—Note—

The effects of a nutritional supplement containing salacinol in neonatal Thoroughbred foals

Atsushi IIDA[1]*, Hitomi SAITO[1], Akihito AMAO[2], Takuya FUJITA[3], Atsushi KATO[3] and Fumitaka UEDA[1]

[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]Shadai Farm, Hokkaido 069-1181, Japan

A nutritional supplement containing salacinol (NSS) was administered to Thoroughbred foals daily beginning 21 days after birth, and clinical signs and intestinal microbiota were analyzed. The average number of days for which foals exhibited a fever between 21 and 110 days after birth was determined. The number of days was significantly reduced, by approximately 1/3, in the NSS group compared with the control group. Furthermore, improved weight gain was observed in the NSS group compared with the control group. By analyzing the intestinal microbiota, it was determined that the ratio of Clostridium cluster XIVa increased after 3 weeks of NSS administration. These results demonstrate that the daily administration of NSS might improve the intestinal environment of neonatal foals and be useful for health.

Key words: *fever, intestinal microbiota, salacinol, weight gain*

J. Equine Sci.
Vol. 31, No. 1
pp. 11–15, 2020

Horses are herbivores; their cecum and colon are highly developed and play a similar role to that of the first stomach in ruminants, such as cattle and sheep. A major carbohydrate source in the feed of horses, such as grass, is cellulose, which is neither digested nor absorbed in the small intestine. Horses use volatile fatty acid products as energy sources; these products are generated by the fermentation of cellulose by protozoa and microorganisms that reside in the hindgut, including the cecum and colon [1]. When the intestinal flora becomes unbalanced, the environment deteriorates, which may lead to intestinal diseases, such as diarrhea and constipation. Neonatal foals may develop fever caused by viral or bacterial infection, and growth may be delayed because of poor feeding [19]. Therefore, maintaining a normal intestinal environment is extremely important for raising healthy horses. Previously, we confirmed that salacinol improves the intestinal environment by increasing the number of beneficial intestinal bacteria in rats and humans [13, 14]. Salacinol is a unique component of plants belonging to the genus *Salacia*; it inhibits α-glucosidase activity and decreases blood glucose levels in rats and humans [6, 9, 11, 15, 16]. *Salacia* is a genus of climbing plants belonging to the family *Hippocrateaceae* and is widely distributed throughout Southeast Asia, including India, Sri Lanka, and Thailand. Extracts of *Salacia* species (such as *Salacia reticulata* and *Salacia oblonga*) have been used for many years in Ayurvedic alternative medical practice to treat the symptoms of diseases such as rheumatism and diabetes [10, 11, 20]. A previous study documented that salacinol increased NK cell activity and thereby ameliorated pneumonia symptoms in mice infected with influenza viruses [18]. In the study described here, we examined the effects of salacinol on the clinical signs and intestinal microbiota in neonatal foals using a nutritional supplement containing salacinol (NSS).

The test food was prepared as described below. Stems and roots of *S. reticulata* grown in and imported from India were dehydrated and pulverized. The powdered material was boiled in water at 70°C for 2 hr. The mixture was then filtered to remove any solid material, and after a process to remove its bitter taste, the resulting filtrate was spray-dried (ADL-310, Yamato Science, Tokyo, Japan). The extract

Received: July 31, 2019
Accepted: January 6, 2020
*Corresponding author. e-mail: atsushi.iida@fujifilm.com

was stored at 4°C until further use. In the test food, dextrin was added to the extract of *S. reticulata*, and fluidized bed granulation was conducted using a 0.2% aqueous solution of guar gum to obtain a granular supplement. The salacinol concentration in the NSS was 0.7 ± 0.1 μg/mg. NSS (3.2 g/day dissolved in water per 100 kg body weight) was orally administered daily using a syringe.

