Short Communication

Received: 23 August 2020 Revised: 28 November 2020 Accepted article published: 10 December 2020 Published online in Wiley Online Library: 20 January 2021

(wileyonlinelibrary.com) DOI 10.1002/jsfa.10999

Biostimulating effect of L-tryptophan on the yield and chemical and microbiological quality of perennial ryegrass (*Lolium perenne*) herbage and silage for ruminant

Sebastian W Przemieniecki,[a*] Cezary Purwin,[b] Jędrzej Mastalerz,[a] Marta Borsuk,[b] Krzysztof Lipiński[b] and Tomasz Kurowski[a]

Abstract

BACKGROUND: This study aimed to evaluate the effect of L-tryptophan (L-TRP) used in the cultivation of *Lolium perenne* on the yield, and chemical and microbiological quality of its herbage and silage. L-Tryptophan was applied in doses of 5, 0.5, 0.05 kg ha^{-1}. The experiment was conducted with a control group (C) and a comparative control group (C+) with higher nitrogen fertilization.

RESULTS: The dose of 5 kg ha^{-1} had a significant effect on herbage yield, which increased by 15% compared to group C and approximated the value achieved in group C+. The treatment with L-TRP caused a significant increase in water-soluble carbohydrate (WSC) content only in Tr5 (165 g kg^{-1} DM), which was reflected in a more beneficial course of fermentation, lower pH (4.59), and a higher sum of fermentation acids, including lactic acid (94.7 g kg^{-1} DM). Ryegrass treatment with a high L-TRP dose effectively reduced the loads of *Clostridium* spp. and fungi, and increased the count of *Bacillus* spp. The L-TRP significantly reduced N-NH_3 content in Tr05 (98.6 g kg^{-1} TN) compared with C+ (123 g kg^{-1} TN) and butyric acid content in Tr05 (from 1.35 g kg^{-1} DM in the C to 0.38 g kg^{-1} DM).

CONCLUSION: The most effective dose turned out to be a dose of 5 kg ha^{-1}, which allowed a higher yield and a better fermentation course to be achieved. This work presents the feasibility of using L-TRP to optimize nutrient consumption by *Lolium perenne* and ultimately to affect the quality of its silage as a feedstuff.

Keywords: *Lolium perenne*; L-tryptophan; silage; microbiota

INTRODUCTION

Abiotic stresses, resulting from water supplies, extremes in temperature, salinity, and acidity, limit the uptake of nutrients and the growth and productivity of grass species.[1] A solution to these problems is offered by natural growth stimulants, like amino acids, which – by being protein constituents – are involved in multiple physiological processes in plants and animals. They affect growth and development and participate in signaling processes and enzymatic transformations. They play the role of immediate or intermediate biostimulants as precursors to secondary metabolites, and their metabolic pathways constitute an integral element of the immune systems of plants.[2,3]

Among its many essential functions in live organisms, L-TRP serves as a precursor of indole-3-acetic acid (IAA), found naturally in plant roots.[4] Nevertheless, ca. 80% of the rhizosphere bacteria exhibit the capability for IAA synthesis.[5] The IAA synthesis is determined by L-TRP content,[6] which depends on the vegetation stage of plants and physiological stage of their cells.[2] Research has shown a beneficial effect from soil or foliar treatment with L-TRP in the cultivation of many crops, including maize,[7] wheat,[8] and lupine.[9] This positive impact has manifested in increased plant growth, green biomass yield, pod number, and improved chemical composition, including increased crude protein (CP) and water-soluble carbohydrate (WSC) content.

The use of IAA has been reported to stimulate the growth and yield of soybean.[10] Among a small number of studies addressing the auxin treatment effect on grasses, Clifford[11] investigated the early bending kinetics in response to unilateral IAA application in *Lolium* nodal segments. Li *et al.*[12] demonstrated enhanced phytoremediation of soil contaminated with phenanthrene by

* *Correspondence to: SW Przemieniecki, Department of Entomology, Phytopathology and Molecular Diagnostics, University of Warmia and Mazury in Olsztyn, Prawocheńskiego 17, 10-720 Olsztyn, Poland. E-mail: sebastian.przemieniecki@uwm.edu.pl*

a *Department of Entomology, Phytopathology and Molecular Diagnostics, University of Warmia and Mazury in Olsztyn, Olsztyn, Poland*

b *Department of Animal Nutrition and Feed Science, University of Warmia and Mazury in Olsztyn, Olsztyn, Poland*

promoting soil enzyme activity and increasing microbial biomass with *Lolium multiflorum* treatment with IAA, which allowed the green biomass of grass to be increased. As an IAA precursor, L-TRP was also expected to affect the yield and quality of fodder grasses intended for the production of silages to be used in ruminant feeding. These issues have rarely been addressed in the scientific literature. In a moderate climate, *Lolium perenne* represents the key species of fodder grasses administered to animals in the form of herbage or silage.[13]

