The MilkQua Project

PRIMA S2 – 2018

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Document information

1. Authors

Organisation name lead contractor: **CSIC**

Author	Organisation	Email
1 Sonia Andrés	CSIC	sonia.andres@eae.csic.es
2 F. javier Giráldez	CSIC	j.giraldez@eae.csic.es
3. Latifa Najar	Idele	Latifa.najar@idele.fr
4. Monia Daaloul	ENMV	moniajd2017@gmail.com

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Executive Summary

Background	*[to be filled out]*
Objectives	*[to be filled out]*
Methods	*[to be filled out]*
Results and implications	*[to be filled out]*

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Description

1. Planification of in vivo trials

The ethical authorization for in vivo experiments will be requested to the local authorities in France (Comité d'éthique régional de Bretagne), Spain (Comité de Ética del CSIC) and Tunisia.

The minimum number of animals to be included in each group (treatment) will be 6. The convenience of using this number of animals (6 cows/group) is based on previous studies (http://dx.doi.org/10.3168/jds.2016-10910) which have allowed to obtain conclusive results.

The animals included in each group will be selected as homogeneous as possible, attending to different parameters such as the age, weight, number of lactation (better in 2nd and 3rd laction for feeding trials), milk production or somatic cell counts (min 250.000 cells for France Protocol). Animals with an important bias in any of these parameters should be discarded for experimental purposes. The animals will be stratified based on these parameters (e.g., live body weight, milk production, somatic cell counts….) and randomly allocated to one of two experimental treatments (n = 8 per dietary treatment) (n=6 per prevention of EO mastitis) during the experimental period.

Animals with foot and leg problems leading to lameness, rumen acidosis or any other metabolic disorder, which could negatively affect feed intake and overall productivity, should be discarded for feed intake trials.

If during the development of the experimental procedure any animal presented febrile syndrome for more than 7 days, severe anorexia and cachexia and/or severe respiratory disorders, euthanasia will be carried out according to the ethical authorisation approved by the local authorities. If only local pain and inflammation is detected in the udder after intrammary infusion of essential oils, then the administration of the corresponding pharmaceutical formulation containing EO will be interrumpted and the veterinarian responsable for animal health in the corresponding experimental unit will apply a local treatment (if required after evaluation) to solve the problem. If the animals do not response to the treatment, the veterinarian will also proceed to euthanasia in the same way as explained above.

2. Data Collection

The forms for data collection will be accorded.

Feeding newborn dairy calves' trial with essential oils

Twenty newborn dairy calves will selected from the flock to minimize the range of both, live body weight (LBW) and day of birth. These animals will be separated from the cows in the first 24 hours of life, penned individually, and allocated randomly to one of two groups (n = 10 per group; control -CTRL- and essential oil -EO-). These animals will be bottle fed milk replacer during the first 15 days, twice a day just after parturition, and then using an automatic feeding machine until weaned (70 d of life). A pure commercial essential oil (88% carvacrol content) will be administered once daily from the first day until 45 days of life. The daily dose of carvacrol will be increased progressively from 65 to 130 mg. All the animals will be weighted weekely throughout the suckling period.

After weaning (aprox 2 months of life), all the dairy calves will be managed in feedlot being fed a total mixed ration (TMR-1) formulated (with no EO) to cover their nutritional requeriments during the first post-weaning period (up to 6 months of life), and a second TMR (TMR-2, with no EO) with a higher amount of fibre during the replacement phase (6-12 months of life). Voluntary feed intake of both TMRs will be recorded individually daily by using control feed intake devices during at least 60 days in each period.

Daily sampling of the TMR after mixing (prior to feeding) and sufficient mixing and subsampling to minimize sampling error will be required. Weekly analysis of daily composited feed samples of the TMR supplied to dairy cows will be performed for DM (ISO 6496:1999), ash (ISO 5984:2002), CP (ISO 5983:2009), amylase-treated neutral detergent fiber [(aNDF), NDF assayed with a heat[1] stable amylase and expressed inclusive of residual ash; Ankom Technology Corp., Macedon, NY, USA] and ADF (ADF expressed inclusive of residual ash; Ankom Technology Corp., Macedon, NY, USA).

