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Ensiling, In Vitro Rumen Digestion and Soaking in Slurry Altered the Germination Capacity of *Rumex obtusifolius* Seeds

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Abstract: This study investigated whether the process of ensiling and in vitro digestion in rumen juice, as well as the response to soaking in pig or cattle slurry, affects the germination rate and germination energy of *Rumex obtusifolius* (broad-leaved dock) seeds. Seeds were subjected to different treatments (200 seeds each) in three experiments: (I) seed ensiling (8 weeks) followed by in vitro rumen digestion (24, 36 and 48 h); (II) the soaking of non-ensiled and ensiled seeds in cattle or pig slurry (2, 4 and 24 weeks); and (III) the in vitro rumen digestion (24, 36 and 48 h) of non-ensiled and ensiled seeds followed by soaking in cattle or pig slurry (24 weeks). The control treatment included untreated seed (0—non-ensiled seed; 0—no in vitro rumen digestion; and 0—no soaking in slurry). Germination tests (germination rate and germination energy) were then conducted in four replicates in the germination chamber under alternating day (20–35 °C for 14 h under light) and night conditions (17–20 °C for 10 h without light) at 75% relative humidity. Experiment I showed that ensiling significantly ($p \leq 0.001$) reduced both the germination rate and germination energy of *R. obtusifolius* seeds. In addition, the length of in vitro digestion duration that the non-ensiled seeds were subjected to significantly ($p \leq 0.001$) reduced their germination energy but not the total germination rate. However, the seeds that were subjected to the process of ensiling and in vitro digestion in the rumen lost their germination completely. The Experiment II investigated the effects of soaking non-ensiled seeds in slurry and showed that germination rates were comparable in pig and cattle slurry. Longer soaking times significantly reduced the germination rate, with no germination observed after 24 weeks. The Experiment III considered the combined effects of in vitro digestion and slurry soaking and showed that rumen digestion reduced the proportion of germinable seeds. Germination was inhibited in pig slurry, while in cattle slurry, a decreasing germination rate was observed with increasing digestion time.

Keywords: broad-leaved dock; in vitro rumen digestion; ensiling; pig and cattle slurry; seed germination

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1. Introduction

The loss of plant community diversity due to weed invasion reduces the resilience of grasslands to disturbance (e.g., drought). Unfortunately, many invasive weeds are prolific seed producers (e.g., *Rumex* species), and it is difficult to remove them from a pasture or meadow once they become established. *Rumex obtusifolius* (broad-leaved dock) is one of the five most common perennial weeds, native to Euroasia but distributed worldwide [1–3]. It is mainly distributed in temperate regions but also occurs within the Arctic Circle and at high altitudes in equatorial regions. It is described by Hultén [1] as a circumpolar plant and gives its northernmost limit as 68° north latitude. In the United Kingdom, *R. obtusifolius* is listed in the 1959 Weeds Act as one of the five noxious weeds whose seed dispersal must be prevented and whose control can be enforced by a landowner [4]. In the USA, it is listed as an invasive weed [5].

Today, it occurs in various plant communities of meadows and pastures, particularly in organic farming systems where the use of herbicides is not allowed and where the swards are severely disturbed. Deep soils rich in nutrients, especially nitrogen, are suitable for *R. obtusifolius* [2,6,7]. The seeds of *R. obtusifolius* remain viable in the soil for many years. Nevertheless, the viability of the seeds decreases over the years (after 20 years, about one-third of the seeds germinate, while after 80 years, the viability decreases to only a few seeds) [8]. Their broad leaves, rapid growth and wide natural distribution make *R. obtusifolius* a strong competitor for other pasture or meadow plants, competing for available nutrients, water and light. If not controlled, it reaches high densities; therefore, it is of great importance in temperate grasslands, where dry matter yield and nutritional value of herbage can be reduced by 10 to 40% [9,10]. The leaves are rich in proteins and minerals, especially magnesium. However, they are worthless in small quantities as fresh and dry feed but can be harmful or toxic in larger quantities due to oxalates [11,12].

Mature plants of *R. obtusifolius* develop a large number of seeds each year (between 30,000 and 60,000) which, once shed, can remain dormant in the soil for many years (>80 years have been recorded) [2,3,6]. Flowering and seed production usually occurs in the second year after seed germination. The main flowering periods of *R. obtusifolius* are March–April and July–October [6]. Six days after the first flowering end, 15% of the seeds are germinable; after 18 days, more than 90% of the seeds can germinate. However, the germinability of *R. obtusifolius* also depends on the time of seed production. Seeds developed in spring have low viability, while the germination rate of seeds developed in summer is much higher. Moreover, the seeds of the second regeneration after cutting have a higher germination rate than those of the third regeneration harvested in autumn [2]. Accordingly, the intensive forage production, the early grazing and the cutting date hardly allow a significant *R. obtusifolius* seed production and its dispersal. More recently, the reasons for higher seed loads are the growing number of agri-environmental measures, i.e., field margin protection programmes and the integration of flowering mixtures into cropping systems [13,14], as well as the use of cattle or pig slurry, compost and the application of green manure as a substitute for farm manure on organic farms with low livestock density [15–18]. Todays, this is also often the cause of a more considerable amount of *R. obtusifolius* seeds present in biomass streams and, thus, into silos and feed rations for ruminants. Some seeds ingested by ruminants can survive passage through the digestive tract, and seedlings of many species have been detected in faeces [19]. Seed resistance, in combination with mass seed production, its longevity and presence in feed rations, means that *R. obtusifolius* can quickly build up a large seed supply in the soil. This is the reason why this weed has been causing major problems in temperate grasslands for several decades.

