Original Article Lactobacillus fermentum V3 ameliorates colitis-associated tumorigenesis by modulating the gut microbiome

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Abstract: *Lactobacillus* spp., a common probiotic used as a dietary supplement, is good for the digestive system. However, its anti-cancer activity still remains unclear. In this study, we aim to examine the effect of *Lactobacillus fermentum*, *Lactobacillus acidophilus* and *Lactobacillus rhamnosus* on azoxymethane/dextran sulfate sodium (AOM/DSS)-induced colitis-associated cancer. Male ICR mice were injected with 10 mg/kg AOM and 2.5% DSS via drinking water, and then fed with different *Lactobacillus* (1×10^8 CFU/day) for 14 weeks. The colonic tissues were collected for biomedical analysis, and gut microbiota profiling was detected by next generation high-throughput sequencing comparing to the 16S rRNA gene. We found that pretreatment with *Lactobacillus fermentum* (Lac. ferm) significantly inhibits colonic tumor formation (P < 0.05) and markedly decreases pro-inflammatory cytokines in AOM/DSS-induced mice. Furthermore, 16S rRNA sequencing data showed that Lac.ferm altered the composition of gut microbiota by reducing the percentage of *Bacteroides*. Moreover, linear discriminant analysis scores revealed that *Lactobacillus fermentum* within phylum *Firmicutes* was the prominent species existing in the Lac.ferm-treated group. Overall, the above findings suggest that dietary Lac.ferm could modulate the gut microbial community, which might be beneficial to alleviating colon cancer progression.

Keywords: Lactobacillus fermentum, azoxymethane, dextran sulfate sodium, gut microbiota, colon cancer

Introduction

Colorectal cancer (CRC) is the third leading cause of cancer mortality worldwide [1]. Westernization of dietary patterns and lifestyles has engendered an increase in the incidence of CRC among individuals aged < 50 years [2]. Genetic and environmental factors-including age, smoking, family history, and dietary habits (e.g., excessive consumption of red meat or processed meat products)-are implicated in CRC occurrence [3]. Conversely, a diet rich in vegetables, fruits, and other fiber-rich foods could obviously decrease the risk of CRC [4]. This is because microorganisms are able to break down non-digestible carbohydrates into short-chain fatty acids (SCFAs) that regulate the intestinal immunity and metabolic functions of the host [5]. As indicated by increasing evidence, the gut microbiota is involved in mucosal immune responses and the pathogenesis of inflammatory bowel disease (IBD) [6]. Dysbiosis of gut microbiota has contributed to impairing intestinal barrier function and causing chronic inflammation by regulating cytokine activity [7]. Besides, pro-inflammatory cytokines were the key predisposing factor to accelerating the development of CRC [8]. Recent studies found that traditional medicines such as Boswellia serrata resin, Ganoderma lucidum, and American ginseng have ability to alter the gut microbiota community and reduce the burden of colon tumors in azoxymethane/dextran sulfate sodium (AOM/DSS)-induced, colitis-associated cancer (CAC) [9-11]. Therefore, maintenance of microbiota homeostasis might be a promising new strategy for cancer chemoprevention.

Probiotics generally refer to live microorganisms that, with moderate daily intake, provide health benefits to the host [12]. Common probiotics-including genus Lactobacillus, Bifidobacterium, and Streptococcus-have been used to manage gastrointestinal (GI) tract functions and the immune system [13]. Numerous studies have noted that parts of probiotics like Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus plantarum, and Lactobacillus fermentum when processed improve the clinical symptoms of IBD in a murine model via decreasing inflammatory cytokines and even shifting the composition of gut microbes [14-17]. In this regard, previous research demonstrated that Lactobacillus spp. were observed to produce lactic acid and inhibit the growth of harmful bacteria in the gut [18]. Although attenuating colitis is thought to be linked to the reduction of colon tumorigenesis, studies have yet to clarify the effects of lactic acid bacteria supplementation on CRC and how it correlates to gut microbiota. In the current study, we aim to screen the effectiveness of probiotic candidates-including Lactobacillus fermentum V3 (Lac.ferm), Lactobacillus acidophilus LA257 (Lac.acid), and Lactobacillus rhamnosus LR132 (Lac.rham)-in an AOM/DSS-induced carcinogenesis model. In addition, we focus on the change of the gut microbiome produced by one Lactobacillus strain, which presented the highest efficiency on inhibiting tumor growth.

