agriculture

MDPI

Article

Screening Lactic Acid Bacteria Strains for Their Tolerance to Increased Osmotic Pressure and Their Suitability to Ensile High Dry Matter Forages

Siriwan D. Martens [1,*], Wolfgang Wagner [2], Mariana Schneider [3], Klaus Hünting [4], Susanne Ohl [5] and Christof Löffler [6]

[1] Department of Animal Husbandry, Saxon State Office for Environment, Agriculture and Geology (LfULG), Am Park 3, 04886 Köllitsch, Germany
[2] Agricultural Technology Center (LTZ) Augustenberg, Nesslerstr. 25, 76227 Karlsruhe, Germany
[3] Bavarian State Research Center for Agriculture (LfL), Institute for Animal Nutrition and Feed Management, Prof.-Dürrwaechter-Platz 3, 85586 Poing, Germany
[4] Experimental and Training Centre Agriculture Haus Riswick, Chamber of Agriculture North Rhine-Westphalia, Elsenpass 5, 47533 Kleve, Germany
[5] Research and Training Centre Futterkamp, Chamber of Agriculture Schleswig-Holstein, Gutshof, 24327 Blekendorf, Germany
[6] Agricultural Centre for Cattle Production, Grassland Management, Dairy Food, Wildlife and Fisheries Baden-Württemberg (LAZBW), Atzenberger Weg 99, 88326 Aulendorf, Germany
* Correspondence: siriwan.martens@smekul.sachsen.de

Abstract: Lactic acid bacteria (LAB) should not only survive, but also perform under increased osmotic pressure in the process of ensiling, which results from the best practice of wilting forage. Simple laboratory protocols are needed to select suitable LAB strains as inoculants for high dry matter (DM) conditions. The aim of this study was to simulate conditions of high osmolality without inducing salt stress and to select a suitable indicator of LAB performance. For that, an MRS medium was enriched with increasing concentrations of glucose and fructose plus a maximum of 28 g KCl/L until achieving an osmolality of 2.4 osmol/kg. Both, growth in the inoculated medium and pH decline, were then compared to the LAB performance in the basic medium. The latter was clearly delayed in the new medium. Finally, the method was validated by comparing the pH of small-scale grass silages of 30–35 and 45–49% target DM after 3–5 days of ensiling to the pH values of the microbiological growth medium. The pH levels of treatments with the homofermentative LAB were clearly attributable to the dry matter or the sugar concentration, respectively. The developed liquid growth medium sufficiently approximates high DM conditions to select for the osmotolerant homofermentative LAB.

Keywords: biological silage additives; ensiling; growth medium; osmolality; protocol; strain selection; wilting; validation

Citation: Martens, S.D.; Wagner, W.; Schneider, M.; Hünting, K.; Ohl, S.; Löffler, C. Screening Lactic Acid Bacteria Strains for Their Tolerance to Increased Osmotic Pressure and Their Suitability to Ensile High Dry Matter Forages. *Agriculture* **2024**, *14*, 825. https://doi.org/10.3390/agriculture14060825

Academic Editor: Siran Wang

Received: 30 April 2024
Revised: 22 May 2024
Accepted: 23 May 2024
Published: 25 May 2024

1. Introduction

The best ensiling practice includes the rapid wilting of forages to above 300 g dry matter (DM)/kg. This reduces silage effluent, decreases respiration and proteolysis, increases the concentration of water-soluble carbohydrates as fermentation substrate, and gives preference to lactic acid bacteria (LAB) compared to enterobacteria and clostridia in order to reduce the risk of butyric acid fermentation under anaerobic conditions [1–4]. Facultatively heterofermentative (colloquially referred to as "homofermentative") LAB are often added to promote lactic acid fermentation during ensiling. *Lactiplantibacillus plantarum* as one representative of this group tends to have a higher resistance to osmotic stress than *Clostridium butyricum*, especially at lower pH [5,6]. Osmotic stress is increased

by wilting [7]. The suppliers of biological silage additives face the challenge of offering LAB strains, which are particularly suitable for high dry matter conditions.

There have been attempts to simulate high osmotic pressure in microbiological culture media by increasing the concentration of osmotically active particles in the solution, which is the osmolality. Hoedtke [7] investigated the osmolality of various temperate forages and their silages. For *Lolium perenne* wilted to 46% DM, she determined an osmolality of 2.4 osmol/kg plant extract. Whereas some components of forages such as mono- and disaccharides and salts have an osmotic effect, others such as proteins and starch have very little effect [8,9]. To simulate the increased osmotic pressure and decreased water activity, salts such as NaCl have been added to a solution [10]. Moreover, in a test to approve biological silage additives for osmotolerance, KCl has been added to MRS agar (97 g KCl/L) as an elective agent and to simulate the reduced water activity at around 45% dry matter in forages [11]. Similarly, this is done in the Rostock fermentation test, a rapid in vitro test to evaluate the effectiveness of silage inoculants using minced forage material in an aqueous solution [12]. However, the observation of the strong inhibition of either the growth or activity of various LAB strains when adding salt was the starting point of the presented study to look for reasons and alternatives. Glasker et al. [13] found that both NaCl and KCl induce salt stress in *Lactiplantibacillus plantarum*, a species which is currently represented in many silage inoculants. Thus, these salts are less suitable to simulate hyperosmotic conditions in a biological system. In order to cultivate osmophilic microorganisms, mostly the use of glucose or sometimes saccharose is recommended [14]. Plate count is favored as a relatively simple and cost-effective standardized method. So, it was used in the above-mentioned approval scheme for ensiling agents [11]. However, in contrast to the objective of pure growth, for ensiling, the fermentation effectiveness is of eminent importance. As metabolic activity is sometimes less inhibited than cell propagation at increased osmolality [15,16], it is advised to rather measure an indicator for activity than to estimate cell counts. The Rostock fermentation test is one example of activity measurement in a simulated ensiling environment [12,17–19]. In the initial phase of its development, it was proven that the main agent that caused a pH decrease was lactic acid produced by the inoculated LAB. Consequently, pH was employed as an indicator of LAB activity in the present study.