As shown, there are many studies on the seed germination of *R. obtusifolius*; often with contradictory results, as the physical and physiological variability of the seeds leads to different responses to external factors. In general, seeds can become viable from the milk stage onwards but may require a short post-ripening period before germinating. It is important to highlight that the experiments conducted primarily focused on assessing the germination capacity of *R. obtusifolius* seeds after various experimental treatments. However, this approach makes it impossible to distinguish between dormant and non-viable seeds, consequently excluding an evaluation of seed viability. Therefore, the main objective of the present study was to investigate the germination of non-ensiled seeds of *R. obtusifolius* and compare them with the germination capacity of seeds subjected to ensiling (feed preservation) and subsequently exposer to in vitro digestion processes in the rumen juice of bulls. Since Masuda et al. [20] found that silage reduced the germination rate of *R. obtusifolius* seeds, we predicted that the non-ensiled seeds of *R. obtusifolius* would germinate better than the silage seeds. Furthermore, we assumed that the combination of ensiling and the time of in vitro digestion and exposure to microbial digestion in rumen juice would additionally have a characteristic effect on the seed germination of *R. obtusifolius* in non-ensiled and ensiled seeds. Since the effects of soaking *R. obtusifolius* seeds in cattle

or pig slurry on their germination rate are not well studied, this study also investigated whether seedlings could be produced when *R. obtusifolius* seeds are subjected to anaerobic fermentation in cattle or pig slurry, as [21–23] reported that seed germination ability can be affected by slurry storage conditions.

2. Materials and Methods

2.1. Seed Sampling and Preparation

The seeds of *R. obtusifolius* used in this experiment were collected in late summer in a region near Maribor in north-eastern Slovenia. The collection sites were late-cut semi-natural meadows (46° 33′29″ N, 15° 48′50″ E; 264 m a.s.l.), where three cuts with autumn grazing take place annually. Seeds were collected from a group of mature plants at three independent sites and an independent *R. obtusifolius* population. Five plants were randomly selected at each site, taking care not to favour either large or small plants. Plants were dried in air and shade for an additional five days [24]. The seeds were then stored in paper bags at room temperature in the dark until the experiment was conducted. At the end of August (the same year of seed collection, and after a short storage period at room temperature), all seeds were divided among twenty permeable polypropylene bags (optimal water, light, and air permeability). Each bag contained about 200 seeds, corresponding to four replicates. The seed samples were subjected to the following treatments divided into three experiments:

Experiment I: Seed ensiling (8 weeks) followed by in vitro rumen digestion (0, 24, 36 and 48 h).
Experiment II: Soaking of non-ensiled and silage seeds in cattle or pig slurry (0, 2, 4 and 24 weeks).
Experiment III: In vitro rumen digestion (0, 24, 36 and 48 h) of non-ensiled and ensiled seeds followed by soaking in cattle or pig slurry (24 weeks).

Control treatment of all experiments consisting of untreated (initial) seed (0—non-ensiled seed; 0—no in vitro rumen digestion; and 0—no soaking in slurry).

2.2. Grass Silage Preparation

The silage was obtained from a semi-natural meadow near Maribor, Slovenia (46°28′ N, 15°38′ E; 300 m a.s.l.), where four annual cuts have taken place since 1995. Annually, 180 kg N, 120 kg P_2O_5 and 180 kg K_2O were applied for fertilization. The mown green mass from the third cut was dried in the field until the moisture content dropped to about 60–75% (the optimal moisture content for grass silage production). The random mass from the meadow was then cut to a length of 3 to 5 cm with a hand cutter. While about 12 kg of grass silage was filled into four buckets of 30 L capacity each and pressed, the bags of *R. obtusifolius* (initial) seeds were randomly placed into the buckets. A plastic sheet filled with about 8 kg of sandy soil was placed over the ensiled mass to press it down before the filled buckets were hermetically sealed with suitable lids. The gaps between the lid and the buckets were sealed with wide adhesive tape. After eight weeks of ensiling, the silage was analysed for dry matter (DM) and pH and the seeds were prepared for the germination test, in vitro digestion and slurry soaking.