Materials and methods

Chemicals and bacterial strain

Azoxymethane was purchased from Sigma Chemical Co., and dextran sodium sulfate (DSS; molecular weight of 36,000-50,000 Da) was purchased from MP Biomedicals (Aurora, OH). Antibodies CD68 were obtained from Abcam (Cambridge, MA). All other reagents used were obtained from Sigma-Aldrich, unless otherwise noted. *Lactobacillus fermentum* V3, *Lactobacillus acidophilus* LA257, and *Lactobacillus rhamnosus* LR132 were isolated from pickled bamboo shoots, milk, and feces samples of healthy adults, respectively. All bacterial strain powders were provided by Syngen Biotech Co., Ltd., Taiwan.

Establishment of experimental animal model

Five-week-old male ICR mice were purchased from BioLASCO (Taipei, Taiwan) and randomly

divided into five groups (n = 6). A mouse model of AOM/DSS-induced CAC was established, as previously reported [19]. The control group served as an untreated control. Mice in the other four groups received a single intraperitoneal injection of 10 mg/kg AOM at the day 0. One week after AOM injection, mice were given two cycles of 2.5% DSS (w/v) in drinking water for 7 days. The first cycle of DSS was followed by two weeks of sterile water. In the experimental group, mice were administered different Lactobacillus strains by oral gavage once a dayincluding Lac.ferm, Lac.acid, and Lac.rham (1 × 10⁸ CFU/day each group)-for 5 days per week at the beginning until the end of the experiment (Supplementary Figure 1). The AOM/DSS group was given the equivalent volume of saline. All protocols were executed in accordance with the guidelines approved by the Institutional Animal Care and Use Committee (IACUC) of the National Taiwan University (NTU-107-EL-00150). Mice were housed at a constant atmosphere (25 \pm 1°C and 50% relative humidity) under 12 h light/12 h dark cycles and fed with standard diet and water ad libitum. At the end of the study at 14 weeks, animals were sacrificed by CO₂ inhalation and dissected. The colon tissues and stool samples were collected and stored at -80°C until used. The other half colon was fixed in 10% buffered formalin for histologic analysis.

Microbial analysis

Fecal samples were collected from the colon under aseptic conditions and immediately stored at -80°C until analysis. Bacterial genomic DNA was extracted using the innuSPEED Stool DNA Kit (Analytik Jena AG, Jena, Germany) and according to manufacturer's recommendations. The primer sequences used to amplify the V3-V4 region of the 16S rDNA gene followed the results of a previous study [20]. Next, the second PCR used the Illumina DNA library preparation kit (Illumina, San Diego, CA, USA) to construct the dual-index barcodes and sequencing adaptors under the following conditions: 95°C for 3 min, 8 cycles of 95°C (30 s), 55°C (30 s), and 72°C (30 s), with a final extension at 72°C for 5 min. The amplicons were extracted from 2% agarose gels and purified with AMPure XP beads. Barcoded amplicons were pooled (1 nM) and combined with 5% PhiX to use as a spike-in control for Illumina sequencing runs. The library was sequenced on an IlluminaHiSeq2500 platform and paired-end reads (250 bp) were generated. Operational taxonomy units (OTUs) were clustered using the UCLUST method and sequenced at least 97% identities. Microbial data analyses including α - and β -diversity were performed with Quantitative Insights into Microbial Ecology (QIIME; version 1.9.1) software.

Histopathology and Immunohistochemistry

Colonic tissues were fixed in 10% buffered formalin overnight and embedded in paraffin. The 4-µm tissue sections were deparaffinized in xylene and rehydrated in ethanol/water. Slides were then stained with hematoxylin-eosin (H&E) or processed for immunostaining. Immunohistochemical staining was performed with a primary antibody rabbit anti-CD68 (1: 500) at 4°C overnight. Secondary antibody incubation and EnVision® + dual link System-HRP (Dako, CA, USA) were then used following the manufacturer's recommendations. Images were captured using a Stereo Investigator® system (MBF Bioscience, Williston, VT, USA).

