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Fermentative parameters and aerobic stability of orange peel silage with pelleted citrus pulp

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ABSTRACT - This study evaluated the effects of pelleted citrus pulp (PCP) added to orange peel on fermentative parameters and aerobic stability of orange peel silages. The treatments were based on different levels of PCP: 0 (control), 10% PCP, 20% PCP, and 30% PCP calculated according to the weight of orange peel (w/w), with five experimental silos per treatment stored for 60 days. Chemical composition, fermentative parameters, microbial population, and dry matter (DM) losses were performed in silages after opening the experimental silos. Furthermore, aerobic stability was evaluated for 12 days. Silages with 10 and 20% PCP presented suitable levels of DM, 226 and 302 g kg^{-1}, respectively, and probably adequate water activity that benefited the lactic acid fermentation, but it jeopardized their aerobic stabilities. The inclusion of 10% PCP did not reduce the effluent loss compared with the control silages. Yet, 30% PCP silage showed the lowest effluent loss (93%), in contrast to the low lactic acid content (35 g kg^{-1}) and short aerobic stability (49 h). Control silages remained stable for a longer period (115 h), but showed greater loss of N as NH_3, and higher losses of DM through gas (354 g kg^{-1}) and effluents (114 g kg^{-1}). In short, we highlighted 20% PCP silage because of its high lactic acid bacteria (6.3 cfu g^{-1}), high lactic acid:acetic acid ratio (1.41), low nitrogen degradation as NH_3, and reduced gas (67%) and effluent (80%) productions. With the highest lactic acid (66.42 g kg^{-1}), these well-preserved silages showed a more intense aerobic degradation, starting after 42h. The inclusion of PCP to orange peel improves the fermentation process probably due to the decrease of water activity, but decreases the aerobic stability of the silage as well.

Keywords: byproduct, citrus pulp pellets, fermentation, short-chain fatty acid

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Introduction

During the harvest season from May to January, Brazil produces around 17.5 million tons of orange per year (IBGE, 2017) and approximately 7 million tons of orange peel. The high nutritive orange peel can be used directly as fodder (Ashbell et al., 1987), but animals cannot consume the entire amount that is produced during the harvest season; therefore, ensiling can be a promising technique to preserve high moisture material as orange peel (Ashbell and Weinberg, 2001; Ítavo et al., 2000).

The main problems described in the literature related to the ensiling of orange peel are low dry matter (DM) level and yeast fermentation during ensiling, which result in high DM losses due to gas production (Ashbell et al., 1987). There is a consensus that high moisture affects the quality of ensiled orange peel, and treatments that increase the DM level of wet orange peel are therefore necessary (Megías et al., 1993). In high-moisture conditions, clostridia, enterobacteria, and other opportunistic

microorganisms, which ferment lactic acid into butyric acid and amino acids into ammonia, can become active (Muck, 2010). This process, known as clostridial fermentation, increases the pH and DM losses in the silage (Muck, 2010).

Recent studies have suggested pre-drying orange peel before ensiling (Valença et al., 2016) or the inclusion of water absorbent additives such as pelleted citrus pulp (PCP) (Grizotto et al., 2017) to increase the initial DM content and, consequently, improve the quality of the silages. The improvements in silage quality make the aerobic deterioration become a significant problem resulting in a post-storage quality reduction due to the growth of yeast and molds that can lead to high dry matter losses (McDonald et al., 1991). Despite the importance of establishing how long a silage can be used as fodder, there is a lack of studies focused on aerobic stability of orange peel.

We hypothesized that the inclusion of PCP would increase the DM content of orange peel to a suitable level that may favor the fermentative pattern into the silos. We expect that the inclusion of PCP may alter the fermentative pattern and reduce the DM losses as effluent and gas and alter the aerobic stability of the silages positively. Therefore, the objective of this study was to evaluate the effect of different levels of PCP added to orange peel on fermentative parameters and aerobic stability of orange peel silage produced in experimental silos.

Material and Methods

About 500 kg of orange peel was collected in May 2014 at Delta Citrus, an orange juice processing plant located in the north of São Paulo State, Brazil. The byproduct was transported to an experimental farm in Colina, SP, Brazil (20°43'05" S latitude, 48°32'38" W longitude) and was used on the same day. Table 1 shows the chemical composition of PCP, orange peel, and the respective mixtures of PCP and orange peel used in the ensiling process.

Twenty experimental silos were prepared according to methodology of Siqueira et al. (2007), adapted by using a plastic bucket (volume of 0.03 m^3, 30 cm in diameter, 30 cm in height). Its bottom was covered with a 10-kg layer of prewashed and dried (55 °C/72 h) coarse sand to collect the effluent produced during the fermentation. A non-woven sheet was placed on top of the sand layer to avoid its contact with the ensiled mass. The treatments were: 0 (control), 10% PCP, 20% PCP, and 30% PCP added to orange peel (w/w), with five experimental silos per treatment. The orange peel was mixed with the corresponding proportion of PCP in a 400-L concrete mixer (CSM, Jaraguá do Sul, Brazil) for 3 min. About 15 kg of the homogeneous material (orange peel with 0 or 10% PCP, 20% PCP, or 30% PCP) was then transferred to the plastic bucket and packed manually until an average density of 830±13 kg m^{-3} was obtained. The experimental silos were sealed with an appropriate plastic lid, weighed, and stored

Table 1 - Characteristics of the pelleted citrus pulp (PCP) and fresh orange peel with four inclusion levels of PCP before ensiling (mean±SD; n = 3 for chemical composition, n = 6 for microbiological analysis)

