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Using canonical correlation analysis to understand the rumen biohydrogenation patterns of linoleic and alpha-linolenic acids in the rumen fluid of bovines

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ABSTRACT. The objective of this study was to determine the multivariate relationship among linoleic acid, alpha-linolenic acid, and their main rumen biohydrogenation (BH) intermediates and products in bovine rumen fluid using canonical correlation analysis (CCA). A dataset consisting of 1177 observations generated by 107 *in vitro* rumen incubation systems of pure and mixed linoleic acid (18:2-c9, c12) and alpha-linolenic acid (18:3-c9, c12, c15) was gathered. Two canonical variates were defined: A: composed of the nine main BH intermediates and products (18:2-c9, t11; 18:2-t11, c15; 18:1-t11; 18:1-t9; 18:1-t6; 18:1-c11; 18:1-c6; 18:1-c9; 18:0) of 18:2-c9, c12 and 18:3-c9, c12, c15 and B: composed of 18:2-c9, c12 and 18:3-c9, c12, c15. Two canonical functions between A and B with significant canonical correlations (R_1=0.990 and R_2=0.738; p <0.01) were obtained. However, only the first function was selected for CCA. Exploration of canonical loadings for first function, revealed the following quantitative significance (absolute value) order for fatty acids (FA) within their respective canonical variates: A: 18:0(0.958)>18:1-t9(0.837)>18:1-c11(0.835)>18:1-c6(0.824)>18:1-t11(0.747)>18:1-c9(0.738)>18:1-t6(0.415)>18:2-t11, c15(0.387)> 18:2-c9, t11(0.239); B: 18:2-c9, c12(0.667)>18:3-c9, c12, c15(0.488). The CCA showed that 18:2-c9, c12 has a greater contribution than that of 18:3-c9, c12, c15 on the production of the aforementioned BH intermediates, in which 18:0, as well as the groups of 18:1 cis and trans-FA were mainly affected.

Keywords: cattle; lipid metabolism; multivariate analysis; polyunsaturated fatty acid; ruminant.

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Introduction

Ruminants can transform polyunsaturated fatty acids (PUFA) from diets into both cis and trans monounsaturated fatty acids (MUFA), as well as into saturated fatty acids (SFA) through the rumen biohydrogenation (BH) process (Jenkins, Wallace, Moate, & Mosley, 2008; Ferlay, Bernard, Meynadier, & Malpuech-Brugère, 2017). Moreover, a great proportion of forage and feedstuffs contains significant amounts of PUFA, in which both linoleic acid (18:2-c9, c12) and alpha-linolenic acid (18:3-c9, c12, c15) have a significant contribution to such fatty acids (FA) composition (Makmur, Zain, Agustin, Sriagtula, & Putri, 2020). Hence, a better understanding of the 18:2-c9, c12 and 18:3-c9, c12, c15 rumen BH process provides interesting findings regarding the use of feedstuffs rich in these FA in ruminant diets, searching for the improvement of the FA composition in ruminant milk and meat.

Reiser (1951) reported for the first time the BH pattern of PUFA by rumen microorganisms and Kepler, Hirons, McNeill and Tove (1966) made an early description of its mechanism. Further studies showed that some FA intermediates of rumen BH have beneficial effects on human health (Eftekhari, Aliasghari, Babaei-Beigi, & Hasanzadeh, 2013; Viladomiu, Hontecillas, & Bassaganya-Riera, 2016), and showed as well that the concentration of such FA can be modified through changes in the 18:2-c9, c12/18:3-c9, c12, c15 ratio of the diet (Ribeiro, Eastridge, Firkins, St-Pierre, & Palmquist, 2007; Vargas, Olivera, Ribeiro, & Daza, 2018). This provided valuable information regarding the mechanism of both 18:2-c9, c12 and 18:3-c9, c12, c15 rumen BH and the influence of their concentrations in the diet on the MUFA and PUFA profile in milk and meat of ruminants (Nudda et al., 2014, Chiofalo, Di Rosa, Lo Presti, Chiofalo, & Liotta, 2020).

