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Increasing Biogas Yield from Fodder by Microbial Stimulation of Propionic Acid Synthesis in Grass Silages

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Abstract: A new direction in the use of lactic acid bacteria inoculants is their application for renewable raw materials ensiling for biogas production. The aim of the study was to demonstrate the possibility of stimulating the synthesis of propionic acid in the process of co-fermentation of selected strains of *Lactobacillus buchneri* and *L. diolivorans* as well as *L. buchneri* and *Pediococcus acidilactici*. *L. buchneri* KKP 2047p and *P. acidilactici* KKP 2065p were characterized by the special capabilities for both synthesis and metabolism of 1,2-propanediol. *L. diolivorans* KKP 2057p stands out for the ability to metabolize 1,2-propanediol to propionic acid. As a result of the co-fermentation a concentration of propionic acid was obtained at least 1.5 times higher in the final stage of culture in comparison to cultivating individual species of bacteria separately. The results of in vitro experiments were applied in agricultural practice, by application of two lactic acid bacteria inoculants in ensiling of grass silage and improving its suitability for biogas production. Grass silages made with the addition of the inoculant were characterized by the content of 1,2-propanediol, 1-propanol and propionic acid ensured extension of the aerobic stability from 4 to 7 days in comparison to untreated silages. It was found that the use of both inoculants resulted in an approximately 10–30% increase in biogas yield from this raw material.

Keywords: 1,2-propanediol; biogas; grass silage; *Lactobacillus*; *Pediococcus*; propionic acid

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1. Introduction

Biodiversity of lactic acid bacteria results not only from a great number of species, but also from a variety of strains isolated from different environments, which may differ at the genetic and phenotypic level, what influences their biochemical properties, and thus their functional characteristics [1]. The biochemical knowledge on lactic acid bacteria (LAB) metabolism can be practically applied when composing bacterial starter cultures in order to stimulate the synthesis of desirable metabolites in biotechnological processes [2–4]. An economically attractive technology to generate bioenergy is the production of biogas that is a mixture of methane and carbon dioxide as the main components. Biogas is a valuable renewable energy and also a secondary energy carrier produced from biodegradable organic materials via anaerobic digestion [5]. Because of a growing demand for renewable energy production grassland biomass can be an excellent feedstock for biogas production, but its ensiling is necessary for the efficient preservation of the energy content of a crop during the bacterial fermentation. Favorable changes in the chemical properties of silages transfer into an increase in the biogas yield from the dry matter of the organic feedstock subject to methane fermentation [6].

Raw plant biomass that can be converted into methane with high process efficiency should be characterized by high lactic and acetic acid concentrations, low concentrations

of butyric acid and ammonia-N [7,8]. Moreover, a wide range of LAB fermentation secondary products formed during ensiling can influence specific methane yield such as 1,2-propanediol and its derivatives. These low-mass metabolites also influence significantly the oxygen stability of ensiled raw material [7]. The fundamental factor determining the methane yield from ensiled plant biomass is the selection of LAB strains composing starter cultures of bacterial preparations intended for the preservation of feed and renewable raw materials [3,4,9].

A number of silage inoculants have been developed with the presence of heterofermentative *Lactobacillus buchneri* species and contribute to the extension of the aerobic stability of ensiled plants [9]. Biochemical changes under the influence of *L. buchneri* species are directed into stimulating the synthesis of acetic acid and 1,2-propanediol. Bacteria of the species *L. buchneri* produce 1,2-propanediol in the phosphogluconate pathway with the release of carbon dioxide [10]. The results of Quidé Elferink et al. [11] showed that *L. buchneri* and *L. parabuchneri* are able to synthesize 1,2-propanediol under anaerobic conditions by converting 1 mole of lactic acid to 0.5 mole of acetic acid and 0.5 mole of 1,2-propanediol [11].

L. diolivorans isolated from maize silage and described by Kroonemann et al. [12] as a new bacterial species utilizes 1,2-propanediol as the only carbon source and metabolizes it into 1-propanol and propionic acid [12]. The presence of cobalamin is an important factor for the course and efficiency of the biochemical route, because the vitamin is a cofactor of the diol dehydratase—an enzyme involved in the metabolism of 1,2-propanediol [13–15].

Zielińska et al. [16,17] described two interesting strains isolated from ensiled maize grain: *L. buchneri* KKP 2047p, which was characterized with the special capabilities for both synthesis and metabolism of 1,2-propanediol and *L. diolivorans* KKP 2057p with the ability to metabolize 1,2-propanediol to propionic acid in the presence of cobalamin. Recently, another uncommon new strain *Pediococcus acidilactici* was isolated from ensiled maize grain and characterized with the ability to grow in medium on 1,2-propanediol as the only carbon source, and thus revealed the ability to metabolize it. The aim of the study was to demonstrate the possibility of stimulating the synthesis of propionic acid in the process of co-fermentation of *L. buchneri* KKP 2047p and *L. diolivorans* KKP 2057p as well as *L. buchneri* KKP 2047p and *P. acidilactici* KKP 2065p. Preliminary experiments outlined the dynamics of 1,2-propanediol metabolites in new strain of *P. acidilactici* KKP 2065p cultured solely or in co-fermentation with *L. buchneri* KKP 2047p. The results of in vitro experiments in flasks cultures were applied in agricultural practice to demonstrate the improvement of physicochemical properties and aerobic stability of grass silage as well as improvement of silage suitability for biogas production by application of two bacterial strains inoculant during the ensiling process.

