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Short communication

Antimethanogenic activity of *Monascus* metabolites in the rumen revealed by the concentration of statins, their diversity and the presence of acid forms

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ABSTRACT

Monascus-fermented cereals reduce methane production from the rumen. The identification of the metabolites responsible of the antimethanogenic effect is important to assess the potential of this strategy as a mitigation option in ruminant production. This study highlights metabolites from *Monascus ruber* associated to methane inhibition. An in vitro rumen screening test was used to rank solid-state fermented wheat samples for their ability to inhibit methane. Four active and four less-active samples were selected for metabolomics analysis using liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS) and the identity of discriminant variables responsible for this group distinction was assigned thanks to tandem mass spectrometry (MS/MS) experiments. A total of 28 discriminating metabolites were putatively identified based on their accurate *m/z* values, fragmentation pathways and information from databases. The chemical structure (identification level 1) was confirmed for 9 of them thanks to the available authentic chemical standards. Most of these metabolites belong to the chemical class of statins and their derivatives (n=13), four of them annotated as statin-like molecules were observed here for the first time. A targeted approach using LC-MS/MS was performed to measure the levels of known metabolites and showed that the lovastatin concentration in active samples was 16-fold greater than in least-active samples. Whereas lovastatin was the major metabolite, up to 40 % of the total statins were represented by other statin molecules. Comparison of the functional capability of lovastatin lactone and lovastatin acid demonstrates that the acid form is responsible for the antimethanogenic activity in the rumen environment. This study shows that *Monascus*-fermented feeds contain a wide variety of statins in both lactone and acid forms. Information from this work provides insight for improving the antimethanogenic efficacy of diets containing bioactive *Monascus* metabolites in ruminants

Abbreviations: HMGCR, 3-hydroxy-3-methyl-glutaryl-CoA reductase; MFW, *Monascus*-fermented wheat; PCA, Principal Component Analysis; OPLS-DA, orthogonal partial least squares discriminant analysis; MS, mass spectrometry; LC-HRMS, liquid chromatography coupled with high-resolution mass spectrometry; LC-MS/MS, liquid chromatography-tandem mass spectrometry; ESI, electrospray ionization; VIP, variable importance in projection.

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1. Introduction

Monascus-fermented cereals are widely used in the food industry as colorants and as nutraceutical dietary supplements for lowering lipidemia levels in humans. *Monascus spp.* produce several secondary metabolites such as statins, pigments, and γ-aminobutyric acid (Zhu et al., 2019). The concentration of secondary metabolites in the fermented product is influenced by many factors, in particular the type of substrate, temperature, time of incubation, and pH (Hajjaj et al., 2001; Kilikian et al., 2007; Zhou et al., 2009). Statins are a major family of metabolites with biological activities such as cholesterol lowering (Cho et al., 2006; Lee and Pan, 2012), anti-inflammatory, and anti-bacterial effects (Zhou et al., 2018). Statins have also been identified as anticancer agents in some cancers (Corcos and Le Jossic-Corcos, 2013; Zhang et al., 2019). Their health benefits have been reviewed (Lin et al., 2008; Xiong et al., 2019).

More recently, *Monascus*-fermented feeds were shown to reduce enteric methane production in ruminants (Morgavi et al., 2013; Wang et al., 2016). This anti-methanogenic effect is mainly ascribed to statins that affect the growth of rumen methanogens through the inhibition of the enzyme 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMGCR) (Miller and Wolin, 2001). Fermented feeds from *Aspergillus terreus*, another fungal species able to produce statins, also decreased methane emissions in goats (Candyrine et al., 2018; Mohd Azlan et al., 2018). Notwithstanding, the antimethanogenic effect could not be clearly ascribed to the dose of lovastatin (also known as monacolin K), the most common naturally produced statin in fermented feeds. And, in some studies, no or minor effects were observed (Klevenhusen et al., 2011; Ramírez-Restrepo et al., 2014). Some of these differences may be due to the animal species (small ruminant vs cattle). Whereas a minimal effective dose and the presence of the statin adsorbed to feeds or free in the medium have also been cited (Abrego-Gacia et al., 2021). Another possible reason for the inconsistent results is whether statins actively inhibit HMGCR in rumen methanogens. Statins occur naturally either as an intact lactone ring or as an open acid form. The open acid form is the active one that reduces cholesterol levels in clinical settings (Sirtori, 2014).

Fermented feeds contain different proportions of lovastatin lactone and lovastatin acid forms but also several other statin molecules with presumably antimethanogenic activity. In addition, other biologically active compounds such as pigments, organic acids and flavonoids (Zhu et al., 2019) might also have a direct or indirect effect on methane production. For instance, pigments produced by *Monascus spp*. also affect HMGCR activity (Zhou et al., 2019). Up to now, fermented feeds and extracts tested on ruminants were characterized for their lovastatin concentration. We hypothesized that other statins, including the proportion of lactone and acid forms, and secondary compounds might also contribute to the methane reduction effect of *Monascus*-fermented feeds and could explain the differences in biological activity of seemingly equivalent products. To use Monascus-fermented cereals as anti-methanogenic feed additives, it is essential to identify the metabolite(s) responsible for reducing enteric methane emissions. In this study, we investigated the composition of various *Monascus* extracts that had contrasting effects on methane production by the rumen microbiota in vitro. A metabolomics approach was used to highlight discriminant metabolites related to methane production.