2.3. Rumen Juice Sampling, Determination and Preparation

Rumen contents were collected from ten Simental bulls (all from the progeny test) freshly slaughtered at a local slaughterhouse (TMI Košaki, Maribor, Slovenia). The animals were 16 to 18 months old, weighed 650 to 700 kg and were fed a standard diet [25]. The materials, equipment and procedure for rumen juice collection at the abattoir were prepared as described by [26]. The rumen juice was strained into preheated thermos flasks, immediately hermetically sealed and transported in the thermic bag to the faculty laboratory. At the laboratory, the previously prepared bags of non-ensiled (initial) seeds (nine bags of 200 seeds each) and ensiled seeds (three bags of 200 seeds each) were added to the bottles with rumen juice for in vitro digestion. Both groups of seeds were subjected to the same in vitro conditions. The thermos bottles were then placed in a heating chamber

where the temperature of the rumen juice was maintained at 39 °C. The contents of the bottles were stirred gently every hour. The seeds were exposed to in vitro digestion of the rumen juice for 24, 36 or 48 h.

2.4. Cattle and Pig Slurry Treatments

The cattle and pig slurry were taken from a moderately intensive dairy and pig farm where the slurry is stored for six months. The cattle slurry had a pH of 7.6, contained 8.4% dry matter (DM) and 701 g organic matter (OM)/kg DM; the pig slurry contained 4.8% DM and 701 g OM/kg DM and had a pH of 6.6. Six sets of non-ensiled (initial) seeds (200 seeds each) and another six sets of in vitro digested seeds (200 seeds each) were immersed in eight 12 L plastic containers—four containing cattle slurry and four containing pig slurry. Each container was completely filled with slurry, the lids screwed on and sealed with plastic tape. Before the cattle and pig slurry was added to the 12 L plastic containers, it was mixed well. The seeds were taken out of the slurry after 2 weeks, 4 weeks and 6 months and rinsed three times in demineralised water before the germination test was carried out.

2.5. Seed Germination Test

Germination tests were carried out for all seed treatments of *R. obtusifolius* in all three experiments. Thirty seeds of each treatment in four replicates were placed in 9 cm diameter Petri dishes on three layers of filter paper (FT-3-101-090, 0.21 mm) previously moistened with deionized water twice the thickness of the paper. The seeds were kept in a germination chamber (LO 600-S) under alternating day (20–35 °C for 14 h under light) and night conditions (17–20 °C for 10 h without light) at 75% relative humidity. Germinated seeds were counted daily for each treatment until 21 days after the start of the test (germination rate) and for another seven days, for a total of 28 days. Normally developed seedlings with at least 0.5 cm plums and primary roots were considered [24]. During this test, the date for the end of the test was also set. The most commonly used parameters for evaluating seed germination [27,28] are listed in Table 1.

Table 1. Formulas for calculating the indicators of seed germination.

Indicators	Calculation Formulae
Germination rate (%)	(Number of germinated seeds ÷ Total number of seeds) × 100
Germination energy (%)	(Number of germinated seeds 4d * ÷ Total number of seeds) × 100

* 4d—on the fourth day after the start of the germination test.

2.6. Statistical Methods

All data were analysed using a two-way analysis of variance (ANOVA). As calculations are given as percentages and are not normally distributed, data were transformed (arcsin transformation) before analysis. Statistically significant differences between treatments were tested with Duncan's test at a p-value ≤ 0.05. However, characteristic interactions were tested with Tukey's HSD test at a 5% risk. Results are reported as estimated means ± standard error of the mean (SEM). The calculations obtained were processed using the Microsoft Excel programme and the Statistical Package for the Social Sciences (IBM SPSS 22.0, Chicago, IL, USA).

3. Results

3.1. Experiment I: Seed Viability after Ensiling and In Vitro Digestion in Rumen Juice

A statistically significant effect of the treatments studied on the proportion of germinating seeds and germination energy is shown in Tables 2 and 3. The differences between the two seed treatments (non-ensiled and ensiled seeds) are statistically significant ($p \leq 0.001$). The germination rate of non-ensiled seeds was 77.7%, while none of the ensiled seeds germinated.

Table 2. The effects of seed treatment followed by in vitro digestion in rumen juice on germination (%) of *R. obtusifolius* seeds (mean of four replicates).

Seed Treatment	In Vitro Digestion in Rumen Juice (h) ns				Average Seed Treatment ***
	0	24	36	48	
Non-ensiled seeds	61.7 ± 14 b	85.0 ± 1.7 a	82.5 ± 2.8 a	81.7 ± 2.9 a	77.7 ± 4.1 A
Ensiled seeds	0.0	0.0	0.0	0.0	0.0 B
Average in vitro digestion ns	30.8 ± 13.3	42.5 ± 16.1	41.3 ± 15.6	40.8 ± 15.5	

*** significant at the 0.001 probability levels; ns—nonsignificant. A, B mean values (± SEM) for each factor level followed by different letters are significantly different (Duncan, $\alpha = 0.05$). a, b mean values (± SEM) within the same row followed by different letters are significantly different (Duncan, $\alpha = 0.05$).

Table 3. The effects of seed treatment followed by in vitro digestion in rumen juice on germination energy (%) of *R. obtusifolius* seeds (mean of four replicates).