This study aimed to determine the effect of exogenous application of L-TRP as a plant growth stimulant on the yield of *Lolium perenne* and to evaluate the quality of silages produced. Hypothesis: L-tryptophan improves the quality of herbage and silage for ruminant.

MATERIALS AND METHODS

The study was conducted with perennial ryegrass (*Lolium perenne* L.), from experimental plots (2 × 5 m) at the experimental station in Tomaszkowo (53° 43′ 04″ N, 20° 24′ 32″ E). A field experiment was performed in a randomized block system, with each experimental variant conducted in three replications. The seed rate was 35 kg ha^{-1}. A standard nitrogen dose was used for fertilization (N/P/K: 60/30/60 kg ha^{-1}, group C) whereas a higher nitrogen dose (N/P/K: 90/30/60 kg ha^{-1}) was used in a comparative control group (C+). Neither control variant (C or C+) was treated with L-TRP. The 99.8% L-TRP (Sigma-Aldrich, St. Louis, Missouri, United States) was applied in doses of 5 kg ha^{-1} (Tr5), 0.5 kg ha^{-1} (Tr05), and 0.05 kg ha^{-1} (Tr005), established based on a preliminary study with seedling pallets. The experiment was carried out on the soil deficient in nitrogen characterized by the following chemical parameters: total nitrogen, 0.09%; P_2O_5, 21.1 mg 100 g^{-1} of soil; K_2O, 19.5 mg 100 g^{-1} of soil; Mg, 4.3 mg 100 g^{-1} of soil, and pH 5.9. Over the experimental period, L-TRP treatments were performed at the onset of plant vegetation (the first half of May) and after the first cut (the second half of June). Herbage was harvested in the second cut (herbage yield was estimated in the first year, whereas herbage yield and chemical composition were determined in the second year). Material for silage production was obtained in the second year of cultivation (second cut), after 30 days of regrowth. Once cut, the material was weighed, and herbage samples were collected from each plot. The crushed herbage (550 g FM) was vacuum-packed in polyethylene bags (38.6 × 27.9 cm) using a vacuum packing machine (Vacutronic 2000, PP 5.4, ZTP TEPRO, Poland). The bags with silage were opened after 90 days. A detailed description of the laboratory chemicals and quantitative polymerase chain reaction (qPCR) analyses has been provided in our previous work.[14] Samples were assayed for chemical composition: dry matter (DM) (method 934.01), CP (method 976.05), and crude ash (method 942.05),[15] water-soluble carbohydrates (WSCs) – by the anthrone method,[16] neutral-detergent fiber (NDF) assayed with heat-stable amylase and expressed exclusive of residual ash, acid detergent fiber (ADF) expressed exclusive of residual ash – as described by Van Soest *et al.*[17] using the ANKOM220 fiber analyzer (ANKOM Technology Corp., Macedon, NY, USA). The ammonia nitrogen ($N\text{-}NH_3$) content was determined by direct distillation using the 2100 Kjeltec distillation unit (FOSS Analytical A/S, Hilleröd,