Item	PCP	PCP inclusion level			
		0%	10%	20%	30%
pH	4.99±0.16	4.58±0.08	4.67±0.07	4.87±0.01	4.88±0.11
DM (g kg^{-1} as fed)	895.70±7.29	186.35±3.70	225.68±1.37	302.46±1.36	329.12±2.47
Ash (g kg^{-1} of DM)	94.29±0.80	39.97±1.38	54.70±1.64	60.63±2.08	61.99±0.39
CP (g kg^{-1} of DM)	75.53±1.11	83.21±0.92	75.54±0.85	73.72±0.81	72.07±1.35
NDF (g kg^{-1} of DM)	472.96±89.72	304.54±66.70	350.77±28.34	383.21±52.71	359.79±64.02
Lignin (g kg^{-1} of DM)	48.40±4.63	20.08±0.61	26.59±5.35	23.91±5.83	27.34±1.43
IVDMD (g kg^{-1} of DM)	930.04±2.29	977.63±4.98	970.80±8.05	960,00±9.99	969.28±7.04
LAB (log cfu g^{-1})	ND	4.72±0.20	5.23±0.10	4.78±0.56	4.95±0.18
Yeast (log cfu g^{-1})	ND	3.21±0.38	2.42±1.24	2.94±1.01	3.12±0.29

DM - dry matter; CP - crude protein; NDF - neutral detergent fiber; IVDMD - *in vitro* dry matter digestibility; LAB - lactic acid bacteria; cfu - colony forming units; ND - not determined.

at room temperature. Three fresh samples of wet orange peel with and without PCP were collected at the beginning of ensiling (d 0).

After 60 days of storage (d 60), the experimental silos were weighed again and opened, and their contents pooled and thoroughly mixed. The weight of the empty silos containing only the coarse sand and effluent was recorded. Three sub-samples of approximately 300 g of fresh silage were taken, and the remaining material (about 9 kg) was used in the aerobic stability test. One sub-sample was analyzed on the same day regarding pH and lactic acid bacteria (LAB) and yeast counts. The other two sub-samples were stored at −18 °C for the subsequent analysis of chemical composition and fermentation products.

Samples of fresh orange peel or silage after fermentation (60 d) were dehydrated in a forced-ventilation oven (model TE394/3, TECNAL, Piracicaba, Brazil) at 55 °C for 72 h and ground through a 1-mm screen in a mill (model MA680, Marconi, Piracicaba, Brazil). Sub-samples were analyzed for DM (method 967.03), ash (method 642.05), and crude protein (CP; method 981.10) according to the AOAC (1990). Neutral detergent fiber (NDF) and lignin were sequentially analyzed according to Robertson and Van Soest (1981) in amylase-treated samples with no added sodium sulfite. *In vitro* DM digestibility (IVDMD) was evaluated in a Daisy-like incubator (model TE-150, TECNAL, Piracicaba, Brazil) according to Goering and Van Soest (1970). All results were expressed in g kg^{-1} of DM, except for DM values, which were given in g kg^{-1} on an as-fed basis.

Dry matter losses through gas and effluent and the rate of DM recovery (DMR) were calculated based on the difference of weight between the initial experimental silos and after 60 days of storage (Siqueira et al., 2007). Effluent was given in kg t^{-1} as is, and gas and DMR were expressed in g kg^{-1} of DM.

The pH and fermentation products (lactic acid, short-chain fatty acids, ammoniacal nitrogen) were determined in an aqueous extract (1:10) prepared from the sampled material (25 g), shaken with 225 g distilled water, and filtered through four layers of sterile cotton gauze according to Silva et al. (2018). The pH was immediately measured with a DM-22 pH meter (Digimed, São Paulo, Brazil). The aqueous extract was centrifuged at 10,000 × *g* for 5 min. Lactic acid was measured by colorimetry in the supernatant in a UV-visible spectrophotometer (700 plus, FEMTO, São Paulo, Brazil) at 565 nm (Pryce, 1969) and was expressed in g kg^{-1} of DM. In the same supernatant, short-chain fatty acids (acetic, propionic, butyric, valeric, and isovaleric) were analyzed in a high-performance gas chromatograph (GC-2014, Shimadzu®, Kyoto, Japan) equipped with an auto-sampler (AOC-20i, Shimadzu®, Kyoto, Japan) and a capillary HP INNOwax column (30 m length × 0.32 mm ID × 0.5 µm). The original results expressed in mL L^{-1} were converted to g kg^{-1} of DM based on their respective molecular weights: 60.05 g mol^{-1} for acetic acid, 74.08 g mol^{-1} for propionic acid, 88.10 g mol^{-1} for butyric acid, and 102.13 g mol^{-1} for valeric and isovaleric acids. Ammoniacal nitrogen ($N\text{-}NH_3$) was measured according to Chaney and Marbach (1962) in a UV-visible spectrophotometer (700 plus, FEMTO, São Paulo, Brazil) at 620 nm, and the results were expressed in g kg^{-1} of total nitrogen.

Samples of 25 g of orange peel with or without PCP before ensiling (d 0) and after 60 days of storage were shaken with 225 mL of sterilized 0.1% peptone water (Peptone type 1, HiMedia Laboratories, Mumbai, India) for 1 min, serially diluted from 10^{-1} to 10^{6}, and spread on Petri dishes by the pour plate method (Tabacco et al., 2009). Man, Rogosa, and Sharpe (MRS) agar and potato dextrose agar (PDA) (HiMedia Laboratories, Mumbai, India) without antibiotics were used to count LAB at 35 °C and yeast at 28 °C, respectively. After three days of incubation, the number of LAB and yeast was determined as colony forming units (cfu) per gram of silage (cfu g^{-1}).

