SOYBEAN PRODUCTS AS A PROTEIN SOURCE IN MILK REPLACER FOR PRERUMINANT CALVES.

by

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SOYBEAN PRODUCTS AS A PROTEIN SOURCE IN MILK REPLACER FOR PRERUMINANT CALVES.

The development of milk substitutes

The first successful substitutes for whole milk were developed around 1950. Previously, milk replacers had really been milk extenders and consisted of linseed oil meal, wheat middlings, wheat red dog and oat flour. These were fed as gruels and were only successful if given together with whole milk (18,64). The early commercial milk replacers were, in fact, largely composed of milk derivatives. Thus, dried skim milk was the principle source of protein and carbohydrate, while tallow provided the lipid fraction. The major problem with these early milk replacers was poor growth rates due to the low energy content; only limited amounts (<10 %) of fat could be added without clogging the batch mixers. Advances in processing technology solved the problem of restricted fat addition by homogenization of the mixture followed by spray drying (59). Another problem with the early milk replacers included excessive scouring following overheating of the skim milk during drying. This problem was solved by preheating to 60-74°C for 30 min before spray drying (59).

In 1966, due to a change in price support policy, the relative cost of skim milk increased. As a result of these changes, manufacturers turned to casein and whey as alternative protein and carbohydrate sources, respectively. These products proved to be of comparable quality to the skim

milk-based products. In 1971 the U.S. government changed policies from subsidized milk production to subsidized slaughter of dairy cows and milk disposal programs. In the same period Australia and New Zealand, both important producers of casein for the world market, suffered drought and reduced casein production. The subsequent increase in casein prices stimulated renewed interest in non-dairy sources of protein and caused milk replacer manufacturers to use whey as the major source of protein.

Carbohydrate replacement in milk replacers

The young preruminant calf can successfully digest only lactose among the oligosaccharides, because lactase (ß-galactasidase) is the only brush border enzyme secreted in significant amounts (36,64). Invertase is absent, consequently calves are unable to utilize sucrose (71). Some maltase has been found (10), but the absence of salivary amylase (10,36,51) and the relatively low secretion of pancreatic amylase (36,51,71) limits the capacity of the preruminant calf to digest starch. According to Thivend (94), calves under 8 wks of age cannot utilize more than 15% of the dry matter in their diets as starch and this has to be reached by gradual increase. Milk replacers generally contain lactose, in the form of whey, as their carbohydrate source. Glucose, though available to the calf, is comparatively expensive. Carbohydrate cannot be used as the major energy source, because hexose absorptive capacity is limited (17). Should excessive carbohydrate, or carbohydrate of a type unavailable to the calf be given, calves become predisposed to fermentative diarrhea (69). Such is the case

(exacerbated by high mineral content) when using large amounts of dried whey, which has not been ultrafiltered, or when a large amount of soy flour is used.

Lipid replacement in milk replacers

Carbohydrate absorption is limited, therefore lipids are needed if a milk replacer is to supply sufficient energy for rapid growth. The digestion of fat in the preruminant calf is accomplished by pregastric esterase and pancreatic lipase (55). Much of the early work on the use of alternative lipids gave poor results because of a deficiency of the fat soluble vitamin E. With supplementation of vitamins the list of viable alternative lipid sources is somewhat increased (68). There are several sources of lipids used in milk replacers, with choice white grease and tallow being the most common (74). Other lipids used in milk replacers include partially hydrogenated fish oil, palm oil and sunflower oil (59,74). Milk replacer trials in which cotton seed oil or soybean oil was the energy source resulted in unthriftiness, excessive scouring and high mortality, this poor response being partially alleviated by hydrogenation of the unsaturated oils (4,52). Homogenization and emulsification of lipids in milk replacers is thought to improve their utilization (71). Soy lecithin is normally added to improve lipid emulsification. After homogenization and emulsification the digestibility of choice white grease and tallow approaches that of butterfat (73). Another benefit of fat addition is a reduction in scours, with the effect being somewhat proportional to the amount of fat (11,59).

Protein replacement in milk replacers

Protein requirements of the preruminant calf. The requirements of the preruminant calf for digestible protein are dependent upon body weight, rate of gain and age (19,69). The recommended concentration of protein in milk replacers is 22 % (54). This recommendation is for milk replacers containing high quality milk protein. Roy et al. (71) proposed utilization of milk replacers with higher protein levels if protein sources of lower biological value than milk protein were used. This theory is supported in work by Akinyele and Harsbarger (1). However, other studies have shown no benefit to feeding milk replacers with higher protein levels to compensate for poor biological value of ingredients (14,56).

In estimating amino acid requirements, the relationship between the carcass content of a reference amino acid and the average dietary requirement for the same amino acid is found. This relationship is used to estimate the requirements of other amino acids relative to their carcass composition. The method assumes the relationship between carcass composition and dietary requirement is proportional, that rates of turnover of all amino acids in the body are similar and absorption of all amino acids is the same. Differences in estimating amino acid requirements were observed when lysine was used as the reference amino acid rather than methionine (102). Methionine is required in methyl transfer reactions and this fraction of the absorbed methionine does not become part of the carcass tissue; also cystine can have a sparing effect on methionine. Radostits and Bell (64) reported that apparent digestibility of methionine

was lower than that of the other essential amino acids. Such factors help explain the range in values given for amino acid requirements. Some of the essential amino acid requirements of the calf have been determined (21,60,102).

Non-milk protein in milk replacer. Huber (35) compared the relative costs of using skim milk powder, fish products and soy flour as protein sources in milk replacers. The economic benefits of these alternative proteins were quite substantial. However, performance of calves has been inadequate when these products supply more than 30-40 % of the dietary protein. Thus, there is an interest in developing or discovering a non-milk protein which is economical, while allowing high calf growth rates. A number of materials have been tested as a non-dairy protein source for milk replacers (59,66,90). Most non-dairy proteins have undesirable effects on calf performance when used as the major protein. Use of some products is discouraged by limited or unreliable supply; many of the animal by-products come into this category. Some of the alternative proteins tested appear to have nutritional potential but require further study and currently lack the production infrastructure needed. Pea (9) and alfalfa protein concentrates (2) come into this category.

The two most extensively studied non-milk protein sources for use in milk replacers are fish and soybeans. These two base products have widespread international production and utilization, with reliable supply. Both fish and soybeans have proved unsuitable as the major protein in milk

replacer in their more common forms, and as a result have undergone extensive study in an attempt to provide a protein devoid of deleterious side effects.

Fish as a protein in milk replacers for calves

Replacement of a major portion of milk replacer protein with fish protein concentrate can result in reduced diet palatability, poor calf growth, and a reduction in the quality, both taste and appearance, of veal (13,27,35,40,58). Huber (35) reviewed some of the effects of using fish products as a protein source. The organic solvent used to extract fish protein affects the quality of the final product. Dichloroethane (DCE) extraction results in a superior product for milk replacers, compared to isopropanol (ISO) extraction, or hexane-heptane (HH) extraction. At least part of the difference between end products is due to the toxicity of residues. Fish protein solubles have an unfavorable amino acid balance. Fish products are deficient in vitamin E; while the iron content is sufficient to reduce the anemia necessary for light colored veal (98). Though inferior to milk, "satisfactory" calf performance has been obtained with the use of ISO extracted herring meal, together with NaOH neutralized acid whey (27).

In summary, ISO extracted fish protein concentrate, with supplemental vitamin E, can be used to supply up to 35% of the protein in a milk replacer for young (<3 wk old) non-veal calves. As calves age beyond 3 wks, particularly when given dry feed, fish protein concentrate can successfully provide around 70 % of the protein in a milk replacer (35).

Soybeans in milk replacers for calves

Soybeans can be divided into three fractions of major interest to a manufacturer of milk replacer; lipid, carbohydrate and protein.

The earliest attempts at using soy products in a milk replacer involved full-fat soybean meal. These early soy-based products resulted in diarrhea, loss of appetite, muscular weakness, poor growth and high mortality (78,103). In studies where soy oil was used as the lipid source in a skim milk based milk replacer, unthriftiness, excessive scouring and high mortality resulted (4,52). In consequence of this and the relatively high value of soy oil, subsequent studies have largely concentrated on lipid extracted soy products.

Carbohydrate composes approximately 1/3 of a soy flour, with roughly half of this being soluble (34). The soluble sugars are unavailable to the calf, but can undergo microbial fermentation with resultant flatulence and diarrhea (69). The insoluble carbohydrate fraction consists largely of pectins, arabinogalactans and cellulose; these are not readily fermentable and therefore not thought to be detrimental to the calf. Enzymatic pre-digestion of the carbohydrates in soy flour failed to improve its utilization by calves (16). Further, the feeding of soy protein concentrates and soy protein isolates (which have their soluble carbohydrates removed during processing) generally fails to produce satisfactory calf performance (56,57). It therefore seems that the carbohydrate component of soybeans has at least the potential for a detrimental effect on calf performance, and that major problems remain with the proteins themselves.

