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## Effects of the combination between selected phytochemicals and the carriers silica and Tween 80 on dry matter and neutral detergent fibre digestibility of common feeds

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### ABSTRACT

The overall objective of the current study was to evaluate the effects of 2 carriers (Silica, SIL, and Tween 80, T80) and their interaction with 8 phytochemicals (PCs), on *in vitro* dry matter and neutral detergent fibre digestibility (DMD, NDFD) of 3 substrates commonly used as feed for dairy cattle (soybean meal, maize meal and total mixed ration –TMR). A total of eight PCs were tested: 4 essential oils (EO) - cinnamon oil (CIN), clove oil (CLO), thyme oil (THY) and oregano oil (ORE) - and 4 essential oil active compounds (EOAC) - cinnamaldehyde (CIN-AC), eugenol (EUG), thymol (THY-AC) and carvacrol (CAR). A positive control with carrier and no PCs was tested for each substrate (CRR). Each PC was tested at 0.5 mg L<sup>-1</sup> of medium on DMD and NDFD in an *in vitro* batch fermentation system. The incubation was performed twice at the intervals of 4 (DMD4, NDFD4) and 24 (DMD24, NDFD24) h. The PCs effect was significant on maize meal and soybean meal DMD24. The carrier's effect was significant on soybean meal DMD24, indicating a depressive effect of T80 on soybean meal. The PC-carrier interactions were significant on both DMD and NDFD of the tested substrates, except for maize meal and soybean meal DMD24. The PCs-SIL combinations generally increased digestibility while the combination with T80 exerted positive effect only on maize DMD4. The PC-carrier combinations variably affect digestibility of different substrates and these interactions should be considered both for their scientific and commercial implications.

### HIGHLIGHTS

- PC-Carrier interactions affect *in vitro* digestibility.
- In general, the PC-SIL combinations increase digestibility while those with Tween 80 exert depressing effect.
- The PC-Carrier effect is variable depending on the degraded substrate.

### ARTICLE HISTORY

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Essential oils; cattle; *in vitro* digestibility; supports

## Introduction

Essential oils (EO) and essential oil active compounds (EOAC) are considered phytochemicals (PCs), and their effects are widely studied, especially after the ban on the use of antibiotics in animal feeding in the European Union, which occurred in January 2006. In ruminant nutrition, PCs are studied as nutritional additives or microbial modulators to improve ruminal fermentation and feed efficiency. However, their effect on the ruminal fermentation processes varies greatly between studies depending also on the *in vivo* or *in vitro* method used, even though most studies are

performed *in vitro*. In particular, the PCs effects are variable concerning the dose tested, their composition, their concentration and the substrate fermented (Benchaar, Chaves, et al. 2007; Calsamiglia et al. 2007; Cobellis et al. 2016).

In several *in vitro* fermentation studies, the PCs have been tested through their direct dilution in the incubation medium (Benchaar, Chaves, et al. 2007; Tager and Krause 2010; Patra and Yu 2014; Roy et al. 2014). but this is not possible when the PCs have to be included in the diet. Moreover, since the EO and EOAC include highly volatile aromatic compounds,

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poorly soluble in water, they need to be adsorbed or emulsified to be added to feedstuff for their commercial use. The carrier thus became a further factor potentially affecting the PCs action.

Literature describes highly technological systems to embed the PCs, including macro-emulsion, liposomes and solid lipid nanoparticles (Edris and Malone 2012). These are generally too expensive for routine use in animal feeding. Consequently, EO and EOAC used as additives in cattle nutrition are normally adsorbed, diluted and/or dissolved in some inorganic clays or clay components (kaolin, vermiculite, bentonite, silica), organic carriers, such as rice hulls (Santos et al. 2010), or solvents as propylene glycol, diethyl phthalate, petroleum ether, methanol and ethanol (Busquet et al. 2006; Benchaar, Chaves, et al. 2007; Bravo et al. 2015; Chaves et al. 2008; Edris and Malone 2012). As reported by Edris and Malone (2012) alcohol acts as an enhancer that improves solubilisation of EO, while non-ionic surfactants (as Tween 20, 80, Span 85), selected for their low toxicity, shelf stability, and low production cost, were tested for their possible use in food and pharmaceutical preparations (Ma et al. 2016). It has been demonstrated by Cong et al. (2009) that Tween 80 *per se* at low doses does not affect the fermentative activity in the rumen. Therefore, it can be considered as a valid alternative carrier for water-insoluble ruminant additives. In analogy, silica-silicon dioxide- *per se*, has been historically described and considered as not affecting digestibility of cattle and unable to interact with feeds (Baker 1966). However, some effects of this additive on growth performance and feed conversion efficiency have been described in avian species (Tran et al. 2015). A study on the influence of different solvents (water, methanol, ethanol, petroleum ether, and propylene glycol) on the effects of EO supplementation on the *in vitro* methane production, showed that carriers can affect gas production, digestibility of nutrients, methane and Volatile Fatty Acids (VFA) production (Wadhwa et al. 2014). In particular, Ma et al. (2016) observed interferences between cinnamon, eugenol and thyme EOs and the supports T80 and soybean oil with effects on their *in vitro* antimicrobial activity. Other trials showed that a possible variability factor of EO activity is the feed composition (Calsamiglia et al. 2007).

In the majority of the *in vitro* studies evaluating the activity of OE and EOAC on ruminal digestibility, the role of the carrier is generally not considered (Patra 2011; Cobellis et al. 2016). The carriers are, in fact, often tested separately from the PCs, and the

evaluation of the possible synergic or antagonist effect on the fermentation process is missing.

The objective of the present work was to evaluate the effects of the combination between 8 PCs and 2 carriers, on *in vitro* dry matter (DM) and neutral detergent fibre (NDF) digestibility (DMD, NDFD) of 3 substrates commonly used as feeds for dairy cattle.

## Materials and methods

This study was carried out in accordance with the Italian Legislation on animal care (DL 26 04/03/2014).

Two commercial carriers were tested in addition to 8 PCs during the fermentation of three substrates at two time-points. For each carrier, a positive control (CRR), consisting of the carrier alone added to the substrate, was also included in the experimental set.

The two carriers employed were silica (Rhodia-Solvay, Bruxell, Belgium -SIL) and polyoxysiethylensorbitan monooleate (Tween 80, polyoxysiethylensorbitan monooleate, Cod: 907739787; A.C.E.F. Spa, Fiorenzuola D'Arda, Piacenza, Italy -T80).

The PCs tested were four EO obtained by Muller & Kostner (Liscate, Italy): cinnamon oil (CIN; 72–82% cinnamaldehyde) and clove oil (CLO; 70–83% eugenol) as phenylpropanoids and thyme oil (THY; 41–43% thymol) and oregano oil (ORE; 64–70% carvacrol) as terpenoids; the other PCs tested were four EOAC obtained by Frey and Lau (Henstedt-Ulzburg, Germany): cinnamaldehyde (CIN-AC), eugenol (EUG), thymol (THY-AC), and carvacrol (CAR).

The fermentation was conducted in an *in vitro* batch system described by Goering and Van Soest (1970) with the modifications reported by Righi et al. (2009); DMD and NDFD were measured in duplicate after 4 and 24 h of incubation as described in Righi et al. (2017). The fermentations were repeated twice in two separate weeks. The PCs were combined with the carriers at the equivalent dose of 1 g/cow/day, as suggested by Benchaar et al. (2006), assuming a hypothetical DM intake of about 20 kg (Righi et al. 2017; Ammer et al. 2018). The final concentration of the PCs in each flask was therefore set at 50 mg kg<sup>-1</sup> of DM (equivalent to 0.5 mg L<sup>-1</sup> considering a substrate of 0.5 g and 50 ml of liquids in each flask). In particular, PCs were diluted in SIL to obtain a concentration of 0.125% wt wt<sup>-1</sup> of the main active compound and this premix was added at the dose of 20 mg to each flask. On the other hand, the PCs were premixed with T80 to obtain a ratio of 1:1.5 wt wt<sup>-1</sup> between the main active compound (MAC) and the carrier. This premix was then diluted in distilled water to obtain a

MAC dilution of 1:3200; the latter solution was then added to each flask in the amount of 0.2 ml.

The fermentation substrates included maize meal and soybean meal, representing common concentrate feed ingredients in dairy cattle diets, supplying starch and protein, respectively; the third fermentation substrate employed was a total mixed ration (TMR) for dairy cows, to represent a typical maize silage based diet of the Padana Plain – Northern Italy (Comino et al. 2015; Righi et al. 2016, 2017). The TMR composition was as follows (g kg<sup>-1</sup> DM): maize silage, 246; maize meal, 180; triticale silage, 108; soybean meal, 79; maize grain flaked, 66; sorghum haylage, 61; alfalfa hay, 49; barley meal, 42; maize distillers, 38; soybean hulls, 33; beet pulp, 33; wheat straw, 10; soybean flakes, 20; fat supplement (Megalac<sup>®</sup>), 18; mineral and vitamin premix, 18. Substrates were dried at 55 °C for 48 h and then ground in a Cyclotec mill (Tecator, Herndon, VA, USA) to pass a 1-mm screen. According to the European Commission regulation 152/2009, crude protein (CP) was determined through the Kjeldhal method, ether extract was quantified through a Soxhlet extraction system, ash was obtained through ignition to 550 °C and starch was determined by the polarimetric method. The neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) contents were analysed by the method described by Van Soest et al. (1991) following the sequential analyses system. The chemical composition of the dried substrates is shown in Table 1.