Table 1. Yield and chemical composition of herbage and silage

Parameter[a]		Unit	C[b]	C+	Tr005	Tr05	Tr5	SEM
Herbage	Yield DM I	mg·ha^{-1}	13.06	13.95	11.84	11.71	13.5	0.32
	Yield DM II		12.26 C	15.62 A**	13.33 BC	12.68 BC	14.1 AB*	0.36
	Dry matter	g·kg^{-1}	317	300	322	333	318	0.45
	Crude ash	g·kg^{-1} DM	87.2	88.5	79.3	79.8	77.3	0.25
	Crude protein		130	139	123	122	122	0.22
	WSC		155 AB	145 B	155 AB	161 AB	165 A	0.23
	NDF		500	484	498	489	491	0.33
	ADF		329	317	313	311	311	0.33
Silage	pH		4.71	4.63	4.65	4.62	4.59	0.01
	Dry matter	g·kg^{-1}	301	280	307	314	307	0.45
	Crude ash	g·kg^{-1} DM	131	110	113	112	104	0.33
	Crude protein		130	151	129	129	128	0.24
	WSC		15.7 B	13.2	22.0 AB	32.0 A**	28.8 A*	0.21
	NDF		526	504	523	513	509	0.33
	ADF		355	335	338	336	335	0.35
	$N\text{-}NH_3$	g·kg^{-1} TN	105 AB	123 A	105 AB	98.6 B	106 AB	4.90
	Ethanol	g·kg^{-1} DM	0.47	0.76	0.57	0.60	0.55	0.08
	Lactic acid		69.7 B	77.4 B	80.4 B	77.5 B	94.7 A**	2.47
	Acetic acid		3.19	4.44	3.43	3.95	3.66	0.29
	Propionic acid		0.05	0.04	0.03	0.04	0.03	0.011
	Valeric acid		0.03	0.02	0.02	0.03	0.03	0.003
	Butyric acid		1.35 A	0.70 AB	0.67 AB	0.38 B*	0.54 AB	0.157

Different letters in the same row indicate significant difference A, B, C $P < 0.05$; The values in the same row with different superscripts are significantly different from the control group (C) * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.
[a] WSC, water soluble carbohydrate; NDF, neutral detergent fiber; ADF, acid detergent fiber.
[b] C, control group; C+, comparative control group; L-TRP was applied in doses of 5 kg ha^{-1} (Tr5), 0.5 kg ha^{-1} (Tr05), and 0.05 kg ha^{-1} (Tr005); SEM, standard error of the mean.