Animals will the weighed once a month in order to measure feed efficiency traits during this phase. Average daily weight gain (ADG, g/d) will be estimated as the regression coefficient (slope) of LBW against time using the REG procedure of the SAS package (SAS Institute Inc., Cary, NC). The feed conversion ratio (FCR) will be obtained by dividing the feed intake per day by the ADG (g/d). Residual feed intake will be calculated using a multiple linear regression model, into which the DMI, ADG and mid-test metabolic body weight (MBW, as $LBW_{0.75}$) data of all the calves will be introduced. The statistical model used will be: $Y_i = \beta_0 + \beta_1$ MBWi + β_2 $ADG_i + e_i$, where Y_i represents the predicted dry matter intake of the i_{th} animal; β_0 is the equation intercept; β_1, the regression coefficient on MBW; β_2, the regression coefficient on ADG; and e_i, the residual of the i_{th} animal. Then, this prediction may be thought of as the "average" or expected value for animals of similar weights and rates of gain. The actual daily feed intake minus the predicted feed intake of each individual corresponds to the residual feed intake.

Four sampling times will be planned (3 days, 2, 6 and 10 months of life) and analysed as follows:

- Plasma samples to measure indicators of health status (e.g., biochemical profile, antioxidant status, acute-phase inflammatory proteins, cytokines…) during the pre- and post-weaning (replacement) phases will be collected and analysed by CSIC.
- Plasma samples will be transferred to UNIMI-WP5 for metabolomics using couriers with capability for chilled transfer at specified (and monitored) temperatures.
- Feces samples (freezed dried) will be submitted (courier transport) to UNIMI-WP5 for microbiome analysis.
- Blood for flow cytometry and cytokine quantification after lympho stimulation (CSIC).

Results will be analysed by a repeated measurement design using the MIXED procedure of SAS.

Feeding lactating dairy cows with essential oils

LPAF (INRAT) will conduct two feeding trials to investigate the effect of different EOs (trial 1 and trial 2) on feed efficiency and milk yield and quality of adult dairy cattle. Multiparous cows in mid lactation will be randomly assigned to two groups which will receive an additive (EOs) enriched diet or the control diet (without additive). A 2x2 cross-over design will be used. At least 2 weeks of a washout period being fed the control diet will be allowed to both groups as transition period and to avoid the carry-over effect of the additive (EOs) when additive-group shifts to the control diet.

Animals will be milked twice daily at 07:30 and 19:30 h. A total mixed ration (TMR) formulated to meet the nutritional requirements will be supplied after milking. The TMR will be composed of gross forage (silage and hay) and a commercial concentrate for dairy cattle. Feed intake will be controlled for each individual penned animal at least during 60 days[2], and avoiding early lactation if possible (better if the measurements are performed from 120 d of lactation onwards, when the animals have returned to neutral energy balance). Fresh drinking water will be always available. All the animals will be fed a TMR ad libitum (allowing refusal rate of 20%) once a day at 9:00 h, or twice a day at 9:00 and 15:00 h, depending on the size of the feeders used in each location. Cows from the additive-group will be daily drenched with 1.2 g EOs. Individual feed intake will be measured by weighing the amount of feed offered and that of individual refusals.

[1] Samples of the orts will be collected weekly for subsequent analyses of DM content after mixing all the subsamples refused in each feeder during one week.

[2] Coons et al. (2019). J. Dairy Sci. 102:9814–9826 https://doi.org/10.3168/jds.2019-16786

Leftovers will be weighted and collected daily, pooled in weekly composites for each animal (when being fed individually in pens[3]), and analysed for DM content by drying in a forced-air oven at 55°C for 24 h. Daily sampling of the TMR after mixing (prior to feeding) and sufficient mixing and subsampling to minimize sampling error will be required. Weekly analysis of daily composited feed samples of the TMR supplied to dairy cows will be performed for DM (ISO 6496:1999), ash (ISO 5984:2002), CP (ISO 5983:2009), NDF (Ankom Technology Corp., Macedon, NY, USA), ADF (Ankom Technology Corp., Macedon, NY, USA), total fat (Acid Hydrolysis Filter Bag Technique using the AnkomHCl Hydrolysis System) and starch according to Hall (2009[4]). All these data collected along the whole experimental period will allow to express proximal chemical composition of the diet supplied in terms of mean ± SE for each parameter on a dry matter basis, and according to these data NE_L, NE_M and NE_G will be estimated.

