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Assessment of the effect and safety of salacinol in horses

Fumitaka UEDA[1]*, Atsushi IIDA[1], Hitomi SAITO[1], Shinobu SEKI[1], Akihito AMAO[2] and Hirotsugu YAMATE[3]

[1]Bio Science & Engineering Laboratory, Research & Development Management Headquarters, FUJIFILM Corporation, Kanagawa 258-8577, Japan
[2]Highly Functional Materials Business Development Headquarters, FUJIFILM Corporation, Tokyo 107-0052, Japan
[3]Yamate Racehorse Clinic, Iwate 020-0103, Japan

We report a study that examined the effect and safety of salacinol from Salacia reticulata extract (SRE) for the intestinal microbiota of horses. We administered SRE to healthy horses and evaluated their intestinal microbiota before and after the test period for changes in composition. Horses that received the SRE showed notable differences in intestinal microbiota composition between before and after administration, with a substantial increase in bacteria of the order Lactobacillales at the end of the test period. Moreover, the Firmicutes-to-Bacteroidetes ratio was elevated. Salacinol was administered as a supplement for 28 days. Physiological and blood tests were conducted in the presence of a veterinarian, and a safety assessment was performed. These evaluations revealed no detrimental findings.
Key words: *Firmicutes, horse, intestinal microbiota, Lactobacillales, salacinol*

Horses are herbivores and have a highly developed cecum and colon. As energy sources, horses depend on volatile fatty acids produced via the degradation of carbohydrates by protozoa and microbes that are present in the hindgut [1]. Compared with other animals, horses are physiologically susceptible to the effects of drugs, feed, and stress. Horses are prone to diarrhea, loose stools, and colic from activities such as long transportation for exercise or leisure. Therefore, proper maintenance of the microbiota in the hindgut is important for equine health [19].

The intestinal microbiota is considered important for overall health, particularly for immune function, evention of bowel disease, efficient nutrient use, and stress relaxation ability. These functions exemplify the diversity of conditions that is impacted by the intestinal microbiota. Racehorses are subjected to high levels of stress from long-distance travel [4], antimicrobial administration [2], training, and racing. Because some horses suffer from intestinal disorders, such

as diarrhea and constipation, the development of health maintenance methods through improvement of the equine intestinal environment is urgently required.

Methods of increasing beneficial bacteria to enhance the horse enteric environment include administration of lactic acid bacteria and bifidobacteria. In particular, Lactobacillales includes *Lactobacillus* species, which have been reported to be useful bacteria that prevent equine diarrhea and contribute to weight gain of foals [21, 22].

We previously reported that salacinol, the active ingredient in *Salacia reticulata* extract (SRE), increased the number of beneficial bacteria commonly found in rat and human intestines and improved immune function [17, 18].

When SRE was administered to rats, changes were observed in the pattern of the intestinal microbiota; initially, gene expression analysis of mRNA extracted from the mucosal cells of the small intestine revealed an upregulation of certain biological defense-related genes, including immune-relevant genes [17].

In a randomized, double-blind, parallel-group study conducted in humans, SRE administration significantly increased the proportion of *Bifidobacterium* in the intestinal microbiota, and the bacteria of the order Lactobacillales showed an upward trend.

In humans, saccharides are assimilated by bacteria such as *Bifidobacterium*. An increase in their number enhances

production of short-chain fatty acids in the intestine [6], which are then absorbed by the body for energy, in addition to promotion of colonic mucus secretion [20] and activation of intestinal motility [7]. Moreover, short-chain fatty acids suppress the growth of bacteria that exert detrimental biological effects [16].

The composition of the intestinal microbiota can be substantially different in different animals, and novel species can be harbored depending on the animal [12]. Therefore, it is necessary to determine the *in vivo* function of the intestinal microbiota in each animal species. The aim of the present study was to investigate the effects of SRE administration on horses, with a focus on their intestinal microbiota. Moreover, the safety of SRE administration was evaluated.

The feed amendment for SRE administration in this study was derived from an extract of *S. reticulata* harvested in Sri Lanka by Eco Tech Create 21 Co., Ltd. (Sri Lanka) and purchased from Eco Tech Create 21 Co., Ltd. (Japan). The harvested plant stems and roots were subjected to comparative botanical identification using *S. reticulata* reference samples in the herbarium at the Industrial Technology Institute in Sri Lanka as well as thin-layer chromatography fingerprinting and were confirmed to be *S. reticulata* at that institute.