2. Materials and methods

2.1. Evaluation of the antimethanogenic activity of Monascus-fermented wheat and lovastatin

Monascus-fermented wheat (MFW) was prepared using *Monascus ruber* (DSMZ, 62748). We used solid-state fermentation batches produced at a pilot scale by an external provider. The pilot plant used standardized solid-state fermentation protocols with controlled temperature, agitation and aeration and the main variable assessed was the time of incubation. Briefly, wheat was macerated in water at 4°C overnight followed by heating at 60 °C for 2 h. After cooling, excess water was removed and the soaked wheat was autoclaved at 121 °C for 20 min. One-week-old cultures of *Monascus ruber* were used for inoculation as described (Morgavi et al., 2013) and incubated at 30 °C for two or three weeks and dried at 50 °C at the end of the process. The homogeneity of the industrial process between batches was monitored using standard procedures based on fungal growth and color development of the MFW. The inhibition of methane production upon rumen microbes by MFW was then tested in vitro using a sequential batch incubation protocol as previously described (Morgavi et al., 2013). Briefly, three consecutive 24-h anaerobic incubations at 39 °C were performed in triplicate using MFW as substrate. Rumen fluid was obtained from three rumen-cannulated Texel wethers before the morning feeding. Wethers were fed a prairie hay diet and had unrestricted access to water. Rumen contents were transferred to the laboratory in isothermal flasks and strained through a polyester monofilament fabric (250-µm mesh aperture) to remove solids. Equal volumes of rumen fluid from each animal were pooled and then 10 mL were immediately inoculated into 120-mL vials containing 30 mL of an anaerobic buffer solution kept at 39 °C, and 500 mg of ground (1-mm sieve) MFW. The time elapsed between the collection of rumen contents and the start of the incubation was less than 30 min and the manipulations in the laboratory were carried out under a stream of oxygen-free CO_2 gas. In controls, MFW was replaced by unfermented wheat. At 24 h of incubation, 10 mL from each incubation were used to inoculate a new vial containing buffer and substrate. Including the first incubation, a total of 3 serial cultures were performed. Several batches of MFW (n= 20) were tested in a blind manner, and the most and least active samples (n= 4 in each group) were selected at the end of the third consecutive batch incubation. Data were statistically analyzed by 1-way ANOVA using the General Linear procedure of SAS (version 9.4, SAS Inst. Inc., Cary, NC) and fermentation vials as experimental units. Differences among means were tested against the control using the Dunnet statement. The antimethanogenic activity was expressed as the percentage decrease in methane production compared to 100 % in controls.

Conversion of lovastatin lactone (Sigma-Aldrich) to the hydroxy acid form was done following the method described by Yang and Hwang (2006). Lovastatin was dissolved in a mixture of MilliQ water/acetonitrile (1:1, v/v), then mixed (1:1, v/v) with the mixture of alkaline 0.1 N NaOH solution and acetonitrile (1:1, v/v) and incubated at 45 °C for 1 hour. Acetonitrile was further evaporated to avoid a negative effect on rumen microbes and fermentation and re-dissolved in ethanol. The effect on rumen in vitro methane production

was tested using a modification of the consecutive batch incubation described above. Briefly, incubations were performed in triplicate in Balch-type tubes (Chemglass Life Sciences, Vineland, NJ) containing 7 mL of buffer, 100 mg of hay and inoculated with 3 mL of pooled rumen fluid from three cows. Treatments contained 200 μL of lovastatin acid (1 mg/mL), lovastatin lactone (1 mg/mL), ethanol control and a control prepared with acetonitrile-NaOH as for the conversion of lovastatin.

2.2. Analytical methods

Sample preparation. Fermented wheat MFW samples were successively extracted with ethanol and acetonitrile to get the broadest possible representation of polar and medium-polar compounds occurring in extract samples. Briefly, fermented wheat samples were homogenized and ground to pass a 1-mm sieve. One gram of powdered sample was weighed into a 50 mL-PP centrifugation tube, followed by the addition of 2 mL of distilled water. After complete absorption of water, 5 mL of absolute ethanol were added. The resulting mixture was shaken horizontally for 15 min at 40 strokes per minute, and then centrifuged (4 696 *g*, 5 min). Five mL of the upper phase were transferred to a 15-mL Falcon tube. The MFW solid residue from the previous extraction was extracted again as above with 5 mL of acetonitrile. After centrifugation, 5 mL of supernatant were transferred into the same 15-mL Falcon tube. An aliquot (0.5 mL) of the combined extract was evaporated (SpeedVac, Thermo Fisher Scientific, Courtaboeuf, France). The dried residues were dissolved in 0.5 mL of 0.1 % formic acid in acetonitrile (1:1, v/v), and vortex-mixed for 20 seconds. The mixture was filtered through a 0.45 μm filter and analyzed by liquid chromatography-mass spectrometry.

The MFW samples were analyzed by liquid chromatography coupled with high-resolution mass spectrometry (LC-HRMS) to produce metabolic fingerprinting and by liquid chromatography-tandem mass spectrometry (LC-MS/MS) for identification and quantification of some selected metabolites. All analytical conditions are described in the Supporting Information.

