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## *Vernonia galamensis and vernolic acid inhibit fatty acid biohydrogenation in vitro*

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Abstract: Substituted long-chain fatty acids may be useful dietary supplements to suppress ruminal biohydrogenation of unsaturated fatty acids (UFA) and thereby increase the flow of UFA to meat and milk. The aim of this study was to determine if Vernonia galamensis (ironweed, a member of the sunflower family) and vernolic acid (cis-12,13-epoxy-cis-9-octadecenoic acid), the main constituent of the seed oil, affected the biohydrogenation of linoleic acid (LA; cis-9,cis-12-18:2) to rumenic acid (CLA; cis-9, trans-11-18:2), vaccenic acid (VA; trans-11-18:1) and stearic acid (SA; 18:0) by ruminal microorganisms. Ruminal digesta from four sheep receiving a mixed hay-concentrate diet were incubated in vitro with LA (0.2 g/L) □ 0.2 g/L vernolic acid or 5 g/L of dried flowers or leaves of V. galamensis, either alone or combined. Vernolic acid had a substantial effect on LA metabolism, causing decreases in cis-9, trans-11 CLA and VA accumulation as well as SA production (P<0.05). Vernolic acid inhibited growth of the rumen fatty acid-biohydrogenating bacterium, Butyrivibrio fibrisolvens but not B. proteoclasticus at 0.025 g/L; neither species grew at 0.05 g/L. An inhibition of the metabolism of LA as well as a decrease in the accumulation of cis-9, trans-11 CLA and a slowdown in its metabolism were observed in the presence of flowers, leaves and a combination of both (P<0.05). However, only incubations with flowers, either alone or with leaves, resulted in higher accumulation of VA (P≤0.05). Vernolic acid, constituted 2% of the total fatty acid content of flowers whereas it was not detected in leaves of V. galamensis. Thus, the greater accumulation of VA observed with flowers of V. galamensis was probably due to other components rather than vernolic acid. It was concluded that vernolic acid and V. galamensis inhibit the biohydrogenation of LA in vitro. V. galamensis could potentially be used as an additive to alter ruminal biohydrogenation, leading to greater concentrations of cis-9, trans-11 CLA in meat and milk. Evaluation of V. galamensis in vivo is required to confirm the present in vitro observations.

Ms. No. ANIFEE-16-7735R1 "Vernonia galamensis and vernolic acid inhibit fatty acid biohydrogenation in vitro"

We are glad to know that our manuscript has been favorably received. In relation to the referee's comments, we have modified the manuscript accordingly. The changes made in the manuscript as well as the responses to the comments are detailed below (in red).

Reviewer #2: The authors have addressed all of the issues I raised in the initial review. I think the revised manuscript is interesting and provides new ideas about fatty acid biohydrogenation. The only comment of disagreement would be about the statement on line 47 and line 304. The authors can certainly speculate that the greater accumulation of VA observed with the flowers of V glamensis may have been due to the presence of other components. Yes, it is even possible that as yet unidentified compounds are present in V glamensis, and that these unidentified compounds are extremely potent even though they might be present at a very low concentration. However, the authors do not seem to have evidence that these putative "other" components are "present in higher amounts". I suggest they delete the statement "present in higher amounts".

AU: We agree with the reviewer and that statement has been deleted in the revised manuscript.

Reviewer #3: I have just read the revised version; I appreciate the authors' efforts to reply to my suggestions. The answers they gave are satisfactory.
In my opinion an item should be more stressed; as you said on lines 308-310 "Our present understanding of ruminal bacteria that biohydrogenate fatty acids is undoubtedly incomplete, because it is likely that certain members of the community have not yet been cultivated".
This is a very important concept that you could emphasise by a sentence about it in the introduction of your paper; I would suggest on the line 60

AU: This information has been added to the revised manuscript (lines 65-68 "The most active biohydrogenating bacteria isolated from the rumen belong to Butyrivibrio group (Lourenco et al., 2010). However, other as yet uncultivated bacteria may play a more predominant role (Boeckaert et al., 2008; Huws et al., 2011)., as suggested.

Line 279: Replaced "Nobel" by "Noble"

Line 476: Replaced "CLA" by "VA"

**Highlights**

*Vernonia galamensis* and vernolic acid inhibit fatty acid biohydrogenation *in vitro*

**Ramos Morales *et al***

- Substituted long-chain fatty acids inhibit ruminal fatty acid biohydrogenation
- We investigated the effects of *V. galamensis* and vernolic acid on biohydrogenation
- Both the plant and pure vernolic acid inhibited biohydrogenation by ruminal digesta
- Vernolic acid inhibited growth of the biohydrogenating bacteria in pure culture

1 *Vernonia galamensis* and vernolic acid inhibit fatty acid
2 biohydrogenation *in vitro*

3

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16

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18

19

20

21 Abbreviations: CLA, conjugated linoleic acid; DHA, docosahexaenoic acid; DM, dry
22 matter; EPA, eicosapentaenoic acid; MS, mass spectrometry; LA, linoleic acid; LNA,
23 linolenic acid; SA, stearic acid; SRF, strained ruminal fluid; UFA, unsaturated fatty
24 acids; VA, vaccenic acid.

## Abstract

Substituted long-chain fatty acids may be useful dietary supplements to suppress ruminal biohydrogenation of unsaturated fatty acids (UFA) and thereby increase the flow of UFA to meat and milk. The aim of this study was to determine if *Vernonia galamensis* (ironweed, a member of the sunflower family) and vernolic acid (*cis*-12,13-epoxy-*cis*-9-octadecenoic acid), the main constituent of the seed oil, affected the biohydrogenation of linoleic acid (LA; *cis*-9,*cis*-12-18:2) to rumenic acid (CLA; *cis*-9, *trans*-11-18:2), vaccenic acid (VA; *trans*-11-18:1) and stearic acid (SA; 18:0) by ruminal microorganisms. Ruminal digesta from four sheep receiving a mixed hay-concentrate diet were incubated *in vitro* with LA (0.2 g/L) ± 0.2 g/L vernolic acid or 5 g/L of dried flowers or leaves of *V. galamensis*, either alone or combined. Vernolic acid had a substantial effect on LA metabolism, causing decreases in *cis*-9, *trans*-11 CLA and VA accumulation as well as SA production ($P$<0.05). Vernolic acid inhibited growth of the rumen fatty acid-biohydrogenating bacterium, *Butyrivibrio fibrisolvens* but not *B. proteoclasticus* at 0.025 g/L; neither species grew at 0.05 g/L. An inhibition of the metabolism of LA as well as a decrease in the accumulation of *cis*-9, *trans*-11 CLA and a slowdown in its metabolism were observed in the presence of flowers, leaves and a combination of both ($P$<0.05). However, only incubations with flowers, either alone or with leaves, resulted in higher accumulation of VA ($P$≤0.05). Vernolic acid, constituted 2% of the total fatty acid content of flowers whereas it was not detected in leaves of *V. galamensis*. Thus, the greater accumulation of VA observed with flowers of *V. galamensis* was probably due to other components rather than vernolic acid. It was concluded that vernolic acid and *V. galamensis* inhibit the biohydrogenation of LA *in vitro*. *V. galamensis* could potentially be used as an

50 additive to alter ruminal biohydrogenation, leading to greater concentrations of *cis*-9,
51 *trans*-11 CLA in meat and milk. Evaluation of *V. galamensis in vivo* is required to
52 confirm the present *in vitro* observations.
53
54 Keywords**:** biohydrogenation, conjugated linoleic acid, rumen, vernolic acid,
55 *Vernonia galamensis*
56
57 **1. Introduction**
58
59 Ruminant products contain conjugated linoleic acids (CLA), of which the most
60 abundant is usually rumenic acid (CLA; *cis*-9, *trans*-11-18:2), and vaccenic acid (VA;
61 *trans*-11-18:1) which are potential health-promoting and disease-preventing agents
62 (Kritchevsky, 2000; Field et al., 2009; Dilzer and Park, 2012). Conjugated linoleic
63 acid and VA are produced as intermediate products in the biohydrogenation of
64 linoleic acid (LA; *cis*-9, ci*s*-12-18:2) present in the feed to stearic acid (SA; 18:0)
65 (Jenkins et al., 2008). The most active biohydrogenating bacteria isolated from the
66 rumen belong to Butyrivibrio group (Lourenco et al., 2010). However, other as yet
67 uncultivated bacteria may play a more predominant role (Boeckaert et al., 2008; Huws
68 et al., 2011). If ruminal biohydrogenation could be controlled by, for example, a
69 dietary additive, the unsaturated fatty acid content, particularly *cis*-9, *trans*-11 CLA
70 and VA, of ruminant products and their healthfulness would be improved.
71 Evidence is increasing that substituted long-chain fatty acids, comprised of a carbon
72 chain with one or more substituent groups, including those from the plant kingdom
73 (Durmic et al., 2008), may be effective feed additives to control biohydrogenation.
74 Coronaric acid is a C-18 epoxy fatty acid that contributes to the inhibitory effect of

75 *Chrysanthemum coronarium* on biohydrogenation (Wood et al., 2010) that leads to an
76 improved fatty acid composition of milk from sheep receiving *C. coronarium*
77 (Cabiddu et al., 2006). Ricinoleic acid (12-hydroxy-*cis*-9-18:1), the main fatty acid
78 component of castor oil, inhibited the biohydrogenation of LA *in vitro* (Ramos
79 Morales et al., 2012). *Vernonia galamensis*, an East African plant of the sunflower
80 family known sometimes as ironweed, contains a high concentration of vernolic acid
81 (*cis*-12,13-epoxy-*cis*-9-18:1; 54-74%), an isomer of coronaric acid, which is present
82 in the seed oil (Baye et al., 2005). The effects of neither the plant nor vernolic acid on
83 fatty acid biohydrogenation have been investigated. The aim of the present
84 experiments was to explore the potential of *V. galamensis* and vernolic acid to
85 function as biohydrogenation inhibitors.
86
87 **2. Material and methods**
88
89 *2.1. Animals and diets*
90
91 Animal experimentation was carried out under conditions governed by a licence
92 issued by the United Kingdom Home Office. Four mature sheep, each fitted with a
93 ruminal cannula, received 800 g dry matter (DM)/day of ration comprising (g/kg
94 DM) grass hay (300), rolled barley (422.5), soybean meal (167.5), molasses (100)
95 and minerals and vitamins (10) as two equal meals (2 × 400 g) at 0800 and 1600.
96 Samples of ruminal digesta were collected from each animal just before the morning
97 feeding. Digesta samples were bubbled with $CO_2$, maintained at 39 °C, and strained
98 ruminal fluid (SRF) was obtained by straining through double-layered muslin gauze.

