Proposal and validation of new indexes to evaluate maize silage fermentative quality in lab-scale ensiling conditions through the use of a receiver operating characteristic analysis

Proposal and validation of new indexes to evaluate maize silage fermentative quality in lab-scale ensiling conditions through the use of ROC analysis

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Abstract

In the context of dairy cow feeding, it is increasingly important to know the quality of the maize silage used in the ration and therefore, it appears to be crucial optimizing the techniques necessary to assess it. The aim of this study was to evaluate whether the Flieg-Zimmer's score (FZS), could properly estimate the quality of fermentations of maize silage made in a lab-scale ensiling system, and to calculate and validate new quality indexes suitable for lab-scale fermentations. The experimental dataset was obtained by analysing through near-infrared spectroscopy 522 samples of whole maize crop ensiled immediately after the harvest, using the vacuum-packing technique. The five (I1 – I5) new indexes were calculated on the basis of seven parameters chosen among pH, lactic, acetic, propionic and butyric acids, ethanol, mannitol and ammonia. All the indexes were tested for normality with the Shapiro–Wilk test. In order to define the accuracy with which the new indexes ranked the maize silage on the basis of its fermentation quality, a ROC analysis was performed, using the FZS as gold standard test and dichotomizing the FZS in two levels according to a cut-off (FZS <

80, non-excellent vs. FZS ≥ 80, excellent). Accuracy was determined through the value of the area under the curve (AUC). Finally, a one-way ANOVA model was used to compare the quality of maize silage with low (< 320 g/kg), medium (320 - 360 g/kg) and high (> 360 g/kg) dry matter (DM). In the lab-scale silages the new indexes were normally distributed, whereas the FZS was not. The new indexes showed values of AUC ranging between 0.78 and 0.89, with the I5 index showing the best combination of sensitivity (0.87) and specificity (0.77) in discriminating between good and poor quality silage. The cut-off of the new indexes ranged between 45.5 and 57.4 points. The lab-scale silages were all excellent, no matter the category of DM. However, while the FZS did not differ among the 3 categories (mean FZS = 98.7), all the other indexes were significantly higher in silages with low DM ($P < 0.001$). Silages with low DM had the highest concentrations of lactic acid (56.4 g/kg DM, $P < 0.001$), ammonia (61.4 g/kg DM, $P < 0.001$) and butyric acid (0.62 g/kg DM, $P < 0.001$) as well. Data confirmed that the new proposed indexes are promising in describing the fermentation quality of maize silage in both field and lab-scale conditions.

Keyword: maize silage, fermentative quality index, Flieg-Zimmer's score, roc analysis, lab-scale silages.

Abbreviations: ADF - ADF expressed inclusive of residual ash; aNDF - NDF assayed with a heat stable amylase and expressed inclusive of residual ash; AUC – area under the curve; CP - crude protein; DM - dry matter; EE – ether extract; FZS – Flieg-Zimmer's score; I1 – I5- new quality indexes to evaluate maize silage; R^2 - coefficient of determination; SD - standard deviation; SEC- standard error of calibration; SECV- standard error of cross-validation; SEM – standard error of means; 1-VR - Coefficient of determination in cross-validation.

Introduction

Maize (*Zea mays*, L.) silage is one of the most widely used feed in cattle rations in great part of the world (Erdman et al., 2011; Marchesini et al., 2017), especially in temperate areas, since it is a very productive crop, characterized by an excellent nutritional profile and it is suitable to be preserved through ensiling (Khan et al., 2015). Although the nutritional composition of silage, expressed in terms of content in dry matter (DM), crude protein (CP), starch, fibre and nutrients digestibility is of primary importance to optimize animal performance (Kuehn et al., 1999; Addah et al., 2011; Krämer-Schmid et al., 2016), several authors stated that the quality of the fermentation during the ensiling process and its aerobic stability are important as well (Woolford, 1984; McDonald et al., 1991; Oude Elferink et al., 2000). In fact, a silage that has undergone an abnormal fermentation has a lower nutritional value, and is often rejected by animals, leading to reduced dry matter intake and lower performance (Ward, 2011). The quality of fermentations occurring during the ensiling process can be determined through the measure of pH and the analysis of the concentration of a wide range of fermentation products such as: lactate, acetate, propionate, butyrate, isobutyrate, ethanol, mannitol and ammonia (Cherney et al., 2004; Nishino et al., 2004; Johnson et al., 2005). The pH, which in a maize silage should range from 3.7 to 4.2 (Kung and Shaver, 2001), is the result of the concentration of acids, urea and ammonia produced by microorganisms and the buffer capacity of the substrate. Among organic acids, lactate should be the most present acid, as it contributes most to the decline in pH and is associated with a lower DM and energy loss during storage (Kung and Shaver, 2001), whereas the high concentration of acetate (> 30 g/kg DM) or the presence of propionate, butyrate and isobutyrate are associated with a higher loss of DM (Ward., 2011). In addition, acetic acid, typically produced during heterolactic fermentations, although having antimycotic properties seems to interfere with cattle dry matter intake (Mc Donald et al., 1991; Nishino et al., 2004) over a certain concentration (> 60 g/kg DM). Furthermore, butyrate suggests the presence of clostridia (Kung and Shaver, 2001) which are undesirable microorganisms, as they degrade proteins and produce ammonia,