The day when the twenty experimental silos were opened (d 60) was defined as day 0 of aerobic exposure. At day 0, three new polyethylene bags (30 cm wide, 45 cm long) with approximately 3 kg of homogeneous silage were prepared from each experimental silo. In total, 60 polyethylene bags (three bags per silo × four treatments × five replicates of the fermentation phase) were prepared and kept in a controlled temperature room at 25±1 °C. These bags (five replicates per treatment) were analyzed on days 4, 8, and 12 of aerobic storage. At the pre-determined time (days 4, 8, and 12), all material was thoroughly mixed in the correspondent bags from which a homogeneous sample was taken for

pH measurements and yeasts counts, and the surplus disposed. The temperature of the silage was recorded at 30-min intervals by a datalogger (Escort MI-NI-D-2-1, Cryopac, New Jersey, USA) placed in the center of the 12th-day bags of each treatment to record the temperature along the 12 days of aerobic stability. These dataloggers remained untouched during the period of study. Three similar dataloggers were positioned at different sites in the same room to record the ambient temperature. Aerobic stability was defined as the number of hours the silage temperature remained stable before increasing more than 2 °C above room temperature (Cherney and Cherney, 2003). The cumulative daily mean differences (CDMD) between the silage temperature and room temperature were measured after four (CDMD-4), eight (CDMD-8), and 12 (CDMD-12) days of aerobic exposure according to Cherney and Cherney (2003).

The results of chemical composition, DM losses, LAB and yeast counts, fermentative parameters, and the aerobic exposure variables (aerobic stability, CDMD-4, CDMD-8, CDMD-12) were analyzed in a completely randomized design using the MIXED procedure of the SAS program (Statistical Analysis System, version 9.4). The model used was:

$$Y_i = \mu + T_i + \varepsilon_i, \quad (1)$$

in which Y_i = response variable, μ = overall mean, T_i = effect of treatment [i = 0 PCP (control), 10% PCP, 20% PCP and 30% PCP], and ε_i = error.

The pH and yeast counts over 12 days of aerobic exposure were analyzed in a completely randomized design as repeated measures using MIXED procedure of SAS, using the following model:

$$Y_{ij} = \mu + T_i + D_j + A_{j:i} + T \times D_{ij} + \varepsilon_{ij}, \quad (2)$$

in which μ = overall mean, T_i = fixed effect of treatment [i = 0 PCP (control), 10% PCP, 20% PCP, 30% PCP], D_j = fixed effect of day of aerobic exposure (pH and counts of yeasts, j = 0, 4, 8, 12), $T \times D_{ij}$ = interaction between treatment and day of aerobic exposure, $A_{j:i}$ = random effects of experimental silo nested within treatment, and ε_{ij} = error.

Different residual covariance structures were tested to determine the structure that best fitted each variable. The covariance structure was chosen using the Bayesian information criteria (BIC), in which the lowest value was used as a selection criterion. The average of all treatments were analyzed as orthogonal contrasts to determine the type of linear (L) or quadratic (Q) effect using the same statistical program. The significance of linear and quadratic contrasts (P-value) was set at P<0.05. The criteria used to choose the equations were curved behavior, coefficient of determination (r^2), and significance.

Results

The increase in PCP level exerted a quadratic effect (P<0.001) on DM and ash contents, with the observation of maximum levels in 30% PCP silages (Table 2). Crude protein and IVDMD decreased linearly (P<0.001) as the level of PCP increased (Table 2), while pH, NDF, and lignin increased linearly (P<0.001) in silages with higher amounts of PCP (Table 2). The mean CP content of the silages was 95.2 g kg^{-1} of DM (Table 2), a value higher than the average 76.1 g kg^{-1} of DM (Table 1) found in the materials with or without PCP before ensiling. Similarly, the average NDF in silages with PCP (415.9 g kg^{-1}, Table 2) was 51.3 g kg^{-1} higher than the average in the same materials before ensiling (364.6 g kg^{-1}, Table 1). In control silages, the increase in NDF was only 3 g kg^{-1} comparing before (304.5 g kg^{-1}, Table 1) and after (307 g kg^{-1}, Table 2) ensiling. The pH of the material before ensiling increased 0.3 units from 4.58 (0 PCP, Table 1) to 4.88 (30% PCP, Table 1) with addition of PCP.

Lactic acid showed a quadratic effect (P<0.001, Table 3) with higher values (66.4 g kg^{-1} DM) found in silages with 20% PCP and the lower (35.2 g kg^{-1} DM) in 30% PCP silages (Table 3). Acetic acid, 53.4 g kg^{-1} on average, did not change (P>0.050) with increasing quantity of the absorbent additive (Table 3). The lactic acid:acetic acid ratio decreased linearly (P<0.050, Table 3), and the lowest ratio (0.58) was found in silages with 30% PCP. Control silages and silages with 10 and 20% PCP showed similar lactic acid:acetic acid ratios, which varied from 1.43 to 1.03.

The growth of LAB during the storage period showed a quadratic effect (P<0.001, Table 3) with larger number of colonies (>6.0 cfu g^{-1} silage) when the raw materials contained 226 and 302 g DM kg^{-1} (Table 1), corresponding to 10 and 20% PCP, respectively.

