**Supplementary Material**

Effects of fermented oat straw as a lovastatin-carrier on *in vitro* methane production and rumen microbiota

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**Appendix 1.** Table SM1. Concentrations of CO2 and CH4 in the atmosphere in 1950 and 2013 (concentration data from Fig. 1 and Fig. 2, US-EPA, 2014).

|  |  |  |
| --- | --- | --- |
|  | CO2 | CH4 |
| Initial concentration | 390a | 1800b |
| Final concentration | 320 a | 1100 b |
| Increase of concentration in the period (%) | 21.88 | 63.64 |
| Average relative rate of increase of concentration (% yr-1) | 0.35 | 1.01 |
| Average rate of increase of concentration | 1.11c | 11.11d |

a in ppm; b in ppb; c ppm yr-1; d ppb yr-1

**Appendix 2**. Lovastatin grades and their costs and considerations of economic impact on livestock production.

We present and discuss some cost calculations. They are preliminary, but they will allow to estimate the order of the costs incurred by either using commercial or FOS Lv as a supplement to animal feed in a CH4 mitigation approach.

Scenario 1. Commercial lovastatin.

The estimated price for commercial Lv is US$ 7.5/g, departing from the datum of US$75/g Lv of 98% purity (Sigma-Aldrich, 2020). We applied the criterion set reported Mulder et al. (2015) and King (1980) that states that a the substance price increases exponentially (potentially, strictly speaking) with the degree of purity, and an improved change of purity category will increase the price by a factor of 10. In our case, we assumed that 98% purity is the immediate superior category to commercial Lv.

Taking as a basis one steer of 450 kg LW, and using previous calculations in Comment 9 that showed that the Lv dose was 22.2 mg/(kg LW.day).

Therefore, writing m’Lv for the mass of Lv fed to a steer, and rounding off the purity to 100% (it would be around an actual 95%)

m’Lv (g Lv/(head.day)) = 22.2 (mg/(kgLW.day)\*450(kgLW/head)\*(1g/1000mg) =

= 10 g Lv/(head.day) [SM2.1]

The finishing cost of steers due to feed in Mexico was Mx 33.73/(head.day) (Lagos et al., 2014). From FIRA (2010), the average proportion of feed cost in the total cost of cattle production is 32%, so the

total cost = feed cost \* 3.12 [SM2.2]

If we represent the total cost by the Greek letter Γ, using an exchange conversion rate to US$ 2014 of Mx$ 13.00/1 US$, and adjusting to 2021 dollars with an average inflation rate of 1.5% (depreciation of the US$ in USA), we obtain

Γ(US$ 2021/(head.day) = 33.73 Mx$/(head.day)\*3.12 (total/feed)\*(1 US$/13 Mx$)\*

\*(1+0.015)7 = US$ 8.98/(head.day) [SM2.3]

If ΓLv represents the cost for the daily *commercial* Lv fed to a steer,

ΓLv (US$/(head.day)) = 10 gLv/(head.day)\* 7.5 US$/gLv =

= US$ 75/(head.day) [SM2.4]

This result confirms that the use of even the less pure commercial lovastatin likely will not be economically feasible for farmers.

Scenario 2 Use of FOS as Lv-carrier.

Purity of Lv in FOS is very very low. It is not a problem when FOS is mixed with the feed and administered to cattle in farm settings.

Departing from the price US$ 7.5/g of commercial Lv along with considerations of very significant savings in handling Lv as a part of a fermented residue compared to industrial grade Lv, we considered a reasonable price of US$ 0.375g of Lv-FOS (Hernández-Mendo, O., 2020, private communication, Livestock Program, Colegio de Posgraduados, Montecillos, Edo. de Méx. México). Indeed, the FOS approach compared to the industrial one saves costs of Lv extraction and purification (separation processes are very expensive, King, 1980), savings in solvent distillation for reuse and solvent waste treatment and disposal, savings in drying, savings in raw materials and product transportation, savings in product packaging, not to mention the savings in environmental impacts of the industrial approach, among others. Thus, a jump of 1/20 in price between commercial and FOS-Lv is very reasonable.