amines and other substances that compromise the palatability of silage (Ward, 2011). Other compounds, such as ethanol and mannitol, are mainly produced in secondary fermentations by yeasts or heterofermentative bacteria (Pahlow et al., 2003; Nishino et al., 2004). Each of these parameters give information only on a certain aspect of fermentation, indicating for example in the case of butyrate, the presence or absence of fermentations by clostridia, but in order to be able to say whether a fermentation was qualitatively better than another, we need an index that takes into account and weighs each of these parameters. In this regard, there are scores, such as the Flieg-Zimmer′s score (FZS) described by Woolford (1984), that are calculated on the basis of the concentration of lactic, acetic and butyric acids, whereas others take into account the concentration of lactate, volatile fatty acids and ammonia, such as the Vanbelle′s score (Vanbelle and Bertin, 1985). These scores are still used in assessing the quality of silage in field trials, but they sometime show poor capability in discriminating between well- or poorly-preserved silages (Gallo et al., 2016a), especially in conditions of limited variability, as those found in laboratory-scale ensiling systems, where the ensiling conditions are strictly controlled (Cherney et al., 2004; Hoedtke and Zeyner, 2011). These lab-scale ensiling systems are often used, because they are suitable for experimental designs which involve numerous variables, allow a more complete control of the ensiling conditions (Johnson et al., 2005; Romero et al., 2017) and are less costly and labour intensive than farm-scale silos, while still achieving a fermentation reasonably similar to that taking place in field-scale silos (Cherney and Cherney, 2003).

The aims of this study were to evaluate the ability of FZS to estimate the quality of fermentations of maize silage made in a lab-scale ensiling system and to calculate and validate new indexes of maize silage fermentation quality, suitable for lab-scale fermentations.

Materials and Methods

Sample collection, preparation and analysis

96 Samples of whole maize crop (n = 522) belonging to early (variety E, FAO class 200, n = 14) and
97 late (variety L, FAO class 600-700, n = 15) ripening cultivars were harvested and ensiled during the
98 summer season 2016. In order to have samples representative of the wide variability in maize silage,
99 plants belonging to 29 cultivars were grown in areas with different pedoclimatic conditions (from
100 ideal to very stressful) and cropped at different ripening phases (from 5 days before, to 5 days after
101 the 33% milk line stage). Two samples (500 ± 50 g) for each freshly harvested whole maize crop
102 (n=1044) were chopped immediately after the harvest and ensiled in vacuum-packed bags (Orved
103 2633040, Orved SpA, Musile di Piave, VE, Italy). Samples were treated according to the procedures
104 recommended by Johnson and colleagues (2005), especially to avoid bloating (Hoedtke and Zeyner,
105 2011) and they were stored at 23°C for 60 days, before being opened for analysis. Bags (300×400
106 mm) were 90 µm thick, were made of polyamide and polyethylene (PA/PE) and had a gas
107 permeability at 23°C ± 2 of 65, 15 and 200 $cm^3\ m^{-2}\ day^{-1}\ atm^{-1}$ to oxygen, nitrogen and CO_2,
108 respectively. Vacuum-packing was performed using a vacuum-packing machine (Cuisson 41, Orved
109 SpA, Musile di Piave, VE, Italy) drawing 25 m^3 of air per hour for 12 s. Bags were then automatically
110 sealed after air extraction. After 60 days of ensiling, the content of each bag was analysed in duplicate
111 using a FOSS NIRSysistem 5000 scanning monocromator (FOSS NIRSystem, Silver Spring, MD,
112 USA) and a very robust calibration curve, whose specifications are reported in Table 1, based on the
113 historical dataset of MAPS department's laboratory. The calibration curve was created on the basis
114 of the results of chemical analyses obtained using the following procedures: #934.01(AOAC, 2003)
115 for dry matter (DM), 2001.11 (AOAC, 2005) for crude protein (CP), #942.05 (AOAC, 2003) for ash;
116 #996.11 (AOAC, 2000) for starch, 2003.05 (AOAC, 2006) for ether extract (EE); ANKOM
117 Technology (2015a) for aNDF and ANKOM Technology (2015b) for ADF. Lactate, volatile fatty
118 acids (VFA), ethanol and mannitol were extracted in acid solution (sulphuric acid 0.6N) and analysed
119 according to the method by Martillotti and Puppo (1985), blending the samples for 4 minutes. The
120 mixture was then centrifuged at 4000 × g for 10 minutes and filtered through a 0.45 µm filter. An
121 aliquot of 20 µl of the remaining solution was analysed using a high-performance liquid

chromatography system (Shimadzu 10AVP HPLC System, Shimadzu, Tokyo, Japan) equipped with a SIL 10 auto-sampler and a RID 10A detector. A Aminex HPX-87H HPLC column, 300 mm× 7.8 mm (Bio-Rad, Hercules, CA, USA) was used at 40°C, with H_2SO_4 0.0025 N as mobile phase, at 0.6 mL min^{-1}. Ammonia was measured using the Megazyme's ammonia assay kit (Megazyme International, Bray, Wicklow, Ireland), according to the Megazyme's assay procedure (Megazyme, 2014).

Definition and calculation of maize silage fermentation quality indexes

In this study three datasets were used: the historical dataset of MAPS department's laboratory (A, n = 2098), on which we calculated the statistics and the variability of the parameters related to composition and fermentation quality of maize silage, a sub-sample of the historical dataset (B, n = 191), made by the samples on which it was possible to calculate the FZS, and the experimental dataset (C, n = 522) on which the FZS and five new quality indexes were calculated and compared (Table 2).