Seed Treatment	In Vitro Digestion in Rumen Juice (h)				Average Seed Treatment ***
	0	24	36	48	
Non-ensiled seeds	1.8 ± 1.0 d	29.2 ± 0.8 b	39.2 ± 4.2 a	20.8 ± 5.2 c	22.7 ± 3.9 A
Ensiled seeds	-	-	-	-	-
Average in vitro digestion ***	0.8 ± 0.5 C	14.6 ± 5.5 AB	19.9 ± 7.6 A	10.4 ± 4.6 B	

*** significant at the 0.001 probability levels. A–C mean values (± SEM) for each factor level followed by different letters are significantly different (Duncan, $\alpha = 0.05$). a–d mean values (± SEM) within the same row followed by different letters are significantly different (Duncan, $\alpha = 0.05$). "-" seeds that have not germinated and have no germination energy.

Although the duration of exposure to microorganisms in the rumen juice showed a decreasing trend in the proportion of germinating seeds (Table 2), the differences observed were not statistically significant. Thus, the germination rate was 42.5% after 24 h, 41.3% after 36 h and only 40.8% after 48 h. Seeds (non-ensiled and ensiled) that had not previously been microbially digested germinated the worst (30.8%), but this difference is also not significant.

The results show that none of the ensiled seeds germinated in the experiment and none germinated after being exposed to microbial digestion. The germination rate of the non-ensiled seeds was 61.7% and was significantly lower than the proportion of germinated non-ensiled seeds exposed to in vitro digestion in rumen juice. We assume that the rumen microorganisms thus had a stimulating effect on the germination capacity of the non-ensiled seeds, since the proportion of germinated non-ensiled seeds subsequently increased by 20 to 24%. However, the duration of in vitro digestion in rumen juice did not have a significant effect on the seed germination rate, although there is a trend towards a decrease in the germination rate. The germination rate of non-ensiled *R. obtusifolius* seeds was 85% after 24 h, 82.5% after 36 h and 81.7% after 48 h (Table 2).

In vitro digestion in rumen juice also had a significant effect on the energy of the germinating seeds (Table 3). The greatest effect was obtained when the seeds were digested for 36 h (19.9%). Statistically, comparable germination energies were obtained when the seeds were exposed to rumen microorganisms for 24 h (14.6%) and 48 h (10.4%), respectively. Four days after the start of the experiment, only 0.8% of the *R. obtusifolius* seeds digested in the rumen juice germinated. This value is statistically the lowest compared to the other germination energies achieved by *R. obtusifolius*. Similarly, many studies [29,30] have confirmed that the seed germination capacity generally decreases with the time spent in the digestive tract and that microorganisms in the rumen significantly reduce seed germination.

The ensiled seeds did not germinate (Table 2) and had no germination energy (Table 3). Even when these seeds were digested in rumen juice, the germination energy did not increase. The germination energy of untreated seeds of *R. obtusifolius* is also low. After a

four-day germination test, only 1.8% of the seeds germinated. An interesting result is the finding that the germination energy of the untreated seeds is stimulated by the in vitro digestion in the rumen juice. Thus, germination energy increased to 29.2% after 24 h and to 39.2% after 36 h of in vitro digestion, when the statistically highest germination energy was reached. After 48 h, a negative effect of in vitro digestion was observed in rumen juice. At this point, only 20.8% of the seeds were still germinating.

3.2. Experiment II: Seed Viability after Ensiling and Soaking in Pig or Cattle Slurry

The results of Experiment I show that the ensiled seeds of *R. obtusifolius* have completely lost their germination capacity. Even after soaking in pig or cattle slurry, the ensiled seeds did not germinate. Therefore, only the results for non-ensiled seeds are presented below (Tables 4 and 5).

Table 4. The effect of the soaking time of *R. obtusifolius* seeds in pig or cattle slurry on the germination rate (%; mean of four replicates).

Slurry	Soaking Time (Weeks)				Average Slurry [ns]
	0	2	4	24	
Pig	61.7 ± 14.1 a	62.5 ± 12.9 a	25.8 ± 8.8 b	0.0 c	37.5 ± 7.7
Cattle	61.7 ± 14.1 a	49.2 ± 9.9 b	65.8 ± 32.7 a	0.0 c	44.2 ± 8.4
Average soaking time ***	61.7 ± 9.2 A	55.8 ± 4.5 A	45.8 ± 10.9 A	0.0 B	Interaction Tukey's limit ± 18.09

*** significant at the 0.001 probability levels; ns—nonsignificant. A, B mean values (± SEM) for each factor level followed by different letters are significantly different (Duncan, $\alpha = 0.05$). a–c mean values (± SEM) within the same row followed by different letters are significantly different (Duncan, $\alpha = 0.05$).

Table 5. The effect of soaking time of *R. obtusifolius* seeds in pig or cattle slurry on germination energy (%; mean of four replicates).