We studied forty-nine 21-day-old healthy foals born between January and May 2018 (March was excluded, as NSS could not be administered) at a farm in Hokkaido, Japan. They were kept in an approximately 5 ha pasture for 13 hr (16:00–05:00). The experimental feed was independently fed together with 0.25 kg of oats mixed with supplements and grass, and the foals were not allowed to eat the feed for the mother horse. The fed amount of oats was increased with growth until the foals were given 0.75 kg at the age of 3 months. To compare weight gain and fever days between foals that were born in the same period, 35 foals born between April 2018 and May 2018 were divided into an NSS treatment group (n=12; 8 males and 4 females) and a control group (n=23; 10 males and 13 females). To analyze the intestinal flora, 14 foals born between January 2018 and February 2018 were divided into an NSS group (n=7; 4 males and 3 females) and a control group (n=7; 3 males and 4 females). The study was approved by the Fujifilm Animal Experiment Committee (No. A-1-180062; September 20, 2018).

The test was conducted with the approval of the responsible veterinarian on the farm, and the purpose and content of the test, restrictions during the test period, and the welfare of the test horses were fully considered and approved. Body weight was measured daily after birth from day 0 to day 10 and was measured thereafter once every 1–3 weeks. Body temperature was measured rectally twice daily in the morning and evening. In either case, the number of days on which foals had a rectal temperature ≥39°C was summed to determine the number of days with fever between 21 days and 110 days after birth. Prior to the experimental period and after 1 and 3 weeks, feces were collected, and the intestinal microbiota was analyzed. The fecal samples were collected and stored using a Feces Sampling Kit (TechnoSuruga Laboratory Co., Ltd., Shizuoka, Japan). Intestinal microbiota analysis was performed by TechnoSuruga Laboratory Co., Ltd. using terminal restriction fragment length polymorphism (T-RFLP) analysis (previously described) [12] with some modifications. The modifications from the original method are described below. Frozen fecal samples were suspended in guanidinium thiocyanate (GTC) buffer (100 mM Tris–HCl, pH 9.0; 40 mM Tris–EDTA, pH 8.0; 4 M GTC; and 0.001% bromothymol blue) and then ground using zirconia beads (5 m/sec, 2 min; FastPrep-24 Instrument, MP Biomedicals, Irvine, CA, U.S.A.). Using 100 μl of the resulting suspension, DNA was extracted using an automated nucleic acid extraction apparatus (Precision System Science, Chiba, Japan). The reagent used for the automated nucleic acid extraction was MagDEA® DNA 200 (GC; Precision System Science). Fluorescein amidite (FAM) was used for 516F labeling of PCR primers instead of HEX, which was used in the reference. PCR products were purified using MultiScreen PCRμ96 plates (Millipore, Billerica, MA, U.S.A.). Fragment analysis was performed using an ABI PRISM 3130x l genetic analyzer (Applied Biosystems, Foster City, CA, U.S.A.) and the GeneMapper® software (Applied Biosystems). The standard size markers used were the MapMarker® X-Rhodamine Labeled 50–1,000 bp DNA sizing standards (BioVentures, Murfreesboro, TN, U.S.A.).

Statistical analysis was performed using JMP version 14.0.0. The Shapiro-Wilk test was used to evaluate the fever days and intestinal microbiota analysis data for normality. If normality was not shown, comparison between values obtained before and after administration of the test food was performed by the Wilcoxon signed-rank test. For intergroup comparisons, statistical analysis was performed by the Wilcoxon rank sum test. When normality was shown, a comparison between values obtained before and after administration of the test food was performed by the paired *t*-test. For intergroup comparisons, *F* tests for equality of variance were performed. Significant differences were analyzed by Student's *t*-test (homoscedasticity) or Welch's *t*-test (heteroscedasticity).