The FZS was calculated according to the method described by Woolford (1984). In order to verify whether the FZS was suitable to assess the quality of the fermentations of maize silage both produced in field (Dataset B) and laboratory conditions (dataset C), the trend of data distribution was verified and statistics (average, standard deviation and coefficient of variation) were calculated. Furthermore, five new indices (I1, I2, I3, I4 and I5), with a range of values from 1 to 100, were proposed for the evaluation of maize silage fermentation quality and were compared to the FZS. These indexes were set taking into consideration various parameters of fermentation quality and giving them a different weight within the index, as reported in Table 3. It was also necessary to establish a range of values for each parameter, to which attribute a minimum and a maximum score. To define this range it was decided to use the dataset A, calculating the range as the mean ± standard deviation. The reliability of the new indexes were tested against a gold standard (FZS), using a dedicated statistical approach. Finally, to verify whether the new indexes can find differences in the quality of fermentation, the samples of the dataset C were divided into 3 categories based on the DM value (DM <320 g/kg; M,

147 320 ≤ DM < 360 g/kg; H, ≥ 360 g/kg) and compared their values of FZS and of the new quality
148 indexes.

149 *Statistical analysis*

150 Statistical analyses were conducted using SAS release 9.4 (SAS Institute Inc., Cary, NC, 2012). All
151 data belonging to the three datasets (A, B and C) and pertaining to fermentation quality (i.e. pH,
152 lactic, acetic, propionic, butyric acids, ethanol, mannitol and ammonia), the FZS and the new quality
153 indexes (I1 - I5) were tested for normality with the Shapiro–Wilk test (for values > 0.9 data were
154 considered normally distributed).
155 In order to find a correspondence between the FZS and the five new indexes, the dataset B was
156 analysed by performing a ROC Analysis (Hajian-Tilaki, 2013), using the FZS as golden standard test
157 and dichotomizing the FZS in two levels (FZS < 80, not excellent quality vs. FZS ≥ 80, excellent
158 quality, as reported by Woolford (1984). Applying the Youden index to each index proposed (I1 -
159 I5), a cut-off value between excellent and not excellent maize silage was found. The Youden index
160 is a method that maximise the sensitivity and specificity of a test and gives the best cut-off point
161 (Hajian-Tilaki, 2013). This analysis is often used to compare the results of diagnostic tests with a
162 gold standard, in order to calculate the accuracy. The ROC curve is the plot of sensitivity and 1-
163 specifity, and the area under that curve (AUC) represents the accuracy of the test. The AUC shows
164 the effectiveness of a test (or index) in discriminating between two categories (in our case, excellent
165 or not excellent maize silage), finding the best cut-off values, and comparing the different indexes (I1
166 - I5), when applied on the same samples (Hajian-Tilaki, 2013).

167 Finally, in order to verify the reliability of the proposed indexes, chemical composition data and the
168 indexes (I1 - I5) calculated on dataset C were submitted to a one-way ANOVA model with the DM,
169 classified in 3 levels (low, medium and high), as fixed effect. Post-hoc pairwise comparisons were
170 run between factor levels using Bonferroni correction. Assumptions of the linear model on the
171 residuals were graphically tested.

172 **Results**

173 Data in Table 2 show the different chemical characteristics of the datasets used, highlighting the best
174 fermentative quality of the silages obtained with the lab-scale method compared to those obtained
175 directly from the field operating practice. The dataset C had a higher FZS, higher concentrations of
176 lactic acid, ethanol and mannitol and lower concentrations of acetic and propionic acids, compared
177 to datasets A and B. Dataset C was also characterised by a lower variation in almost all composition
178 and fermentation parameters and in the FZS which showed a lower coefficient of variation (CV)
179 compared to datasets A and B (1.6 vs. 21 vs. 21%), respectively.

180 In Table 3, the parameters used to calculate each of the new quality indexes (I1 – I5), and their
181 percentage weight within each index were reported. Four parameters were used by all the indexes
182 and in order of importance they are: lactic acid (41%), ammonia (18%), ethanol (18%) and acetic acid
183 (9%). The pH was considered in all indexes as well, except for I5, even though it had different weights
184 (from 4 to 8%) in the different indexes. Butyric acid was also used in all indexes, with the exception
185 of I1, and had a weight ranging from 2 to 14%. Finally, the mannitol was considered in 3 indexes out
186 of 5 and its weight ranged from 3 to 6%.

187 Table 4 shows the average, standard deviation (SD), coefficient of variation (CV) and the value of
188 the Shapiro-Wilk's test calculated for FZS and the new fermentation quality indexes on both datasets
189 C (lab-scale silage) and B (in field made silage). Whereas Skapiro Wilk's values of the new indexes
190 were always higher than 0.96, indicating their normal distribution in both datasets C and A, the FZS
191 data resulted normally distributed only in dataset B. All the indexes showed higher values, indicating
192 higher fermentation quality, and lower CV in dataset C compared to dataset B.

193 The ROC analysis performed on dataset B (Table 5) shows that the AUC average values of the new
194 indexes are all between 0.78 and 0.89, indicating a moderate accuracy between the indexes I1 - I5
195 and FZS. Among the new indexes, the one with the highest AUC is I5, followed by I2 and I3, whereas

196 I1 shows the lowest value. I5 showed the best combination of sensitivity (0.87) and specificity (0.77)
197 in discriminating between good and poor quality silages. The cut-off, that is the equivalent of 80
198 points in the Flieg score, has a range between 45.5 and 57.4 points among the five new indexes.