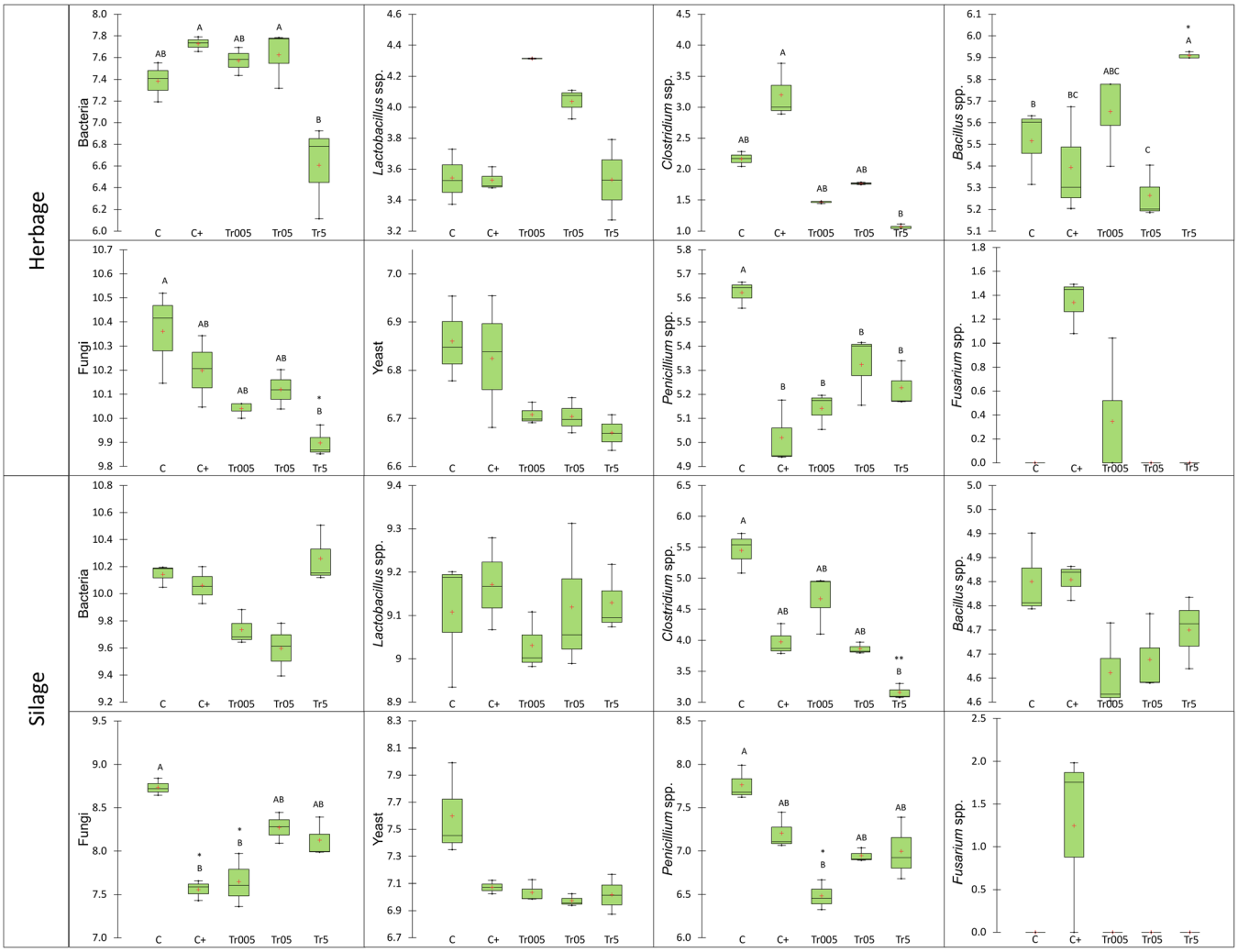

Figure 1. Boxplots for microbial qPCR analysis (gene copies per 1 g of dry matter). Abbreviations: C – control, C+ – high nitrogen fertilization, Tr5 – 5 kg tryptophan ha^{-1}, Tr05 – 0.5 kg tryptophan ha^{-1}, Tr005 – 0.05 kg tryptophan ha^{-1}; Different letters in the same boxplot set indicate significant difference A, B, C ($P < 0.05$); the values in the same boxplot set with different superscripts are significantly different from the control group (C) * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Denmark) after increasing the pH of the samples by adding MgO; acidity was measured with the HI 8314 pH meter (Hanna Instruments, Woonsocket, RI, USA). The concentrations of lactic acid and volatile fatty acids were determined as described by Kostulak-Zielińska and Potkański.[18] The volatile fatty acids and ethanol were separated and determined by gas chromatography on the Varian 450 gas chromatograph with the Varian CP-8410 autosampler, flame-ionization detector (FID), CP-FFAP capillary column (length – 25 m, inner diameter – 0.53 mm, film thickness – 1.0 μm), sample size – 1 μL, detector temperature – 260 °C, injector temperature – 200 °C, column temperature – 90 to 200 °C, carrier gas – helium (flow rate 5.0 mL min^{-1}). Lactic acid content was determined by high performance liquid chromatography (HPLC, Shimadzu, Kyoto, Japan) with isocratic flow. Separation was carried out using the Varian, Palo Alto, USA, Metacarb 67H column (Organic Acids Column), mobile phase: 0.002 mmol L-1 solution of sulfuric acid in deionized water, flow rate of 1 mL min^{-1}, UV detector, 210 nm. External fatty acid standards were supplied by Supelco, and the lactic acid standard – by FLUKA (Buchs, Switzerland). The qPCR results are presented as a gene copy number of each of the analyzed groups of microorganisms g^{-1} (DM). Statistical analysis of the results was preceded by the evaluation of distribution normality (Shapiro–Wilk test) and variance homogeneity (Brown–Forsythe test). Afterward, an ANOVA was performed at $P = 0.05$, $P = 0.01$, and $P = 0.001$. A Tukey test was used for the results that met the criteria for normal distribution, whereas the Kruskal–Wallis test was used for the remaining results (Statistica 13.5 TIBCO Software Inc., Palo Alto, CA, USA). Principal component analysis (PCA) was carried out with PAST 4.02 software[19] based on results centralized according to the following formula: [(value – mean) standard deviation^{-1}]. Distances between points on the biplot were computed based on the Pearson correlation matrix, between groups, represented by replications of a given variable.

RESULTS

The herbage was more differentiated in terms of microbiological than chemical composition. In the case of biomass yield, significant differences were observed in the second year of the study. The highest yields were recorded in variants C+ (a highly significantly higher increase by 27% compared to the control variant)

