The Journal of Veterinary Medical Science

FULL PAPER

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Comparative evaluation of three different formulas for predicting the parturition date of German Shepherds following somatic cell nuclear transfer

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ABSTRACT. Several studies have reported methods to estimate the parturition date of dogs using ultrasonographic measurements. However, these prediction models were mainly determined using ultrasonographic measurements of naturally pregnant small- and medium-sized dogs, and no such studies have been performed using dogs carrying cloned fetuses produced via somatic cell nuclear transfer. The present study evaluated the abilities of three reference formulas (Luvoni and Grioni, Milani *et al*., and Groppetti *et al*.), all of which were developed using data from naturally occurring pregnancies, to accurately predict the parturition date in surrogates carrying cloned German Shepherd (GS) fetuses. All three formulas were based on the use of inner chorionic cavity diameter (ICC) measurements, obtained via ultrasonography. For evaluation, a total of 54 ICC measurements were collected from 14 pregnant bitches carrying cloned GS fetuses. We found that the clinical accuracy of the breed-specific Groppetti *et al*. formula was highest among those of the three formulas tested, with 87% and 100% of the estimated parturition dates (calculated based on the ICC measurements) being within 1 and 2 days, respectively, of the actual delivery date. By contrast, the Luvoni and Grioni formula showed relatively low accuracy, and the Milani *et al*. formula showed higher accuracy than that reported previously for natural pregnancies.

KEY WORDS: dog cloning, German Shepherd, parturition date, pregnancy, ultrasonography

J. Vet. Med. Sci.
83(9): 1448–1453, 2021
doi: 10.1292/jvms.21-0154

Received: 13 March 2021
Accepted: 23 June 2021
Advanced Epub:
6 August 2021

For dog cloning, early pregnancy diagnosis is an important step in confirming whether the cloning process has been successful. However, despite the publication of dozens of scientific papers on dog cloning, little is known about how cloned embryos implant and develop after transplantation into surrogate mothers. Ultrasonography is a useful, non-invasive technique for the diagnosis of pregnancy and monitoring of fetal development, and can be used to estimate parturition and determine the need for intervention. Ultrasonographic observation of fetal organs and structures at different gestational periods has been reported previously in canines [2, 6]. In normal pregnancies, certain ultrasonographic features can be used to identify the stage of gestation [5]. Indeed, measurement of the inner chorionic cavity diameter (ICC) of the gestational sac during early pregnancy provides clinically useful information for the estimation of gestational age [8, 15, 17]. However, in dog cloning, it is difficult to conduct multiple dog cloning and offspring production due to biotechnological difficulties such as a low cloning efficiency and high rate of miscarriage in the early of pregnancy in dog cloning. Therefore, no studies have been reported on the measurement of the estimation of parturition date of cloned dogs to date.

Accurate prediction of the parturition date in canine species is difficult due to ambiguous timing of the fertilization event. After ovulation, canine oocytes take 2–3 days to mature to a fertilizable state [13], and sperm are capable of sustaining their motility in the female reproductive tract during this period [22]. Therefore, the time between ovulation and fertilization can vary, leading to difficulty in determining the commencement of pregnancy in canines. Nevertheless, a number of veterinarians have tried to determine the parturition date of bitches using the anatomical structures of the fetus obtained from ultrasonography. Several mathematical formulas for estimating parturition date have been developed for use in small- and medium-sized dogs [16], although they are reportedly applicable to large-sized dogs also [24].

Ethnicity affects fetal biometry in humans, and various growth charts based on phenotypes and race-related factors are typically

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used to assess pregnancy and parturition [12, 18, 23]. Similarly, in dogs, the fetal morphology can vary substantially across breeds, even those of a similar weight, highlighting the need for research into breed-specific formulas for estimating parturition. Furthermore, dogs are multiparous animals, and the duration of gestation is typically longer for pregnancies of smaller litters (fewer than four puppies) [8]. Small litters of fewer than four puppies are reported commonly in dog cloning, and the average gestation period of cloned fetuses is usually longer than that of those conceived naturally [14]. Several studies also reported that the gestation period is prolonged for animals carrying cloned fetuses compared to with naturally pregnant animals [10, 11]. High plasma and placental levels of TGF-beta 1 protein and a hormonal imbalance caused by a low proportion of active estrogen in cloned cows contribute to delayed delivery of cloned fetuses [10, 11]. However, to our knowledge, there are no reported methods to estimate the parturition date of cloned dogs.