As previously reported, the DMD and NDFD were then evaluated twice at 4 and 24 h of fermentation (DMD4 and DMD24, NDFD4 and NDFD24, respectively). As described in Comino et al. (2015), rumen fluid was collected from a 6-year old, Italian Holstein dry cow of about 660 kg of body weight fed 2 kg d<sup>-1</sup>

of concentrate (on a DM basis: maize meal 36.0%, maize germ meal 19.0%, wheat flour 18.0%, sunflower meal 10.0%, wheat bran 6.0%, soybean meal 3.0%, sugarcane molasses 2.6% and mineral-vitamin mix 5.4%) and given *ad libitum* access to grass and alfalfa mixed hay (55% NDF; 14% CP), stirred and filtered through eight layers of cheesecloth under continuous flushing of CO<sub>2</sub>. Filtered rumen fluid was mixed with the pre-incubated Van Soest buffer at a ratio of 1:4 (Goering and Van Soest 1970). Flasks containing the substrate (0.5 g) and the PCs on the two different carriers were inoculated with 50 mL of diluted fluid and were incubated in a water bath at 39 °C.

Pre-selected flasks were removed at 4 and 24 h of incubation. For each treatment, out of four flasks, two were used for DM and two for NDF analysis. DMD was determined similarly to Righi et al. (2009): the fermentation content of each flask was filtered through crucibles (Robu Glass Filter–ROBU H3, Borosilicate 3.3, 30 mL–Por. 2, Hattert, Germany), rinsed three times with boiling water and dried overnight at 105 °C. For NDFD determination, the fermentation content of each flask was transferred to a Raw Fibre Extractor (FIWE, VELD Scientifica, Usmate Velate, Italy) and boiled for 1 h with the addition of heat-stable amylase (A3306, Sigma Chemical Co., St. Louis, MO). The residues were then rinsed 3 times with boiling water and NDFD was expressed on a DM basis including residual ash, as described by Van Soest et al. (1991). Then, DMD4, DMD24, NDFD4, and NDFD24 were calculated by difference and expressed as a proportion of the initial value.

Statistical analyses were performed with the RStudio (1.2.1335) interface of the R software (version 3.5.3, 2019-03-11). Results of DMD and NDFD were analysed using the ANOVA (2-way test) with the

**Table 1.** Chemical composition and digestibility of the substrates employed in the study.

	Maize meal	Soybean meal	TMR <sup>a</sup>
Dry matter (DM; g kg <sup>-1</sup> as fed)	882	896	475
Nutrients, g kg <sup>-1</sup> DM			
Crude protein	98	457	168
Ether extract	41	28	41
Starch	681	–	293
Ash	17	69	62
Neutral detergent fibre	161	210	348
Acid detergent fibre	29	89	160
Acid detergent lignin	5	9	2
Dry matter digestibility, (4 h)	494	512	412
Dry matter digestibility, (24 h)	872	863	725
Fibre digestibility, g kg <sup>-1</sup> aNDF <sup>b</sup>			
Neutral detergent fibre digestibility, (4 h)	443	348	353
Neutral detergent fibre digestibility, (24 h)	682	837	663

<sup>a</sup>TMR – total mixed ration (g kg<sup>-1</sup> DM): maize silage, 246; maize meal, 180; triticale silage, 108; soybean meal, 79; maize grain flaked, 66; sorghum haylage, 61; alfalfa hay, 49; barley meal, 42; maize distillers, 38; soybean hulls, 33; beet pulp, 33; wheat straw, 10; soybean flakes, 20; fat supplement - Megalac<sup>®</sup>, 18; mineral and vitamin premix, 18.

<sup>b</sup>aNDF: amylase-treated neutral detergent fibre (Mertens et al.; 2002).

interaction between variables to study the effects of the PCs, carriers and PC\*carrier interaction. For each carrier-PC combination, 4 observations were available. The analysis was repeated for each substrate separately: maize meal, soybean meal and TMR, with DMD4, DMD24, NDFD4 and NDFD24 as the dependent variable. Differences were declared significant at  $P \leq 0.05$ .

## Results

In almost all the cases considered, the ANOVA test implemented shows that the PC-carrier interaction is significant. Some exceptions were found regarding DMD.

### DM digestibility

The interaction between carriers and PCs was significant for almost all the substrates at 4 and 24 h intervals. Exceptions were represented by the maize meal and soybean meal DMD at 24 h of incubation ( $p = .231$  and  $p = .240$  respectively). In the case of maize meal, the PC was the main variable affecting DMD24, while both the PC and the carrier influenced DMD24 of soybean meal. These results are reported in Table 2.

Most of the PC-carrier interactions increased maize meal DMD at the 4 h interval (originally equal to 49.4% DM). The maize meal DMD4 was improved in particular by the PCs-T80 combinations, which induced a higher digestibility compared to the homologous treatment carried by SIL. An exception was represented by the associations CIN-T80 and THY-T80 which showed lower values than the same PCs carried by SIL. The most favourable combination for maize meal DMD4 was EUG-T80, while the worst one was the EUG-SIL association, as shown in Figure 1.

Figure 2 shows the effects of the PC-carrier combinations on the DMD24 of maize meal; only few differences were found in comparison to the pure substrate digestibility at 24 h of fermentation (originally equal to 87.2% DM). The carrier and the interaction of the carrier with PCs were not significant. Thus, all the differences were due to the PCs ( $p = .024$ ), with CIN-AC, CLO and CAR inducing no effects or a general increase in the maize digestibility compared to THY, THY-AC and ORE showing no or depressive effects. The ORE showed depressive effects on DMD24 of maize meal.

Concerning soybean meal DMD4, the majority of the tested combinations showed lower digestibility in comparison to the pure substrate (originally equal to 51.2% DM). However, CIN-AC-SIL, CLO-SIL and EUG-SIL (combinations including phenylpropanoic compounds)

**Table 2.** Effect of carriers and phytochemicals (PCs) essential oils (EO) and essential oils active compounds (EOAC) on *in vitro* dry matter (DM) digestibility (%) of maize meal, soybean meal and total mixed ration (TMR) at 4 and 24 h of fermentation.

Item <sup>a</sup>	Maize		Soybean		TMR <sup>b</sup>	
	4	24	4	24	4	24
	% of DMD <sup>c</sup>					
CIN.T80	48.1	85.0	49.7	76.0	35.9	66.1
CIN.SIL	54.8	87.2	47.9	81.7	46.3	75.3
CIN-AC.T80	61.8	85.8	48.1	80.2	41.4	70.9
CIN-AC.SIL	57.6	87.6	57.7	86.7	46.5	73.8
CLO.T80	56.4	86.1	50.2	81.5	39.0	72.3
CLO.SIL	50.3	88.7	54.2	91.7	44.4	76.1
EUG.T80	65.0	86.7	49.1	81.4	37.3	68.2
EUG.SIL	45.6	84.7	56.6	91.9	46.5	73.1
THY.T80	52.3	85.7	50.0	77.8	38.2	70.6
THY.SIL	58.4	85.2	48.4	84.7	42.9	74.9
THY-AC.T80	53.6	84.6	49.7	75.8	38.3	70.2
THY-AC.SIL	51.1	85.2	51.4	89.4	41.9	75.4
ORE.T80	56.6	84.2	55.1	77.9	43.7	70.0
ORE.SIL	55.3	80.7	49.7	76.7	43.8	61.6
CAR.T80	55.5	85.8	54.9	81.8	42.8	72.9
CAR.SIL	49.2	89.5	49.3	89.8	44.2	75.8
CRR.T80	59.8	85.8	49.9	86.8	40.3	72.5
CRR.SIL	53.7	88.3	59.7	87.5	46.1	72.0
SEM	0.50	0.30	0.18	0.59	0.25	0.49
Variables	p Value					
PCs <sup>d</sup>	.030	.024	$\leq .001$	.005	.010	.027
Carriers	.002	.246	$\leq .001$	$\leq .001$	$\leq .001$	.020
PCs*Carriers	$\leq .001$	.231	$\leq .001$	.240	.002	.046

<sup>a</sup>Treatments combinations: CIN = cinnamon oil, CIN-AC = cinnamaldehyde, CLO = clove oil, EUG = eugenol, THY = thyme oil, THY-AC = thymol, ORE = oregano oil, CAR = carvacrol, CRR = negative control (substrate plus carrier), T80 = Tween 80, SIL = Silica.