SRE was initially prepared as follows. The stem and root parts of *S. reticulata* were dried and broken into chips. After sufficient drying, an extract was obtained from the chips by placing them in hot water for 1 hr. After the chips were removed by filtration, and the liquid was treated with activated carbon, cooled, and subjected to spray drying using an ADL-310 spray dryer (Yamato Science Co., Ltd., Tokyo, Japan) at 4°C to obtain a powdery extract. The extract was analyzed for its components and found to contain 60.9% carbohydrates, 17.4% polyphenols, 14.7% ash, 3.7% water, 2.6% protein, and 0.7% lipids.

The SRE thus obtained was subjected to quality analysis for salacinol content using an overall activity evaluation index based on the α-glucosidase concentration. The index was expressed as the 50% inhibitory concentration (IC_{50} value) of α-glucosidase, which was negatively correlated with the salacinol content. Salacinol was quantified using a liquid chromatograph mass spectrometer (LC-MS), as per the method described by Muraoka *et al.* [14]. The results revealed that the SRE contained 0.5% salacinol.

The study was planned with the approval of the Fujifilm Corporation Animal Experiment Committee. Approval to conduct the study was obtained from the responsible veterinarian who was explained the study purpose and content as well as the constraints during the study period.

Thirteen racehorses were subjected to health examinations by a veterinarian. In addition, changes in feed, administra-

tion of drugs that may affect outcomes, long-distance travel, and exercise adjustments within the 3 weeks prior to study initiation were examined. Overall, 11 healthy horses (male and female horses aged 3–7 years, body weight 401–530 kg) were selected for the study. Horses were excluded from the study if there were any changes in feed, if any drug was administered that may affect outcomes, if long-distance travel or exercise adjustments were noted, or if the animal contracted any disease during the 3-week study period. Training continued during the SRE administration period.

SRE equivalent to 10 mg of salacinol was administered as a feed amendment twice daily in 5-mg portions.

This dose was calculated by converting the effective amount in humans [18] to the weight of a horse.

Fecal samples obtained before and 6 days and 14days after study initiation were subjected to intestinal microbiota analysis using the terminal restriction fragment length polymorphism (T-RFLP) method and compared to determine changes in bacterial distribution due to SRE administration. To investigate the safety of SRE, veterinarians observed the condition of the horses and performed blood biochemistry analyses.

After selecting 10 healthy male and female thoroughbred horses aged 5–26 years, an overdose test was conducted wherein 50 mg of salacinol supplement was fed twice daily (morning and evening) together with the feed. Prior to overdose study initiation, the horses were deemed healthy by a veterinarian. The horses were raised in Morioka City, Iwate Prefecture, Japan. The amount of feed ingested and health-related observations were recorded during the 4-week overdose study period. Blood tests, blood biochemistry examinations, and diagnostic evaluations were performed by the veterinarian.

In addition, for the 11 healthy horses (male and female horses aged 3–7 years) fed 10 mg of salacinol supplement twice daily (morning and evening) together with feed, fecal samples were collected during the 3-weeks and their pH was measured to detect conditions such as hindgut acidosis.

The equine fecal samples collected were diluted 10-fold with purified water and homogenized. The pH of the supernatant was measured twice for each horse using a simple pH meter (PH-222, Sato Shouji, Inc., Kawasaki, Japan). The mean pH values were adopted.

Analysis of intestinal microbiota in equine fecal samples was performed by Techno Suruga Lab (Shizuoka, Japan) using T-RFLP method [15]. The procedures described by Tanabe *et al.* [21] were modified as follows: Feces collected in a Feces Sampling Kit (Techno Suruga Lab, Shizuoka, Japan) were crushed with zirconia beads using a FastPrep FP100A Instrument (MP Biomedicals, Santa Ana, CA, U.S.A.) at 5 m/sec for 2 min. DNA extraction from 100-μl aliquots of the fecal suspension was performed using an

automated nucleic acid extractor (Precision System Science, Chiba, Japan). The reagent employed for the automated nucleic acid extraction was GC series Genomic DNA whole blood (Precision System Science). FAM was used for 516F labeling of polymerase chain reaction (PCR) primers instead of HEX as described by Tanabe *et al.* [21]. PCR products were purified using MultiScreen-PCR$_{\mu96}$ Filter Plates (Millipore, Billerica, MA, U.S.A.).