Problems associated with soy proteins in milk replacers for calves

There are numerous anti-nutritional factors associated with soy proteins. Some of these have been studied extensively, while others have received little attention. The less well known effects include: inhibition of iodine uptake by the thyroid, resulting in goiter; having antithyrotoxic, estrogenic and rachitogenic properties (105); and reducing uptake of dietary copper and zinc (33). Other effects of soybeans have received greater attention.

Hemagglutinin. Raw soybeans contain the toxin hemagglutinin. Hemagglutinin is readily inactivated by heat, acid, alkali or pepsin. It is therefore, not considered to be of importance in processed soy products (5).

Urease. Present in raw soybeans, urease is inactivated by heat, with moisture an important cofactor, in a manner similar to trypsin inhibitor (106). Urease has no physiological activity in the monogastric animal and is therefore of little importance in milk replacers. The importance of urease lies in the correlation between its activity and those of trypsin inhibitor and lipase inhibitor. This, together with the simple assay procedure for urease, led to its use as a means of determining the efficacy of heat processing (5,83,106,). However, the use of urease in quality control is limited, it having been shown that urease activity does not always reflect trypsin inhibitor activity (22).

Lipase inhibitor. Soybean lipase inhibitor acts by binding to the substrate, rather than the enzyme (73). Purified lipase inhibitor is inactivated at temperatures above 50°C unless the substrate is emulsified, whereupon it remains active at 60°C (73). Little is known about the occurrence and activity of lipase inhibitor in oil-extracted soy products.

Trypsin inhibitor. The problem with trypsin inhibitor (TI), or more correctly trypsin inhibitors, is more complex than with the other inhibitors. Reviews by Flavin (20) and Wright (106) indicate there are at least 5 different TIs in soybeans. Differences between these TIs include their ability to inhibit trypsin and their susceptibility to inactivation by processing. To further complicate matters, the assay used to measure TI only registers the activity of TI in solution. It has been shown that inactive-bound TI can become active following a change in conditions, such as pH (67).

The mode of action of TI has been established in the rat (20). In this case the TI binds with trypsin, forming an inactive enzyme-inhibitor complex. This in turn decreases both proteolytic activity and negative feedback on trypsin secretion via colecystekinin. The results are a decrease in protein digestion and pancreatic hypertrophy due to sustained stimulation of pancreatic secretion. The effects of TI in the calf differs from those of the rat, as indicated by a lack of pancreatic hypertrophy (28,30). Further, there is some evidence to suggest reduced pancreatic secretion following the feeding of soy proteins to calves (28,30,104). These facts indicate

pancreatic activity in the rat and calf have different regulatory mechanisms. This is not to say that soy TI does not inhibit the proteolytic activity of bovine trypsin. In vitro inhibition of bovine trypsin by soy TI is 100% at a 1:1 molar ratio, compared to 35% inhibition of human trypsin under similar conditions (20).

Several authors have correlated calf performance to the TI activity of milk replacer; some studies find a significant inverse relationship (28,101), while others fail to show any significant relationship, even when adding exogenous TI (43,66). The controversy may in part be due to limitations of the TI assay by its reliance upon the soluble-active portion of the total TI. Alternatively, calf performance may be related to some other component of the soy product, which sometimes reflects TI activity but is essentially independent of it. There is no clear evidence that the inactivation of TI during processing accounts for the improved protein digestion and calf performance generally seen with heated soy flours. Evidence from rats suggests that removal of TI without heating results in 40% of the improvement seen with heating (42). It seems likely there would be some benefit, to the preruminant calf, from the inactivation of TI.

TI activity is reduced by heating, and some form of heat treatment is standard for soy products used in milk replacers. TI activity is inversely related to time and temperature of heating, with moisture an important cofactor. Most researchers find a marked improvement in calf performance with heated soy products over non-heated soy products (5,15,43,56,101). However, excessive heating causes poor product quality by reducing both

palatability and amino acid availability through the Maillard reaction (15,19).

Antigenicity of the soy proteins. Soy protein products, which have undergone processing to remove or inactivate the anti-nutritional factors mentioned above, do not generally produce milk replacers of the predicted high quality. One possible reason for this is an allergic sensitivity of the preruminant calf to soy proteins. Such a reaction was first indicated in 1965, with the discovery of antibody formation in response to diets containing soy flours (97). That an immune hypersensitivity can occur has been confirmed and further studied by several authors (6,7,8,44,85,88). The antigenic proteins have been isolated and identified as glycinin and ß-conglycinin, two of the major storage proteins of soybeans. Further evidence for the involvement of soy protein antigens in the malnutrition of soy-fed calves comes from studies of the small intestine. The effects of diets, based on soy protein, on the rates of flow and composition of digesta were studied in the abomasum and small intestine of fistulated preruminant calves (84). Feeding soy proteins resulted in an inhibition of abomasal emptying, decreased transit time through the small intestine, abnormal salt and water exchange and decreased nitrogen absorption. When first introduced, the soy diets gave comparatively "normal" digesta movements; abnormalities were only exhibited in animals previously fed soy protein and generally coincided with demonstrable serum antibodies against soy proteins. Associated with the development of the immune response and the digestive

disturbances are changes in the intestinal mucosa. These include villous atrophy, crypt hyperplasia, mononuclear cellular infiltration of the lamina propria and villi, and edema and hemorrhage within the laminal tissue (8,61,76,77,79,80). There is also a breakdown in the integrity of the mucosal barrier, allowing passage of macromolecules into the circulatory systems (45). Changes in the intestinal mucosa, particularly the decrease in surface area associated with villous atrophy, together with the decreased transit time of digesta across the small intestine, are thought to be responsible for a decrease in the absorptive ability of the small intestine (13,76,77,79).

Barrat and Porter (6,7,8) studied the immunoglobulins involved in the immune response of calves to soy proteins. Antigen precipitating, complement fixing, IgG (immunoglobulin G) antibody is the major anti-soy immunoglobulin in the serum of soy fed calves. A small percentage of calves develop detectable serum IgE antibody. Immunoglobulin secretions into the intestinal lumen differ in immunoglobulin class and specific activity. IgM and IgA are the predominant immunoglobulins secreted into soy-perfused intestinal loops of soy-sensitive calves, with IgG and IgE sometimes present. Complement, activated by IgG-soy antigen complexes, causes the tissue damage of the small intestine associated with feeding soy based milk replacer. There is evidence suggesting the immune response by calves to the soy protein is related to maternal (colostral) levels of anti-soy immunoglobulins.

Studies in mice with transient dietary hypersensitivity have implicated a cell-mediated immune reaction (91). A comparable etiology has

been proposed to explain dietary hypersensitivity and post-weaning diarrhea in the pig (48). No such response has been reported in calves given soy milk replacer.

Amino acids of soy protein. Soy protein is considered relatively rich in essential amino acids for a plant protein, only methionine and possibly lysine being deficient. The extent of an amino acid deficiency in a milk replacer is dependent upon the proportion of the protein supplied by soy products and the source of the remaining protein. Williams and Hewitt (102) suggest that up to 50% of a high quality milk protein can be replaced by soy protein without an amino acid deficiency resulting. This is contradictory to calculations by Jenkins and Emmons (39), who determined lysine to be 14% and methionine 24% deficient with 55% replacement of milk protein by soy protein. Similar controversy exists as to the effects of supplemental amino acids; results range from a detrimental response (5), through no response (26,89), to a beneficial response (39). These discrepancies could be due to the range of values given for amino acid requirements, the variable response of calves to apparently similar soy products and differences in the availability of essential amino acids (28,49).

Evidence from rats, pigs, chicks and humans all indicate a methionine deficiency in soy protein (5,48). A comparable deficiency of methionine for the preruminant calf seems probable.

Soybean products

Full-fat soy flour. Whole soybeans are dehulled and ground to produce the basic full-fat soy flour. Products range from those given no additional heat up to a toasted or fully cooked product. Toasted products are heated to improve nutritional value. With the type of heat processing currently given soy products this is the case when the destruction of antinutritional factors is balanced by the loss of available essential amino acids (83). The three important variables in standard heat processing are heat, moisture and duration; they interact to determine the effectiveness of the heat treatment (63). One result of this complexity in the heat treatment is that each mill has individual processing conditions. Unfortunately, there is variability in the final product, both between producers and between batches from the same producer. There are several tests used to indicate the effectiveness of heat treatment including the levels of urease, TI and available lysine; the nitrogen solubility index (NSI); protein dispersable index (PDI); and protein efficiency ratio (PER). The advantages, disadvantages and use of these tests are reviewed by Horan (34). Full-fat soy flours are not generally used in milk replacers due to the detrimental effects of the carbohydrates and polyunsaturated oil.