<sup>b</sup>TMR: Total mixed ration (see food notes of Table 1 for the composition);

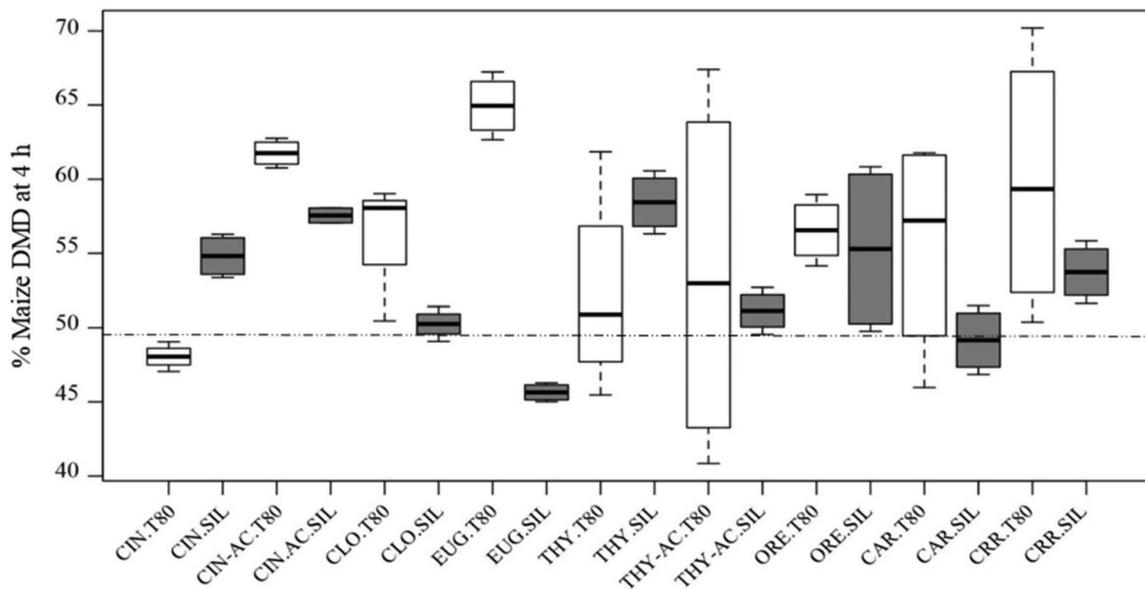
<sup>c</sup>DMD: Dry matter digestibility.

<sup>d</sup>PCs = Phytochemicals.

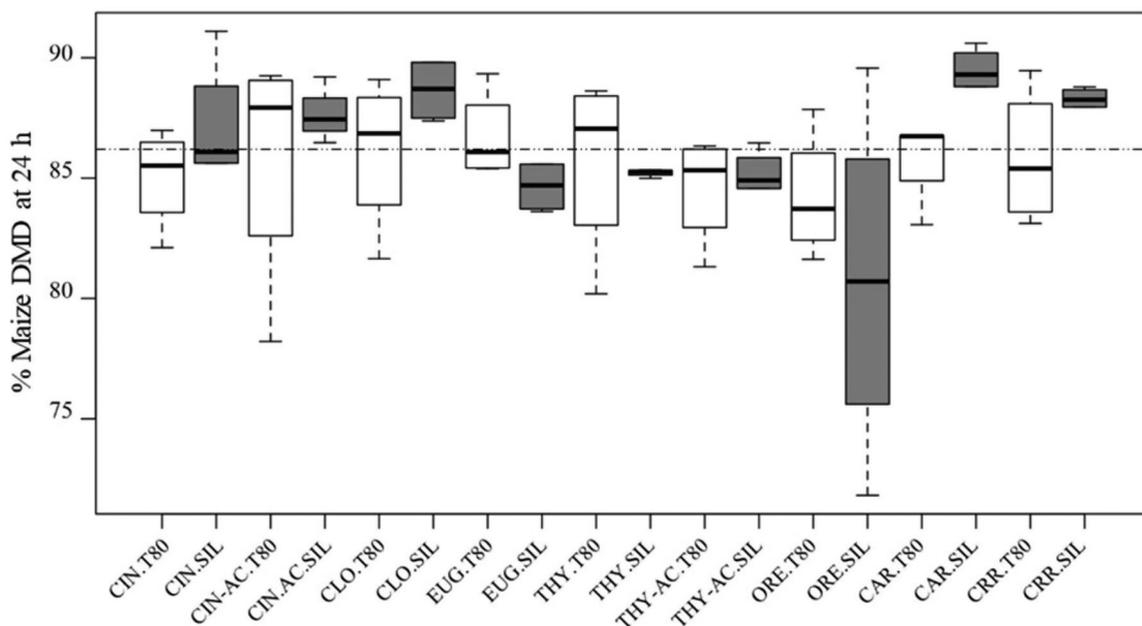
and ORE-T80 and CAR-T80 (combinations including terpenoids) showed higher digestibility than pure soybean meal. The highest DMD4 was found, as represented in Figure 3, both when SIL was tested alone and together with CIN-AC, while the lowest value was found testing the combination CIN-SIL.

Figure 4 shows the effects of the different associations of PCs and carriers on soybean meal DMD24. Both PCs and carriers had significant effects while their interaction was not significant. Regarding the carrier effect, SIL showed an increased DMD24 in comparison to the T80. Regarding the PCs, the major effects were observed for CAR, CLO, EUG and THY-AC. In particular, CLO and EUG combined with SIL gave the maximum improvement of the DMD24.

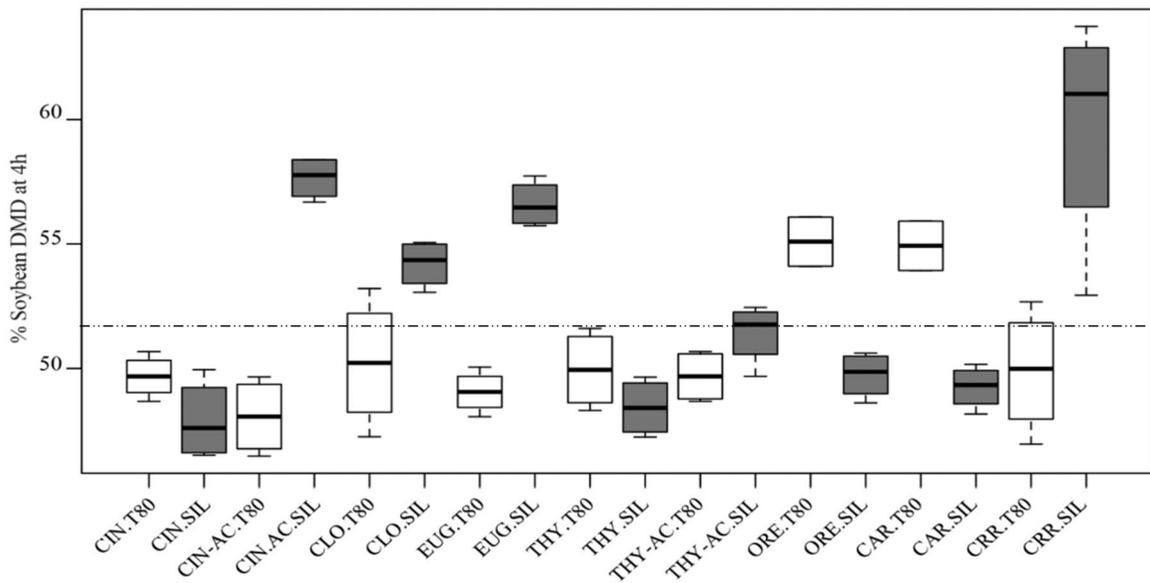
The combination between SIL and all the phenylpropanoid compounds (CIN, CIN-AC, CLO and EUG) as well as with THY and THY-AC gave higher digestibility values than the same PCs combined with T80. Particularly, the associations which led to the highest TMR DMD4 were CIN-SIL, CIN-AC-SIL, and EUG-SIL, while the lowest TMR DMD4 was obtained by the addition of CIN-T80, as demonstrated in Figure 5.



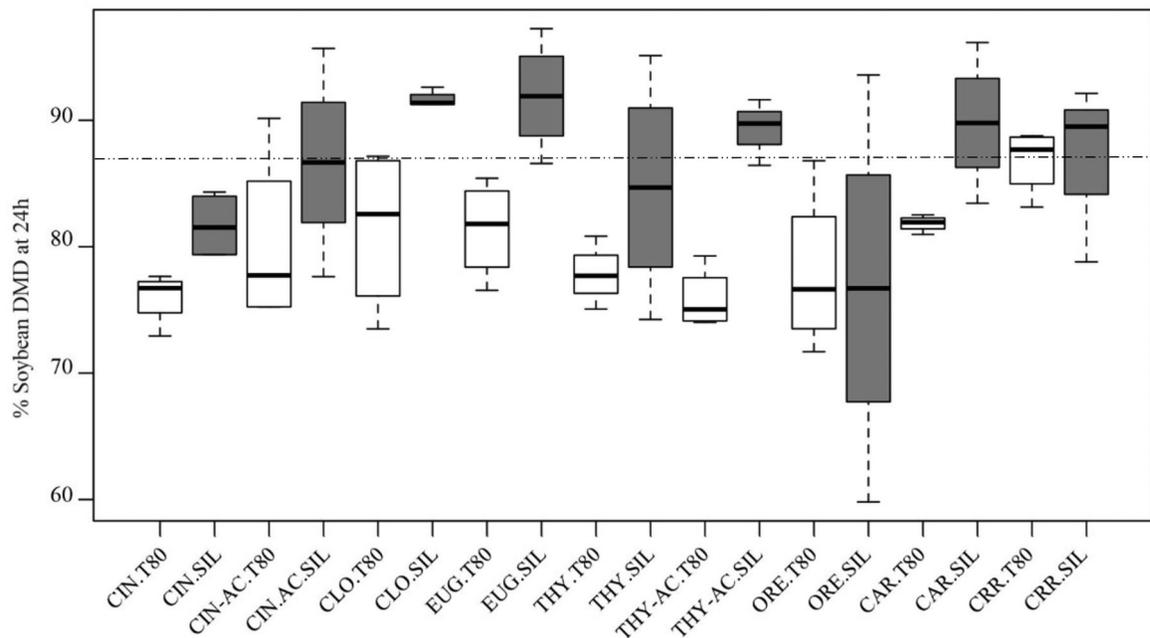
**Figure 1.** Boxplot comparing the effects across all combination between phytochemicals (PC) and carrier on maize meal dry matter digestibility (DMD) at 4 h of fermentation. The white boxes express the DMD distribution affected by the PC emulsified (T80), while the grey boxes express the DMD distribution affected by the PC adsorbed on silica (SIL). No outliers were detected then no points of values were plotted individually. The horizontal line in the middle indicates the median of the sample, the top and the bottom of the rectangle (box) represents the 75<sup>th</sup> and 25<sup>th</sup> percentiles. The whiskers at either side of the rectangle represent the lower and upper quartile. The dotted line represents the substrate digestibility. Treatments combinations: CIN = cinnamon oil, CIN-AC = cinnamaldehyde, CLO = clove oil, EUG = eugenol, THY = thyme oil, THY-AC = thymol, ORE = oregano oil, CAR = carvacrol, CRR = negative control (substrate plus carrier), T80 = Tween 80, SIL = Silica.



