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DEVELOPMENT OF RAPID ANALYTICAL METHODS BASED ON ELECTRONIC NOSE TECHNOLOGY AND NEAR INFRARED SPECTROSCOPY TO ADVANCE DAIRY PRODUCTION

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1. BACKGROUND OF RESEARCH AND OBJECTIVES

Worldwide, dairy production continues to follow a trend towards increased intensification of small, medium and larger farms, into more specialized dairy production units. In intensive dairy farms, the importance of efficient feeding or nutrition cannot be underestimated, since feeding influences the overall growth and development of the animals, and also accounts for almost 70% of dairy production cost. Efficient feeding plays a huge role in the building of cows' body reserves for next lactation cycle, which success largely depends on the quality and quantity of feed supplied. Also, various chemical and physical dietary factors such as neutral detergent fiber (NDF) concentration and particle size can affect rumen fermentation, and consequently milk production and composition. It is important to state, that livestock farmers are aware of the importance of feed quality, but until the introduction of rapid analytical technologies such as near infrared (NIR) spectroscopy, it was not easy to obtain real time quantitative information at an affordable price.

Physiologically, the nutritional composition of milk or dairy products may be influenced by the transfer of specific chemical compounds in the form of nutrients from the feed to the milk through metabolism. Therefore, quality testing and control of milk during and after production is important. Ideally, dairy products quality is not only about the nutritional composition, but also includes organoleptic or sensory properties. Oxidation is an important chemical process which influences the organoleptic properties such as the aroma or odor of dairy products. Off-flavor or odor in dairy products originate mostly from bacterial metabolism, enzymatic activity, photo-oxidation, heat, and oxidation catalysed by chemicals such as pro-oxidants.

For example, headspace of milk typically presents a complex mixture of organic volatiles (e.g. acetone at overwhelming concentration, hexanal, 2-butanone, toluene, limonene, heptanal, styrene, chloroform, etc.) at varying

concentrations and with a high percentage of relative humidity. The aforementioned volatile organic compounds make milk vulnerable to oxidation, and may likely produce off-flavor or odor in milk. This may likely affect the consumer preference for milk, and further reduce the quality of processed milk products.

Through supplementary feeding, i.e. the addition of other nutritive sources to the base feed, milk nutritive value can be improved. In a situation where that is not successful, fortification or enrichment is mostly used to improve the product quality. Dairy product fortification involves the addition of specific micronutrients which are evidently deficient in the dairy product, in order to improve its nutritive value. Fortification of dairy products has been widely used in the dairy industry to achieve desirable nutritional level, flavour and aroma, to increase product acceptability by consumers.

Thus, the composition of feed and dairy products qualities (nutritive and organoleptic) have to be objectively determined, using fast and reproducible analytical methods in order to ensure acceptable final product quality to consumers. Emerging rapid analytical methods such as near infrared (NIR) spectroscopy has significantly addressed the issue of cost and time, since the technique can provide reliable estimates of feed and dairy product quality at an affordable cost in real time. Similarly, the electronic nose (e-nose) technology or machine olfaction has made it easier to detect off odors in milks and dairy products, in order to guarantee quality.

This doctoral work focused on the possible applications of correlative analytical methods (i.e. NIR and e-nose) in quality assessment of feeds and dairy products.

From this overall aim, the following **specific objectives** which form the main chapters of this thesis were considered:

1. To demonstrate the applicability of near-infrared (NIR) spectroscopy in the evaluation of the qualitative and quantitative characteristics of newly introduced forage mixtures in dairy cattle farming.

2. To check the feasibility of electronic nose (e-nose) technologies to evaluate the quality of some feeds and dairy products, i.e. describing the odor profile of: **(a)** alfalfa and rye silages used in dairy feeding, **(b)** raw bovine milk as affected by feeding, and **(c)** value-added functional food fortified with health promoting additive.

2. MATERIALS AND METHODS

2.1. NIR study of dairy feeds

2.1.1. Forage mixture samples

The compositions of the four forage mixtures used in the study were (commercial products, producer: Agroteam S.p.a., Torrimpietre (RM), Via di Granaretto, 26, 00054 Italy):

Mixture A: 40% of two cultivars of winter triticale + 30% of two cultivars of winter oats + 20% of winter barley + 10% of winter wheat.
Mixture B: 50% of two cultivars of winter triticale + 40% of winter barley + 10% of winter wheat.
Mixture C: 55% of three types of Italian ryegrass + 45% of two cultivars of winter oat.
Mixture D: 40% of three types of Italian ryegrass + 30% of two cultivars of winter oat + 15% of two cultivars of winter triticale + 10% of winter barley + 5% of winter wheat.

The percentages of the forage mixtures composition reflect the seed weight ratio of the various winter cereals (WC) and Italian ryegrasses (IRG) used in the study.

2.1.2. Harvesting of the forages

The four forage mixtures (Mixture A, B, C and D) were harvested on five (5) phases, with one week interval between each harvest (Cut 1, 2, 3, 4 and 5), at each harvest, 5 samples of each of the 4 mixtures (510 g) were taken on the field, totaling 20 samples per each cut. The total number of samples at the end of the harvest period was 100 (20 by 5).

2.1.3. Forage preservation, storage and preparation

One part of the forage samples (fresh) were stored frozen at -20 °C in sealed polyethylene bags (ca. 1000 g), and the other part was dried at 60°C until mass constancy (ca. 600 g) using a Memmert UFE400 oven (Memmert GmbH, Buechenbach, Germany). The dried samples were ground with a Retsch Rotor Beater Mill SR 200 with a bottom sieve of 1 mm aperture size (Retsch GmbH, Haan, Germany).

2.1.4. Ensiling of forages

Wilted and chopped materials of 510 g were packed into laboratory silos (anaerobic glass jars capacity of 0.72 litre [729 kg/m3]) using a mechanical hand packer. On days 0, 7, 14, and 90 after ensiling, five laboratory silos per mixtures (A, B, C and D) were opened and samples of silages were stored frozen at -20 °C in sealed polyethylene bags. Samples were thawed at 4 °C for 24 hours until being measured with NIR spectroscopy in fresh or moist form, without grinding the chopped samples. Then the silages were dried and ground analogously to the forages, and NIR spectra of the dried silages were also recorded.

2.1.5. Chemical analyses of forages

Chemical analyses were performed in the analytical laboratory of Kaposvár Campus. The crude protein (CP), ether extract (EE), crude ash, crude fiber (CF) and total sugar were determined according to AOAC (2006). Neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) were analyzed according to the procedure of Van Soest et al. (1991). The statistical evaluation of the chemical data was performed with one-way analysis of variance (ANOVA) including Tukey's post-hoc test to check the group differences, using the SPSS v26 (IBM Corp., Armonk, USA).

