ لمدة 20 دقيقة، كان توزيع الحجم وzetapotential أصغر من تلك ذات السعة 40٪. يتأثر إنتاج مستحلب النانومتري بشدة بدرجات حرارة التحضير العالية (أكثر من 70 درجة مئوية) وانخفاض مستويات المادة المستحلبة؛ كما وجد أن تخزين مستحلب النانو لزيوت الطعام في درجة حرارة الغرفة لمدة تصل إلى 15 يومًا أمر مقبول. كان من الواضح أيضًا أن استخدام Tween 80 حتى 11.2٪ من المستحلب قد قلل من حجم قطرات المستحلب النانومتري؛ ومع ذلك ، فقد أثر سلبا على تخمرات الكرش ، وخاصة تركيز الأسيتات والبروبيونات عند مقارنته بمستوى Tween 80 المعتدل (5.6٪ من المستحلب). في الختام، تعتبر الموجات فوق الصوتية مثالية لإنتاج مستحلبات نانومترية، خاصة عند استخدام السعة المناسبة ومستوى مناسب من المستحلب ودرجة الحرارة ، خاصة وأن تحضير مستحلب النانومتري لم يكن له تأثير ملموس على تركيبة الاحماض الدهنية للزيت.

الكلمات المفتاحية: الزيوت ، المستحلبات النانومترية ، الأحماض الدهنية ، حجم القطرات ، جهد زيتا