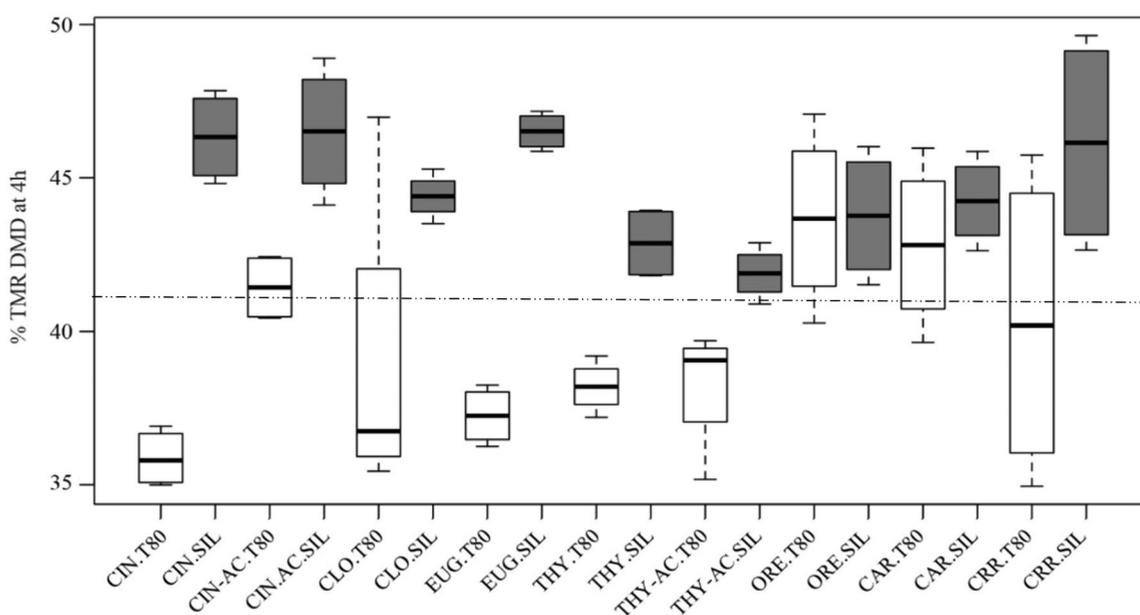
**Figure 2.** Boxplot comparing the effects across all combination between phytochemicals (PC) and carrier on maize meal dry matter digestibility (DMD) at 24 h of fermentation. The white boxes express the DMD distribution affected by the PC emulsified (T80), while the grey boxes express the DMD distribution affected by the PC adsorbed on silica (SIL). No outliers were detected then no points of values were plotted individually. The horizontal line in the middle indicates the median of the sample, the top and the bottom of the rectangle (box) represents the 75<sup>th</sup> and 25<sup>th</sup> percentiles. The whiskers at either side of the rectangle represent the lower and upper quartile. The dotted line represents the substrate digestibility. Treatments combinations: CIN = cinnamon oil, CIN-AC = cinnamaldehyde, CLO = clove oil, EUG = eugenol, THY = thyme oil, THY-AC = thymol, ORE = oregano oil, CAR = carvacrol, CRR = negative control (substrate plus carrier), T80 = Tween 80, SIL = Silica.



**Figure 3.** Boxplot comparing the effects across all combination between phytochemicals (PC) and carrier on soybean meal dry matter digestibility (DMD) at 4 h of fermentation. The white boxes express the DMD distribution affected by the PC emulsified (T80), while the grey boxes express the DMD distribution affected by the PC adsorbed on silica (SIL). No outliers were detected then no points of values were plotted individually. The horizontal line in the middle indicates the median of the sample, the top and the bottom of the rectangle (box) represents the 75<sup>th</sup> and 25<sup>th</sup> percentiles. The whiskers at either side of the rectangle represent the lower and upper quartile. The dotted line represent the substrate digestibility. Treatments combinations: CIN = cinnamon oil, CIN-AC = cinnamaldehyde, CLO = clove oil, EUG = eugenol, THY = thyme oil, THY-AC = thymol, ORE = oregano oil, CAR = carvacrol, CRR = negative control (substrate plus carrier), T80 = Tween 80, SIL = Silica.



**Figure 4.** Boxplot comparing the effects across all combination between phytochemicals (PC) and carrier on soybean meal dry matter digestibility (DMD) at 24 h of fermentation. The white boxes express the DMD distribution affected by the PC emulsified (T80), while the grey boxes express the DMD distribution affected by the PC adsorbed on silica (SIL). No outliers were detected then no points of values were plotted individually. The horizontal line in the middle indicates the median of the sample, the top and the bottom of the rectangle (box) represents the 75<sup>th</sup> and 25<sup>th</sup> percentiles. The whiskers at either side of the rectangle represent the lower and upper quartile. The dotted line represents the substrate digestibility. Treatments combinations: CIN = cinnamon oil, CIN-AC = cinnamaldehyde, CLO = clove oil, EUG = eugenol, THY = thyme oil, THY-AC = thymol, ORE = oregano oil, CAR = carvacrol, CRR = negative control (substrate plus carrier), T80 = Tween 80, SIL = Silica.



**Figure 5.** Boxplot comparing the effects across all combination between phytochemicals (PC) and carrier on total mixed ration (TMR) dry matter digestibility (DMD) at 4 h of fermentation. The white boxes express the DMD distribution affected by the PC emulsified (T80), while the grey boxes express the DMD distribution affected by the PC adsorbed on silica (SIL). No outliers were detected then no points of values were plotted individually. The horizontal line in the middle indicates the median of the sample, the top and the bottom of the rectangle (box) represents the 75<sup>th</sup> and 25<sup>th</sup> percentiles. The whiskers at either side of the rectangle represent the lower and upper quartile. The dotted line represents the substrate digestibility. Treatments combinations: CIN = cinnamon oil, CIN-AC = cinnamaldehyde, CLO = clove oil, EUG = eugenol, THY = thyme oil, THY-AC = thymol, ORE = oregano oil, CAR = carvacrol, CRR = negative control (substrate plus carrier), T80 = Tween 80, SIL = Silica.

At 24 h of fermentation the differences in the TMR DMD were reduced compared with the results found at 4 h interval. All the PCs adsorbed on SIL gave higher values in comparison to those emulsified with T80, except for ORE. Indeed, the combination ORE-SIL reduced the TMR digestibility giving the lowest value. The highest TMR DMD<sub>24</sub> was obtained with CLO-SIL as shown in Figure 6.

### NDF digestibility

The interaction between carriers and PCs expressed a highly significant effect on the NDFD for all the substrates tested, as shown in Table 3.

The NDFD<sub>4</sub> of the pure maize meal was 44.3% of NDF, and the interaction PCs-carriers showed in almost all the cases an increased effect on this parameter. However, as depicted in Figure 7, CIN-AC, THY-T80 and ORE-T80 had depressive effects on NDFD<sub>4</sub>. The highest digestibility was observed with CIN-SIL, while the lowest was found when the combination THY-T80 was tested.