2.1.6. NIR spectroscopic measurement of forages and silages

NIR spectroscopy measurements were performed in the NIRS laboratory of Kaposvár Campus. In all cases, samples were at room temperature (25 °C) during the NIRS measurements. The reflectance NIR spectra (log R^{-1}) of the moist silages (n = 80) were recorded with a FOSS DS2500 spectrometer (FOSS Analytical A/S, Hillerod, Denmark) in the range of 400-2500 nm, at 2 nm spectral steps. To avoid spectral variation caused by heterogeneity of the silages, the large cup (FOSS 60056582) was rotated eight times during the successive scans, and the acquired spectrum of an individual sample was the average of spectra of two refilled subsamples. FOSS Mosaic Solo v.8.0.4.10 (FOSS Analytical A/S, Hillerod, Denmark) and FOSS ISIScan Nova (FOSS Analytical A/S, Hillerod, Denmark) software packages were used for the operation of the spectrometer and data acquisition.

The NIR spectra of the dried and ground forages (n = 100) and ensiled forages (n = 80) were collected in reflectance mode using a NIRSystems 6500 spectrometer (FOSS NIRSystems, Laurel, MD, USA) equipped with a sample transport module and small ring cup (FOSS IH-0307). Log R^{-1} spectra were recorded in the 400-2500 nm range with 2 nm spectral step. The WinISI II version 1.5 spectral analytical software (InfraSoft International LLC, State College, PA, USA), was utilized for the operation of the spectrometer and acquiring data.

2.2. E-nose study of dairy feeds

2.2.1. Alfalfa and rye silage samples

The study used alfalfa and rye silages (n = 22 and 38, respectively) derived from large scale commercial dairy farms in Hungary. Alfalfa forages were prepared for ensiling with and without wilting (n = 10 and 12, respectively), while rye silages were harvested before heading and in heading (n = 17 and

21, respectively). The fresh samples were dried at 70 °C for 8 hours, then homogenized with a laboratory mill (Peppink Mills, Netherlands) and analyzed for dry matter (DM), CP, EE, CF, crude ash, pH, acetic acid and lactic acid concentrations by means of NIR spectroscopy using a Quant FT-NIR spectrometer (Q-Interline, Denmark) and an internationally recognized calibration database (Samplinq®, Eurofins Agro Inc., Wageningen, Netherlands) at Livestock Performance Testing Ltd, Gödöllő, Hungary. The quality of samples was evaluated based on the pH and the lactic acid/acetic acid ratio. Each freshly collected sample was equally distributed into six plastic bags for the odor measurement. The prepared sub-samples (n = 360) were stored at -20 °C in sealed bags under vacuum, until the odor measurements. Frozen storage of any sample did not last longer than 30 days.

2.2.2. Odor measurement of alfalfa and rye silages

The odor measurement was performed at Kaposvár University (now Hungarian University of Agriculture and Life Sciences, Kaposvár Campus, Kaposvár, Hungary) for six days where, the six sets of sub-samples were analyzed daily (n = 60 per day). The odor of each sample was examined with an Alpha MOS FOX4000 electronic nose based on the metal oxide semiconductor (MOS) sensor array technology (Alpha M.O.S., Toulouse, France) within 30 days after sampling. 2 g of subsamples (n=360) were filled into 20 mL glass vials then sealed with polytetrafluoroethylene septa. The headspace samples containing the volatiles of the silages were generated above the solid sample during three-minute incubation at 40 °C. 5 mL of the headspace was injected into the continuous flow of synthetic air. The relative resistance changes (ΔR/R0) of 18 MOS sensors caused by the injected volatiles were measured after each injection and saved as sensitivity values. One measurement cycle took 20 minutes, including two minutes data acquisition and 18 minutes cleaning phase when pure synthetic air was used

to rinse the sensors and the syringe. The maximal sensitivity experienced on each sensor was saved in each measurement cycle, thus, all sub-samples were described with 18 variables.

2.2. E-nose study of improved milk

2.3.1. Milk samples

The total mixed rations (TMR) used in the experimental feed trial were formulated using the forage mixtures described in Chapter 2.1. The study was carried out at the dairy farm (Fészerlak) of the Hungarian University of Agriculture and Life Sciences (MATE), Kaposvár Campus. The experimental design used was a single-blinded randomized efficacy study divided into 5 periods conducted between August 2019 and March 2020.

The dairy cows used for the study were 32 multiparous Holstein-Friesian cows (>150 days in milking, average milk production: <25 kg/day), fed with 4 experimental diets (EXP-1, 2, 3, 4) and a control (CTR) (Table 1). Mix A, B, C, D within the experimental diets as mentioned below are identical with those described earlier (Chapter 2.1).

Table 1. The control (CTR) and experimental diets (EXP-1, 2, 3 and 4)

Parameter	CTR	EXP-1	EXP-2	EXP-3	EXP-4
Ingredient, kg/ cow/ day					
Corn silage	11	11	11	11	11
Alfalfa haylage	7	7	7	7	7
Vetch-triticale haylage	7	-	-	-	-
Mix A[1]	-	-	-	-	7
Mix B[2]	-	-	-	7	-
Mix C[3]	-	7	-	-	-
Mix D[4]	-	-	7	-	-
Concentrate[5]	6	6	6	6	6
Grass hay	2	2	2	2	2
Molasses (liquid)	1.2	1.2	1.2	1.2	1.2

[1]Mix A: 40% of two cultivars of winter triticale + 30% of two cultivars of winter oats + 20% of winter barley + 10% of winter wheat
[2]Mix B: 50% of two cultivars of winter triticale + 40% of winter barley + 10% of winter wheat
[3]Mix C: 55% of three types of Italian ryegrass + 45% of two cultivars of winter oat)
[4]Mix D: 40% of three types of Italian ryegrass + 30% of two cultivars of winter oat + 15% of two cultivars of winter triticale + 10% of winter barley + 5% of winter wheat

[5]Vitafort Co., Dabas, Hungary ("533-614", dry matter: 88.00%, crude protein: 16.00%, NE_l MJ kg^{-1}: 6.74, crude fiber: 5.00%, ether extract: 2.90%, ash: 8.30%, starch: 42.71%, sugar: 2.34%, calcium: 1.71%, phosphorus: 0.57%, sodium: 0.66%, magnesium: 0.37%, vitamin A: 22,800 IU kg^{-1}, vitamin D: 4,500 NE kg^{-1}, vitamin E: 128 mg kg^{-1}

Milk collection started after 2 weeks of dairy cow adaptation to each feeding trial. During this adaptation period, cows were fed their assigned diet and then studied for two weeks in order to facilitate the cows' physiological adjustment to the feed, as well as to avoid the influence of a preceding trial on a succeeding one. The cows' udder health was continually monitored with Mastatest (Mastaplex Ltd., Dunedin, New Zealand) in order to rule out mastitis. The milk of unhealthy cows was not included in the test sample. For each trial or period (5 trials in total), 8 separate collections of homogenized milk from each group were sampled into 0.5 L bottles (n = 40), which were then stored frozen (-20 °C) for subsequent e-nose measurement.

