



## Research article

## Preliminary assessment of probiotic *Bacillus subtilis* C-3102 in feces: evaluation of their survival after oral supplementation in goats

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### Abstract

The study's objective was to demonstrate the ability of *Bacillus subtilis* to survive gastrointestinal transit after oral supplementation assessed in fecal samples is considered an inherent property of potential probiotics. Six, rumen-fistulated, 3.5-year-old, non-lactating female Saanen goats (average initial body weight of  $65 \pm 8$  kg) were assigned to two treatments: basal rations (CON) and basal rations supplemented with *B. subtilis* probiotic product (BS) in a cross-over design. Each experimental period lasted 21 days. On the last day of each experimental period, rumen fluid and fecal samples were collected. Body weights were recorded weekly throughout the experiment. Bodyweight and rumen pH were found to be similar between dietary treatments. The goats that received BS had higher numbers of *B. subtilis* in fecal samples than CON. Therefore, it was concluded that *B. subtilis* met a prerequisite of probiotics to survive the passage through the gastrointestinal tract. The current result also provides a factual basis for future research involving any effects after supplementing probiotic *B. subtilis* in small ruminants.

**Keywords:** *Bacillus subtilis*, Probiotic, Goats

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## INTRODUCTION

In recent years, the feeding of additives containing live microorganisms (i.e., direct-fed microbials or probiotics) is generally considered beneficial in animal nutrition to improve hosts' health (FAO, 2016). While the wide array of bacteria has been used as probiotics, spore-forming bacteria, particularly many species from the genus *Bacillus*, are increasingly being used in animal feed due to their spores' robustness during feed processing (FAO, 2016). In dairy cattle, supplementation of feed with *Bacillus subtilis* fermentation products promoted of ruminal bacteria (Sun et al., 2013), increased nutrient utilization (Peng et al., 2012; Sun et al., 2013; Song et al., 2014), improved rumen fermentation (Peng et al., 2012; Sun et al., 2013; Sun et al., 2016) and enhanced milk production (Peng et al., 2012; Sun et al., 2013; Choonkham et al., 2020). Moreover, in periparturient cows, supplementation with *B. subtilis* decreases NEFA concentrations (Peng et al., 2012) and improves the animals' oxidative status (Choonkham and Suriyasathaporn, 2018). In lactating ewes, supplemental *B. subtilis* has been shown to decrease mortality during the suckling period, increase milk yield, and improve the milk composition (Kritis et al., 2006). In weaned lambs, immune- and oxidative statuses were improved (Mousa et al., 2019).

Viability after transit through the gastrointestinal tract is considered an inherent property of potential probiotics. Therefore, a preliminary assessment of the presence of *B. subtilis* in fecal samples after oral supplementation was performed to demonstrate the ability of *B. subtilis* to survive passage through the gastrointestinal tract.

## MATERIALS and METHODS

### Ethical considerations and animal welfare

The experimental protocol was approved by the Animal Experiments Committee of the Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands (Ethics Committee approval no. 10803-2019-3).

### Animals, experimental design, and housing

The study was conducted at the Department of Population Health Sciences, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands. Six rumen-fistulated, 3.5-year-old, non-lactating female Saanen goats with an average initial body weight of  $65 \pm 8$  kg were used. The trial had a cross-over design, two experimental periods lasting 21 days with a 7-day washout period. Samples were collected on the last day of each period. The animals were randomly assigned to two treatments, i.e., a basal ration (CON) and a basal ration supplemented with *B. subtilis* probiotic product (BS). The goats were individually housed in pens with automated temperature monitoring and control. Environmental enrichment (jolly balls) and wall-mounted grooming brushes were also provided in the pens. Throughout the study, goats had free access to a salt block and fresh drinking water.

## Experimental rations

The goats were fed iso-energetically (on net energy basis), and energy intake was calculated to be 6.4 MJ NE/day to meet the energy requirement for maintenance (CVB, 2012). The daily amounts of feed (as fed basis) provided to the animals were as follows; 100 g hay, 100 g unpelleted straw, 630 g pelleted straw (B. V. Oldambt, Oostwold, The Netherlands), and 630 g pelleted concentrate either without or with *B. subtilis* C-3102 (Research Diet Services B. V., Wijk bij Duurstede, The Netherlands). The pelleted concentrate containing *B. subtilis* C-3102 (Calsporin®, Orffa Additives B. V., Werkendam, The Netherlands)  $9.7 \times 10^6$  CFU/g were measured by plate count (Mérieux NutriSciences, Barcelona, Spain). The hay, pelleted straw, and pelleted concentrate were offered in two meals at 7:00 and 19:00, while the unpelleted straw was provided once daily at 12:00. The feed was offered and monitored by the animal caretaker to ensure complete consumption. The chemical composition of the experimental feedstuffs is presented in Table 1.