Soy flour. More correctly called defatted soy flour, soy flours are similar to full-fat soy flours, except for the absence of the lipid fraction. The efficiency of lipid removal is dependant upon the extraction procedure. The majority of manufacturers use the more efficient solvent extraction,

reducing lipids to around 1% in the flour (34,83). Mechanical extraction leaves around 5% lipids in the flour and is produced mainly as a speciality item (34,83). Similar to the full-fat products, additional heat treatment ranges from none up to toasting to optimum nutritional value. The same tests of the efficacy of heat treatment can be used for full-fat and oil extracted products. For use in a milk replacer a soy flour should be "fully cooked". Soy flours receiving less than optimal heat treatment generally produce correspondingly inferior results. However, even a fully cooked soy flour results in poor calf performance if used to replace more than about 30% of the milk protein (16,57,71,78,90). The digestibility of soy flour protein in milk replacer by 4-6 wk old calves has been estimated at 50% (57).

Soy protein concentrate. Protein concentrates are made by heating defatted soy flakes and then leaching out soluble materials with either alcohol or acidic water (34). Most of the protein is retained in a protein concentrate, while soluble oligosaccharides (sucrose, stacyose, raffinose) are removed, as is much of the beany flavor associated with soy flours. Insoluble sugars are not removed in concentrate processing, they remain forming ca 20% of the product. Despite the increased cost of protein concentrates, they are currently the most common soy protein products found in milk replacers. The widespread use of soy protein concentrates in milk replacers reflects increased calf performance over heated soy flours (28,57). Though a soy concentrate allows a superior milk replacer to soy

flour, it remains inadequate for high level protein replacement (13,28,50,57). The digestibility of protein from a soy protein concentrate in milk replacer fed 4-6 wk old calves has been estimated at 75% (57).

Soy protein isolate. Isolates are made by extracting unheated defatted soy flakes with mild alkali (pH 7-8.5). The extract is acidified to pH 4.5 (isoelectric point of the major soy proteins) to precipitate the protein. The protein is then washed and either ground to form isoelectric isolate, or neutralized and ground to form neutral isolate. Isolates are ca 96% protein with ash forming most of the remainder. The procedure is approximately 60% efficient in terms of total protein recovery. The more complex processing results in greater costs for isolates than flours or concentrates. The increased cost of an isolate does not necessarily translate into improved calf performance. Because of increased costs without reliable benefit, soy isolates are seldom used in milk replacers.

Ethanol treated soy flour. Fukushima (24) showed that soy products were denatured by heating with aqueous ethanol. Apparently the hydrophobic centers of the globular protein molecules become exteriorized, altering the protein configuration and stability. Aqueous ethanol extraction can be used to eliminate the antigenicity of soy proteins (85). The ability of ethanol extraction to inactivate antigenic factors was questioned by others (6,7) who continued to find immune hypersensitive reactions. Subsequently, it was found that processing conditions, such as ethanol concentration,

temperature and duration of extraction were critical in determining the efficacy of the process (81). Use of ethanol extracted, non-immunogenic soy flour in milk replacer results in calf performance superior to soy concentrate but inferior to milk protein (79,85).

Enzyme treated soy products. It has been shown that peak activities of protease in gastric and pancreatic secretions of preruminant calves are not achieved until 1 month of age (28,29,104). Sissons & Thurston (82) reported that in vitro pepsin digestion destroys the antigenic activity of glycinin, but not that of ß-conglycinin, while trypsin has little effect on either protein. In the belief that the calves digestive system could be augmented by the use of exogenous enzymes, both supplementation and pre-digestion by enzymes has been tried. The addition of pepsin to milk replacer containing soy proteins failed to improve calf performance (23,47). Digestion with individual enzymes or combinations of enzymes, prior to feeding, failed to improve soy flour-based milk replacer (16,23).

Acid/Alkaline treated soy products. Incubating a "fully cooked" soy flour at pH 4 or pH 10.6 for 5 hr at 36°C improved the product for use in milk replacer (16). A similar improvement was found with a thermo-alkaline treatment (pH 10.5, 85°C, 5 min;15). Careful control of conditions during heating is needed to prevent methionine loss or the production of toxic peptides (15). Though these products proved superior to the pretreated

forms of soy flour, they remain inferior to milk protein for use in milk replacer.

Thermoplastically extruded soy products. Thermoplastic extrusion, or simply extrusion processing can use any of the aforementioned soy products as a raw material. When using the more basic soy flours a comparatively inexpensive end product can result. Thermoplastic extrusion works by the interaction of temperature, pressure and the large shear forces exerted upon material being forced through the extruder barrel. There is an approximate knowledge of the gross physical conditions within an extruder, while the microenviroment and the chemical reactions of the substrate within the barrel of an extruder are largely unknown. Studies of the effects of extrusion have largely been of an empirical nature. The majority of research effort in this field has been devoted to the study of texturing soy proteins for human consumption. Hamdy (31), in a review of the human nutritional aspects of textured soy products, concludes "under the moist-heat-pressure conditions of processing textured soy proteins, antimetabolites in soybeans are inactivated to have practically no effect on the latter's nutritive value". In respect to the calf, texturing is not of direct interest; however, the reactions of the process are of interest. The fact that a major portion of the total protein in soybean is allergenic to the calf prevents removal of this antimetabolite from being a viable method of processing. An alternative is to modify the proteins such that they have different biochemical activities. The change in physical properties during

texturing reflects a change in the structure and therefore the biological activity of the proteins. Thus, extrusion offers the possibility of a product with a similar amino acid profile to raw soybeans, while having a different biochemical activity. Little is known about the potential for extruded soy products in milk replacer. Srihara (88) showed an extruded soy flour to be superior to a heated soy flour, but inferior to milk protein, when used in milk replacers for calves.

EXPERIMENT 1

SOY PROTEIN CONCENTRATE, COMMERCIAL HEATED SOY FLOUR AND AN EXPERIMENTAL SOY FLOUR AS PROTEIN SOURCES IN MILK REPLACERS FOR CALVES.

ABSTRACT

Metabolic, physiological and immunological parameters were monitored in preruminant calves fed one of four milk replacers. Protein sources for the milk replacers were all milk (AM) or 25% milk protein and 75% protein from either soy protein concentrate (SPC), commercial soy flour (CSF) or experimental soy flour (ESF). The experimental soy flour had trypsin inhibitor activity of <1 unit/mg. Using the nitrogen retained at 3 and 6 wks, the presence of abnormal intestinal mucosa, growth rates and the development of circulatory immunoglobulins specific to soy proteins, the three soy-based milk replacers were inferior to the AM milk replacer. The soy-based diets induced a marked antibody-mediated allergic response in the calves, with no major difference in this response between soy products. There was no cell-mediated immune response to any of the soy products. Calves showed improved performance with age, particularly on the soy-based diets. Milk replacers SPC and ESF gave comparable calf performance, both being superior to CSF.

INTRODUCTION

The cost of production and the value of milk as a human food makes its use as animal feed relatively expensive. The vulnerability of the young calf to stress and its reliance upon liquid feed presents a difficult problem to those looking to provide an alternative to milk. Protein is the major cost ingredient of a milk replacer and it has proved difficult to find a satisfactory alternative source to milk. Soybean protein has potential for use in a milk replacer and has received much attention. It is recognized that commercial soy flours and protein concentrates require further processing to inactivate antinutritional factors prior to their intensive inclusion in high quality milk replacers for calves. It has been shown that trypsin inhibitor (TI) inhibits the activity of bovine trypsin (20). The reduced proteolytic activity probably contributes to the poor digestibility of soy proteins in the young calf.

The objectives of this study were to use metabolic, growth, health and immunological responses of calves to determine the relative quality of four milk replacers. The milk replacers differed mainly in the source of the major portion of protein. In effect the objective was to compare milk, heated soy flour, soy protein concentrate and an experimental moist heat processed soy flour as protein sources in calf milk replacer. The experimental soy flour received moist heat treatment sufficient to reduce TI activity <1 TI unit/mg.

MATERIALS AND METHODS

Management of calves

Eight Holstein bull calves, 2 per treatment, housed in metabolism crates, were used to measure nitrogen balance and xylose tolerance at 3 and 6 wks of age and intestinal morphology at 6 wks of age. A further 24 calves, 6 per treatment, housed in hutches, were used from birth through 6 wks to monitor growth and immune reactions.

