High Fat Feeding of Lactating Mice Causing a Drastic Reduction in Fat and Energy Content in Milk without Affecting the Apparent Growth of Their Pups and the Production of Major Milk Fat Globule Membrane Components MFG-E8 and Butyrophilin

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Lactating mice were fed either a low fat or a high fat diet. Milk samples were collected and the composition was examined. Triglyceride and free fatty acid contents were greatly reduced in the milks of high fat diet group, while protein and lactose contents were almost the same between both diet groups. Although the energy content of each component was also lower in milk of high fat diet group, there was apparently no significant difference in the growth of the pups raised by either diet group. This discrepancy might be in part explained by a hypothesis that the pups might monitor calorie content in milk and keep suckling until the energy intake reaches their satisfaction. Moreover, nearly the same amounts of major milk fat globule membrane proteins MFG-E8 and butyrophilin were shown to be present in the milks from both diet groups and gene expression of both proteins in the mammary glands were also indistinguishable, suggesting that production of major MFGM components is not simply related to fat production and secretion.

Key words: high fat diet; lactation; mammary gland; milk fat globule membrane

During gestation and lactation periods, drastic changes in protein, fat, and carbohydrate metabolism are required to provide for functional and structural development of the mammary gland leading to substantial milk production. For example, genes coding for caseins and whey proteins are predominantly expressed during these periods under regulation of lactogenic hormones such as insulin, glucocorticoid, and prolactin. Likewise, fat metabolism in lactating mammary gland is also hormonally regulated. Milk is mainly composed of fat, protein, and lactose with slight differences in amount of each component in various species, and is the sole nutritional source for a newborn infant mammal. For the growth of newborn infants, quality as well as quantity of milk should be considered.

Several research groups reported dietary control of milk composition. Food deprivation for 24 h results in inhibition of mammary gland lipogenesis, which is rapidly reversed by refeeding chow for 2 h. It was shown by Robinson et al. that these changes correlate with changes in circulating insulin. It has also been reported that a high fat (HF) diet feeding throughout lactation considerably decreases the rate of lipogenesis in rat and mouse mammary glands. This lipogenic inhibition keeps through the isolation of mammary acini and can be completely reversed by incubation of these cells with insulin in vitro. This result well agrees with the results that administration of insulin to rats in vivo can partially reverse the inhibition of mammary gland lipogenesis brought by HF feeding. However, little information about relationship between dietary status and the growth of newborn infants, from the nutritional standpoint is available.

In this study, we investigated the effects of HF feeding of lactating mother mice throughout lactation immediately after parturition on the milk composition such as fat, protein, and lactose, and showed that milk fat yield and energy level of milk was greatly reduced, but protein and lactose levels were not affected by high fat feeding. Despite reduction in the energy content in the milk, there was apparently no significant difference in growth of the pups raised by the mice fed either a HF or a low fat (LF) diet. Furthermore, the level of major milk fat globule membrane (MFGM) glycoproteins, MFG-E8 and butyrophilin, and sugar chain structure of milk proteins were examined by Western blotting and RT-PCR amplification.

Materials and Methods

Diets and animals. Female pregnant ddY mice, 10 weeks of age, were purchased from Japan SLC (Hamamatsu, Japan). Until parturition mice were fed laboratory chow (Japan SLC) ad libitum. Litter size was standardized to 8 pups within 24 h postpartum. Lactating mice were fed one of following two semipurified diets: The LF diet contains in grams per 100 g: casein, 25.0; d,L-methionine, 0.3; mineral mixture, 3.5; vitamin mixture, 1.0; choline chloride, 0.4, cellulose powder, 4.0; corn oil, 5.0; corn starch, 40.8; and sucrose, 20.0. The HF diet was formulated by replacing 25 g of corn starch with 25.0 g of corn oil. During lactation, the mice

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Abbreviations: MFGM, milk fat globule membrane; HF, high fat; LF, low fat; TG, triglyceride; FFA, free fatty acid; RCA-I, Ricinus communis agglutinin-I; WGA, wheat germ agglutinin; SBA, soybean agglutinin
of each group were pair-fed to be supplied with the same amount of energy. The mice were individually housed in a room maintained at 22 to 24°C and about 50% relative humidity, and had free access to tap water.