Slurry	Soaking Time (Weeks)				Average Slurry [ns]
	0	2	4	24	
Pig	1.8 ± 1.0 b	55.8 ± 3.7 a	0.0 ± 8.8 b	0.0 b	14.4 ± 6.2
Cattle	1.8 ± 1.0 b	49.2 ± 4.9 a	5.0 ± 5.0 b	0.0 b	13.9 ± 5.5
Average soaking time ***	1.8 ± 0.6 A	52.5 ± 3.1 A	2.5 ± 2.5 A	0.0 B	Interaction Tukey's limit ± 8.51

*** significant at the 0.001 probability levels; ns—nonsignificant. A, B mean values (± SEM) for each factor level followed by different letters are significantly different (Duncan, $\alpha = 0.05$). a, b mean values (± SEM) within the same row followed by different letters are significantly different (Duncan, $\alpha = 0.05$).

The differences in germination rate between the different types of slurry (pig or cattle) are statistically comparable ($p > 0.05$; Table 4). The average germination rate of seeds soaked in pig slurry was 37.5%, while seeds soaked in cattle slurry had a slightly better but similar germination rate (44.2%).

The time of soaking in pig or cattle slurry significantly influenced the germination rate ($p \leq 0.001$; Table 4) of *R. obtusifolius* seeds. On average, seeds reached a germination rate of 55.8% after two weeks of soaking in slurry. This was statistically comparable to the germination rate after four weeks (45.8%). However, a decreasing trend in germination rate was observed with increasing soaking time. We found that after twenty-four weeks of soaking the seeds in slurry (the estimated storage time of the slurry in the containment systems on the farm), they were no longer capable of germination. The statistically comparable and highest percentage of germinating seeds was recorded after soaking in cattle slurry for four weeks (65.8%) and after soaking in pig slurry for two weeks (62.5%). These values were completely comparable to the control treatment (0—no soaking in slurry; 61.7%), which was not significantly different from the percentage of germinating seeds (49.2%) soaked in cattle slurry for a fortnight (Tukey's limit for the interaction was ± 18.09). After four weeks of soaking in cattle slurry, the germination rate was significantly lower, with only 25.8% of

the seeds remaining viable. Interestingly, the percentage of germinating seeds decreases with increasing soaking time in slurry, because after half a year of soaking, not a single seed germinated.

The differences in germination energy between the different types of slurry (pig or cattle) are statistically comparable ($p > 0.05$; Table 5). The average germination energy of seeds soaked in pig or cattle slurry was 14.4% and 13.9, respectively.

Statistically, the highest germination energy was achieved in seeds soaked in slurry for a fortnight (52.5% of seeds germinated in the first 4 days; 55.8% and 49.2% in pig and cattle slurry, respectively). Again, germination energy decreases the longer the seed is soaked in the slurry. After four weeks, only 2.5% of the seeds germinated (counting germinated seeds after four days; germination energy in pig and cattle slurry was 0.0% and 5.0%, respectively), and after twenty-four weeks, not a single seed germinated. We suspect that the high ammonia content of the pig slurry may have contributed to the lower germination rate. The Tukey limit for the interaction was ± 8.51.

3.3. Experiment III: In Vitro Rumen Digestion Followed by Soaking in Cattle or Pig Slurry

The influence of in vitro digestion in cattle rumen juice and subsequent soaking (24 weeks—the estimated storage time of slurry in containment systems on farms) in cattle or pig slurry showed statistically significant differences ($p \leq 0.001$) in the germination rate and germination energy of *R. obtusifolius* seeds (Tables 6 and 7). We find that the proportion of germinable seeds decreases with the increasing duration of exposure to microorganisms in the rumen juice. The highest proportion of germinable seeds of *R. obtusifolius* (32.9%) was observed after 24 h of in vitro rumen digestion, while the germination rate was 19.6% and 15.0% after 36 and 48 h, respectively.

Table 6. The effects of in vitro digestion in rumen juice followed by soaking in slurry on germination rate (%) of *R. obtusifolius* seeds (mean of four replicates).

Slurry	In Vitro Digestion in Rumen Juice (h)				Average Slurry ***
	0	24	36	48	
Pig	0.0	0.0	0.0	0.0	0.0 B
Cattle	0.0 c	65.8 ± 5.0 a	39.2 ± 7.4 b	30.0 ± 8.2 b	33.8 ± 6.2 A
Average in vitro digestion ***	0.0 D	32.9 ± 12.5 A	19.6 ± 7.6 B	15.0 ± 5.9 C	

*** significant at the 0.001 probability levels. A–D mean values (± SEM) for each factor level followed by different letters are significantly different (Duncan, α = 0.05). a–c mean values (± SEM) for interaction followed by different letters are significantly different (Tukey, α = 0.05).

Table 7. The effects of in vitro digestion in rumen juice followed by soaking in slurry on germination energy (%) of *R. obtusifolius* seeds (mean of four replicates).

Slurry	In Vitro Digestion in Rumen Juice (h)				Average Slurry ***
	0	24	36	48	
Pig	-	-	-	-	-
Cattle	-	24.2 ± 7.1 a	12.5 ± 2.8 ab	5.8 ± 3.7 b	10.6 ± 2.3
Average in vitro digestion ***	-	12.1 ± 5.6 A	6.3 ± 2.7 BC	2.9 ± 2.0 C	

*** significant at the 0.001 probability levels. A–C mean values (± SEM) for each factor level followed by different letters are significantly different (Duncan, α = 0.05). a, b mean values (± SEM) for interaction followed by different letters are significantly different (Tukey, α = 0.05). "-" seeds that have not germinated and have no germination energy.