All calves received colostrum for 3 days after birth. Treatments were assigned to calves by randomized block, a block being the 4 treatment diets. The appropriate milk replacer was fed warm, by pail, half in the morning, half in the afternoon. No dry feed was given, water was available ad libitum. Calves in metabolism crates had no bedding and were housed in a temperature controlled room. The remaining calves were housed outdoors in individual hutches and bedded on straw.

Each calf was twice daily assigned a value for general appearance (good, fair or poor) and consistency of feces (1, normal, through 4, watery)(46). No treatment for scours was necessary during the trial.

Milk replacers

The ingredient composition and nutritional composition of the milk replacers are shown in Tables 1 and 2, respectively. The four milk replacers were similar in all aspects except the source of the major portion

TABLE 1. Ingredient composition of milk replacers.

Item	Milk Replacer			
	AM	SPC	CSF	ESF
	(%)			
Spray dried whey	19.32	16.25	16.00	16.00
Whey protein concentrate	48.25	...	...	...
Soy protein concentrate[a]	...	26.25	...	...
Commercial soy flour[b]	...	...	33.15	...
Experimental soy flour[c]	...	...	...	33.15
L-Lysine	...	.02	...	...
DL-Methionine	...	.04	...	...
Lactose	...	22.51	15.92	15.92
10/50 Fat[d]	31.25	33.75	33.75	33.75
Limestone	.63	.63	.63	.63
Na mineral premix	.50	.50	.50	.50
Chloratetracycline 100	.05	.05	.05	.05
	I.U./Kg			
Vitamin A	33069	33069	33069	33069
Vitamin D_3	6613	6613	6613	6613
Vitamin E	200	200	200	200

[a] Procon, A.E. Staley Manufacturing Co., Decatur, IL 62526.

[b] Lauhoff soy flour (50%), Lauhoff Grain Co., Danville, IL.

[c] Experimental soy flour (<1 unit TI/mg) see text.

[d] 10/50 Made from fat (50%), dried whey (45%), and caseinate (5%) and contains 10% protein and 50% fat.

TABLE 2. Nutrient composition of milk replacers.

Item	Milk Replacer			
	AM	SPC	CSF	ESF
	(%) of DM			
Protein (N X 6.25)	21.98	22.56	21.98	21.98
Lipid	17.26	17.11	17.35	17.35
Ash	7.01	4.14	4.82	4.82
Calcium	1.07	.62	.64	.64
Phosphorus	.68	.32	.52	.52
Lysine	2.06	1.62	1.54	1.54
Methionine	.41	.45	.33	.33

of protein. Diet AM was a positive control for the soy based milk replacers, with all of its protein being milk protein. The three remaining diets each had 25% of their protein derived from milk and 75% from either soy protein concentrate (SPC), commercial heated soy flour (CSF), or an experimental soy flour (ESF). The experimental soy flour was prepared by steam heating (100°C) 9 kg batches of dehulled, defatted, raw soy flakes, with 10% added moisture, for 60 min. The "cooked" soy was dried overnight in a forced air oven (50°C) before grinding. The resultant product had TI activity of <1 unit/mg. TI activity was measured using the method of Ramsey and Willard (67).

Milk replacer was given at 1.25 kg DM/100 kg body weight, fed as a 12.8% solution for the first 2 wks and 1.75 kg DM/100 kg body weight, fed as a 17% solution thereafter. The amount of milk replacer fed was adjusted

weekly. If a calf lost weight, milk replacer was fed at the level appropriate to the weight before such loss.

Nitrogen retention

The percent nitrogen retained by calves kept in metabolism crates was monitored over the last 72 hrs of wks 3 and 6. Urine and feces were collected together in tubs placed below the metabolism crates, into which 25 ml of concentrated sulfuric acid had been placed. Collections were made over three consecutive 24 hr periods. The material collected was thoroughly mixed before a 1% subsample was taken and frozen. Subsamples from consecutive 24 hr periods were pooled to give the sample for one calf for that collection period. The nitrogen content of both feeds and excreta were measured using standard macro-kjeldahl procedures (3). Percent nitrogen retained by the calf was calculated as the nitrogen fed minus nitrogen in excreta expressed as a percentage of the nitrogen fed.

Xylose tolerance.

Following the nitrogen collection periods, calves in metabolism crates uderwent xylose absorption studies. D(+)-xylose does not normally occur in any significant amount in blood and urine, is not degraded in the intestine and is apparently absorbed by diffusion (87). Following oral ingestion of xylose the observation of a low level of xylose in the blood and urine is considered indicative of a defect in the absorptive function of the small intestine (87). The method involves omission of an afternoon and

morning feeding before oral administration of 0.5 g D(+)-xylose/Kg body weight as a 10% solution. Jugular blood was sampled before and hourly for 5 hr after xylose administration. Blood samples were refrigerated overnight before serum was collected and frozen until analyzed for xylose content. Urine was collected during the 5 hrs following xylose administration and a quantitative sample frozen until analyzed for xylose content. Xylose content of samples were measured using the method of Henry (32).

Intestinal morphology

One of the symptoms of an allergic response to ingested items is atrophy of the intestinal villi. At the end of 6 wks, calves from the metabolism crates were euthanized and sections taken from the duodenun (10 cm distal to the pylorus), jejunum (220 cm distal to the pylorus) and middle of the small intestine. Samples were preserved in 10% buffered formalin before being viewed using light and scanning electron (SEM) microscopes, according to the methods of Seegraber and Morrill (76).

Serum antibodies to soy proteins

Blood samples were obtained from calves in hutches on the day a treatment was assigned and weekly thereafter. The serum was frozen until analyzed for anti-soy immunoglobulins. Following the recommendations of Pitts et al. (62) an enzyme linked immuno-sorbent assay (ELISA) method was used to find the levels of serum immunoglobulin G (IgG) specific to the

soybean proteins glycinin and ß-conglycinin. The immunogenic soy proteins were extracted and purified by the method of Thanh and Shibasaki (93).

The ELISA procedures used in this experiment were as given below.

a) The wells of microtitre plates[1] were used as the solid phase and coated with purified glycinin or ß-conglycinin, by incubating 200 µl/well of a solution of 100 µg protein/ml in carbonate buffer (pH 9.6) for 1 hr at 37°C.

b) Plates were washed 3 times, using phosphate buffered saline (PBS) containing 1% bovine serum albumen (BSA), to remove unbound soy protein.

c) The fluid from the last wash was left in the wells and incubated for 30 min. at 37°C (this was to allow the BSA to bind to and thereby block any areas of the solid phase not occupied by the soy protein antigens).

d) Serum samples from experimental calves were diluted 1:200 in PBS with 0.5% BSA and 0.1% tween 80 (solution W). After removing the blocking fluid, diluted serum was added (200 µl/well) to each of three antigen coated wells. Plates were incubated for 1 hr at 37°C, to allow serum antibodies specific to the soy protein to bind to the antigen.

e) Plates were washed 3 times with solution W, to remove unbound antibodies.

f) 200 µl/well of peroxidase labeled, affinity purified, goat

[1]Dynatech laboratories Inc. 900 Slaters Lane, Alexandria, Virginia.

antibovine IgG[2], at 1:2000 in solution W, was incubated for 1 hr at 37°C (the peroxidase labeled goat antisera reacts with the IgG from the test serum to form a solid phase-antigen-antibody-peroxidase labeled complex).

g) Plates were washed 3 times with solution W, to remove unbound goat serum.

h) 200 µl/well of peroxidase substrate [0.012% H_2O_2, 0.0001% 2-2-Azino-di(3-ethyl-benzthiazoline sulfonic acid) (ABTS) in 0.045% Citrate buffer, pH4] was added and incubated for 30 min. at 37°C (the enzyme and substrate react giving a colored end product).

i) The optical density of each well was measured[3] at 405 nm.

Optical density is positively correlated to anti-soy antibody concentration in the test serum.

A positive control (serum from a calf given intramuscular injections of glycinin and ß-conglycinin homogenized with Freund's complete adjuvent), a negative control (fetal calf serum), and a blank (PBS) were run with each plate.

[2]Kirkegard and Perry Laboratories Inc., 2 Cessna Court, Gaithersburg, MD 20879.

[3]Biotek EIA Reader, Model EL 307, Biotek Instruments Inc., Highland Park, Box 998, Winooski, VT 05404.