2.3.2. Odor measurement of milks

A Heracles Neo (Alpha MOS, Toulouse, France) flash gas chromatograph with two columns (MXT-5 and MXT-1701, Restek, USA) equipped with a HS 100 (PAL Systems, Switzerland) auto-sampler was used for the odor measurements in the Correltech® Laboratory of ADEXGO Kft., Herceghalom, Hungary. The hydrogen used as carrier gas during the

measurement was in FID Grade. After 5-minute incubation (50 °C) of 20 mL sealed vials with UltraCleanTM polytetrafluoroethylene/silicone septum (Supelco, Inc., Merck KGaA, Darmstadt, Germany) containing 1 mL of individual milk samples (5 measurement per sample (n = 25)). A 5 mL headspace injection was done, and acquisition time per sample was 110 s, with analysis period per sample being 9 minutes, respectively.

2.3.3. Analysis of milk fatty acids

Milk (n=40) was homogenized (IKA T25 Digital Ultra Turrax, Staufen, Germany) in a 20-fold volume of chloroform:methanol (2:1 v:v). Furthermore, the total lipid content was extracted (Folch et al., 1957). The solvents were ultrapure grade (Sigma-Aldrich, St. Louis, MO, USA); moreover, 0.01% w/v butylated hydroxytoluene was added in order to prevent FA oxidation. The samples were then evaporated to dryness under a nitrogen stream, and were transmethylated through a base-catalyzed $NaOCH_3$ method (Christie, 1982).

After cooling, the total lipid was extracted with chloroform and then trans-methylated with an acid-catalyzed method (Christie, 2003), while using H_2SO_4 (1 v/v%) in methanol as a methyl donor, and toluene as a solvent at 50 ^{o}C overnight. FA methyl-esters were extracted into 300 μL ultrapure n-hexane for the purposes of gas chromatography (AOC 20i automatic injector; Shimadzu 2030, Kyoto, Japan); then equipped with a Phenomenex Zebron ZB-WAXplus capillary GC column (30 m × 0.25 mm ID, 0.25 μm film, Phenomenex Inc., Torrance, CA, USA); and also subjected to a flame ionization detector (FID). The characteristic operating conditions were as follows: injector temperature: 220 °C; detector temperature: 250 °C; and helium flow: 28 cm/s. The oven temperature was then graded from 60 (2 min holding) to 150 °C, from 150 to 180 °C; 2 °C/min and 10 min at 180 °C, from 180 to 220 °C; and then 2 °C/min and 16 min at 220 °C. In addition, the

makeup gas used was nitrogen. Calculation was then performed with the LabSolutions 5.93 software, using the PostRun module (Shimadzu, Kyoto, Japan) with manual peak integration. FA results were expressed as the weight % of the total FA-methyl esters.

2.3. E-nose study of a fortified dairy product

2.4.1. Fortified milkshake powder samples

Milk-based vanilla shake powder was fortified with micro-encapsulated microalgae oil (brand: S17-P100, life's DHA, DSM Nutritional Products Inc., Heerlen, Netherlands) to increase the omega-3 (n3) fatty acid content. A 10-step oil-enrichment protocol was performed from 0.2 up to 2 w/w% inclusion rate (Table 2), to increase the DHA in the shake product, and based on this a flash-GC-based e-nose was used to describe the product's odor.

Table 2. The graded food additive enrichment protocol (product composition)

	shake powder	S17-P100	total mass	calc. DHA	calc. EPA	calc. EPA+DHA
No.	mg in 10 g					
1	9980	20	10000	4.9	1.5	6.4
2	9960	40	10000	9.8	3.0	12.8
3	9940	60	10000	14.7	4.4	19.1
4	9920	80	10000	19.6	5.9	25.5
5	9900	100	10000	24.5	7.4	31.9
6	9880	120	10000	29.4	8.9	38.3
7	9860	140	10000	34.3	10.4	44.7
8	9840	160	10000	39.2	11.8	51.0
9	9820	180	10000	44.1	13.3	57.4
10	9800	200	10000	49.0	14.8	63.8

S17-P100, brand name of the primarily docosahexaenoic acid enrichment used; EPA, eicosapentaenoic acid (C20:5 n3); DHA, docosahexaenoic acid (C22:6 n3)

2.4.2. Odor measurement of milkshakes

The same electronic nose was used as described in 2.3.2. Three-times 1 g aliquots of each sample were placed into three 20-mL headspace vials (n=30), sealed with a magnetic cap and an UltraCleanTM polytetrafluoroethylene/silicone septum (Supelco, Inc., Merck KGaA, Darmstadt, Germany), with 5 mL headspace injection. Incubation: 80°C for 10 min.

2.4.3. Analysis of fatty acids

Samples (shake powder, FA additive, complemented shake powder) were homogenized (IKA T25 Digital Ultra Turrax, Staufen, Germany) in a 20-fold volume of chloroform:methanol (2:1 vol:vol) and total lipid content was extracted according to (Folch et al. 1957). Solvents were ultrapure-grade (Carl Roth, Karlsruhe, Germany) and 0.01 % w:v butylated hydroxytoluene was added to prevent FA oxidation. Directly to the raw, dry sample, C19:0 internal standard was added (Merck cat. No.: 72332). The internal standard used was a solution of 1 mg/ml in chloroform:methanol (2:1 vol:vol). The total amount added was ca. 1/20 mass of the extracted fat, i.e. to 1 g raw sample (ca. 100 mg crude extract) 5 mg C19:0 was added.

Total lipid extract (including the internal standard as well) was dried fully on a rotary evaporator, under a nitrogen stream and was trans-methylated with the acid-catalyzed method (Christie, 2003), using H2SO4 (1 vol/vol%) in methanol as a methyl donor, and toluene was used as a solvent. For the quantitative analysis, C19:0 methyl ester standard calibration was used at 6 points (Merck cat. No.: 74208) to assess detector response, and the concentration of analyte in the calibration was between 5 and 500 microg/ml. The correlation coefficient was not less than 0.999, proving the linearity of analysis. Fatty acid methyl-esters were extracted into ultrapure n-hexane for

gas chromatography. This was performed on a Shimadzu GCMS-QP2010 apparatus (AOC 20i automatic injector), equipped with a Phenomenex Zebron ZB-WAX Capillary GC column (30 m x 0.25 mm ID, 0.25 micrometer film, Phenomenex Inc., Torrance, CA, USA). Characteristic operating conditions were: injector temperature: 270 °C, detector temperature: 300 °C, helium flow: 28 cm/sec. The oven temperature was graded: from 80 to 205 °C: 2.5 °C/min, 5 min at 205 °C, from 205 to 250 °C 10 °C/min and 5 min at 210 °C. FA results were expressed as mg/g of raw sample mass and as well as weight% of the total FAs. All samples were analyzed in duplicate, and results are means of 2 analyses. The Limit of Detection was determined as three times the signal-to-noise ratio (3S/N), while the Limit of Quantification 10S/N. The range of LOD was between 0.1– 0.5 μg/ml for the FAs; C4:0 and C24:0).