199 The FZS and the other indexes were applied to the C dataset to find differences in the quality of
200 fermentation between three different levels of DM. As can be seen in Table 6, silages with different
201 levels of DM differed significantly for the concentration of lactic acid, butyric acid, ammonia, ethanol
202 and for pH, but did not differ for propionic acid and mannitol. For all the indexes, FZS included, the
203 silage of all 3 categories were considered excellent, that is they had an index value higher than the
204 cut-off. An exception is the I1 index for which the silages with high DM were not classified as
205 excellent because their average value was lower than the cut-off (57.4). However, while the FZS did
206 not differ among the 3 categories and had an average value of 98.7, all the other indexes were
207 significantly higher in silages with low DM, followed by that with medium and high DM,
208 respectively. Compared to other categories, silages with low DM had the highest concentrations of
209 lactic acid (56.4 g/kg DM), ammonia (61.4 g/kg DM) and butyric acid (0.62 g/kg DM).

210 **Discussion**

211 Given the importance of maize silage in cattle feeding and the impact that its quality can have on
212 animal health and animal productions, in this study we wanted to test some new indexes for the
213 evaluation of maize silage quality, comparing them with the Flieg- Zimmer's Score, an index widely
214 used for the evaluation of maize silage (Borowiec et al., 2001; Gallo et al., 2016a). We also wanted
215 to test the reliability of FZS on lab-scale maize silages, as these silages are now widely used in
216 research, to test for example different cultivars, the use of inocula, nutrient losses, etc. (Cherney et
217 al., 2004; Romero et al., 2017). Data on which FZS and the other indexes were calculated and applied
218 were in line with what reported in the literature regarding the composition of maize silage (Gallo et
219 al., 2016b), pH and fermentation by-products such as lactic acid, volatile fatty acids, ammonia, etc.
220 (Nishino et al., 2004; Gallo et al., 2017b). Since, on the basis of FZS, maize silage is evaluated as

221 good when the FZS is between 60 and 79 and excellent when is > 80 (Woolford, 1984), it can be
222 stated that overall, silages from dataset C, made through vacuum packing, were excellent and that the
223 ones belonging to dataset B, were both good and excellent. Although the dataset C consisted of maize
224 silage belonging to 29 cultivars, of different precociousness, cultivated in areas with different
225 pedoclimatic characteristics and collected at different ripening stages, the variability of its parameters
226 was much lower than that of the dataset B, as in C the ensiling process always occurred under the
227 same controlled conditions, and not in field conditions as in B. The ensiling conditions in the field,
228 the type of silo used, the technique used to consolidate the mass, the speed in filling the silo and the
229 sampling point, are all factors that strongly impact on the variability of the characteristics of the maize
230 silage (Gallo et al., 2016b). While FZS was formulated to evaluate homolactic, heterolactic and
231 clostridia fermentations, through the measure of lactic, acetic and butyric acids, respectively
232 (Woolford, 1984), the new indexes were thought to take into account proteolytic activity as well, by
233 measuring ammonia (Ward, 2011) and to deepen the information related to the fermentation by yeasts
234 and heterofermentant lactic acid bacteria through the measure of ethanol and mannitol (Nishino et al.,
235 2004).
236 With the purpose of finding the fermentative quality index most suitable to properly classify poorly-
237 or well preserved maize silages, on a scale from 0 to 100, we decided to assign, for all the new indexes,
238 the great part of the points (86) to parameters well known to be indicative of the extent of
239 homofermentant and heterofermentant lactic acid bacteria fermentations and proteolytic activity. The
240 remaining 14 points were differently shared, according to the index in question (I1 – I5), between
241 parameters that give more comprehension on suitability of the environment to undesirable
242 microorganisms (pH), activity of clostridia (butyric acid), and the use of fructose by heterofermentant
243 lactic acid bacteria (mannitol), as reported in literature (Kung and Shaver 2001; Nishino et al., 2004).
244 Among the 4 parameters common to all the indexes, the higher weight was assigned to the high
245 concentration of lactic acid (41 points), because it is a good index of the extent of homolactic
246 fermentation and its concentration and the consequent accumulation of hydrogen ions discourages