Cell-mediated immune response

Jugular blood collected in heparinized tubes from two calves per treatment was used within the hour following collection. 2 ml of blood mixed with 2 ml of phosphate-buffered saline (PBS) were layered on 4 ml of Histopaque-1077[4] containing ficoll and sodium diatrizoate, then centrifuged at 400 X g for 30 min. Lymphocytes sedimented at the interphase were collected with a pasteur pipette, washed twice with RPMI-1640[5] medium and resuspended in RPMI-1640 containing 25 mM Hepes buffer and 10% fetal calf serum (FCS). Cells were counted in a hemocytometer, and the suspension adjusted to contain $1X10^5$ cells/ml. The cell suspension lacking or containing various concentrations of glycinin or ß-conglycinin was distributed (200 µl/well) in quadruplicate in a flat bottom tissue culture plate. Cultures were incubated at 37°C in a humidified carbon dioxide incubator. Viability of lymphocytes was >95% during the course of the experiment as determined by trypan blue dye exclusion test. After 48 hr of incubation 0.2 µCi/culture tritiated thymidine[6] was added (specific activity 6.7 Ci/mM). Cultures were harvested 24 hr later on glass fiber filters in an automated cell counter[7]. Filters were counted in a liquid scintillation

[4]Sigma Chemical Co., St. Louis, MO 63178

[5]Grand Island Biological Co., Grand Island, NY 14072

[6]Schwarz/Mann, Dickinson and CO., Orangeburg, NY 10962.

[7]PHD Cell harvesting system, Cambridge Technology, Cambridge, MA.

counter[8] to determine the incorporation of labeled thymidine in lymphocytes. Lymphocyte stimulation indices (LSI) were calculated as: LSI = Disintegrations per minute (DPM) of stimulated lymphocyte cultures/DMP of control cultures.

Cutaneous immune response

At 6 wks, after the final serum sample for ELISA had been taken, all calves were given intradermal injections of purified proteins. Calves were prepared by shaving the hair from an area of white skin and 1 mg of purified protein was injected as a 2 mg/ml emulsion. Proteins injected were glycinin, ß-conglycinin, bovine serum albumen and whey, each at a separate site. The first two are the soybean proteins shown to elicit an allergic response in the calf, while the latter two were negative controls. The sites of injection were observed for swelling, frequently during the first hour, hourly to 6 hrs and once the following morning.

Statistical analysis

Data were analyzed using statistical analysis system's (SAS) general linear models (GLM) procedures (72), where the four diets were the treatments. Treatments were blocked by time started on experiment and with birth weight as a covariate.

[8]LS 6800 Model, Beckman Instruments Inc., Fullerton, CA 92634.

RESULTS AND DISCUSSION

Calf growth.

Mean weekly weights for the 4 treatment groups are in Figure 1. Calves given diet AM gained more weight in the first 3 wks than calves on the soy diets. This early weight advantage was maintained to the end of the experimental period at 6 wks. Among the soy diets, the SPC group gained weight during the first 2 wks, while the ESF and CSF groups lost weight. In the third week the ESF group did show compensatory growth sufficient to equal and continue to equal the SPC group. Calves are particularly susceptible to stress during the first few weeks. If the inferior response in the first 2 wks of calves on ESF, compared to SPC, proved to be consistent, it would be grounds for choosing SPC over ESF. In weeks 4, 5 and 6 the rates of gain for the AM, SPC and ESF groups were similar, though the actual weights of the AM group remained higher. Though the CSF group gained weight in the last 4 wks it did so at a rate slightly lower than the other groups.

Nitrogen retention.

Nitrogen retention by calves as a percent of nitrogen fed is shown in Table 3. Calves fed diets SPC and ESF had superior nitrogen retention to calves on the CSF diet, but inferior nitrogen retention to the AM calves; this was true at both 3 and 6 wks. All 4 treatment groups showed

Figure 1. Growth of calves fed milk replacers with all milk (AM) protein, or with 70% of the protein from soy protein concentrate (SPC); commercial heated soy flour (CSF); or an experimental heated soy flour (ESF). Values are the mean for five calves per treatment, different superscripts denote significant differences ($P<.05$) within a week.

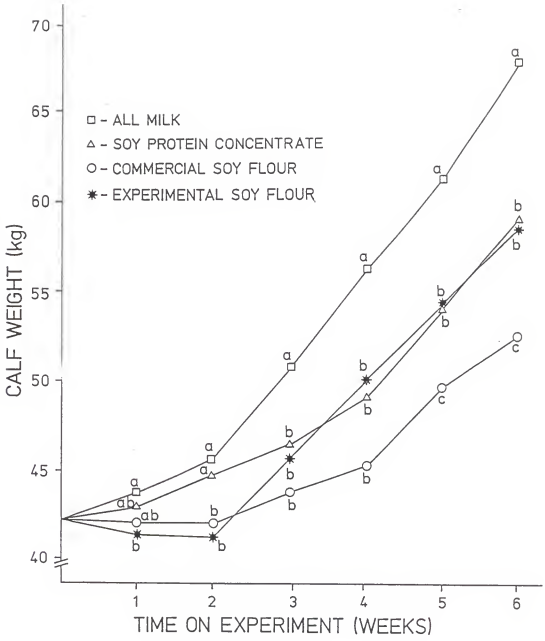

□ - ALL MILK
△ - SOY PROTEIN CONCENTRATE
○ - COMMERCIAL SOY FLOUR
✱ - EXPERIMENTAL SOY FLOUR
CALF WEIGHT (kg)
70
65
60
55
50
45
40
1
2
3
4
5
6
TIME ON EXPERIMENT (WEEKS)

TABLE 3. Nitrogen retained by calves at 3 and 6 weeks of age.

Milk replacer	% Dietary nitrogen retained[a]	
	3 weeks	6 weeks
AM	47.0 ± .2	61.5 ± 2.3
SPC	30.3 ± 1.4	51.1 ± 2.1
CSF	22.5 ± [b]	38.2 ± 8.1
ESF	33.0 ± 11.1	46.5 ± 1.5

[a] Mean of two calves ± SE.

[b] Data from one calf only.

improved dietary nitrogen retention when calves were 6 wks old, compared to 3 wks old, this is thought to reflect maturation of the digestive system. Calves receiving the all milk protein milk replacer (AM) showed relatively small improvement in nitrogen retained with aging. The small improvement with diet AM was due to the initial nitrogen retention being relatively high, leaving little room for improvement. In calves receiving a soy-based diet, development of a gastro-intestinal immune response acted against improvements in digestibility due to the maturing digestive system. One of the symptoms of a food allergy is reduced absorption of nutrients. Calves on diets SPC and ESF showed greater improvement in nitrogen retained with age than calves on either diet AM or CSF. The relatively small

improvement in nitrogen retained by calves on the CSF diet may have been associated with a more severe immune reaction.

Serum antibodies to soy proteins.

The mean serum IgG levels specific to the soy proteins glycinin and ß-conglycinin are illustrated in Figures 2 and 3, respectively. There was a marked elevation in soy directed antibodies in all soy-fed groups by the third week on trial. Elevated serum antibodies specific to a protein is a strong indication of an allergic response. The AM group maintained their anti-soy antibodies at a level similar to that in the initial pre-trial serum samples. There were statistically significant differences in the antibody response between soy-fed groups. The differences between soy diets are not consistent between the two antigenic proteins and may not represent biologically significant differences in the calves response. It is possible that the different processing given the soy products resulted in different activities of the two antigens.

Cell-mediated immune response.

We did not detect a cell-mediated immune reaction to either glycinin or ß-conglycinin, as determined by in vitro lymphocyte stimulation. This method is relatively sensitive to mitogenic activity, suggesting that there was no significant cell-mediated immune response in the calves tested. It remains possible that a cell-mediated immune response may occur in some cases, but it seems not to be a general response.

Figure 2. Serum antibodies (IgG) to the soy protein glycinin in calves fed milk replacers with all milk (AM) protein, or with 75% of the protein from soy protein concentrate (SPC); commercial heated soy flour (CSF); or an experimental heated soy flour (ESF). Values are the mean for five calves per treatment, different superscripts denote significant differences ($P<.05$) within a week.