2. Materials and Methods

2.1. Bacterial Strains

In the study following strains of lactic acid bacteria were used: *L. buchneri* KKP 2047p, *L. diolivorans* KKP 2057p and *P. acidilactici* KKP 2065p. Strains were obtained from the Collection of Industrial Microorganisms at the prof. Waław Dąbrowski Institute of Agricultural and Food Biotechnology—State Research Institute in Warsaw and has been stored in the atmosphere of liquid nitrogen.

Genetic identification of *L. buchneri*, *L. diolivorans* and *P. acidilactici* species was performed by sequencing the 16S RNA gene fragment on the basis of results indicating a 99% degree of identity with the sequences for the species in the databases (www.ncbi.nlm.nih.gov/BLAST, accessed on 1 May 2021). Experimental strains identified as *L. buchneri* and *L. diolivorans* have been subjected for strain-specific reaction using RAPD-PCR (Random Amplification of Polymorphic DNA-Polymerase Chain Reaction) with M13 primer as described Andrighetto et al. [18] (5'-GAGGGTGGCGTTCT-3'). PCR products were separated on a 1.5% agarose gel (Sigma-Aldrich, St. Louis, MO, USA) stained with fluorescent DNA stain (GelRed, Biotium Inc., Hayward, CA, USA) [19,20]. The RAPD analysis of those strains

generated different bands profiles which indicated that each strain is genetically diverse (data not shown).

2.2. Flask Cultures Experiments

Lactic acid bacteria were cultivated for 21 days on modified MRS broth medium at 30 °C and under aerobic conditions inoculated with 3% v/v 24-h culture provided in Lactobacilli MRS broth (Difco, Franklin Lakes, NJ, USA). Modified MRS media contained 8.0 g/L (0.11 M) of 1,2-propanediol and glucose in the concentration of 2.5, 5.0 and 10.0 g/L when *Lactobacillus* co-fermentations were provided. Modified MRS media contained 6.0 g/L (0.09 M) or 8.0 g/L (0.11 M) of 1,2-propanediol and alternatively glucose in the concentration of 5.0 g/L, when *Pediococcus* species was cultured singly or along with *L. buchneri*. Vitamin B12 (100 µg/L) was added in each trial. In order to determine the dynamics of synthesis of the studied metabolites, supernatant samples were taken for analysis after 3, 7, 14 and 21 days. Bacterial biomass was centrifuged using a Jouan CR 3i centrifuge (Jouan S.A., Saint Herblain, France) (10,000 rpm, 10 min, 15 °C).

2.3. Grass Silage Production

Silage experiments were carried out under production conditions at the experimental station of the Institute of Technology and Life Sciences in Poland. Bacterial inoculants were prepared as described previously [21]. Biomass of bacteria was grown in biofermentors, at a temperature of 30 °C, with pH adjusted with the use of ammonia water to the value of 5.7 for 24 h. After centrifugation the biomass was mixed with carriers and biostimulants and freeze-dried. The finished preparation contained a bacterial count of 2×10^{10} CFU/g. Experimental silages from grasses were sprayed with the inoculant Biogas A which consisted of *L. buchneri* KKP 2047p and *L. diolivorans* KKP 2057p (50%: 50%) strains or inoculant Biogas B which consisted of *L. buchneri* KKP 2047p and *P. acidilactici* KKP 2065p (50%: 50%). Control silages were prepared without the bacterial inoculant. All silages were made from plants withered after cutting with a dry matter content of approximately 40–50%. The preparation, at a dose of 2×10^5 CFU/g of fresh plants was given after dissolving in water, in form of spraying using an applicator, before the plants were rolled up and bales were formed with an average weight of about 500–900 kg in stationary wrapping machine Metal-Racch Z-237. After 60 days of ensiling the probes were taken for analyses and pH value, lactic, acetic, 3-hydroxybutyric and propionic acids, 1-propanol and 1,2-propanediol content were determined. Control and experimental silages were subjected for biogas production.

2.4. Biogas Yield and Biogas Content Determination

Biogas yield was determined by methane fermentation method according to analytical procedure of Institute of Technology and Life Sciences in Poland. Biogas composition was determined by GA 2000 Geotechnical Instruments and Multi Gas gauge (United Kingdom). Determination of biogas yield from dry organic matter of agricultural raw material by five-week laboratory methane fermentation of samples with saturation of 10% organic substrates was performed according to German standard method DIN 38414-8 [22]. Unit of biogas yield—NI /kg organic dry matter (odm) is a standardized net liter of biogas obtained from the 1 kg of organic dry material of a given sample and refers to the stated dry matter content and organic dry matter content.

2.5. Analytical Methods

The extract of macerated silage was prepared with distilled water as described previously [21]. An adjusted dry matter content was determined according to ISO standard (PN-ISO 6496: 2002) and pH using the potentiometric method. Determination of L- and D-lactic acid content, acetic and 3-hydroxybutyric acid contents were conducted by enzymatic methods according to the manufacturer's protocols (Boehringer, Mannheim, Germany). Propionic acid, 1-propanol and 1,2-propanediol were determined by gas chromatography

according to Zielińska et al. [21]. Lactobacilli cells were enumerated by pour plating of Lactobacilli MRS agar medium according to ISO standard (PN-ISO 15214:2002). Aerobic stability test was performed according to Honig [23]. Determination of organic dry matter of silage was provided during combustion at 550 °C in Carbolite RWF 1200 muffle furnace. Biogas yield from ensiled renewable raw materials was performed by methane fermentation method, analysis of biogas composition was done using the GA 2000 Geotechnical Instruments and Multi Gas meter, Monitor 1312 Innova.