The aim of this study was to develop a laboratory method to screen lactic acid bacteria for their lactic acid fermentation capacity in highly wilted forage. This approach included three steps:

(1) Developing a standardized medium (liquid as broth and solid with agar) for LAB with an osmolality of 2.4 osmol/kg (simulating about 45% DM in a temperate grass) without provoking salt stress.
(2) Testing the growth of different LAB strains on the solid medium developed in step (1) compared to an unmodified MRS (de Man, Rogosa, Sharpe [20]) medium, and measuring the acidification in the liquid medium (from step (1)) compared to the unmodified MRS broth. This two-track approach was followed to indicate metabolic activity in possible contrast to cell propagation.
(3) Validating the ensiling capacity of the selected LAB strains in low and high DM forage compared to the laboratory results, i.e., whether there were statistical effects of the DM level or medium on the final pH across all strains.

Hypothesis: *The modified medium provides a sufficiently accurate approach to simulate high osmolality conditions, which prevail in high DM forages, to reflect the metabolic activity of LAB strains in the initial anaerobic ensiling phase, indicated by pH development.*

2. Materials and Methods

2.1. Step 1 Development of a Medium for LAB with Increased Osmolality and Limited Salt Concentration

As a starting point, the minimum, maximum, and mean contents of sugars and mineral salts (Na, K, and Cl) as osmotically active substances in fresh grass of 70 samples from Saxony for the years 2018–2019 were calculated (Table 1).

Table 1. The content of sugars and mineral salts in fresh grass from pastures in Saxony (2018–2019), n = 70, calculated for 45% dry matter (DM).

Item	Mean	SEM	Median	Minimum	Maximum
Sugar (g/kg)	83.5	3.09	82.9	34.9	153
Sodium (g/kg)	0.34	0.039	0.20	0.05	1.80
Potassium (g/kg)	11.7	0.36	11.8	5.51	18.0
Chloride (g/kg)	2.56	0.178	2.06	0.53	6.09
Salts (sum) (g/kg)	14.6		14.1	6.09	25.8

To cover the nutrient requirements of LAB, an MRS medium was chosen as the basic medium (MRS Broth pH 5.7, Art. No. HP64.1, Carl Roth GmbH + Co. KG, Karlsruhe, Germany).

In a step-by-step approach, the osmolality of the medium was adapted to 2.4 osmol/kg using the increasing concentrations of glucose (D(+)-glucose-monohydrate, Art. No. 108342, Merck KGaA, Darmstadt, Germany) and fructose (D(−)-fructose, Art. No. 4981.4, Carl Roth GmbH + Co. KG, Karlsruhe, Germany) plus a maximum of 28 g KCl (Potassium chloride for analysis, Art. No. 1.04936, Merck KGaA, Darmstadt, Germany) per liter distilled water (Table 2).

Table 2. Increasing concentrations of glucose, fructose, and KCl (g) added to the MRS broth (containing 20 g glucose/L) and 1.0 L of distilled water to obtain ~2.4 osmol/kg in the medium.

Level	+Glucose	+Fructose	+KCl
0	0	0	0
1	10	0	0
2	10	10	0
3	10	20	0
4	10	30	0
5	10	30	14
6	10	30	28
7	30	30	0
8	30	60	0
9	30	60	14
10	30	60	28
11	60	30	0
12	60	60	0
13	60	60	14
14	60	60	28
15	80	80	28
16	100	100	28
17	110	110	28
18	120	120	28
19	130	130	28

The osmolality was determined using Gonotec® Osmomat® (Model 010, ELITechGroup Inc., Logan, UT, USA).

2.2. Step 2 Evaluation of Growth of and Acidification by Different LAB Strains in the Modified Media

The final medium was prepared both as a solid medium using bacteriological agar (GranaCult^{TM} MRS-Agar, Art. No. 1.10660, Merck KGaA, Darmstadt, Germany) and as a broth (see step 1). In total, LAB from 31 biological silage additives were grown. Of those, 10 products contained obligate heterofermentative LAB exclusively or predominantly (no. 10) (Table 3), in the following referred to as "heterofermentative". The other 21 products containing facultatively heterofermentative and obligately homofermentative strains are referred to as "homofermentative" in the following. The products were diluted in serial dilutions and plated on Petri dishes by the pour plate method. They were incubated aerobically at 30 °C for 3 d in the case of the standard MRS and 3 to 10 d in the high-sugar (HS) medium, depending on the growth of colonies, before counting. For pH measurement, the amount of product was inoculated in 10 mL broth as recommended by the manufacturer (g/g fresh matter = g/mL broth), and the tubes were incubated for a maximum of 72 h at 30 °C [20]. The pH was determined at 0, 24, 48, and 72 h using a pH meter (inoLab pH 720, electrode SenTix 81, WTW GmbH, Weilheim, Germany).