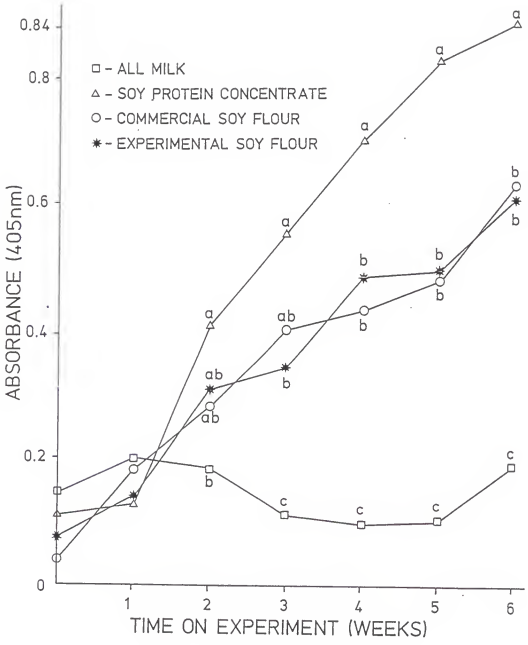
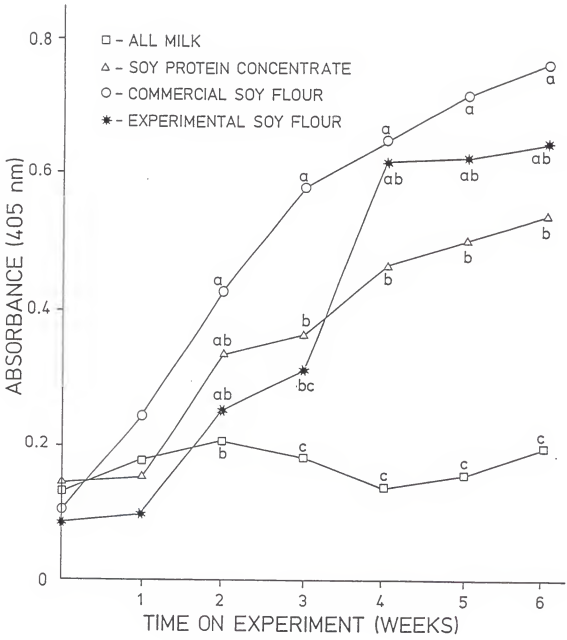

0.84
0.8
0.6
0.4
0.2
0
ABSORBANCE (405nm)
□ - ALL MILK
△ - SOY PROTEIN CONCENTRATE
○ - COMMERCIAL SOY FLOUR
✱ - EXPERIMENTAL SOY FLOUR
1
2
3
4
5
6
TIME ON EXPERIMENT (WEEKS)

Figure 3. Serum antibodies (IgG) to the soy protein ß-conglycinin in calves fed milk replacers with all milk (AM) protein, or with 75% of the protein from soy protein concentrate (SPC); commercial heated soy flour (CSF); or an experimental heated soy flour (ESF). Values are the mean for five calves per treatment, different superscripts denote significant differences ($P<.05$) within a week.


□ - ALL MILK
△ - SOY PROTEIN CONCENTRATE
○ - COMMERCIAL SOY FLOUR
✱ - EXPERIMENTAL SOY FLOUR
ABSORBANCE (405 nm)
0
0.2
0.4
0.6
0.8
1
2
3
4
5
6
TIME ON EXPERIMENT (WEEKS)

Cutaneous immune response.

The cutaneous injection of purified proteins failed to induce any detectable immune response, with one exception. One calf on diet ESF developed severe scours and lost weight during its late second and early third week on trial. Because of its weak condition the calf was removed from the experiment after 17 days. This calf was subsequently given diet AM and rapidly improved in condition. One week after being taken off experiment it was given cutaneous injections of glycinin and ß-conglycinin in the same manner as the other calves. By 4 hrs after the injections a clear swelling had developed in response to both glycinin and ß-conglycinin. The swelling was indicative of IgG mediated, type III hypersensitivity. That there was a systemic IgG response to the soy diets is indicated by the serum ELISA results. It seems that this cutaneous test has insufficient sensitivity to detect this IgG mediated response in most cases. It is possible that there was some additional component to the immune response in this one calf that caused its exceptional severity. Severe responses to soy proteins by a minority of calves have been reported by others (7,67). It is not uncommon to have an immune reaction confined to either the humoral or cell-mediated immune system. It appears that the calf, unlike the mouse and piglet, does not have a cell-mediated component to this dietary intolerance.

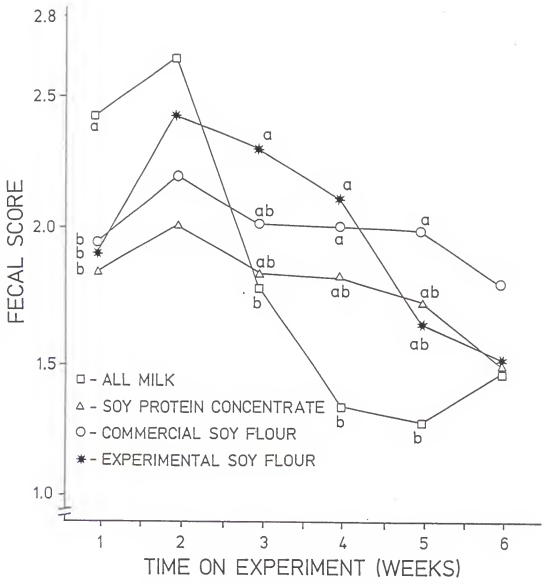
Intestinal morphology.

Observations of the small intestinal mucosa indicated some villous atrophy in calves receiving diets containing soy protein. Villous atrophy was not as severe as noted in some studies (76,80) and there were no marked differences between the three soy diets. No detailed microscopic analysis of the tissues was done and there may have been minor differences between the soy groups. Calves receiving diet AM had comparatively normal villi.

Fecal consistency.

The mean weekly fecal scores are in Figure 4. The AM group had the softest feces for the first 2 wks and the driest for the last 4 wks. It was unexpected for the AM fed group to have looser feces at any time. It may be that the higher level of whey minerals in the AM diet (diet composition Table 2) resulted in the soft feces. The reduction in fecal score by week 3 may reflect adaptation to these levels of whey. Work by Bush et al. (11) indicates calves adapt to high levels of whey in their diet within 2 wks. All treatment groups showed an increase in fecal score from the first to second week. This was due mainly to the low fecal score in the early part of the first week as calves excreted their colostral feces. From the second week on to the end of the experimental period all treatment groups showed a decrease in fecal score, presumably due to adaptation to the feeding regime and maturation of the intestine. The CSF group showed the least improvement in fecal score; this may reflect a more severe effect of this diet on calves.

Figure 4. Fecal score (1=normal, 4=watery) of calves fed milk replacers with all milk protein (A M), or with 75% of the protein from soy protein concentrate (SPC); commercial heated soy flour (CSF); or an experimental heated soy flour (ESF). Values are the mean of five calves per treatment, different superscripts denote significant differences (P<.05) within a week.


2.8
2.5
2.0
1.5
1.0
FECAL SCORE
□ - ALL MILK
△ - SOY PROTEIN CONCENTRATE
○ - COMMERCIAL SOY FLOUR
✱ - EXPERIMENTAL SOY FLOUR
1
2
3
4
5
6
TIME ON EXPERIMENT (WEEKS)

Xylose tolerance.

The percent urinary xylose clearance by calves over 5 hrs following administration are in Table 4. Serum xylose concentrations for the 5 hrs following oral xylose administration are illustrated in Figures 5 and 6 for wks 3 and 6, respectively. We expect absorptive capacity to be positively correlated with calf performance. Based on other results in this trial the AM group was expected to have the greater absorptive capacity and the CSF group the least. In fact, the SPC fed calves had significantly higher blood xylose concentrations at both 3 and 6 wks, with no difference among the other three treatment groups. There were no significant differences in urinary xylose concentrations. These results suggest the SPC group had

TABLE 4. Percent of oral xylose dose excreted in urine in five hours.

Milk Replacer	Urinary xylose: % of dose[a]	
	3 weeks	6 weeks
AM	10.8 ± 1.7	17.2 ± 7.6
SPC	5.8 ± 2.0	15.6 ± 4.9
CSF	7.2 ± 3.4	8.9 ± 1.1
ESF	8.4 ± 1.3	9.8 ± 3.9

[a]Mean of two calves ± SE.

Figure 5. Serum xylose concentration following oral administration of .5g xylose/Kg body weight in 3 wk old calves fed milk replacers with all milk (AM) protein, or with 75% of the protein from soy protein concentrate (SPC); commercial heated soy flour (CSF); or an experimental heated soy flour (ESF). Values are the mean of two calves per treatment, different superscripts denote significant differences ($P<.05$) at a sample period.