99 Each set of incubations was carried out using ruminal digesta from four sheep
100 incubated individually (four replicates).
101
102 *2.2. Vernonia galamensis samples*
103
104 Freeze dried samples of flowers and leaves of *V. galamensis* were obtained from the
105 African Centre for Crop Improvement (University of KwaZulu-Natal, South Africa).
106 Samples were ground to pass a 1 mm screen.
107
108 *2.3. Incubations with ruminal digesta in vitro*
109
110 In order to determine the effect of vernolic acid on the metabolism of LA, SRF was
111 incubated with 0.2 g/L of LA (Sigma–Aldrich Co. Ltd., UK) in the presence or
112 absence of vernolic acid (Larodan Fine Chemicals, Sweden) added at 0.2 g/L.
113 Additionally, incubations of SRF with 0.2 g/L of either *cis*-9, *trans*-11 CLA or VA
114 (Sigma–Aldrich Co. Ltd., UK), as substrates for the biohydrogenating bacteria, with
115 or without vernolic acid (0.2 g/L), were carried out with the aim of studying where in
116 the biohydrogenation sequence the inhibition by vernolic acid occurred. Likewise, in
117 order to determine the effect of flowers or leaves of *V. galamensis* on the metabolism
118 of LA, SRF was incubated with 0.2 g/L of LA in the presence or absence of 5 mg of
119 ground and freeze dried flowers or leaves. Furthermore, in an attempt to study the
120 possible synergistic effect between flowers and leaves on the metabolism of LA,
121 incubations of SRF with LA (0.2 g/L) with or without a mixture of flowers and leaves
122 (2.5 mg of each) of *V. galamensis* were carried out. Fatty acids were incubated as an
123 oil in-water suspension obtained by sonication for about 4 min.

124 Tubes were removed at 0, 1, 3, 6, 9 and 24 h for fatty acid analysis. Reactions were
125 stopped by heating in a heating block at 100°C for 10 min and tubes were stored at -
126 20°C.

127

128 *2.4. Incubations with pure cultures*

129

130 Two species of ruminal bacteria were used. *Butyrivibrio fibrisolvens* JW11 was
131 originally isolated from sheep as a proteolytic species (Wallace and Brammall, 1985).
132 *Butyrivibrio proteoclasticus* P18 is a SA-producing bacterium isolated from grazing
133 sheep (Wallace et al., 2006). These two species are the main cultivated species known
134 to be involved in fatty acid biohydrogenation (Lourenço et al., 2010).
135 *Propionibacterium acnes* G449 was isolated in the same study as *B. proteoclasticus*
136 P18. *Megasphaera elsdenii* J1 was isolated from a sheep. These ruminal bacteria are
137 held in the culture collection maintained at the Rowett Institute of Nutrition and
138 Health.
139 In order to study the effect of vernolic acid on growth, incubations were carried out
140 under $O_2$-free $CO_2$ at 39°C in Hungate-type tubes in the medium M2 (Hobson, 1969)
141 without agar. Inoculum volumes were 5% (v/v) of a fresh overnight culture into 5 mL
142 of medium. Vernolic acid was added to a final concentration of 0.0025, 0.025 and
143 0.05 g/L. Fatty acids were prepared as a separate solution, sonicated for 4 min in a
144 small volume of medium and added to the medium before dispensing and autoclaving.
145 Growth of bacteria was measured in triplicate from the increase in optical density at
146 650 nm of the control tubes using a Novaspec II spectrophotometer (Amersham
147 Biosciences, UK).

148

149 *2.5. Fatty acid extraction and analysis*

150

151 Extraction of total fatty acids was based on the method of Folch et al. (1957),
152 incorporating the modifications of Devillard et al. (2006). Nonadecanoic acid (0.1 mL
153 of 0.2 g/L in methanol) was used as internal standard. Solid-phase extraction
154 (Kalunzy et al., 1985) was used to separate free fatty acids from other lipids following
155 Folch extraction.

156 Fatty acid methyl esters were prepared under mild conditions using methanolic $H_2SO_4$
157 (Wąsowska et al., 2006) as a catalyst and quantified using a gas chromatograph
158 (model 6890, Agilent Technologies) equipped with a flame-ionization detector,
159 quadrupole mass-selective detector (model 5973N) and a 100-m fused silica capillary
160 column CP Sil 88 (i.d., 0.25 mm) coated with 0.2 μm film of cyanopropyl
161 polysiloxane (Varian Analytical Instruments, Walton-on-Thames, Surrey, UK) using
162 Helium as the carrier gas. The fatty acid methyl esters profile in 1 μl of sample at a
163 split ratio of 20:1 was determined using a temperature gradient programme (initial
164 temperature 80$^o$C for 1 min; increased at a rate of 25$^o$C/min to 160$^o$C, which was held
165 for 3 min, then increased at a rate of 1$^o$C/min to 190$^o$C, then further increased at a rate
166 of 10$^o$C/min to 230$^o$C, held for 40 min). Injector and MS detector temperatures were
167 maintained at 250 and 230$^o$C, respectively. Peaks were routinely identified by
168 comparison of retention times with authentic fatty acid methyl esters standard
169 obtained from Sigma (Poole, Dorset, UK) and Matreya (Pleasant Gap, PA, USA).
170 Identification was validated based on electron impact ionization spectra of fatty acid
171 methyl esters obtained under an ionization voltage of 2247 eV.

172 Vernolic acid concentration in flowers and leaves was determined at the University of
173 the Free State (South Africa). Fatty acids were extracted following the method of

174 Folch et al. (1957) and a base-catalysed transesterification with sodium methoxide
175 was carried out as described by Park et al. (2001), Kramer et al. (2002) and Alfaia et
176 al. (2007). Vernolic acid was determined using a Varian 430 flame ionization gas
177 chromatograph with a fused silica capillary column Chrompack CP Sil 88 (100 m ×
178 0.25 mm i.d. × 0.2 μm film thickness) and hydrogen as the carrier gas. Vernolic acid
179 was determined using a temperature program that initially started at 40°C for 2 min,
180 increased at a rate of 4°C/min to 230 which was held for 10 min. Injector and detector
181 temperatures were maintained at 250°C.
182
183 *2.6. Data Analysis*
184
185 Mixed-culture data were analysed at each time point separately by randomized
186 complete block ANOVA, with individual sheep as a blocking term, according to the
187 model:
188 $Y_{ijk} = \mu + D_i + A_j + e_{ijk}$
189 Where $Y_{ijk}$ is the dependent, continuous variable (n =4), μ is the overall mean; $D_i$ is
190 the fixed effect of the treatment, $A_j$ is the random effect of the animal inoculum (j= 1
191 to 4) and $e_{ijk}$ is the residual error. Individual comparisons were determined by post
192 hoc *t*-tests using the unprotected LSD. Pure-culture data were analysed by ANOVA,
193 again compared at each sampling time. Genstat 10th edition (VSN International, UK)
194 was used.
195
196 **3. Results**
197
198 *3.1. Incubations of ruminal digesta with C-18 fatty acids and vernolic acid*

199

200 *In vitro* incubations of SRF and LA either alone or with 0.2 g vernolic acid/L were
201 carried out in order to study the influence of vernolic acid on the metabolism of LA
202 (Fig. 1). An inhibition ($P<0.05$ at 1 and 3 h) of LA disappearance as well as an
203 inhibition of the accumulation of *cis*-9, *trans*-11-CLA ($P<0.05$ at 1, 3 and 6 h) and
204 VA ($P<0.05$) was observed in the presence of vernolic acid. Subsequently, SA
205 accumulation over time decreased ($P<0.05$) with vernolic acid.
206 Incubations of SRF with *cis*-9, *trans*-11 CLA as substrate (Fig. 2a) confirmed a
207 slightly slower metabolism compared to LA (Fig. 1a), as 29.6% and 60% of the initial
208 amount of CLA and LA added, respectively, disappeared after 1 h of the incubation.
209 Vaccenic acid accumulated and SA increased in much the same way as with LA.
210 Vernolic acid caused a slowdown of the loss of *cis*-9, *trans*-11 CLA ($P<0.05$ at 3 and
211 9 h), in comparison with the control incubations. Also, the accumulation of VA was
212 lower ($P<0.05$ at 1, 9 and 24 h), as was the increase of SA over time ($P<0.05$) in the
213 presence of vernolic acid (Fig. 2).
214 Vaccenic acid was metabolised more slowly than the dienoic acids, indeed its
215 concentration reached a plateau level at 12 h which was not changed at 24 h (Fig. 3).
216 Vernolic acid did not appear to impair the metabolism of VA; if anything the
217 percentage of VA that disappeared in the presence of vernolic acid was slightly
218 greater (38.5% vs. 30 and 39.8% vs. 29.5% at 9 and 24 h, respectively, $P<0.05$),
219 although less SA was produced over time as compared with the control ($P<0.05$).

220

221 *3.2. Incubations of ruminal digesta with linoleic acid and leaves and flowers of*
222 *Vernonia galamensis.*

223

224 Strained rumen fluid was incubated *in vitro* with LA (0.2 g/L) in the presence or
225 absence of flowers or leaves of *V. galamensis* (Fig. 4). Incubations of *V. galamensis*
226 leaves with LA led to an inhibition of the disappearance of LA ($P$<0.05) and lower
227 accumulation of *cis*-9, *trans*-11-CLA ($P$<0.001 at 1 h) with slightly increased
228 accumulation of VA and no effect on SA production. Incubations with flowers of *V.*
229 *galamensis* and LA also resulted in an inhibition of disappearance of LA ($P$<0.05),
230 although to a less extent than that observed with the leaves. A decrease in the
231 accumulation of *cis*-9, *trans*-11 CLA was also shown in incubations with flowers
232 ($P$<0.001 at 1 h), values being higher than those observed in the presence of leaves.
233 However, VA concentration increased by 16-23% (VA tended to be higher, $P$=0.054
234 and 0.057, at 1 and 9 h of the incubation, compared with the control). A similar effect,
235 inhibition of the loss of LA ($P$<0.05) as well as decreased *cis*-9, *trans*-11 CLA
236 accumulation ($P$<0.05 at 1 h) and increased accumulation of VA ($P$=0.033 at 1 h of
237 the incubation), was observed when flowers and leaves were combined (Fig. 5), but
238 no signs of synergy were evident. Oleic acid and LA were present at a higher
239 concentration in flowers (0.618 and 2.92 mg/g DM) than leaves (0.367 and 2.08 mg/g
240 DM). Also, the proportion of oleic and LA in the non-esterified form was higher in
241 flowers than leaves (Table 1). Leaves of *V. galamensis* had a much higher
242 concentration of linolenic acid (LNA; 13.9 mg/g DM) in comparison with flowers
243 (1.98 mg/g DM). Vernolic acid, comprised 2% of the total fatty acids in flowers
244 whereas it was not detected in leaves of *V. galamensis* (data not shown).
245
246 *3.3. Influence of vernolic acid on growth of biohydrogenating ruminal bacteria*
247