After indicated days of lactation, mother mice were separated from their pups. After 3 h they were injected intraperitoneally with 50 μl of Nembutal (Abbott Laboratories, USA) and 10 units of oxytocin and then milked. Milk samples were stored at −80°C until use.

Chemical analyses. The milk was diluted 5-fold with distilled water and an equal volume of methanol-chloroform (2:1) was added. The lipids were recovered by evaporation of the chloroform layer and redissolved in chloroform for lipid analyses. Triglyceride and free fatty acid of chloroform extract of the milk were enzymatically measured using a Triglyceride G-test kit (Wako Pure Chem. Ind., Osaka, Japan) and a NefA C-test kit (Wako Pure Chem. Ind., Osaka, Japan), respectively.

To measure the protein content, the milk was diluted 500-fold with distilled water and then assayed by the method of Bradford et al. To measure the lactose content, the trichloroacetic acid-insoluble fraction was obtained and then enzymatically assayed using a F-kit lactose/galactose (Boehringer Mannheim, Germany).

SDS-PAGE and Western and lectin blotting. Proteins were separated by SDS-PAGE by the method of Laemmli. For Western blot analysis, samples were separated by SDS-PAGE and electrophoretically transferred to a PVDF membrane, essentially by the method of Towbin et al. Antiserum against mouse butyrophilin and MFG-E8 were prepared as described in ref. 13 and 14, respectively. Lectin blotting using Ricinus communis agglutinin-I (RCA-I) and wheat germ agglutinin (WGA) was carried out as described.51

RNA preparation. Total RNA was prepared from lactating mammary glands at the indicated days with Isogen reagent (Nippon Gene, Japan) according to the manufacturer's instructions. Briefly, tissue was homogenized in the Isogen solution followed by centrifugation at 10,000 × g for 15 min at 4°C. Aqueous phase was carefully recovered and then precipitated with 2-propanol. Poly (A) + RNA was isolated using Oligotex-dT30 super (Takara, Japan). The RNA samples were stored at −80°C until use.

RT-PCR. Both sense and antisense primers used in this study were selected based on the reported cDNA sequences. The primers are as follows; MFG-E8 (M38337), nucleotide residues 112–993: 5′-GGG-TCT-GGT-GAC-TTC-TGT-GAC-TCC-ACC-CTG-3′ and 5′-ATT-CTT-CAG-GCC-CAG-GGG-CTC-GAG-ACA-TCC-3′, butyrophilin (U67065, nucleotide residues 568–1359): 5′-AAA-CTA-TAC-AAG-GAA-AGA-TCC-AGT-3′ and 5′-TGA-AGC-TGG-AAT-ATT-TGT-TAT-GGT-3′, β-casein (M26940, nucleotide residues 95–702): 5′-CCA-AGA-GAG-CTT-CCT-ATT-GAG-3′ and 5′-AGT-AGT-TCT-AGG-TAC-TGG-AGA-3′, GAPDH (M32599, nucleotide residues 42–1059): 5′-ACA-AAA-

TGG-TGA-AGG-GTT-TCT-TAC-TCC-3′ and 5′-TCC-AGG-GTT-TCT-TAC-TCC-112

Statistical analysis. In all experiments, the group means were analyzed by means of one-way ANOVA.

Results

Effects of high fat feeding on milk composition and the growth of pups

Over a period of lactation (20 days), one group of mice was allowed access to a LF diet ad libitum. Another group of mice was fed a HF diet with the same energy level as the mice fed a LF diet. Their demand for food gradually increased during lactation (data not shown). Body weights of lactating mother mice were monitored immediately after parturition until the end of lactation. As shown in Fig. 1A, no significant difference in body weight was observed between the diet groups throughout lactation. At the indicated days of lactation, the mother mice were killed, and then mammary tissues were excised and weighed. There was also no significant difference between both diet groups (Fig. 1B). Other tissues were excised and weighed, but no significant differences were also observed (data not shown).