The concentration of propionic acid decreased linearly (P<0.010, Table 3) as the PCP level increased. The average butyric (1.10 g kg^{-1}), isovaleric (0.99 g kg^{-1}), and valeric (11.92 g kg^{-1}) acids did not change with increasing PCP (P>0.050, Table 3). The sum of short-chain fatty acids (acetic + propionic + butyric + valeric + isovaleric) of silages was 70.34 g kg^{-1} (control), 70.76 g kg^{-1} (10% PCP silages), 62.76 g kg^{-1} (20% PCP silages), and 77.19 g kg^{-1} (30% PCP silages). The short-chain fatty acid acid:lactic acid ratios were higher in control (1.19), 10% PCP (1.31), and 30% PCP (2.19) silages, but lower in 20% PCP silages (0.95).

Table 2 - Chemical composition of orange peel silage with four inclusion levels of pelleted citrus pulp (PCP) after 60 days of storage

Item	PCP inclusion level				SEM	P-value	
	0%	10%	20%	30%		L	Q
pH[1]	3.48	3.59	3.65	3.77	0.02	<0.001	0.993
DM (g kg^{-1} as fed)[2]	144.67	198.54	232.69	298.86	0.28	<0.001	<0.001
Ash (g kg^{-1} of DM)[3]	51.88	73.55	74.64	77.71	0.85	<0.001	<0.001
CP (g kg^{-1} of DM)[4]	103.80	97.84	92.64	86.50	0.91	<0.001	0.920
NDF (g kg^{-1} of DM)[5]	307.27	393.41	397.15	457.28	15.07	<0.001	0.392
Lignin (g kg^{-1} of DM)[6]	21.01	31.59	33.94	39.83	2.00	<0.001	0.251
IVDMD (g kg^{-1} of DM)[7]	955.64	945.17	947.90	939.73	2.13	<0.001	0.594

DM - dry matter; CP - crude protein; NDF - neutral detergent fiber; IVDMD - *in vitro* dry matter digestibility; SEM - standard error of the mean; P - probability; L and Q - effects of linear and quadratic order concerning the inclusion of PCP in orange peel silages significant at P<0.05.
Regression equations: [1] $\hat{Y} = 3.48 + 0.01X$ ($r^2 = 0.98$); [2] $\hat{Y} = 148.06 + 4.02X + 0.03X^2$ ($r^2 = 0.99$); [3] $\hat{Y} = 53.01 + 2.18X - 0.05X^2$ ($r^2 = 0.94$); [4] $\hat{Y} = 103.76 - 0.57X$ ($r^2 = 0.99$); [5] $\hat{Y} = 320.71 + 4.54X$ ($r^2 = 0.90$); [6] $\hat{Y} = 22.77 + 0.59X$ ($r^2 = 0.93$); [7] $\hat{Y} = 953.86 - 0.45X$ ($r^2 = 0.77$); in which X is the level of PCP and r^2 is the coefficient of determination.

Table 3 - Fermentation products, microbial counts, and dry matter (DM) loses of orange peel silage with four inclusion levels of pelleted citrus pulp (PCP)

Item	PCP inclusion level				SEM	P-value	
	0%	10%	20%	30%		L	Q
Lactic acid (g kg^{-1} of DM)[1]	59.12	53.89	66.42	35.20	4.11	0.008	0.008
Acetic acid (g kg^{-1} of DM)	49.12	53.31	50.04	61.25	8.34	0.345	0.698
Propionic acid (g kg^{-1} of DM)[2]	3.32	3.28	2.59	2.10	0.32	0.005	0.496
Butyric acid (g kg^{-1} of DM)	1.09	1.52	1.08	0.718	0.19	0.090	0.061
Valeric acid (g kg^{-1} of DM)	15.72	11.60	8.11	12.25	3.10	0.283	0.206
Isovaleric acid (g kg^{-1} of DM)	1.09	1.05	0.94	0.87	0.34	0.581	0.963
Lactic:acetic acid ratio[3]	1.43	1.03	1.41	0.58	0.18	0.018	0.268
Ammonia nitrogen (g kg^{-1} of total N)[4]	10.87	9.01	7.47	7.48	1.20	0.035	0.444
LAB (log cfu g^{-1})[5]	5.39	6.11	6.30	5.89	0.08	<0.001	<0.001
Yeast (log cfu g^{-1})[6]	2.56	2.00	3.24	3.56	0.28	0.003	0.137
Effluent (kg t^{-1})[7]	114.27	101.88	22.31	7.48	1.85	<0.001	0.519
Gas (g kg^{-1} of DM)[8]	353.72	194.57	115.28	115.69	17.53	<0.001	<0.001
DMR (g kg^{-1} of DM)[9]	642.31	747.60	735.89	890.51	6.70	<0.001	<0.001

cfu - colony forming units; LAB - lactic acid bacteria; DMR - dry matter recovery; SEM - standard error of the mean; P - probability; L and Q - effects of linear and quadratic order concerning the inclusion of PCP in orange peel silages significant at P<0.05.
Regression equations: [1] $\hat{Y} = 56.04 + 1.36X - 0.06X^2$ ($r^2 = 0.64$); [2] $\hat{Y} = 3.47 - 0.04X$ ($r^2 = 0.92$); [3] $\hat{Y} = 1.44 - 0.02X$ ($r^2 = 0.49$); [4] $\hat{Y} = 10.46 - 0.12X$ ($r^2 = 0.88$); [5] $\hat{Y} = 5.38 + 0.10X - 0.003X^2$ ($r^2 = 0.99$); [6] $\hat{Y} = 2.42 - 0.02X - 0.002X^2$ ($r^2 = 0.75$); [7] $\hat{Y} = 121.48 - 4.00X$ ($r^2 = 0.90$); [8] $\hat{Y} = 353.71 - 19.90X + 0.40X^2$ ($r^2 = 1.00$); [9] $\hat{Y} = 656.48 + 3.63X + 0.12X^2$ ($r^2 = 0.87$); in which X is the level of PCP and r^2 is the coeficiente of determination.