2.6. Statistical Analyses

The results were analyzed using STATISTICA 13.1 software (TIBCO Software Inc., Palo Alto, CA, USA). Standard deviations were calculated. To verify the statistical hypothesis about the normality of the distribution of experimental data, the Shapiro–Wilk test was used, and the hypothesis about the homogeneity of variance was verified using the Levene test and the Brown–Forsythe test. The ANOVA (analysis of variance) was used to assess the significance of differences. Tukey post-hoc test was applied to organize data in homogenous groups [24]. The significance level $\alpha = 0.05$ was chosen. Experiments were provided at least in triplicates.

3. Results

3.1. Stimulation of Propionic Acid Synthesis in the Co-Fermentation of *L. buchneri* KKP 2047p and *L. diolivorans* KKP 2057p

The aim of the preliminary flask culture experiment was to demonstrate the possibility of stimulating the synthesis of propionic acid in the process of co-fermentation of *L. buchneri* KKP 2047p and *L. diolivorans* KKP 2057p. There were provided three independent co-fermentations in modified MRS media, in which 1,2-propanediol was applied at a constant concentration (8.0 g/L) and variables amounts of glucose, respectively; 2.5; 5.0, and 10.0 g/L were used. The research has tended to focus on the importance of the second carbon source (glucose) presence in medium in the context of maximizing the biotransformation of 1,2-propanediol into carboxylic acid. The characteristics of the biochemical features of *L. buchneri* KKP 2047p and *L. diolivorans* KKP 2057p strains are presented in Table 1 and related to their ability to synthesize and/or metabolize 1,2-propanediol to propionic acid. Table S1 and Figure 1 report the dynamics of synthesis and production of carboxylic acids and their derivatives during 21 days of culture.

Table 1. Ability of strains species *L. buchneri*, *L. diolivorans* and *P. acidilactici* to synthesize and/or metabolize 1,2-propanediol.

Lactobacillus Strain	Source of Species/Strain Isolation	Specific Features		Reference
		1,2-propanediol * Content, Synthesized from Glucose, mg/100 mL	Propionic Acid Content, mg/100 mL	
<i>L. buchneri</i> KKP 2047p	maize silage	400.00 *	174.9 ¹	17,18
<i>L. buchneri</i> KKP 907p	meadow sward	120.00 *	no detected	24,37
<i>L. diolivorans</i> LMG 19667	maize silage	no	125.8 ²	13
<i>L. diolivorans</i> KKP 2057p	maize silage	no	125.4 ¹	18
<i>P. acidilactici</i> KKP 2065p	buckwheat grain	no	248.20 ³	—

* bacteria grown in MRS medium, ¹ bacteria grown in modified MRS medium (1,2-propanediol—8.0 g/L; glucose 10.0 g/L), ² bacteria grown in modified MRS medium (1,2-propanediol—2.9 g/L), 21 days, 30 °C, ³ bacteria grown in modified MRS medium (1,2-propanediol—8.0 g/L; glucose 5.0 g/L).

The general trend was observed for all variants of the experiment (Figure 1). The content of acetic acid and the intermediate metabolites of its conversion—propionic acid and 1-propanol, increased until the 14th day of culture. After then the concentration of 1-propanol decreased but the content of propionic acid increased to the end, reaching on 21st day the level of 223.0 mg/100 mL in medium containing 10.0 g/L glucose. The degree of 1,2-propanediol utilization reached 90.0%. When glucose content was lowered twice to 5.0 g/L, the propionic acid content reached on the 21st day of culture a higher level by 20% than that in medium with 10.0 g/L of glucose i.e., 270.09 mg/100 mL. The utilization rate

of 1,2-propanediol was 93.0%. Lowering the glucose concentration in the medium to the value of 5.0 g/L did not negatively affect the development of bacterial growth (Figure 1). On the 3rd day of culture, the number of bacterial cells grown in both media reached 4.0×10^9 CFU/mL (Table S1).

Figure 1. Metabolite profile of supernatant resulting from co-fermentation of strains *L. buchneri* KKP 2047p and *L. diolivorans* KKP 2057p in medium containing 8.0 g/L 1,2-propanediol and glucose as a carbon sources in concentration: (a) 2.5 g/L; (b) 5.0 g/L; (c) 10.0 g/L. There was showed a standard deviation for each measurement.

In the experimental variant with a 2.5 g/L glucose content in medium the content of propionic acid in the final day of research was 285.20 mg/100 mL, it increased only by 15 mg/100 mL in relation to the results obtained during the cultivation of lactobacilli in medium with 5.0 g/L of glucose. The growth of bacterial cells on the 3rd day of culture was three times less intensive, and the degree of the use of 1,2-propanediol decreased to 85.0% (Figure 1, Table S1).

It was found that the addition of glucose at the level of at least 5.0 g/L turned out to be essential for the optimal growth of 2-strain bacterial starter culture consisted of *L. buchneri* KKP 2047p and *L. diolivorans* KKP 2057p. Therefore, almost all loadings of 1,2-propanediol were used for lactobacilli growth and biotransformation to expected metabolites. Further reduction of glucose to the level of 2.5 g/L inhibited the growth and metabolism of bacterial cells. Moreover, it has been shown that the process of propionic acid synthesis depends to a large extent on the carbon source used in the culture medium and as a consequence the

interaction of the *L. buchneri* KKP 2047p and *L. diolivorans* KKP 2057p. The complementary ability of these bacteria to synthesize and to metabolize 1,2-propanediol to propionic acid was proved phenotypically and investigated during ensiling of grass in the next step of the study.