Fragment analysis was performed using an ABI PRISM 3130xl genetic analyzer (Thermo Fisher Scientific, Waltham, MA, U.S.A.), and the GeneMapper® (Thermo Fisher Scientific) software was used for evaluation. MapMarker® X-Rhodamine Labeled 50–1,000 bp (BioVentures, Murfreesboro, TN, U.S.A.) was used as standard size markers.

Blood was collected on the last of the 7 days of repeated SRE administration and used for blood tests and blood biochemistry analysis at Fujifilm Monolith Co., Ltd., Tokyo, Japan. Blood indices examined included white blood cell count (WBC), red blood cell count (RBC), hemoglobin amount (HGB), hematocrit value (HCT), mean corpuscular volume (MCV), mean cell hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet count (PLT), lymphocyte percent (LY), monocyte percent (MO), eosinophil percent (EO), and granulocytes (GR). Blood biochemistry indices examined included total protein concentration (TP), glucose (GLU), blood urea nitrogen (BUN), creatine (CRE), triglyceride (TG), calcium (Ca), inorganic phosphorus (IP), albumin concentration (ALB), alkaline phosphatase (ALP), glutamic pyruvic transaminase (GPT), glutamic oxaloacetic transaminase (GOT), total bilirubin (TBIL), and creatine phosphokinase activity (CPK).

For WBC, RBC, HGB, HCT, MCV, MCH, MCHC, PLT, LY, MO, EO, and GR was added to the blood as an anticoagulant, and plasma was obtained via centrifugation at 3,000 rpm for 10 min. The serum was analyzed for these indices using a comprehensive hematology laboratory system (Sysmex XT-2000iV, Sysmex Co., Ltd., Hyogo, Japan). For TP, GLU, BUN, CRE, TG, Ca, IP, ALB, ALP, GPT, GOT, TBIL, and CPK was used as an anticoagulant, and plasma was obtained via centrifugation at 3,000 rpm for 10 min. The serum was analyzed for these indices using an automatic biochemistry analyzer (Hitachi H 7070, Hitachi Ltd., Tokyo, Japan).

The Shapiro-Wilk test was used to evaluate the blood test data, intestinal microbiota analysis data, and the ratio of Bacteroidetes to Firmicutes for normality. If normality was not confirmed, comparison between values obtained before and after ingestion of the test food was performed by the Wilcoxon signed-rank test. When normality was confirmed, comparison between values obtained before and after ingestion of the test food was performed by the paired t-test.

Statistical analysis was performed with JMP version 14.0.0.

Analysis of the intestinal microbiota using the T-RFLP method revealed changes in the enteric bacterial composition of the group receiving SRE. Notably, a significant increase in the proportion of bacteria classified as Lactobacillales was observed on the sixth day, indicating that SRE ingestion considerably changed the equine intestinal microbiota (Fig. 1).

Analysis focusing on Firmicutes and Bacteroidetes in the intestinal microbiota revealed that SRE administration reduced the proportion of Bacteroidetes and increased that of Firmicutes. The ratio of Firmicutes to Bacteroidetes (F/B ratio) significantly increased (Fig. 2).

During the SRE administration period, no changes in feed preference or body weight were observed, and equine health conditions remained unchanged. Abnormalities in the results of blood tests and blood biochemistry analysis were not observed (Table 1). In the overdose study conducted with the salacinol supplement, no abnormalities in equine health or in the results of blood tests and blood biochemistry analysis during the dosing period were observed (Table 2).

Although the blood Ca^{2+} concentration tended to increase, it did not appear to cause abnormalities in the findings of the general diagnostic evaluation by the veterinarian. The results of pH measurements of equine feces indicated little change in values during the administration period.