Table 6 shows that none of the seeds germinated after exposure to pig slurry. In contrast, the seeds soaked in cattle slurry germinated, but the percentage of germination decreased with the duration of in vitro digestion in rumen juice. We suspect that the main

reasons for this are the influence of microorganisms in the rumen juice that stimulate or inhibit seed germination. The highest germination rate was observed in seeds soaked in rumen juice for 24 h (65.8%). The percentage of germinating *R. obtusifolius* seeds decreased statistically significantly to 39.2% after 36 h and to 30.0% after 48 h.

The duration of exposure of *R. obtusifolius* seeds to in vitro digestion of rumen juice and the type of slurry had a significant effect on germination energy (Table 7). On average, seeds exposed to the in vitro digestion of rumen juice reached the highest germination energy (12.1%) after 24 h. The longer the seed is exposed to the microorganisms in the rumen juice, the lower the germination energy. Thus, only 6.3% and 2.9% of the seeds of *R. obtusifolius* germinated after 36 and 48 h, respectively.

In cattle slurry, we recorded the statistically highest germination energy on average (10.6%). In the first four to six days of the germination trials, none of the seeds exposed to in vitro digestion in rumen juice and pig slurry germinated. Meanwhile, germination energy in cattle slurry statistically decreased from an initial 24.2% after 24 h to 12.5% after 36 h and finally to 5.8% after 48 h of rumen juice digestion.

4. Discussion

4.1. Experiment I: Seed Viability after Ensiling and In Vitro Digestion in Rumen Juice

Several authors found in similar experiments that the seeds of *Stipa aliena* [30], *Saussurea japonica* and *Saussurea iodostegia* [31] and *Vicia angustifolia* [29] exposed to microbial digestion in the rumen did not germinate or that seed germination was significantly reduced, whereas in the present experiment, only the germination of *R. obtusifolius* was studied. Even if the rumen juice in the present Experiment I had a stimulating effect on seed germination (the microbes thin the cell wall of the seeds with their microbial activity and thus accelerate germination), the discrepancies between the studies are most likely due to the fact that different plant species react differently to microbial digestion.

Gardener, Mcivor and Jansen [19] note that the longer microbial digestion takes, the less chance the seed has of germinating. However, it is not only the microbes in the rumen that influence seed germination but also the pH fluctuations in the rumen that significantly affect the success of seed germination. The pH value in the rumen drops sharply when the animal eats and stabilizes again when the animal ruminates. The composition of the animal's feed ration also has a decisive influence on the composition of the microorganisms in the rumen because there are different microorganisms for different types of feed, and all this has an effect on the degradation time of the ration. The intensity of feeding shortens the retention time of the feed in the gastrointestinal tract and increases the outflow from the rumen. Under anaerobic conditions in the digestive tract, bacteria secreting proteolytic and cellulolytic enzymes attach themselves to the surface of the seed and break down the proteins into amino acids and the cellulose into simple sugars. In addition, swallowing and chewing the seeds can cause mechanical damage, which also affects the subsequent germination of the seed.

The results of Experiment I are similar to those of Blackshaw et al. [32] and Hahn, et al. [33]. They observed that the ensiling process severely affected the germination of certain plant species. In their study, the seeds lost germination capacity significantly and, in some cases, completely. This makes it unlikely that these germinated seeds will develop into seedlings. Our research findings that the most important reason for the loss of germination of ensiled seeds is the elevated temperature ($\geq$60 °C) that occurs at the beginning of ensiling, as well as the long-term exposure to lactic acid (a fermentation product during ensiling), are also confirmed by Blackshaw and Rode [32] and Hahn, de Mol and Müller [33]. Masuda, Nishimura, Kobayashi, Yamano, Nakano and Goto [20] found that storage in silage reduced but did not prevent germination of mature seeds of *R. obtusifolius*. However, they tested the seeds in silage with a dry matter content (DM) of 412 g/kg. Such a silage would be typical for silages produced under Slovenian conditions. In Slovenia, a content of 350 to 450 g DM/kg fresh mass is recommended for grass silages [34]. The silages in our experiments had an average dry matter content between 421.75 g and 428.11 g DM/kg,

with a pH between 4.76 and 4.81. The low pH was probably responsible for the destruction of *R. obtusifolius* seeds and their germination capacity.

It appears that the germination energy of *R. obtusifolius* seeds decreases after a sufficiently long exposure to rumen microorganisms. Germination energy is of great importance for the maintenance of the species. In our study, we are dealing with seeds of plants from the third cutting (late summer). At this time, the favourable period for plant germination is shorter. Faster germination is thought to increase the seeds' chances of survival in the wild, as they have a shorter window of favourable environmental conditions for germination and emergence. This adaptation enables them to produce a greater number of new plants.