Previous reports have evaluated the effects of HF feeding on fat metabolism by assaying the activity of fat synthesis-related enzymes, whereas little information about the effect of high fat feeding on milk composition have been available from the standpoint of nutritional aspects. To analyze this, we collected milk from the lactating mother mice of both diet groups at days 2, 7, 14, and 17 after parturition and the content of four components measured triglyceride (TG), free fatty acid (FFA), protein, and lactose was. In a preliminary experiment, we confirmed that no significant changes in mouse milk composition was observed during milking or the time to milk in a day (data not shown). Table 1 summarizes the results. Throughout lactation, TG and FFA content of milks collected from the lactating mice fed a HF diet were reduced by 40 to 70% and 60 to 80%, respectively, compared to those collected from the mice fed a LF diet. These results might reflect the previous reports that rates of fatty acid synthesis in vivo and in vitro were inhibited by HF feeding. On the other hand, no significant difference in the content of protein and lactose from the diet groups was observed.

Based on the determination of TG, FFA, protein, and lactose, the energy content in the milk collected at indicated days was calculated using a criterion of 9 kcal/g
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Fig. 1. Body Weight (Panel A) and Mammary Gland Tissue Weight (Panel B) of Lactating Mice Fed a LF or a HF Diet throughout Lactation.

The lactating mice of each group were pair-fed to be supplied with the same amount of energy and weighed at indicated days after parturition. At indicated days of lactation, mammary tissues were removed from each mouse and weighed. Results are shown as means±SEM $(n=5)$.

Table 1. TG, FFA, Protein and Lactose Content of the Milk from Lactating Mice Fed a Low Fat (LF) Diet or a High Fat (HF) Diet

<table>
<thead>
<tr>
<th>Day postpartum</th>
<th>TG (mg/ml)</th>
<th>FFA (mg/ml)</th>
<th>Protein (mg/ml)</th>
<th>Lactose (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LF</td>
<td>HF</td>
<td>LF</td>
<td>HF</td>
</tr>
<tr>
<td>2</td>
<td>64.5±10.5</td>
<td>28.5±7.4*</td>
<td>0.59±0.04</td>
<td>0.12±0.04*</td>
</tr>
<tr>
<td>7</td>
<td>67.2±10.4</td>
<td>33.8±5.3*</td>
<td>0.75±0.03</td>
<td>0.16±0.10*</td>
</tr>
<tr>
<td>14</td>
<td>92.6±9.1</td>
<td>27.3±6.2*</td>
<td>0.58±0.19</td>
<td>0.12±0.09*</td>
</tr>
<tr>
<td>17</td>
<td>104.1±12.6</td>
<td>41.2±2.8*</td>
<td>0.86±0.14</td>
<td>0.32±0.12*</td>
</tr>
</tbody>
</table>

The content of each component was assayed as described under Materials and Methods. All values are expressed as mean±SEM $(n=5-7)$.

* Values that are significantly different from those fed a LF diet are shown at $p<0.05$.

for triglyceride and free fatty acid, and 4 kcal/g for protein and lactose. As shown in Table 2, the energy content in the milks of HF group was reduced by 20 to 34% compared to those of LF group.

To discover the effect of changes in milk composition on the growth of pups, body weight of pups raised by the mice fed either diet was monitored (Fig. 2). It was surprising that almost the same growth curves for both diet groups were obtained, although the calorie content of the milk of HF group was much lower than that of LF group. Immediately after weaning day, the pups were killed, serum was collected, and various tissues such as liver, lung, heart, and kidney were removed and weighed. Like body weight, no significant difference in tissue weight was observed between both diet group (Table 3). Instead, serum triglyceride and free fatty acid level of the pups raised by the mother mice fed a HF diet were significantly reduced. This might reflect the low fat content of the milk from the HF group.

Table 2. Energy in the Milk from Lactating Mice Fed a Low Fat (LF) Diet or a High Fat (HF) Diet

<table>
<thead>
<tr>
<th>Day postpartum</th>
<th>Total energy per ml of milk (kcal)</th>
<th>LF</th>
<th>HF</th>
<th>ratio (HF/LF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>1.14±0.12</td>
<td>0.82±0.12*</td>
<td>0.72</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>1.32±0.12</td>
<td>1.12±0.05*</td>
<td>0.85</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>1.56±0.10</td>
<td>1.03±0.07*</td>
<td>0.66</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>1.78±0.15</td>
<td>1.43±0.15*</td>
<td>0.80</td>
<td></td>
</tr>
</tbody>
</table>

Energy was estimated based on the criteria; 4 kcal/g protein and carbohydrate, and 9 kcal/g fat (triglyceride and free fatty acid). All values are expressed as means±SEM $(n=5-7)$.