Administration of SRE to horses increased the proportion of enteric bacteria belonging to the order Lactobacillales. Lactobacillales includes *Lactobacillus* species, which have previously been reported to be beneficial to horses [21, 22], suggesting that in our study, the equine intestinal

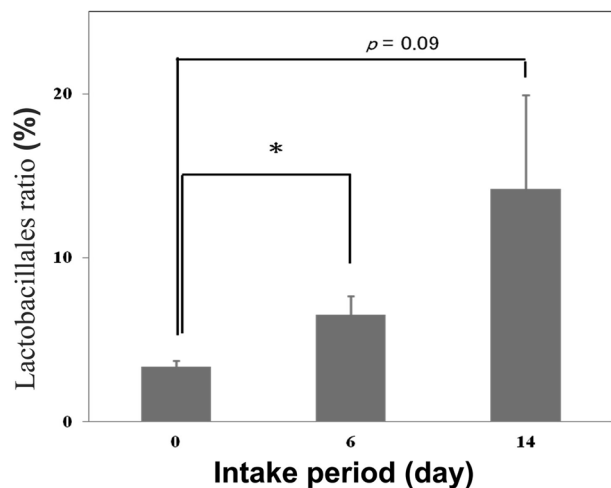**Fig. 1.** Changes in enteric bacterial ratio in the feces analyzed by T-RFLP when *Salacia reticulata* extract was fed to racehorses. Feeding the racehorses *Salacia reticulata* extract significantly increased the Lactobacillales ratio. *P<0.05.
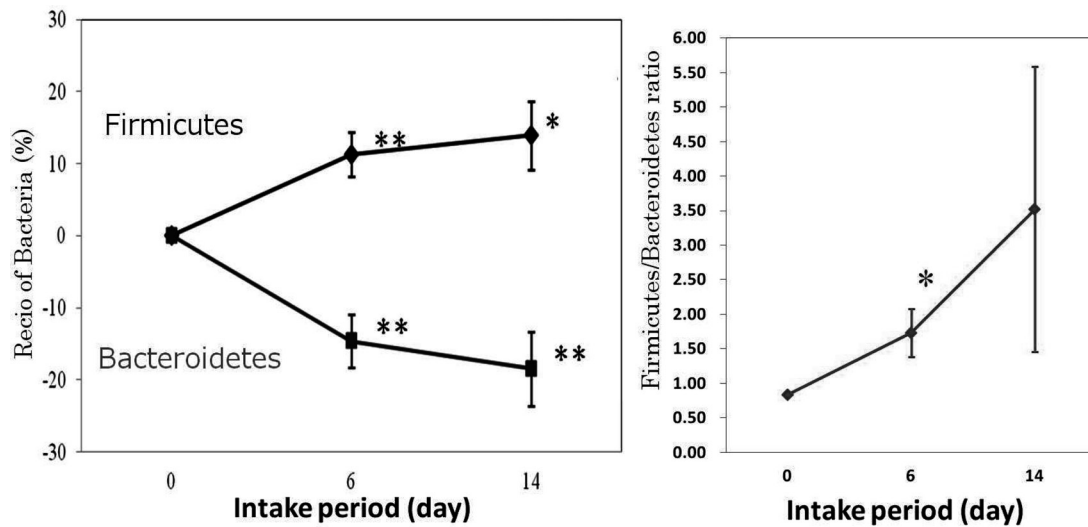
Fig. 2. Feeding the racehorses *Salacia reticulata* extract increased and decreased the proportions Firmicutes and Bacteroidetes, respectively, in the intestinal flora of the Firmicutes/Bacteroidetes ratio. *$P<0.05$; **$P<0.01$.

Table 1. Blood biochemistry test results when *Salacia reticulata* extract was ingested at the effective dose for 4 weeks