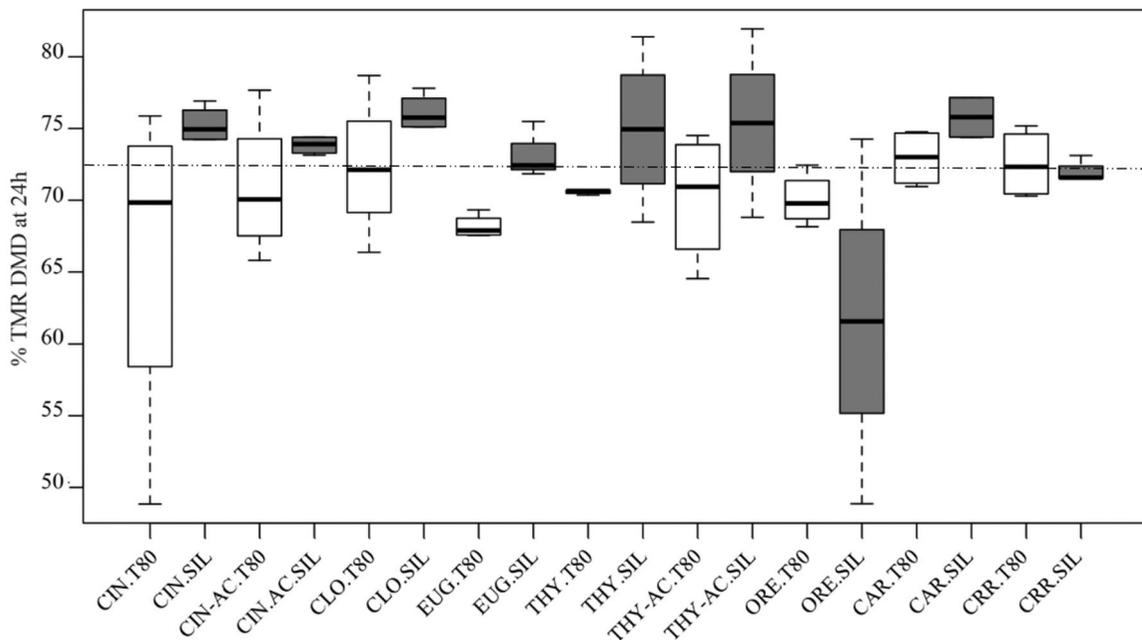
The PCs-SIL combinations gave higher NDFD<sub>24</sub> values than the PCs-T80 on the maize meal whose digestibility was 68.2% DM, while the opposite effect was observed in the case of CLO combinations. The highest value was observed, as represented in Figure

8, using SIL alone or in combination with CAR, while the THY-AC-T80 had the most depressive effect on NDFD<sub>24</sub>.

The PC-carrier combinations tested on soybean meal NDFD<sub>4</sub> are represented in Figure 9 and resulted in similar or higher values than the NDFD<sub>4</sub> of the pure substrate (equal to 34.8% DM). However, the data obtained clearly suggest that the combinations PCs-SIL lead to higher digestibility values than the PCs combined with T80. The highest NDFD<sub>4</sub> was induced by the combinations THY-SIL and CAR-SIL, while the lowest value was found when the combination THY-AC-T80 was tested.

The results previously reported for the soybean meal NDFD<sub>4</sub> were confirmed also at 24 h of fermentation, as shown in Figure 10. Almost all the PC-SIL associations gave higher NDFD<sub>24</sub> in comparison to the PC-T80 combinations. Exceptions are represented by CLO and EUG, whose combinations showed an opposite effect on substrate fibre degradation, increasing the digestibility when carried by T80. The maximum value of NDFD<sub>24</sub> was obtained on soybean meal with the combination THY-AC-SIL, ORE-SIL and CAR-SIL while this parameter was minimised by THY-T80.

The NDFD<sub>4</sub> of the TMR (originally equal to 35.3% DM) was generally depressed or not affected by the tested PC-carrier combinations as depicted in Figure



**Figure 6.** Boxplot comparing the effects across all combination between phytochemicals (PC) and carrier on total mixed ration (TMR) dry matter digestibility (DMD) at 24 h of fermentation. The white boxes express the DMD distribution affected by the PC emulsified (T80), while the grey boxes express the DMD distribution affected by the PC adsorbed on silica (SIL). No outliers were detected then no points of values were plotted individually. The horizontal line in the middle indicates the median of the sample, the top and the bottom of the rectangle (box) represents the 75<sup>th</sup> and 25<sup>th</sup> percentiles. The whiskers at either side of the rectangle represent the lower and upper quartile. The dotted line represents the substrate digestibility. Treatments combinations: CIN = cinnamon oil, CIN-AC = cinnamaldehyde, CLO = clove oil, EUG = eugenol, THY = thyme oil, THY-AC = thymol, ORE = oregano oil, CAR = carvacrol, CRR = negative control (substrate plus carrier), T80 = Tween 80, SIL = Silica.

11. Nonetheless, the results show higher NDFD4 of the PCs associated with SIL compared to the same plant products carried by T80. The sole exception was EUG-T80 that gave similar value to EUG-SIL, even if the latter showed higher variability of the results. The highest improvement of the TMR NDFD4 was obtained with CLO-SIL and CAR-SIL. Whereas, CAR-T80 showed the most depressive effect on digestibility.

The differences between the digestibility induced by the PC-carrier combinations on the NDFD24 of the pure TMR were reduced in comparison to the 4 h interval. Also data on TMR NDFD24 show higher values when the PCs are associated with SIL, even if CIN-AC shows the opposite effect. The most depressive effect was induced by CIN-T80, while the highest increase was obtained by the CLO-SIL combination as depicted in Figure 12.

## Discussion

This study was performed to evaluate the effects of PCs, carriers and their interaction on the *in vitro* digestibility of some worldwide employed ruminant feeds.

The effect of PCs was significant on both maize meal and soybean meal DMD24. Maize meal DMD24 seems not to be affected by the addition of CIN, CIN-AC, CLO and EUG. These results agree with those reported by Ornaghi et al. (2017), who tested the effects of clove and cinnamon essential oils on DMD and NDFD of a diet for young bulls comprised of 79% of cracked maize. Similar observations can also be done for the other PCs; no effects or a slight increase was observed with adding CAR, while a clear decrease in maize meal DMD24 was induced with adding ORE. Similar results were obtained testing oregano oil on organic matter, true matter and NDF digestibilities of maize silage and barley grain as reported by Temizkan et al. (2011). It can be speculated that ORE in addition to amylaceous feed inhibits the fermentation process, as also hypothesised by the cited authors, who highlighted a strong dose-dependent negative effect of the oregano oil. It has in fact been shown that the PCs effects on DM and NDF digestibility are substrate-dependent (Kilic et al. 2011; Khiaosa-Ard and Zebeli 2013). The effects of EO on several rumen fermentation parameters have been reviewed by Benchaar et al. (2008), who indicated thymol (administered at a dose of 1 g/d) as the strongest inhibitor of the amino

**Table 3.** Effect of carriers and phytochemicals (PCs) essential oils (EO) and essential oils active compounds (EOAC) on *in vitro* neutral detergent fibre (NDF) digestibility (%) of maize meal, soybean meal and total mixed ration (TMR) at 4 and 24 h of fermentation.

Item <sup>a</sup>	Maize		Soybean		TMR <sup>b</sup>	
	4	24	4	24	4	24
	% of NDFD <sup>c</sup>					
CIN.T80	48.4	68.4	42.3	82.9	26.8	54.4
CIN.SIL	67.7	89.0	45.4	94.1	35.0	70.9
CIN-AC.T80	42.5	65.7	35.4	85.3	30.0	63.0
CIN-AC.SIL	66.5	86.3	51.6	96.3	34.0	54.6
CLO.T80	53.1	74.5	35.2	88.7	34.4	66.3
CLO.SIL	57.9	66.3	49.0	87.2	37.4	74.2
EUG.T80	54.4	68.2	37.3	86.4	36.4	64.8
EUG.SIL	59.8	81.5	47.0	87.9	35.0	70.1
THY.T80	34.7	64.9	37.2	79.0	25.8	61.1
THY.SIL	60.6	85.4	54.6	95.1	35.0	62.8
THY-AC.T80	48.2	55.3	35.7	87.1	27.1	60.4
THY-AC.SIL	55.4	85.9	51.1	98.8	35.5	64.6
ORE.T80	40.0	60.0	37.3	85.2	27.8	64.4
ORE.SIL	59.4	80.7	49.7	99.5	35.6	72.1
CAR.T80	50.4	61.6	38.4	85.1	24.7	61.2
CAR.SIL	56.7	90.1	54.5	99.7	38.1	67.9
CRR.T80	50.2	66.9	41.5	84.2	26.8	59.2
CRR.SIL	58.1	92.3	48.5	98.7	34.4	70.0
SEM	0.49	0.32	0.37	0.49	0.26	0.59
Variables	<i>p</i> Value					
PCs <sup>d</sup>	.001	<.001	.193	.058	<.001	.005
Carriers	<.001	<.001	<.001	<.001	<.001	<.001
PCs*Carriers	<.001	<.001	.002	.003	<.001	.010

<sup>a</sup>Treatments combinations: CIN = cinnamon oil, CIN-AC = cinnamaldehyde, CLO = clove oil, EUG = eugenol, THY = thyme oil, THY-AC = thymol, ORE = oregano oil, CAR = carvacrol, CRR = negative control (substrate plus carrier), T80 = Tween 80, SIL = Silica.

<sup>b</sup>TMR: Total mixer ration (see food notes of Table 1 for the composition).

<sup>c</sup>NDFD: Neutral detergent fibre digestibility.