All animals will be weighed every two weeks immediately after the 07:30 h milking. Predicted daily BW (BW_{pred}) will be estimated by fitting a linear model of individual periodic BW using the equation:

$$BW_{pred} = b_0 + b_1 \times DIM + b_2 \times (DIM)^2,$$

where b_0 = intercept, and b_1, b_2 = coefficients for linear and quadratic effects of DIM (days in milk), respectively. Using the BW_{pred} values, ADG will be calculated by subtracting BW_{pred} on the first day of available feed intake data from BW_{pred} on the last day of available feed intake data and dividing by the number of days in the test period (Connor et al., 2013).

Commenté [A1]: Different procedure to estimate ADG when compared to the previous experiment.

Digestibility will be determined using acid insoluble ash (AIA) as an internal marker. Feed and faeces will be sampled for 9 d once the animals are completely adapted to being fed the TMR. Samples of offered and refused feed will be taken daily from the first to the seventh day of the collection period; daily rectal grab samples will be collected once a day before the morning meal for the last 7 d of the collection period. Samples of both feed and faeces of each animal will be weighed and pooled to form composite samples and stored at –30°C until AIA analysis (Van Keulen and Young, 1977[5]).

According to Connor et al (2013[6]), daily ME intake will be calculated from weekly feed composite TDN values using the following equations from NRC (2001):

$$DE(Mcal/kg) = TDN\ (\%DM) \times 0.04409$$
$$ME\ (Mcal/kg) = (DE \times 1.01) - 0.45$$
$$Energy\ intake\ (Mcal) = ME \times DMI\ (kg)$$

Commenté [A2]: Formula for Total Digestible Nutrientes needed.

Milk yields will be recorded indivicually at every milking from d 0 to 60 of each period and used to calculate average daily yield. Milk samples for each cow will be obtained once weekly alternating morning and evening milking periods, preserved (how), and stored at 4°C until sent to a commercial laboratory (XXXX) and analyzed for fat percentage, true protein percentage), and somatic cell conunt (SCC, Foss).

Somatic cell count will be transformed and analyzed as SCS according to Shook (1993[7]) using the equation: $SCS = \log_2 (SCC/100) + 3$, where SCC is in units of 1,000 cells/mL.

[3] https://afs.ca.uky.edu/dairy/ten-factors-which-impact-dairy-feed-efficiency
[4] Hall, M. B. 2009. Determination of starch, including maltooligosaccharides, in animal feeds: Comparison of methods and a method recommended for AOAC collaborative study. J. AOAC Int. 92:42–49.
[5] Hall, M. B. 2009. Determination of starch, including maltooligosaccharides, in animal feeds: Comparison of methods and a method recommended for AOAC collaborative study. J. AOAC Int. 92:42–49.
[6] Connor et al., 2013. J. Anim. Sci. 2013.91:3978–3988 doi:10.2527/jas2012-5977
[7] Shook, G. E. 1993. Genetic improvement of mastitis through selection on somatic cell count. Vet. Clin. North Am. Food Anim. Pract. 9:563–581.

According to Connor et al. (2013), predicted daily milk protein ($protein_{pred}$) and fat yield (fat_{pred}) will be estimated for each animal and milking period (morning and evening) by fitting a linear model of individual periodic milk composition using this equation:

$$fat_{pred} \text{ or } protein_{pred}, \text{ kg} = b_0 + MP + b_1 \times DIM + b_2 \times (DIM)_2,$$

where b_0 = intercept, and b_1, b_2 = coefficients for linear and quadratic effects of DIM, respectively; MP = milking period defined as morning (1) or evening (2); DIM = days in milk. Finally, energy-corrected milk (ECM) yield will be calculated from the sum of the morning and evening milking periods using the equation ECM yield = (0.327 × daily milk, kg) + (12.95 × fat_{pred}, kg) + (7.2 × $protein_{pred}$, kg) from Orth (1992[8]).

Commenté [A3]: A little bit differnt form the paper published by Coons et al (2019), where ECM is calculated using the equation: ECM = [(0.327 × kg of milk yield) + (12.95 × kg of fat yield) + (7.65 × kg of protein yield)] according to Tyrrell and Reid (1965).