4.2. Experiment II: Seed Viability after Ensiling and Soaking in Pig or Cattle Slurry

Recent research on the effects of manure or slurry handling on weed seed germination ability is limited. Although some studies have examined the effects of seed treatments with various organic amendments, including animal manure, on the germination rate of other plant species, none of these studies have examined the effects of soaking *R. obtusifolius* seeds in cattle or pig slurry on the germination rate and energy. However, earlier work provides valuable insights, some of which are almost comparable to the results of our research. Oswald [35] was one of the first to report on the effects of animal digestion and manure treatment on weed seed germination capacity in the early 20th century. The study included two sources of weed seeds in manure: either contaminated feed ingested by the animals or seeds in the bedding that bypassed the animals. These seeds of 52 weed species were placed in piles of horse manure, cow manure or a mixture of horse and cow manure. After 60 days, the seeds of all 52 weeds had completely lost their ability to germinate. In modern containment systems, sediment from solid separators or settling ponds can concentrate weed seeds in the slurry, resulting in very high numbers of viable weed seeds in the slurry [36].

Kimura, Umetsu and Takahata [23] came to similar conclusions as those in our study. The aim of their study was to investigate the survival of *R. obtusifolius* subjected to anaerobic fermentation in slurry. Two fermentation temperatures (35 and 42.5 °C) were used to determine the percentage of seed germination, and three periods (10, 15 and 20 days) were used, during which the seeds were exposed to fermentation. The authors of the study conclude that anaerobic fermentation reduces the seed germination of *R. obtusifolius*. Fewer seeds germinate at a temperature of 42.5 °C than at 35 °C, and the combination of time and temperature destroys their ability to germinate.

Zaller [17] investigated how different methods of manure composting (conventional or biodynamic with a temperature of up to 63 °C and compost with earthworms with a temperature of up to 35 °C) affect the germination of *R. obtusifolius* seeds. The author notes that after one month of composting, the germination rate of *R. obtusifolius* seeds was higher in the compost with earthworms (48%), while the percentage of germinating seeds was 18% in the biodynamic compost and 28% in the conventional compost. After three and four months, only the trial with *R. obtusifolius* seeds in compost with earthworms was carried out. Between 22% and 3% of the seeds germinated. Seeds stored at room temperature germinated up to 89%. The results show that the highest temperature is not the main factor reducing the germination of *R. obtusifolius* seeds.

4.3. Experiment III: In Vitro Rumen Digestion Followed by Soaking in Cattle or Pig Slurry

We believe that the lack of germination of seeds after exposure to pig slurry and the decreasing percentage of germination of seeds soaked in cattle slurry when digested in vitro for a prolonged period in the rumen juice is due to the influence of the microorganisms present in the rumen juice. These microorganisms are probably responsible for stimulating or inhibiting seed germination. It is well known that the seed coat is one of the most important determinants of seed germination [37]. Understanding its properties and characteristics can explain, predict or even modify seed performance under specific environmental conditions. There is growing evidence that seed coat properties are related

to specific seed problems. For example, susceptibility to mechanical damage is related to seed coat lignin content, while tolerance to environmental stress depends on seed coat integrity. We believe that in our experiment the cellulitic, proteolytic and amylolytic activities of the microbes influence the seed germination [38–40]. It is assumed that the microbes accelerate the thinning or degradation of the cell wall of the seeds. However, to confirm this assumption, further studies would be needed to more precisely define the mechanisms of action of the rumen microorganisms on the seeds of *R. obtusifolius*.

The studies [22,41] came to similar conclusions as ours. According to the authors of [42], the seeds of *R. obtusifolius* found in cattle slurry lose their germination capacity during the ripening process. In contrast, the experiment observed by Humphreys, Culleton, Jansen, ORiordan and Storey [22] used seeds of *R. obtusifolius* collected between June and November. These seeds were exposed to silage for 100 days and then soaked in rumen juice for 72 h and in cattle slurry for 100 days. Seeds collected in early June did not germinate. Seeds collected in late June had a germination rate of 10% and seeds collected in early August had a germination rate of 85%. Seeds that were ensiled did not germinate. Seeds that were soaked in slurry retained 64.9% germination. Fermentation in the rumen clearly had a negative effect on the germination of *R. obtusifolius*, as only 24.9% of the seeds germinated. The results are comparable to ours, as we also found a deterioration in germination of *R. obtusifolius* seeds after fermentation in rumen juice.

In several studies (from the late 20th to the early 21st century), the researchers mentioned below investigated the effects of the digestion of various animals and their faeces on the germinability of weed seeds. They found that the digestion of animals and their faeces can be an important source for the spread of weeds.