3.2. Stimulation of Propionic Acid Synthesis in *L. buchneri* KKP 2047p and *P. acidilactici* KKP 2065p Co-culture

The newly isolated *P. acidilactici* KKP 2065p with the unexpected ability to synthesize propionic acid was a subject of further investigations aimed to provide evidence of its usefulness in composing LAB starter cultures. Firstly, *Pediococcus* strain was cultured solely in media with 1,2-propanediol or glucose as carbon sources. Table S2 and Figure 2 illustrates changes in the content of metabolites measured after 3, 7, 14 and 21 days. What can be observed, independently from the level of 1,2-propanediol used (6.0 or 8.0 g/L) the content of 1-propanol and propionic acid increased, while acetic acid and 1,2-propanediol concentration decreased. On 21st day of the experiment, the utilization rate of 1,2-propanediol reached 98% for all variants. The highest change in the content of propionic acid was observed between 3rd and 7th day when cultures reached the stationary growth phase (from 47.17 to 119.13 mg/100 mL in the medium containing 6.0 g/L 1,2 propanediol without glucose, from 87.05 to 150.40 mg/100 mL in medium containing 6.0 g/L 1,2 propanediol and 5.0 g/L glucose, and from 112.10 to 180.40 mg/100 mL in the medium containing 8.0 g/L 1,2 propanediol and 5.0 g/L glucose).

The third medium was characterized by an increased content of 1,2-propanediol to 8.0 g/L with the addition of glucose in the same dose as in the second medium (5.0 g/L). Under these conditions the strain of *P. acidilactici* was grown separately, as well as in the combined co-fermentation with *L. buchneri* KKP 2047p to demonstrate the synergy of their action towards stimulating the synthesis of propionic acid from 1,2-propanediol. The use of glucose in the amount of 5.0 g/L provided a good start for the growth of the bacterial inoculum (Figure 2). On the third day of cultivation, their number in both experiments was 3×10^9 CFU/mL (Table S2). During the culture with *P. acidilactici* KKP 2065p single strain, the level of propionic acid on the 21st day reached the value of 248.2 mg/100 mL (Figure 2).

The results of the co-fermentation revealed the dynamic growth of bacteria resulting in an increase of the acetic acid content approximately 1.5 times compared to previous experimental results (0.34 mg/100 mL in 3rd day in comparison to 0.20 in second medium when *P. acidilactici* KKP 2065p was grown solely). On the third day of the combination cultivation, the content of propionic acid reached the value of 162.10 mg/100 mL and at the end of the culture, its content increased to 352.00 mg/100 mL, with the 1,2-propanediol utilization rate accounting for 99.9%. In the case of co-fermentation of the synergistically acting strains of *L. buchneri* KKP 2047p and *P. acidilactici* KKP 2065p, a higher propionic acid content was obtained in the same medium by 30% compared to the cultivation of a single strain of the genus *P. acidilactici* (Figure 2).

3.3. Improvement of Aerobic Stability of Grass Silage and Biogas Yield under the Influence of the Lactic Acid Bacteria Inoculants

Promising results of the in vitro studies were next used in the farm practice. The action of the inoculant Biogas A containing *L. buchneri* KKP 2047p and *L. diolivorans* KKP 2057p strains in a 1:1 ratio and inoculant Biogas B containing *L. buchneri* KKP 2047p and *P. acidilactici* KKP 2065p in a 1:1 ratio was observed at a production scale. In order to assess the effectiveness of ensiling the chemical composition, oxygen stability and biogas yield of experimental silages, treated with the bacterial inoculants and untreated control silages were compared.

The oxygen stability test was carried out for 11 days after opening the silo by temperature analysis. Experimental silages with the addition of Biogas A inoculant were characterized by extended aerobic stability up to 7 days, while control silages without the addition of the preparation were stable for only 4 days (Figure 3). The similar observations were made for the second bacterial starter culture Biogas B (data not shown).

Figure 2. Metabolite profile of supernatant resulting from *P. acidilactici* KKP 2065p (a–c) culture and co-fermentation of strains *L. buchneri* KKP 2047p and *P. acidilactici* KKP 2065p (d) in medium containing glucose and/or 1,2-propanediol as a carbon sources (a)—medium containing 6.0 g/L 1,2-propanediol; (b) medium containing 6.0 g/L 1,2-propanediol and 5.0 g/L glucose; (c,d) medium containing 8.0 g/L 1,2-propanediol and 5.0 g/L glucose. There was showed a standard deviation for each measurement.

Figure 3. The effect of inoculant Biogas A consisted of *L. buchneri* KKP 2047p and *L. diolivorans* KKP 2057p on the aerobic stability test of grass silages.

The high content of acetic acid, 1,2-propanediol and propionic acid ensures silage an extension of aerobic stability [6,25]. Under the influence of the bacterial preparations Biogas A and B, the experimental silages were characterized by high quality, lack of butyric acid, higher content by 25% lactic acid and about two times acetic acid and more than ten times higher content of propionic acid in relation to the concentration of those metabolites in control silage (116.4 mg/100 g and 138.32 when Biogas A and B inoculants were used respectively) (Table 2). In addition, 1,2-propanediol and 1-propanol were found in silages treated with Biogas A reaching 97.8 and 90.6 mg/100 g and 100.8 and 90.4 mg/100 g in Biogas B inoculated silages.

Table 2. The content of organic acids, 1,2-propanediol and 1-propanol in grass silages, prepared with and without the addition of the bacterial inoculant Biogas A consisted of *L. buchneri* KKP 2047p and *L. diolivorans* KKP 2057p or bacterial inoculant Biogas B consisted of *L. buchneri* KKP 2047p and *P. acidilactici* KKP 2065p. There was showed a standard deviation for each measurement (after the symbol “±”), n.d.—not detected.