Using multicompartmental models, Ribeiro et al. (2007) and Vargas et al. (2018) showed that 18:2-c9, c12 concentration influences the isomerization and BH rates of the main steps of 18:3-c9, c12, c15 rumen BH and vice versa. Also, Lee and Jenkins (2011) revealed that 18:2-c9, c12 and 18:3-c9, c12, c15 have common BH intermediates. Thus, 18:2-c9, c12 and 18:3-c9, c12, c15 interact during BH, and therefore, a multivariate approach is suitable to understand the relationship among 18:2-c9, c12, 18:3-c9, c12, c15, and their main BH intermediates in a more realistic way than this process is commonly studied (i.e., under a univariate approach). However, there are no studies in this regard, despite the availability of different multivariate statistical techniques. One of these techniques is the canonical correlation analysis (CCA).

The CCA calculates the multivariate correlations between two sets of variables (Hair, Black, Babin, & Anderson, 2014). This statistical technique has been used in ruminant nutrition studies with satisfactory results exploring the association between ruminal FA and bacteria taxa in dairy cows (Zubiria et al., 2019). Hence, CCA could be useful to understand the multivariate association between 18:2-c9, c12, 18:3-c9, c12, c15, and their main intermediates and products of their BH in the rumen as well, which can provide valuable findings regarding the quantitative associations between those FA during the rumen BH. Therefore, this study determined the strength of the multivariate association among 18:2-c9, c12, 18:3-c9, c12, c15, and their main BH rumen FA intermediates and products in the rumen fluid of bovines using CCA. The hypothesis of this study is that CCA provides multivariate quantitative correlations with biological meaning among 18:2-c9, c12, 18:3-c9, c12, c15, and their main FA rumen BH intermediates and products.

Materials and methods

Ethical considerations

All procedures on animals were approved by the Bioethics Committee of the Department of Veterinary and Animal Science of the National University of Colombia (Act 001 of 2010).

Dataset construction

A dataset consisting of 1177 observations generated by 107 rumen incubation systems from an *in vitro* study (Vargas et al., 2018) with pure and mixed 18:2-c9, c12 and 18:3-c9, c12, c15 was gathered (Table 1). Briefly, each incubation system was composed by 500 mg of kikuyu grass (*Cenchrus clandestinus*) mixed with different 18:2-c9, c12: 18:3-c9, c12, c15 (i.e., 100:0, 75:25, 50:50, 25:75, and 0:100) mixtures and 50 mL of pre-warmed rumen inoculum (composition: 40 mL buffer McDougall (McDougall, 1948) and 10 mL rumen fluid) from a rumen-fistulated Holstein Steer. The incubations were performed in 100 mL tubes sealed with one-hole rubber stoppers (Fisherbrand, Pittsburgh, PA, USA) during 0, 2, 4, 6, 8, and 16 h in a water bath at 39ºC (Blue Island, Illinois, USA) (Tilley & Terry, 1963).

The study was conducted at the Animal Nutrition Laboratory of the National University of Colombia (Bogota Campus, Colombia) and more details regarding the experimental conditions can be found in Vargas et al. (2018). The dataset built comprised 18:2-c9, c12, 18:3-c9, c12, c15, and their nine main FA rumen BH intermediates and products (18:2-c9,t11; 18:2-t11, c15; 18:1-t11; 18:1-t9; 18:1-t6; 18:1-c11; 18:1-c6; 18:1-c9; 18:0) according to the BH pathway patterns proposed by Vargas et al. (2018) under normal rumen physiological conditions. The proportions of FA were expressed as g 100^{-1} g total FA.

Table 1. Descriptive statistics of the dataset used for the canonical correlation analysis (n = 107).