Table 3. List of biological additives tested *in vitro* and partly *in situ*, and their declared active lactic acid bacteria (LAB) species.

Additive No.	[1] *L. buchneri*	*L. plantarum*	*E. faecium*	*P. acidilactici*	*L. paracasei*	*L. lactis*	*L. rhamnosus*	*P. pentosaceus*	[1] *L. brevis*	[1] *L. diolivorans*	*L. kefir.*
1	x										
2	x										
3	x										
4	x										
5 *	x										
6	x										
7 #	x										
8	x										
9	x										
10	x						x			x	
11		x									
12 +#		x	x					x			
13		x						x			
14 *		x									
15	x	x					x				
16 +#				x	x	x					
17	x	x						x			
18	x	x					x	x	x		
19		x	x					x			
20 +#		x	x					x			
21		x						x			
22 +#		x	x					x			
23 *	x	x						x			x
24 +#		x	x								
25		x	x					x			
26	x	x					x				
27 +#		x									
28	x	x									
29		x									
30		x									
31		x									

[1] obligately heterofermentative species, x species contained in the additive, * additives were tested in situ at 4 institutes in 2022, [+] additives were tested in situ at 1 institute in 2022, and [#] additives were tested in situ at 3–5 institutes in 2023.

2.3. Step 3 Comparison of In Vitro pH Decreases in Growth Medium and In Situ in Temperate Grass at Two Wilting Levels

Year 1: From the 31 products tested in step 2, three additives were selected (nos. 5, 14, and 23) and tested on the grass at four different locations (institutes, see authors affiliations) in the south, east, north, and west of Germany (48°10′–54°17′ N; 6°10′–13°7′ E; 15–516 m ASL) in 2022. The forage was cut from different types of grassland. All four institutions located in Bavaria, North Rhine-Westphalia, Schleswig-Holstein, and Saxony used first-cut grass from from the vegetative to the generative phase. One of the tested additives was a heterofermentative LAB (no. 5, Table 3). Additionally, another two products were tested in one of the three institutes each (nos. 22, 27; 20, 24; 12, 16). Thus, a total of 9 additives were tested in situ in laboratory-scale silages. A DM content of around 30–35% was aimed at for a low osmolality, while a DM content of 45–47% was set as the target for high osmolality. It was then wilted to achieve the target DM (field-dried up to 48 h) and chopped to about 2–4 cm in length. The additives were diluted with water and the different treatments were applied according to the dosage recommended by the manufacturer using a pump sprayer. The treatments comprised a negative control without inoculants (CON) to check the potential of the forage with the native microflora. After thorough mixing, the ensiling material was either manually compacted in WECK® jars (0.5–1.0 L volume) and then closed using a rubber seal and clips or filled in small vacuum bags (13 × 22 cm) and vacuum-sealed in triplicates. After three days at around 22–25 °C the samples were removed from the storage room. The silos were opened and the pH was measured in an aliquot of the forage (10 g) soaked in distilled water (100 mL).

Year 2: In 2023, seven products (nos. 7, 12, 16, 20, 22, 24, and 27; Table 3) containing one purely obligately heterofermentative (no. 7) were grown in vitro in the sterilized standard MRS broth and the HS medium at 30 °C for 48 h (n = 3). The change in pH was measured at 0, 24, and 48 h. Grass silages were prepared at five different locations, see year 1, plus Baden-Wuerttemberg (47°57′ N; 9°38′ O; 590 m ASL), including the first to the fourth cut from May to September. For low osmolality, a DM of about 30–35% was aimed at, and for high osmolality, 47–49% in order to obtain a clear differentiation between the levels. The heterofermentative product (no. 7, Table 3) was inoculated at all five trial sites. All other products (nos. 12, 16, 20, 24, and 27) were applied at three sites each, and product no. 22 at four sites. The ensiling procedure was the same as described for year 1. The only difference was that there was a second opening date on day 5 for the heterofermentative treatments (another three replicates). The theoretical ensilability of the fresh forage material was characterized by analyzing the sugar content (WSC) and buffering capacity (BC) [21] and calculating the fermentability coefficient (FC) [22].

2.4. Statistical Analysis

For step 3, a variance analysis considering the osmolality, adjusted by either wilting or sugar level of the medium, respectively, as effect on final pH was performed using the procedure GLM, followed by a Tukey test post hoc (SAS Studio 3.82, SAS Institute, Cary, NC, USA).

$$Y_i = \mu + OSMOL_i + \varepsilon_i$$

where μ = general mean, i = 1, 2, 3, 4 (osmolality adjusted by low/high DM or sugar level respectively), and ε_i = residual error.

3. Results

3.1. Step 1 A Medium for LAB with Increased Osmolality

The final medium with an increased osmolality (2.41 osmol/kg) consisted of MRS as the dehydrated culture media (54 g) plus 100 g glucose and fructose each, along with 28 g KCl dissolved in 1.0 L distilled water (Figure 1, medium 16).