2.3. Data processing and statistical analysis

Raw data was converted to NetCDF format using the file converter tool of Xcalibur 2.0.7 software (Thermo Fisher Scientific, Courtaboeuf, France), and processed using the open-source Workflow4Metabolomics platform running under R (Giacomoni et al., 2015). After filtering, a data matrix containing mass and retention time with associated signal intensities for all detected peaks was generated.

MS data were normalized to the total intensity and analyzed after pareto scaling by multivariate data analysis using SIMCA-P software (Umetrics, v. 13.01, Sweden). Principal component analysis (PCA) was first performed to visualize information in a dataset, and orthogonal partial least squares discriminant analysis (OPLS-DA) model was built to further highlight discriminant metabolites between active and non-active samples. A permutation test with 200 iterations was used to validate these models. The ratio of metabolite levels between active and less-active fermented samples was calculated for discriminant variables, and visualized in a heatmap using Metaboanalyst (http://www.metaboanalyst.ca).

Discriminant metabolites were subsequently subjected to one-way Anova (XLStat, Addinsoft, Paris, France), and $P > 0.05$ was considered as significant. The relations between individual statins and methane production were assessed by linear regression with methane yield as dependent variable and statins as independent variables. In addition, to investigate whether the statin profile can predict methane mitigation, a PLS regression was done with the identified statins and pigments and the methane yield calculated as the percentage inhibition compared to control.

2.4. Metabolite annotation

Discriminant variables with a variable importance in projection (VIP) > 1 (n = 254) were selected as potential metabolite markers, clustered by their retention time and then subjected to annotation/identification. Detection of fragment ions and/or adducts can facilitate their identity assignment, especially to confirm their chemical formula (identification according to the five-level classification system of Schymanski et al. 2014). As statins are the major active compounds in MFW, we attempted to identify them first. The identification level 1 was achieved for only nine of them. Due to unavailability of authentic standard compounds for most of them, their characterization was carried out based on their accurate m/z values, their fragmentation patterns and comparison with data available in public databases such as the Human Metabolome Database (www.hmdb.ca), KEGG (http://www.genome.jp/kegg/), and Metlin (http://metlin.scripps.edu/) (identification level 2 or 3).

3. Results and discussion

The tested MFWs had a wide range of effects on methane production; at the end of the third 24-h in vitro consecutive batch test they ranged from no effect (0 % decrease) up to 82 % decrease compared to controls (Supplementary Fig. S1). From the 20 different lots tested, we selected the four most active MFWs that decreased methane production by about 80 % (range between 82 % and 77 %), whereas the four least active samples selected ranged from 0 % to 22 % (mean= 14 %). Compared to controls, no negative effect on gas production, used as a proxy of rumen fermentation, was observed in any treatment. We then compared the metabolic profiles of these most and least active MFWs to discover metabolites that could explain the biological differences observed in the methane production. Note that most active samples were all from 2-week incubations and least active samples were incubated for 3 weeks.

3.1. Metabolite profiles differ between active and non-active MFW samples

Unsupervised analysis of MS data by PCA revealed a clear separation of the two groups (Fig. 1A; Supplementary Fig. S2 shows chromatograms from untargeted and targeted methods from selected samples). The first two components of PCA explained 32.3 and 21.4 % of the variance, respectively. The supervised OPLS model also showed a clear group separation (Fig. 1B). In both PCA and OPLS models, the active samples were grouped in a compact cluster suggesting similar individual metabolic profiles whereas the profile of least-active samples exhibited a larger variability (Figs. 1A and 1B).

Potential markers were selected in the S-plot (n = 254, Fig. 1C), clustered by retention time, and then subjected to annotation. Detection of fragment ions and/or adducts could increase confidence in the proposed identity. After removing these redundant variables (clusters and fragment ions), 53 putative metabolites were selected for further identification (Table 1 and S1). Based on their measured accurate *m/z* values and fragmentation patterns, 13 statins were identified belonging to 5 chemical groups. These are characterized by the presence of lactone or an acid form, as well as the lack of double bonds (i.e. dihydro statins) or dehydrolyzed statins (i.e. dehydro-monacolin K) (Supplementary information). The major statins have been previously described (Li et al., 2004; Mornar et al., 2013), but four out of the 13 are reported here for the first time (Table 1). We also identified the pigments, monascine and ankafalvin, and several amino acids and derivatives (Table 1).