**Table 1** Chemical composition of the components of the experimental rations provided to the goats.

Items	Components of the experimental rations				
	Hay	Unpelleted Straw	Pelleted straw	Concentrate	Concentrate + BS
DM (g/kg)	930.9	942.5	911.6	897.2	899.9
CP (g/kg)	67.5	47.0	45.3	174.7	178.8
NDF (g/kg)	670.0	715.3	718.4	312.3	352.3
Ash (g/kg)	77.8	90.1	67.4	71.4	74.8
<i>B. subtilis</i> (CFU/g)	-	-	-	< 10 <sup>3</sup>	$9.7 \times 10^6$

Concentrate + BS, the pelleted concentrate containing *B. subtilis*  $9.7 \times 10^6$  CFU/g

Chemical composition on dry matter (DM) basis; CP, crude protein; NDF, neutral detergent fiber

The NDF value of 670.0 g/kg of hay was derived from the CVB (2012).

## Sample collection and analysis

The representative samples of feed were collected and stored at  $-20^{\circ}\text{C}$  until further processing. At the end of the experiment, the feed samples were ground to pass a 1-mm screen in a mill and stored at room temperature until chemical analysis for dry matter (DM), crude protein (CP), neutral detergent fiber (NDF), and ash. Body weights were recorded weekly for each period in the same manner throughout the experiment. On the last day of each experimental period, rumen fluid and fecal samples were collected. Rumen fluid samples were collected through the rumen fistula from 3 sites of the rumen using an evacuated drainage bottle at 2, 4, 6, 12 hours after the morning feeding and measured the pH value immediately after sampling using benchtop pH meter. Fecal samples were collected directly from each animal's rectum in the morning and stored at  $-20^{\circ}\text{C}$  in sealed aluminum trays until the number of *B. subtilis* in fecal samples. According to BSI British Standards (2009), the pelleted concentrates and fecal samples were prepared in 0.2% sodium hydroxide solution for initial suspension and heat-treated at  $80^{\circ}\text{C}$  for 10 minutes. The heat-treated samples were prepared in peptone salt solution (0.1% casein, 0.85% NaCl, pH 7.0  $\pm$  0.2) for serial dilutions, spread plated

on tryptone soy agar (tryptone 1.5%, NaCl 0.5%, soya peptone 0.5%, agar 1.5%, pH 7.3 ± 0.2), and incubated at 37°C for 16 to 24 hours aerobically for enumeration of colony-forming units (CFU) of *B. subtilis* in samples. The detection limit was  $1.0 \times 10^3$  CFU/g of sample.

### Statistical analysis

Data for body weight and rumen pH were analyzed as repeated measures using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC, USA) with a model containing fixed effects of sequence, treatment, period, sampling time, the interaction between treatment and sampling time, and goat within the sequence as a random effect. The best covariance structure was selected based on Akaike's information criterion and Schwarz's Bayesian criterion among first-order autoregressive, compound symmetry, and unstructured. The values were presented as least squares means and standard errors of the mean. Descriptive statistical analysis was completed using the UNIVARIATE procedure of SAS. The numbers of *B. subtilis* more than the detection limit,  $1.0 \times 10^3$  CFU/g of fecal sample between treatments were tested using Chi-square test.

## RESULTS

After one week of the experiment, one goat in CON was excluded due to severe sickness. No difference was observed in body weight and rumen pH with the supplementation of *B. subtilis* (Table 2). Descriptive data of numbers of *B. subtilis* in fecal samples are presented in Table 3. After three weeks of supplementation, BS had higher numbers of *B. subtilis* in fecal samples than CON. Numbers of *B. subtilis* in fecal samples from all goats demonstrated to lower than the detection limit,  $1.0 \times 10^3$  CFU/g within 7 days after cessation of probiotic supplementation in the washout period.