In our current study, we evaluated the abilities of the following models to accurately estimate the parturition date of the cloned German Shepherd (GS): the medium-sized dog-specific Luvoni and Grioni formula and the GS breed-specific Milani *et al*. and Groppetti *et al*. formulas [8, 15, 17]. All three models were based on the use of ICC measurements. The use of cloned dogs is particularly suitable for evaluating these models because, unlike in natural pregnancies, the gestational age can be estimated accurately based on the date of embryo transfer.

MATERIALS AND METHODS

Chemicals

Unless otherwise specified, all chemicals were purchased from Sigma (St. Louis, MO, USA), and culture media were purchased from Gibco Life Technologies (Gaithersburg, MD, USA).

Experimental design

A total of 1,434 reconstructed somatic cell nuclear transfer (SCNT) embryos were transferred into 89 surrogates following the experimental design. Pregnancy was confirmed using real-time transabdominal ultrasonography 22–35 days after embryo transfer. Nineteen pups were obtained from 14 surrogates, and 54 ICC measurements were acquired during the initial ultrasound for pregnancy diagnosis. Using the ICC and parturition date, the accuracy of parturition date prediction was analyzed with three different fetometric formulas (Luvoni and Grioni, Milani *et al*., and Groppetti *et al*.), which were previously reported [8, 15, 17, 24].

Care and use of animals

Female mixed-breed dogs aged between 1 and 7 years (body weight, 20–25 kg) were housed in indoor kennels, fed standard commercial dog food once a day, and given water *ad libitum*. A total of 95 bitches were used as oocyte donors, and 89 surrogates were used for embryo transfer. All animal procedures were conducted in accordance with animal study guidelines approved by the ethics committee at the Abu Dhabi Biotech Research Foundation, Korea (permit no. C-12-01).

Ovulation determination

All donors and recipients employed in the study exhibited spontaneous estrous cycles. The estrous stage was examined weekly by observing vulvar bleeding to detect the onset of the heat period. During heat, a 2 ml blood sample was collected daily by cephalic venipuncture, and serum progesterone levels were measured using an electrochemiluminescence immunoassay (Cobas e411; Roche Diagnostics, Mannheim, Germany). The intra- and inter-assay coefficients of variation of the assay were both <4%. Ovarian ultrasonographies were performed twice a day periodically, when serum P4 levels were higher than 4 ng/ml. The luteinizing hormone (LH) surge was predicted by measuring P4, and the LH peak was considered to be when the P4 level had increased by more than 2 ng/ml [8]. The time of ovulation was defined as the stage at which (i) the ovaries became difficult to find via transabdominal ultrasonography or displayed an apparent decrease in the number or contour of anechoic follicles, and (ii) ≥90% of epithelial cells from vaginal swabs were cornified, as determined by staining with Diff Quik (Sysmex Co., Kobe, Japan) following standard protocols [1].

Preparation of donor cells

Dermal skin tissue samples measuring approximately 3 cm^2 were collected from a male GS under light sedation with 2.5 ng/kg tiletamine and zolazepam (Zoletil; Virbac SA, Carros, France) and local anesthesia (Daehan lidocaine HCl 2%; Dai Han Pharm Co., Ltd., Seoul, Republic of Korea). To obtain fibroblasts, sections of the tissues were cut into small pieces and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. The tissues were maintained at 37°C in an atmosphere of 5% CO_2 and air. Explants were maintained in the culture until they approached 90% confluence.