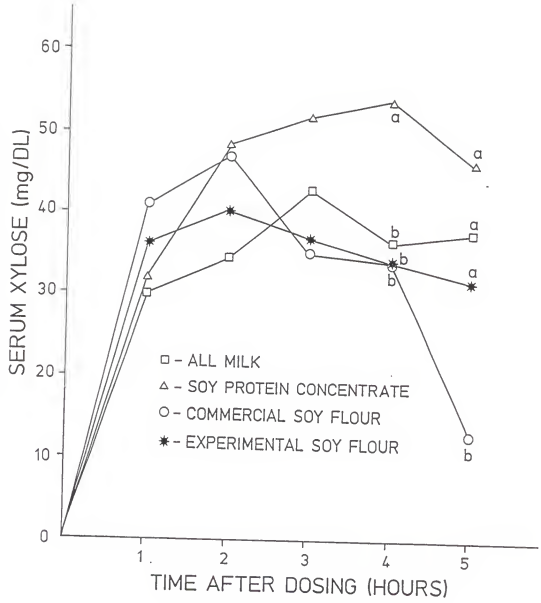
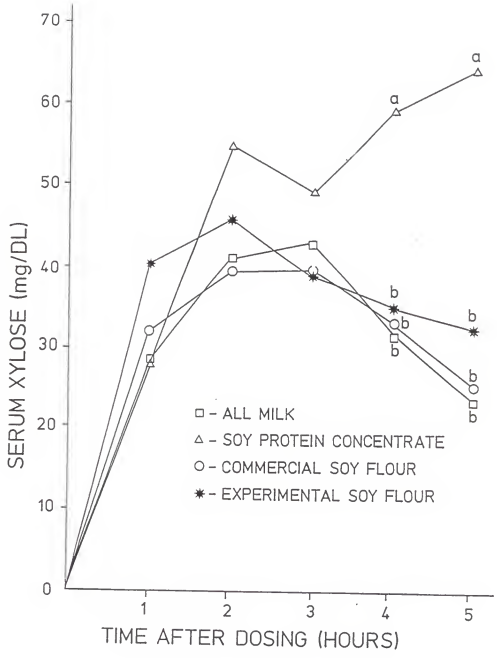

SERUM XYLOSE (mg/DL)
60
50
40
30
20
10
0
1
2
3
4
5
TIME AFTER DOSING (HOURS)
□ - ALL MILK
△ - SOY PROTEIN CONCENTRATE
○ - COMMERCIAL SOY FLOUR
✱ - EXPERIMENTAL SOY FLOUR
a
a
b
a
b
a
b
b

Figure 6. Serum xylose concentration following oral administration of .5g xylose/Kg body weight in 6 wk old calves fed milk replacers with all milk (AM) protein, or with 75% of the protein from soy protein concentrate (SPC); commercial heated soy flour (CSF); or an experimental heated soy flour (ESF). Values are the mean of two calves per treatment, different superscripts denote significant differences ($P<.05$) at a sample period.


70
60
50
40
30
20
10
0
SERUM XYLOSE (mg/DL)
1
2
3
4
5
TIME AFTER DOSING (HOURS)
□ - ALL MILK
△ - SOY PROTEIN CONCENTRATE
○ - COMMERCIAL SOY FLOUR
✱ - EXPERIMENTAL SOY FLOUR
a
a
b
b
b
b
b
b

superior absorptive capacity to the other groups and is contrary to other parameters of calf performance we measured. No satisfactory reason was found for this, though it is noted that the xylose absorption method has been found unreliable in detecting food allergies (32).

SUMMARY

In this experiment, utilization of milk replacers improved as calves aged, particularly over the first three weeks. This phenomenon is compatible with the theory that a neonatal calf has an immature, but developing digestive system. The improvement in performance on the soy-based diets was greater than that seen on the milk-based diet, mostly because the soy based diets were notably inferior for calves <3 wks of age. Performance of calves on the all milk protein was superior to that on soy protein concentrate and experimental soy flour which in turn were superior to the commercial heated soy flour. All soy-fed groups had a humoral, but no cell-mediated immune reaction to soy proteins. An alternative method of processing has to be found before soy can be used as the major protein in a quality milk replacer for young calves.

EXPERIMENT 2.

THERMOPLASTIC EXTRUSION PROCESSING OF SOY FLOUR FOR USE AS A PROTEIN SOURCE IN MILK REPLACER FOR CALVES.

ABSTRACT

Enzyme active soy flour was used as the starting material for thermoplastic extrusion processing. Five processing conditions were varied in a 5 X 2 factorial arrangement, resulting in 32 products. All extrusion products had >90% reduction in trypsin inhibitor (TI) activity. The extrusion products had a wide range of antigenic (glycinin and ß-conglycinin) activities; these were not closely related. Only one product had no detectable activity for both glycinin and ß-conglycinin. This "antigen free" extrusion product had very low TI activity (1 unit/mg) and was used as the major protein source (70%) in three experimental milk replacers. The effects of amino acid supplementation and acidification of milk replacers based on the extruded soy were also studied. Metabolic and growth response of calves were used to compare the experimental milk replacers with an all milk protein control in an in vivo study. The extruded soy was inferior to milk as a protein source for milk replacers. AA supplementation of soy was detrimental to calf performance. Acidification of soy based milk replacer had some benefit in the young (<3 wks old) calf, but was not beneficial later.

INTRODUCTION

There have been many studies to find an inexpensive yet high quality alternative to milk as a protein source in liquid calf feed. To date such studies have been partially successful, manufactures being able to replace a minor portion of the total protein.

There has been considerable research into the use of thermoplastic extrusion as a means of texturing soy proteins for human consumption. Extrusion can alter the physical and biochemical properties of a protein without detriment to its nutritional value. Human studies have concentrated on physical modification of the soy proteins to provide meat analogues. The calf, however, requires modification of the biochemical properties of soy proteins to inactivate antinutritional factors. Because of the different requirements of the end product, much of the work on soy extrusion to date is of limited value to the calf. One area where human orientated extrusion research might be of use would be in identifying processing variables having a marked effect on the end product.

The aim of this study was to examine the effects of thermoplastic extrusion on the suitability of soy flour as a protein source for calf milk replacer. Trypsin inhibitor and antigenic glycinin and ß-conglycinin were monitored in extrusion products. The extrusion product with the lowest activity of these antinutritional factors was tested as the major protein in three experimental milk replacers. Calf health, growth and nitrogen

retention were measured. Also, the effects of amino acid supplementation and acidification of the soy dist were evaluated.

MATERIALS AND METHODS

Extrusion processing

Enzyme active soy flour[9] was chosen as the raw material for extrusion processing. This product is oil-extracted, desolventized soy flour, without additional heat treatment. A Wenger X-20 Extruder[10] was used throughout the experiment. Processing parameters changed were temperature, moisture, ionic concentration, pH and sulfur concentration. There were 32 treatments produced by arranging the processing variables in a 5 X 2 factorial arrangement. To minimize the number of barrel temperature changes (a relatively slow process to stabilize) extrusion processing was carried out over two consecutive days; high temperature combinations on one day and low temperature combinations the next. Barrel temperature was maintained at a relatively high or low temperature, within the range at which a stable product could be obtained, approximately 140°C and 80°C, respectively (temperature measured at the seventh/last head). Similarly, the quantity of steam added to the soy flour in the barrel was changed from a large amount to a small amount, within the range at which a stable product

[9]Industrial Grain Products, Saint Joseph, Mo.

[10]Wenger Mixer MFG., Sabetha, Kansas.

could be obtained, approximately 25% and 19% moisture, respectively (moisture of product at the die). Ionic concentration was changed in half the treatments by the addition of 2% $CaCl \cdot 2H_2O$. Calcium chloride was chosen as the modifier of ionic concentration because of its subsequent potential as a calcium source for the calf. Calcium chloride was added at 2% because this concentration had been shown to be optimal in some textural studies (personal communication, J. Huber, Wenger MFG, Sabetha, Kansas). The pH of the soy flour was reduced in half the treatments by the addition of 3% fumaric acid. An organic acid was chosen because of its potential nutritional value to the calf. Fumaric acid was chosen from among the three most common organic acids, because it was less expensive, it is a stronger acid than citric and lacks the unpleasant odor of butryic. We added 3% fumaric acid, this being the amount needed to bring a solution of the soy flour to approximately pH 4.2, the isoelectric point of soy protein. The presence of organic sulfur is thought to affect the secondary structure of the extruded proteins by the formation of disulfide bonds. Thus, 0.19% organic sulfur was added to half the samples before extrusion. This concentration of sulfur had been shown to be optimal in some textural studies (personal communication, J. Huber, Wenger MFG, Sabetha, Kansas). For each set of extrusion conditions as soon as the product at the die appeared stable a sample was taken to determine dry matter at the die. A second sample was air dried and used to find dry matter, TI and antigen activities.

On the basis of the in vitro tests, one set of processing conditions was chosen to produce the material for the in vivo test. Sufficient material was produced to provide 70% of the protein in three soy-based experimental milk replacers. Before being used in the diets this second extrusion product was tested in vitro for antinutritional factors.