Enzyme-linked immunosorbent assay (ELISA)

Colonic tissues were weighed and homogenized for 30 sec in cold lysis buffer containing protease inhibitors. After incubated on ice for 30 min, the mixture was centrifuged at 10,000 × g for 30 min at 4°C. The supernatants were collected and used for quantified cytokine levels of IL-1 α , IL-1 β , and IL-6 by using Multi-Analyte ELISArray Kits (QIAGEN) according to the manufacturer's instructions.

Statistical analysis

All data were presented as the means \pm SE. SPSS 12.0 (SPSS, Inc., Chicago, IL, USA) was used for statistical analyses. Differences between groups were assessed by one-way ANOVA and followed by Tukey's post hoc tests. A two tailed *p*-value < 0.05 was considered statistically significant.

Results

Different Lactobacillus strains affect the clinical signs induced by AOM/DSS

The AOM/DSS model is applied to study tumor progression driven by colitis [21]. The severity of colitis is characterized by body weight loss

and enlarged spleen. In the present study, receiving DSS lead to temporary weight loss in all groups, especially after the first cycle (P <0.001; Figure 1). During the second DSS exposure cycle, the ratio of body weight loss in the Lac.ferm group was less than that for the AOM/ DSS-treated mice. At the end of the period, the final body weight among all groups showed no significant difference (Figure 1A). Furthermore, we also observed that spleen weight and size are both increased in AOM/DSS-induced groups compared to the control group (P < 0.001; Table 1 and Figure 1B). On the other hand, the appearance and weight of other essential organs such as liver and kidney were not significantly different between all groups (Table 1 and Figure 1B). Moreover, after feeding three kinds of Lactobacillus species for 14 weeks, water intake and food consumption were not affected (Figure 1C and 1D). Also, the other uncomfortable signs were not found. The results suggested that dietary these three candidates of Lactobacillus strains (Lac.ferm, Lac. acid, and Lac.rham) did not appear to present any noticeable effects or acute toxicity.

Lac.ferm suppressed AOM/DSS-induced colorectal tumorigenesis in mice

To evaluate the chemopreventive efficacy of different Lactobacillus strains on colitis-associated CRC, the AOM/DSS carcinogenic mouse model was used. DSS administration has been reported to induce ulceration and bowel wall thickening, which result in shortening the colon length [22]. In the current study, the colon length in AOM/DSS-treated mice (8.11 \pm 0.76 cm) was significantly shorter than that in the control group (10.13 \pm 1.30 cm) (P < 0.001; Table 2 and Figure 2A). Combined treatment with AOM and DSS led to tumor formation and increase of colon weight. We found that the colon weight of mice in the AOM/DSS group $(0.50 \pm 0.13 \text{ g})$ was heavier than that of the control group $(0.25 \pm 0.03 \text{ g})$ (*P* < 0.001; Table 2). Furthermore, the notable finding was that the administration of Lac.ferm resulted in a significant reverse in the colon weight-to-length (W/L) ratio (P < 0.05; Table 2 and Figure 2B). To confirm the cancer suppressive effects, tumor multiplicity was evaluated. Tumor incidence in the AOM/DSS group was 100%, and the average number of tumors in the mid and distal colon were 18.50 ± 3.85 per mouse. After administration of the oral probiotics candidates



Figure 1. Effect of different *Lactobacillus* strains on body weight and clinical signs in AOM/DSS-treated ICR mice. (A) Body weight, monitored weekly for 14 weeks, with average body weight of each group expressed as means \pm SE (n = 6 per group); (B) appearance of organs; (C) daily food intake; and (D) water intake in each group. Data were expressed as the means \pm SE (n = 6 per group). n.s., no significant difference; (*) P < 0.05, (***) P < 0.001 compared with the control group.

Table 1. Effect of	different Lactobacillus	strains
on organ weight		

	Liver	Kidney	Spleen
	weight (g)	weight (g)	weight (g)
Control	2.27 ± 0.30	0.74 ± 0.07	0.10 ± 0.01***
AOM/DSS	2.61 ± 0.26	0.84 ± 0.06	0.19 ± 0.03
Lac.ferm	2.36 ± 0.31	0.81 ± 0.09	0.17 ± 0.04
Lac.acid	2.48 ± 0.17	0.82 ± 0.15	0.18 ± 0.10
Lac.rham	2.36 ± 0.09	0.80 ± 0.09	0.15 ± 0.03

Values are expressed as means \pm SE (n = 6 per group). (***) P < 0.001 compared with the AOM/DSS-treated group.