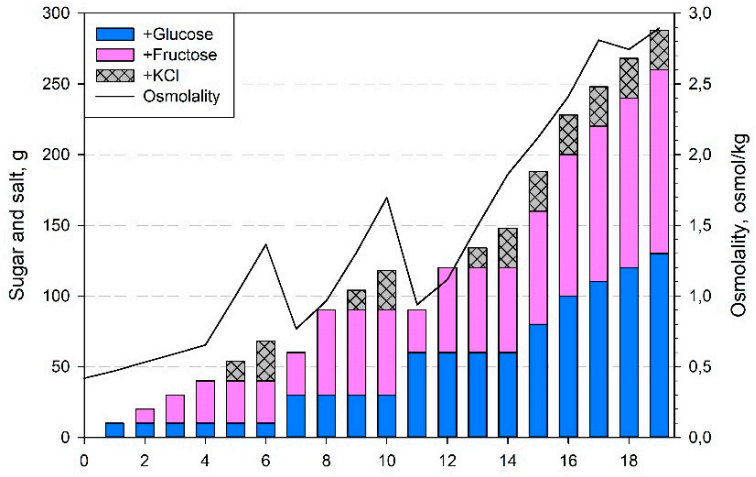

Figure 1. Amount of sugars and KCl added to 1.0 L distilled water and MRS broth and the resulting osmolality of the medium (composition see Table 2).

3.2. Step 2 Growth of and Acidification by Different LAB Strains in the Modified Media

On the high-sugar MRS agar, all of the 10 heterofermentative products exhibited similar growth compared to the standard MRS agar (Figure 2). The colony counts were at least 55% of those on the standard agar. In contrast, only two-thirds of the homofermentative products met this criterion, while two products (nos. 26 and 28) had less than 15% of the colony counts on the high-sugar agar compared to the standard agar (Figure 2).

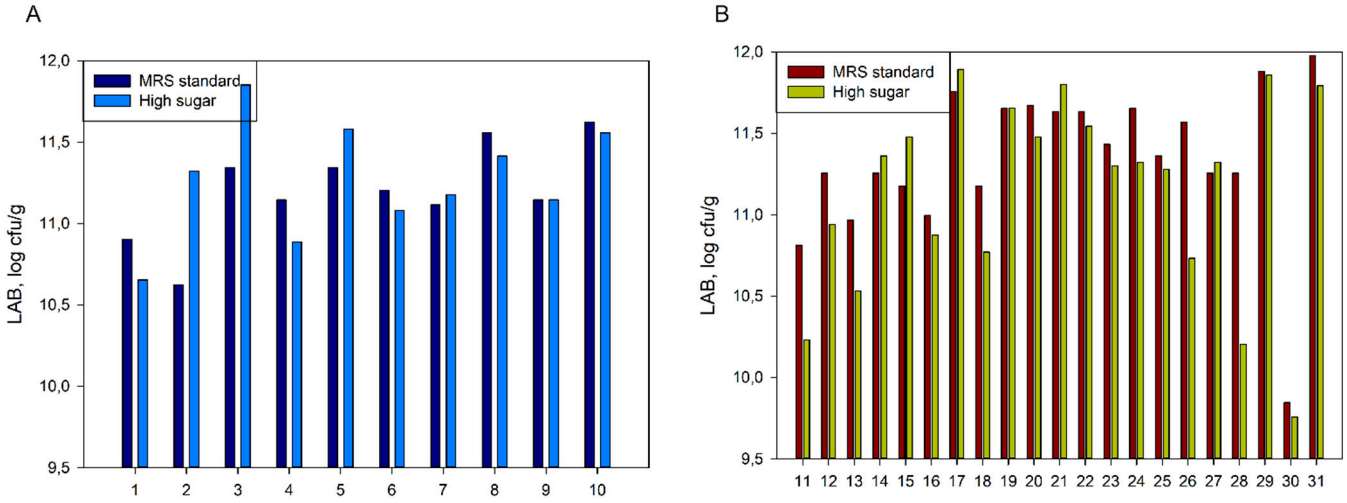

Figure 2. Numbers of lactic acid bacteria (LAB) colonies (cfu/g product, see Table 3) counted on standard and high-sugar MRS agar, (**A**) heterofermentative products, (**B**) homofermentative products.

The initial pH of the standard medium was 5.7 while it was 5.2 in the high-sugar medium. The pH decline was generally more rapid with the homofermentative products in contrast to the heterofermentative ones and obviously delayed in the high-sugar medium (Figure 3). For the homofermentative products, the impeding effect had almost vanished by the end of the second day while for most of the heterofermentative products, there were still differences at that time.