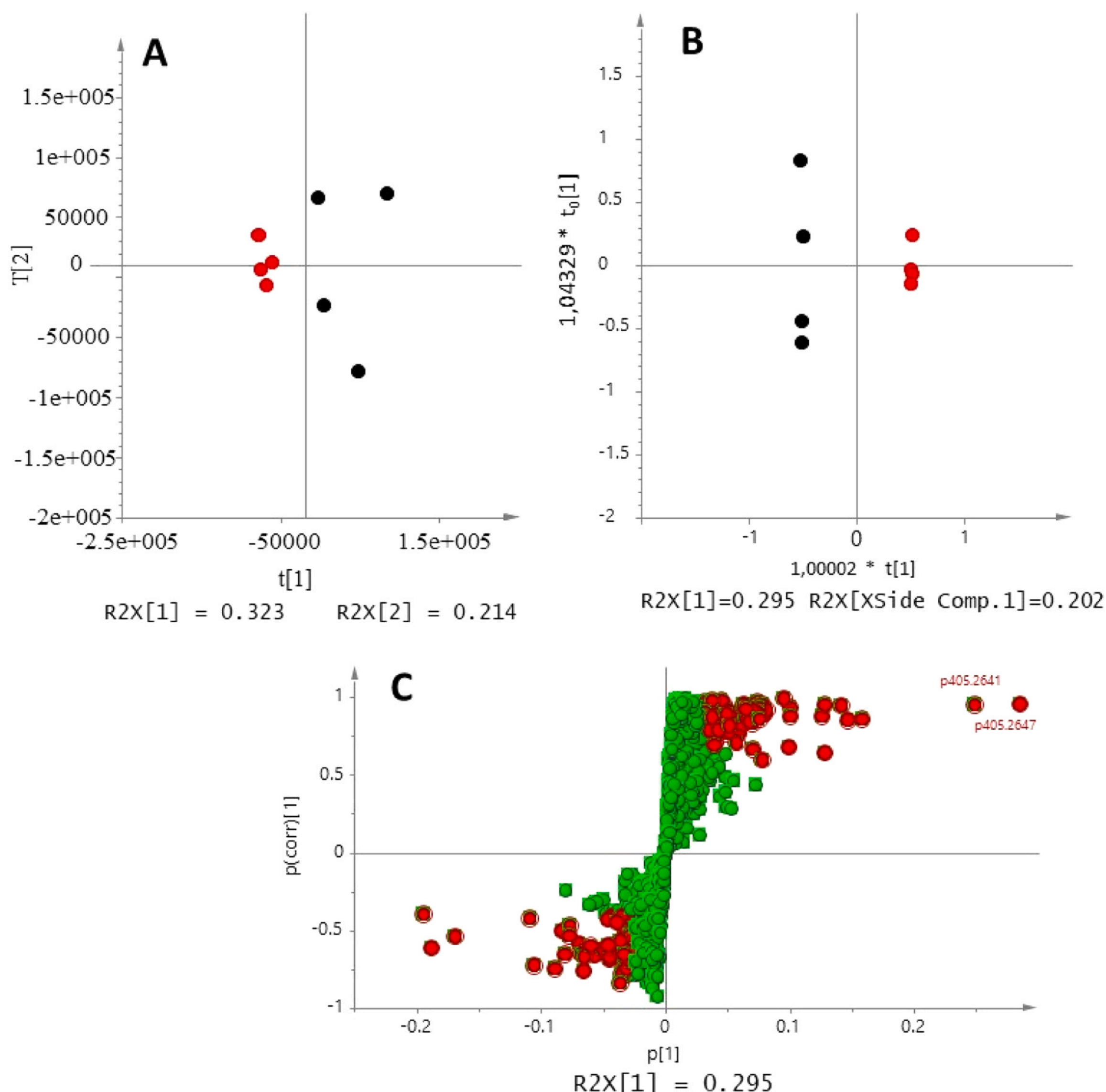

Fig. 1. Principal component analysis (A) and orthogonal projections to latent structures discriminant analysis (OPLS-DA) (B) score plots of mass spectrometry data, showing two distinct groups from active (red) and less active (black) *Monascus*-fermented wheat samples. (C) S-plot from the OPLS-DA model highlighting the most discriminating variables in red (n= 254). The labels correspond to lovastatin lactone and lovastatin acid.

Table 1
Putative metabolites identified by LC-HRMS (Orbitrap) and MS^n analyses in *Monascus*-fermented wheat.