248 Vernolic acid did not inhibit growth of *B. fibrisolvens* or *B. proteoclasticus* at 0.0025
249 g/L ($P$>0.05; Fig. 6a). When added at 0.025 g/L, vernolic acid completely inhibited
250 growth of *B. fibrisolvens* ($P$<0.001) while *B. proteoclasticus* grew after a lag time of 5
251 h (Fig. 6b). Neither species grew when vernolic acid was added at 0.5 g/L (Fig. 6c).
252 Vernolic acid did not have any effect on *M. elsdenii* or *P. acnes* (not shown).
253
254 **4. Discussion**
255
256 Unsaturated fatty acids at high concentration inhibit their own biohydrogenation
257 because they are toxic to the main species involved in biohydrogenation, namely those
258 related to *Butyrivibrio* (Maia et al., 2007). The more unsaturated the fatty acid
259 molecule, the greater seems to be its inhibitory effects (Maia et al., 2007). The fish oil
260 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are
261 particularly effective (Fievez et al., 2003; AbuGhazaleh and Jenkins, 2004; Wąsowska
262 et al., 2006; Maia et al., 2007); these fatty acids originate in algae, which has also
263 shown to inhibit fatty acid biohydrogenation (Boeckaert et al., 2007; Vlaeminck et al.,
264 2008; Toral et al., 2012). Few studies, however, have pointed out the potential of
265 unsaturated hydroxy- and epoxy- fatty acids as manipulators of rumen
266 biohydrogenation (Wood et al., 2010; Ramos-Morales et al., 2012).
267 *Vernonia galamensis* is a widely distributed weed in Eastern Africa, but a potential
268 industrial oilseed crop. The seed oil of *V. galamensis* contains vernolic acid, a natural
269 epoxy fatty acid that can replace the expensive synthetic epoxy compounds used in
270 the plastic and coating industries. Our interest in vernolic acid stemmed from studies
271 carried out with coronaric acid (Wood et al., 2010), an isomer of vernolic acid, which
272 showed its effectiveness in inhibiting biohydrogenation. Also, we hypothesised that

273 the high LNA content in *V. galamensis* leaves reported by others (Baye et al., 2005)
274 could potentially have an inhibitory effect on fatty acid biohydrogenation.
275 The rapid loss of LA and transient accumulation of *cis*-9, *trans*-11 CLA and VA in
276 control incubations, followed by the slower accumulation of SA, are features that
277 have been observed previously (Waşowska et al., 2006; Ramos Morales et al., 2012).
278 They reflect the relative rates associated with the different steps in the pathway, *viz.*
279 LA >CLA>VA>SA (Noble et al., 1974). Vernolic acid slowed the disappearance of
280 LA and decreased the accumulation of *cis*-9, *trans*-11 CLA and VA and also the
281 increase in SA concentration. The inhibition of an accumulation of these
282 intermediates is therefore also reminiscent of previous studies with fish oil, EPA,
283 DHA and ricinoleic acid (Waşowska et al., 2006; Ramos Morales et al., 2012). From
284 these results, vernolic acid appears to be an inhibitor of LA metabolism and
285 biohydrogenation although it did not lead to the accumulation of *cis*-9, *trans*-11 CLA
286 or VA. This effect seems to be greater than that reported for coronaric acid (Wood et
287 al., 2010). It should be noted that Fig. 2 a) (also Fig. 3 a)) illustrates the metabolism
288 of esterified LA in the feed, for which the rate-limiting step will be lipolysis
289 (Lourenço et al., 2010). Vernolic acid did not appear to influence this rate,
290 presumably because it did not inhibit the rate-limiting step.
291 We hypothesised that flowers of *V. galamensis* would possibly have an inhibitory
292 effect on biohydrogenation due to their content in vernolic acid which has been found
293 to be present in seeds of *V. galamensis* (Baye et al., 2005). Our hypothesis is
294 consistent with previous work on the fatty acid composition of the seed oil of the
295 variety of *V. galamensis* used in our study (Shimelis et al. 2006). However, the
296 analysis of the fatty acid composition of the flowers revealed that vernolic acid
297 accounted for about 2% of total lipid (data not shown). The unexpected low vernolic

298 acid content in the flowers could be explained by differences in the mature stage of
299 the seeds as it has been reported that seeds harvested at full maturity had significantly
300 higher oil and vernolic content than comparable samples harvested at a less mature
301 stage (Thompson et al., 1994). Also, it should be borne in mind that entire flowers
302 rather than seeds were used in our incubations and thus the content of vernolic acid
303 would have been lower than that of the seeds alone. Irrespective of the inhibitory
304 effect on biohydrogenation shown in incubations with vernolic acid, the greater
305 accumulation of VA observed with flowers of *V. galamensis* must have been due to
306 other components rather than vernolic acid. Fatty acid analysis confirmed the
307 discovery by Baye et al. (2005) that the leaves contained high concentrations of LNA,
308 which would account for some inhibition of LA biohydrogenation (Maia et al., 2007).
309 Our present understanding of ruminal bacteria that biohydrogenate fatty acids is
310 undoubtedly incomplete, because it is likely that certain members of the community
311 have not yet been cultivated (Boeckaert et al., 2008; Huws et al., 2011). Nonetheless,
312 our best understanding is that members of the *B. fibrisolvens* group convert LA to VA
313 via *cis*-9, *trans*-11-CLA, while *B. proteoclasticus* converts LA all the way to SA, also
314 via *cis*-9, *trans*-11-CLA (Jenkins et al., 2008; Lourenço et al., 2010). Typically, *B.*
315 *proteoclasticus* is more sensitive to the toxic effects of unsaturated fatty acids than *B.*
316 *fibrisolvens* (Wallace et al., 2006; Ramos Morales et al., 2012); this is the first time
317 that we have observed the opposite. *M. elsdenii* (Kim et al., 2002) and *P. acnes*
318 (Devillard and Wallace, 2006) have both been implicated in the formation of *trans*-10,
319 *cis*-12 CLA, with the latter being considered more likely to be the main contributor
320 (Lourenço et al., 2010). They are important because *trans*-10, *cis*-12 CLA causes milk
321 fat depression in lactating ruminants (Griinari et al., 1998; Lock et al., 2007).
322

323 **4. Conclusions**

324 *Vernonia galamensis* and vernolic acid inhibit LA biohydrogenation in vitro. Pure
325 culture incubations with vernolic acid would suggest that this fatty acid may not be
326 useful in preventing the formation of *trans*-10-18:1, which has been associated with
327 milk fat depression in dairy cows. *Vernonia galamensis* could potentially be used as a
328 manipulator of ruminal biohydrogenation, leading to greater concentrations of *cis*-9,
329 *trans*-11 CLA in meat and milk. Further studies are required to confirm that *V.*
330 *galamensis* alters biohydrogenation *in vivo*.

331

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333

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342

343

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345

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457 **Figure captions**

458

459 **Figure 1.** Influence of vernolic acid on metabolism of linoleic acid (LA) in ruminal
460 fluid from sheep receiving a mixed grass hay/concentrate diet. Linoleic acid and
461 vernolic acid were added to an initial concentration of 0.2 g/L. (a) LA. (b) *cis*-9,
462 *trans*-11 CLA. (c) VA. (d) SA. Black symbols are from incubations with LA alone;
463 open symbols are from incubations with LA + vernolic acid. Results are mean ± SE
464 from four sheep.

465

466 **Figure 2.** Influence of vernolic acid on metabolism of *cis*-9, *trans*-11 CLA in ruminal
467 fluid from sheep receiving a mixed grass hay/concentrate diet. *cis*-9, *trans*-11 CLA
468 and vernolic acid were added to an initial concentration of 0.2 g/L. (a) LA. (b) *cis*-9,
469 *trans*-11 CLA. (c) VA. (d) SA. Black symbols are from incubations with *cis*-9, *trans*-
470 11 CLA alone; open symbols are from incubations with *cis*-9, *trans*-11 CLA +
471 vernolic acid. Results are mean ± SE from four sheep.

472

473 **Figure 3.** Influence of vernolic acid on metabolism of VA in ruminal fluid from sheep
474 receiving a mixed grass hay/concentrate diet. Vaccenic acid and vernolic acid were
475 added to an initial concentration of 0.2 g/L. (a) LA. (b) VA. (d) SA. Black symbols
476 are from incubations with VA alone; open symbols are from incubations with VA +
477 vernolic acid. Results are mean ± SE from four sheep.

478

479 **Fig. 4.** Influence of flowers or leaves of *V. galamensis* on metabolism of LA in
480 ruminal fluid from sheep receiving a mixed grass hay/concentrate diet. LA was added
481 to an initial concentration of 0.2 g/L and either flowers or leaves to 5 g/L. (a) LA. (b)

482 *cis*-9, *trans*-11 CLA. (c) VA. (d) SA. Black symbols are from incubations with LA

483 alone, diagonally striped symbols are from incubations with LA + flowers, and open

484 symbols are from incubations with LA + leaves. Results are mean ± SE from 4 sheep.

485

486 **Figure 5.** Influence of a mixture of flowers and leaves of *V. galamensis* on

487 metabolism of LA in ruminal fluid from sheep receiving a mixed grass

488 hay/concentrate diet. LA was added to an initial concentration of 0.2 g/L and flowers

489 and leaves were added at 2.5 g/L each. Results are mean ± SE from four sheep. (a)

490 LA. (b) *cis*-9, *trans*-11 CLA. (c) VA. (d) SA. Black symbols are from incubations

491 with LA alone and open symbols are from incubations with LA and the mixture of

492 flowers and leaves. Results are mean ± SE from 4 sheep.

493

494 **Figure 6.** Influence of vernolic acid on growth of *B. fibrisolvens* JW11 (squares) and

495 *B. proteoclasticus* P18 (triangles). Black symbols are from incubations of pure

496 cultures with no fatty acid added; open symbols are from incubations with vernolic

497 acid a) 0.0025 g/L. b) 0.025 g/L. c) 0.05 g/L. Results are means from three separate

498 cultures. $OD_{650}$ is the optical density of the culture at 650 nm.