**Table 2** Effect of dietary supplementation of *Bacillus subtilis* (BS) on body weight and rumen pH

Items	Time	Treatment		SEM	P-value				
		CON	BS		Sequence	Treatment	Period	Time	Treatment × Time
<b>Bodyweight (kg)</b>	Day 7	67.2	66.7	3.11	0.30	0.37	0.04	0.03	0.23
	Day 14	68.0	69.7	3.11					
	Day 21	68.2	68.5	3.11					
<b>Rumen pH</b>	2 Hours	6.0	6.0	0.09	0.62	0.34	0.04	0.08	0.38
	4 Hours	6.0	6.0	0.09					
	6 Hours	6.1	6.1	0.09					
	12 Hours	6.3	6.1	0.09					

Hours = hours after the morning feeding

CON, no supplemental BS; BS, approximately  $6 \times 10^9$  CFU BS/goat per day

P-value of fixed effects

**Table 3** Effect of dietary supplementation of *Bacillus subtilis* (BS) on numbers of *B. subtilis* in fecal samples

Period	Treatment	Numbers of <i>B. subtilis</i> (CFU/g)				Numbers of tested samples with <i>B. subtilis</i> > 1.0 × 10 <sup>3</sup> CFU/g	P-value
		Mean	SD	Minimum	Maximum		
Period 1	CON (n=2)	< 1.0 × 10 <sup>3</sup>	-	-	-	0/2	0.03
	BS (n=3)	1.5 × 10 <sup>6</sup>	0.40	1.1 × 10 <sup>6</sup>	1.9 × 10 <sup>6</sup>	3/3	
Washout Period	CON (n=5)	< 1.0 × 10 <sup>3</sup>	-	-	-	0/5	-
Period 2	CON (n=3)	< 1.0 × 10 <sup>3</sup>	-	-	-	0/3	0.03
	BS (n=2)	2.0 × 10 <sup>6</sup>	0.49	1.6 × 10 <sup>6</sup>	2.3 × 10 <sup>6</sup>	2/2	

CON, no supplemental BS; BS, approximately 6 × 10<sup>9</sup> CFU BS/goat per day

P-value of Chi-square test

All fecal samples from CON had numbers of *B. subtilis* less than 1.0 × 10<sup>3</sup> CFU/g (detection limits)

The calculation of risk factor with death was based on logistic regression model. The clinical variables (ages, body weight, azotemia, uterine rupture, the presenting of vaginal discharge and the amount of total white blood cell count before surgery) that were significant (P<0.05) based on univariate analysis were selected to perform multivariate analysis. Finally, the only one factor that related with suspected pyometra death was uterine rupture. The fluid filled in uterus dog that presented with uterine rupture had 7.38 times risk to death than dog without uterine rupture (adjusted odd ratio 7.38 (95% CI =2.73,19.93)) as shown in the Table 3.

## DISCUSSION

The current results indicate that *B. subtilis* can survive gastrointestinal transit that fulfills to qualify as a probiotic (Borchers et al., 2009). A higher number of *B. subtilis* in fecal samples as a consequence of supplementing *B. subtilis* was also observed in pigs (Menegat et al., 2019 and 2020). Lower than the detection limit, 1.0 × 10<sup>3</sup> CFU/g after cessation of probiotic supplementation agrees with the characteristic of Bacillus-based probiotics that spores can germinate but cannot proliferate in the gastrointestinal tract (Buchanan et al., 1974). No differences in body weight were also observed in sow (Menegat et al., 2019), nursing piglets (Menegat et al., 2020), and dairy cattle (Souza et al., 2017) supplemented with *B. subtilis*. No differences in body weight may be due to no alteration in dry matter intake (Peng et al., 2012, Sun et al., 2013), chewing activity, and nutrient digestibility (Souza et al., 2017) after supplementation of *B. subtilis*. The rumen pH values were within the normal range (Table 2). Desnoyers et al. (2008) and Nur et al. (2018) demonstrated that goats could adapt their feeding behavior around the feedings to maintain rumen pH within a normal range (6.0–7.0). In dairy cattle, Sun et al. (2013) found that pH decreased from 6.6 to 6.5 after supplementation of *B. subtilis*, while no difference in rumen pH was observed by Peng et al. (2012).

Further exploration needs to confirm the underlying physiological mechanisms on rumen pH after supplementation of *B. subtilis*.

## CONCLUSION

*B. subtilis* met a prerequisite of probiotics. They can survive the passage through the gastrointestinal tract. The current results also provide a factual basis for future research involving any effects after supplementing probiotic *B. subtilis* in small ruminants.

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## AUTHORS CONTRIBUTION

Conceptualization, W. C., A. R., and J. T. S.; methodology, W. C., A. R., and J. T. S.; validation, J. T. S.; formal analysis, W. C. and A. R.; data curation, W. C. and A. R.; writing—original draft preparation, W. C. and A. R.; writing—review and editing, J. T. S.; supervision, J. T. S. and W. S. All authors have read and approved of the final version of the manuscript before submission to the journal.

## CONFLICT OF INTEREST

The authors declare that no conflict of interest.

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