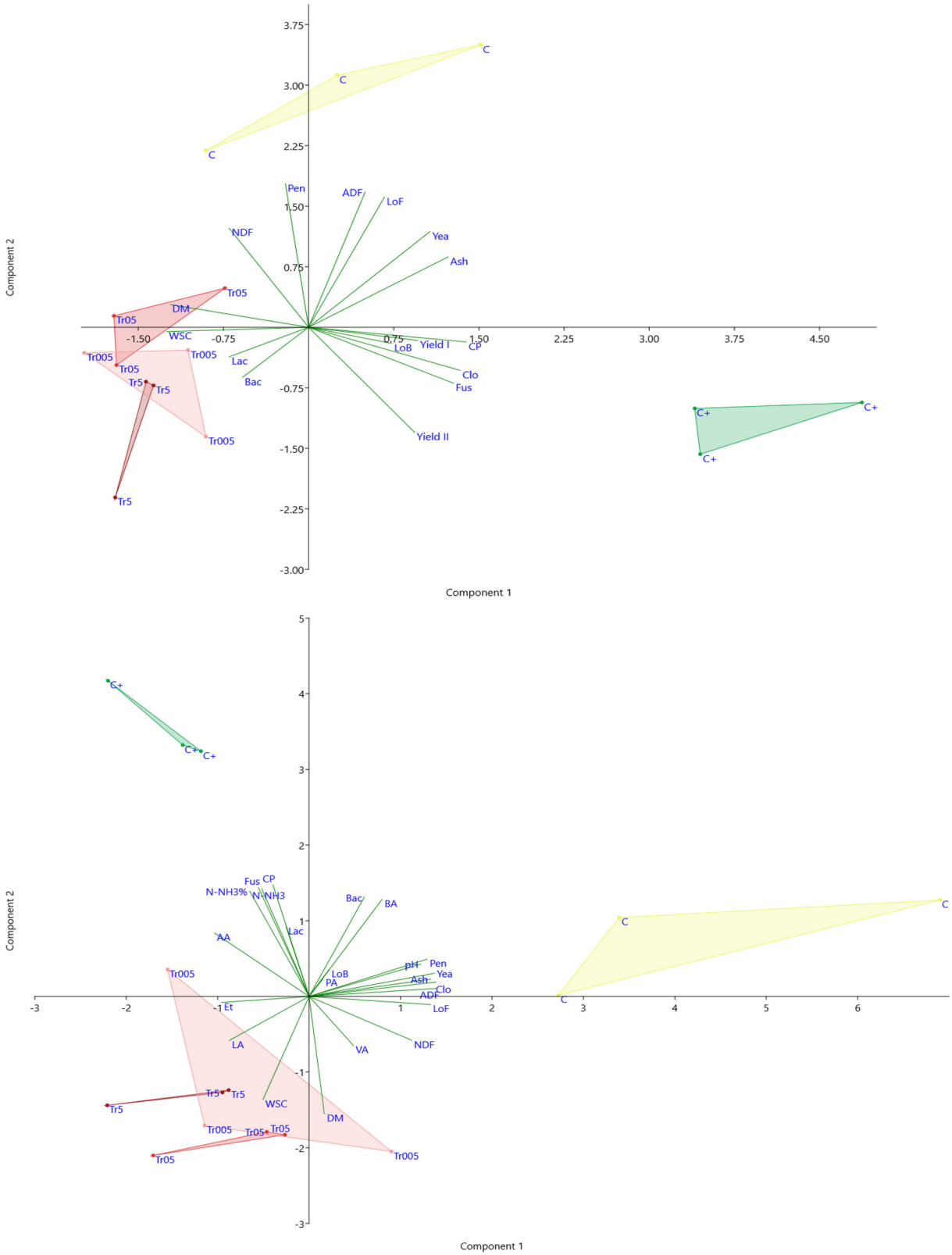

Figure 2. Biplot for A: biomass yield and chemical properties of herbage; B: chemical properties of silage. Abbreviations: C – control, C+ – high nitrogen fertilization, Tr5 – 5 kg tryptophan ha^{-1}, Tr05 – 0.5 kg tryptophan ha^{-1}, Tr005 – 0.05 kg tryptophan ha^{-1} Yield I – yield in the first year of experiment, Yield II – yield in the second year of experiment, DM – dry matter, CP – crude protein, NDF – neutral detergent fiber, ADF – acid detergent fiber, WSC – water-soluble carbohydrates, LA – lactic acid, AA – acetic acid, PA – propionic acid, Et – ethanol, BA – butyric acid, VA – valeric acid, LoB – load of bacteria, Lac-Lactic acid bacteria, Clo – *Clostridium* spp., Bac – *Bacillus* ssp., LoF – load of fungi, Yea – yeast, Fus – *Fusarium* ssp. (with Tri5 gene), Pen – *Penicillium* spp.