499

500

501 **Table 1.** Concentrations (mg/g DM) of oleic (*cis*-9-18:1), linoleic (*cis*-9, *cis*-12-18:2) and linolenic (*cis*-9,*cis*-12,cis-15-18:3)

502 acids in total and non-esterified forms in samples of flowers and leaves of *Vernonia galamensis*.

503

| | *cis*-9-18:1 | | | | *cis*-9, *cis*-12-18:2 | | | | *cis*-9,*cis*-12,*cis*-15-18:3 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Non esterified | | Total | | Non esterified | | Total | | Non esterified | | Total | |
| | Mean[a] | SE[a] | Mean | SE | Mean | SE | Mean | SE | Mean | SE | Mean | SE |
| Flowers | 0.227 | 0.002 | 0.618 | 0.003 | 1.01 | 0.009 | 2.92 | 0.012 | 0.516 | 0.004 | 1.98 | 0.027 |
| Leaves | 0.035 | 0.001 | 0.367 | 0.004 | 0.203 | 0.001 | 2.08 | 0.031 | 0.865 | 0.008 | 13.9 | 0.183 |

504

505 [a]Mean and SE from three replicate analyses.

*Conflict of Interest Statement

**Author declaration template**

We wish to confirm that there are no Known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of the authors listed in the manuscript has been approved by all of us.

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1 *Vernonia galamensis* and vernolic acid inhibit fatty acid
2 biohydrogenation *in vitro*
3
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16
17
18
19
20
21 Abbreviations: CLA, conjugated linoleic acid; DHA, docosahexaenoic acid; DM, dry
22 matter; EPA, eicosapentaenoic acid; MS, mass spectrometry; LA, linoleic acid; LNA,
23 linolenic acid; SA, stearic acid; SRF, strained ruminal fluid; UFA, unsaturated fatty
24 acids; VA, vaccenic acid.

## Abstract

Substituted long-chain fatty acids may be useful dietary supplements to suppress ruminal biohydrogenation of unsaturated fatty acids (UFA) and thereby increase the flow of UFA to meat and milk. The aim of this study was to determine if *Vernonia galamensis* (ironweed, a member of the sunflower family) and vernolic acid (*cis*-12,13-epoxy-*cis*-9-octadecenoic acid), the main constituent of the seed oil, affected the biohydrogenation of linoleic acid (LA; *cis*-9,*cis*-12-18:2) to rumenic acid (CLA; *cis*-9, *trans*-11-18:2), vaccenic acid (VA; *trans*-11-18:1) and stearic acid (SA; 18:0) by ruminal microorganisms. Ruminal digesta from four sheep receiving a mixed hay-concentrate diet were incubated *in vitro* with LA (0.2 g/L) ± 0.2 g/L vernolic acid or 5 g/L of dried flowers or leaves of *V. galamensis*, either alone or combined. Vernolic acid had a substantial effect on LA metabolism, causing decreases in *cis*-9, *trans*-11 CLA and VA accumulation as well as SA production ($P<0.05$). Vernolic acid inhibited growth of the rumen fatty acid-biohydrogenating bacterium, *Butyrivibrio fibrisolvens* but not *B. proteoclasticus* at 0.025 g/L; neither species grew at 0.05 g/L. An inhibition of the metabolism of LA as well as a decrease in the accumulation of *cis*-9, *trans*-11 CLA and a slowdown in its metabolism were observed in the presence of flowers, leaves and a combination of both ($P<0.05$). However, only incubations with flowers, either alone or with leaves, resulted in higher accumulation of VA ($P\leq0.05$). Vernolic acid, constituted 2% of the total fatty acid content of flowers whereas it was not detected in leaves of *V. galamensis.* Thus, the greater accumulation of VA observed with flowers of *V. galamensis* was probably due to other components rather than vernolic acid. It was concluded that vernolic acid and *V. galamensis* inhibit the biohydrogenation of LA *in vitro*. *V. galamensis* could potentially be used as an

50 additive to alter ruminal biohydrogenation, leading to greater concentrations of *cis*-9,
51 *trans*-11 CLA in meat and milk. Evaluation of *V. galamensis in vivo* is required to
52 confirm the present *in vitro* observations.
53
54 Keywords**:** biohydrogenation, conjugated linoleic acid, rumen, vernolic acid,
55 *Vernonia galamensis*
56
57 **1. Introduction**
58
59 Ruminant products contain conjugated linoleic acids (CLA), of which the most
60 abundant is usually rumenic acid (CLA; *cis*-9, *trans*-11-18:2), and vaccenic acid (VA;
61 *trans*-11-18:1) which are potential health-promoting and disease-preventing agents
62 (Kritchevsky, 2000; Field et al., 2009; Dilzer and Park, 2012). Conjugated linoleic
63 acid and VA are produced as intermediate products in the biohydrogenation of
64 linoleic acid (LA; *cis*-9, ci*s*-12-18:2) present in the feed to stearic acid (SA; 18:0)
65 (Jenkins et al., 2008). The most active biohydrogenating bacteria isolated from the
66 rumen belong to Butyrivibrio group (Lourenco et al., 2010). However, other as yet
67 uncultivated bacteria may play a more predominant role (Boeckaert et al., 2008; Huws
68 et al., 2011). If ruminal biohydrogenation could be controlled by, for example, a
69 dietary additive, the unsaturated fatty acid content, particularly *cis*-9, *trans*-11 CLA
70 and VA, of ruminant products and their healthfulness would be improved.
71 Evidence is increasing that substituted long-chain fatty acids, comprised of a carbon
72 chain with one or more substituent groups, including those from the plant kingdom
73 (Durmic et al., 2008), may be effective feed additives to control biohydrogenation.
74 Coronaric acid is a C-18 epoxy fatty acid that contributes to the inhibitory effect of

75 *Chrysanthemum coronarium* on biohydrogenation (Wood et al., 2010) that leads to an
76 improved fatty acid composition of milk from sheep receiving *C. coronarium*
77 (Cabiddu et al., 2006). Ricinoleic acid (12-hydroxy-*cis*-9-18:1), the main fatty acid
78 component of castor oil, inhibited the biohydrogenation of LA *in vitro* (Ramos
79 Morales et al., 2012). *Vernonia galamensis*, an East African plant of the sunflower
80 family known sometimes as ironweed, contains a high concentration of vernolic acid
81 (*cis*-12,13-epoxy-*cis*-9-18:1; 54-74%), an isomer of coronaric acid, which is present
82 in the seed oil (Baye et al., 2005). The effects of neither the plant nor vernolic acid on
83 fatty acid biohydrogenation have been investigated. The aim of the present
84 experiments was to explore the potential of *V. galamensis* and vernolic acid to
85 function as biohydrogenation inhibitors.
86
87 **2. Material and methods**
88
89 *2.1. Animals and diets*
90
91 Animal experimentation was carried out under conditions governed by a licence
92 issued by the United Kingdom Home Office. Four mature sheep, each fitted with a
93 ruminal cannula, received 800 g dry matter (DM)/day of ration comprising (g/kg
94 DM) grass hay (300), rolled barley (422.5), soybean meal (167.5), molasses (100)
95 and minerals and vitamins (10) as two equal meals (2 × 400 g) at 0800 and 1600.
96 Samples of ruminal digesta were collected from each animal just before the morning
97 feeding. Digesta samples were bubbled with $CO_2$, maintained at 39 °C, and strained
98 ruminal fluid (SRF) was obtained by straining through double-layered muslin gauze.

99 Each set of incubations was carried out using ruminal digesta from four sheep
100 incubated individually (four replicates).
101
102 *2.2. Vernonia galamensis samples*
103
104 Freeze dried samples of flowers and leaves of *V. galamensis* were obtained from the
105 African Centre for Crop Improvement (University of KwaZulu-Natal, South Africa).
106 Samples were ground to pass a 1 mm screen.
107
108 *2.3. Incubations with ruminal digesta in vitro*
109
110 In order to determine the effect of vernolic acid on the metabolism of LA, SRF was
111 incubated with 0.2 g/L of LA (Sigma–Aldrich Co. Ltd., UK) in the presence or
112 absence of vernolic acid (Larodan Fine Chemicals, Sweden) added at 0.2 g/L.
113 Additionally, incubations of SRF with 0.2 g/L of either *cis*-9, *trans*-11 CLA or VA
114 (Sigma–Aldrich Co. Ltd., UK), as substrates for the biohydrogenating bacteria, with
115 or without vernolic acid (0.2 g/L), were carried out with the aim of studying where in
116 the biohydrogenation sequence the inhibition by vernolic acid occurred. Likewise, in
117 order to determine the effect of flowers or leaves of *V. galamensis* on the metabolism
118 of LA, SRF was incubated with 0.2 g/L of LA in the presence or absence of 5 mg of
119 ground and freeze dried flowers or leaves. Furthermore, in an attempt to study the
120 possible synergistic effect between flowers and leaves on the metabolism of LA,
121 incubations of SRF with LA (0.2 g/L) with or without a mixture of flowers and leaves
122 (2.5 mg of each) of *V. galamensis* were carried out. Fatty acids were incubated as an
123 oil in-water suspension obtained by sonication for about 4 min.

124 Tubes were removed at 0, 1, 3, 6, 9 and 24 h for fatty acid analysis. Reactions were
125 stopped by heating in a heating block at 100°C for 10 min and tubes were stored at -
126 20°C.
127
128 *2.4. Incubations with pure cultures*
129
130 Two species of ruminal bacteria were used. *Butyrivibrio fibrisolvens* JW11 was
131 originally isolated from sheep as a proteolytic species (Wallace and Brammall, 1985).
132 *Butyrivibrio proteoclasticus* P18 is a SA-producing bacterium isolated from grazing
133 sheep (Wallace et al., 2006). These two species are the main cultivated species known
134 to be involved in fatty acid biohydrogenation (Lourenço et al., 2010).
135 *Propionibacterium acnes* G449 was isolated in the same study as *B. proteoclasticus*
136 P18. *Megasphaera elsdenii* J1 was isolated from a sheep. These ruminal bacteria are
137 held in the culture collection maintained at the Rowett Institute of Nutrition and
138 Health.
139 In order to study the effect of vernolic acid on growth, incubations were carried out
140 under $O_2$-free $CO_2$ at 39°C in Hungate-type tubes in the medium M2 (Hobson, 1969)
141 without agar. Inoculum volumes were 5% (v/v) of a fresh overnight culture into 5 mL
142 of medium. Vernolic acid was added to a final concentration of 0.0025, 0.025 and
143 0.05 g/L. Fatty acids were prepared as a separate solution, sonicated for 4 min in a
144 small volume of medium and added to the medium before dispensing and autoclaving.
145 Growth of bacteria was measured in triplicate from the increase in optical density at
146 650 nm of the control tubes using a Novaspec II spectrophotometer (Amersham
147 Biosciences, UK).
148

149 *2.5. Fatty acid extraction and analysis*

150

151 Extraction of total fatty acids was based on the method of Folch et al. (1957),
152 incorporating the modifications of Devillard et al. (2006). Nonadecanoic acid (0.1 mL
153 of 0.2 g/L in methanol) was used as internal standard. Solid-phase extraction
154 (Kalunzy et al., 1985) was used to separate free fatty acids from other lipids following
155 Folch extraction.