247 the activity of undesirable microorganisms like clostridia and enterobacteria (Kung and Shaver,
248 2001). Subsequently, equal importance was given to ammonia and ethanol which, in well preserved
249 silages, should be both low in concentration. Ammonia tends to accumulate due to the degradation of
250 amino acids and peptides, in part during homolactic fermentation and in part by proteolytic
251 enterobacteria and clostridia (McDonald et al., 2011; Ward, 2011), whereas ethanol is produced by
252 both heterofermentant lactic acid bacteria and by yeasts, in anaerobic conditions (Nishino et al.,
253 2004). Finally, acetic acid, is mainly produced during heterolactic fermentations (Gallo et al., 2016a)
254 and by enterobacteria (McDonald et al., 2011). High concentrations of both ethanol and acetic acid
255 are associated with high loss of DM and energy (Ward, 2011).
256 Lactic, acetic and butyric acids, ammonia and pH were already used in one or more indexes as FZS,
257 the Index 2 formulated by Gallo et al. (2016 a), Vanbelle's score and DLG score (Gallo et al., 2016a),
258 whereas ethanol and mannitol were measured individually to characterised the quality of the
259 fermentation (Nishino et al., 2004), but they were never used to formulate an index before.
260 When the indexes were applied to the B dataset, which refers to samples of maize silage produced
261 under field conditions, it can be noticed that the new indexes, with the exception of I1, had a
262 coefficient of variation on average higher than that of FZS and this means that they better represented
263 the variability of the different samples of maize, probably guaranteeing greater differentiation
264 between one sample and another. This difference between the new indexes and FZS was even more
265 accentuated in the dataset C, where the more limited variability among samples, due to the same
266 ensiling conditions, led the FZS to have a very low coefficient of variation, equal to about one seventh
267 of that of the other indexes. The new indexes therefore, if compared to FZS, amplified the distance
268 between well- and poorly preserved maize silages, even in silages made in standard conditions, a
269 criterion already used in the formulation of other indexes (Gallo et al 2016a). Besides, in C the
270 average of FZS was 97.7 and was therefore close to the maximum value of the index. However, it
271 seems unlikely that the samples were so little differentiated, considering that they belonged to samples
272 derived from varieties with different precociousness, with different DM levels, cultivated under

273 stressful or very favourable conditions and collected both before or after the 33% milk line phase. It
274 seems therefore that FZS was able to sufficiently detect differences in the quality of fermentations
275 between samples obtained in field conditions, as also noted by other authors (Borowiec et al., 2001),
276 but it was not suitable in detecting differences between samples obtained under standardized or
277 slightly differentiated conditions, as also detected by Gallo and colleagues (2016b) for the difference
278 between samples collected at the centre or at the sides of the silo.
279 Among the new indexes identified, the one that apparently had a greater accuracy according to the
280 ROC analysis was the I5, with a value of AUC equal to 0.89, which according to the scale given by
281 Swets (1989) for which AUC values are respectively non-informative (AUC = 0.5), inaccurate (0.5
282 < AUC ≤ 0.7), moderately accurate (0.7 < AUC ≤ 0.9) or highly accurate (0.9 < AUC ≤ 1.0), appears
283 to be at the highest end of the range in which the test is considered moderately accurate. This index
284 had a good sensitivity and specificity in correctly classifying silage samples either as excellent or not
285 excellent. Its cut-off was equal to 48 out of 100 points, showing a greater range of points above the
286 cut-off (51.8) which therefore allowed a better differentiation between samples of maize silage
287 considered excellent, compared to the 20 points above the cut-off (80) of the FZS. This index,
288 compared to the others, did not consider the pH and mannitol, but gave greater importance to the
289 concentration of butyric acid, with values that reached up to 14 points. I2 and I3, which were the
290 indexes that had the highest value of AUC after I5, assigned to pH up to 4 points and a to butyric
291 acid, assigned 10 and 7 points, respectively. I1, the index with the lowest AUC value, was the one
292 that gave the highest value to pH and to mannitol, but did not take into account butyric acid. These
293 results were predictable, as I2, I3 and I5 were the indices which most closely resemble the FZS,
294 regarding the parameters taken into consideration and their percentage weight.
295 When the indexes were applied to the silages produced with the vacuum packing, FZS was not
296 affected by the different level of DM of the samples and showed values very close to the maximum
297 score of the index for all the categories of DM. This is in line with what reported by other authors
298 (Gallo et al., 2016a), who showed that the FZS scores had limits in finding quality differences

between various points of the same silo. The other indexes were all significantly affected by the level of DM, showing the highest, medium and the worse values for silages with low, medium and high DM, respectively. This result was mainly linked to the highest concentration of lactic acid in silages with low DM. In these silages the higher weight of lactic acid in the calculation of the indexes prevailed on the fact that values of ammonia, acetic acid and butyric acid, were higher and therefore unfavourable. Although ethanol was higher in low DM silages, it did not affect the difference between the indexes because in all the categories it exceeded the maximum threshold and therefore the maximum score (18 points) was assigned to the silages of all the DM categories. In this case, none of the new indexes proved to be better than the others in distinguishing the different categories of silage by quality, even though I1 was the only one to attribute a value below the excellence to silages with high DM. The excellent quality of all the silages from a fermentative perspective is confirmed by the fact that the concentrations of lactic, acetic, butyric acids, ammonia and the pH value were in line with the concentrations recommended in the literature (Kung and Shaver, 2001; Ward, 2011). Even the concentration of ethanol, in spite of the fact that was beyond the maximal value considered for the calculation of the indexes, were below the threshold of 3% DM recommended by Kung and Shaver (2001) for good quality silage.

Conclusions

From the results obtained it can be stated that the Flieg-Zimmer's Score was able to differentiate the quality of the silage in field conditions, but was not sensitive enough to differentiate silages prepared in standard conditions such as those on a laboratory scale used for research. The new quality indexes, calculated taking into account the concentrations of lactic acid, ammonia, ethanol, acetic acid and other parameters such as pH and mannitol, gave results in line with those of FZS in field conditions, but proved to be better suited to differentiate the quality of silages, both made in field conditions and

323 through the vacuum packing technique. Although there is still a lot of work to be done to refine the
324 techniques of silage quality evaluation, these indices promise to be useful tools in this regard.