Examinations were performed on foals of the same age that were born during the same season to remove any influence of these factors on the results. The average number of days on which foals given NSS had a fever (≥39°C) was significantly reduced during the administration period of 90 days (P<0.01), with the number decreasing to 3.3 days (Fig. 1). Furthermore, the distribution of days of fever was overall less in the NSS group than that in the control group. The proportion of foals that did not experience fever during the experimental period was 0% in the control group, whereas it was 41.6% in the NSS group. The number of foals diagnosed as having a suspected infection (*Rhodococcus equi* or rotaviruses) was 2 out of 23 in the control group and 2 out of 12 in the NSS group, respectively. However, the 2 foals in the control group had 10 and 24 days with fever, respectively, whereas the 2 foals in the NSS group had 3 and 8 days with fever, respectively.

The average body weights of the control group and NSS group were plotted for every 10 days, and individual growth curves were produced to compare weight gain (Fig. 2). The average body weight at birth was 56.5 ± 5.2 kg for the control group and 56.1 ± 6.2 kg for the NSS group; there was no significant difference (P=0.83) between the two groups. When comparing the individual growth curves,

steady weight gain was observed in the NSS group, whereas in the control group, 5 foals were far below the average body weight curve.

Intestinal microbiota analysis indicated that the ratio of *Clostridium* cluster XIVa in the NSS group showed a significant increase from 13.5% ± 3.1% before administration to 25.6% ± 2.2% after 3 weeks (Fig. 3). There was no change in the ratio of *Clostridium* cluster XIVa when comparing before and after administration in the control group (P=0.40).

We observed a marked decrease in the number of days with fever in foals receiving NSS. The ratios of foals suspected of being infected were similar in the NSS treatment group and the control group. These results suggest that NSS is effective in suppressing some factor that induces fever. It has been reported that the main causes of fever

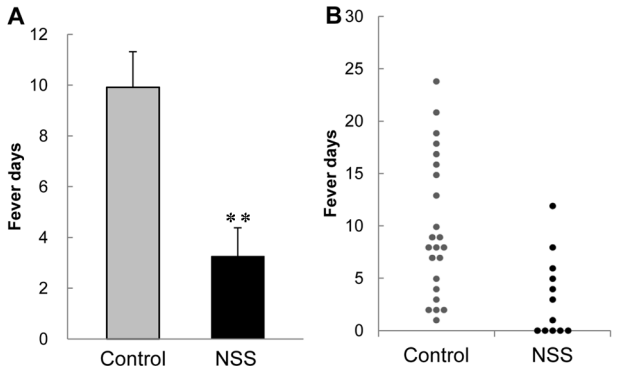

Fig. 1. Body temperature was measured daily during the 90 day administration period, and the number of days with fever (≥39°C) in the control and nutritional supplement containing salacinol (NSS) groups was determined. A. The mean numbers of days with fever in all foals in the control and NSS groups were compared. The results are expressed as the mean ± SE (control group, n=23; NSS group, n=12). **P<0.01 compared with the control group. B. The number of days for which fever lasted in all foals in the control and NSS groups was plotted.

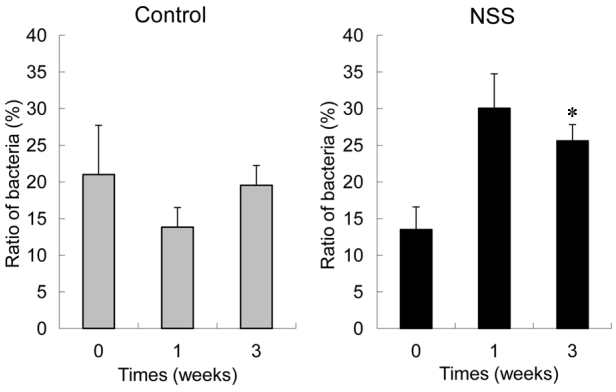

Fig. 3. The changes in the ratios of *Clostridium* cluster XIVa in the control and nutritional supplement containing salacinol (NSS) groups among three time points, before the start of the test (administration) and after 1 and 3 weeks. The results are expressed as mean ± SE values (control group, n=7; NSS group, n=7). *P<0.05 compared with 0 week.