Tyrrell, H. F., and J. T. Reid. 1965. Prediction of the energy value of cow's milk. J. Dairy Sci. 48:1215–1223

Calculation of RFI during Lactation according to Connor et al., 2013
The GLM procedure (SAS Inst. Inc., Cary, NC) will be used to predict average energy intake for each animal by fitting this regression model (adapted from Van Arendonk et al., 1991):

$$\text{Predicted Energy Intake} = b_0 + \text{Parity (1,2,3+)} + b_1 \times \text{metabolic BW} + b_2 \times \text{ADG} + b_3 \times \text{ECM yield} + \text{RFI},$$

where b_0 = intercept; Parity = animal parity (1 = first; 2 = second; 3+ = third or greater); b_1 = partial regression coefficient of intake on average metabolic BW [$(BW_{pred})0.75$, kg]; b_2 = partial regression coefficient of intake on ADG (kg/d); and b_3 = partial regression coefficient of intake on ECM yield (kg/d).

The RFI (Mcal ME/d) for each animal will be calculated as the difference between actual and predicted average energy intakes during the trial.

Coons et al., 2019 also calculated the following parameters:

3.5% FCM (fat corrected milk) = [(0.432 × kg of milk yield) + (16.216 × kg of fat yield)];

SCM (solids-corrected milk) = [(12.3 × kg of fat yield) + (6.56 × kg of SNF (solids-non fat)) – (0.0752 × kg of milk yield)];

Feed efficiency also will be calculated by dividing milk yield by the dry matter intake (DMI).

Feed efficiency (kg/kg) will be also estimated and expressed as actual milk/DMI, 3.5% FCM/DMI, and SCM/DMI.

Milk N efficiency will be calculated as (kg of milk N/kg of N intake) × 100.

The profile of fatty acids in milk will be determined on GC (Gas Chromatography).

Commenté [A4]: Details should be provided

Urine samples will be taken during two consecutive days for the analyses of purine derivatives and also for urea, creatinin, calcium and magnesium.

Two blood sampling times will be planned (0 days, 60 d) for each experiment with dairy cows (two trials). Animals will be blood sampled after morning milking for metabolomics analysis early in the morning before TMR distribution by jugular venipuncture into tubes containing lithium-heparin, placed in iced water and centrifuged at 3,520 × g for 16 min at 5ºC. Then, plasma samples will be stored at -80ºC until metabolome analyses. Moreover, blood samples will be collected into tubes containing no anti-coagulant. Tubes with no anti-coagulant will be allowed to clot at room temperature, and then centrifuged at 3,520 × g for 16 min at 4ºC. Thereafter, serum samples obtained will be stored at -80ºC until used to measure biochemical profile

[8] Orth, R. 1992. Sample day and lactation report. DHIA 200 Fact Sheet A-2. Mid-states DRPC, Ames, IA.

[total protein, creatinine, urea, calcium, magnesium, phosphorus, β-hydroxybutyrate (BHB) and non-esterified fatty acids (NEFA)]. The measurements will be peformed on serum by sequential competitive immunoassay (Immulite®/Immulite® 1000 Progesterone).

Milking trials with essential oils

IDELE: A small scale study of the curative effect of EOs on mastitis at the level of controlled experimental conditions. The trial will be performed in a herd located in the experimental farm of Méjusseaume (INRAE, France). Twenty-eight Holstein lactating cows will be included in two experimental studies. Cows suffering from mild or moderate clinical mastitis according to the definition given from the International Dairy Federation (International Dairy Federation. Suggested interpretation of mastitis terminology: International Dairy Bulletin - Federation, 1999:3–26). Those suffering from mastitis in more than one mammary gland and with recurrent mastitis will be excluded from the study. The randomized trial will compare each time the effectiveness of t two different treatment strategies (EOs treatment VS Control or EO+antibiotics VS control) . (in 2020 and 2021) control group (no treatment). The adequate dose of EOs will be determinate based on the preliminary in vitro study (WP3) and used after a preliminary skin irritation dose on control animals (sheep). The dosage of antibiotic will be in accordance with manufacture and optimized properly by the veterinarian following the experimentation at Méjusseaume.
The trial will last 30 days.

Commenté [A5]: We can not have 24 cows on the same time and 12 of them killed due to Eos administration

A trial 2 will be conducted (subject to INRAE accreditation) in 2021 to compare the antibiotic + essential oil combination group with a control groupWhole blood samples (5 ml) shall be collected and placed in test tubes and EDTA tubes at T0, T24 and T48 after the last EO administration. The samples will be transported in a cooler to the laboratory for hematological and blood chemistry analysis.