* Values that are significantly different from those fed a LF diet are shown at $p<0.05$.

MFGM components in milk and their expression in mammary gland

After milk fat is produced in mammary epithelial cells...
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Fig. 2. Effects of HF Feeding of Lactating Mice on the Growth for Their Pups.

Body weight of the pups raised by the lactating mice fed a LF or a HF diet was measured at indicated days throughout lactation. This figure shows a representative of five mother mouse-pups groups for each diet group and results are shown as means ± SEM.

Table 3. Some Parameters of the Pups Raised by Mother Mice Fed a Low Fat (LF) Diet or a High Fat (HF) Diet

<table>
<thead>
<tr>
<th></th>
<th>LF</th>
<th>HF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver (g)</td>
<td>0.565 ± 0.045</td>
<td>0.595 ± 0.051</td>
</tr>
<tr>
<td>Lung (g)</td>
<td>0.090 ± 0.008</td>
<td>0.094 ± 0.004</td>
</tr>
<tr>
<td>Heart (g)</td>
<td>0.067 ± 0.015</td>
<td>0.067 ± 0.011</td>
</tr>
<tr>
<td>Kidney (g)</td>
<td>0.145 ± 0.025</td>
<td>0.141 ± 0.011</td>
</tr>
<tr>
<td>Serum TG (mg/ml)</td>
<td>1.15 ± 0.02*</td>
<td>0.80 ± 0.04</td>
</tr>
<tr>
<td>Serum FFA (mg/ml)</td>
<td>1.53 ± 0.08*</td>
<td>0.96 ± 0.12</td>
</tr>
</tbody>
</table>

All values are expressed as means ± SEM of 8 pups raised by the mother mice. This shows a representative of five mother mouse-pups groups for each diet group.

* Values that are significantly different from those fed a LF diet are shown at p < 0.05.

as a small droplet, the droplets gather and resultant large droplets fuse to an apical plasma membrane. Finally the milk fat is secreted into milk fluid after being surrounded by the plasma membrane referred to as milk fat globule membrane (MFGM).\textsuperscript{16,17} It has been suggested that some components in MFGM play a critical role in milk fat secretion, but the mechanisms has remained to be clarified. If MFGM protein secretion is related to the secretion of milk fat, the quantity or quality of the glycoproteins could change when the amount of milk fat changes. As mentioned above, HF feeding causes a dramatic decrease in milk fat content. This prompted us to measure MFGM components in milk from both diet groups. We found that two components, MFG-E8 and butyrophilin, are major components in mouse MFGM as well as the bovine one, using specific polyclonal and monoclonal antibodies.\textsuperscript{13,14,18,19} Using these antibodies, Western blot analyses were done to measure the amount of these two proteins. A sample of total milk was centrifuged and resultant cream and whey fractions were retained. An aliquot of cream fraction was solubilized with SDS sample buffer and separated by SDS-PAGE under reducing conditions followed by Western blotting using specific antisera against MFG-E8 and butyrophilin (Fig. 3). These figures show that both MFG-E8 and butyrophilin increased in amount as lactation proceeded and that no obvious difference in the profiles was observed between the diet groups. On the other hand, a major milk protein, β-casein, in the whey fraction was constantly detected throughout lactation periods in both diet groups.

Gene expression of MFG-E8 and butyrophilin in mammary gland was further investigated. At the same days as milking was done, mammary tissues were excised and total RNA was prepared. Poly (A)\textsuperscript* RNA was purified and random-primed 1st strand cDNA was used for subsequent PCR amplification using a set of specific primers for MFG-E8 and butyrophilin. All the amplified bands were sequenced and confirmed to be corresponding sequences by BLAST search, and the amplification was shown to depend on the initial amount of the cDNAs as well as the numbers of cycles for PCR reaction (data not shown). MFG-E8 (upper band)\textsuperscript{20} was shown to be expressed constantly throughout lactation and the expression profiles in the diet groups were very similar (Fig. 4). Butyrophilin slightly increased as lactation proceeded, but the expression profiles in both diet groups were nearly the same (Fig. 4). These results are consistent with protein content in milk samples (Fig. 3) and suggest that HF diet has no effect on the production of major milk fat globule membrane proteins.