156 Fatty acid methyl esters were prepared under mild conditions using methanolic $H_2SO_4$
157 (Wąsowska et al., 2006) as a catalyst and quantified using a gas chromatograph
158 (model 6890, Agilent Technologies) equipped with a flame-ionization detector,
159 quadrupole mass-selective detector (model 5973N) and a 100-m fused silica capillary
160 column CP Sil 88 (i.d., 0.25 mm) coated with 0.2 μm film of cyanopropyl
161 polysiloxane (Varian Analytical Instruments, Walton-on-Thames, Surrey, UK) using
162 Helium as the carrier gas. The fatty acid methyl esters profile in 1 μl of sample at a
163 split ratio of 20:1 was determined using a temperature gradient programme (initial
164 temperature 80$^{o}$C for 1 min; increased at a rate of 25$^{o}$C/min to 160$^{o}$C, which was held
165 for 3 min, then increased at a rate of 1$^{o}$C/min to 190$^{o}$C, then further increased at a rate
166 of 10$^{o}$C/min to 230$^{o}$C, held for 40 min). Injector and MS detector temperatures were
167 maintained at 250 and 230$^{o}$C, respectively. Peaks were routinely identified by
168 comparison of retention times with authentic fatty acid methyl esters standard
169 obtained from Sigma (Poole, Dorset, UK) and Matreya (Pleasant Gap, PA, USA).
170 Identification was validated based on electron impact ionization spectra of fatty acid
171 methyl esters obtained under an ionization voltage of 2247 eV.

172 Vernolic acid concentration in flowers and leaves was determined at the University of
173 the Free State (South Africa). Fatty acids were extracted following the method of

174 Folch et al. (1957) and a base-catalysed transesterification with sodium methoxide
175 was carried out as described by Park et al. (2001), Kramer et al. (2002) and Alfaia et
176 al. (2007). Vernolic acid was determined using a Varian 430 flame ionization gas
177 chromatograph with a fused silica capillary column Chrompack CP Sil 88 (100 m ×
178 0.25 mm i.d. × 0.2 μm film thickness) and hydrogen as the carrier gas. Vernolic acid
179 was determined using a temperature program that initially started at 40$^o$C for 2 min,
180 increased at a rate of 4$^o$C/min to 230 which was held for 10 min. Injector and detector
181 temperatures were maintained at 250$^o$C.
182
183 *2.6. Data Analysis*
184
185 Mixed-culture data were analysed at each time point separately by randomized
186 complete block ANOVA, with individual sheep as a blocking term, according to the
187 model:
188 $Y_{ijk} = \mu + D_i + A_j + e_{ijk}$
189 Where $Y_{ijk}$ is the dependent, continuous variable (n =4), μ is the overall mean; $D_i$ is
190 the fixed effect of the treatment, $A_j$ is the random effect of the animal inoculum (j= 1
191 to 4) and $e_{ijk}$ is the residual error. Individual comparisons were determined by post
192 hoc *t*-tests using the unprotected LSD. Pure-culture data were analysed by ANOVA,
193 again compared at each sampling time. Genstat 10th edition (VSN International, UK)
194 was used.
195
196 **3. Results**
197
198 *3.1. Incubations of ruminal digesta with C-18 fatty acids and vernolic acid*

*In vitro* incubations of SRF and LA either alone or with 0.2 g vernolic acid/L were carried out in order to study the influence of vernolic acid on the metabolism of LA (Fig. 1). An inhibition ($P<0.05$ at 1 and 3 h) of LA disappearance as well as an inhibition of the accumulation of *cis*-9, *trans*-11-CLA ($P<0.05$ at 1, 3 and 6 h) and VA ($P<0.05$) was observed in the presence of vernolic acid. Subsequently, SA accumulation over time decreased ($P<0.05$) with vernolic acid.

Incubations of SRF with *cis*-9, *trans*-11 CLA as substrate (Fig. 2a) confirmed a slightly slower metabolism compared to LA (Fig. 1a), as 29.6% and 60% of the initial amount of CLA and LA added, respectively, disappeared after 1 h of the incubation. Vaccenic acid accumulated and SA increased in much the same way as with LA. Vernolic acid caused a slowdown of the loss of *cis*-9, *trans*-11 CLA ($P<0.05$ at 3 and 9 h), in comparison with the control incubations. Also, the accumulation of VA was lower ($P<0.05$ at 1, 9 and 24 h), as was the increase of SA over time ($P<0.05$) in the presence of vernolic acid (Fig. 2).

Vaccenic acid was metabolised more slowly than the dienoic acids, indeed its concentration reached a plateau level at 12 h which was not changed at 24 h (Fig. 3). Vernolic acid did not appear to impair the metabolism of VA; if anything the percentage of VA that disappeared in the presence of vernolic acid was slightly greater (38.5% vs. 30 and 39.8% vs. 29.5% at 9 and 24 h, respectively, $P<0.05$), although less SA was produced over time as compared with the control ($P<0.05$).

### *3.2. Incubations of ruminal digesta with linoleic acid and leaves and flowers of Vernonia galamensis.*

224 Strained rumen fluid was incubated *in vitro* with LA (0.2 g/L) in the presence or
225 absence of flowers or leaves of *V. galamensis* (Fig. 4). Incubations of *V. galamensis*
226 leaves with LA led to an inhibition of the disappearance of LA ($P<0.05$) and lower
227 accumulation of *cis*-9, *trans*-11-CLA ($P<0.001$ at 1 h) with slightly increased
228 accumulation of VA and no effect on SA production. Incubations with flowers of *V.*
229 *galamensis* and LA also resulted in an inhibition of disappearance of LA ($P<0.05$),
230 although to a less extent than that observed with the leaves. A decrease in the
231 accumulation of *cis*-9, *trans*-11 CLA was also shown in incubations with flowers
232 ($P<0.001$ at 1 h), values being higher than those observed in the presence of leaves.
233 However, VA concentration increased by 16-23% (VA tended to be higher, $P=0.054$
234 and 0.057, at 1 and 9 h of the incubation, compared with the control). A similar effect,
235 inhibition of the loss of LA ($P<0.05$) as well as decreased *cis*-9, *trans*-11 CLA
236 accumulation ($P<0.05$ at 1 h) and increased accumulation of VA ($P=0.033$ at 1 h of
237 the incubation), was observed when flowers and leaves were combined (Fig. 5), but
238 no signs of synergy were evident. Oleic acid and LA were present at a higher
239 concentration in flowers (0.618 and 2.92 mg/g DM) than leaves (0.367 and 2.08 mg/g
240 DM). Also, the proportion of oleic and LA in the non-esterified form was higher in
241 flowers than leaves (Table 1). Leaves of *V. galamensis* had a much higher
242 concentration of linolenic acid (LNA; 13.9 mg/g DM) in comparison with flowers
243 (1.98 mg/g DM). Vernolic acid, comprised 2% of the total fatty acids in flowers
244 whereas it was not detected in leaves of *V. galamensis* (data not shown).
245
246 *3.3. Influence of vernolic acid on growth of biohydrogenating ruminal bacteria*
247

248 Vernolic acid did not inhibit growth of *B. fibrisolvens* or *B. proteoclasticus* at 0.0025
249 g/L ($P$>0.05; Fig. 6a). When added at 0.025 g/L, vernolic acid completely inhibited
250 growth of *B. fibrisolvens* ($P$<0.001) while *B. proteoclasticus* grew after a lag time of 5
251 h (Fig. 6b). Neither species grew when vernolic acid was added at 0.5 g/L (Fig. 6c).
252 Vernolic acid did not have any effect on *M. elsdenii* or *P. acnes* (not shown).
253
254 **4. Discussion**
255
256 Unsaturated fatty acids at high concentration inhibit their own biohydrogenation
257 because they are toxic to the main species involved in biohydrogenation, namely those
258 related to *Butyrivibrio* (Maia et al., 2007). The more unsaturated the fatty acid
259 molecule, the greater seems to be its inhibitory effects (Maia et al., 2007). The fish oil
260 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are
261 particularly effective (Fievez et al., 2003; AbuGhazaleh and Jenkins, 2004; Wąsowska
262 et al., 2006; Maia et al., 2007); these fatty acids originate in algae, which has also
263 shown to inhibit fatty acid biohydrogenation (Boeckaert et al., 2007; Vlaeminck et al.,
264 2008; Toral et al., 2012). Few studies, however, have pointed out the potential of
265 unsaturated hydroxy- and epoxy- fatty acids as manipulators of rumen
266 biohydrogenation (Wood et al., 2010; Ramos-Morales et al., 2012).
267 *Vernonia galamensis* is a widely distributed weed in Eastern Africa, but a potential
268 industrial oilseed crop. The seed oil of *V. galamensis* contains vernolic acid, a natural
269 epoxy fatty acid that can replace the expensive synthetic epoxy compounds used in
270 the plastic and coating industries. Our interest in vernolic acid stemmed from studies
271 carried out with coronaric acid (Wood et al., 2010), an isomer of vernolic acid, which
272 showed its effectiveness in inhibiting biohydrogenation. Also, we hypothesised that