2.4. Statistical Analysis

2.4.1. Multivariate analysis of NIR data

The multivariate data of the NIR spectroscopic measurements were exported from WinISI and Mosaic software packages in text file format and The Unscrambler 11.0 (CAMO Analytics AS, Oslo, Norway) was applied for data processing and analysis. Moving average smoothing was applied with 5 spectral points before further data pre-treatments. SNV transformation (Barnes et al., 1989) and 2^{nd} order Norris derivatives with 5-point gap (Hopkins, 2001; Norris, 2001) were calculated to decrease additive and multiplicative effects of light scatter. PCA (Cowe and McNicol, 1985) was used to describe the basic multidimensional characteristics of the NIR data matrix and to visualize the differences of the sample groups. Calibrations for the chemical constituents were conducted with the forage NIR data and chemical (reference) data using PLS regression, and models were tested with full cross-validation (Næs et al., 2002). The precision and accuracy of the

chemometric models were evaluated by the determination coefficient (R^2, R^2_{CV}) and the root mean square error (RMSEC, RMSECV) of calibration and cross-validation, respectively (Næs et al., 2002).

2.4.2. Multivariate analysis of e-nose data

The recorded data of the electronic nose were analyzed with multivariate classification methods using AlphaSoft v12 (Alpha M.O.S., Toulouse, France). Discriminant analysis was applied to test the possibility of group identification based on the odor properties (Næs et al., 2002). Cross-validation was used to test the supervised classification models when all sub-samples of a single sample were left out of the modelling iteratively and were used for testing the classification capability of the model. The classification models were evaluated based on the confusion matrices where hit rates and misclassifications were indicated.

In the e-nose studies involving milk and milkshake samples, the AlphaSoft v12 (Alpha M.O.S., Toulouse, France) was again used for multivariate data analysis approaches. The chromatogram peaks were used as sensor data representing the smell fingerprints of the milk samples. Data were analyzed with multivariate data classification methods: principal component analysis (PCA) and discriminant factor analyis (DFA) as unsupervised and supervised classification methods, respectively. The PCA was used to describe the general multidimensional patterns of e-nose data, and the DFA to establish the possibility of group identification based on odor properties of the milk and shake samples (Næs et al., 2002). The PLSR (Næs et al., 2002) was used to fit calibration models describing the relation of the odor signals and the concentration of S17-P100 food additive.

The accuracy of the DFA and PLSR models was tested with leave-one-out cross-validation, when a single record was left out of the modeling process and was used for testing by predicting its qualitative or quantitative

properties; this process was repeated iteratively until all samples had been used for validation once (Næs et al., 2002). The sensor selection function of AlphaSoft was used to identify the most distinctive variables during the qualitative and quantitative approaches.

In addition, DFA and PLSR calculations based on the selected sensors were performed. The volatile compounds described by the selected sensors were identified using the AroChemBase database. The retention time, defined as the amount of time a compound spends in the column after it has been injected (Burian et al., 2010) was converted to Kováts retention index (RI) (Guardino et al., 1976). The most abundant compounds were also identified based on the retention indices using the AroChemBase (Alpha MOS, Toulouse, France) database.

3. RESULTS

3.1. NIR study of dairy feeds

3.2.Qualitative analysis (harvesting days and forage types)

The PCA score plots (Figure 1a and 1b) below calculated from SNV NIR spectra, show the differences between the days of harvest (cut:1-5) and the types of mixture forages (mixture: A-D) respectively. The score plot (b) highlights the biggest difference between mixtures containing Italian ryegrass (mixtures C and D) and those not containing it (mixtures A and B). The difference between these groups appeared in the time of the 3rd cut (Figure 1 a) which indicates that mixtures C and D maturated earlier than mixtures A and B.

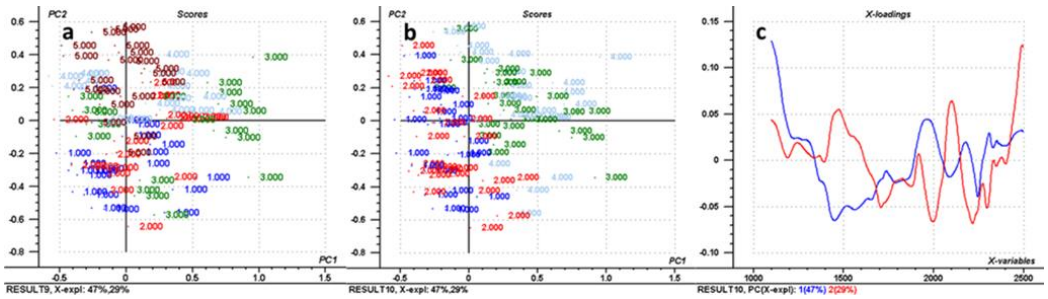

Figure 1. PCA score plots of the dried forages calculated from the SNV corrected NIR spectra (1100-2500 nm) coloured by the five harvesting days (a) (1: cut 1, 2: cut 2, 3: cut 3, 4: cut 4, 5: cut 5) or by the four mixtures (b) (1: mixture A, 2: mixture B, 3: mixture C, 4: mixture D), and the loading vectors (c) of the first two principal components (PC1, PC2) highlighting the dominant spectral regions responsible for the distribution of samples along PC1 and PC2 in the score plots.

3.2.1. Silage qualitative study (NIR)

In the wet samples (Figure 2a) only the D0 was different from others (D7, D14, and D90) as described by the first two principal components (PCs) that described 96% of the total variance of the NIR spectral data. The absorption regions of water are dominant in the loadings responsible for the two PCs. This reflects that the biggest difference seen in the wet samples was related to water spectral changes between the initial stage (D0) and the other sampling days during the fermentation. In the dry samples (Figure 2c) however, the different fermentations days separated within the PCA, revealing age dependent variation between the sampling days.

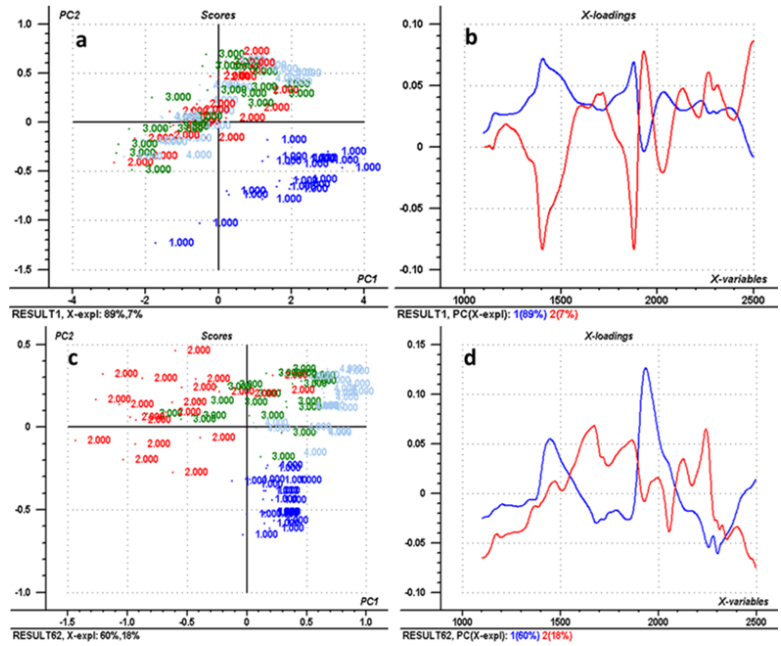

Figure 2. PCA score plots calculated from the SNV corrected NIR spectra (1100-2500 nm) of the wet (a) and dried (c) silages indicating the fermentation days (1: Day 0; 2: Day 7; 3: Day 14; 4: Day 90), and the loading vectors (b, d) of the first two principal components defining the planes of the score plots.