IUPAC name of the FA	Formula	Mean of FA concentration (g 100^{-1} total FA)	Standard deviation	Minimum	Maximum
cis-9, cis-12-octadecadienoic acid	18:2-c9, c12	17.6	14.7	0.77	58.2
cis-9, cis-12, cis-15-octadeca trienoic acid	18:3-c9, c12, c15	17.9	11.8	3.42	48.6
cis-9, trans-11-octadeca dienoic acid	18:1-c9, t11	2.42	2.39	0.00	12.2
trans-11, cis-15-octadeca dienoic acid	18:2-t11, c15	2.59	1.79	0.09	6.67
trans-11-octadecenoic acid	18:1-t11	10.6	5.34	2.39	20.8
cis-9-octadecenoic acid	18:1-c9	0.97	0.26	0.52	1.68
trans-6-octadecenoic acid	18:1-t6	0.14	0.05	0.05	0.26
trans-9-octadecenoic acid	18:1-t9	0.36	0.21	0.08	0.90
cis-6-octadecenoic acid	18:1-c6	1.10	0.63	0.26	3.23
cis-11-octadecenoic acid	18:1-c11	0.25	0.09	0.12	0.50
Stearic acid	18:0	24.2	7.25	12.6	44.9

The FA proportions in the *in vitro* incubation systems were determined by GC-FID (gas chromatography coupled to a flame ionization detector). Briefly, the FA of freeze-dried content incubation systems (Alpha 1-4 Christ® plus LO lyophilizer) were extracted and methylated in a one-step method (Garcés & Mancha, 1993), being the methyl esters analyzed in a Shimadzu GC-2014 gas chromatograph (Shimadzu Manufacturing, Inc., Canby, OR, USA) under the chromatographic conditions stated elsewhere (Vargas et al., 2018).

Statistical analysis

At first, multivariate normality in the dataset was verified by using the test proposed by Mardia (1974) with the aid of the %MULTNORM macro of SAS (Statistical Analysis System [SAS], 2010). Thereafter, the relationship among 18:2-c9, c12, 18:3-c9, c12, c15, and their main rumen BH intermediates was determined using CCA. The CCA was performed applying the PROC CANCORR of the Statistical Analysis Software (version 9.4) and the following multivariate relationship was explored: 18:2-c9, c12 and 18:3-c9, c12, c15 (g FA 100^{-1} g total FA) with their main rumen BH intermediates and products (g FA 100^{-1} g total FA).

The objective of CCA is to explore the multivariate correlations between two sets of linear combinations of variables (intermediates plus products (canonical variate Ai; set one; dependent variables) vs precursors (canonical variate Bi; set two; independent variables)). In the case of the current study, the pair of linear combinations of the canonical variates can be mathematically defined as follows (Equations 1 and 2):

$$Ai = a_{i1} \times 18{:}2\text{-}c9, t11 + a_{i2} \times 18{:}2\text{-}t11, c15 + a_{i3} \times 18{:}1\text{-}t11 + a_{i4} \times 18{:}1\text{-}t9 + a_{i5} \times 18{:}1\text{-}t6 + \\ + a_{i6} \times 18{:}1\text{-}c11 + a_{i7} \times 18{:}1\text{-}c6 + a_{i8} \times 18{:}1\text{-}c9 + a_{i9} \times 18{:}0 \quad (1)$$

$$Bi = b_{i1} \times 18{:}2\text{-}c9, c12 + b_{i2} \times 18{:}3\text{-}c9, c12, c15 \quad (2),$$

in which, ai and bi are the standardized canonical weights, and Ai and Bi are the canonical variates. The total number of canonical pairs is defined by the minimum number of original variables included in a canonical variate being studied (i.e., canonical variate Bi). Thus, in this study, the number of pairs is equal to one (i = 2). Therefore, the pair of canonical correlations can be defined as (Equations 3 and 4):

$$R_1 = \frac{C\hat{o}v\,(A_1,B_1)}{\sqrt{(V\hat{a}r(A_1)V\hat{a}r(B_1)}} \quad (3)$$

$$R_2 = \frac{C\hat{o}v\,(A_2,B_2)}{\sqrt{(V\hat{a}r(A_2)V\hat{a}r(B_2)}} \quad (4)$$

The significance of the canonical correlations (R) was determined by the Wilks' Lambda test, and the proportion in the canonical variate Ai that is explained by the canonical variate Bi was assessed using the determination coefficient (R^2). The confidence level adopted was 95% ($\alpha = 0.05$).