We previously reported that reactivity of soybean agglutinin (SBA) to MGP53/57, which are bovine homologues of MFG-E8 and CD36 (=PAS-V) of bovine MFGM changed in early stages of lactation.\textsuperscript{15,19} In this study, the same approaches were done using some lectins to elucidate the changes in sugar chain structure of MFGM glycoproteins throughout lactation. The cream fraction rich in MFGM components was used, because it is difficult to purify MFGM quantitatively from small

Fig. 3. Western Blot Analysis of MFGM Glycoproteins, MFG-E8 and Butyrophilin in the Milk from Mice Fed a LF or a HF Diet.

For the detection of MFG-E8 and butyrophilin, a sample of the cream fraction corresponding to 50 μl of milk was used. For the detection of β-casein, a sample (10 μl) of the whey fraction corresponding to 50 μl of milk was used. Molecular mass of each protein is indicated at the right side of the figure. This figure shows a representative of milk samples from 3 independent mother mice fed either diet.
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Fig. 4. RT-PCR Analysis of MFG-E8 and Butyrophilin in Lactating Mammary Gland from Mice Fed a LF or a HF Diet.

At indicated days, mammary tissues were excised and RNA was prepared followed by cDNA synthesis. PCR amplification was done as described in Materials and Methods and a sample was separated on a 1.0% agarose gel followed by ethidium bromide staining. cDNAs for β-casein and GAPDH were also amplified to verify the quality of the cDNAs. The upper band for MFG-E8 corresponds to the reported sequence and the lower band has been shown to be an alternative splice variant of MFG-E8. This figure shows a representative of 3 tissue samples from 3 independent mother mice fed either diet.

Fig. 5. Lectin Blot Analysis of the Milk from Mice Fed a LF or a HF Diet.

A sample of the cream fraction corresponding to 50 μl of milk was separated by SDS-PAGE followed by blotting to a PVDF membrane. The membrane was sequentially incubated with WGA or RCA-I lectin, rabbit anti-lectin antibody and POD-linked anti-rabbit IgG. Color development was conducted using chromogenic reagent 4-chloro-1-naphotol. The same samples were separated by SDS-PAGE followed by CBB staining to ensure that nearly the same amount of protein was included in the samples. This figure shows a representative of milk samples from 3 independent mother mice fed either diet.

amounts of mouse milk and preliminary data revealed that lectin blotting patterns with MFGM and cream fractions (prepared on day 14 of lactation) were indistinguishable (data not shown). The cream fractions were separated by SDS-PAGE under reducing condition followed by blotting to a PVDF membrane. When the membrane was incubated with WGA, which binds oligosaccharides terminating with sialic acid, two distinct bands with the high molecular mass of 168 K and 120 K was detected in all samples of both diet groups, but the intensity of both bands, especially 120 K protein, of HF diet group is stronger than that of LF diet group (Fig. 5, upper panel). When the membrane was incubated with RCA-I, which binds oligosaccharides terminating with the Galβ1→4GlcNAc group, 120 K protein was detected in the samples of both diet group but the reactivity was weaker in the milk of LF diet group (Fig. 5, middle panel), while only 168 K glycoprotein in the milk of HF diet group bound the lectin. The CBB staining pattern showed that nearly the same amount of proteins were included in the samples for lectin blotting (Fig. 5, lower panel).