273 the high LNA content in *V. galamensis* leaves reported by others (Baye et al., 2005)
274 could potentially have an inhibitory effect on fatty acid biohydrogenation.
275 The rapid loss of LA and transient accumulation of *cis*-9, *trans*-11 CLA and VA in
276 control incubations, followed by the slower accumulation of SA, are features that
277 have been observed previously (Waşowska et al., 2006; Ramos Morales et al., 2012).
278 They reflect the relative rates associated with the different steps in the pathway, *viz.*
279 LA >CLA>VA>SA (Noble et al., 1974). Vernolic acid slowed the disappearance of
280 LA and decreased the accumulation of *cis*-9, *trans*-11 CLA and VA and also the
281 increase in SA concentration. The inhibition of an accumulation of these
282 intermediates is therefore also reminiscent of previous studies with fish oil, EPA,
283 DHA and ricinoleic acid (Waşowska et al., 2006; Ramos Morales et al., 2012). From
284 these results, vernolic acid appears to be an inhibitor of LA metabolism and
285 biohydrogenation although it did not lead to the accumulation of *cis*-9, *trans*-11 CLA
286 or VA. This effect seems to be greater than that reported for coronaric acid (Wood et
287 al., 2010). It should be noted that Fig. 2 a) (also Fig. 3 a)) illustrates the metabolism
288 of esterified LA in the feed, for which the rate-limiting step will be lipolysis
289 (Lourenço et al., 2010). Vernolic acid did not appear to influence this rate,
290 presumably because it did not inhibit the rate-limiting step.
291 We hypothesised that flowers of *V. galamensis* would possibly have an inhibitory
292 effect on biohydrogenation due to their content in vernolic acid which has been found
293 to be present in seeds of *V. galamensis* (Baye et al., 2005). Our hypothesis is
294 consistent with previous work on the fatty acid composition of the seed oil of the
295 variety of *V. galamensis* used in our study (Shimelis et al. 2006). However, the
296 analysis of the fatty acid composition of the flowers revealed that vernolic acid
297 accounted for about 2% of total lipid (data not shown). The unexpected low vernolic

298 acid content in the flowers could be explained by differences in the mature stage of
299 the seeds as it has been reported that seeds harvested at full maturity had significantly
300 higher oil and vernolic content than comparable samples harvested at a less mature
301 stage (Thompson et al., 1994). Also, it should be borne in mind that entire flowers
302 rather than seeds were used in our incubations and thus the content of vernolic acid
303 would have been lower than that of the seeds alone. Irrespective of the inhibitory
304 effect on biohydrogenation shown in incubations with vernolic acid, the greater
305 accumulation of VA observed with flowers of *V. galamensis* must have been due to
306 other components rather than vernolic acid. Fatty acid analysis confirmed the
307 discovery by Baye et al. (2005) that the leaves contained high concentrations of LNA,
308 which would account for some inhibition of LA biohydrogenation (Maia et al., 2007).
309 Our present understanding of ruminal bacteria that biohydrogenate fatty acids is
310 undoubtedly incomplete, because it is likely that certain members of the community
311 have not yet been cultivated (Boeckaert et al., 2008; Huws et al., 2011). Nonetheless,
312 our best understanding is that members of the *B. fibrisolvens* group convert LA to VA
313 via *cis*-9, *trans*-11-CLA, while *B. proteoclasticus* converts LA all the way to SA, also
314 via *cis*-9, *trans*-11-CLA (Jenkins et al., 2008; Lourenço et al., 2010). Typically, *B.*
315 *proteoclasticus* is more sensitive to the toxic effects of unsaturated fatty acids than *B.*
316 *fibrisolvens* (Wallace et al., 2006; Ramos Morales et al., 2012); this is the first time
317 that we have observed the opposite. *M. elsdenii* (Kim et al., 2002) and *P. acnes*
318 (Devillard and Wallace, 2006) have both been implicated in the formation of *trans*-10,
319 *cis*-12 CLA, with the latter being considered more likely to be the main contributor
320 (Lourenço et al., 2010). They are important because *trans*-10, *cis*-12 CLA causes milk
321 fat depression in lactating ruminants (Griinari et al., 1998; Lock et al., 2007).
322

323 **4. Conclusions**

324 *Vernonia galamensis* and vernolic acid inhibit LA biohydrogenation in vitro. Pure
325 culture incubations with vernolic acid would suggest that this fatty acid may not be
326 useful in preventing the formation of *trans*-10-18:1, which has been associated with
327 milk fat depression in dairy cows. *Vernonia galamensis* could potentially be used as a
328 manipulator of ruminal biohydrogenation, leading to greater concentrations of *cis*-9,
329 *trans*-11 CLA in meat and milk. Further studies are required to confirm that *V.*
330 *galamensis* alters biohydrogenation *in vivo*.

331

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333

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342

343

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345

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457 **Figure captions**

458

459 **Figure 1.** Influence of vernolic acid on metabolism of linoleic acid (LA) in ruminal
460 fluid from sheep receiving a mixed grass hay/concentrate diet. Linoleic acid and
461 vernolic acid were added to an initial concentration of 0.2 g/L. (a) LA. (b) *cis*-9,
462 *trans*-11 CLA. (c) VA. (d) SA. Black symbols are from incubations with LA alone;
463 open symbols are from incubations with LA + vernolic acid. Results are mean ± SE
464 from four sheep.

465

466 **Figure 2.** Influence of vernolic acid on metabolism of *cis*-9, *trans*-11 CLA in ruminal
467 fluid from sheep receiving a mixed grass hay/concentrate diet. *cis*-9, *trans*-11 CLA
468 and vernolic acid were added to an initial concentration of 0.2 g/L. (a) LA. (b) *cis*-9,
469 *trans*-11 CLA. (c) VA. (d) SA. Black symbols are from incubations with *cis*-9, *trans*-
470 11 CLA alone; open symbols are from incubations with *cis*-9, *trans*-11 CLA +
471 vernolic acid. Results are mean ± SE from four sheep.

472

473 **Figure 3.** Influence of vernolic acid on metabolism of VA in ruminal fluid from sheep
474 receiving a mixed grass hay/concentrate diet. Vaccenic acid and vernolic acid were
475 added to an initial concentration of 0.2 g/L. (a) LA. (b) VA. (d) SA. Black symbols
476 are from incubations with VA alone; open symbols are from incubations with VA +
477 vernolic acid. Results are mean ± SE from four sheep.

478

479 **Fig. 4.** Influence of flowers or leaves of *V. galamensis* on metabolism of LA in
480 ruminal fluid from sheep receiving a mixed grass hay/concentrate diet. LA was added
481 to an initial concentration of 0.2 g/L and either flowers or leaves to 5 g/L. (a) LA. (b)

482 *cis*-9, *trans*-11 CLA. (c) VA. (d) SA. Black symbols are from incubations with LA
483 alone, diagonally striped symbols are from incubations with LA + flowers, and open
484 symbols are from incubations with LA + leaves. Results are mean ± SE from 4 sheep.
485
486 **Figure 5.** Influence of a mixture of flowers and leaves of *V. galamensis* on
487 metabolism of LA in ruminal fluid from sheep receiving a mixed grass
488 hay/concentrate diet. LA was added to an initial concentration of 0.2 g/L and flowers
489 and leaves were added at 2.5 g/L each. Results are mean ± SE from four sheep. (a)
490 LA. (b) *cis*-9, *trans*-11 CLA. (c) VA. (d) SA. Black symbols are from incubations
491 with LA alone and open symbols are from incubations with LA and the mixture of
492 flowers and leaves. Results are mean ± SE from 4 sheep.
493
494 **Figure 6.** Influence of vernolic acid on growth of *B. fibrisolvens* JW11 (squares) and
495 *B. proteoclasticus* P18 (triangles). Black symbols are from incubations of pure
496 cultures with no fatty acid added; open symbols are from incubations with vernolic
497 acid a) 0.0025 g/L. b) 0.025 g/L. c) 0.05 g/L. Results are means from three separate
498 cultures. $OD_{650}$ is the optical density of the culture at 650 nm.
499
500

501 **Table 1.** Concentrations (mg/g DM) of oleic (*cis*-9-18:1), linoleic (*cis*-9, *cis*-12-18:2) and linolenic (*cis*-9,*cis*-12,cis-15-18:3)

502 acids in total and non-esterified forms in samples of flowers and leaves of *Vernonia galamensis*.

503

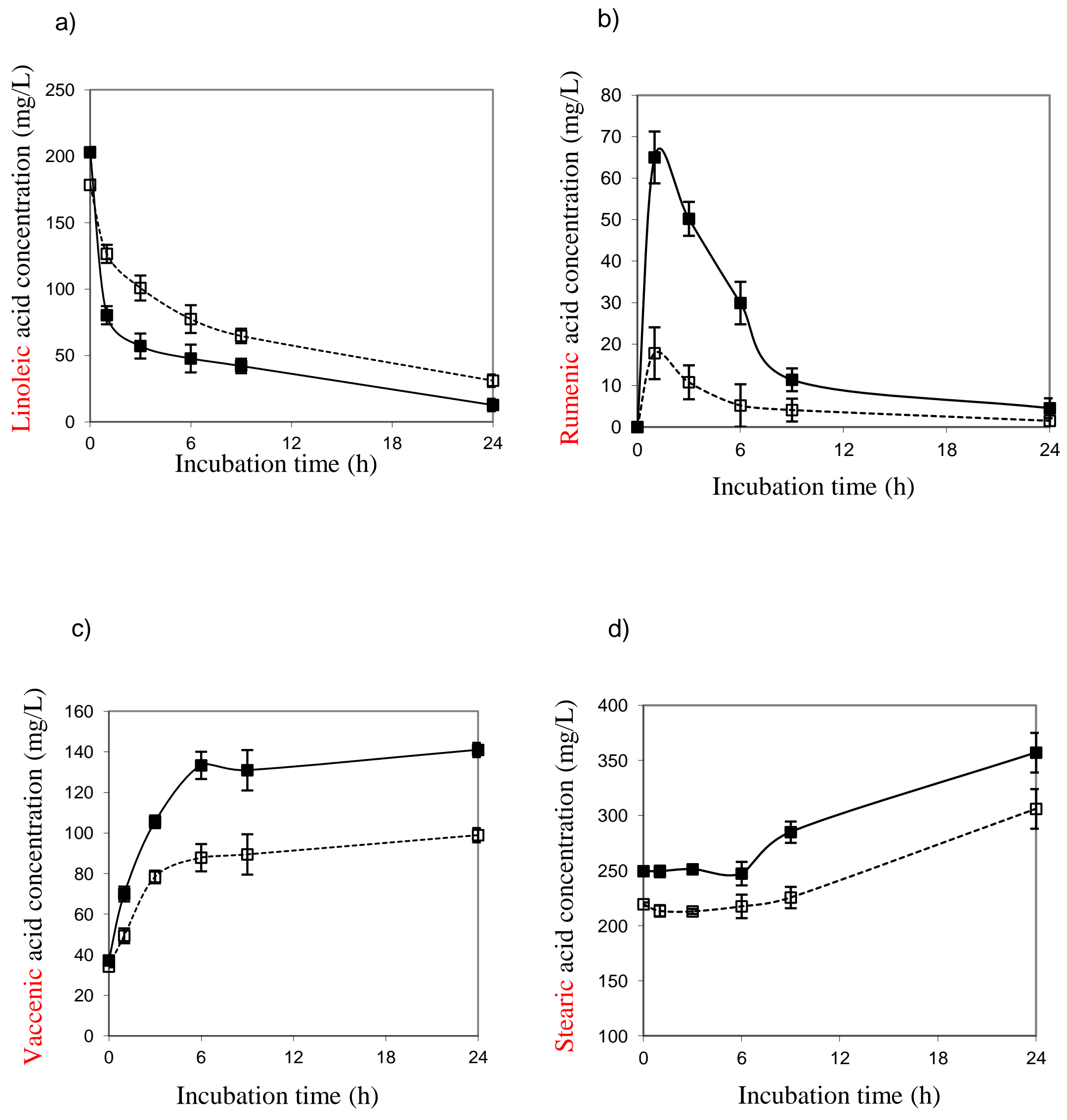
| | *cis*-9-18:1 | | | | *cis*-9, *cis*-12-18:2 | | | | *cis*-9,*cis*-12,*cis*-15-18:3 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Non esterified | | Total | | Non esterified | | Total | | Non esterified | | Total | |
| | Mean[a] | SE[a] | Mean | SE | Mean | SE | Mean | SE | Mean | SE | Mean | SE |
| Flowers | 0.227 | 0.002 | 0.618 | 0.003 | 1.01 | 0.009 | 2.92 | 0.012 | 0.516 | 0.004 | 1.98 | 0.027 |
| Leaves | 0.035 | 0.001 | 0.367 | 0.004 | 0.203 | 0.001 | 2.08 | 0.031 | 0.865 | 0.008 | 13.9 | 0.183 |

504

505 [a]Mean and SE from three replicate analyses.