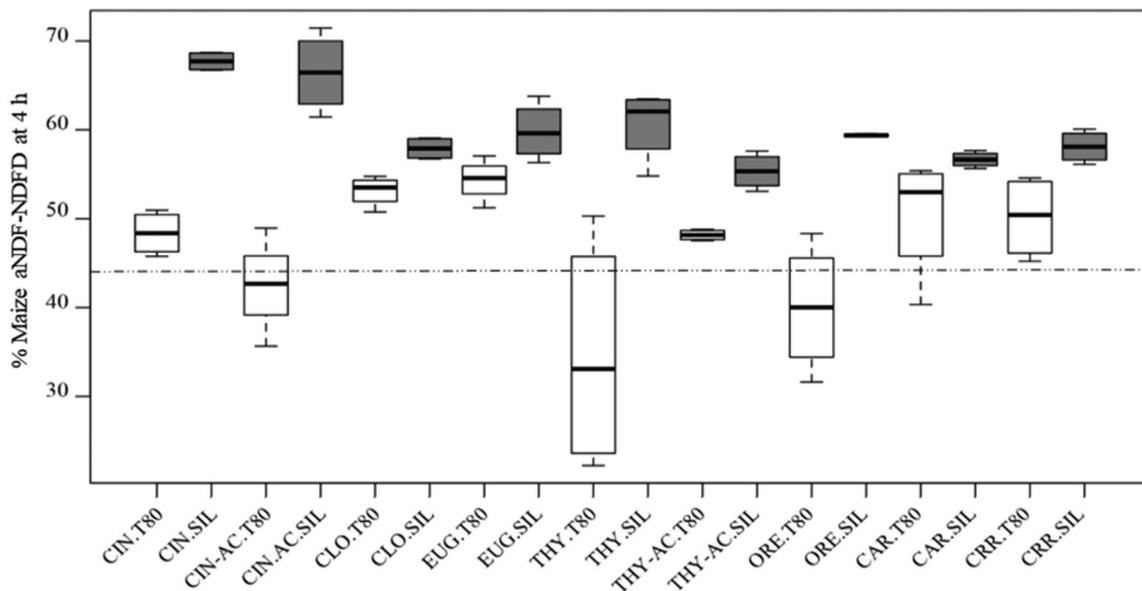
<sup>d</sup>PCs: Phytochemicals.

acids deamination by ruminal bacteria which could have affected protein degradability. The same authors reported also a depressive effect on soybean meal digestibility when supplemented with the commercial mixture of cresol, resorcinol, thymol, guaiacol and eugenol patented by Rossi (1996). Similarly, in the present trial, soybean meal digestibility was reduced in presence of THY, THY-AC, CLO and EUG. It should be highlighted, however, that the addition of silica as a carrier was able to reverse their effects.

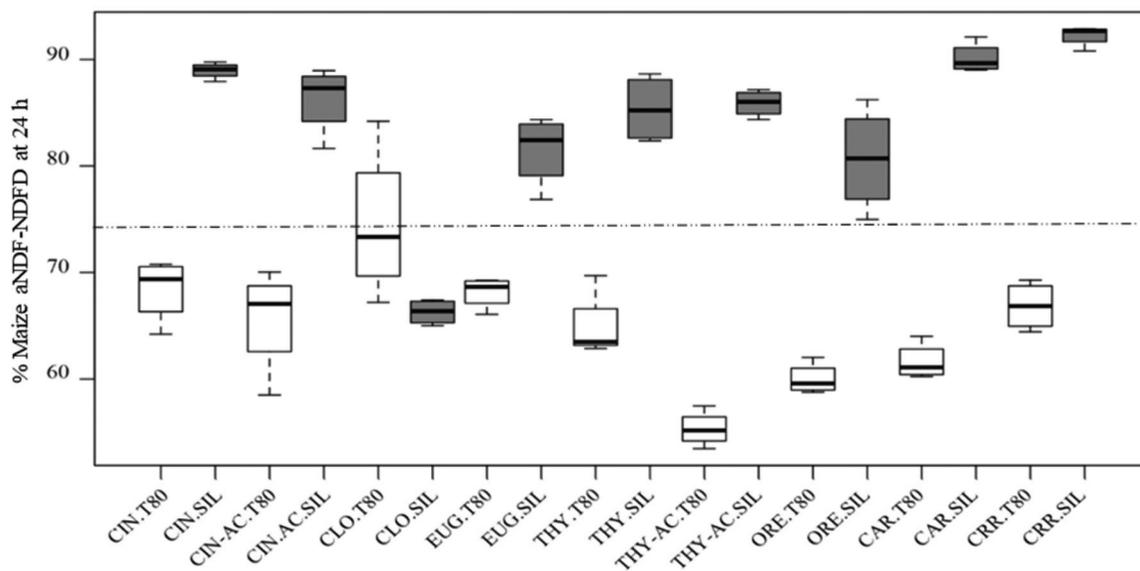
Our study revealed, in fact, the presence of a carrier effect on the *in vitro* soybean meal DMD24, indicating in particular a positive influence of SIL and a general null or depressive effect of T80 on this parameter. The SIL is a mineral clay component recognised as a feed additive, characterised by spherical particles of about 0.1 to 1.5 mm in diameter with a microporous surface that tends to absorb the small drops of EO (Rossi 1996). The EO absorbed in SIL micropores, could be tightly retained and probably released slowly. This could reduce their concentration in the medium at shorter intervals with positive effects on the microbiome. In a

meta-analytic study, Khiaosa-Ard and Zebeli (2013) reported, in fact, an increase of the protozoa number with low doses of EO bioactive compounds. Moreover, the same EO doses led to a modification of the VFA proportion in beef cattle and small ruminants, with increased propionate, indicating a possible modification of the microbiome composition towards non-structural carbohydrate degrading bacteria. These results differ from the conclusions drew by Baker (1966) which fed SIL as an additive to cattle diet. In the cited work, no effect on digestibility was attributed to SIL when added to the concentrate of beef cattle.

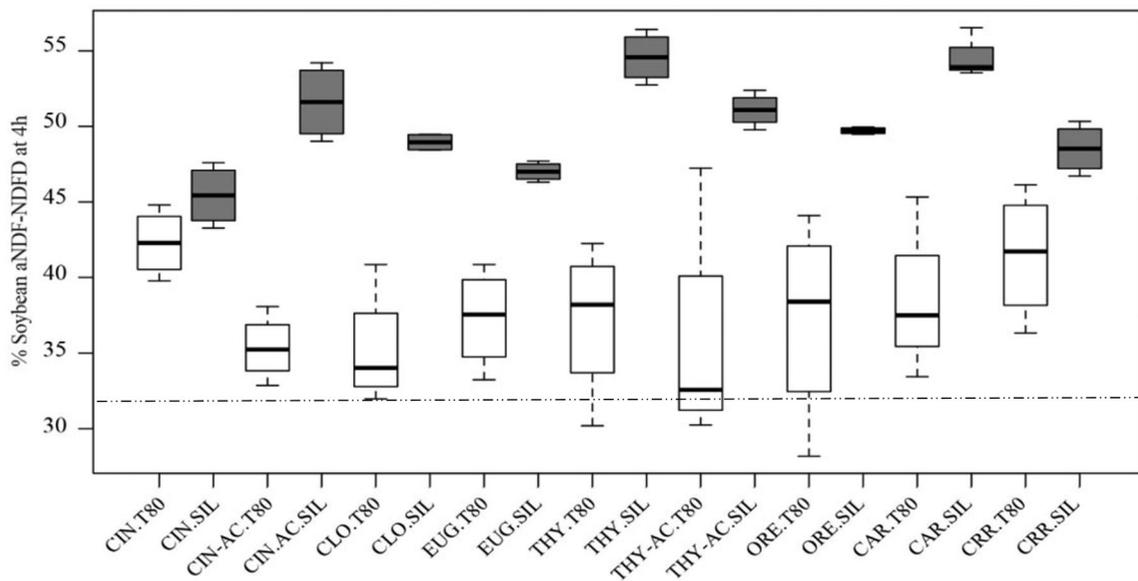
However, the most interesting result of the present trial is the significant effect of the interaction found between PCs and carriers, that was particularly strong on the NDFD parameter. Consistently with our findings, testing the effects of several EO (cinnamaldehyde, carvone and limonene) dissolved in different liquid supports, Wadhwa et al. (2014) found a significant interaction between the EO and the solvents used, on both NDFD and total organic matter digestibility. The authors indicated cinnamaldehyde-water followed by cinnamaldehyde-methanol and carvone-methanol as the most promising combinations improving the wheat straw digestibility. The presence of significant PC-carrier interaction indicates that the effect of the tested PCs on *in vitro* digestibility is, at least partially, carrier-dependent. In fact, it is well known that phenolic and non-phenolic compounds of the EO are able to interact differently, due to their chemical composition, with chemical groups of proteins or other active biological molecules as for example the enzymes (Calsamiglia et al. 2007). Additionally, also the carrier can interact differently with other molecules as well as with the PCs. Unlike from SIL, that adsorb and probably slowly release the EO, T80 is a high molecular weight organic surfactant that creates bridges at the interface between polar and non-polar molecules. Its main mechanism of action consists in the rise of the microbial endogenous enzymatic release and activities, as well as in the increase of the affinity between the enzymes and the specific substrates (Liu et al. 2013). As reported by the latter authors, T80 appeared to stimulate microbial population activity in the rumen and to increase *in vitro* DMD; thus, an improvement in the degradability of roughages is expected by adding surfactants as feed additives. An increase of *in vitro* DMD and of *in vitro* organic matter digestibility was also observed by Cong et al. (2009) adding non-ionic surfactants to different cereal straws at the dose of 0.1%. The only exception in the latter study was represented by T80