#	Metabolites	m/z[a]	Suggested ion formula	Δ ppm	Rt (min)	VIP	P-value	Annotation	ID level[b]	FC[c]
	Monacolins and derivatives									
1	Lovastatin (lactone form)	405.2641	$C_{24}H_{37}O_5$	-1.30	16.5	10.6	0.015	$[M+H]^+$	1	10.5
		831.5015*			16.5	1.6	0.062	$[2M+Na]^+$		5.4
		826.5477*			16.5	3.2	0.073	$[2M+NH_4]^+$		66.0
		422.2895*			16.5	4.1	0.002	$[M+NH_4]^+$		9.3
		267.1443**			16.5	0.8	0.100	$[MH-C_5H_{14}O_4]^+$		20.6
		199.1463**			16.5	1.5	0.283	$[MH-C_9H_{18}O_5]^+$		39.6
2	**Lovastatin derivative**	450.3215	$C_{26}H_{44}O_5N$	-0.3	16.5	2.9	0.076	$[M+H]^+$	3	3.0
3	Monacolin J	321.2061	$C_{19}H_{29}O_4$	-0.2	11.8	1.3	0.008	$[M+H]^+$	2	22.8
4	Monacolin L	305.2119	$C_{19}H_{29}O_3$	-2.5	14.3	0.3	0.708	$[M+H]^+$	1	2.2
		287.1998**			14.2	1.0	0.595	$[M-H_2O]^+$		46.1
	Dehydroxy-Monacolins and derivatives									
5	Dehydroxy-monacolin K	387.2529	$C_{24}H_{35}O_4$	-0.33	18.6	6.2	0.075	$[M+H]^+$	1	20.2
		795.4812*			18.6	1.8	0.135	$[2M+Na]^+$		195.2
		790.5263*			18.6	2.2	0.153	$[2M+NH_3]^+$		238.6
		409.2347*			18.6	1.5	0.015	$[M]+Na]^+$		7.4
		404.2791*			18.6	3.2	0.023	$[M+NH_4]^+$		16.7
		285.1840**			18.6	1.7	0.243	$[MH-C_5H_{10}O_2]^+$		40.6
		199.1482**			18.6	1.0	0.193	$[MH-C_5H_{10}O_2-C_4H_6O_2]^+$		126.5
6	**Dehydroxy-MK derivative**	432.3100	$C_{26}H_{42}O_4N$	1.9	18.6	3.2	0.039	$[M+H]^+$	3	6.7
	Monacolins-acides and derivatives									
7	Lovastatin (acid form)	423.2736	$C_{24}H_{39}O_6$	-1.3	15.1	1.5	0.043	$[M+H]^+$	2	10.6
		445.2561*			15.1	1.1	0.017	$[M+Na]^+$		3.8
		405.2647**			15.1	12.1	0.015	$[MH-H_2O]+$		8.1
		321.2061**			15.1	0.9	0.026	$[MH-C_5H_{10}O_2]^+$		7.5
		303.1958**			15.1	3.1	0.011	$[MH-C_5H_{10}O_2-H_2O]^+$		11.1
		285.1857**			15.1	2.2	0.046	$[MH-C_5H_{10}O_2-2H_2O]^+$		9.7
		199.1483**			15.1	2.1	0.174	$[MH-C_5H_{10}O_2-2H_2O-C_4H_6O_2]^+$		19.4
8	Monacolin X acid form	437.2522	$C_{24}H_{37}O_7$	2.6	13.5	1.4	0.046		1	27.1
	Dihydro-Monacolins and derivatives									
9	Dihydro monacolin K	407.2784	$C_{24}H_{39}O_5$	1.9	17.7	4.2	0.023	$[M+H]^+$	2	31.9
		835.5344*			17.7	1.0	0.040	$[2M+Na]^+$		238.1
		429.2622*			17.7	1.3	0.015	$[M+Na]^+$		6.0
		424.3065*			17.7	2.7	0.015	$[M+NH_4]^+$		22.6
		305.2119**			17.7	1.1	0.054	$[MH-C_5H_{10}O_2]^+$		17.6
10	**Dihydro MK derivative**	452.3372	$C_{26}H_{46}O_5N$	-0.3	17.7	2.7	0.026	$[M+H]^+$	3	9.7
11	dihydro monacolin J	323.2221	$C_{19}H_{31}O_4$	-1.3	11.4	3.4	0.015	$[M+H]^+$	1	36.9
		340.2483*			11.4	1.4	0.110	$[M+NH_4]^+$		13.2
		305.2113**			11.4	6.0	0.018	$[MH-H_2O]^+$		12.9
		287.1981**			11.4	1.2	0.279	$[MH-2H_2O]^+$		15.5
		201.6015**			11.4	1.0	0.010	$[MH-C_5H_{10}O_2]^+$		14.0
	Dehydroxy-Dihydro-Monacolins and derivatives									
12	Dehydroxy-dihydro-monacolin K	389.2690	$C_{24}H_{37}O_4$	-1.0	19.9	2.5	0.069	$[M+H]^+$	1	51.4
		411.2503*			19.9	1.3	0.075	$[M+Na]^+$		20.7
		406.2958*			19.9	2.2	0.047	$[M+NH_4]^+$		70.9
		287.2001**			19.9	1.0	0.103	$[MH-C_5H_{10}O_2]^+$		29.7

(*continued on next page*)

Table 1 (*continued*)

#	Metabolites	*m/z*[a]	Suggested ion formula	Δ ppm	Rt (min)	VIP	P-value	Annotation	ID level[b]	FC[c]
13	**Dehydroxy-dihydro MK derivative**	434.3264	$C_{26}H_{44}O_4N$	-0.2	19.9	2.1	0.047		3	36.0
	Pigments									
14	Monascine	359.1846	$C_{21}H_{27}O_5$	1.9	16.9	2.6	0.646	$[M+H]^+$	1	0.2
15	Ankaflavin	387.2181	$C_{23}H_{31}O_5$	-4.0	18.6	0.6	0.706	$[M+H]^+$	1	2.8
16	Porphobilinogen	227.1026	$C_{10}H_{15}O_4N_2$	0.20	4.4	3.8	0.193	$[M+H]^+$	3	0.0
	Amino acids and derivatives									
17	Aminoadipic acid	162.0766	$C_6H_{12}O_4N$	3.3	0.4	1.3	0.706	$[M+H]^+$	3	0.5
18	Gamma-Aminobutyric acid	104.0701	$C_4H_{10}O_2N$	4.9	0.5	1.0	0.752	$[M+H]^+$	1	1.6
19	Alanine	90.05458	$C_3H_8O_2N$	4.2	0.5	2.0	0.508	$[M+H]^+$	3	1.9
20	Proline	116.0705	$C_5H_{10}O_2N$	0.7	0.6	7.2	0.435	$[M+H]^+$	3	0.4
21	Leucyl-Glycine	189.1232	$C_8H_{17}O_3N_2$	0.7	0.6	4.6	0.581	$[M+H]^+$	3	0.1
22	γ-Glutamyl-β-aminopropiononitrile	200.1041	$C_8H_{14}O_3N_3$	-5.7	0.6	3.4	0.047	$[M+H]^+$	3	4.6
23	Adenosine	268.1042	$C_{10}H_{14}O_4N_5$	-0.6	0.6	5.4	0.056	$[M+H]^+$	3	7.7
24	Phenylalanine	166.0865	$C_9H_{12}O_2N$	-1.2	0.7	8.0	0.359	$[M+H]^+$	3	0.3
25	N-Acetyl-3-hydroxy-L-tyrosine	240.0869	$C_{11}H_{14}O_5N_1$	-0.9	7.4	2.6	0.092	$[M+H]^+$	3	2.6
26	Propenoylcarnitine	216.1229	$C_{10}H_{18}O_4N$	0.8	7.8	3.1	0.561	$[M+H]^+$	3	1.2
27	3, 5-Tetradecadiencarnitine	368.2802	$C_{21}H_{38}O_4N$	-1.8	12.4	2.8	0.052	$[M+H]^+$	3	10.2
28	LysoPC(18:1(9Z))	522.3567	$C_{26}H_{53}O_7NP$	-2.5	16.8	2.0	0.612	$[M+H]^+$	3	3.4