The highest level of NH_3 was 10.87 g kg^{-1} of total N found in control silages and decreased linearly (P<0.050, Table 3) with increasing PCP.

The effluent production decreased linearly (P<0.001, Table 3) with increase of DM. The lowest effluent productions were observed in silages with 20% PCP (22.3 kg t^{-1}) and 30% PCP (7.48 kg t^{-1}), which correspond to 80 and 93% of reduction compared with control silages (114 kg t^{-1}) (Table 3). Silages with low level of PCP (10%) showed losses through effluents about 100 kg t^{-1} equivalent to the control silage (Table 3).

There was a quadratic effect on DM losses through gas (P<0.001, Table 3) with increasing PCP level, which was higher in the control silage (354 g of gas per kg of DM) and lower in silages with 20 and 30% PCP (approximately 115 g of gas per kg of DM). The reduction on gas production from 354 to 115 g kg^{-1} represents 67% of decrease for both treatments (20 and 30% PCP). In 10% PCP silages, the decrease in gas production was 45%, from 354 to 194 g kg^{-1} DM. A quadratic effect was observed for DMR (P<0.001, Table 3), with the highest value (890 g kg^{-1} of DM) in silage with 30% PCP and the lowest (642 g kg^{-1} of DM) in the control silage.

A quadratic effect (P<0.001) was observed in most variables related to aerobic exposure with the exception of aerobic stability (EA), which showed a linear decrease (P<0.001, Table 4). Silages with 20% PCP were more susceptible to aerobic degradation, as demonstrated by the lowest AS (42 h) and the highest cumulative daily temperature (2.84 °C) along the 12 days of air exposure (Table 4), heating the mass to approximately 33 °C. On the other hand, the control silage remained stable for a longer period (115 h) with negative temperature accumulation (−0.34 °C) over the 12 days of air exposure (Table 4).

We observed interaction (P<0.001) between treatment and days of aerobic exposure for silage pH (Figure 1a) and yeast population (Figure 1b). There was a reduction in pH values up to the eighth day of aerobic exposure in all silages, followed by an increase in pH until the end of the analyzed period (12 days) (Figure 1a). Lower pH values were observed in the control silages throughout aerobic exposure, while silages with 30% PCP showed the highest values (Figure 1a). The yeast count decreased over the first four days, with the lowest counts being observed in silages with extreme DM levels, i.e., in the control silages and silages with 30% PCP (Figure 1b). From the fourth day on, yeast count increased in the silages with 10, 20, and 30% PCP, reaching values of about 6.0 cfu g^{-1}. The control silages had lower yeast count (4.1 cfu g^{-1}) at the end of the aerobic exposure period (12 days).