Lac.ferm, Lac.acid, and Lac.rham, the average number of tumors was 12.17 \pm 3.92, 16.60 \pm

5.73, and 15.00 \pm 5.22, respectively (**Figure 2C** and **2D**). Notably, we found that daily supplementation with Lac.ferm markedly inhibited tumor growth (*P* < 0.05), but this was not the case with the Lac.acid or Lac.rham group (**Figure 2D**). Based on these results, the protective effects of the Lac.ferm probiotic needs to be examined more thoroughly.

Lac.ferm altered gut microbial community in AOM/DSS-treated mice

Accumulating evidence suggests that microbiota dysbiosis was associated with the colonic inflammation and even progression of CRC [23]. To determine the changes of gut microbio-

and weight				
	Colon length (cm)	Colon weight (g)	Weight/length ratio	
Control	10.13 ± 1.30**	0.25 ± 0.03***	0.024 ± 0.01***	
AOM/DSS	8.11 ± 0.76	0.50 ± 0.13	0.062 ± 0.02	
Lac.ferm	8.37 ± 0.98	0.40 ± 0.04	0.048 ± 0.01*	
Lac.acid	8.24 ± 0.56	0.47 ± 0.18	0.057 ± 0.02	
Lac.rham	8.25 ± 0.83	0.42 ± 0.14	0.052 ± 0.02	

Table 2. Effect of different Lactobacillus strains on colon length

Values are expressed as means \pm SE (*n* = 6 per group). (*) *P* < 0.05, (**) *P* < 0.01, and (***) P < 0.001 compared with the AOM/DSS-treated group.



Figure 2. Effect of different Lactobacillus on AOM/DSS-induced colonic shortening and polyp formation. (A) Representative images of colon length in each group; (B) colon weight-to-colon length (W/L) ratio; (C) representative images of colon tissue in each group; and (D) minimum and maximum observations and median polyp number. (*) P < 0.05, (***) P < 0.001compared with the AOM/DSS-treated group.

ta, we detected the microbial community diversity in fecal samples using next-generation high-throughput sequencing of the 16S rRNA gene. In this study, the amounts of operational taxonomic units (OTUs) were similarly detected in three groups. The Venn diagram showed the overlap of OTUs between each groups (Figure 3A). Results revealed that a total of 363 OTUs were shared by all groups, 386 OTUs presented in both control and AOM/DSS groups, and 374 between AOM/DSS and Lac.ferm groups. In addition, the clustering tree based on the

unweighted pair group method with arithmetic mean (UPGMA) analysis displayed that the gut microbiota composition of the Lac.ferm group showing a clear separation of other groups (Figure 3B). Furthermore, the partial least squares discriminant analysis (PLS-DA) plot also indicated an obvious difference in three groups (Figure 3C). For further taxonomic analysis, the relative abundance of bacterial communities appears in Figure 3D and 3E. At the phylum level, there are no significant difference in the Firmicutes and Bacteroidetes ratio between control and AOM/DSS groups. In the Lac.ferm group, however, a decrease in the proportions of phyla Bacteroidetes was observed. At the order level, histogram representation showed that Lactobacillales displayed species richness via treatment with Lac.ferm (19.64%) compared to control (12.75%) and AOM/DSS groups (5.29%). These results implied that Lac. ferm-suppressed inflammationassociated tumorigenesis might be linked to the alteration of specific bacterial taxa.