10 mg salacinol/day	Day 0		Day 28		t test	Reference range [3]
Blood indices	AVG	SD	AVG	SD		
WBC (10²/μl)	64.5	16.1	58.8	13.5	0.452	54–143
RBC (10⁴/μl)	824.0	123.0	828.6	100.0	0.935	680–1290
HGB (g/dl)	13.3	1.6	13.3	1.3	0.974	11–19
HCT (%)	37.2	4.8	38.2	3.8	0.662	32–53
MCV (fl)	45.3	2.0	46.2	2.1	0.390	37–58.5
MCH (pg)	16.2	0.8	16.1	0.9	0.795	12.3–19.7
MCHC (g/dl)	35.7	0.5	34.7**	0.8	0.010	31–37
PLT (10⁴/μl)	11.3	1.3	12.0	1.9	0.411	10–35
LY (%)	32.9	3.7	33.4	1.4	0.746	17–68
MO (%)	4.0	2.5	2.4	1.6	0.135	0–7
EO (%)	3.0	2.4	3.6	2.3	0.601	0–10
GR (%)	60.1	4.0	60.6	1.7	0.719	22–78
TP (g/dl)	5.7	0.3	6.1*	0.3	0.011	5.5–7.3
GLU (mg/dl)	86.3	6.5	97.3**	7.2	0.006	76–127
BUN (mg/dl)	19.3	2.3	20.5	2.9	0.364	12–26
CRE (mg/dl)	1.1	0.1	1.0	0.1	0.128	1–1.9
TG (mg/dl)	11.5	8.0	16.6	11.0	0.304	4–44
Ca (mg/dl)	14.4	0.4	14.7	0.4	0.262	10.6–13
IP (mg/dl)	2.5	0.4	2.9	0.6	0.130	2–4.3
ALB (g/dl)	3.0	0.2	3.1	0.2	0.208	2.7–4.2
ALP (U/l)	342.5	76.1	302.8	58.1	0.260	102–257
GPT (U/l)	7.9	1.4	7.5	1.3	0.583	4–12
GOT (U/l)	280.5	49.6	296.5	47.1	0.519	152–294
TBIL (mg/dl)	1.7	0.5	1.6	0.3	0.684	0.5–2.1
CPK (U/l)	152.6	63.6	142.1	37.7	0.694	113–333

WBC, white blood cell count; RBC, red blood cell count; HGB, hemoglobin amount; HCT, hematocrit value; MCV, mean corpuscular volume; MCH, mean cell hemoglobin; MCHC, mean corpuscular hemoglobin concentration; PLT, platelet count; LY, lymphocyte percent; MO, monocyte percent; EO, eosinophil percent; GR, granulocytes; TP, total protein concentration; GLU, glucose; BUN, blood urea nitrogen; CRE, creatine; TG, triglyceride; Ca, calcium; IP, inorganic phosphorus; ALB, albumin concentration; ALP, alkaline phosphatase; GPT, glutamic pyruvic transaminase; GOT, glutamic oxaloacetic transaminase; TBIL, total bilirubin; CPK, creatine phosphokinase activity; AVG, average; SD, standard deviation. *$P<0.05$; **$P<0.01$.

Table 2. Blood biochemistry test results when *Salasia reticulata* extract was ingested at 5 times the effective dose for 4 weeks

50 mg salacinol/day		Day 0		Day 28		*t* test	Reference range [3]
Blood indices		AVG	SD	AVG	SD		
WBC	$(10^2/\mu l)$	57.0	9.3	71.1	24.8	0.109	54–143
RBC	$(10^4/\mu l)$	855.0	114.7	1041.1	268.4	0.059	680–1290
HGB	(g/dl)	14.1	2.0	17.5	3.9	0.026	11–19
HCT	(%)	40.0	5.7	49.2	11.5	0.037	32–53
MCV	(fl)	46.8	2.2	47.5	2.1	0.497	37–58.5
MCH	(pg)	16.5	0.9	16.9	1.0	0.314	12.3–19.7
MCHC	(g/dl)	35.2	0.4	35.6*	0.5	0.043	31–37
PLT	$(10^4/\mu l)$	12.4	1.7	9.6	4.2	0.065	10–35
LY	(%)	34.4	3.9	47.9*	15.1	0.014	17–68
MO	(%)	2.3	0.9	2.5	0.9	0.688	0–7
EO	(%)	2.7	1.7	1.9	2.3	0.430	0–10
GR	(%)	60.6	4.3	47.8*	13.8	0.011	22–78
TP	(g/dl)	6.0	0.5	6.2	0.4	0.309	5.5–7.3
GLU	(mg/dl)	84.4	4.6	82.6	8.3	0.555	76–127
BUN	(mg/dl)	20.6	3.2	18.5	2.0	0.102	12–26
CRE	(mg/dl)	1.2	0.1	1.1	0.2	0.127	1–1.9
TG	(mg/dl)	14.5	6.9	15.8	4.0	0.611	4–44
Ca	(mg/dl)	13.1	0.5	13.7	0.4	0.006	10.6–13
IP	(mg/dl)	3.6	0.3	2.7**	0.3	0.000	2–4.3
ALB	(g/dl)	3.2	0.1	3.2	0.1	0.175	2.7–4.2
ALP	(U/l)	279.8	33.4	266.6	39.2	0.429	102–257
GPT	(U/l)	8.1	1.4	7.7	1.5	0.551	4–12
GOT	(U/l)	253.3	44.4	276.0	53.0	0.313	152–294
TBIL	(mg/dl)	1.9	0.5	1.6	0.4	0.180	0.5–2.1
CPK	(U/l)	140.9	46.0	152.3	54.5	0.619	113–333