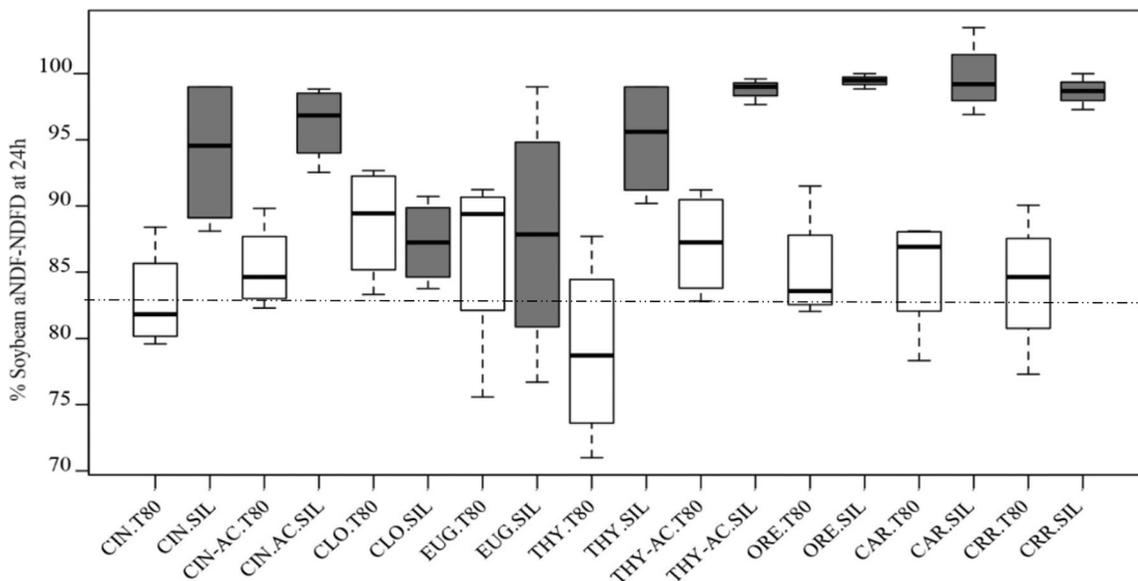
**Figure 7.** Boxplot comparing the effects across all combination between phytochemicals (PC) and carrier on maize meal neutral detergent fibre digestibility (NDFD) at 4 h of fermentation. The white boxes express the NDFD distribution affected by the PC emulsified (T80), while the grey boxes express the NDFD distribution affected by the PC adsorbed on silica (SIL). No outliers were detected then no points of values were plotted individually. The horizontal line in the middle indicates the median of the sample, the top and the bottom of the rectangle (box) represents the 75<sup>th</sup> and 25<sup>th</sup> percentiles. The whiskers at either side of the rectangle represent the lower and upper quartile. The dotted line represent the substrate digestibility. Treatments combinations: CIN = cinnamon oil, CIN-AC = cinnamaldehyde, CLO = clove oil, EUG = eugenol, THY = thyme oil, THY-AC = thymol, ORE = oregano oil, CAR = carvacrol, CRR = negative control (substrate plus carrier), T80 = Tween 80, SIL = Silica.



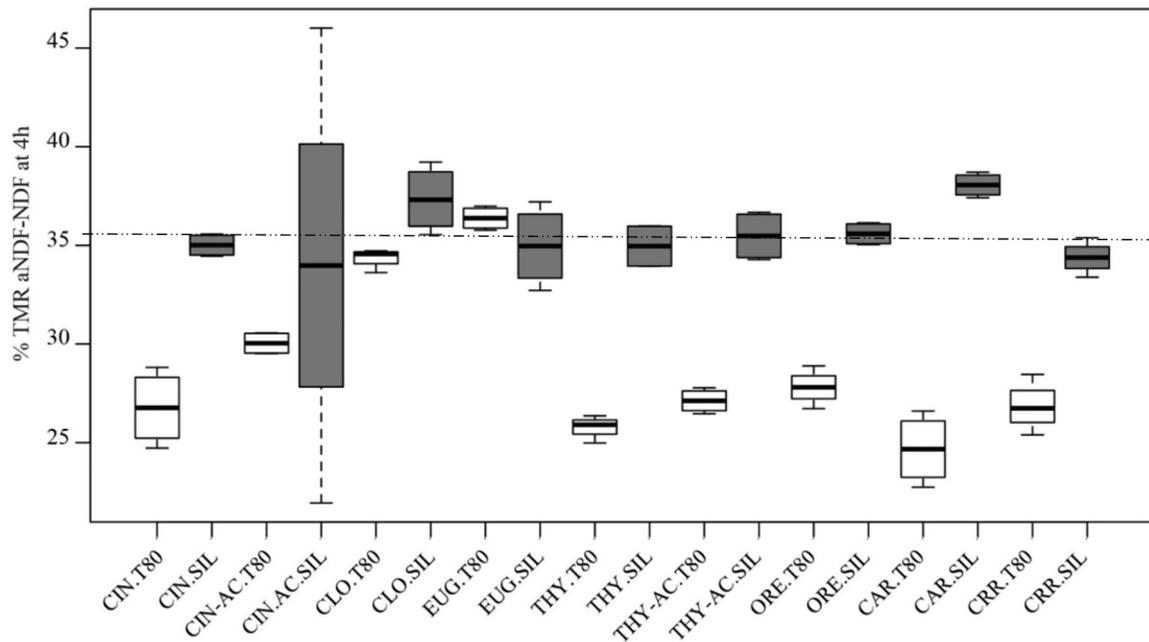
**Figure 8.** Boxplot comparing the effects across all combination between phytochemicals (PC) and carrier on maize meal neutral detergent fibre digestibility (NDFD) at 24 h of fermentation. The white boxes express the NDFD distribution affected by the PC emulsified (T80), while the grey boxes express the NDFD distribution affected by the PC adsorbed on silica (SIL). No outliers were detected then no points of values were plotted individually. The horizontal line in the middle indicates the median of the sample, the top and the bottom of the rectangle (box) represents the 75<sup>th</sup> and 25<sup>th</sup> percentiles. The whiskers at either side of the rectangle represent the lower and upper quartile. The dotted line represent the substrate digestibility. Treatments combinations: CIN = cinnamon oil, CIN-AC = cinnamaldehyde, CLO = clove oil, EUG = eugenol, THY = thyme oil, THY-AC = thymol, ORE = oregano oil, CAR = carvacrol, CRR = negative control (substrate plus carrier), T80 = Tween 80, SIL = Silica.



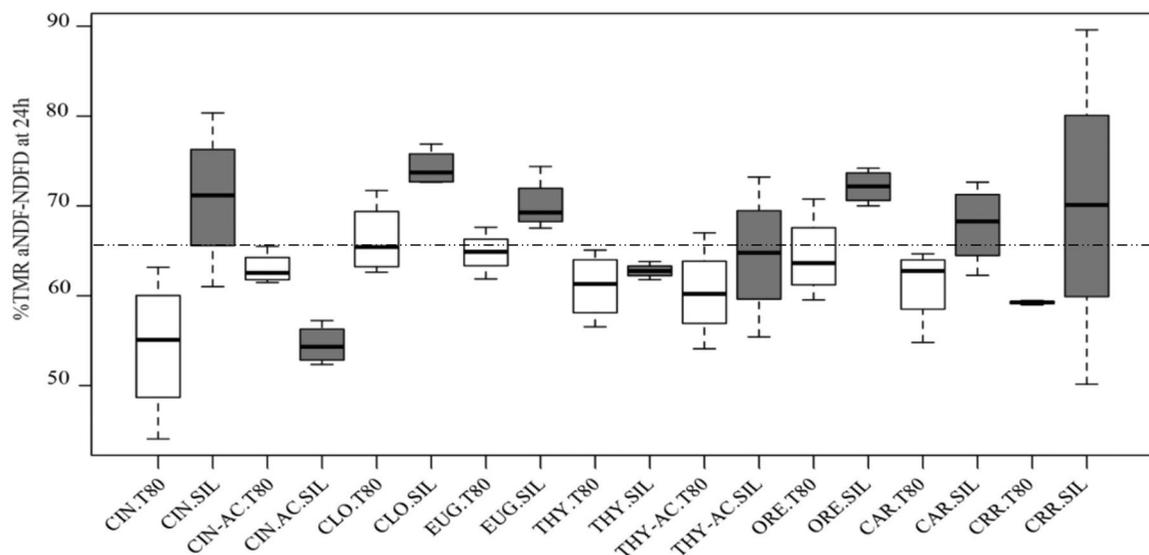
**Figure 9.** Boxplot comparing the effects across all combination between phytochemicals (PC) and carrier on soybean meal neutral detergent fibre digestibility (NDFD) at 4 h of fermentation. The white boxes express the NDFD distribution affected by the PC emulsified (T80), while the grey boxes express the NDFD distribution affected by the PC adsorbed on silica (SIL). No outliers were detected then no points of values were plotted individually. The horizontal line in the middle indicates the median of the sample, the top and the bottom of the rectangle (box) represents the 75<sup>th</sup> and 25<sup>th</sup> percentiles. The whiskers at either side of the rectangle represent the lower and upper quartile. The dotted line represents the substrate digestibility. Treatments combinations: CIN = cinnamon oil, CIN-AC = cinnamaldehyde, CLO = clove oil, EUG = eugenol, THY = thyme oil, THY-AC = thymol, ORE = oregano oil, CAR = carvacrol, CRR = negative control (substrate plus carrier), T80 = Tween 80, SIL = Silica.