and Tr5 (a significant increase by 15% compared to the control variant). Herbage yields determined in the other Tr variants were significantly lower than the C+ variant. The results of chemical analyses showed increased WSC content in Tr5 herbage (by 20 g kg^{-1} DM) compared to C+ (Table 1). The results of microbiological analyses demonstrated the highest bacterial loads in the C+ and Tr05 variants and the lowest one in the Tr5 variant. The load of *Clostridium* spp. was the highest in variant C+. It was lower in the other variants (at least by one order of magnitude); however, a significantly lower count of this bacterial genus was determined only in variant Tr5. Results and significant differences analogous to these shown for *Clostridium* load were reported for fungi load. Results obtained for *Bacillus* spp. demonstrated the highest load of these bacteria in variant Tr5 and a significantly lower load (by 57%) in variant C. Whereas the *Bacillus* spp. load determined in variants C+ and Tr05 was significantly lower compared to variant Tr5. In the case of *Penicillium* spp. load, a highly significant ($P = 0.001$ or 0.01) reduction was observed regardless of L-TRP dose (by 62% on average compared to the control variant). The loads of lactic acid bacteria, yeast, and *Fusarium* spp. did not differ statistically among the variants (Fig. 1).

Perennial ryegrass treatment with various L-TRP doses did not influence the proximate composition of the respective silages (Table 1). The higher L-TRP doses (Tr5 and Tr05) had a significant effect on the WSC loss reduction during ensilaging. In turn, the increased WSC content in Tr herbage enhanced acid production during fermentation and increased the rate of pH decline, thereby inhibiting protein degradation. The lowest pH was measured in the Tr5 variant; however, there were no statistically significant differences in its value among groups. In contrast, differences were observed in the N-NH_3 content of silages. Compared with the other variants treated with L-TRP, the differences were not statistically significant; however, a downward tendency was observed compared with the C+ variant. The L-TRP treatment allowed achieving a significantly higher sum of acids in silage, being the highest in variant Tr5. Regarding contents of volatile acids and ethanol, differences were only observed for butyric acid, the highest content of which was determined in variant C, the lowest one in variant Tr05, and a downward tendency in variant Tr5. This proves the positive effect of L-TRP treatment on the course of clostridial fermentation. In general, the above results are consistent with the *Clostridium* spp. load, but a highly significant decrease was noted in the load of these bacteria in variant Tr5 and a downward tendency in variant Tr05 (Table 1, Fig. 1). Considering the other microbiological parameters tested in the study, the load of fungi generally decreased in each variant compared to the control variant but this reduction proved significant only in variants C+ and Tr005. In the case of *Penicillium* spp. gene copy number, a significant decrease of its load was observed in variant Tr005 and a downward tendency in variants Tr05 and Tr5 (Fig. 1).

The first two principal components in the PCA of the herbage explained in total 77.6% of the variance, with component 1 (the horizontal axis) explaining 49.4% and component 2 (the vertical axis) explaining 28.2% of the variance. The PCA results demonstrated that all variants treated with tryptophan (Tr) were highly similar to each other but significantly different from the control variant (C) and the variant with an increased nitrogen dose (C+). All Tr variants were similar, as evidenced by their close arrangement on the coordinate axis. The analysis of vector loading and orientation showed that the high *Penicillium* spp. load, high ADF content, and high fungi load, that were positively correlated with each other, were typical of variant C. The vectors CP, Clo, Fus, Yield I, and LoB (with a slightly lower loading) were strongly correlated with each other, and their high values were determined in variant C+. The WSC vectors and two vectors with slightly lower loadings – Lac and Bac – were typical of all Tr variants, and all were moderately correlated with each other. In turn, the high DM content was shown mainly in Tr05 variants. Moreover, high values of NDF for Tr and C, Yea and crude ash for C and C + and Yield II for Tr and C + were observed (Fig. 2(A)).

The first two principal components of silages explained 76.3% of the variance, with component 1 explaining 42.6% and component 2 explaining 33.7%. As in the case of the herbage, the PCA results demonstrated that all variants treated with tryptophan (Tr) were very similar to each other but significantly different from the control variant (C) and the variant with a high nitrogen dose (C+).