Harmon et al. [42] studied the effects of digestive tract and manure on weed seeds. The weed seeds were fed to calves, horses, sheep, pigs, or chickens. Almost 25% of the seeds fed to pigs and cattle were found in the faeces, compared to only 10 to 12% in horses and sheep. Chickens were the most effective at destroying weed seeds. Only 2% of the fed seeds of *Abutilon theophrasti* were germinated, while none of the fed seeds of *Convolvulus arvensis*, *Melilotus* sp., *Rumex altissimus*, *Arabidopsis thaliana*, *Rosa acicularis* and *Lepidium latifolium* were germinated. Of the seeds obtained from calves, horses, sheep or pigs, an average of 25% germinated. Although few in number, 62% of the seeds that survived consumption by a chicken germinated, suggesting that the gizzard may have scratched the seeds and stimulated germination. Combining the recovered seeds and the germination of the fed weed seeds, sheep, horses, pigs and calves each had 6–10% germinable seeds, while poultry had only 1% germinable seeds, which could be due to crushing in the gizzard.

Similar to this study, Atkeson, et al. [43] investigated the effects of cattle digestion and subsequent manure storage on weed seed germination. Dairy cows were fed 2 L of weed seed in one day. The manure was collected, mixed with straw to simulate manure, and stored for 3 months. The seeds were ingested by the animals for 4 days. According to the authors, weeds with soft seed coats were more affected than those with hard ones. Digestion alone reduced the germination capacity of *Avena fatua*, *Melilotus officinalis*, *Plantado major* and *Medicago sativa* by more than 80% but had a significantly lower effect on *Setaria viridis*, *Linaria vulgaris*, *Rumex crispus*, *Centaurea maculosa* and *Heracleum maximum*. After 3 months of storage in manure very few germinated weed seeds were found. Only the seeds of *Amaranthus* sp., *Rumex* sp. and *Eleusine indica* survived both digestion and storage in the manure. In contrast, all seeds of *Melilotus officinalis*, *Plantago lanceolata*, *Setaria viridis*, *Equisetum arvense*, *Briza media*, *Avena fatua*, *Brassica rapa* and *Cirsium discolor* germination capacity. Olson et al. [44] found that only 4% of mature seeds of *Euphorbia esula* were germinated when fed to sheep.

Jeyanayagam et al. [45] compared weed seed germination in simulated fermenters with 3-L containers and daily feeding at 35 °C. After simulated rumen treatment followed by 15 to 20 days in a digester, the weed seed germination of *Sorghum halepense* decreased by 18 and 82% and of *Panicum dichtomiflorum* by 24 and 76% for dormant and non-dormant

seeds, respectively. Anaerobic digestion killed about three to five times more non-dormant seeds than dormant seeds.

Katovich et al. [46] investigated the effects of anaerobic slurry digestion on weed seed germination ability. Seeds of six weed species were subjected to rumen fermentation and a subset of the seeds was stored in a plug flow anaerobic digester for 20 days (this is the time it takes a batch of slurry to pass through the digester), while another subset was stored in the slurry pit for the same period before entering the digester. During a germination test in the field, the sward of a long-established *Poa pratensis* plot was removed to expose the bare soil. The recovered seed and digested or undigested manure were spread on the bare soil in autumn. The emergence of weeds was monitored during the following two growing seasons. The germinability of weed seeds used in this study ranged from 82% for *Panicum miliaceum* to 99% for *Abutilon theophrasti* and germination rates ranged from 1 to 14% in initial tests. The rumen treatment appeared to kill all seeds of *Setaria faberi*, *Panicum miliaceum* and *Alopecurus pratensis*, as no seeds germinated in the inorganic fertiliser control. Temperatures in the anaerobic digester where the seeds were introduced were between 35 and 38 °C, well below the 60 °C required to kill weed seeds. The anaerobic digestion of manure did not kill or reduce weed seed germination in this study. The possibility that anaerobic digestion kills seeds in slurry was not tested because all seeds were exposed to rumen digestion.

Compare this with the results of the study by Šarapatka, et al. [47], in which the weed seeds of eight species were placed at two depths in simulated anaerobic digester tanks for about 30 days. Passage through the dairy cows did not kill all weed seeds but effectively reduced the germination of *Chenopodium album* and *Echinochloa crus-galli*. Some weed seeds at 40 cm depth survived digestion, but no viable seeds were found at 175 cm depth near the bottom of the tank. These differences were partly attributed to the higher initial temperatures at 175 cm depth.

5. Conclusions

In line with the research objectives, we found that ensiling is an effective but often overlooked method to reduce the germination of *R. obtusifolius* seeds. However, the treatments used in this study that reduce the germination of *R. obtusifolius* seeds do not necessarily affect their viability. As already described in:

- Experiment I: Ensiled seeds did not germinate at all. Seeds that are not ensiled have a high germination capacity.
- Experiment II: In vitro rumen digestion affects the germination energy of non-ensiled seeds but not the overall percentage of germination.
- Experiment III: Soaking in pig and cattle slurry does not promote germination and reduces the percentage and energy.

Ensiling and digestion in the rumen help to displace the seeds of *R. obtusifolius* and reduce their density, which delays their germination. However, these processes can also activate non-ensiled seeds without increasing their germination rate. Ensiling and rumen digestion can be effective ecological control methods if the seeds are part of the biomass destined for storage. However, further studies are needed to understand the causes and consequences of this issue and to compare them with other research results.

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