Silage	pH	Volatile Carboxylic Acids Content, g/100 g of Fresh Mass Silage			Metabolite Content, mg/100 g of Fresh Mass Silage		
		Lactic	Acetic	3-Hydroxy Butyric	1,2-Propanediol	1-Propanol	Propionic Acid
Untreated (control A)	5.10 ± 0.1	1.18 ^a ± 0.2	0.22 ^a ± 0.1	0.09 ^a ± 0.15	n.d. ^a	n.d. ^a	10.8 ^a ± 3.4
Treated with bacterial inoculant Biogas A	4.48 ± 0.1	1.48 ^a ± 0.3	0.45 ^{ab} ± 0.15	n.d. ^b	97.8 ^b ± 11.3	90.6 ^b ± 11.4	116.4 ^b ± 6.8
Untreated (control B)	4.55 ± 0.1	1.25 ^a ± 0.2	0.27 ^a ± 0.1	0.18 ^a ± 0.15	n.d. ^a	n.d. ^a	8.2 ^a ± 3.4
Treated with bacterial inoculant Biogas B	4.53 ± 0.1	1.40 ^a ± 0.3	0.67 ^b ± 0.15	n.d. ^b	100.8 ^b ± 9.3	90.4 ^b ± 9.4	138.32 ^c ± 6.5

^{a, b, c}—homogeneous groups designated on the basis of Tukey’s test.

Biogas yields from untreated and treated silages were compared by means of five-week laboratory methane fermentation in order to evaluate the usefulness of bacterial inoculants for biogas purposes. The substrate loading of the fermenting mixture was 10% in these experiments. The results for biogas and methane yield are presented in Table 3. Compared to the biogas yield obtained from control silage, the methane fermentation of experimental silage resulted in biogas yield higher by 160.1 and by 45.1 NI /kg organic dry matter, additionally with a higher methane content by 8.5 and 6.7% in respect to Biogas A and B inoculants (Table 3).

Table 3. Biogas yield and biogas purity produced from grass silage: untreated (control) and treated with the bacterial inoculant Biogas A consisted of *L. buchneri* KKP 2047p and *L. diolivorans* KKP 2057p or bacterial inoculant Biogas B consisted of *L. buchneri* KKP 2047p and *P. acidilactici* KKP 2065p. There was showed a standard deviation for each measurement (after the symbol “±”).

Silage	Dry Matter, %	Loading the Fermentation Mixture with Organic Dry Matter odm *, %	Biogas Yield NI/kg odm *	Methane in Biogas, %
Untreated (control A)	45.0 ± 0.9	70.4 ± 2.0	367.3 ± 18.7	58.4 ± 1.4
Treated with bacterial inoculant Biogas A	44.1 ± 0.9	69.8 ± 2.1	527.4 ± 25.5	66.9 ± 2.1
Untreated (control B)	50.8 ± 3.6	66.4 ± 2.1	461.9 ± 23.1	61.9 ± 2.2
Treated with bacterial inoculant Biogas B	41.5 ± 0.9	66.6 ± 2.1	507.3 ± 23.5	68.6 ± 1.5

* odm—organic dry matter.

The use of the bacterial inoculants for grass ensiling positively influenced the biogas potential of silages. Experimental grass silages with the addition of the preparation used as a substrate for biogas production showed accelerated and anticipating dynamics of the methane fermentation process in comparison to the control silage, without its addition. The effect was more visible to the bacterial starter culture consisted of two *Lactobacillus* strains, but despite the closer levels of biogas yields obtained from meadow grass silages treated with *Pediococcus* cells, the usefulness of Biogas B inoculant was still promising.

4. Discussion

Based on the results described by Zielińska et al. [16] it was evaluated that in the case of *L. buchneri* KKP 2047p and *L. diolivorans* KKP 2057p being cultured for 21 days separately, under comparable conditions to the study presented in this article, different propionic acid concentrations were obtained. For the culture of *L. buchneri* KKP 2047p, the final content of propionic acid was 174.9 mg/100 mL. *L. diolivorans* KKP 2057p grown in modified MRS medium with 1,2-propanediol (0.8 g/100 mL) was characterized by the ability to synthesize propionic acid in concentration of 122.8 mg/100 mL (Table 1). Kroonemann et al. [12] obtained a similar final level of propionic acid 125.8 mg/100 mL, after 21 days of *L. diolivorans* LMG 19667 strain culture in medium containing 1,2-propanediol as the only carbon source (Table 1).

The results of model studies presented by the authors highlighted the synergistic effect of bacterial strains of the studied *L. buchneri* and *L. diolivorans* species what was not reported in the literature before. The finding points to complementary ability of the species to synthesize and/or metabolize 1,2-propanediol to propionic acid. As a consequence of the co-fermentation of these species, under comparable bacterial culture conditions, at least 1.5 times more propionic acid was identified in culture supernatant than in the case of individual culture of bacterial species. Further molecular studies should be provided in order to estimate the efficiency of conversion of 1,2-propanediol synthesized by *L. buchneri* to propionic acid synthesized by *L. diolivorans*.

In addition, the results have further strengthened our confidence that the level of complementary carbon source—glucose, which is a starter substrate for proliferating of lactic acid bacteria cells, has a significant impact on the synthesis of propionic acid from 1,2-propanediol. In the medium with a glucose content of 5.0 g/L, the level of propionic acid after 21 days of co-fermentation was 270.09 mg/100 mL, and after further reduction of the glucose content to 2.5 g/L, the final level of propionic acid reached 285.20 mg/100 mL. With this culture the concentration of glucose affected bacterial growth and caused a decrease in the use of the second substrate—1,2-propanediol from 93 to 85% (Figure 1). It was found that supplementary glucose addition to the culture medium (in the amount of 5.0 g/L), in which the main carbon source was 1,2-propanediol, turned out to be necessary for development of bacterial inoculum and thus for metabolizing 1,2-propanediol to propionic acid by bacterial cells. Depending on the tested bacterial strain, under the same culture conditions and the availability of the basic carbon source—1,2-propanediol, a different level of the final product of its metabolism, i.e., propionic acid, could have been obtained. The same phenomenon was observed in the case of fermentation of *P. acidilactici* KKP 2065p. The content of propionic acid in the supernatant was much higher when glucose was a medium ingredient. Unfortunately, there are no other similar studies to compare or confront our own results.