Results and discussion

The BH of 18:2-c9, c12 and 18:3-c9, c12, c15 produce a diversity of PUFA, MUFA, and SFA, which are interrelated to each other by complex biochemical mechanistic processes that take place in the rumen (Ferlay et al., 2017). This agrees with the results of this study, in which two significant canonical correlations were found with values greater than that of 0.73 ($p < 0.01$; Table 2), between the canonical variates Ai (i.e., the main rumen BH intermediates of 18:2-c9, c12 and 18:3-c9, c12, c15) and Bi (i.e., 18:2-c9, c12 and 18:3-c9, c12, c15). Hence, the multivariate relationship among 18:2-c9, c12, 18:3-c9, c12, c15, and their main BH intermediates can be analyzed with confidence under CCA.

Table 2. Canonical correlations between linoleic acid, alpha-linolenic acid, and their main rumen biohydrogenation intermediates and products.

Number of the canonical function	Canonical correlation (R)	Squared canonical correlation (R^2)	Eigenvalue[a]	Approximate F statistics	p-value
1	0.990	0.981	51.57	104.04	<0.0001
2	0.738	0.545	1.199	14.54	<0.0001

[a] Eigenvalue of Inv(E)*H = $R^2/(1-R^2)$, in which Inv(E)*H is the product of the inverse (Inv) of the error matrix (E) and the model matrix (H).

An exploration of the two canonical correlation functions revealed that the eigenvalue for the first one (51.1) was significantly larger than that for the second one (1.19), which suggests that the variance in the canonical variate Ai explained by the canonical variate Bi can be better explained by the first canonical

function (98.1%; Figure 1a) than that for the second canonical function (54.5%; Figure 1b). Hence, considering the aforementioned, in this study, only the first canonical correlation function was interpreted.

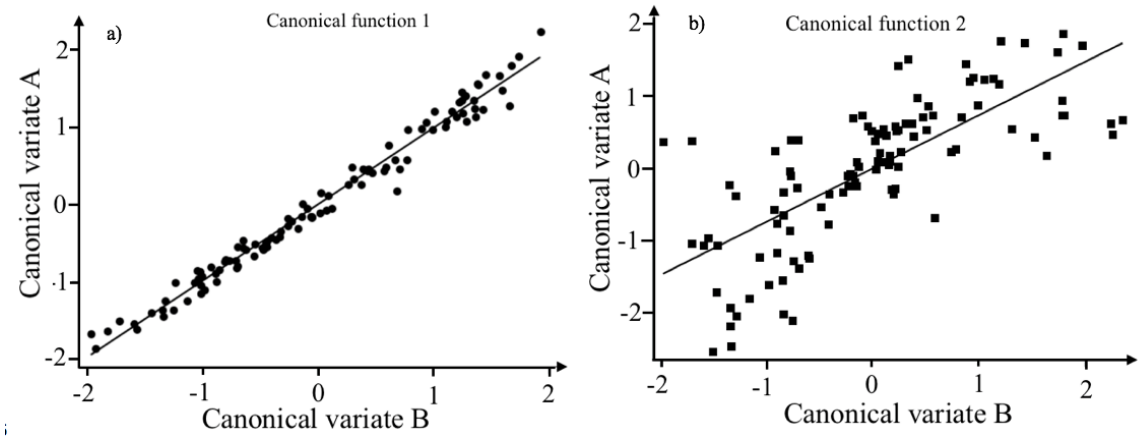

Figure 1. Scores of significant canonical correlation functions

Canonical weights of the original variables reveal their relative contribution to the corresponding Ai and Bi canonical variates. However, they may show high variability and multicollinearity producing unreliable estimations of variable contributions to canonical variates (Hair et al., 2014). Possibly, the above-mentioned fact took place in the current study, because low canonical weights for most of the main FA intermediates were observed (Table 3), despite the great FA abundance in the rumen BH, as reported in previous studies (Jouany, Lassalas, Doreau, & Glasser, 2007; Makmur et al., 2020). Therefore, to overcome this limitation, a deep exploration of the canonical loadings and cross-loadings was preferred in this study, instead of evaluating the canonical weights.