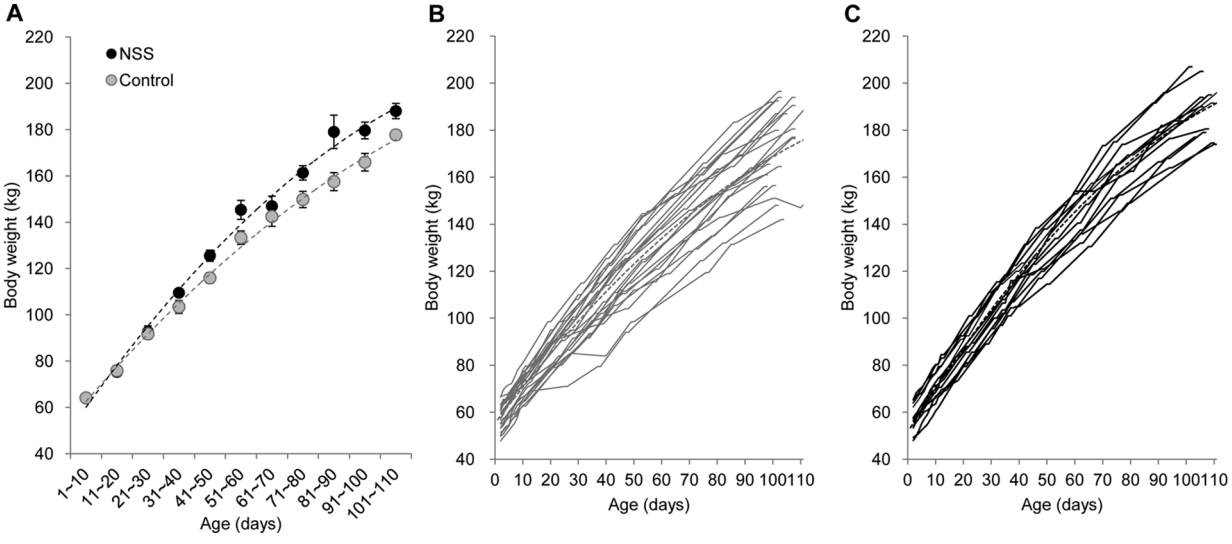

Fig. 2. The growth curves of the control group and the nutritional supplement containing salacinol (NSS) group were compared. (A) The average values of the control group and the NSS group for every 10 days from day 1 to day 110 were plotted, and the mean curves were drawn (control group, n=23; NSS group, n=12). Results are expressed as the mean ± SE. Individual growth curves are shown for the control group (B) and the NSS group (C), respectively. The mean curves are shown as dotted lines.

of unknown origin in horses are infection, neoplasia, and immune-mediated diseases [8]. It is possible that NSS administration results in enhanced immune function and increased resistance to infection. In this study, an increase in the proportion of *Clostridium* cluster XIVa species was observed in the intestinal microbiota in foals receiving NSS. Bacteria belonging to *Clostridium* cluster XIVa have been shown to ferment dietary fiber and produce butyrate in the intestinal tract [2]. Butyrate has been demonstrated to induce the differentiation of regulatory T (Treg) cells, suppress excessive immune responses to reduce inflammation, and maintain the intestinal mucosa and barrier functions [5]. We have previously reported that salacinol increases beneficial intestinal microbiota and enhances immune function in rats [13]. Lindenberg *et al*. demonstrated that intestinal microbiota may be related to the expression of genes that signify regulatory immunity in horses [7]. By examining the expression of immune-related genes and NK cell activity, it may become clear whether there is a similar effect of NSS for regulatory immunity in horses.