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329 **References**

330 Addah, W., Baah, J., Groenewegen, P., Okine, E.K., McAllister, T.A., 2011. Comparison of the
331 fermentation characteristics, aerobic stability and nutritive value of barley and corn silages
332 ensiled with or without a mixed bacterial inoculant. Can. J. Anim. Sci. 91, 133-146.
333 ANKOM Technology. 2015a. Method 12, 4/10/15: Acid Detergent Fiber in Feeds - Filter Bag
334 Technique (for A2000 and A2000I). ANKOM Technology, Macedon, NY.
335 https://www.ankom.com/sites/default/files/document-files/Method_12_ADF_A2000.pdf
336 (accessed 20.03.16).
337 ANKOM Technology. 2015b. Method 13, 4/10/15: Neutral Detergent Fiber in Feeds - Filter Bag
338 Technique (for A2000 and A2000I). ANKOM Technology, Macedon, NY.
339 https://www.ankom.com/sites/default/files/document-files/Method_13_NDF_A2000.pdf
340 (accessed 20.03.16).
341 AOAC, 2000. Official methods of analysis, 17th ed. Association of Official Analytical Chemists,
342 Gaithersburg, MD.
343 AOAC, 2003. Official methods of analysis. 17th ed., 2nd revision. Association of Official
344 Analytical Chemists, Gaithersburg, MD.
345 AOAC, 2005. Official methods of analysis. 18th ed. Association of Official Analytical Chemists,
346 Gaithersburg, MD.

AOAC, 2006. Official method of analysis. 18th ed. AOAC International, Gaithersburg, MD.

Borowiec, F., Zając, T., Kowalski, Z.M., Micek, P., Marciński, M., 2001. The effect of additives on palatability, voluntary intake and digestibility of lucerne and whole-crop oat silages. J. Animal Feed Sci. 10, Suppl. 2, 345–350.

Cherney, J.H., Cherney, D.J.R., 2003. Assessing silage quality, in: Buxton, D.R., Muck, R., Harrison, J. (Eds), Silage Science and Technology. ASA, CSSA, SSSA, Madison, WI, pp. 141–198.

Cherney, D.J., Cherney, J.H., Cox, W.J., 2004. Fermentation characteristics of corn forage ensiled in mini-silos. J. Dairy Sci. 87, 4238–4246.

Erdman, R.A., Piperova, L.S., Kohn, R.A., 2011. Corn silage versus corn silage:alfalfa hay mixtures for dairy cows: effects of dietary potassium, calcium, and cation-anion difference. J. Dairy Sci. 94, 5105–5110.

Gallo, A., Bertuzzi, T., Giuberti, G., Moschini, M., Bruschi, S., Cerioli, C. Masoero, F., 2016b. New assessment based on the use of principal factor analysis to investigate corn silage quality from nutritional traits, fermentation end products and mycotoxins. J. Sci. Food Agric. 96, 437–448.

Gallo, A., Giuberti, G., Bruschi, S., Fortunati, P., Masoero, F., 2016a. Use of principal factor analysis to generate a corn silage fermentative quality index to rank well- or poorly preserved forages. J. Sci. Food Agric. 96, 1686–1696.

Hajian-Tilaki, K., 2013. Receiver Operating Characteristic (ROC) Curve Analysis for Medical Diagnostic Test Evaluation. Caspian J. Intern. Med. 4, 627–635.

Hoedtke, S., Zeyner, A., 2011. Comparative evaluation of laboratory-scale silages using standard glass jar silages or vacuum-packed model silages. J. Sci. Food Agric. 91, 841–849.

Johnson, H.E., Merry, R.J., Davies, D.R., Kell, D.B., Theodorou, M.K., Griffith, G.W., 2005. Vacuum packing: a model system for laboratory-scale silage fermentations. J. Appl. Microbiol, 98, 106–113.

373 Khan, N.A., Yu, P., Ali, M., Cone, J.W., Hendriks, W.H., 2015. Nutritive value of maize silage
374 in relation to dairy cow performance and milk quality. J. Sci. Food Agric. 95, 238–252.
375 Krämer-Schmid, M., Lund, P., Weisbjerg, M.R., 2016. Importance of NDF digestibility of whole
376 crop maize silage for dry matter intake and milk production in dairy cows. Anim. Feed
377 Sci. Technol. 219, 68–76.
378 Kuehn, C.S., Linn, J.G., Johnson, D.G., Jung, H.G., Endres, M.I., 1999. Effect of Feeding Silages
379 from Corn Hybrids Selected for Leafiness or Grain to Lactating Dairy Cattle. J. Dairy Sci.
380 82, 2746–2755.
381 Kung, L., R. Shaver., 2001. Interpretation and use of silage fermentation analysis reports. Focus
382 on Forage. 3, 1–5. https://fyi.uwex.edu/forage/files/2014/01/Fermentation.pdf (accessed
383 01.09.17).
384 Marchesini, G., Serva, L., Garbin, E., Mirisola, M., Andrighetto, I., 2017. Near-infrared
385 calibration transfer for undried whole maize plant between laboratory and on-site
386 spectrometers. Ital. J. Anim.Sci. doi.org/10.1080/1828051X.2017.1345660
387 Martillotti F., Puppo S., 1985. Liquid chromatographic determination of organic acids in silages
388 and rumen fluids. Ann. dell'Istituto Sper. Zootec., 18, 1–10.
389 McDonald, P., Henderson, N., Heron, S., 1991. The Biochemistry of Silage, second ed.
390 Chalcombe Publications, Aberystwyth.
391 McDonald, P., Edwards, R.A., Greenhalg, J.F.D., Morgan, C.A., Sinclair, L.A., Wilkinson, R.G.,
392 2011. Silage, in: Animal Nutrition, 7th ed. Pearson Education Limited, Harlow, England,
393 pp. 499–520.
394 Megazyme, 2014. Urea/ammonia assay procedure.
395 https://secure.megazyme.com/files/Booklet/K-URAMR_DATA.pdf. (accessed
396 01.09.17).
397 Nishino, N., Wada, H., Yoshida, M., Shiota, H., 2004. Microbial Counts, Fermentation Products,
398 and Aerobic Stability of Whole Crop Corn and a Total Mixed Ration Ensiled With and