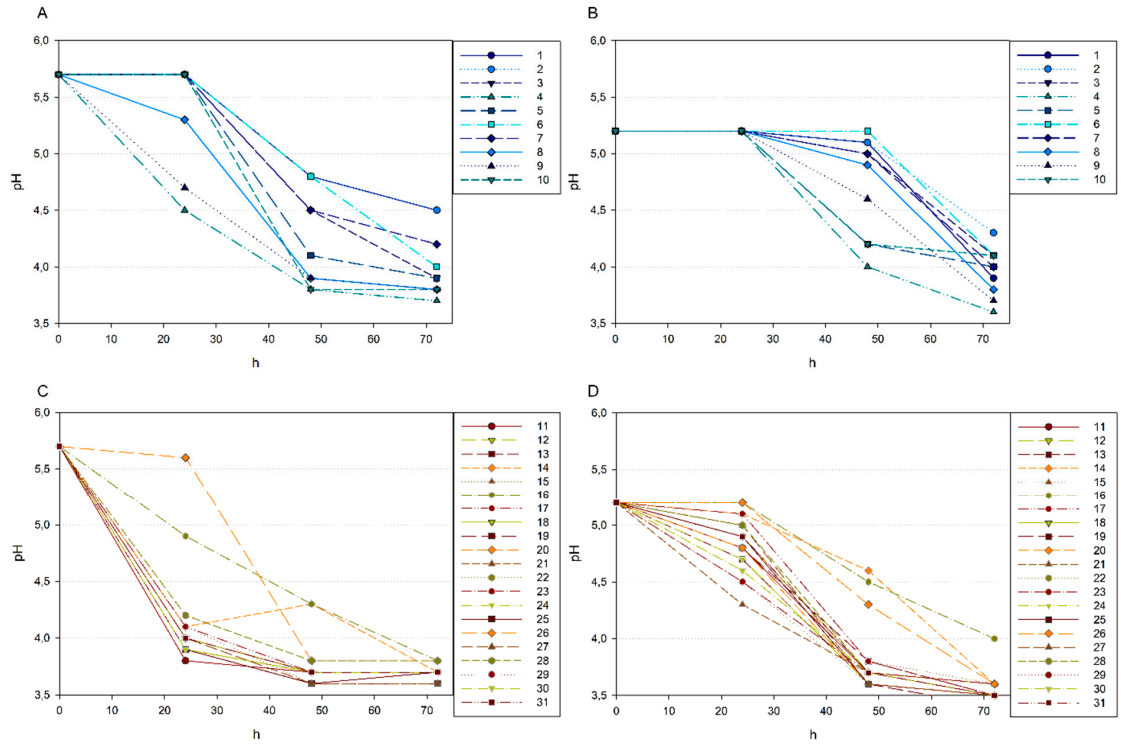

Figure 3. pH development in MRS broth of heterofermentative LAB (**A**,**B**) and homofermentative LAB (**C**,**D**), and in standard MRS (**A**,**C**) and high-sugar MRS (**B**,**D**). LAB species see Table 3.

3.3. Step 3 In Vitro pH Decrease in Growth Medium and In Situ

In the first experimental year, the target DM levels both in the low (30–35%) and in the high level (45–47 or 47–49%) were met more exactly with a mean of 336 and 475 g DM/kg than in the second year with 347 and 532 g DM/kg (Supplementary file S1: Table S1). Here, the limit of 500 g DM/kg was mostly exceeded at the high wilting level. The fermentability coefficient, which considers sugar content, buffering capacity, and DM content as a measure for a potentially butyric acid-free fermentation, varied widely even within the same DM level (35–74 for low DM and 56–82 for high DM) (Supplementary file S2: Table S2).

3.3.1. Products Containing Homofermentative LAB Species

With respect to the achieved forage DM, the correlation to the pH of the inoculated silages after three days of ensiling is presented in Figure 4.

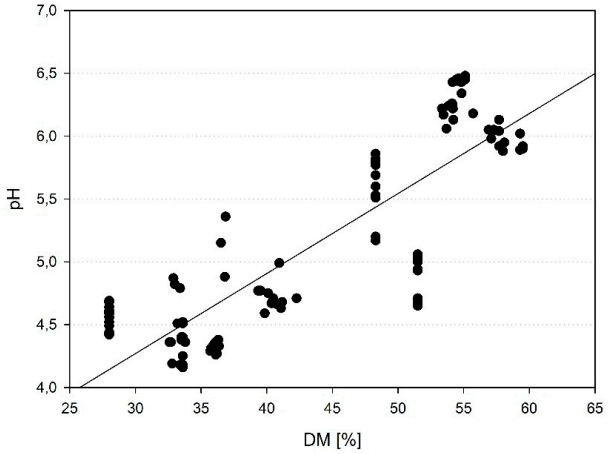

Figure 4. pH value of inoculated grass silages (homofermentative LAB) after 3 days of ensiling as a function of DM concentration (years 2022–2023, n = 117): f(x) = 0.064 x + 2.362, r^2 = 0.70, $p < 0.001$.

Table 4 summarizes the comparison of the pH after 3 d of all inoculated silages in the two different DM levels to the pH values after 24 h of all inoculated media (standard and HS MRS). In the first year, the standard medium and the low DM were similar with a pH of around 4.1. On the other hand, the HS medium and the high DM ranged around pH 4.8. In the second year, there was the following ranking between the four groups: high DM (5.8) > HS medium (5.0) > low DM (4.5) > standard medium (4.2).

Table 4. Dry matter (DM) and pH (3 d) of grass silages and pH of growth medium (24 h) inoculated with homofermentative LAB in years 2022 and 2023.

	2022		**2023**		**2022–2023**	
Medium/DM Level	**DM (g/kg)**	**pH**	**DM (g/kg)**	**pH**	**DM (g/kg)**	**pH**
Standard medium		4.08 ^{b}		4.19 ^{d}		4.13 ^{c}
Low DM	336 ^{b}	4.12 ^{b}	343 ^{b}	4.51 ^{c}	339 ^{b}	4.32 ^{c}
High-sugar medium		4.72 ^{a}		4.95 ^{b}		4.83 ^{b}
High DM	472 ^{a}	4.83 ^{a}	533 ^{a}	5.79 ^{a}	503 ^{a}	5.31 ^{a}
SEM	0.377	0.039	0.308	0.033	0.235	0.028
p-value						
Medium (DM level)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Year					<0.001	<0.001
Medium × Year					<0.001	<0.001

$^{a–d}$ different superscript letters within a column mean significant differences ($p < 0.05$).