3.2.2. Quantitative analysis (predicting forage chemical constituents)

The results of the best PLSR models showed below in Table 3 were built by applying a second derivative math treatment to the NIR data. The least precise model was built for the total sugar content. The R^2 and R^2_{CV} for the other constituents were greater than 0.9, except the R^2_{CV} for EE (0.87). The RMSECV ranged between 0.2-2.4, lower than the standard deviations (SD) of the reference chemical data and with LV values (3-7) lower than 1/10th of the total sample number used in the study.

Table 3. The PLSR calibration and cross-validation results for the chemical constituents of the dried forages (n = 100) on dry weight basis (%) with second derivative (smoothing 5, gap 5) pre-treatment.

Constituent (%DM)	LV	R^2	RMSEC (%)	R^2_{CV}	RMSECV (%)
CP	3	0.96	0.57	0.95	0.59
CF	3	0.95	0.70	0.95	0.76
EE	7	0.93	0.16	0.87	0.22
Ash	6	0.96	0.25	0.93	0.31
Total sugar	3	0.77	2.19	0.74	2.36

CP: Crude protein; CF: Crude fiber; EE: Ether extract; LV: number of latent variables in the PLSR calibration model; R^2: determination coefficient of calibration; RMSEC: root mean square error of calibration; R^2_{CV}: determination coefficient of cross-validation; RMSECV: root mean square error of cross-validation.

3.3. E-nose study of dairy feeds

Both the alfalfa and rye silages separated according to the different harvest technologies of ensiling. In the case of alfalfa, the application of wilting before ensiling caused significant odor variations, while in the case of rye,

the maturity of the crop upon ensiling resulted in detectable characteristic variations of the odor profile. In average, more than 65% of samples were classified correctly in the cross-validations in both types of silages (Table 4).

Table 4. Modelling and cross-validation results of the odor-based classification of silages and according to the applied harvest technologies

	Alfalfa silages			Rye silages		
	Processing groups	(A)	(B)	Phenological phase groups	(C)	(D)
Model	(A) Direct-cut	68.1%	31.9%	(C) Before heading	78.0%	22.0%
	(B) Wilted	28.3%	71.7%	(D) Heading	41.7%	58.3%
Cross-validation	(A) Direct-cut	66.7%	33.3%	(C) Before heading	77.4%	22.6%
	(B) Wilted	30.0%	70.0%	(D) Heading	41.7%	58.3%

Table 5. Modelling and cross-validation results of the odor-based classification of silages and haylages according to the quality groups defined by the pH values

		Alfalfa silages and haylages		Rye silages	
	pH groups (3)	< 4.4 (n = 6)	≥ 4.4 (n = 16)	< 4.4 (n = 26)	≥ 4.4 (n = 12)
Model	< 4.4	73.3%	26.7%	67.3%	32.7%
	≥ 4.4	42.2%	57.8%	35.2%	64.8%
Cross-validation	< 4.4	66.7%	33.3%	66.0%	34.0%
	≥ 4.4	46.1%	53.9%	37.0%	63.0%

The Table 5 shows the cross-validation results of the developed models for the pH of the silages. In the case of rye silages, 64.5% of the cross-validation (CV) samples were classified correctly according to the pH groups, while the

classification of alfalfa silages was less accurate (average hit rate in CV: 60.3%).

3.4. E-nose study of improved milk

The Figure 4 below shows the odor differences of the milk samples caused by the feeds from different forage compositions. DF1 and DF2 helped to explain 58.58% and 41.42% of the total variance, respectively, with the milk of groups associated with feeding diets containing winter cereals and Italian ryegrass (WC+IRG). These milks, which make up the EXP-1 and EXP-2 groups, were separated from the milk of groups associated with feeding diets containing winter cereals (WC), which make up EXP-3 and EXP-4, alongside DF1.

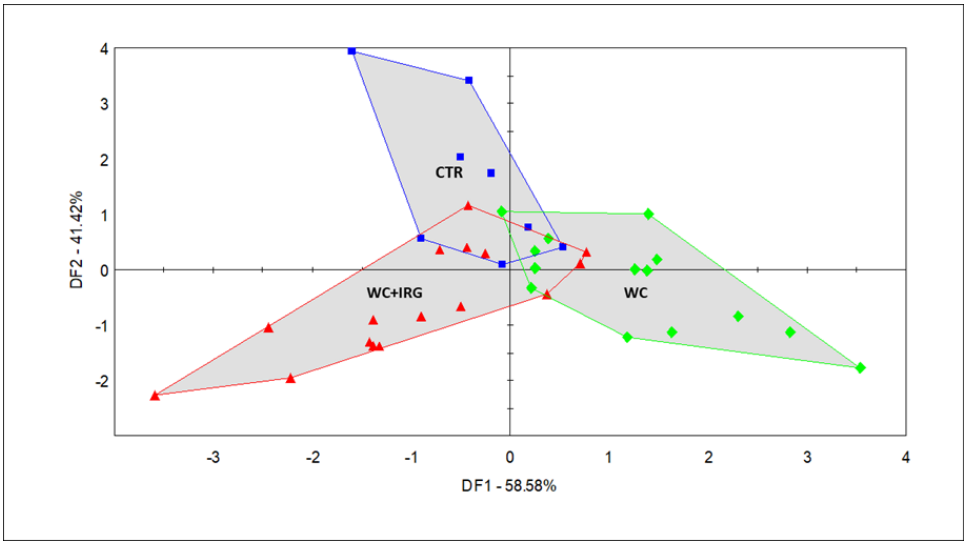

Figure 3. Discriminant factor analysis (DFA) score plot of milk samples collected from cows fed with the five diets containing the different forage sources, as performed via using the selected sensors (CTR (blue square): control diet; WC+IRG (red triangle): winter cereals and Italian ryegrass containing diets; and WC (green diamond): winter cereals containing diets).

The milk of the CTR group separates from that of the WC+IRG and WC groups, alongside DF2. This reveals that the odor variation between the milk samples of the WC+IRG and WC groups was bigger than the variation that existed between the milk samples of the CTR group and the milk samples of the experimental groups. Furthermore, the CTR and WC+IRG milks entirely overlap in the plane of the most dominant discriminating factor (DF1), while WC shows little overlapping with the aforementioned. Thus, the WC milks proved to possess the most different odor when compared with the other two main classes (i.e., WC+IRG and CTR).