Specific phylotypes of gut microbiota modulated by AOM/ DSS and Lac.ferm

To explore the specific bacterial taxa characterized in each group, linear discriminant analysis (LDA) effect size (LEfSe)

analysis was applied (Figure 4A and 4B). According to LDA scores, data showed that five taxa were affected by AOM/DSS treatment, and six taxa were enriched in the Lac.ferm group. In particular, we found that Lactobacillus fermentum within the phylum Firmicutes was the prominent species existing in the Lac.fermtreated group. Furthermore, the abundance distribution of the dominant 35 OTUs for genus level were represented as colors in a heatmap among the different groups. The results demonstrated that bacterial taxa such as



Figure 3. Dietary Lac.ferm transformed the microbiota diversity in each groups. (A) Venn diagram of shared and unique OTUs among three groups; (B) hierarchical clustering tree analysis at OTU levels on each sample; (C) PLS-DA plot analyzed by OTU abundance from each sample; (D) relative abundance of microbiota profiling at phylum level; and (E) relative abundance of microbiota profiling at order level.

Bacteroides, Erysipelatoclostridium, and others in Figure 5A were associated with the AOM/ DSS treatment, but negatively to another two groups. Moreover, in Figure 5B, the relative abundance of genus Lactobacillus in the Lac. ferm group was higher than in the AOM/DSS group. Otherwise, recent studies indicated that the Gram-negative bacteria Akkermansia were correlated with colonic tumor burden in mice [24, 25]. Consistent with our results, Akkermansia had arisen in the AOM/DSS group and significantly decreased after administration of Lac.ferm for 14 weeks (Figure 5C). Overall, the above findings suggested that dietary Lac.ferm could modulate the gut microbial community, which might be beneficial to alleviating colon cancer progression.

Lac.ferm regulated colonic inflammation in AOM/DSS-induced CRC

Histological analysis with H&E staining revealed manifest crypt destruction, multiple adenomas, and even large adenocarcinomas in the colonic sections of the AOM/DSS group (Figure 6A). In contrast, the Lac.fermtreated mice showed a marked reduction of these symptoms. Growing data suggested that microbial endotoxins might promote macrophage infiltration, and chronic inflammation is a determining factor in the promotion phase of CRC [26]. To evaluate tumor-infiltrating macrophages, immunostaining for CD68-positive cells in tumor areas was performed. In our results, there were markedly more CD68⁺ macrophages in tumor tissues from AOM/DSStreated mice compared with the Lac.ferm group. Furthermore, proinflammatory cyto-

kines such as IL-1 and IL-6 produced by immune cells had to activate tumor cell proliferation [8]. Figure 6B showed that the levels of IL-1 α , IL-1 β ,



Figure 4. Variations on gut microbiota presented in AOM/DSS and Lac.ferm-treated mice. (A) Taxonomic cladogram obtained using LEfSe analysis of the 16S sequences in fecal samples (n = 5 per group); and (B) histogram of the LDA scores computed for taxa differentially abundant among three groups. Thresholds > 2.0 are shown.

and IL-6 in the AOM/DSS group were all higher than in the control group (P < 0.001). Conversely, cytokines IL-1 α and IL-6 were significantly downregulated after feeding with Lac.ferm. These results indicated that dietary Lac.ferm alleviated colitis-associated CRC, possibly through lowering the infiltration of macrophages and decreasing proinflammatory cytokines in colonic tissue.

Discussion

Accumulating evidence has suggested the beneficial functions of probiotics in cancer prevention [27]. *Lactobacillus* spp. is the most dominant probiotic strain used as a dietary supplement that is good for both the digestive and the immune systems. Numerous studies have noted that parts of probiotics such as *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, and *Lactobacillus fermentum* when processed improve the clinical symptoms of IBD and even attenuate cancer at early stage [17, 28-30]. In this study, we evaluated the protective role of these three potential *Lactobacillus* spp. on anticancer activity in AOM/DSS-induced colon cancer. Our results showed that dietary administration of Lac.ferm isolated

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Figure 5. Dietary Lac.ferm altered gut microbiota in AOM/DSS-treated mice. (A) Heatmap showing the abundance of high-confidence OTUs in fecal samples from mice. Relative abundance of (B) *Lactobacillus* and (C) *Akkermansia* at genus level (n = 5 per group). (*) P < 0.05 compared with the AOM/DSS-treated group.

from pickled bamboo shoots alone for 14 weeks markedly ameliorates tumor formation in AOM/DSS-treated mice (Figure 2C and 2D). These results are in agreement with those of a previous study reporting that feeding with *Lactobacillus fermentum* reduced the number of aberrant crypt foci (ACF) in 1,2-dimethylhy-drazine-induced Swiss mice [30]. Notably, a novel finding in our study is that oral Lac.ferm, working against colon tumorigenesis, might act in part through altering the gut microbial community and reducing the pro-inflammatory cyto-kines surrounding colonic tissues (Figures 3-6).