399 Without Inoculation of Lactobacillus casei or Lactobacillus buchneri. J. Dairy Sci. 87,
400 2563–2570.
401 Oude Elferink, S.J.W.H., Driehuis, F., Gottschal, J.C., Spoelstra, S.F., 2000. Silage fermentation
402 processes and their manipulation. FAO Plant P. 161, 17–30.
403 Pahlow, G., Muck, R.E., Driehuis, F., Oude Elferink, S.J.W.H., Spoelstra, S.F., 2003.
404 Microbiology of ensiling, in: Buxton, D.R., Muck, R., Harrison, J. (Eds), Silage Science
405 and Technology. ASA, CSSA, SSSA, Madison, WI, pp. 31–93.
406 Romero, J.J., Zhao, Y., Balseca-Paredes, M.A., Tiezzi, F., Gutierrez-Rodriguez, E., Castillo,
407 M.S., 2017. Laboratory silo type and inoculation effects on nutritional composition,
408 fermentation, and bacterial and fungal communities of oat silage. J. Dairy Sci. 100, 1812–
409 1828.
410 Swets, J.A., 1989. Measuring the accuracy of diagnostic systems. Science, 240, 1285–1293.
411 Vanbelle, M., Bertin, G., 1985. L'ensilage, Aspects Biologiques Nouveaux. Sanofi sante animale,
412 Paris.
413 Ward, R., 2011. Analyzing silage crops for quality: what is most important? In: Proceedings of
414 Western alfalfa and forage conference, Las Vegas, NV.
415 Woolford, M.K., 1984. The Silage Fermentation. Marcel Dekker, NewYork.
416

417 **Table 1**

418 Specifications of the calibration curve used for maize silage analyses (n = 2098).

Constituents (g/kg DM)	Mean	SD [a]	SEC [b]	R^2 [c]	SECV [d]	1-VR[e]
DM (g/kg)	333	56.9	15.0	0.93	15.4	0.93
Ash	14.5	2.40	1.60	0.55	1.60	0.52
Crude protein	25.4	4.90	1.70	0.89	1.70	0.88
Ether extract	10.7	3.00	1.80	0.64	1.90	0.61
aNDF	153	22.0	9.80	0.80	10.1	0.79
ADF	84.9	11.5	6.30	0.70	6.40	0.69
Starch	89.8	30.6	14.3	0.78	14.7	0.77
pH [f]	3.80	0.16	0.09	0.70	0.09	0.68
Lactic acid	13.8	6.20	3.70	0.65	3.80	0.63
Acetic acid	5.70	3.30	1.60	0.77	1.60	0.76
Propionic acid	1.20	1.10	0.70	0.58	0.70	0.55
Butyric acid	0.20	0.10	0.10	0.21	0.10	0.17
Ethanol	1.80	1.10	0.60	0.70	0.60	0.67
Mannitol	2.20	1.90	1.20	0.64	1.20	0.61
Ammonia	19.6	7.70	4.10	0.71	4.20	0.70

419 [a]Standard deviation.

420 [b] Standard error of calibration.

421 [c] Coefficient of determination.

422 [d] Standard error of cross-validation.

423 [e] Coefficient of determination in cross-validation.

424 [f] It is expressed as a pure number.

425

426 **Table 2**

427 Chemical composition and fermentation parameters of maize silage samples belonging to the datasets
428 A, B and C (Mean ± SD).

Constituents (g/Kg DM)	Dataset A n = 2098	Dataset B n = 191	Dataset C n = 522
DM g/kg	333 ± 56.9	339 ± 66.3	346 ± 54.1
Ash	43.9 ± 7.60	38.5 ± 13.7	33.9 ± 3.50
Crude protein	76.1 ± 9.20	74.0 ± 11.9	72.7 ± 5.50
Ether extract	31.7 ± 8.30	31.3 ± 9.10	21.2 ± 3.60
aNDF	464 ± 53.9	452 ± 65.4	403 ± 30.7
ADF	259 ± 38.5	253 ± 41.8	213 ± 21.3
Starch	261 ± 57.9	286 ± 44.2	328 ± 42.8
pH [a]	3.81 ± 0.16	3.79 ± 0.41	3.85 ± 0.06
Lactic acid	41.3 ± 18.7	35.7 ± 17.0	51.1 ± 9.20
Acetic acid	17.3 ± 10.1	16.2 ± 10.0	9.20 ± 2.70
Propionic acid	3.80 ± 4.00	3.10 ± 3.30	0.30 ± 0.40
Butyric acid	0.50 ± 0.50	0.60 ± 0.30	0.60 ± 0.10
Ethanol	5.40 ± 3.30	4.90 ± 3.20	11.4 ± 5.10
Mannitol	6.80 ± 5.90	6.00 ± 5.30	9.50 ± 2.80
Ammonia	58.7 ± 23.3	57.5 ± 18.3	58.4 ± 5.50
Flieg-Zimmer's score [a]	79.8 ± 16.9	79.8 ± 16.9	98.7 ± 1.54

429 [a] It is expressed as a pure number.