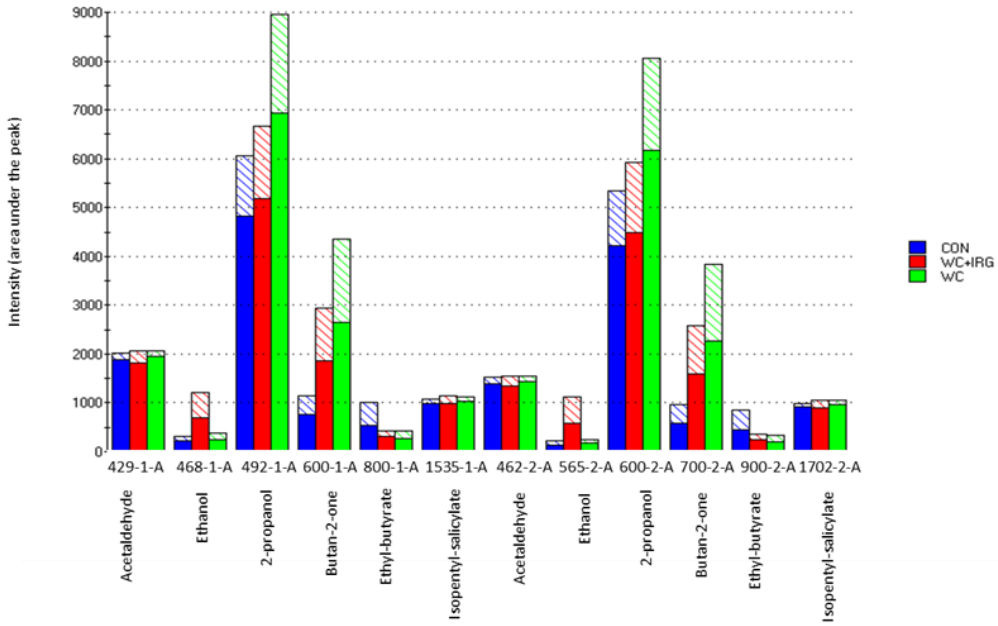

Figure 4. Bar graph of detection intensities, as measured with electronic nose at retention indices (RI) that corresponded to certain identified odor, which produced volatile compounds as detailed in column 1 ("-1-A") and 2 ("-2-A") of the milk samples obtained from dairy cows who were fed with the control (CTR, first bar graph from left) diet, as well as the diets containing winter cereal and Italian ryegrass (WC+IRG, middle bar graph), or winter cereals only (WC, first bar graph from right).

The main volatile compound responsible for the identification of the odor of the CTR milk was ethyl-butyrate; whereas 2-propanol and butan-2-one dominated in WC milks; and the milks of the WC+IRG groups were influenced by ethanol (Figure 5).

3.5. E-nose study of a fortified dairy product

To describe the multivariate odor patterns, principal component analysis (PCA) was performed with all the sensor signals that were derived from the chromatograms. The first (PC1) and second (PC2) principal components explained 72.2% and 19.4% of the e-nose data variation, respectively, without any systematic (increased or decreased) pattern of odor differences between the supplemented samples and the control (Figure 6).

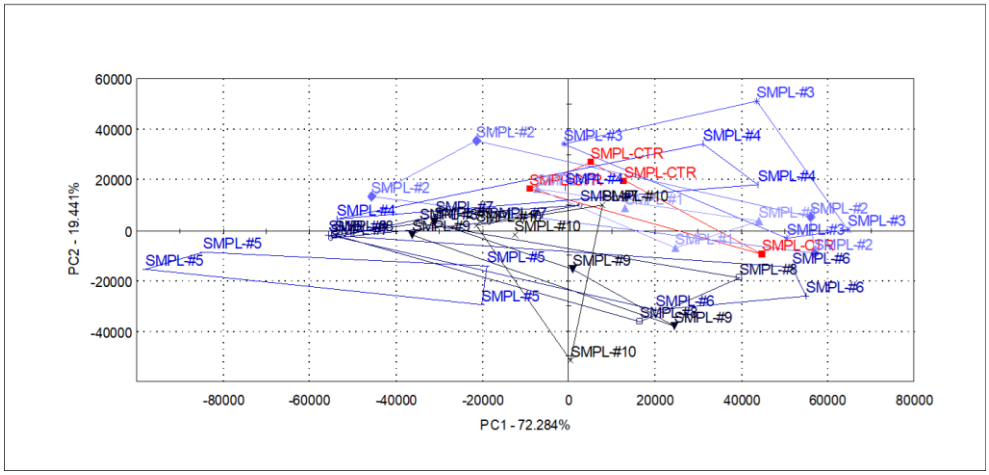

Figure 5. Score plots of PCA performed with data from all sensors, showing the planes of the 1st and 2nd principal components (n= 4 replicate/sample, SMPL-CTR: milkshake with no supplementation (control); SMPL#1 to SMPL#10: milkshake samples with increasing level of microalgae oil supplementation (0.2 to 2%).

However, when the relation between the sensor data and the supplementation level was described with partial least squares regression (PLSR) in figure 7 below, an accurate (R^2 = 0.833) calibration model was built, which indicated a possibility to find supplementation-dependent odor variations.

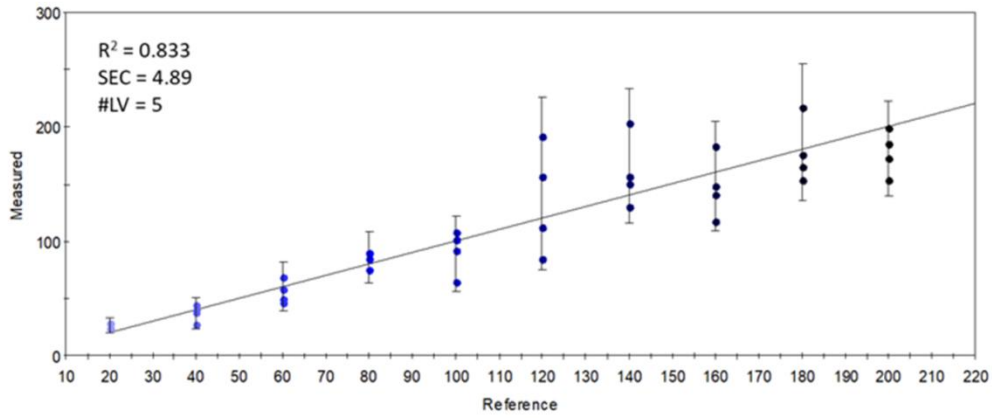

Figure 6. Y-fit of the PLSR calibration on the microalgae oil concentration (mg/in 10 g product) using data of all sensors (R^2: determination coefficient; SEC: standard error of calibration, #LV: number of latent variables).

To further investigate this seeming possibility, specific sensors which had the biggest impact on the classifications were selected. These qualitative sensors represent the most dominant odors of the samples in general, without considering DHA levels during the sensor selection. Although the data of the selected qualitative sensors (targeted modeling) holds enough information to differentiate the samples by the groups of DHA inclusion levels of the S17-P100 supplementation, this differentiation is not based on volatiles that are in direct relation with the supplemented concentration. This is demonstrated in the below PLSR calibration on the supplementation level using the data of the qualitative sensor set. The weak results (R^2 = 0.509) indicate that there is

no linear combination of the qualitative sensors' signals that could accurately describe the supplemented concentration variation (Figure 8).