Oocyte retrieval and nuclear transfer

Donors and surrogates were matched based on synchronization of their estrus cycles. Oocytes were retrieved surgically at 72–84 hr after ovulation. The maturation stage of the retrieved oocytes was determined as described previously [25]. Subsequently, metaphase II oocytes were enucleated by aspirating the first polar body and the metaphase II plate into a small amount of surrounding cytoplasm using a glass pipette. Donor cells were prepared and treated as described previously [25]. Using a fine pipette, a trypsinized fetal fibroblast with a smooth cell surface was transferred into the perivitelline space of an enucleated oocyte. The couplets were equilibrated with 0.26 M mannitol solution containing 0.5 mM HEPES, 0.1 mM $CaCl_2$, and 0.1 mM $MgSO_4$

for 4 min. Subsequently, the couplets were then transferred to a chamber with two electrodes and covered with the mannitol solution. Fusion was performed with two DC pulses of 1.75 to 1.85 kV/cm for 15 µsec using a BTX Electro-Cell Manipulator 2001 (BTX, Inc., San Diego, CA, USA). After simultaneous fusion and activation, groups of five to six embryos were cultured in 25 µl microdrops of modified synthetic oviduct fluid medium (mSOF) covered with mineral oil for 1 hr or less at 39°C in a humidified atmosphere (5% O_2, 5% CO_2, and 90% N_2) until embryo transfer.

Embryo transfer

Surrogates underwent embryo transfer 84–96 hr after ovulation. The surrogates were anesthetized with a mixture of xylazine hydrochloride (1 mg/kg body weight; Bayer Korea, Ansan, Republic of Korea) and ketamine HCl (4 mg/kg body weight; YuHan Corp., Seoul, Republic of Korea). The fat layer covering the ovary was grasped gently with forceps and suspended with a suture to exteriorize the fimbriae and the left oviduct. Reconstructed embryos were loaded into a tomcat catheter (Sherwood Medical, St. Louis, MO, USA) with 4 µl transfer media and then transferred gently into the 2/3 distal position of the left oviduct through the infundibulum.

Pregnancy diagnosis and fetal measurement

Ultrasonography was performed in the dorsal recumbency position, and the dogs were sedated mildly with 2.5 ng/kg tiletamine and zolazepam (Zoletil). Rubbing alcohol or transmission gel was applied copiously to the shaved ventral abdomen. Two-dimensional gray-scale, real-time ultrasound images were produced using mechanical and phased-array sector curved-linear transducers with frequencies of 3.5 MHz (Sonace R7; Samsung Medison, Seoul, Korea). The uterus was located by moving the transducer cranially from the edge of the pubic bone, using the anechoic urinary bladder and crescent-shaped hyperechoic descending colon as landmarks. After examining the entire uterus, extra-fetus structure were measured during each examination. Fetal measurements were made by one sonographer without knowledge of the date of embryo transfer. A total of 54 ICC measurements were collected from 14 bitches. Measurements were either taken at the time of examination using electronic calipers incorporated in the ultrasound apparatus, or were made from prints of the image using mechanical calipers and the centimeter electronic scale on the margin of the ultrasound image. The diameter of the longitudinal axis, designated lICC, and that of the transverse axis, designated tICC, were determined based on the fetal ICC of the gestational sac (Fig. 1). At the time of identification of the pregnancy by ultrasonography, the position of the fetal head within the gestational sac was determined. The long axis from the head to the tail was defined as lICC, and the length in the perpendicular direction from the lICC was defined as tICC (Fig. 1). Both lICC and tICC were measured after embryonic vesicles appeared regularly spherical with a clearly defined margin. For each examination, the ICC was determined by taking the average of two measurements made perpendicular from one side of the trophoblastic decidual reaction to the other.

Data analysis and statistics

Data analysis was performed using SPSS (version 15; SPSS Inc., Chicago, IL, USA), and graphs were prepared with GraphPad Prism (version 4.0). The abilities of the three formulas (Luvoni and Grioni, Milani *et al*., and Groppetti *et al*.) to estimate the parturition date based on the ICC measurements were determined. Accuracy was evaluated based on the difference between the parturition date estimated using the formula and the actual delivery date. We changed the three different formulas to a homogenous form following methods reported previously [24]. The reference formulas were as follows: Luvoni and Grioni: days before parturition (DBP)=45.628–0.556 × ICC (mm); Milani *et al*.: DB*P*=48.121–0.5237 × ICC (mm); Groppetti *et al*.: DB*P*=44.76–0.434 × ICC (mm). When comparing the accuracies of the three formulas, ICC was the independent variable, and DBP was the dependent variable. We calculated the standard error estimate (SEE) to evaluate the degree of compliance of the formulas with the empirical data. After calculating the average absolute difference (MAD) between the actual delivery date and the parturition date estimated by each formula, pairwise comparisons of the performances of the formulas were performed using Student's *t*-tests.