430

Table 3

Characterization of the new quality indexes I1 – I5: parameters used, range of values used for scoring and score interval of each parameter.

Parameters	Range of values	Score interval				
	(g/kg DM)	I1	I2	I3	I4	I5
Lactic acid	22.6 – 60.0	0 - 41	0 - 41	0 - 41	0 - 41	0 - 41
Ammonia	82.0 - 35.4	0 - 18	0 - 18	0 - 18	0 - 18	0 - 18
Ethanol	8.70 - 2.10	0 - 18	0 - 18	0 - 18	0 - 18	0 - 18
Acetic acid	27.4 - 7.20	0 - 9	0 - 9	0 - 9	0 - 9	0 - 9
pH [a]	3.97 - 3.65	0 - 8	0 - 4	0 - 4	0 - 6	-
Butyric acid	1.00 - 0.00	-	0 - 10	0 - 7	0 - 2	0 - 14
Mannitol	12.7 - 0.90	0 - 6	-	0 - 3	0 - 6	-
Index maximum score	-	100	100	100	100	100

[a] It is expressed as a pure number.

436 **Table 4**

437 Mean, standard deviation (SD), Shapiro-Wilk test and coefficient of variation (CV) of Flieg-
438 Zimmer's score (FZS) and the new quality indexes (I1 – I5) in both datasets B (in field made silages)
439 and C (lab-scale silages).

	Dataset B			Dataset C		
	Mean ± SD	Shapiro-Wilk	CV	Mean ± SD	Shapiro-Wilk	CV
FZS	79.8 ± 16.9	0.91	0.21	97.7 ± 1.54	0.75	0.02
I1	57.3 ± 12.2	0.97	0.21	57.2 ± 7.90	0.98	0.14
I2	47.8 ± 13.3	0.99	0.28	56.7 ± 7.49	0.97	0.13
I3	47.1 ± 13.0	0.99	0.27	54.6 ± 7.69	0.98	0.14
I4	47.7 ± 12.7	0.99	0.27	54.2 ± 8.05	0.98	0.15
I5	46.4 ± 13.4	0.99	0.29	55.2 ± 7.26	0.97	0.13

440

441 **Table 5**

442 Results of the ROC analysis performed on dataset B (n = 191): accuracy of the new quality indexes
443 (I1 – I5) in discriminating between excellent and non-excellent silages on the basis of the results of a
444 reference test that in this case is the Flieg-Zimmer's score. Accuracy is expressed as AUC.

Index	AUC ± SE [a]	95% CI [b]	Cut-off [c]	Sensitivity	Specificity
I1	0.78 ± 0.03	0.71 - 0.84	57.4	0.80	0.63
I2	0.87 ± 0.03	0.81 - 0.91	49.5	0.87	0.73
I3	0.84 ± 0.03	0.78 - 0.89	46.3	0.77	0.79
I4	0.80 ± 0.03	0.74 - 0.86	45.5	0.68	0.78
I5	0.89 ± 0.02	0.83-0.93	48.2	0.87	0.77

445 [a] Area under the curve ± standard error.

446 [b] Confidence interval at 0.95.

447 [c] The value of the index above which the silage is considered excellent.

448

Table 6

Comparison of mean values of fermentation parameters, Flieg-Zimmer's score (FZS) and quality indexes (I1 – I5) between silages with different concentration of dry matter (DM) in dataset C (lab-scale silage)

DM range	< 320 (g/kg)	320 – 360 (g/kg)	> 360 (g/kg)	SEM [b]	P-value
N.	192	132	198		
Constituents (g/kg DM)					
DM (g/kg)	318 [z]	344 [y]	375 [x]	2.100	<0.001
pH [a]	3.84	3.85	3.86	0.005	0.041
Lactic acid	56.4 [x]	50.7 [y]	46.4 [z]	0.600	<0.001
Acetic acid	9.90	9.20	8.40	0.267	0.037
Propionic acid	0.30	0.30	0. 30	0.043	0.608
Butyric acid	0. 62 [x]	0.58 [y]	0.54 [z]	0.0004	<0.001
Ethanol	12.6	11.2	10.3	0.043	0.034
Mannitol	9.60	9.60	9.70	0.267	0.590
Ammonia	61.4 [x]	58.6	55.2 [z]	0.043	<0.001
Quality indexes					
FZS	98.9	98.7	98.6	0.190	0.738
I1	62.1 [x]	57.4 [y]	52.6 [z]	0.627	<0.001
I2	60.8 [x]	56.7 [y]	52.6 [z]	0.613	<0.001
I3	59.2 [x]	54.8 [y]	50.3 [z]	0.630	<0.001
I4	59.2 [x]	55.3 [y]	51.4 [z]	0.650	<0.001
I5	59.1 [x]	54.3 [y]	49.5 [z]	0.607	<0.001

[a] It is expressed as a pure number

[b] Standard error of means

[xyz] Means within lines not sharing a common superscript are significantly different at the 1% level of probability