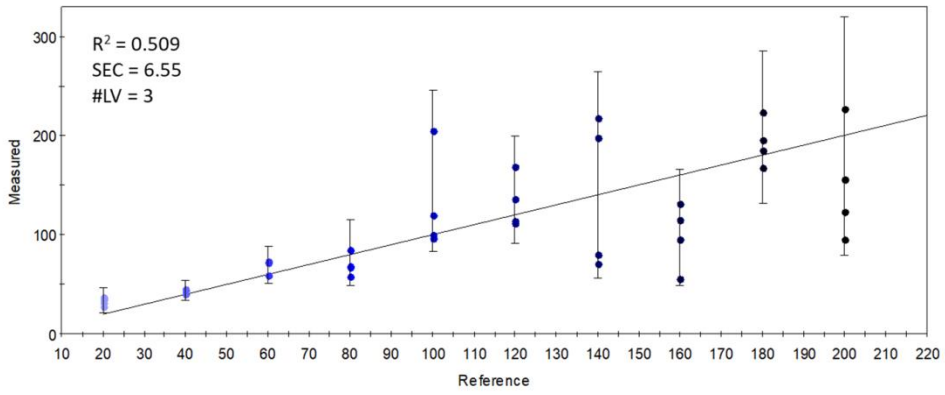

Figure 7. Y-fit of the PLSR calibration on the S17-P100 concentration (mg/10 g product) using data of the selected qualitative sensors (R^2: determination coefficient; SEC: standard error of calibration, #LV: number of latent variables)

The PLSR calibration and sensor selection based on PLSR calibration are targeted approaches to find specific odor differences focusing on predefined parameters or constituents, thus, finding the relevant odor variations is possible even if the causing odorants are very weak. However, an approach that is non-targeted like PCA is relevant in identifying the general odor patterns, focusing on the major odor variations, which in this paper revealed non-dependent odor differences in relation to increased level of DHA supplementation.

4. CONCLUSIONS DRAWN FROM THE RESEARCH AND SUGGESTIONS

Based on the results of the first experiment, it can be concluded that near-infrared (NIR) spectroscopic analysis could be helpful in the evaluation of the quality differences between mixture forages of winter cereals and Italian ryegrass, during harvesting especially at different phenological phases of the forages. Again, the fermentation quality of silages made from these novel forage mixtures could be monitored during the various days or phases of ensiling, to enable the production of good quality silages on time, to meet the preference or demand of dairy farms. Quantitatively, the predictive models developed could be used for quick analysis of some essential nutritive constituents such as crude protein, crude fiber, and ash contents with high accuracy and precision, at low cost whiles avoiding the laborious conventional laboratory methods.

As a preliminary test, the use of an e-nose with metal oxide semi-conductor (MOS) gas sensors in the evaluation of rye and alfalfa silages based on different harvest technologies, phenological phase at harvest, and the pH proved promising in the second experiment. The application of the e-nose technology for quick screening of silages could ensure safe selection of silages with good characteristic smell or aroma to meet acceptable preference. The best harvesting and processing method could also be selected through the smell characteristics of the silages, thereby ensuring efficient good silage production. Based on this study, the application of wilting to alfalfa forages before ensiling caused odor variations as compared to ensiling direct cut alfalfa forages, while, harvesting rye crop before heading caused variations of the odor profile upon ensiling. Thus, these outcomes could provide silage producers a fair idea of which technology or processing method and phenological phase of harvest to depend on to ensure good silage

production. This preliminary study affirms instrumental odor testing methodology to be promising in the field of quality testing of fermented forages.

The application of an ultra-fast flash GC-FID e-nose to evaluate bovine milk from different feed regimes proved effective. Based on the results, it can be concluded that milk odor and fatty acid composition can be altered through feeding dairy cows with feeds made from the novel mixtures of winter cereals and Italian ryegrass silages while also the level of odor alteration can be monitored and the best option of mixed silages may be selected based on the odor pattern of the produced milk.

Also, the effectiveness of the e-nose technology to test the odor stability of a bioactive improved milkshake proved efficient. The e-nose could therefore serve as a rapid analytical tool to complement human sensory evaluation in the production and development of milk and milk-based products to ensure high quality standard, integrity, and to meet consumer preference or choice. The limitation of the technology is its inability to determine dairy product preference by the consumer, notwithstanding its efficient detection of aroma pattern of the products. Further studies on the human sensory preference and acceptability of the milks and bioactive improved shakes are recommended.

It is important to mention, that at this current stage, the e-nose methods investigated are not applicable in current daily routine, as there is no version of these e-noses that could function well in routine practice. However, the e-nose studies done in the thesis were necessary to investigate how these technologies with high precision could work. We decided not to focus on the actually cost effective technologies only, but investigate the needs and possible outputs of still expensive ways, so we can direct the developments on a way to create the cheaper technologies that are really needed.

5. NEW SCIENTIFIC RESULTS

1. The near-infrared (NIR) spectroscopic measurement coupled with principal component analysis (PCA) as a chemometric tool was able to qualitatively differentiate between the mixture forages of winter cereals only, and winter cereals plus Italian ryegrass, harvested at different phenological phases of growth, and ensiled at different days.

2. The partial least square (PLS) regression models developed for predicting the chemical constituents of the mixture forages of winter cereals and Italian ryegrass from their NIR spectra are robust with high accuracy (R^2 and $R^2_{CV} > 0.9$) with root mean square error of cross-validation (RMSECV = 0.59%, 0.76% and 0.31%) and latent variables (LV= 3, 3, 6) for crude protein, crude fiber, and ash, respectively.

3. The applied MOS e-nose was capable of identifying the odor differences in alfalfa silages caused by wilting, and can therefore be used for the efficient monitoring of the quality changes that occur in silages due to wilting (75% hit rate in CV).

4. Based on the odor patterns measured, the MOS e-nose proved to be useful in the detection and description of the quality groups of alfalfa and rye silages objectively as defined by the pH of the silages (average hit rate in CV= 60.3% and 64.5% respectively).

5. The applied ultra-fast flash GC-FID e-nose measurement revealed the odor differences in milks from the different feeding regimes. As detected by the e-nose, milk which originated from cows fed corn silage and alfalfa haylage based control diet was largely influenced by ethyl-butyrate, whereas milk associated with supplementation with silage of winter cereals was influenced by 2-propanol and butan-2-

one, and that of winter cereals plus Italian ryegrass was influenced by ethanol.

6. The applied ultra-fast flash GC-FID e-nose revealed that to produce bovine milk with less organoleptic differences, the inclusion of winter cereals plus Italian ryegrass silage (7 kg/day) in cows' diet could be more beneficial over that of winter cereals only, as it caused less prominent odor alterations, but still caused changes in the milk fatty acid composition.
7. As detected by the applied ultra-fast flash GC-FID e-nose, an increased DHA level in vanilla milkshake up to 412 mg/100 g with the aim of improving the bioactive content of the product using micro-encapsulated algae oil would not cause off odor in the product.