Several foals in the control group were well below the average body weight curve, whereas steady weight gain was observed in the foals of the treatment group. Previous studies demonstrated that approximately 60% of foals experience diarrhea in their first 6 months of life [4]. When diarrhea persists, the nutritional absorption from the intestines becomes inadequate, and foals do not gain sufficient weight. A previous study showed that when the intestinal environment was improved immediately after birth, diarrhea was prevented and foals gained weight adequately [21]. Butyrate, produced by *Clostridium* cluster XIVa species, serves as the major respiratory fuel in colonic epithelial cells in rats [17]. For horses, butyrate is a major source of energy and plays an important role in the maintenance of intestinal health [3]. However, in this study, we could not prove the direct effects of NSS on weight gain.

In conclusion, the present study suggests that NSS changed a certain bacterial group in the intestinal microbiota and reduced the number of days with fever, which might affect the growth of foals. Furthermore, NSS may have contributed to the reliable weight gain of foals. Further studies are needed to clarify these mechanisms of NSS.

References

1. Argenzio, R.A., and Hintz, H.F. 1972. Effect of diet on glucose entry and oxidation rates in ponies. *J. Nutr.* **102**: 879–892. [Medline] [CrossRef]
2. Barcenilla, A., Pryde, S.E., Martin, J.C., Duncan, S.H., Stewart, C.S., Henderson, C., and Flint, H.J. 2000. Phylogenetic relationships of butyrate-producing bacteria from the human gut. *Appl. Environ. Microbiol.* **66**: 1654–1661. [Medline] [CrossRef]
3. Bergman, E.N. 1990. Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. *Physiol. Rev.* **70**: 567–590. [Medline] [CrossRef]
4. Frederick, J., Giguère, S., and Sanchez, L.C. 2009. Infectious agents detected in the feces of diarrheic foals: a retrospective study of 233 cases (2003–2008). *J. Vet. Intern. Med.* **23**: 1254–1260. [Medline] [CrossRef]
5. Furusawa, Y., Obata, Y., Fukuda, S., Endo, T.A., Nakato, G., Takahashi, D., Nakanishi, Y., Uetake, C., Kato, K., Kato, T., Takahashi, M., Fukuda, N.N., Murakami, S., Miyauchi, E., Hino, S., Atarashi, K., Onawa, S., Fujimura, Y., Lockett, T., Clarke, J.M., Topping, D.L., Tomita, M., Hori, S., Ohara, O., Morita, T., Koseki, H., Kikuchi, J., Honda, K., Hase, K., and Ohno, H. 2013. Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. *Nature* **504**: 446–450. [Medline] [CrossRef]
6. Kajimoto, O., Kawamori, S., Shimoda, H., Kawahara, Y., Hirata, H., and Takahashi, T. 2000. Effects of a diet containing *Salacia reticulata* on mild type 2 diabetes in humans. A placebo-controlled, cross-over trial. *J. Jpn. Soc. Nutr. Food Sci.* **53**: 199–205. [CrossRef]
7. Lindenberg, F., Krych, L., Fielden, J., Kot, W., Frøkiær, H., van Galen, G., Nielsen, D.S., and Hansen, A.K. 2019. Expression of immune regulatory genes correlate with the abundance of specific Clostridiales and Verrucomicrobia species in the equine ileum and cecum. *Sci. Rep.* **9**: 12674. [Medline] [CrossRef]
8. Mair, T.S., Taylor, F.G., and Pinsent, P.J. 1989. Fever of unknown origin in the horse: a review of 63 cases. *Equine Vet. J.* **21**: 260–265. [Medline] [CrossRef]
9. Matsuura, T., Yoshikawa, Y., Masui, H., and Sano, M. 2004. [Suppression of glucose absorption by various health teas in rats]. *Yakugaku Zasshi* **124**: 217–223 (in Japanese). [Medline] [CrossRef]
10. Muraoka, O., Morikawa, T., Miyake, S., Akaki, J., Ninomiya, K., and Yoshikawa, M. 2010. Quantitative determination of potent α-glucosidase inhibitors, salacinol and kotalanol, in *Salacia* species using liquid chromatography-mass spectrometry. *J. Pharm. Biomed. Anal.* **52**: 770–773. [Medline] [CrossRef]
11. Muraoka, O., Morikawa, T., Miyake, S., Akaki, J., Ninomiya, K., Pongpiriyadacha, Y., and Yoshikawa, M. 2011. Quantitative analysis of neosalacinol and neokotalanol, another two potent α-glucosidase inhibitors from Salacia species, by LC-MS with ion pair chromatography. *J. Nat. Med.* **65**: 142–148. [Medline] [CrossRef]
12. Nagashima, K., Mochizuki, J., Hisada, T., Suzuki, S., and Shimomura, K. 2006. Phylogenetic analysis of 16S ribosomal RNA gene sequences from human fecal microbiota and improved utility of terminal restriction fragment length polymorphism profiling. *Biosci. Microflora* **25**: 99–107. [CrossRef]