**Figure**

1




2 **Figure 1. Ramos Morales et al.**

Figure

1

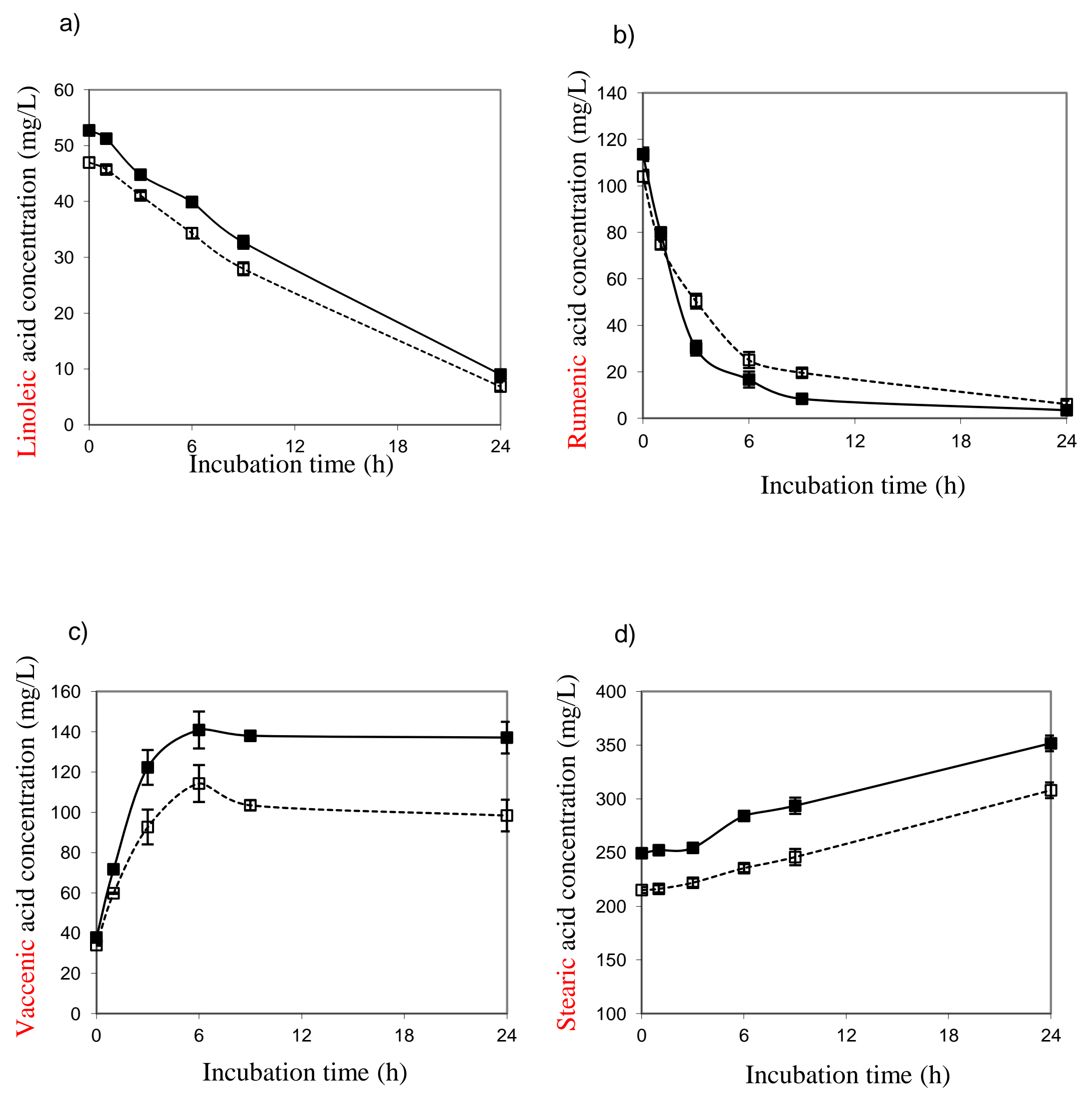
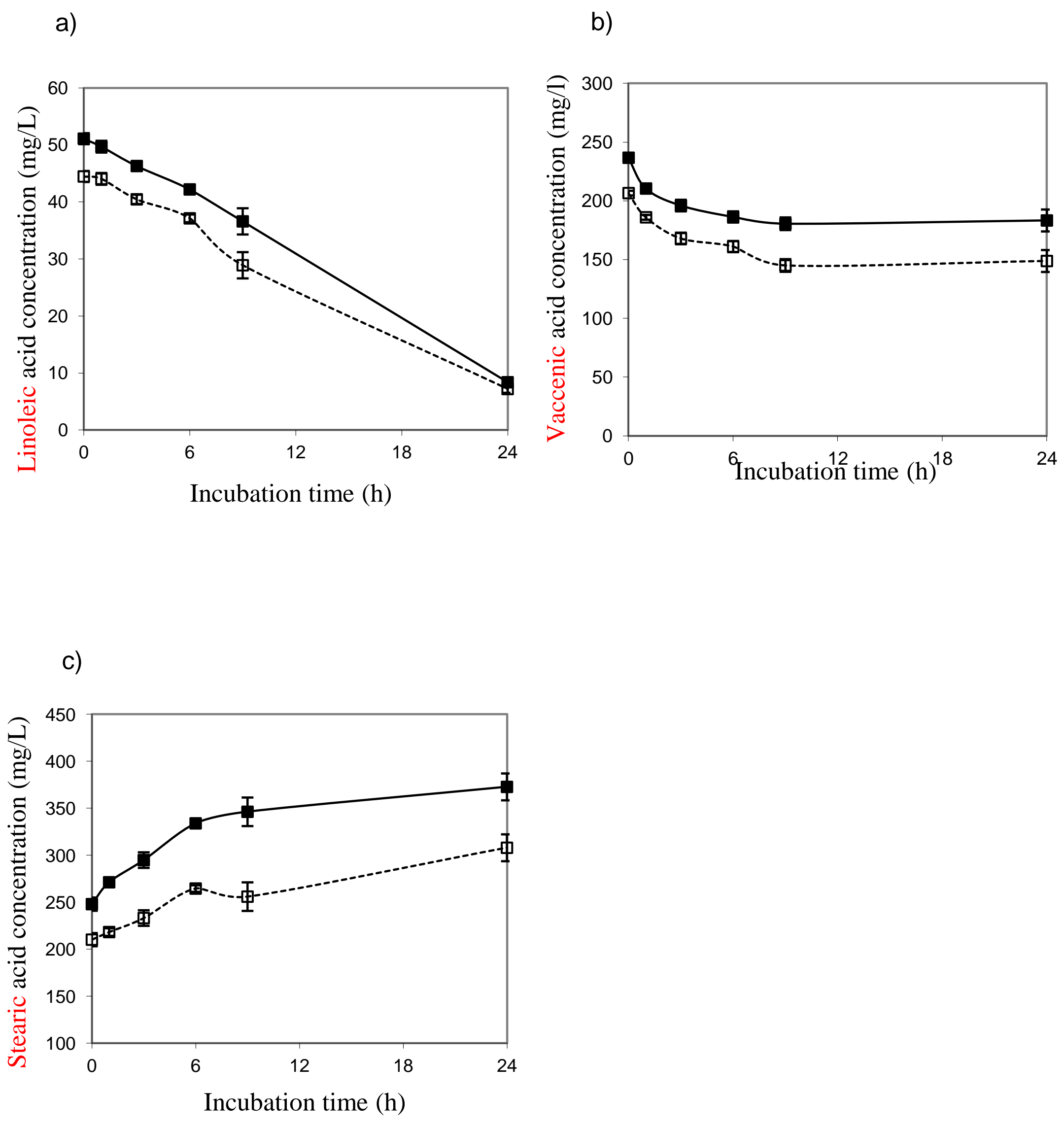