Microbiota dysbiosis leads to chronic inflammation and acts as a driving force for tumor progression [31]. As recent studies described, an increase of *Bacteroides*, *Parabacteroides*, and *Akkermansia* in the feces had highly correlated to colon tumorigenesis [24]. Gram-negative genus *Bacteroides* and *Akkermansia* are mucin-degrading bacteria having large endo-

toxins at their outer wall, which result in mucosa damage and trigger tumorigenesis [24, 25]. In our results, the relative abundances of Bacteroides and Akkermansia were higher in the AOM/DSS model group. In contrast, oral Lac. ferm significantly decreased the abundance of Bacteroides and Akkermansia in the feces (Figure 5). In addition, previous studies demonstrated that the growth of beneficial bacteria in the gut, such as part of Lactobacillus strains, have been good for managing diarrhea, allergies, and IBD [32]. Consistent with our results, Lactobacillus fermentum (5 \times 10⁸ and 1 \times 10⁹ CFU/day, respectively) has been reported to exert anti-inflammatory effects and restore the gut microbial homeostasis in DSS-induced colitis [17, 33]. In this study, we also found that treatment with Lactobacillus fermentum (1 × 10⁸ CFU/day) for 14 weeks markedly increased Lactobacillus at the genus level compared to the AOM/DSS group (Figures 4, 5). Moreover, Wu et al. [34] found that the abundance of



Lactobacillus increased by treating with Bacillus subtilis daily, which involved in tumor suppression. The above evidences suggest that the oral probiotic Lac.ferm might attenuate colon cancer progression partly by manipulating the gut microbiota.

Chronic inflammation plays a curial role in tumor growth due to the pro-inflammatory cyto-

kines (e.g., IL-1, IL-6, and TNF- α) accelerating tumor progression [35]. Current evidence suggests that novel anticancer therapies have targeted the tumor microenvironment, particularly because macrophage activations are involved in inflammatory cytokine production therein [36]. A previous study suggested macrophage depletion as a therapeutic target of CAC in AOM/DSS-induced mice [37]. In the present

study, we found that the infiltration of CD68+ macrophages were decreased in the tumors of CAC mice treated with Lac.ferm (Figure 6A). Meanwhile, the pro-inflammatory cytokines such as IL-1 α , IL-1 β , and IL-6 were also markedly reduced in the Lac.ferm group (Figure 6B). Similar to our study, Jang et al. demonstrated that Lactobacillus fermentum isolated from the feces of healthy adults ameliorates DSSinduced colitis by inhibiting the expression of some immune cytokines (e.g., IL-1ß, IL-6, and TNF- α) and altering the gut microbial composition [17]. Based on these results, we speculate that supplementation with Lac.ferm inhibited tumor growth and may contribute to reducing harmful bacteria, blocking macrophage recruitment in the gut. Notably, for the first time, we found that the probiotic Lac.ferm caused macrophage depletion and changed the tumor microenvironment, and that these effects would eliminate the tumorigenic factors during CAC development.

Conclusion

In conclusion, dietary Lac.ferm might rebalance the enteric microbiome community, which were beneficial to suppressing colon tumorigenesis in mice. Hence, we consider the probiotic Lac. ferm to have great potential for antitumor growth and in turn as a biotherapeutic agent for the future. However, to determine these properties in clinical patients, further verifications are still required.

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Disclosure of conflict of interest

None.

Abbreviations

AOM/DSS, azoxymethane/dextran sulfate sodium; CAC, colitis-associated cancer; CRC, colorectal cancer; IBD, inflammatory bowel disease; Lac.acid, *Lactobacillus acidophilus* LA257; Lac. ferm, *Lactobacillus fermentum* V3; Lac.rham, *Lactobacillus rhamnosus* LR132; LDA, linear discriminant analysis; OUT, operational taxonomic unit; SCFA, short-chain fatty acid.

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Supplementary Figure 1. Schematic of *Lactobacillus* spp. administration in AOM/DSS animal model.