3.3.2. Products Containing Predominantly Heterofermentative LAB Species

After 24 h of the in vitro incubation of product no. 5 and 7, the mean pH in the HS medium (5.14 ± 0.05) was equal or very close to the original at 0 h, which was pH 5.2. Overall, this was similar for the in situ high DM treatments after 3 d of ensiling (mean of 5.58 ± 0.72), having only marginally decreased the pH of the fresh forage of >6.0. Thus, in the second year, 48 h of incubation and 5 d of ensiling were additionally compared using product no. 7. While in the overall evaluation there was a clear difference in pH between the high DM level (mean of 532 g DM/kg) versus the low DM level (mean of 347 g DM/kg) and the standard medium, the pH of the HS medium was lower than in the high DM level (Table 5).

Table 5. Descriptive statistics comparing the pH values of in vitro (after 48 h incubation in growth medium at two osmolality levels) and in situ (after 120 h of anaerobic storage at two DM levels) when inoculated with an obligately heterofermentative silage additive (no. 7, Table 3) in year 2.

Product No. 7	**Mean**	**SD**
Standard medium	4.60 ^{b}	0.232
Low DM	4.64 ^{b}	0.100
High-sugar medium	4.90 ^{b}	0.232
High DM	5.67 ^{a}	0.100
p	<0.001	

^{a,b} different superscript letters within a column mean significant differences ($p < 0.05$). DM dry matter.

4. Discussion

The concentration of sugars and salts chosen initially (levels 1–6) for the development of the modified medium were derived from the concentrations prevailing in fresh grass wilted to 450 g DM/kg. However, the results showed that the increased osmolality measured in wilted grass [7] is obviously not only a result of the inherent sugar and salt contents. Hoedtke [7] introduced the unit osmol/kg DM as a measurand to compare different plant species independent from their DM content with regard to their osmolality. She found out that even varieties and cultivars of the same species could differ as a result of their inherent metabolic peculiarities, while location, fertilization, and weather conditions could

play another role. In the study presented here, sugars had to be increased by a multiple to achieve the targeted osmolality while KCl was restricted to 28 g to avoid salt stress. The final sugar concentration of the HS medium is similar to MY20 agar or glucose agar with 25% glucose [14], which were developed for the cultivation of osmophilic bacteria.

The HS medium obviously delayed the decrease in pH. This shows that the increased osmolality had the expected effect on the lactic acid production of the LAB. On the other hand, the effect was not a total inhibition, which can be a problem for microorganisms if electrolytes such as NaCl or KCl are used as osmolytes [23–25].

The connection between a reduced percentage of growth and the rate of pH reduction in the HS medium in general was not clear. The product no. 26, containing both homo- and heterofermentative strains, was the only one with both poor colony growth on HS medium (15%) compared to the standard agar, and a slow pH decline. Two other homofermentative products were rather slow in pH decrease (no. 14 and 16). However, there was no noticeable growth depression observed in the solid high-sugar medium. All other homofermentative products had a comparable pH development on the HS medium, irrespective of colony numbers (Figures 2 and 3). Thus, there seems to be no stringent connection between the growth and fermentation activity of LAB, which again confirms the observations of Marauska et al. and Beker et al. [15,16]. Their investigation showed that the lactic acid production of *L. plantarum* ceased at a significantly higher osmolality than its cell growth. This shows the necessity to work with a meaningful and robust indicator for the metabolic activity of LAB, which is the focus of interest when developing silage starter cultures, such as the pH.

The aim of ensiling grass at two different wilting levels with the selected products was to validate whether the developed medium could sufficiently simulate high dry matter conditions to evaluate in vitro the potential osmotolerance of LAB products in the future. The MRS standard medium can be considered as a medium, which provides optimal growth conditions for the majority of LAB species in silage additives. Thus, the fermentation capacity should be unlimited until the nutrients are depleted or until a too-low pH (autoacidification) has become the limiting factor [26]. In the high-sugar medium, the osmotic pressure should be the only initial obstacle to overcome. In contrast, a heterogeneous material such as grass consisting of different species at varying growth stages from diverse locations offers the LAB variable growth and fermentation conditions. Moreover, inoculants also have to compete with the native microflora. The forage utilized at the different institutions showed a high variability in its potential ensilability characterized by the sugar content and buffering capacity. Furthermore, it was not very easy to achieve the desired target DM contents due to the variations in solar radiation and wind strength. This was especially true for the second experimental year, highlighting the need for standardized conditions when selecting LAB for high osmotolerance. In the first year, there was no significant pH difference between the low DM and standard medium, and between the high DM and HS medium, indicating a high degree of comparability. In the second year, the statistical grouping resulted in four groups. However, as in the first year, the pH values in the low DM/standard medium groups were lower than in the high DM/HS medium groups. It has to be taken into account that the realized DM was above the targeted one, resulting in a higher osmolality than in the HS medium. For example, *Lolium perenne* with 600 g DM/kg had an osmolality of around 3.7 osmol/kg DM [7]. Consequently, this resulted in a higher pH in the high DM silage. Considering the high variation in the forage material, still specific pH ranges could be attributed to the different DM levels and compared to the growth media with different osmolality levels.