Table 3. Standardized canonical weights, canonical loadings, and cross-loadings of the canonical correlation function 1.

Variable	Standardized canonical weights	Canonical loadings	Canonical cross-loadings
Canonical variate A (intermediates and products)			
cis-9, trans-11-octadeca dienoic acid	0.2229	0.2385	0.2362
trans-11, cis-15-octadeca dienoic acid	0.3155	0.3869	0.3832
trans-11-octadecenoic acid	-0.0040	0.7472	0.7400
cis-9-octadecenoic acid	-0.0199	0.7379	0.7308
trans-6-octadecenoic acid	0.1016	0.4152	0.4112
trans-9-octadecenoic acid	0.0384	0.8372	0.8292
cis-6-octadecenoic acid	-0.0604	0.8239	0.8160
cis-11-octadecenoic acid	0.1483	0.8347	0.8267
Stearic acid	0.7245	0.9580	0.9489
Canonical variate B (Precursors)			
cis-9, cis-12-octadecadienoic acid	-0.9227	-0.6669	-0.6606
cis-9, cis-12, cis-15-octadeca trienoic acid	-0.7878	-0.4882	-0.4835

Canonical loadings reveal the association degree (correlation) between the original variables (i.e., FA proportions) and their respective canonical variates (i.e., Ai or Bi). The data showed a decreasing order of canonical loadings for the canonical variate Ai (Table 3) as follows: 18:0 (0.958) > 18:1-t9 (0.837) > 18:1-c11 (0.835) > 18:1-c6 (0.824) > 18:1-t11 (0.747) > 18:1-c9 (0.738) > 18:1-t6 (0.415) > 18:2-t11,c15 (0.387) > 18:2-c9,t11 (0.239). The pattern of values for canonical loadings in Ai agrees with several studies that revealed an association among these FA during the rumen BH of 18:2-c9, c12 and 18:3-c9, c12, c15 (Shingfield & Wallace, 2014; Ferlay et al., 2017). Additionally, the positive value of canonical loadings conforms to the fact that these FA are being produced during the rumen BH from the initial time at 18:2-c9, c12 and 18:3-c9, c12, c15 reach the rumen inoculum (Dewanckele, Toral, Vlaeminck, & Fievez, 2020).

A similar exploration of the canonical loadings for the variate Bi (Table 3), showed that the canonical loading (in absolute value) for 18:2-c9, c12 (-0.667) was greater than that of 18:3-c9, c12, c15 (-0.488). This suggests that 18:2-c9, c12 had a greater contribution in the canonical variate Bi than that observed for 18:3-c9, c12, c15. This

occurred by the fact that regardless of 18:2-c9, c12: 18:3-c9, c12, c15 ratio in the lipid mixtures evaluated in the incubation systems, 18:2-c9, c12 tends to BH slower than 18:3-c9, c12, c15 (Jouany et al., 2007). Hence, 18:2-c9, c12 tends to be more accumulated in the rumen than 18:3-c9, c12, c15. The negative sign in both canonical loadings for 18:2-c9, c12 and 18:3-c9, c12, c15 suggests that these FA are consumed during the rumen BH, which agrees with several FA kinetic studies (Jouany et al., 2007; Ribeiro et al., 2007; Vargas et al., 2018) that found a similar response to the one observed in this study. Additionally, the same sign for 18:2-c9, c12 and 18:3-c9, c12, c15 canonical loadings revealed that these FA are positively correlated, which may be due to a synergistic interaction of 18:2-c9, c12 and 18:3-c9, c12, c15 during their BH (Vargas et al., 2018), considering that both processes are metabolically linked (Lee & Jenkins, 2011).