Applying Eq B12.1 and B12.4 with this price, we obtain a

ΓLv = 3.75 US$/(head.day) [SM2.5]

Therefore, the ratio of Lv-to-total costs in the finishing of steers is

(ΓLv /Γ)\*100 = (3.75 US$/(head.day)/ US$ 8.98/(head.day))\*100 =

= 41.76% [SM2.6]

If we express the relationship of the cost of Lv in terms of the total cost incurred, the number is lower, then

(ΓLv /(Γ + ΓLv)\*100 = (3.75 US$/(head.day)/ US$ 12.73/(head.day))\*100 =

= 29.45% [SM2.7]

*The cost of the Lv approach with the FOS Lv carrier is still considerable, but not prohibitive if we consider other factors and expenses in a context of public policies that control CH4 emissions from catlle growing facilities.*

Indeed, the “fine-avoidance” or “farm-closure” cost, prorated during the growing season of the steers can be considerable. Fines and/or the cost of the farm shut-down because of environmental violations can mean significant increases of production costs, even the bankruptcy of the cattle farm as a business unit. The costs of “fines or shut-down” expenses will add to the denominator of Eq.B12.6; this, in turn, will lead to significantly lowering the relative cost of the Lv approach.

Fines or possible shut-down of farm operations are expected to be the consequences of implementing a scientific and serious policy of CH4 emissions for the cattle-grower sector. So far, in Mexico such a system of fines or inspections leading to farm closures is not set yet. In fact, to the best of our knowledge, estimations of CH4 regulations dealing with emissions cut-backs for the cattle-grower sector have not been calculated and less released.

But we are in the verge of their implementation of such regulations if we want to succeed in abating climate change and warming, to contribute to the sustainable development of Mexico, and to the survival of the cattle-growing sector since business as usual is not feasible anymore.

**Appendix 3.** Table SM2. Effect of lovastatin on *in vitro* rumen fermentation.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Source of lovastatin | Donor animal and experimental diet | *In vitro* technique | Dose | Methane inhibition (%) | HTST  rumen microbiota analysis | Substrate of in vitro rumen fermentation | Reference |
| Fermented rice straw | Animal: bovine Diet: f:c4 ratio (40:60) | Technique: calibrated glass syringes  Inoculum: ruminal fluid | 4.3 | 24 | No | Fermented rice straw | 1 |
| Fermented purple  corn cob | Animal: Dairy steers  Diet: NR | Technique: serum bottles  Inoculum: ruminal fluid | 32 | 3.5 | No | Fermented purple corn cob | 2 |
| Fermented rice | Animal: sheep  Diet: Hay (twice per day) | Technique: Hungate tubes  Inoculum: ruminal fluid | 40 | 9.4 | No | Fermented rice | 3 |
| Lovastatin, Sigma-Aldrich,  St Louis, MO | Animal: Bovine Diet: f:c ratio (50:50) | Technique: fermentation bottles  Inoculum: ruminal fluid | 5 | NS | No | Diet f:c ratio (50:50) | 4 |
| Lovastatin, >98% purity, Sigma-Aldrich, St Louis, MO | Animal: bovine Diet: f:c ratio (60:40) | Technique: serum bottles  Inoculum: ruminal fluid | 3.2 | NS | No | Ryegrass, silage and barley, f:c (50:50) | 5 |
| Lovastatin, >98% purity,  Sigma-Aldrich, GmbH, Buchs, Switzerland | Animal: bovine Diet: hay, ryegrass, and concentrate | Technique: RUSITEC system  Inoculum: ruminal fluid | 150 | 40 | No | Ryegrass hay, soybean, and barley, f:c (50:50) | 6 |
| Fermented  Oat straw | Animal: bovine Diet: f:c ratio (60:40) | Technique: serum bottles  Inoculum: ruminal fluid | 150 | 38 | YES | Diet: f:c ratio (60:40) | 7 |

NS, not significant; 1, Jahromi et al. 2013; 2, Khonkhaeng and Cherdthong 2020; 3, Morgavi et al. 2013; 4, Busquet et al. 2005; 5, O Brien et al. 2014; 6, Soliva et al. 2011; 7, this work.

**Appendix 4**. Conditions of solid-state fermentation of oat straw and determination of lovastatin.

Five grams of oat straw were ground (5 mm), placed in 250 mL Erlenmeyer’s flask, and moisture content adjusted to 70 % with the *SSF* medium (concentrations in g L-1, unless otherwise stated): CaCl2, 0.3; KH2PO4, 2.1; ZnSO4, 0.3; MgSO4, 0.3; NaNO3, 0.5; methionine, 1.4 and glycerol, 20 mL L-1 (Jirasatid et al., 2013). The culture medium pH was set to 5.5 with 1 M H2SO4. Then, the flasks were sterilized at 121 °C/15 min, cooled to room temperature, and seeded with 2 mL of spore suspension (CDBB H-194). Abiotic controls were carried out with inactive *Aspergillus* strains. Experiment was performed in triplicate. The cultures were kept at 30 °C/72 h. Afterwards, the temperature was reduced to 28 °C/24 h and then to 26 °C until the end of the incubation period (Xu et al., 2005). The flasks were shaken twice a day. Sampling was performed at 0, 6, 12 and 16 days.