The control variant (C) was characterized with high values of pH, Pen, Yea, crude ash, Clo, ADF, and LoF (which were strongly positively correlated with each other but negatively correlated with LA and Et). Typical of the C+ variant were the strongly positively correlated CP, Fus, N-NH_3, and N-NH_3/TN as well as the less strongly correlated Lac. However, their values were moderately negatively correlated with DM, VA, and NDF. In this variant, analyses showed the presence of the Tri5 gene typical of the genus *Fusarium*. Typical of all Tr variants were high contents of lactic acid and WSC but also, partly, ethanol and DM contents. This is important because grass treatment with L-TRP during vegetation increases WSC content and in this way increases fermentation effectiveness by reducing lactic acid production and pH (Fig. 2(B)).

DISCUSSION

Literature data confirm enhanced plant yield after L-TRP treatment.[7,8,9] Similar observations of increasing WSC content in herbage were made by Khalifa *et al.*[9] for L-TRP-treated lupine. However, Khalifa *et al.*[9] we did not confirm a CP content increase upon L-TRP treatment. The positive effect of increasing the WSC content in herbage on the course of fermentation is consistent with previous findings reported by Owens *et al.*[20] The Tr05 dose allowed achieving its significantly lower N-NH_3 content, which points to the lesser extent of proteolytic transformation during ensilaging.[21] Silage from Tr5 variant also had the highest contents of lactic acid and WSC residue, which may be indicative of the higher effectiveness of lactic acid fermentation.[22] The silages from variant Tr05 had the lowest butyric acid and N-NH_3/TN content, i.e. indicators of this fermentation type, which are strongly correlated with each other.[22]

Feedstuffs, and particularly silages, often have undesirable loads of *Clostridium* spp., fungi, and *Bacillus* spp. Good practice is to use ensilaging additives; however, their effectiveness is not always satisfactory.[14,23,24] In the present study, using a biostimulant not only allowed increasing herbage yield but also contributed to minimizing the risk posed by *Clostridium* spp. and *Bacillus* spp., and to reduced fungal growth in the silages. This approach proves more effective and economically viable than the use of ensilaging additives.

CONCLUSIONS

In summary, the results of this study prove that perennial grass treatment with L-TRP with a dose of 5 kg ha^{-1} significantly affected herbage yield, making the amount of biomass produced similar to that obtained from the variant with a high nitrogen dose used for fertilization. Although the high nitrogen fertilization caused an increase in CP, grass treatment with L-TRP contributed

to a higher WSC content. The increased WSC content of Tr5 herbage resulted in a more favorable course of fermentation. Interestingly, L-TRP used in medium and high doses effectively reduced the loads of *Clostridium* spp. and fungi, and increased the *Bacillus* spp. load. It is worth emphasizing that although they are antagonists of plant pathogens,[25] the latter bacteria lead to the deterioration of ready silages under aerobic conditions.[22] The positive effect of L-TRP treatment during plant vegetation affected the key parameters of silage, including pH value, a high lactic acid content, and a high WSC content. This study points to the feasibility of using L-TRP to optimize nutrient consumption by *Lolium perenne* at stands with more inferior soil quality. It also provides pilot results that will allow the L-TRP dose to be optimized for future application in large-scale field investigations.

ACKNOWLEDGEMENTS

Project financially supported by the Ministry of Science and Higher Education in the range of the program entitled 'Regional Initiative of Excellence' for the years 2019-2022, Project No. 010/RID/2018/19, amount of funding 12.000.000 PLN.