Cows will be milked twice daily (08:30, 19:00 h) in a double-12 parallel milking parlor. Milk sampling will be performed according to the following procedure:
Milk
- Pathogens will be identified by using a standard mastitis diagnostic test. The somatic cell count will be also assessed. Teats will be desinfected using 70% alcohol and after, forestripping the first streams of milk, a composite sample of 10 ml of milk from the four udders will be collected in a labelled aseptic container (after rigorous cleaning of the udder and aseptic sampling) will be taken morning and evening on infected udders at 0, 24h, 48h, D7, D14, D21 and D30 days. The various samples will be sent in optimal conditions of conservation to Villers Bocage technological laboratory for bacteriological, biochemical and sensory analyses (Fatty acid composition and sensorial properties of UHT milk issued of EOs treated animals will be analyzed (IDELE, France) in cohesion with WP6 (PhD study).Samples will be sent to STLO (INRAE) for technological analyses of milk and to the University of Milan for microbiome analysis.

ENMV A large scale study of the curative effect of EOs on mastitis at the level of the farm

A 12-month blind randomized controlled trial will be performed in Tunisian farms in agreement and cooperation of the livestock and pasture office (OEP) and regional veterinarians. Three Veterinary students from ENMV will be involved in the following of the study and data collection.

The calculation of the sample size will be determined according to specific statistics tests (http://hedwig.mgh.harvard.edu/sample_size/size.html). The protocol will be adjusted according to WP2 epidemiological study (task 2.1) and the results of in vitro trials. The treatments that will be applied to animals will be the same as in Task 5.3 (EOs treatment, antibiotics treatment, EOs+ antibiotics treatment).

We will register monthly in the herd individual milk yield and somatic cell count. Milk samples will be collected before and 21 days after the end of the treatment to perform bacteriological studies accordingly to the standard methods of bacteriology (following the Good Laboratory Practices) and/or as explained above

for IDELE trial. Api galleries and other bacteriological tests on milk (staphylococcal agglutination kit, streptococcal grouping kit, Listeria mobility test) will be performed to allow the determination of the specific bacteria. The antimicrobial resistance to antibiotic will be determined according to the method of antibiogram by swabbing or diffusion on agar medium (Mueller-Hinton agar) the rules for the technical implementation of the antibiogram in animal health are defined in the AFNOR standard NF U47-107. The critical thresholds presented in these veterinary recommendations are adapted to this standard. The interpretative reading refers also to this AFNOR norme of CA-SFM veterinarian version 2017.

UHT Milk samples will be transferred to LPAM by courier transport to determine the sensorial properties of milk after EOs treatment (link with WP7).

Statistical analysis will be performed by Data State Unit of both ENMV and IDELE

Commenté [A6]: This part should be disussed with monia as OEP will not be involved but only veterinian school. Please sent her for sharing

3. Identificacion of samples, storage and delivery conditions

A consistent **identification** procedure for samples across the whole project will be implemented:

The first digit in the box containing the samples will be related to the partner producing the sample.

1. IDELE
2. ENMV
3. CSIC
4. LPAF (INRAT)

The second digit in the box containing the samples will describe the type of experiment that the sample comes from:

1. Milking experiment
2. Feeding experiment

The third digit in the box containing the samples will describe the type of sample:

1. Milk
2. Plasma
3. Feed
4. Feaces

The fourth digit in the box containing the samples will describe the type of analyses that the samples is used for:

1. Metabolomics
2. Microbiome
3. Biochemical profile
4. Chemcial composition

The first digit in the sample container will describe the sampling time (using two figures like this: 10, 20, 30,… for the first, second or third sampling times, respectively).

The second digit in the sample container will describe the number of the animal included in the experiment (using two figures like this: 01, 02, 03, 04…).

Thus, milk samples produced in IDELE during the milking experiment, wich are going to be used for studying the microbiome should be contained in a box called “MilkQua 1112”. If we are looking into the box for the second sampling time of the cow number 04 we must find the Eppendorf identified like “2004”.

Storage and delivery conditions

Milk for microbiological analsysis, chemical composisiton and sensorial charcteristics should be kept at 4ºC after collection and immediately transfered to the laboratory analysis were it will be aliquoted for bacteriological studies or kept at -20ºC for chemical analysis, or sensorial atributes.

Milk and plasma samples for metabolome must be kept at -80 ºC. At least two different replicates in two different ultrafreezers. Delivery details; dry ice, courier transport.

Feaces samples for digestibility and microbiome should be freeze dried. Delivery details; room temperature courier transport.