**Figure 10.** Boxplot comparing the effects across all combination between phytochemicals (PC) and carrier on soybean meal neutral detergent fibre digestibility (NDFD) at 24 h of fermentation. The white boxes express the NDFD distribution affected by the PC emulsified (T80), while the grey boxes express the NDFD distribution affected by the PC adsorbed on silica (SIL). No outliers were detected then no points of values were plotted individually. The horizontal line in the middle indicates the median of the sample, the top and the bottom of the rectangle (box) represents the 75<sup>th</sup> and 25<sup>th</sup> percentiles. The whiskers at either side of the rectangle represent the lower and upper quartile. The dotted line represents the substrate digestibility. Treatments combinations: CIN = cinnamon oil, CIN-AC = cinnamaldehyde, CLO = clove oil, EUG = eugenol, THY = thyme oil, THY-AC = thymol, ORE = oregano oil, CAR = carvacrol, CRR = negative control (substrate plus carrier), T80 = Tween 80, SIL = Silica.



**Figure 11.** Boxplot comparing the effects across all combination between phytochemicals (PC) and carrier on total mixed ration (TMR) neutral detergent fibre digestibility (NDFD) at 4 h of fermentation. The white boxes express the NDFD distribution affected by the PC emulsified (T80), while the grey boxes express the NDFD distribution affected by the PC adsorbed on silica (SIL). No outliers were detected then no points of values were plotted individually. The horizontal line in the middle indicates the median of the sample, the top and the bottom of the rectangle (box) represents the 75<sup>th</sup> and 25<sup>th</sup> percentiles. The whiskers at either side of the rectangle represent the lower and upper quartile. The dotted line represents the substrate digestibility. Treatments combinations: CIN = cinnamon oil, CIN-AC = cinnamaldehyde, CLO = clove oil, EUG = eugenol, THY = thyme oil, THY-AC = thymol, ORE = oregano oil, CAR = carvacrol, CRR = negative control (substrate plus carrier), T80 = Tween 80, SIL = Silica.



**Figure 12.** Boxplot comparing the effects across all combination between phytochemicals (PC) and carrier on total mixed ration (TMR) neutral detergent fibre digestibility (NDFD) at 24 h of fermentation. The white boxes express the NDFD distribution affected by the PC emulsified (T80), while the grey boxes express the NDFD distribution affected by the PC adsorbed on silica (SIL). No outliers were detected then no points of values were plotted individually. The horizontal line in the middle indicates the median of the sample, the top and the bottom of the rectangle (box) represents the 75<sup>th</sup> and 25<sup>th</sup> percentiles. The whiskers at either side of the rectangle represent the lower and upper quartile. The dotted line represents the substrate digestibility. Treatments combinations: CIN = cinnamon oil, CIN-AC = cinnamaldehyde, CLO = clove oil, EUG = eugenol, THY = thyme oil, THY-AC = thymol, ORE = oregano oil, CAR = carvacrol, CRR = negative control (substrate plus carrier), T80 = Tween 80, SIL = Silica.

on maize stove digestibility which was not affected by the treatment.

Conversely, in our study, the PC-T80 combinations had generally no or depressive effect on digestibility in most of the cases. A similar decreasing effect was reported by Baah et al. (2005) on orchardgrass digestibility using 0.2% of T80. Moreover, these contrasting results indicate a different effect of the surfactants depending on the substrate tested, in analogy to our findings on digestibility when using T80. Our study revealed significant interactions between the type of carrier and the PC tested on DMD4, NDFD4 and NDFD24 for all the tested substrates and also for TMR DMD24. Based on the results, the PCs effect changes depending on the carrier used; moreover, as previously reported, the carrier effect can be substrate-dependent. This create a wide possible range of variation of the results connected with the use of PCs that should be considered also both in experimental trials and in formulating commercial products. The carrier effect could be related to its ability in modulating the release of the PCs or to its chemical interaction with the substrate or, again, to its direct effect on bacteria cytoplasmic membrane (Al-Adham et al. 2000, 2013). Furthermore, the latter authors indicate that oil/water micro-emulsions in general exert a biocidal activity against yeast and fungi. The micro-emulsion-cell wall interaction leads to the membrane and cell wall damage and to a consequent leakage of substances, rendering the fungal cell wall sensitive to osmotic lysis (Al-Adham et al. 2013).

With the present study we can therefore provide some suggestions regarding the effect of some PCs-carrier combinations on the main feeds used in the bovine nutrition. Phenylpropanoid PCs induce higher DMD combined with SIL when added to the soybean meal and TMR; this was evident at 4 h of the fermentation, compared with the results obtained by the combinations with T80. This effect seems to be in contrast with the general effect of the phenylpropanoids which usually act, according to Besle et al. (1994), as a physical-chemical barrier to the action of microbial enzymes and express antimicrobial activity against rumen microbes. On the other hand, ORE and CAR (both terpenoid PCs) showed stimulatory activity on soybean meal DMD4 when combined with T80, and the opposite effect when adsorbed in SIL, in comparison with the relative CRR. This could be related to a variable interaction between the active molecules that are part of the PCs and the different carriers used, probably affecting both their solubility and stability in

the mixture tested, and consequently their effect on the *in vitro* digestibility.

More in detail, among the PC-carrier combinations, CIN-AC and EUG combined with T80 improved the maize meal DMD4 and in combination with SIL increased both soybean meal and TMR DMD4. Our results on TMR DMD24 apparently agree with those reported by Tager and Krause (2010), in which both eugenol and cinnamaldehyde, tested without a support, had no effect on the TMR DMD24. Similarly, in the work of Benchaar (2016), the combination cinnamon oil-SIL and cinnamaldehyde-SIL (administered at a dose of 50 mg/Kg DMI of EOAC) showed no effects on *in sacco* TMR DMD24. The PC EUG showed wide differences in the DMD4 results, for all the substrates, depending on the carrier used. The highest digestibility values were obtained with SIL which probably only absorbed the EO without explicating any effect on the bacteria wall. However, an opposite result was found in maize meal DMD4 when the T80 was employed. As reported by Goto et al. (2003), T80 *per se* increases the accessibility of enzymes to the substrate and the rate and extent of enzymatic degradation. However, eugenol (mixed with rolled barley grain and top-dressed on TMR) seems to have no effect on *in vivo* organic matter and starch digestibility (Yang et al. 2010). Furthermore, depressive effects of both eugenol and carvacrol (mixed with 99.5% ethanol) were observed on TMR on both *in vitro* DMD24 and NDFD24 (Benchaar, Petit, et al. 2007). These results indicate that the combination EUG-SIL can be employed to increase soybean and TMR digestibility in short intervals, while improving starch digestibility when associated with T80. Moreover, ORE-SIL combination depressed the DMD24 of the TMR while an opposite effect was observed on the NDFD24 of the same substrate. Partially in contrast with these results, Zhou et al. (2020) reported an increase of the *in vitro* digestibility of both DM, NDF and ADF of a maize-silage based TMR with adding oregano oil (especially at 52 mg/L and 91 mg/L) supported by lactic acid, cobalt carbonate and clinoptilolite. Whereas, no effects on both DMD24 and NDFD24 were observed by Benchaar (2020) on TMR adding both carvacrol and oregano oil supported by SIL (50 mg/Kg). In the present study, CAR-SIL showed similar effects to those reported by the latter author on TMR. These results are not supported by the literature. Carvacrol has in fact reported to possess different modes of action and biological activities, improving nutrient utilisation, digestion and metabolism (Alagawany, 2015).

The combination terpenoids-SIL appeared to be more effective in improving NDFD in comparison to the combinations with T80. This results were observed especially on maize meal NDFD24, on soybean meal NDFD4 and NDFD24 and on TMR NDFD4. The cinnamaldehyde-SIL combination tended to decrease the NDFD24 compared to cinnamon oil-SIL (Benchaar 2016). In agreement with this evidence, in the present study CIN-AC-SIL had lower value of the TMR NDFD compared to the CIN-SIL at 4 h, and the differences became greater at 24 h of incubation. Similar results were observed also on maize meal NDFD, while an opposite trend was shown for the soybean meal NDFD. The CAR-SIL induced the highest value of soybean NDFD4 and NDFD24 and of maize meal NDFD24. We can speculate that CAR-SIL is the best association in order to increase the fibre digestibility of high starch and high protein concentrates. Moreover, also THY and THY-AC demonstrated a different effect on NDF digestibility of maize meal depending on the carrier employed. This latter evidence further confirms the hypothesis that the effects of the EO and EOAC can be deeply influenced by the carrier used.

## Conclusions

The type of carrier, the PC and their interaction affect the *in vitro* digestibility of feeds also as a function of the substrate degraded.

In general, PCs-SIL appear to enhance the digestibility while PCs-T80 shows a negative effect especially on NDFD. However, most of the tested combinations increased maize meal DMD4 and NDFD4, soybean meal NDFD4 and NDFD24, and TMR DMD4. Among them, EUG-SIL can be employed to improve soybean meal and TMR digestibility in the short intervals, EUG-T80 improve maize digestibility and CAR-SIL increase the fibre digestibility of both the high starch and the high protein concentrates tested.

Since the interaction between PC and the carrier was significant, it is possible to hypothesise that specific affinities between certain molecules included in PCs and the carriers employed can exist, and those affinities can change according to the substrate tested. These concepts should be taken into account when testing PCs and carriers and when developing products for commercial purposes.

Further investigations are needed to better understand the PC-carrier interactions in determining the digestibility of the different feeds/substrate and the variations of other fermentation parameters as well as the microbiome.

## Disclosure statement

No conflicts of interest have been declared.

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