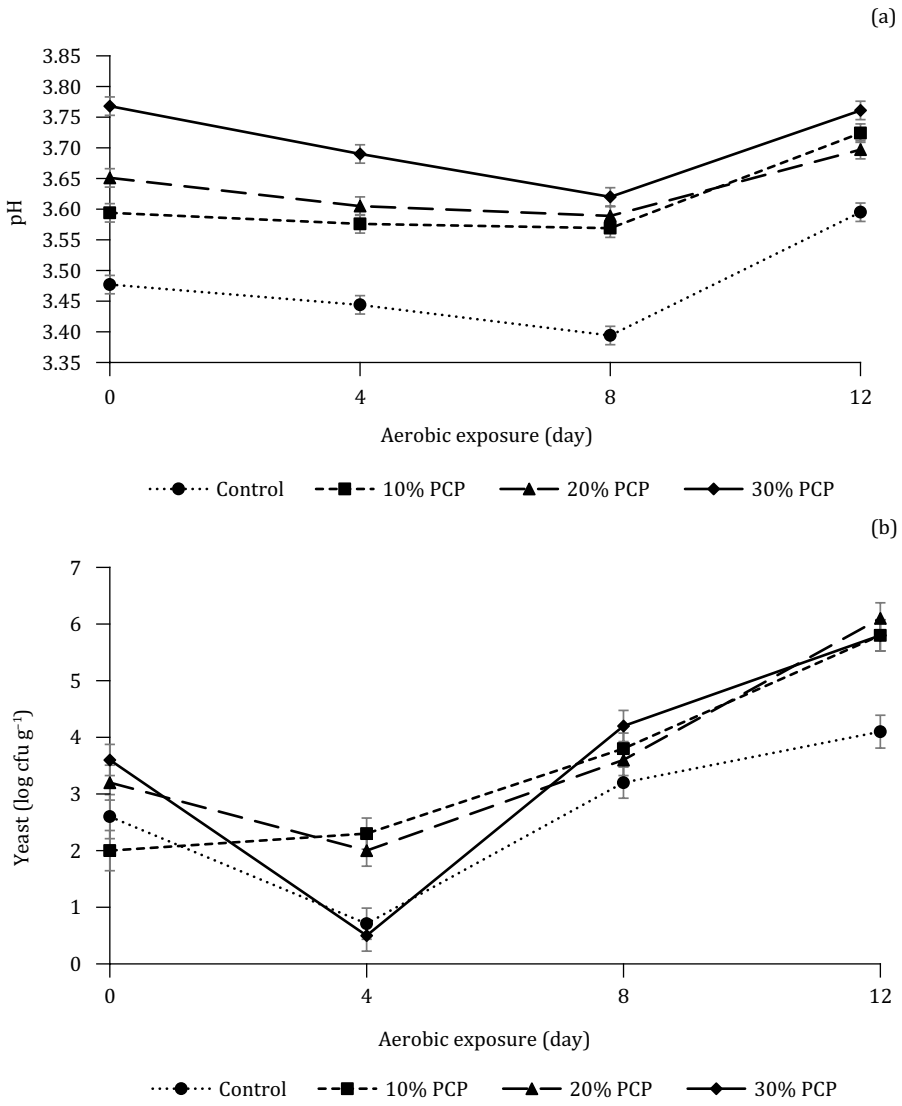
Discussion

The increase in DM content due to the inclusion of PCP probably decreased the water activity of the ensiled mass that altered the fermentation process into the silos and confirmed the hypothesis of this study. The inclusion of 10 and 20% PCP increased the DM to a range between 226 and 302 g kg^{-1}, respectively, which seems to be optimal for LAB growth, since the population declined at values

Table 4 - Aerobic exposure of orange peel silage with four inclusion levels of pelleted citrus pulp (PCP) after 60 days of storage

Item	PCP inclusion level				SEM	P-value	
	0%	10%	20%	30%		L	Q
Aerobic stability (h)[1]	115.40	62.00	42.00	48.90	14.13	<0.010	0.052
CDMD-4 (°C)[2]	−1.02	0.51	2.05	1.37	0.32	<0.001	<0.001
CDMD-8 (°C)[3]	−0.72	1.99	2.53	2.32	0.21	<0.001	<0.001
CDMD-12 (°C)[4]	−0.34	2.54	2.84	2.11	0.21	<0.001	<0.001

CDMD-4 - cumulative daily medium difference between silage temperature and environmental temperature after four days of aerobic exposure; CDMD-8 - CDMD after eight days of aerobic exposure; CDMD-12 - CDMD after 12 days of aerobic exposure; SEM - standard error of the mean; P - probability; L and Q - effects of linear and quadratic order concerning the inclusion of PCP in orange peel silages significant at P<0.05. Regression equations: [1] $\hat{Y} = 100.00 - 2.19X$ ($r^2 = 0.72$); [2] $\hat{Y} = -1.16 + 0.26X - 0.006X^2$ ($r^2 = 0.95$); [3] $\hat{Y} = -0.65 + 0.31X - 0.007X^2$ ($r^2 = 0.98$); [4] $\hat{Y} = -0.26 + 0.35X - 0.009X^2$ ($r^2 = 0.98$); in which X is the level of PCP and r^2 is the coefficient of determination.



SEM - standard error of the mean.
(a) SEM = 0.01; (b) SEM = 0.15. (a, b) effect of PCP inclusion, P<0.001; effect of day of aerobic exposure period, P<0.001; interaction effect between PCP level and day of aerobic exposure period, P<0.001.
Error bars indicate SEM in each aerobic exposure day.

Figure 1 - Silage pH (a) and yeast counts (b) over 12 days of orange peel silage with four inclusion levels of pelleted citrus pulp (PCP).

above or below this range. Probably, LAB found better growth conditions in an environment with adequate DM (302 g kg^{-1}) and slightly higher pH due to the alkalinity of PCP related to the calcium hydroxide (CaOH) in its formulation (Carvalho, 1995). The higher concentration of lactic acid found in 20% PCP silages confirms the best adaptation of LAB bacteria in the medium. Calcium hydroxide is a coadjutant in the pelleting process that is used to facilitate the release of water from the material (Agry, 2015) and contributed to increase ash, as noticed by Yasuoka et al. (2015) in Xaraés grass silage with 30% PCP.

The increase in NDF and lignin in the orange peel before ensiling may be associated to the inclusion of PCP, which is rich in both components. However, the most interesting results occurred during the fermentation of orange peel silages with PCP (high DM and possible low water activity). During the anaerobic fermentation period of orange peel, facultative and mandatory anaerobic microorganisms such as enterobacteria, clostridia, certain bacilli, and yeast compete with the LAB flora for nutrients (Pahlow et al., 2003). The growth of these microorganisms that convert soluble carbohydrates into

fermentation-derived acids (Megías et al., 1993) might be related to the increase of 51.3 g kg^{-1} of NDF in the silages with PCP. Probably, bacteria degrading soluble carbohydrates might have found better conditions for their development in the anaerobic fermentation of orange peel with PCP. On the other hand, in the absence of the water-absorbent additive, the high moisture environment (and probably high water activity) did not benefit the growth of the same bacteria, resulting in a very small increase in NDF, only 3 g kg^{-1}.

The changes in fiber fractions probably decreased the digestibility of silages with PCP, as the additive originally contained higher amounts of lignin and NDF compared with the levels found in wet orange peel. Yet, the CP levels of all silages were higher than the 70 to 75 g kg^{-1} usually found in wet orange peel silages (Rego et al., 2012; Valença et al., 2016). Probably, the increase in protein concentration may be due to soluble carbohydrate consumption during the fermentation process that led to an indirect effect of protein concentration (Bernardes et al., 2005). Similar increases in CP in silages were often found in orange peel silages (Pinto et al., 2007; Valença et al., 2016; Grizotto et al., 2017).