RESULTS

GS cells were used as donor nuclei for somatic cell nuclear transfer. Overall, 1,434 reconstructed embryos were transplanted into 89 surrogate dogs. A total of 21 pregnancies were observed, and 19 live births occurred (Table 1).

Based on the actual delivery dates of the puppies, we compared the experimental DBP with those predicted by three different mathematical formulas (Luvoni and Grioni, Milani *et al*., and Groppetti *et al*.) using the experimental ICC measurements. For this analysis, we used 54 ICC measurements collected from 14 bitches. As shown in Table 2, the Groppetti *et al*. formula showed the highest accuracy in predicting the delivery date (± 1 day: 87.0%, ± 2 days: 100.0%; SEE: 0.7 days), whereas the Luvoni and Grioni formula showed the lowest accuracy (± 1 day:

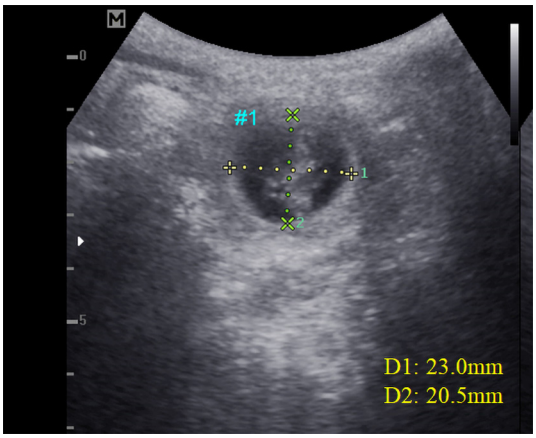

Fig. 1. A representative ultrasonography image of the internal chorion diameters (tICC and lICC) at 35 days before parturition in a cloned German Shepherd.

0.0%, ± 2 days: 44.4%; SEE: 2.9 days). Figure 2 shows a comparison of the actual DBP values and the regression lines of those predicted by the three formulas using the ICC measurements.

Next, the average absolute difference (MAD) between the actual and predicted delivery dates was determined for each formula, and pairwise comparisons were made. The MAD values for the Luvoni and Grioni, Milani *et al*., and Groppetti *et al*. formulas were 0.9, 0.7, and 0.5 days, respectively, and there were significant differences between all groups in pairwise comparisons (Table 3).

Table 1. Pregnancy rates of the surrogates

Breed	No. of embryos transferred	Embryo transfer			Parturition	
		No. of surrogates	No. of pregnancies[a]		No. of pups (%)[b]	Gestational period (days)[c]
			At early-term (%)	To term (%)		
GS	1,434	89	21 (23.6)	19 (21.3)	19 (21.3)	68.3 (1.5)

GS: German Shepherd. [a]The pregnancy rates was calculated as the number of pregnancies divided by the total number of surrogates. [b]The cloning efficiency was calculated as the number of pups born divided by the total number of surrogates. [c]The gastational period was calculated as the period from the estimated luteinizing hormone surge to the date of parturition. Data are presented as the mean (SD) of all independent experiments.

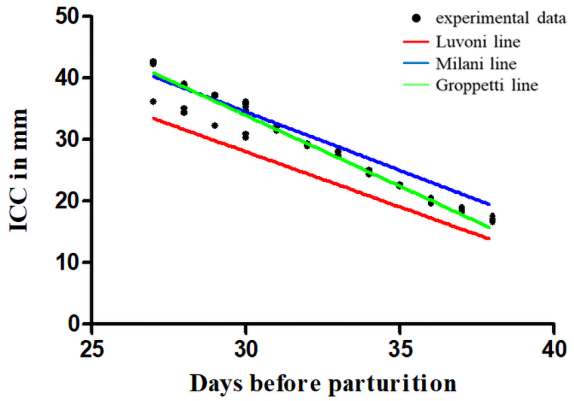

Fig. 2. The relationship between the inner chorionic cavity diameter (ICC) and the number of days before parturition in pregnant dogs carrying cloned German Shepherd fetuses. The graph compares the experimental data with the regression lines determined using the three formulas.