Obligately heterofermentative LAB exhibit a slower decrease in pH due to their metabolic pathway, which is split into the production of both lactic and acetic acid, the latter representing a weaker acid, or ethanol, depending on the substrate [27]. Heterofermentative LAB are used as silage additives with the aim to enhance aerobic stability, mainly because of their acetic acid production in the long run, which decelerates yeast activity [28]. When evaluating their osmotolerance based on the pH decrease within the first days of

fermentation, it can be questioned whether this is the appropriate indicator to measure their metabolic activity under the given conditions as they exhibit their desired properties only after 7–8 weeks of ensiling [29]. In the presented *in vitro* trial, the lag phase was clearly pronounced. In situ, other products than no. 7 could be evaluated in the future after 5 d of ensiling with the mentioned reservation. An alternative here could be to continue the plate counting method on the two types of solid medium to evaluate colony growth at low and high osmolality.

5. Conclusions

For homofermentative LAB, evaluating the pH decrease after 24 h of incubation in the newly developed liquid HS medium is a suitable method to select strains for fermentation activity at high osmotic pressure compared to a standard MRS medium, which confirms the initial hypotheses. The HS medium provides a sufficiently accurate approximation. It simulates high DM conditions in forages as a first step to select for osmotolerance in the laboratory prior to field evaluations. For obligately heterofermentative LAB, this method needs further evaluation.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture14060825/s1, Table S1: DM contents of the silages of the low and high DM level in 2022 and 2023 at the participating institutes; Table S2: Ensilability parameters of forages in 2023.

Author Contributions: Conceptualization, S.D.M. and W.W.; methodology, S.D.M. and W.W.; validation, S.D.M., W.W., M.S., K.H., S.O. and C.L.; formal analysis, S.D.M. and M.S.; investigation, S.D.M., W.W., M.S., K.H., S.O. and C.L.; writing—original draft preparation, S.D.M.; writing—review and editing, S.D.M., W.W., M.S., K.H., S.O. and C.L. All authors have read and agreed to the published version of the manuscript.

Funding: The DLG Service GmbH supported parts of the trials financially.

Institutional Review Board Statement: Not applicable.

Data Availability Statement: The original data can be provided by the corresponding author upon reasonable request.

Acknowledgments: We kindly thank the DLG (German Agricultural Society) Technical Committee for Silage Additives and Preservatives for providing the platform for the scientific exchange to develop the protocol. Thanks go to Barbara Misthilger (LfL) who organized the osmolality measurement of the media.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

ASL, above sea level, BC, buffering capacity [g lactic acid/kg DM]; DM, dry matter; *E. faecium*, *Enterococcus faecium*; FC, Fermentability coefficient [FC = 8 * S/BC + DM, %]; HS, high sugar; LAB, lactic acid bacteria; *L. brevis*, *Levilactobacillus brevis*; *L. buchneri*, *Lentilactobacillus buchneri*; *L. diolivorans*, *Lentilactobacillus diolivorans*; *L. kefir.*, *Lactobacillus kefiranofaciens*; *L. paracasei*, *Lacticaseibacillus paracasei*; *L. plantarum*, *Lactiplantibacillus plantarum*; *L. rhamnosus*, *Lacticaseibacillus rhamnosus*; MRS, growth medium according to de Man, Rogosa, Sharpe [20]; *P. acidilactici*, *Pediococcus acidilactici*; *P. pentosaceus*, *Pediococcus pentosaceus*; S/BC, Sugar: buffering capacity quotient

References

1. Buxton, D.R.; O'Kiely, P. Preharvest Plant Factors Affecting Ensiling. In *Silage Science and Technology*; Buxton, D.R., Muck, R.E., Harrison, J.H., Eds.; American Society of Agronomy, Crop Science Society of America, Soil Science Society of America: Madison, WI, USA, 2003; pp. 199–250.
2. Muck, R.E.; Moser, L.E.; Pitt, R.E. Postharvest Factors Affecting Ensiling. In *Silage Science and Technology*; Buxton, D.R., Muck, R.E., Harrison, J.H., Eds.; American Society of Agronomy, Crop Science Society of America, Soil Science Society of America: Madison, WI, USA, 2003; pp. 251–304.