**Figure 2. Ramos Morales et al.**

2

Figure

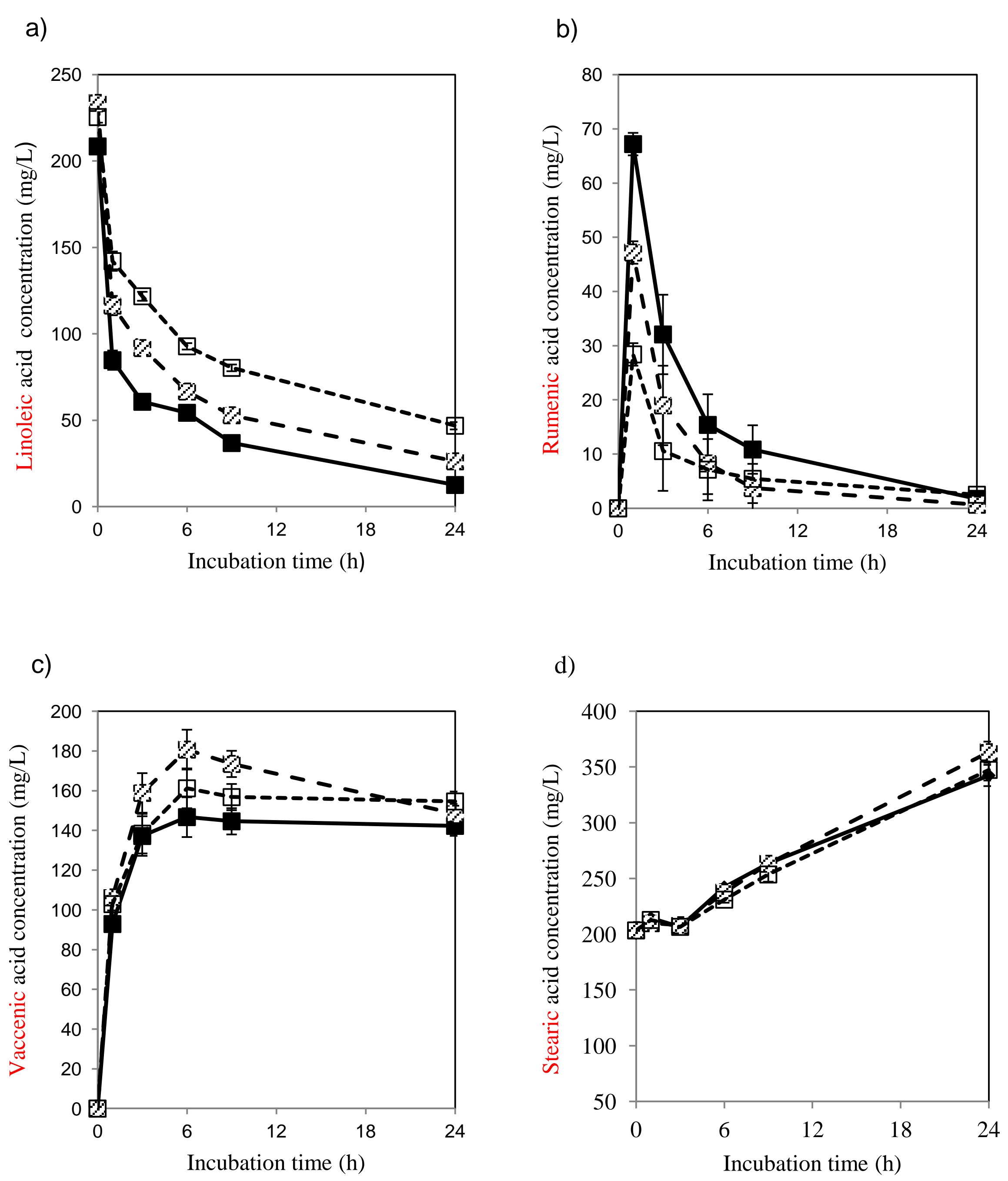
1



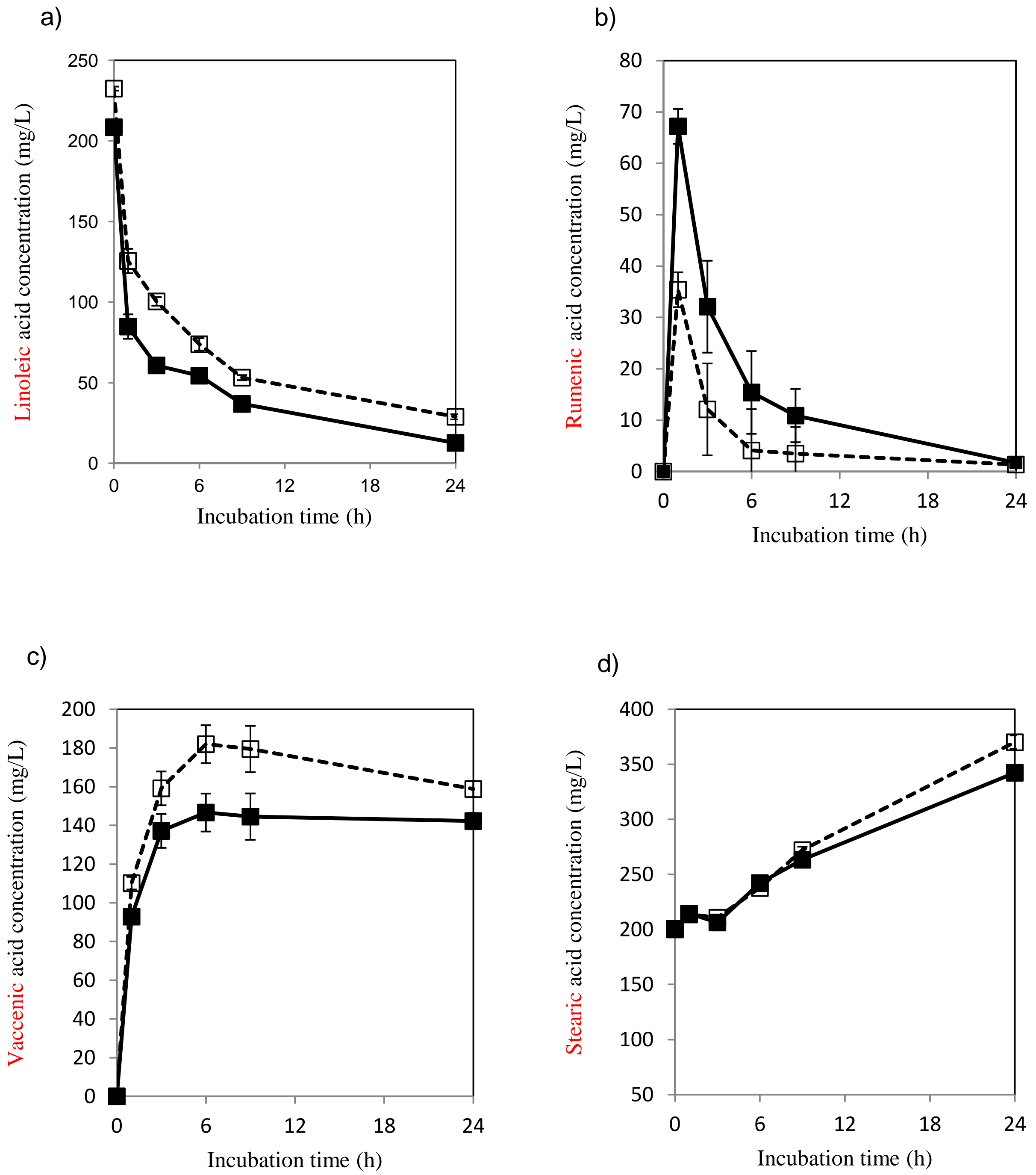

2 **Figure 3. Ramos Morales et al.**

**Figure**

1

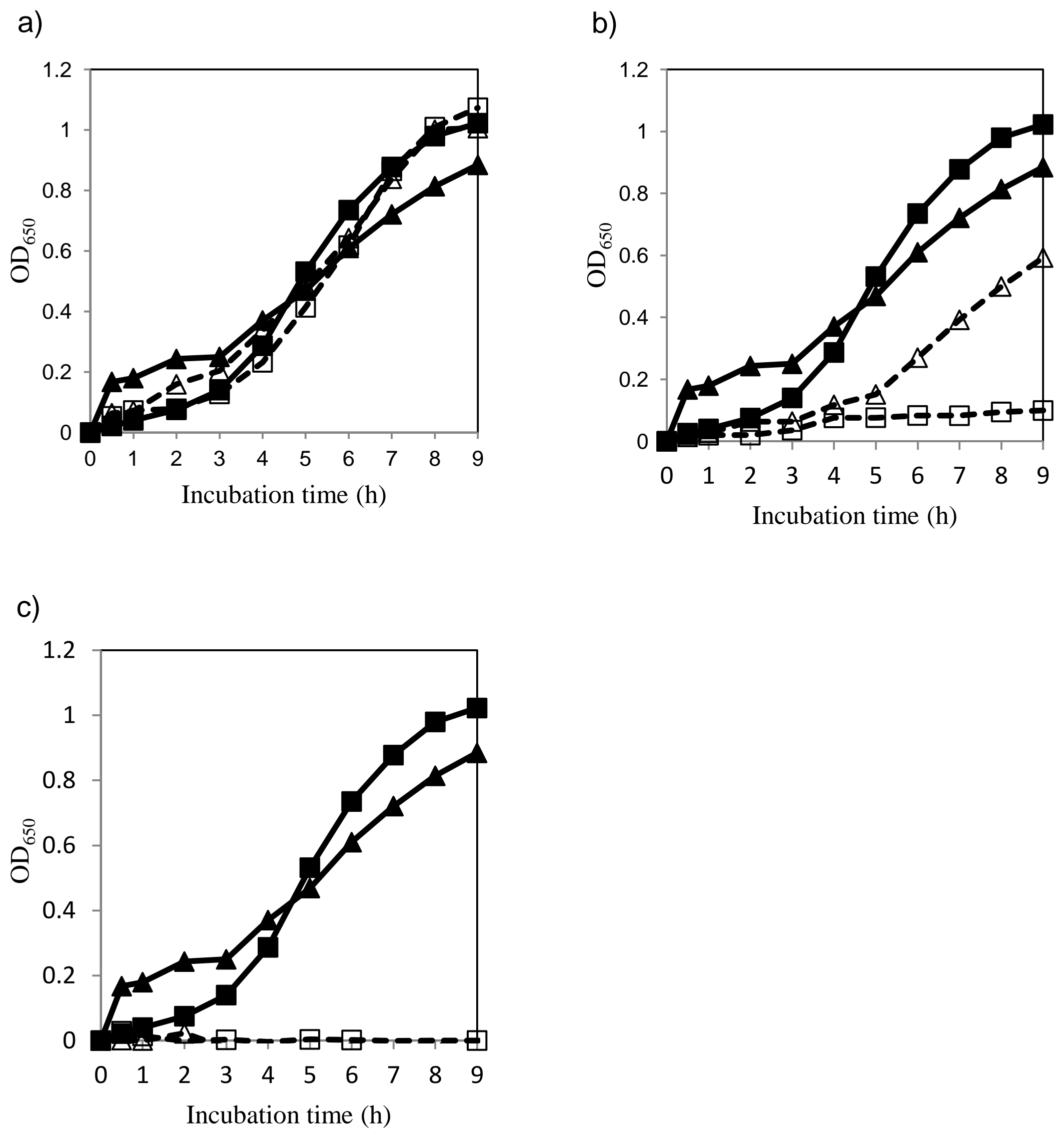



2 **Figure 4. Ramos Morales et al.**




1 **Figure 5. Ramos Morales et al.**

1




2 **Figure 6. Ramos Morales et al.**