Table 2. The accuracies of the three formulas for estimating the parturition date of cloned German Shepherds

	Accuracy	Luvoni and Grioni [16]	Milani *et al*. [17]	Groppetti *et al*. [8]
ICC	± 1 day	0/54 (0.0%)	25/54 (46.3%)	47/54 (87.0%)
	± 2 days	24/54 (44.4%)	50/54 (92.6%)	54/54 (100.0%)
	SEE (days)	2.9	1.2	0.7

ICC: inner chorionic cavity diameter; SEE: standard error estimate; DPB: days before parturition. Reference formulas: Luvoni and Grioni: DBP=45.628−0.556 × ICC (mm); Milani *et al*.: DBP=48.121−0.5237 × ICC (mm); Groppetti *et al*.: DBP=44.76−0.434 × ICC (mm).

Table 3. Pairwise comparisons of the three different formulas for estimating parturition date of cloned German Shepherd fetuses

	Average 1	Average 2	*P*-value
ICC	Luvoni and Grioni [16] (MAD: 0.9)	Miliani *et al*. [17] (MAD: 0.7)	<0.001
	Luvoni and Grioni [16] (MAD: 0.9)	Groppetti *et al*. [8] (MAD: 0.5)	<0.001
	Milani *et al*. [17] (MAD: 0.7)	Groppetti *et al*. [8] (MAD: 0.5)	<0.001

ICC: inner chorionic cavity diameter; MAD: average of the absolute difference between the actual delivery date and that predicted using the specified formula and the ICC measurements.

DISCUSSION

Ultrasonography is a useful tool for the observation of canine pregnancies [8, 16, 17], as it is non-invasive and can be used to identify both the developmental stage and health status of the fetus. Accurate prediction of the parturition date in dog cloning is important to manage pregnant bitches and to prepare for delivery. It has been reported that ICC measurement in early pregnancy is the most accurate approach to estimate parturition date through ultrasonographic examination [24]. However, studies of parturition date prediction in dog cloning are limited because the characteristics of reproduction differ between dogs and other mammals, the cloning efficiency is low, and maternal management is essential [14]. In this study, we evaluated the abilities of three formulas to predict the expected parturition date of cloned dogs using ICC measurements collected via ultrasonographic examination. To our knowledge, this study is the first such analysis in cloned dogs.

There have been many attempts to predict the delivery dates of various pregnant animals using ultrasonography measurements [19, 26, 27]. As dogs vary in size, there is a need to develop accurate formulas to measure and monitor fetal development according to breed. To date, two breed-specific formulas for predicting gestational age or parturition date in the GS have been proposed by Milani *et al*. in 2013 and Groppetti *et al*. in 2015 [8, 17]. Milani *et al*.'s formula is based on measurements of seven pregnant female dogs [17], whereas Groppetti *et al*. developed their formula using measurements of 40 pregnant dogs [8]. In a similar analysis to that performed here, Socha *et al*. evaluated the use of fetometric measurements to predict the parturition date of naturally pregnant GSs using breed-specific formulas as well as a non-specific formula for medium-sized bitches [24].

Most formulas using ultrasonographic fetometry are designed to calculate the date of parturition. The estrus cycle of female dogs begins with vaginal bleeding [7]. From 3 days after this time, ovarian follicles begin to grow alongside the development of sexual receptivity, and take approximately 6–12 days to mature into fully grown follicles [9]. After a LH surge, immature oocytes are released from the ovaries and mature in the oviducts, where the progesterone level is high [4]. Previous studies have shown that oocytes mature 60 hr after the LH surge [3]. Accurate prediction of the gestation length of naturally bred dogs is difficult because of the ambiguity related to the onset of fertilization. This ambiguity does not occur for cloned fetuses as the reconstructed embryos are transferred into the surrogate immediately after reprogramming. Therefore, we believe that the use of cloned fetuses enables a more reliable fetometric analysis and evaluation of formulas to predict the parturition date.