REFERENCES

1 Tester M and Bacic A, Abiotic stress tolerance in grasses. From model plants to crop plants. *Plant Physiol* **137**:791–793 (2005).
2 Hildebrandt TM, Nunes Nesi A, Arau´jo WL and Braun HP, Amino acid catabolism in plants. *Mol Plant* **8**:1563–1579 (2015).
3 El-Awadi ME, Dawood MG and Assan EAH, Role of amino acids as plant bio-stimulants (review). *World Rural Obs* **11**:1–12 (2019).
4 Villareal SQ, Hernández NZ, Romero IL, Lazcano EA and Dorantes AR, Assessment of plant growth promotion by rhizobacteria supplied with tryptophan as phytohormone production elicitor on *Axonopus affinis*. *Agric Sci Res J* **2**:574–580 (2012).
5 Idris E, Bochow ES, Ross H and Borriss R, Use of Bacillus subtilis as biocontrol agent. VI Phytohormone like action of culture filtrates prepared from plant growth-promoting *Bacillus amyloliquefaciens* FZB24, FZB42, FZB45 and Bacillus subtilis FZB37. *J Plant Dis Prot* **111**:583–590 (2004).
6 Patten C and Glick BR, Bacterial biosynthesis of indole-3- acetic acid. *Can J Microbiol* **42**:207–220 (1996).
7 Ahmad R, Khalid M, Naveed M, Shahzad SM, Zahir ZA and Khokhar SN, Comparative efficiency of auxin and its precursor applied through compost for improving growth and yield of maize. *Pak J Bot* **40**: 1703–1710 (2008).
8 El-Hosary AA, Hammam GY, El-Morsi A, Hassan EA, El-Awadi ME and Abdel Baky YR, Effect of some bio-regulators on growth and yield of some wheat varieties under newly cultivated land conditions. *Sci Nat* **2**:75–83 (2013).
9 Khalifa YAM, Abd El-Naem GF and Mahmoud MA, Effect of tryptophan and ascorbic acid on yield and some chemical constituents of lupine (lupines termis L.) plants. *Egypt J Agron* **42**:47–61 (2020).
10 Sarkar PK, Haque MH and Karim MA, Effects of GA3 and IAA and their frequency of application on morphology, yield contributing characters and yield of soybean. *Pak J Agron* **1**:119–122 (2002).
11 Clifford PE, Early bending kinetics in response to geostimulation or unilateral IAA application in *Lolium* nodal segments. *Plant Cell Environ* **11**:621–628 (1988).
12 Li W, Wang D, Hu F, Li H, Ma L and Xu L, Exogenous IAA treatment enhances phytoremediation of soil contaminated with phenanthrene by promoting soil enzyme activity and increasing microbial biomass. *Environ Sci Pollut Res* **23**:10656–10664 (2016).
13 Lübberstedt T, Schejbel Andreasen B and Bach Holm P, Development of ryegrass allele-specific (GRASP) markers for sustainable grassland improvement – a new EU framework V project. *Czech J Genet Plant Breed* **39**:125–128 (2003).
14 Fijałkowska M, Przemieniecki SW, Purwin C, Lipiński K, Kurowski TP and Karwowska A, The effect of an additive containing three *Lactobacillus* species on the fermentation pattern and microbiological status of silage. *J Sci Food Agric* **100**:1174–1184 (2020).
15 AOAC, *Official Methods of Analysis*, 15th edn. Association of Official Analytical Chemists, Washington, DC (2005).
16 Thomas TA, An automated procedure for the determination of soluble carbohydrates in herbage. *J Sci Food Agric* **28**:639–642 (1977).
17 van Soest PJ, Robertson JB and Lewis BA, Symposium: carbohydrate methodology, metabolism and nutritional implications in dairy cattle. *J Dairy Sci* **74**:3583–3597 (1991).
18 Kostulak-Zielińska M and Potkański A, Quality of baled grass-clover silages ensiled with chemical additives. Chemical composition. *Ann Anim Sci* **1**:153–165 (2001).
19 Hammer Ø, Harper DAT and Ryan PD, PAST: paleontological statistics software package for education and data analysis. *Palaeontol Electron* **4**:1–9 (2001). http://palaeo-electronica.org/2001_1/past/issue1_01.htm.
20 Owens VN, Albrecht KA, Muck RE and Duke SH, Protein degradation and fermentation characteristics of red clover and alfalfa silage harvested with varying levels of total non-structural carbohydrates. *Crop Sci* **39**:1873–1880 (1999).
21 Davies DR, Merry RJ, Williams AP, Bakewell EL, Leemans DK and Tweed JK, Proteolysis during ensilage of forages varying in soluble sugar content. *J Dairy Sci* **81**:444–453 (1998).
22 McDonald P, Henderson AR and Heron SJE, *The Biochemistry of Silage*, 2nd edn. Chalcombe publications, Marlow Bucks (1991).
23 König W, Lamminen M, Weiss K, Tuomivirta TT, Sanz Muñoz S, Fritze H *et al.*, The effect of additives on the quality of white lupin–wheat silage assessed by fermentation pattern and qPCR quantification of clostridia. *Grass Forage Sci* **72**:757–771 (2017).
24 Seppälä A, Heikkilä T, Mäki M and Rinne M, Effects of additives on the fermentation and aerobic stability of grass silages and total mixed rations. *Grass Forage Sci* **71**:458–471 (2016).
25 Przemieniecki S, Kurowski T, Damszel M, Krawczyk K and Karwowska A, The effectiveness of the *Bacillus* sp. SP-A9 strain as a biological control agent for spring wheat (*Triticum aestivum* L.). *J Agric Sci Technol* **20**:609–619 (2018).