[a] Adduct ions are marked with an asterisk (*) and product ions generated from decomposition of $[M+H]^+$ species are marked with two asterisk (**)
[b] ID level indicates the degree of confidence in the metabolite identification according to Schymanski et al. (2014): Level **1** for structure confirmed with data from an authentic standard such as accurate *m/z*, retention time and MS/MS spectra; Level **2** for probable structure based on library data match (e.g., presence of specific fragment ions); Level **3**: for tentative candidate such as compound class, sub-structure; Level **4**: for unequivocal determination of the chemical molecular formula with the detection of adducts and/or isotope; Level **5**: for *m/z* of interest accurately measured. Metabolites in bold, reported for the first time.
[c] FC, fold change was calculated by the average value of the most active *Monascus*-fermented wheat samples to that of the less active.

3.2. Relationship between identified metabolites and methane inhibition

In our study, a total of 28 metabolites, including statins (n = 13), pigments (n = 3), and amino acids and derivatives (n = 12), associated to methane inhibition were identified. The other 25 metabolites that were discriminant for their methane inhibition effect could not be identified (Supplementary Table S1). Hierarchical cluster analysis revealed two clusters corresponding to most-active and least-active samples and heatmap visualization of the identified metabolites showed that all statins as well as pigments were predominantly present in the active samples, whereas the less active samples were richer in amino acids and their derivatives (Supplementary Fig. S3). A PLS regression between the y-response (i.e. methane production) and the different independent variables, i.e. the main metabolites (statins, pigments monascine and ankaflavin) revealed the involvement of some specific metabolites with VIP values greater than 1.5. The identified metabolites could predict the effect of MFW on methane production with high accuracy ($R^2 = 0.98$, Fig. 2). Linear regressions of individual compounds show a negative relationship between all statins and methane production, whereas the relationships for the pigments, monascine and ankaflavin were weak or absent (Supplementary Fig. S4). Taken together, the PLS results (higher VIPs) and linear regression analyses indicate that methane production was mainly affected by a mixture of statins. Among these, two structures of lovastatin, the lactone form and the acid form, represented 28 and 33 %, respectively, of the total intensity of statins detected in active FMW samples using the untargeted method (Fig. 3A). In addition, the targeted method measured higher concentrations of these two statins in active samples compared to less-active samples, approximately 24 and 11 times higher for lovastatin and lovastatin acid, respectively (Fig. 3B). In contrast, the best regression models were obtained with lovastatin acid ($R^2 = 0.93$) and monacolin X acid ($R^2 = 0.91$), indicating that statins in acid form have a more significant effect (Supplementary Fig. S3). γ-Aminobutyric acid was also detected at a mean concentration of 212 μg/g but with no differences between groups. The other statins, mevastatin and pravastatin that could be identified by the targeted method, as well as citrinin and Sudan red G, were not present at detectable levels.

The average decrease in methane production observed in this study with the active MFW samples was similar to that obtained previously with extracts from *Monascus*-fermented rice using the same in vitro consecutive batch culture method (Morgavi et al., 2013). However, other in vitro studies (summarized in Abrego-Gacia et al., 2021) show an inconsistent relationship between statin dose and methane inhibition activity. Information from animal studies also show a lack of uniformity between the statin dose and the antimethanogenic effect. Fermented feeds added to the diet that delivered a dose of lovastatin of ~4 mg /kg BW (Body Weight) decreased methane emissions in goats from a range of 13–32 % (Wang et al., 2016; Candyrine et al., 2018; Mohd Azlan et al., 2018). In sheep, a dose of ~2.3 mg lovastatin/kg BW decreased enteric methane emissions by 30 % (Morgavi et al., 2013) and in beef cattle doses of up to 2.3 mg lovastatin/kg BW were not effective (Ramírez-Restrepo et al., 2014). In beef cattle, a higher dose was not tested because higher inclusion of fermented rice used as a bioactive feed negatively affected animal intake and health (Ramírez-Restrepo et al., 2014). Discrepancies between these studies could be due to different factors as type of fungus and strain, type of substrate used to grow them, fermentation temperature, and incubation time could significantly affect the concentration and type of statins produced. For animal studies, physiological differences between ruminant species, including the rumen environment and its microbiota, are also