There are some characteristic differences between natural canine pregnancies and those of dogs with cloned fetuses. DNA demethylation and epigenetic reprogramming, which are important for programmed gene expression during early embryonic development, are reported to be abnormal in cloned animals [21]. In canine, estrogen in plays an important role in pregnancy and induction of parturition [15, 20]. However, parturition is reportedly delated due to abnormal expression of TGF-β1 protein and active estrogen following excessive estrogen sulfoconjugation in cattle [11]. Furthermore, smaller litters are usually reported during dog cloning, and accordingly, the gestation period of dogs carrying cloned fetuses is typically longer than that of naturally pregnant dogs [8, 14]. Our results are consistent with these previous findings. The gestational period of cloned GS (68.3 days) in the present study is longer than that previously reported for naturally pregnant dogs (65.5 days) [8]. Additionally, the pattern of ICC measurements based on DBP differed between the current study and a previous report [24]. Therefore, it is essential to determine whether models for predicting the parturition date of naturally pregnant dogs are also applicable to dogs carrying cloned fetuses.

The present study analyzed the accuracies of three formulas in estimating the parturition date of cloned GSs. These formulas are all based on the use of ICC measurements. Analysis of the regression line of each estimation model confirmed that there was a close relationship between the Groppetti *et al*. formula and the experimental data (Fig. 2), whereas the regression line of the Luvoni and Grioni formula was more displaced. We found that the clinical accuracies of the Milani *et al*. and Groppetti *et al*. formulas, defined as their abilities to predict parturition within 2 days, were greater than 80%, and thus adequate, as reported in a previous study of naturally pregnant dogs [24]. The clinical accuracy of the breed-specific Groppetti *et al*. formula was the highest among those of the three formulas tested, with 87% and 100% of the estimated parturition dates (calculated based on the ICC measurements) being within 1 and 2 days, respectively, of the actual delivery date (Table 2). These results are similar to the reported clinical accuracy of the Groppetti *et al*. formula when the litter size is less than three [8]. Notably, the Luvoni and Grioni formula showed the lowest accuracy in estimating the parturition date (Table 2), and the accuracy of the Milani *et al*. formula (92.6%) for estimation of parturition within 2 days of the actual delivery date was higher than that described previously for naturally pregnant GSs (88.8%) [17]. The ICC values in dogs carrying cloned GS were similar to those previously reported for naturally pregnant dogs [24]. However, the Luvoni and Grioni formula showed the highest accuracy and the Milani *et al*. formula showed the lowest accuracy in naturally pregnant GS [24]. Our results imply that the accuracies of both breed-specific and non-specific formulas differ between naturally pregnant dogs and dogs carrying cloned fetuses. These differences in accuracy could be explained by the fact that pregnant dogs carrying cloned GS fetuses typically have longer gestational periods than naturally pregnant dogs. As DBP was calculated based on parturition date, even when DBP was equivalent, the fetal development of cloned dogs was prolonged compared with that of naturally conceived dogs. So even if the DBP were the same, the period of fetus development in the surrogate was prolonged in cloned dogs compared to that of natural pregnant dogs.

However, our results for prediction of the parturition date for cloned GS have several limitations. The present study only measured the ICC at early pregnancy. The biparietal diameter (BP), which is an accurate indicator of estimation for parturition date in late pregnancy and is useful for identifying developmental differences between cloned offspring and offspring produced through natural mating, was not evaluated in this study. Furthermore, there were no results of time dependent developmental changes of cloned GS fetus throughout the full gestation period. The observed extended gestational period of cloned canines has also been reported in other species [10, 11], but the causal elements underlying these observations remain poorly understood. An in-depth study designed to evaluate various factors including the number of fetuses per pregnancy, variation in epigenetic and cellular reprograming, and the maternal-fetal interplay would help to

determine why gestation is prolonged for cloned animals.

In summary, the results presented here demonstrate that, of the three formulas examined, the Groppetti *et al*. formula is the most suitable for predicting parturition in dogs carrying cloned GS fetuses and small litter numbers. In contrast to the results reported for naturally pregnant dogs, the Luvoni and Grioni formula was unable to predict parturition accurately in pregnant dogs carrying cloned GS fetuses. Although, further studies are needed to characterize the features of developing cloned GS fetuses on ultrasonographic examinations, the Groppetti *et al*. formula can be used to predict the parturition date of cloned GS.

POTENTIAL CONFLICTS OF INTEREST. The authors have no conflicts of interest to declare.

ACKNOWLEDGMENTS. This work was supported by the Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry and Fisheries (IPET), through the Agri-Bio Industry Technology Development Program, funded by the Ministry of Agriculture, Food and Rural Affairs (MAFRA; grant number: 318016-5).

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