3. DLG e.V. *Praxishandbuch Futter—Und Substratkonservierung*; DLG-Verlag: Frankfurt, Germany, 2012; p. 416.
4. Mitrik, T. *Ensilage*; Wageningen Academic Publishers: Wageningen, The Netherlands, 2021; p. 280.
5. Wieringa, G.W. The Effect of Wilting on Butyric Acid Fermentation in Silage. *Neth. J. Agric. Sci.* **1958**, *6*, 204–210. [CrossRef]
6. Lanigan, G.W. Silage Bacteriology I. Water Activity and Temperature Relationships of Silage Strains of *Lactobacillus plantarum*, *Lactobacillus brevis*, and *Pediococcus cerevisiae*. *Aust. J. Biol. Sci.* **1963**, *16*, 606–615. [CrossRef]
7. Hoedtke, S. Die Quantifizierung der Osmolalität in Futterpflanzen und ihre Veränderung in verschiedenen Stadien der Silierung. Ph.D. Thesis, University of Rostock, Rostock, Germany, 2008.
8. Atkins, P.W. *Einführung in Die Physikalische Chemie. Ein Lehrbuch Für Alle Naturwissenschaftler*; VCH Verlagsgesellschaft: Weinheim, Germany, 1994; p. 472.
9. Levine, I.N. *Physical Chemistry*; McGraw-Hill: New York, NY, USA, 2022; p. 989.
10. Weissbach, F. Beziehungen zwischen Ausgangsmaterial und Gärungsverlauf bei der Grünfuttersilierung. Post-Doctoral Thesis, University of Rostock, Rostock, Germany, 1968.
11. DLG TestService GmbH. *DLG Testing Guidelines for the Award and Use of the DLG Quality Mark for Ensiling Agents*; DLG TestService GmbH: Frankfurt, Germany, 2018.
12. Pieper, B.; Hoedtke, S.; Wensch-Dorendorf, M.; Korn, U.; Wolf, P.; Zeyner, A. Validation of the Rostock Fermentation Test As an *in-vitro* method to estimate ensilability of forages using glass jar model silages as a basis for comparison. *Grass Forage Sci.* **2016**, *72*, 568–580. [CrossRef]
13. Glaasker, E.; Tjan, F.S.B.; Ter Steeg, P.F.; Konings, W.N.; Poolman, B. Physiological response of *Lactobacillus plantarum* to salt and nonelectrolyte stress. *J. Bacter.* **1998**, *180*, 4718–4723. [CrossRef] [PubMed]
14. Atlas, R.M. *Handbook of Microbiological Media*; CRC Press: Boca Raton, FL, USA, 2010; p. 2040.
15. Beker, M.E.; Vigant, A.K.; Marauska, M.K.; Klintsare, A.A. Osmotic sensitivity of the bacterium *Lactobacillus casei var. alactosus*. *Prikl. Biokhimiia Mikrobiol.* **1998**, *34*, 161–163.
16. Marauska, M.; Vigants, A.; Klincare, D.; Upite, D.; Kaminska, E.; Bekers, M. Influence of Water Activity and Medium Osmolality on the Growth and Acid Production of *Lactobacillus casei* var. *alactosus*. *Proc. Latv. Acad. Sci. Sect. B* **1996**, *50*, 144–146.
17. Schuster, M.; Kolln, K.; Baranowski, A.; Richter, W.I.F.; Spiekers, H. Method and range of applications of the "Rostocker Fermentation Test". *Ubers. Zur Tierernährung* **2007**, *35*, 103–116.
18. Zierenberg, B. *In Vitro Method to Determine the Fermentation Performance of Lactic Acid Bacteria and Their Influence on the Metabolic Activity of Other Microorganisms Relevant for Ensiling Under Varying Fermentation Conditions*; University of Rostock: Rostock, Germany, 2000.
19. Pieper, B.; Müller, T.; Robowsky, K.-D.; Seyfarth, W. Rapid Fermentation Test as a Method for Assessing the Ensiling Potential of Herbage. In Proceedings of the XIth International Silage Conference, Wales, UK, 8–11 September 1996; pp. 120–121.
20. de Man, J.C.; Rogosa, M.; Sharpe, M.E. A medium for the cultivation of *Lactobacilli*. *J. Appl. Bacteriol.* **1960**, *23*, 130. [CrossRef]
21. Weissbach, F. Die Bestimmung der Pufferkapazität in Futterpflanzen und ihre Bedeutung für die Beurteilung der Vergärbarkeit. *Tagungsbericht* **1967**, *92*, 211–220.
22. Pahlow, G. Application of a New Concept for the Estimation of the Ensiling Potential of Forages for a Range of Crops. In Proceedings of the XIIIth International Silage Conference, Auchincruive, Scotland, UK, 11–13 September 2002; pp. 361–371.
23. Baird-Parker, A.C.; Freame, B. Combined effect of water activity, pH and temperature on the growth of *Clostridium botulinum* from spore and vegetative cell inocula. *J. Appl. Bacteriol.* **1967**, *30*, 420–429. [CrossRef] [PubMed]
24. Rehacek, J.; Sozzi, T.; Studer, P. Effect of water activity on the development of lactic acid bacteria and yeast utilized in the food industry. *Milchwissenschaft* **1982**, *37*, 151–154.
25. Strong, D.H.; Foster, E.F.; Duncan, C.L. Influence of water activity on the growth of *Clostridium perfringens*. *Appl. Microbiol.* **1970**, *19*, 980–987. [CrossRef] [PubMed]
26. Vinderola, G.; Champagne, C.P.; Desfosses-Foucault, E. The Production of Lactic Acid Bacteria Starters and Probiotic Cultures: An Industrial Perspective. In *Lactic Acid Bacteria*; CRC Press: Boca Raton, FL, USA, 2019; pp. 317–336.
27. von Wright, A.; Axelsson, L. Lactic Acid Bacteria. An Introduction. In *Lactic Acid Bacteria: Microbiological and Functional Aspects*, 5th ed.; Vinderola, G., Ouwehand, A., Salminen, S., von Wright, A., Eds.; CRC Press: Boca Raton, FL, USA, 2019; Chapter 1, pp. 1–16.
28. Elferink, S.J.W.H.O.; Driehuis, F.; Krooneman, J.; Gottschal, J.C.; Spoelstra, S.F. *Lactobacillus buchneri* can improve the aerobic stability of silage via a novel fermentation pathway: The anaerobic degradation of lactic acid to acetic acid and 1,2-propanediol. In Proceedings of the XII International Silage Conference, Uppsala, Sweden, 5–7 July 1999; pp. 266–267.
29. Kleinschmit, D.H.; Kung, L. The effects of *Lactobacillus buchneri* 40788 and *Pediococcus pentosaceus* R1094 on the fermentation of corn silage. *J. Dairy Sci.* **2006**, *89*, 3999–4004. [CrossRef] [PubMed]