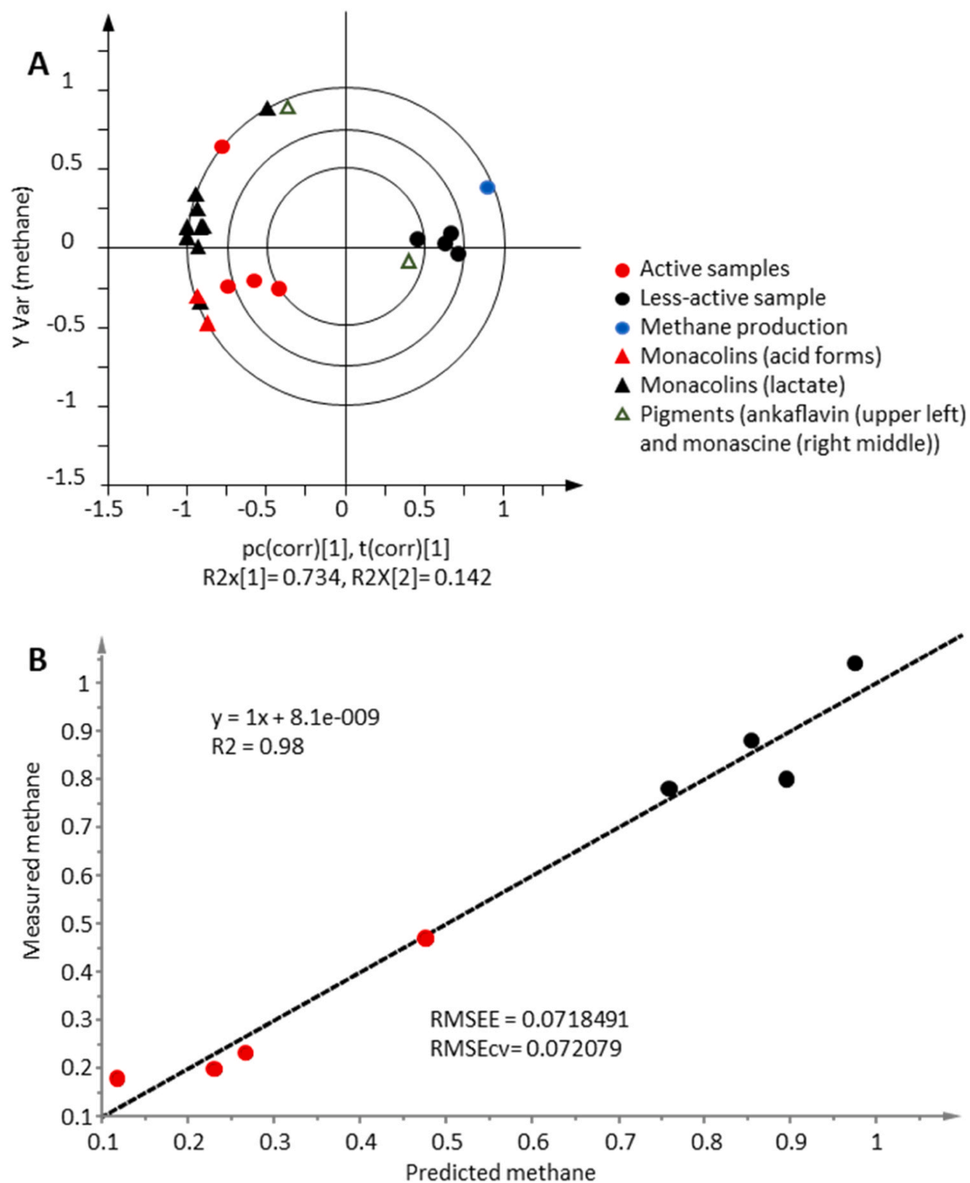

Fig. 2. Partial least square (A) and regression (B) for the prediction of the antimethanogenic effect of *Monascus*-fermented wheat based on concentration of statins and pigments.

possible explanations for differences across studies. For instance, low rumen pH may increase the absorption of statins, reducing their activity in the gastrointestinal tract (Hubert et al., 2018). Although pure statin preparations have been investigated, most studies reported the use of various fermented feeds such as straw, rice, wheat, or palm kernel cake that provided the substrate for different species and strains of *Monascus* and *A. terreus*.

Statin-containing fermented feeds and extracts that were tested to decrease enteric methane in ruminants were typified by the concentration of the main statin lovastatin but the proportion of the acid and lactone forms varied between studies (Wang et al., 2016; Candyrine et al., 2018), whereas this information is missing in others studies (Morgavi et al., 2013; Ramírez-Restrepo et al., 2014; Mohd Azlan et al., 2018). In mammalian cells it is known that the acid statin is the physiologically active form affecting cholesterol synthesis in the mevalonate pathway through inhibition of the HMGCR (Alberts, 1988; Sirtori, 2014). The same pathway is used by archaea to synthesize archaeol for their cell membranes. However, for methane mitigation there are contrasting results regarding whether the lactone or acid form is most active in the gastrointestinal environment. The acid form of lovastatin was found to be ineffective in inhibiting methane production in humans (Gottlieb et al., 2016; Hubert et al., 2018). The lactone form is more stable than the acid form and is more lipophilic. Thus, it is better suited to get though archaeal membranes and once inside the cell the lactone ring is broken up (Gottlieb et al., 2016). Also, there are indications that lactone forms may affect other targets in the methanogenesis