6. SCIENTIFIC PAPERS AND LECTURES ON THE SUBJECT OF THE THESIS

6.1. Peer-reviewed papers published in foreign scientific journals

Yakubu, H. G., Kovacs, Z., Toth, T., and Bazar, G. (2022). The recent advances of near-infrared spectroscopy in dairy production– a review. *Critical Reviews in Food Science and Nutrition*, 62(3), 810-831. doi: 10.1080/10408398.2020.1829540, **D1, IF: 11.208**.

Yakubu, H.G., Kovacs, Z., Toth, T., and Bazar, G. (2022). Trends in artificial aroma sensing by means of electronic nose technologies to advance dairy production – a review. *Critical Reviews in Food Science and Nutrition*, 63(2), 234-248. doi: 10.1080/10408398.2021.1945533, **D1, IF: 11.208**.

Yakubu, H.G., Worku, A., Tóthi, R., Tóth, T., Orosz, S., Fébel, H., Kacsala, L., Húth, B., Hoffmann, R., and Bazar, G. (2023). Near-infrared spectroscopy for rapid evaluation of winter cereals and Italian ryegrass forage mixtures. *Animal Science Journal*, 94(1), 1-13.doi: 10.1111/asj.13823, **Q2, IF: 2.000**

Yakubu, H.G., Ali, O., Szabó, A., Tóth, T., and Bazar, G. (2023). Feeding mixture silages of winter cereals and Italian ryegrass can modify the fatty acid and odor profile of bovine milk. *Agriculture,* 13, 381, 1-15.doi: 10.3390/agriculture13020381, **Q1, IF 3.600 (2022)**.

Yakubu, H.G., Ali, O., Ilyés, I., Vigyázó, D., Bóta, B.; Bazar, G., Tóth, T., and Szabó, A. (2022). Micro-encapsulated microalgae oil supplementation has no systematic effect on the odor of vanilla shake– test of an electronic nose. *Foods*, 11, 3452. 1-19, doi: 10.3390/foods11213452, **Q1, IF: 5.200**.

6.2. Peer-reviewed paper published in Hungarian scientific journal

Yakubu H.G., Bázár, G., Radó-Nyiczky, É., Orosz S., and Tóth T. (2022). Description of the odor profile of fermented alfalfa and ryegrass silages using

an electronic nose. Állattenyésztés és Takarmányozás (Hungarian Journal of Animal Production), 71(1), 1-10.

6.3. Scientific papers on the subject of the dissertation but being not incorporated for some reasons

Yakubu, H.G., Kovács, Z., Vitális, F., and Bázár, G. (2021). Near-infrared spectroscopy: rapid and effective tool for measuring fructose content. Élelmiszervizsgálati Közlemények (Journal of Food Investigation) (67) 1, 3259-3268. doi: 10.52091/JFI-2021/1- 1-ENG, **Q4**.

Worku, A., Tóth, T., Orosz, S., Fébel, H.; Kacsala, L., Húth, B., Hoffmann, R., **Yakubu, H.G**., Bazar, G., and Tóthi, R. (2021). Aroma Profile, Microbial and Chemical Quality of Ensiled Green Forages Mixtures of Winter Cereals and Italian Ryegrass. *Agriculture,* 11,512. doi: 10.3390/agriculture11060512, **Q2, IF: 2.925 (2021)**.

Bazar, G., Hajnalka, H., Éva,C., Csaba, P., **Yakubu, H.G**., Carlos, P.V., Didier, D., Francesco, C., Alessandra, B., and Toth, T. (2022). Machine Olfaction to Evaluate the Stability of the Odor Profile of Pancakes Enriched with Docosahexaenoic Acid and Anthocyanins. Food Analytical Methods, 15(7), 1961-1967. doi: 10.1007/s12161-022-02232-3, **Q2, IF: 3.498**.

6.4. Abstracts

Yakubu, H.G., Tóth, T., and Bazar, G. Rapid method to monitor the effect of feeding on the odor profile of bovine milk, 14th Conference on Rapid Methods Europe, Amsterdam, The Netherlands, 3-5 October, 2022, poster presentation.

Yakubu, H.G., Aguinaga-Bósquez, J.P., Kovács, Z., Roszkos, R., Tóth, T., and Bazar, G. Establishing the impact of improved feeding on the quality of dairy products using correlative analytical technology (E-nose), 4th

International Conference on Food Science and Technology, MATE, Budapest, Hungary, 10-11 June, 2022, poster presentation.

6.5. Oral Presentations

Yakubu, H. G. Feed related quality differences of cheese described with near-infrared (NIR) spectroscopy. Innovative scientific workshops in the Hungarian agricultural higher education (Innovatív tudományos műhelyek a hazai agrár felsőoktatásban), EFOP-3.6.3-VEKOP-16-2017-00008 project, MATE, Kaposvar, Hungary, 4 May, 2021, oral presentation.

Yakubu, H. G., Tóth T., Tóthi R., Worku, A., and Bázár G. Feed-related quality differences of dairy products described with near-infrared spectroscopy (NIRS) and electronic nose (E-nose). 4th International Conference on Biosystems and Food Engineering, Lurdy Conference and Event Centre, Budapest, Hungary, 4 June, 2021, oral presentation.

7. CURRICULUM VITAE

Mr. Haruna Gado Yakubu was born in Tamale, Ghana, on the **31st of March, 1992**, into the Mabunwura Yakubu family of Kpembe.

Mr. Yakubu had his basic education between the years **1997 to 2007,** at Kpembe Local Government Basic Schools, and passed the Basic Education Certificate Examination (BECE) in **2007,** for enrolment into Senior High School.

Between the years **2007 to 2011**, Mr. Yakubu attended Salaga Senior High, where he studied General Agriculture, and passed the West Africa Senior High Certificate Examination in **June 2011**.

In **August 2012**, he was admitted into the School of Agriculture, University of Cape Coast, Cape Coast, Ghana, to read BSc Agriculture.

Mr. Yakubu, in **May 2016**, successfully completed the Bachelor of Agriculture degree, having passed all required examinations and dissertation, and graduated with **First Class Honours.**

Between the years **2016 to 2017**, he worked as a Teaching Assistant at the School of Agriculture, University of Cape Coast, Cape Coast, Ghana.

In **September 2017**, he was awarded the Stipendium Hungaricum Scholarship, tenable in Hungary, where he studied and acquired a Master of Animal Nutrition and Feed Safety Engineering (top of his class) in **June 2019,** from Kaposvár University, now Hungarian University of Agriculture and Life Science, MATE. During the **2019** Students' Scientific Cycle Competition, Mr. Yakubu was adjudged the 2nd Best Student Researcher, in the Life Science Session, amongst 12 competitors.

In **September 2019**, Mr. Yakubu was admitted into the Doctoral School of Animal Science, for his Ph.D studies under the supervision of Dr. György Bázár. His Ph.D theme was "the development of rapid analytical methods based on electronic nose technology and near infrared spectroscopy to advance dairy production."

Mr. Yakubu also worked as a Research Assistant at the Institute of Physiology and Animal Nutrition, Hungarian University of Agriculture and Life Sciences, Kaposvár Campus, from **January 2020 to November 2022**. Mr. Yakubu joined Agrofeed Kft, **in April 2023**, as Export Manager in charge of the Ghanaian Market.