However, the high amount of protein in control silages does not mean an advantage. The point to presume is the highest loss of nitrogen as ammonia in these silages indicating undesired fermentation. Some opportunistic microorganisms such as *Clostridium* sp or other bacteria such as enterobacteria can transform amino acids into ammonia (Muck, 2010) and amines (Rook and Hatfield, 2003). Furthermore, many amines have bioactive properties and may reduce feed intake and, in high concentration, can be toxic (Rook and Hatfield, 2003). Low nitrogen losses in the form of NH_3 observed in silages with high DM levels (20 and 30% PCP) is probably associated with control of fermentation by opportunistic microorganisms.

The DM increase did not alter the growth of acetic acid microorganisms, as its level was similar in both control silages and those with increasing levels of PCP. The average acetic acid concentration (53.4 g kg^{-1}) of our silages was higher than 24 g kg^{-1} (Weinberg et al., 1989), but lower than 120 g kg^{-1} (Megías Rivas et al., 1992; Rego et al., 2012) found in orange peel silages. These silages were packed with lower DM levels (*ca.* 200 g kg^{-1}) than our silages with PCP. Our findings, supported by Megías et al. (1993), Rego et al. (2012), and Weinberg et al. (1989), suggest that the growth of acetic acid microorganisms is not limited by different DM contents.

Silages with the highest DM (299 g kg^{-1}) probably had low water activity (aw) which may be related to the decreasing of LAB population and, as a result, low lactic acid content. The low level of lactic acid resulted in the lowest lactic acid:acetic acid ratio (0.58) in the 30% PCP silages. Lactic acid bacteria, as well as most bacteria, are more sensitive to dry conditions, generally growing at water activity levels below 0.9 (McDonald et al., 1991).

The increase of yeasts in 20 and 30% PCP silages may be related to their high substrate content, possible low water activity, and the low propionic acid concentration that altogether generated conditions for yeast growth. Most yeasts are less sensitive to dry conditions; for example, some osmophilic yeasts can grow in very low aw as 0.6 (McDonald et al., 1991). Silages with higher PCP inclusion (20 and 30%) exhibited the lowest concentration of propionic acid. These results are an indicator of growth control of *Propionibacterium* or other bacteria such as *Clostridium propionicum* and *Selenomonas ruminantium* (Muck, 2010). According to Muck (2010), propionic acid is the end-product of the fermentation of these bacteria and well-known inhibitors of yeast at low pH, but more effective than acetate and lactate (Moon, 1983). Unfortunately, we did not find any explanation in the literature for the high quantities of valeric acid in our study.

In general, there is no evidence of clostridial fermentation in orange peel silages with or without PCP based on low contents of butyric and isovaleric acids (Muck, 2010). Butyric acid was lower than the 5 g kg^{-1} expected in silages with clostridial activity (Muck, 2010). Isovaleric acid, which is a result of oxidation/reduction of leucine by proteolytic clostridium (McDonald et al., 1991), can be another indicator of the absence of clostridial fermentation in orange peel silages. Simultaneously to the low levels of butyric and isovaleric acids, other indicators of lack of contribution of clostridium in orange peel silages include low loss of N in the form of NH_3 (below 10.87 g kg^{-1}), pH (3.48 to 3.77), and high

lactic acid concentration. These results agree with Ashbell et al. (1987), who did not detect clostridia in wet orange peel silage.

It is possible to infer that orange peel silages, except those with 20% PCP, had predominance of heterofermentative LAB and other opportunistic microorganisms such as clostridia (Megías Rivas et al., 1992) due to a higher quantity of short-chain fatty acids compared with lactic acid. Within 20% PCP silages, the analysis of short-chain fatty acids indicated a main homolactic conversion of the sugars to rather high amounts of lactic acid (Weinberg et al., 1989). According to Muck (2010), heterofermenters produce less lactic acid (1 mol) compared with homofermenters, which produce more lactic acid (2 mol) from the same amount of glucose (1 mol).

The high water-absorption capacity of PCP contributed to a significant reduction in effluent production, 80 to 93%, provided that the DM level before ensiling was adjusted to a minimum of 302 g kg^{-1} of DM, which corresponds to the inclusion of 20% PCP. Below this DM concentration, the losses through effluents are equivalent to the control silage. In short, the DM increase to 226 g kg^{-1} in orange peel with 10% PCP had no benefits on reducing effluent production.

Reductions in effluents are environmentally friendly because the slurry produced in wet orange peel silage has high biological oxygen demand, approximately 78,000 mg O_2 per liter (Ashbell et al., 1987). This fact can have a significant environmental impact, increasing the risk of contamination of groundwater and natural springs (Muck, 2010). Besides, the release of greenhouse gases during the breakdown of organic material, the proliferation of crop pests, and the development of microorganisms can cause diseases (Muck, 2010). In view of effluent reduction, silages with 30% PCP showed the lowest loss of effluent, but the high DM content of the silages and, consequently, low water activity could have changed the environment, making it unsuitable for lactic acid fermentation. Thus, we highlighted the inclusion of 20% PCP because it was the treatment that resulted in low effluent production and high nutritive value silages without damaging the LAB growth that are relevant for both animal nutrition and aspects of environmental conservation.

The intensity of gas release was very high in control silages. Such high losses could be risen from the extensive production of carbon dioxide, hydrogen, and ethanol from the fermentation of lactate or hexoses (McDonald et al., 1991). Additionally, the highest loss of nitrogen in the form of NH_3 found in control silages compared with those with PCP could be an indicator of proteolytic clostridial activity or other enterobacteria capable of producing ammonia during ensilage of orange peel (Muck, 2010). From the inclusion of 20% PCP onward, there was 67% decrease on gas production compared with control silages, which could be related to the suitable DM and decrease of water activity (Bernardes et al., 2005) of these silages that may have controlled the growth of clostridium with less ammonia and butyric acid and, consequently, less gas production. The high amounts of gas release and effluent lost in control silages may have decreased their quality, since these components are rich in high digestibility compounds as well as soluble carbohydrates, organic acid, minerals, and nitrogen compounds (Ashbell et al., 1987).