Fermented oat straw was dried in a convective, forced-air oven (55 °C/24 h) and powdered using a mortar and pestle. Dry powdered substrates (1 g) were extracted with 40 mL of ethyl acetate in 250 mL Erlenmeyer’s flasks and they were agitated for 2 h in a shaker at 200 rpm. The mixture was separated by filtration through a membrane filter (0.22 Durapore, Millipore, MA, USA)*.* The solvent was evaporated in a rotary evaporator Model R3 (Büchi Labortechnik, AG, Switzerland) under vacuum at 60 °C. The dry residual was re-dissolved in 5 mL of acetonitrile and further filtered through 0.22 μm acrodisc syringe filters (Millipore, MA, USA)before HPLC analysis (Jaivel and Marimuthu, 2010).

The hydroxy acid form standard of *Lv* was prepared departing from *Lv* lactone form standard (Sigma-Aldrich, St. Louis, MO, USA) according to Nyilasi *et al*. (2014). Lovastatin was determined as reported by Yang and Hwang (2006) using a HPLC (Varian Analytical Instruments, Model 9010, CA, USA). Chromatographic separation was performed in a reversed phase (Gemini 5U C18 column, Phenomenex, CA, USA) with acetonitrile: H2O (70:30, v/v) as a mobile phase containing H3PO4 at 0.1 % (v/v) (0.5 mL min-1 flowrate). Wavelength of detection was 237 nm (UV Spectro Monitor, Thermo Separation Products, model no. 3200, MA, USA); the sample injection volume was 50 μL. Finally, *Lv* was identified in lactone and hydroxy acid forms and both were calculated as the *Lv* yield.

**Appendix 5**. Explanation on the implementation of the experimental treatments and the units of expression.

First, and for instance, in the target dose of 150 mg Lv/L, L stands for liter of final mixture to be fermented (mixture of ration (ground corn grain, soybean meal, urea, mineral mix), FOS, compensation oat straw, ruminal fluid, and liquid medium). This is the Lv target concentration that will be “seen” by the methanogenic archaea in the fourth treatment.

Second, now we refer the Reader to Table 1. The solids that are concocted to give that concentration, are calculated per kg of dry matter of the solids mixture. According to Table 2, 5th column, the solids mixture are made of 566 g of dry matter of ground corn grain, 120 g of dry matter of soybean meal, 10 g DM urea, 20 g dray matter mineral premix, 0 g dry matter oat straw (compensating oat straw), and 284 of dry matter of FOS (fermented oat straw that carries the Lv)

This solid mixture adds to 1000 g dry matter total, i.e., 1 kg of DM total of solids mixture.