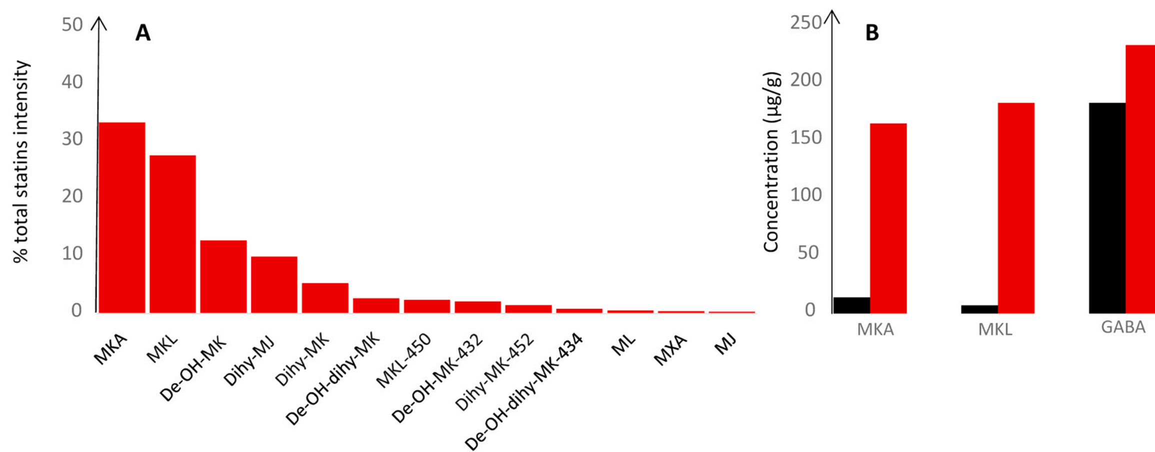

Fig. 3. Statins in Monascus-fermented wheat. A) Proportion of main statins detected in active antimethanogenic samples by untargeted liquid chromatography coupled with high resolution mass spectrometry. B) Differences in lovastatin concentration between active (red) and less-active (black) samples by targeted liquid chromatography- tandem mass spectrometry. MKA: lovastatin acid or monacolin K acid, MKL: lovastatin (lactone form) or monacolin K, De-OH-MK: dehydromonacolin K, Dihy-MJ: dihydro monacolin J, Dihy-MK: dihydro monacolin K, De-OH-dihy-MK: dehydro-dihydro-monacolin K, MKL-450: lovastatin derivative (*m/z* 450.3215), De-OH-MK-432: dehydro-MK derivative (*m/z* 432.3100), Dihy-MK-452: dihydro MK derivative (*m/z* 452.3372), De-OH-dihy-MK-434: dehydro-dihydro-monacolin K (*m/z* 434.3264), mL: monacolin L, MXA: Monacolin X acid form, MJ: Monacolin J, and GABA: γ-Aminobutyric acid.

pathway such as the F_{420}-dependent methylenetetrahydromethanopterin dehydrogenase (Muskal et al., 2016). In contrast, extracts from rice straw fermented by *Aspergillus terreus* containing 75 % of the acid form of lovastatin inhibited growth and methane production in pure cultures of *Methanobrevibacter smithii* more effectively than equivalent concentrations of lovastatin lactone (Faseleh Jahromi et al., 2013). For refining what would be the most active form in the rumen environment, we tested in vitro pure lovastatin lactone and the converted hydroxyacid form (Yang and Hwang, 2006) at a concentration of 25 μg/mL. At the end of the third sequential 24-h batch incubation, methane production decreased by 63 % for lovastatin acid but remained the same as control for the lactone form. There is no information on the fate of statins in the rumen environment. It has been reported that under temperature and pH comparable to those in the gastrointestinal tract, there is an abiotic conversion of lactone to the acid form (Beltran et al., 2019). However, this was observed over periods longer than the normal residence time of digesta in the rumen. Gut microbes do not seem to be involved in this first step but they can degrade the active acid form (Beltran et al., 2019), potentially reducing the antimethanogenic effect.

4. Conclusion

This study shows that Monascus-fermented feeds contain a rich mixture of statins in different chemical forms that collectively contribute to the antimethanogenic activity on the rumen microbiota. This also shows that acid lovastatin is the active form in the rumen environment, a finding that probably extends to all statins. Although lovastatin was the most abundant natural statin, minor statin fractions that are not usually monitored in standard analysis, contribute to the activity of the fermented feed. The proportion of lactone or acid form and the presence of statins other than the major statin lovastatin may account for the discrepant results reported in the literature. These results are relevant for further exploring the use of HMGCR inhibitors for reducing enteric methane production.

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CRediT authorship contribution statement

Estelle Rathalao-Paris: Writing – review & editing, Validation, Methodology, Formal analysis. **Ulli Hohenester**: Validation, Methodology, Formal analysis. **Hamid Boudra**: Writing – original draft, Validation, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Diego Morgavi**: Writing – original draft, Methodology, Investigation, Funding acquisition, Conceptualization. **Mounir Traikia**: Methodology, Formal analysis. **Marine Gauthier**: Methodology.

Declaration of Competing Interest

The authors declare no conflict of interest in this study.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.anifeedsci.2024.116013.

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