In the current attempt, there was revealed that the newly isolated and characterized strain of *P. acidilactici* KKP 2065p was more effective in the process of 1,2-propanediol metabolism than the known and described strains of the species *L. diolivorans*, as evidenced by the data presented in Table 1 and Figure 2. This encouraged to use *P. acidilactici* strain in starter culture in ensiling process with the combination of other widely used and described in such studies—*L. buchneri*. Effects of two inoculant Biogas A and Biogas B were set together in order to compare the effect at farm conditions to prepare silages with high potential for biogas production. It is probably first attempt to apply *P. acidilactici* in co-fermentation experiments with *L. buchneri* species and one of a few using starter culture containing *L. diolivorans* and *L. buchneri* species.

Our results share a number of similarities claimed by other authors, who describe improvement of aerobic stability of maize silage under the influence of bacteria of the *L. buchneri* species, as well as *L. buchneri* followed by the addition of commonly used lactic acid bacteria e.g., *L. plantarum* [17,26,27]. Moreover, Nishino et al. [2] found that when *L. buchneri* inoculants were used to ensilage maize, a significant improvement in the oxygen stability was observed due to the accumulation of 1,2-propanediol in the ensiled plant

material. The positive effect of propionic acid production synthesized as a consequence of 1,2-propanediol accumulation in *L. buchneri* and *L. diolivorans* co-fermentation of sourdough was also described by Zhang et al. [28]. Selwet showed that maize silages inoculated with *Lactobacillus* strains revealed better aerobic stability than control samples due to the higher acetic and propionic acid concentrations. Silages inoculated with starter culture consisted of *L. buchneri* ATCC 4005, *L. diolivorans* LGM 19667 and *L. reuteri* ATCC 23272 contained 1.4 g/100 g dm. No 1,2-propanediol and 1-propanol were found in the control samples [29]. The same observations were made in our research.

Many studies have focused on improved silage quality e.g., increased silage digestibility, dry matter intake and prevention from spoilage microorganisms followed by rumen fermentation parameters [30]. Interestingly some bacterial inoculants are intentionally added prior decreasing methane production in rumen. Khota et al. [31] confirmed that *L. casei* TH14 could improve sorghum silage fermentation, inhibit protein degradation and decrease methane fermentation during in vitro studies. At the same time, the scientific literature lacks data on bacterial inoculants impact on biogas yield from silages. Sparse studies are in agreement with our results. McEniry et al. [8] claimed that grass silage fermentation characteristics appear to have relatively little effect on specific methane yield. However, the relatively high concentrations of fermentation products such as butyric acid in the heterofermentative LAB treatments by inoculant Pioneer 11A44 containing *L. buchneri* resulted in a numerically higher specific methane yield.

What can be also underlined is the fact that the common energy crops are maize or sorghum. Meanwhile the methane content achieved in grass silages in this study was more or of the same amount than other authors' results e.g., with one ton of corn silage with a content of 30% to 40% of the dry matter, biogas with a content of 50–55% methane could be obtained. With 1 ton of sorghum silage, it produced biogas with 54% methane content. The highest cumulative values of biogas production were obtained for Elvira beet, the methane content was 64 % [32]. In our study the average methane content in grass silages was 66.9% and 68.6% for Biogas A and Biogas B inoculants, respectively.

5. Conclusions

Model experiments on co-fermentation of bacterial strains from the species: *L. buchneri* and *L. diolivorans* or *P. acidilactici* indicated the possibility of controlling and stimulating the synthesis of acetic acid, its conversion to 1,2-propanediol and metabolizing this compound to propionic acid. The utilization of subsequent substrates during the culture of the studied bacteria (1,2-propanediol, glucose), followed by the synthesis of 1-propanol and propionic acid, allowed their transformation in the growth environment. As a result of the co-fermentation a 1.5 times higher concentration of propionic acid was obtained at least in the final stage of culture when compared with individual species of bacteria grown separately.

Lactic acid and volatile organic acids such as acetic and propionic acids are suitable methane fermentation substrates, not necessary often appreciated in order of possessing high-yield biogas. Ensiled green biomass can be rich in those volatile short-chain fatty acid and though it can be considered as a good substrate for agriculture biogas plants. In the paper we presented a method of boosting microbial processes in grass silages in order to increase especially propionic acid content and thus increasing the biogas yield in comparison to the non-inoculated fodder.

The successful transfer of microbial inoculants from the laboratory to the field remains a challenge. This is primarily due to the presence of many crop species and crop varieties, variable environmental conditions between fields, and the exponential increase in the number of microbial isolates. The limitations of collecting data during in vivo experiments on microbial culture reconstruction experiments and model organisms for understanding plant–microbe interactions also limit the progress in this field [33]. There was a significant positive effect acknowledged by our results. Grass silages made with the addition of an inoculant containing the starter culture of *L. buchneri* KKP 2047p and *L. diolivorans* KKP 2057p (Biogas A) or *P. acidilactici* KKP 2065p (Biogas B) were characterized by the content

of 1,2-propanediol, 1-propanol and propionic acid at a level ensuring the extension of their aerobic stability from 4 to 7 days in comparison to untreated silages. It was found that the use of inoculants resulted in an approximately 10–30% increase in biogas yield, including methane from this raw material. The combination of bacterial species used in the inoculant had a positive effect on the quality parameters of silages, in terms of their use in a biogas plant and therefore this direction of research is worth continuing. These results raise many questions regarding the biochemical background of interactions between the strains present in the bacterial starter culture. There is also a need to discuss whether other combinations of lactic acid bacteria strains could refine green biomass preservation for energy purposes. These issues should be further studied, and molecular biology tools should be used.