Canonical cross-loadings are used to explore the multivariate association between the original variables and their opposite canonical variates. Therefore, in the present study, canonical cross-loadings for 18:2-c9, c12 and 18:3-c9, c12, c15 explored the multivariate correlations between these FA and the canonical variate Ai. Complementarily, canonical cross-loadings for intermediates and products evaluated the multivariate correlation between these FA and the canonical variate Bi. Thus, the aforementioned correlations can be used to understand the association among 18:2-c9, c12, 18:3-c9, c12, c15, and their respective rumen BH intermediates and products under a multivariate quantitative approach.

The data showed a similar pattern between canonical loadings and cross-loadings. The canonical cross-loading (in absolute value) for 18:2-c9, c12 (- 0.661) was greater than that of 18:3-c9, c12, c15 (- 0.484) and both correlations were negative (Table 3). The canonical cross-loadings for the rumen BH intermediates and products demonstrated the following decreasing pattern: 18:0 (0.949) > 18:1-t9 (0.829) > 18:1-c11 (0.827) > 18:1-c6 (0.816) > 18:1-t11 (0.740) > 18:1-c9 (0.731) > 18:1-t6 (0.411) > 18:2-t11,c15 (0.383) > 18:2-c9,t11 (0.236) and all of these were positive (Table 3). This suggests that a decrease in both 18:2-c9, c12 and 18:3-c9, c12, c15 concentration tends to increase the concentration of FA intermediates of products, which is the expected biological response for the 18:2-c9, c12 and 18:3-c9, c12, c15 rumen BH (Vargas et al., 2018).

Canonical cross-loadings also revealed a greater contribution of 18:2-c9, c12 than that of 18:3-c9, c12, c15 in the production of BH intermediates and products, in which 18:0, as well as the groups of 18:1 cis and trans-FA were mainly affected. These results agree with Jouany et al. (2007) who showed that 18:2-c9, c12 tended to be completely converted into 18:0, whereas 18:3-c9,c12,c15 tended to be mainly transformed into 18:1 trans-FA. Similar to Shingfield and Wallace (2014) and Ferlay et al. (2017) findings, which proposed the 18:1-t11 as the main intermediate of 18:2-c9, c12 and 18:3-c9, c12, c15 rumen BH and the presence of cis-18:1 FA during both processes. Therefore, in the designing of ruminant diets, a greater proportion of 18:2-c9, c12 than that of 18:3-c9, c12, c15 (60:40 approximately) may favor the production of 18:1-t11, the endogenous precursor for 18:2-c9,t11 production (Griinari et al., 2000; Vargas, Pabón, Olivera, & Carulla, 2010).

The evaluation of the dynamics of 18:2-c9, t11, 18:1-t11, and 18:0 production in the rumen has recently gained special attention due to beneficial effects on human health of 18:2-c9, t11 (i.e., CLA or conjugated linoleic acid) (Viladomiu et al., 2016). The data showed the lowest canonical cross-loading for 18:2-c9, t11, whereas 18:0 and 18:1-t11 showed the highest one among the aforementioned FA. This agrees with the 18:2-c9, t11, 18:0, and 18:1-t11 already known mechanisms of production in the rumen, which show that 18:1-c9, t11 is mainly produced by 18:2-c9, c12 (Shingfield & Wallace, 2014). However, 18:1-c9, t11 is rapidly converted into 18:1-t11 (Ribeiro et al., 2007), which tends to accumulate (Vargas et al., 2018), but it is finally transformed into 18:0 (Dewanckele et al., 2020). Hence, CCA explained the dynamics of 18:2-c9, c12, 18:3-c9, c12, c15, and their main BH intermediates and products with biological meaning, thereby, it is here being proposed its use on future studies aiming to understand the intricated patterns of 18:2-c9, c12 and 18:3-c9, c12, c15 rumen BH.

Conclusion

The results of the current study showed for the first time that the CCA technique can be used to understand the multivariate relationship between PUFA and their main rumen BH intermediates and products in the rumen fluid of bovines under an *in vitro* approach. This study also revealed that 18:2-c9, c12 has a greater contribution than that of 18:3-c9, c12, c15 on the production of the main FA rumen BH intermediates and products, in which 18:0, as well as the groups of 18:1 cis and trans-FA were mainly affected.

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