WBC, white blood cell count; RBC, red blood cell count; HGB, hemoglobin amount; HCT, hematocrit value; MCV, mean corpuscular volume; MCH, mean cell hemoglobin; MCHC, mean corpuscular hemoglobin concentration; PLT, platelet count; LY, lymphocyte percent; MO, monocyte percent; EO, eosinophil percent; GR, granulocytes; TP, total protein concentration; GLU, glucose; BUN, blood urea nitrogen; CRE, creatine; TG, triglyceride; Ca, calcium; IP, inorganic phosphorus; ALB, albumin concentration; ALP, alkaline phosphatase; GPT, glutamic pyruvic transaminase; GOT, glutamic oxaloacetic transaminase; TBIL, total bilirubin; CPK, creatine phosphokinase activity; AVG, average; SD, standard deviation. *$P<0.05$; **$P<0.01$.

environment may have improved. In horses, SRE appears to have promoted the growth of Lactobacillales via the same mechanism of α-glucosidase inhibition, similar to rats and humans.

The mechanism of action of salacinol involves the inhibition of α-glucosidase in the intestinal epithelium, thereby suppressing the degradation of disaccharides and related compounds to glucose. Water-soluble oligosaccharides that are prevented from decomposing and being absorbed in the small intestine flow into the cecum, where the growth of intestinal microbiota, including Lactobacillales, is encouraged. Specific species of the order Lactobacillales have been identified in the intestines of healthy horses [13], and it can be inferred that salacinol functions to assist the growth of these bacteria.

Compared with several α-glucosidase inhibitors, extracts from plants of the genus *Salacia* are reported to exert strong *in vivo* effects [8]. This may be due to the active ingredient salacinol remaining unabsorbed in the small intestine, thereby functioning more effectively in animals with long intestines, such as horses. Further, the proportion of Firmicutes (an upper classification of Lactobacillales) increased substantially in horses, with a considerable elevation in the F/B ratio. Changes in F/B ratio were shown in mice to affect the energy absorption efficiency, as bacteria belonging to the Firmicutes typically decompose dietary fibers and similar substances that are expelled undigested as feces [9].

Differences in intestinal microbiota among animal species have been noted. In humans, the proportion of *Bifidobacterium* was significantly increased by SRE intake [18], whereas in rats, the F/B ratio tended to decrease [17]. Differences between these observations and the results of the present study are attributed to the long cecum of horses and their herbivorous nature of horses, which contributes to a population of cellulolytic bacteria (which generate compounds for energy conversion) in the hindgut.

No detrimental findings or blood abnormalities were

noted when SRE or salacinol was administered at the effective doses or at five times the effective dose, indicating that at these levels, although the active ingredient affected the intestinal microbiota, it was not toxic [3]. The blood Ca^{2+} concentration tended to increase, suggesting that organic acids produced by fermentation of oligosaccharides and flowing from the cecum to the colon facilitated the absorption of minerals [10].

Abnormal fermentation in the cecum and symptoms of acidosis have been reported when horses are administered large amounts of cereal [11]. When acidosis occurs, cellulolytic bacteria inhabiting the cecum and colon cannot survive, causing gastrointestinal disorders such as dyspepsia and colic [5]. The possibility of promoting hindgut acidosis through SRE ingestion was considered, because α-glucosidase inhibitory activity in the small intestine facilitates more oligosaccharides to flow into the cecum and colon. However, no decrease in fecal pH following administration of the salacinol supplement was observed, and an increase in Firmicutes (which include cellulolytic bacteria) was noted, indicating that the probability of promoting hindgut acidosis was low. No issues were noted with regard to safety in this study. Based on these outcomes, SRE feed amendment and salacinol supplementation were deemed safe in Thoroughbreds.

This is the first study to show that administration of SRE alters the intestinal flora of horses.

In the future, we would like to study how increasing lactobacillales in intestinal bacteria affects on horse health.

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