6. Patents

Patent PL no. 236769 “Nowy szczep bakterii *Pediococcus acidilactici* o zdolności metabolizowania 1,2-propanodiolu, zawierająca go kultura starterowa oraz preparat.” Zielińska K., Piasecka-Józwiak K., Świątek M., Miecznikowski A. (2018) [in Polish].

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/en14102843/s1>, Table S1. Metabolite profile of supernatant resulting from co-fermentation of strains *L. buchneri* KKP 2047p and *L. diolivorans* KKP 2057p in medium containing 1,2-propanediol and glucose as a carbon sources; Table S2. Metabolite profile of supernatant resulting from *P. acidilactici* KKP 2065p culture and co-fermentation of strains *L. buchneri* KKP 2047p and *P. acidilactici* KKP 2065p in medium containing glucose and/or 1,2-propanediol as a carbon source.

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References

1. Sun, Z.; Yu, J.; Dan, T.; Zhang, W.; Zhang, H. Phylogenesis and Evolution of Lactic Acid Bacteria. In *Lactic Acid Bacteria*; Metzler, J.B., Ed.; CRC Press: Boca Raton, FL, USA, 2014; pp. 1–101.
2. Nishino, N.; Yoshida, M.; Shiota, H.; Sakaguchi, E. Accumulation of 1,2-propanediol and enhancement of aerobic stability in whole crop maize silage inoculated with *Lactobacillus buchneri*. *J. Appl. Microbiol.* **2003**, *94*, 800–807. [CrossRef]
3. Jatkauskas, J.; Vrotniakienė, V.; Ohlsson, C.; Lund, B. The effects of three silage inoculants on aerobic stability in grass, clover-grass, lucerne and maize silages. *Agric. Food Sci.* **2013**, *22*, 137–144. [CrossRef]
4. Soucaille, P.; Meynial-Salles, I.; Voelker, F.; Figge, R. Microorganisms and Methods for Production of 1,2-Propanediol and Acetol. U.S. Patent Application 9051588, 9 June 2015.
5. Awe, O.W.; Zhao, Y.; Nzihou, A.; Minh, D.P.; Lyczko, N. A Review of Biogas Utilisation, Purification and Upgrading Technologies. *Waste Biomass Valorization* **2017**, *8*, 267–283. [CrossRef]
6. Herrmann, C.; Idler, C.; Heiermann, M. Improving aerobic stability and biogas production of maize silage using silage additives. *Bioresour. Technol.* **2015**, *197*, 393–403. [CrossRef] [PubMed]
7. Amon, T.; Amon, B.; Kryvoruchko, V.; Zollitsch, W.; Mayer, K.; Gruber, L. Biogas production from maize and dairy cattle manure—Influence of biomass composition on the methane yield. *Agric. Ecosyst. Environ.* **2007**, *118*, 173–182. [CrossRef]
8. McEniry, J.; Allen, E.; Murphy, J.; O’Kiely, P. Grass for biogas production: The impact of silage fermentation characteristics on methane yield in two contrasting biomethane potential test systems. *Renew. Energy* **2014**, *63*, 524–530. [CrossRef]
9. Muck, R.; Nadeau, E.; McAllister, T.; Contreras-Govea, F.; Santos, M.; Kung, L. Silage review: Recent advances and future uses of silage additives. *J. Dairy Sci.* **2018**, *101*, 3980–4000. [CrossRef]
10. Kung, L.; Taylor, C.; Lynch, M.; Neylon, J. The Effect of Treating Alfalfa with *Lactobacillus buchneri* 40788 on Silage Fermentation, Aerobic Stability, and Nutritive Value for Lactating Dairy Cows. *J. Dairy Sci.* **2003**, *86*, 336–343. [CrossRef]
11. Elferink, S.J.W.H.O.; Krooneman, J.; Gottschal, J.C.; Spoelstra, S.F.; Faber, F.; Driehuis, F. Anaerobic Conversion of Lactic Acid to Acetic Acid and 1,2-Propanediol by *Lactobacillus buchneri*. *Appl. Environ. Microbiol.* **2001**, *67*, 125–132. [CrossRef]