13. Oda, Y., Ueda, F., Kamei, A., Kakinuma, C., and Abe, K. 2011. Biochemical investigation and gene expression analysis of the immunostimulatory functions of an edible Salacia extract in rat small intestine. *Biofactors* **37**: 31–39. [Medline] [CrossRef]
14. Oda, Y., Ueda, F., Utsuyama, M., Kamei, A., Kakinuma, C., Abe, K., and Hirokawa, K. 2015. Improvement in human immune function with changes in intestinal microbiota by salacia reticulata extract ingestion: a randomized placebo-controlled trial. *PLoS One* **10**: e0142909. [Medline] [CrossRef]
15. Premakumari, S., Kowsalya, S., Sailaavanya, S., and Mujumdar, V. 2009. Sub-chronic toxicity of Ekanayakam (*Salacia reticulata*) in albino rats. *Indian J. Nutr. Diet.* **46**: 1–13.
16. Radha, R., and Amrithaveni, M. 2009. Role of medicinal plant *Salacia reticulata* in the management of type II diabetic subjects. *Anc. Sci. Life* **29**: 14–16. [Medline]
17. Roediger, W.E. 1982. Utilization of nutrients by isolated epithelial cells of the rat colon. *Gastroenterology* **83**: 424–429. [Medline]
18. Romero-Pérez, G.A., Egashira, M., Harada, Y., Tsuruta, T., Oda, Y., Ueda, F., Tsukahara, T., Tsukamoto, Y., and Inoue, R. 2016. Orally administered *Salacia reticulata* extract reduces H1N1 Influenza clinical symptoms in murine lung tissues putatively due to enhanced natural killer cell activity. *Front. Immunol.* **7**: 115. [Medline] [CrossRef]
19. Tanabe, S., Suzuki, T., Wasano, Y., Nakajima, F., Kawasaki, H., Tsuda, T., Nagamine, N., Tsurumachi, T., Sugaya, K., Akita, H., Takagi, M., Takagi, K., Inoue, Y., Asai, Y., and Morita, H. 2014. Anti-inflammatory and intestinal barrier-protective activities of commensal lactobacilli and bifidobacteria in thoroughbreds: role of probiotics in diarrhea prevention in neonatal Thoroughbreds. *J. Equine Sci.* **25**: 37–43. [Medline] [CrossRef]
20. Yoshikawa, M., Morikawa, T., Matsuda, H., Tanabe, G., and Muraoka, O. 2002. Absolute stereostructure of potent α-glucosidase inhibitor, Salacinol, with unique thiosugar sulfonium sulfate inner salt structure from *Salacia reticulata*. *Bioorg. Med. Chem.* **10**: 1547–1554. [Medline] [CrossRef]
21. Yuyama, T., Takai, S., Tsubaki, S., Kado, Y., and Morotomi, M. 2004. Evaluation of a host-specific *Lactobacillus* probiotic in training-horses and neonatal foals. *J. Intestinal Microbio.* **18**: 101–106.