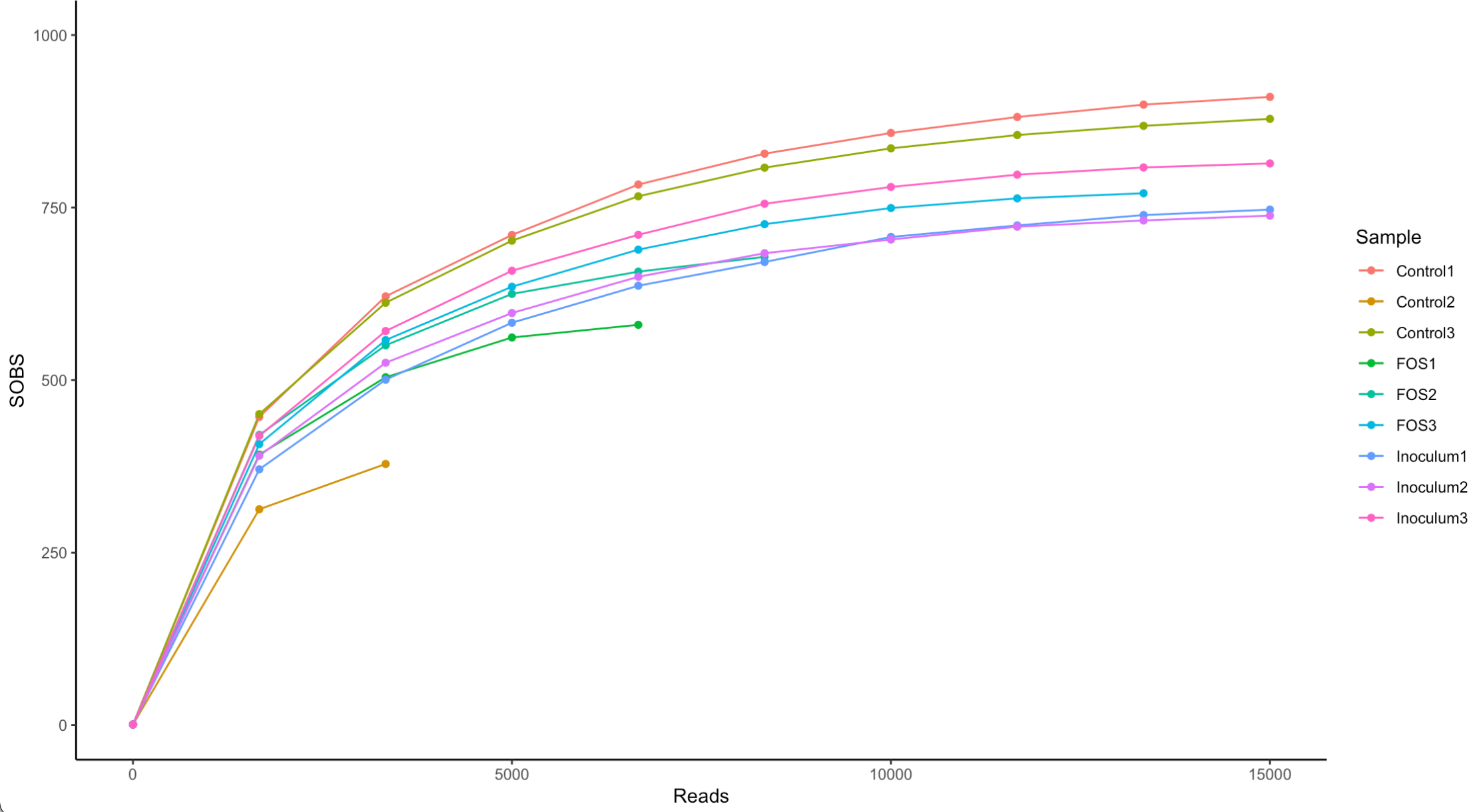
Third, when we state 284 g/kg DM in the text of the manuscript, we intend to express 284 g FOS-DM/kg solid mixture-DM. As it can be appreciated, this unit is very long, and we have used a shorter expression g /kgDM, in the understanding that the grams in the numerator correspond to dry matter of FOS, and the kg DM in the denominator corresponds to the 1000 g solid mixture dry matter.

Fourth, please note that we have been very meticulous to compensate for the nutritional presence of oat straw in each mixture (or dose). The objective was to keep the total amount of dry matter of oat straw *constant*. The total oat straw that corresponds to the sum of oat straw and fermented oat straw dry matter, is always 284 g DM-oat straw. In this way we avoid a potential bias associated with different loads of fermented oat straw in each treatment.

For instance, for the target concentration 50 mg Lv/L mixture suspension (3rd column of Table 1), the total oat straw is 189 g of DM-oat straw plus 95 g DM-FOS, for a total of 284 g DM-oat straw.

Furthermore, for the target concentration 150 mg Lv/L mentioned above (5th column of Table 1), the total oat straw is 0 g of DM-oat straw plus 284 DM-FOS, for a total oat straw of 284 DM-oat straw.

**Appendix 6.** FigureSM6. Rarefaction curve analysis for observed number of OTUs. SOBS, the number of species observed in a sample.



FigureSM6. Rarefaction curve analysis for observed number of OTUs. SOBS, the number of species observed in a sample.

**Notation**

DM Dry matter

FOS Fermented oat straw, lovastatin-carrier

HTST High throughput sequency technology

Lv Lovastatin

LW Live weight

OTUs Operational taxonomic units

SOBS The number of species observed in a sample

SSF Solid-state fermentation

*Greek characters*

ηc-t percent decrease of relative abundance of MA between the control (0

mgLv/L) and the “treatment” with 150 mgLv/L

ηin-t decrease of relative abundance of MA between the inoculum and

the “treatment” with 150 mgLv/L

Γ production cost for head of cattle in the finishing stage without Lv

treatment

ΓLv production cost for head of cattle in the finishing stage only due to Lv

supplementation to the ration

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