Discussion

Milk is the sole nutrient for a newborn infant. If the composition of milk can be regulated by feeding, it might be helpful for lactating females to find what kind of food is better for their milk production and their newborn infant during gestation and lactation periods. Since HF feeding causes obesity leading to severe diseases such as hypertension, diabetes, and arteriosclerosis, and it has been reported that the amount and type of dietary fat can significantly influence the development and/or growth of mammary tumors in rodents, we decided to investigate the effects of HF feeding on the milk composition and the growth of pups. We selected corn oil as the fat source, as a diet enriched in vegetable oil such as corn oil markedly increases mammary tumorigenesis. Some researchers have reported the effects of HF feeding on the lipogenesis in lactating mammary gland in vivo and in vitro, where triglyceride with long chain fatty acids was more effective in inhibiting mammary gland lipogenesis than those with predominantly medium-chain fatty acids. However, they have not conducted the experiments from the nutritional point of view; for instance, energy intake by mother mice between HF and LF diet group was not considered and several parameters for the pups raised by both diet groups were not available. We showed here that fat components such as triglyceride and free fatty acid in milk was considerably reduced by HF feeding and accordingly the energy content of the milk was reduced, because mouse milk has a high fat content compared with other species (cf. human milk contains 3.8% fat). Nonetheless, the pups raised by the mice fed a HF diet grew as well as ones raised by the mice fed a LF diet, which has a standard diet composition containing...
5% fat for an experimental animal. This apparently contradicted results might be reasoned by a hypothesis that pups can monitor the fat or calorie level in milk and continue to suck until the level reaches their satisfaction, even though the exact amount of milk intake could not be measured. In this study, we used corn oil as a fat source but it would be interesting that similar effect occurs by other fat sources such as lard, rich in saturated fatty acids.

Other point of this study is that it is unlikely that milk fat secretion is not simply related to the secretion of major MFGM components. We have shown that MFG-E8 and butyrophilin are major components in mouse MFGM and assumed that both glycoproteins might play a critical role in milk fat secretion.14,15,16,18,19 In the milk of LF diet group, triglyceride level increased as lactation proceeded (Table 1) and MFG-E8 and butyrophilin also increased in amount in a similar manner (Fig. 3), which is nearly consistent with our previous observation.10 From this result, it seems likely that milk fat secretion is related to the glycoprotein secretion. However, we cannot accept this result as a conclusion, because almost the same amount of both proteins was detected even in the milk of the HF diet group (Fig. 3) and their gene expression in mammary gland was very similar between both diet groups (Fig. 4). These results suggest that MFGM components might be produced and secreted into milk during lactation irrespective of the dietary fat content and/or that some other minor components which have not yet been identified might have a critical regulatory role in milk fat secretion.

In our previous studies,15,19 SBA lectin binding to MGP53/57 and CD36 of bovine MFGM increased during early stages of lactation. Although most of bovine MFGM glycoproteins contain SBA-positive sugar chains,20 we could not detected any SBA-positive glycoproteins of mouse milk cream rich in MFGM components. Among those examined, obvious binding was observed with WGA and RCA-I lectins. Two bands of 168 K and 120 K were shown to be positive to WGA lectin and the binding was stronger for the proteins in milks from HF diet group throughout lactation. The 168 K glycoprotein in the milk of HF diet group, not LF diet group, bound RCA-I lectin especially at the late lactating stage, while the 120 K glycoprotein of both diet groups was reactive to the lectin with slight differences in binding strength. The reactivity of RCA-I to both bands did not change even after treatment with A. ureafaciens silaidase, which cleaves all siaiy linkages, and these lectin bindings disappeared when the membrane was treated with N-glycanase before incubation with each lectin (data not shown), suggesting that both WGA- and RCA-I-positive oligosaccharides are linked to asparagine residue(s) and silaylation and galacatosylation on the N-linked sugar chains of the 168 K and 120 K proteins were stimulated by HF feeding. We have not identified the 168 K and 120 K glycoproteins, because they could not be detected by CBB staining, indicating they are minor components in the milk. Affinity purification of these glycoproteins on WGA-Sepharose column with a combination of other fractionation methods is in progress.

In conclusion, HF feeding caused a drastic reduction in fat and energy content of milk but the apparent growth of the pups raised by mice fed a HF or a LF diet was indistinguishable. Moreover, such drastic reduction in fat content of milk was not accompanied with that in content of the major MFGM components, MFG-E8 and butyrophilin, and modification of sugar chains on the 168 K and 120 K proteins in milk was stimulated by HF feeding.

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