12. Krooneman, J.; Faber, F.; Alderkamp, A.C.; Elferink, S.J.H.W.O.; Driehuis, F.; Cleenwerck, I.; Swings, J.; Gottschal, J.C.; Vancanneyt, M. *Lactobacillus diolivorans* sp. nov., a 1,2-propanediol-degrading bacterium isolated from aerobically stable maize silage. *Int. J. Syst. Evol. Microbiol.* **2002**, *52*, 639–646. [[CrossRef](#)]
13. Toraya, T. The structure and the mechanism of action of coenzyme B12-dependent diol dehydratases. *J. Mol. Catal. B Enzym.* **2000**, *10*, 87–106. [[CrossRef](#)]
14. Taranto, M.P.; Vera, J.L.; Hugenholtz, J.; De Valdez, G.F.; Sesma, F. *Lactobacillus reuteri* CRL1098 Produces Cobalamin. *J. Bacteriol.* **2003**, *185*, 5643–5647. [[CrossRef](#)]
15. Sriramulu, D.D.; Liang, M.; Hernandez-Romero, D.; Raux-Deery, E.; Lünsdorf, H.; Parsons, J.B.; Warren, M.J.; Prentice, M.B. *Lactobacillus reuteri* DSM 20016 Produces Cobalamin-Dependent Diol Dehydratase in Metabolosomes and Metabolizes 1,2-Propanediol by Disproportionation. *J. Bacteriol.* **2008**, *190*, 4559–4567. [[CrossRef](#)]
16. Zielińska, K.; Fabiszewska, A.; Świątek, M.; Szymanowska-Powałowska, D. Evaluation of the ability to metabolize 1,2-propanediol by heterofermentative bacteria of the genus *Lactobacillus*. *Electron. J. Biotechnol.* **2017**, *26*, 60–63. [[CrossRef](#)]
17. Zielińska, K.J.; Fabiszewska, A.U. Improvement of the quality of maize grain silage by a synergistic action of selected lactobacilli strains. *World J. Microbiol. Biotechnol.* **2018**, *34*, 9. [[CrossRef](#)] [[PubMed](#)]
18. Andrighetto, C.; Zampese, L.; Lombardi, A. RAPD-PCR characterization of lactobacilli isolated from artisanal meat plants and traditional fermented sausages of Veneto region (Italy). *Lett. Appl. Microbiol.* **2001**, *33*, 26–30. [[CrossRef](#)] [[PubMed](#)]
19. Williams, J.G.; Kubelik, A.R.; Livak, K.J.; Rafalski, J.A.; Tingey, S.V. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* **1990**, *18*, 6531–6535. [[CrossRef](#)] [[PubMed](#)]
20. Oneca, M.; Irigoyen, A.; Ortigosa, M.; Torre, P. PCR and RAPD identification of *L. plantarum* strains isolated from ovine milk and cheese. Geographical distribution of strains. *FEMS Microbiol. Lett.* **2003**, *227*, 271–277. [[CrossRef](#)]
21. Zielinska, K.; Fabiszewska, A.; Stefanska, I. Different aspects of *Lactobacillus* inoculants on the improvement of quality and safety of alfalfa silage. *Chil. J. Agric. Res.* **2015**, *75*, 298–306. [[CrossRef](#)]
22. German National Standard. *German Standard Methods for the Examination of Water, Waste Water and Sludge; Sludge and Sediments (Group S); Determination of the Amenability to Anaerobic Digestion (S 8)*; DIN 38414-8; Deutsches Institut Fur Normung E.V.: Berlin Germany, 1985.
23. Honig, H. *Determination of Aerobic Deterioration*; System Volkenrode-Institut für Grünland und Futterpflanzenforschung der Bundesforschungsanstalt für Landwirtschaft Braunschweig-Volkenrode (FAL): Berlin, Germany, 1985.
24. Nelson, W. *Applied Life Data Analysis*; Wiley: Hoboken, NJ, USA, 1982.
25. Danner, H.; Holzer, M.; Mayrhuber, E.; Braun, R. Acetic Acid Increases Stability of Silage under Aerobic Conditions. *Appl. Environ. Microbiol.* **2003**, *69*, 562–567. [[CrossRef](#)]
26. Zhang, T.; Li, L.; Wang, X.-F.; Zeng, Z.-H.; Hu, Y.-G.; Cui, Z.-J. Effects of *Lactobacillus buchneri* and *Lactobacillus plantarum* on fermentation, aerobic stability, bacteria diversity and ruminal degradability of alfalfa silage. *World J. Microbiol. Biotechnol.* **2009**, *25*, 965–971. [[CrossRef](#)]
27. Lindsey, J.R.; Kung, L. Effects of combining *Lactobacillus buchneri* 40788 with various lactic acid bacteria on the fermentation and aerobic stability of corn silage. *Anim. Feed Sci. Tech.* **2010**, *159*, 105–109. [[CrossRef](#)]
28. Zhang, C.; Brandt, M.J.; Schwab, C.; Gänzle, M.G. Propionic acid production by cofermentation of *Lactobacillus buchneri* and *Lactobacillus diolivorans* in sourdough. *Food Microbiol.* **2010**, *27*, 390–395. [[CrossRef](#)]
29. Selwet, M. Influence of inoculation with *Lactobacillus* on fermentation, production of 1,2-propanediol and 1-propanol as well as Maize silage aerobic stability. *Open Life Sci.* **2020**, *15*, 373–378. [[CrossRef](#)] [[PubMed](#)]
30. Ellis, J.; Bannink, A.; Hindrichsen, I.; Kinley, R.; Pellikaan, W.; Milora, N.; Dijkstra, J. The effect of lactic acid bacteria included as a probiotic or silage inoculant on in vitro rumen digestibility, total gas and methane production. *Anim. Feed. Sci. Technol.* **2016**, *211*, 61–74. [[CrossRef](#)]
31. Khota, W.; Pholsen, S.; Higgs, D.; Cai, Y. Fermentation quality and in vitro methane production of sorghum silage prepared with cellulase and lactic acid bacteria. *Asian Australas. J. Anim. Sci.* **2017**, *30*, 1568–1574. [[CrossRef](#)] [[PubMed](#)]
32. Szlachta, J.; Prask, H.; Fugol, M.; Luberański, A. Effect of Mechanical Pre-Treatment of the Agricultural Substrates on Yield of Biogas and Kinetics of Anaerobic Digestion. *Sustainability* **2018**, *10*, 3669. [[CrossRef](#)]
33. Saad, M.M.; Eida, A.A.; Hirt, H. Tailoring plant-associated microbial inoculants in agriculture: A roadmap for successful application. *J. Exp. Bot.* **2020**, *71*, 3878–3901. [[CrossRef](#)] [[PubMed](#)]