The significant reduction in DM losses through gas and effluent that occurred in silages with higher levels of PCP has a direct impact on the increase of DMR and agrees with Valença et al. (2016).

In view of the findings above, silages with 20% PCP can be considered well-preserved due to the highest amount of lactic acid within the lactic acid range of 60 to 80 g kg^{-1} established for well-preserved alfalfa silages (Borreani et al., 2018). Silages with 20% PCP also showed a high lactic acid:acetic acid ratio (1.41), low nitrogen degradation as NH_3, and low DM losses through gas and effluent. We infer that the increase in DM content up to 302 g kg^{-1} by the inclusion of 20% PCP favored lactic acid fermentation, resulting in silages of better quality despite the lack of control over propionic acid microorganisms that control yeast. The increase in DM up to 302 g kg^{-1} also promoted significant reductions in gas and effluent production (67 and 80%, respectively), and a good DMR rate (736 g kg^{-1}). However, when exposed to air, the well-preserved 20% PCP silages had a fast increase in temperature resulted from an intense aerobic degradation (Davies et al., 2007; Kung Jr., 2010). Although the 20% PCP silages showed high concentrations of lactic acid, this acid has poor antifungal attributes (Muck, 2010). Acetic

and propionic acids are good antifungal acids. However, they were not enough to control yeasts during aerobic exposure. The presence of nutrients available for the growth of yeast, spores, or conidia of certain molds, which are the most common microorganisms responsible for the onset of aerobic instability as well as the growth of acetic acid bacteria (McDonald et al., 1991; Cherney and Cherney et al., 2003), resulted in fast deterioration when exposed to air. The control silage exhibited less intense aerobic deterioration, which can be attributed to butyric acid and ammonia N that are positively correlated with aerobic stability (McDonald et al., 1991). Additionally, propionic acid may have contributed to the control of yeast during aerobic exposure due to its high antifungal activity (Tabacco et al., 2009) and consequently prevented the heating of these silages.

Unexpectedly, we observed decrease in the number of yeasts and the reduction in pH in all orange peel silages (with or without PCP), during the first four days of aerobic exposure. From the fourth day on, the growth of yeast in the silages with 10, 20, and 30% PCP assumed the general pattern of aerobic deterioration with increase of yeast (Borreani et al., 2018; Davies et al., 2007) consuming sugars and fermentation acids and raising silage temperature and pH as described in literature (Balieiro Neto et al., 2009; Kung Jr, 2010; Siqueira et al., 2007; Tabacco et al., 2009). We suppose that, in the beginning of aerobic exposure, the decrease in the number of yeast and acidification of the medium may be related to the newly established anaerobic environment resulting from the physical characteristics of orange peel, including its high density and easy compaction. This temporary anaerobic condition supposedly allowed the growth of LAB, such as *L. plantarum*, which competed for nutrients and produced enough acids to inhibit yeast growth. After the initial period, regrowth of yeast occurred because of the temporary acidification of the medium. More studies are crucial to confirm this hypothesis.

In summary, the inclusion of PCP in orange peel showed improvement in the fermentation and decrease of DM losses as gas and effluents. Inclusion of 10% PCP is not interesting due to lack of control over microorganisms that resulted in high losses of gas and effluents. Additionally, its aerobic stability decreased 53 h compared with control. The higher inclusion of PCP (30%) practically eliminated effluent. Although the silages with 30% PCP showed good fermentative pattern, they still have some limitation such as low lactic acid production (probably due to the low aw) and short aerobic stability (only 49 h), which should be overcome as in silages with 20% PCP. Therefore, the inclusion of surplus of PCP above the 20% PCP may not be justified in view of its limited results. After having weighed up all pros and cons, we suggest the inclusion of 20% PCP to orange peel because of its fermentative patterns, low losses of DM (gas and effluent), although it still has problems such as intense aerobic degradation starting after 42 h.

Conclusions

The addition of pelleted citrus pulp to orange peel benefits the fermentative process probably due to the low water activity, which controls the microbial growth, and decreases dry matter losses as gas and effluent. The inclusions of 20 and 30% pelleted citrus pulp significantly reduces both production of gas (67%) and effluent (80% and 93%, respectively). However, the increase in dry matter decreases the aerobic stability. Silages with pelleted citrus pulp remain stable for less time (two to three days), while the control silages remain stable for a longer period (five days) after silo opening. However, ensiling orange peel with pelleted citrus pulp is promising. We suggest the inclusion of 20% pelleted citrus pulp because it favors the lactic acid fermentation and reduces dry matter losses.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization: G.R. Siqueira, A.F. Campos and F.D. Resende. Data curation: R.K. Grizotto. Formal analysis: R.K. Grizotto, G.R. Siqueira and A.F. Campos. Funding acquisition: R.K. Grizotto and F.D.

Resende. Investigation: R.K. Grizotto, A.F. Campos and R.T. Modesto. Methodology: R.K. Grizotto, A.F. Campos and R.T. Modesto. Project administration: A.F. Campos. Resources: R.K. Grizotto and G.R. Siqueira. Supervision: G.R. Siqueira, A.F. Campos and F.D. Resende. Writing-original draft: R.K. Grizotto and G.R. Siqueira. Writing-review & editing: R.K. Grizotto, G.R. Siqueira and F.D. Resende.

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