Antinutritional factors

Trypsin inhibitor. TI activities of the 32 products were tested by the method of Ramsey & Willard (67).

Antigens. The activities of the recognized antigens (glycinin and ß-conglycinin) were measured in the 32 products, using the procedures below.

The immunogenic soy proteins were extracted and purified by the method of Thanh and Shibasaki (94). Using these purified proteins, antisera were developed in rabbits. Four New Zealand White rabbits, 8-10 weeks of age, were injected intramuscularly, or into the foot pad, with 2 mg solutions of either glycinin or ß-conglycinin homogenized with Freund's complete adjuvant. Three of these injections were given at 2 wk intervals. A week after the final injection, approximately 50 ml of blood was collected from the ear vein of each rabbit and the serum frozen until purified. Rabbit serum proteins were purified by ammonium sulfate precipitation, by the method of Garvey et al. (25). Approximately half of the purified antisera were frozen until used in the ELISA assay. The

remaining antisera were conjugated to horse radish peroxidase by the method of Nakane and Kawaoi (53) and frozen until used in the ELISA assay. Extracts of the experimentally processed soy flours were prepared by extracting 3.0 g of each ground extruded product with 25 ml 0.9% NaCl for 1 hr, at pH 6.7. Soluble proteins were recovered by centrifugation.

Enzyme linked immuno-sorbent assay (ELISA) procedure a) The wells of microtitre plates[11] were used as the solid phase and coated with purified rabbit antisera to glycinin or ß-conglycinin. Plates were coated by incubating 200 µl/well of antisera, diluted 1:50 for glycinin or 1:100 for ß-conglycinin in carbonate buffer (pH 9.6) overnight at 4°C.

b) Plates were washed 3 times, using phosphate buffered saline (PBS) with 0.1% Tween 20 (solution Y), to remove unbound antisera.

c) The soy extracts to be tested were diluted to 1:25 for glycinin and 1:50 for ß-conglycinin, in solution Y. Diluted extract (200 µl/well) was added to the antisera coated wells, 3 wells per sample. Plates were incubated overnight at 4°C, allowing the soy antigens to complex with the appropriate bound antibody.

d) Plates were washed 3 times with solution Y, to remove unbound soy protein.

e) Peroxidase labeled rabbit antiglycinin or antiß-conglycinin

[11]Dynateck Laboratories Inc., 900 Slaters Lane, Alexandria, Virginia.

antibody was added (200 µl/well) as appropriate, diluted 1:100 in solution Y and incubated for 1 hr at 37°C.

f) Plates were washed 3 times with solution Y, to remove unbound conjugate.

g) Peroxidase substrate [0.012% H_2O_2, 0.0001% 2-2-Azino-di(3-ethyl-benzthiazoline sulfonic acid) (ABTS) in 0.045% Citrate buffer, pH4] was added (200 µl/well) and incubated for 30 min. at 37°C (the enzyme and substrate react giving a colored end product).

h) The optical density of each well was measured at 405nm[12]. Optical density is positively correlated to soy antigen activity in the test extract.

A positive control (extract from enzyme active soy flour), a negative control (whey protein extract), and a blank (PBS) were run with each plate.

Experimental milk replacers

On the basis of the TI and antigen assays, one of the 32 extruded products was chosen as a protein source for three experimental milk replacers, for use in an in vivo study. The extrusion product chosen was processed with high moisture, high temperature, high ionic concentration, high sulfur concentration and low pH. The antigenic activities of glycinin and ß-conglycinin were zero and TI activity was 1 unit/mg.

Four milk replacers were prepared differing as follows: MR1, a

[12]Biotek EIA reader, Model EL 307, Biotek Instruments Inc, Highland Park, Box 998, Winooski, VT 05404.

positive control with all milk protein; MR2, 30% of the total protein from milk and 70% from the extruded soy flour; MR3, similar to MR2, except 1.4% of the protein was supplied by methionine (0.7%) and lysine (0.7%) rather than milk protein; MR4, similar to MR3, except for the addition of 1% citric acid. The milk replacers were formulated to have 22% protein (N X 6.25), 20% lipids, 0.95% calcium, 15909 IU/kg vitamin A, 3409 IU/kg vitamin D_3, and 9.09 IU/kg vitamin E. Ingredient composition of the milk replacers are shown in Table 5.

In vivo study

Management of calves. Eight Holstein bull calves, 2 per treatment, housed in metabolism crates, were used to monitor nitrogen balance at 2, 4 and 6 wks. All calves received colostrum for 3 days after birth. Treatments were assigned calves by randomized block, a block being the four treatment diets. The appropriate milk replacer was fed warm, by pail, half in the morning, half in the afternoon. The amount of milk replacer given was 1.25 kg DM/100 kg body weight, for the first 2 wks and 1.75 kg DM/100 kg body weight thereafter. Milk replacer was given at 12.5% DM the first week, 13.5% DM the second week, 16% DM for weeks 3 and 4, and 18% DM for weeks 5 and 6. The four increases in dry matter content of milk replacers as fed over time were to avoid either large rapid changes in dry matter concentration or excessively large volumes of milk replacer as the calves gained weight. No dry feed was given, water was available ad libitum, and the amount of milk replacer fed was adjusted weekly. If a calf

TABLE 5. Milk replacers: Ingredient composition.

INGREDIENT (%)	MILK REPLACER			
	MR1	MR2	MR3	MR4
Spray dried whey	12.5	-	-	-
Whey protein concentrate	49.0	-	-	-
Extrusion product 16[a]	-	32.5	32.5	32.5
Na Caseinate	-	3.2	1.4	1.4
Skim milk	5.0	5.0	5.0	5.0
L-Lysine	-	-	.7	-
DL-Methionine	-	-	.7	-
Lactose	-	25.8	26.2	25.2
7/60 Fat[b]	31.5	31.5	31.5	31.5
Limestone	.5	1.0	1.0	1.0
Dicalcium Phosphate	1.0	.5	.5	.5
Mineral/vitamin premix	.5	.5	.5	.5

[a]Extruded at high temperature and moisture, with high salt and sulfur, and at low pH (see table 6).

[b]7/60 A product containing 7% protein and 60% fat.

lost weight, milk replacer was fed at the level appropriate to the weight before such loss. Calves had no bedding and were housed in a temperature-controlled room.

Each calf was twice daily scored for general appearance (good, fair or poor) and consistency of feces (1=normal, 4=watery)(46). No treatment for scours was given during the trial.

Nitrogen retention

Nitrogen balance studies were carried out at the end of 2,4 and 6 weeks on trial. If for any reason a calf refused part of its milk replacer

during or immediately before a nitrogen balance period then that period was abandoned and an alternate nitrogen balance study was inserted the following week. Excreted material was collected and analyzed as described in Experiment 1.

RESULTS AND DISCUSSION

Extrusion processing

The 32 extrusion treatments are shown in Table 6. Some additional processing data (rate of flow at the die and volume change) are given in the Appendix. Some general trends were noted among treatments. It was easier to obtain a stable extrusion product from those treatments with high sulfur and they tended to form a light puffy product. There was a slight sulfurous odor/flavor associated with the extrusion of sulfur containing treatments, becoming less noticeable on cooling. Addition of acid gave extrusion products with a tangy taste.

Trypsin inhibitor

The enzyme active soy flour had a TI activity of 147.5 units/mg. The levels of TI in the 32 extrusion products are shown in Table 6. All extrusion treatments reduced TI activity >90% and several treatments eliminated detectable TI activity. There were four extrusion products which retained TI activities between 7 and 13 TI units/mg. This compares with 7-8 TI

TABLE 6. Processing conditions and antinutrients of extrusion products

	PROCESSING CONDITIONS					ANTINUTRIENTS		
Product #	Acid 3%[a]	Temp. °C[b]	Salt 2%[c]	Moist. %[d]	Sulfur 0.19%	TI[e]	Glycinin A[f]	ß-Conglycinin A[f]
1	-	150	-	26.66	-	-	.033	.060
2	-	150	-	17.48	-	-	.087	.017
3	+	140	-	26.58	-	7.1	.117	.041
4	+	140	-	18.07	-	3.2	.098	.021
5	-	140	+	24.76	-	-	.199	.038
6	-	140	+	20.82	-	.6	.090	.012
7	+	150	+	24.76	-	11.8	.152	.050
8	+	130	+	23.76	-	6.9	.096	.026
9	-	125	-	26.40	+	-	.085	.058
10	-	110	-	19.85	+	.3	.066	.010
11	+	125	-	24.73	+	.5	.039	.013
12	+	118	-	19.48	+	.6	.059	.038
13	-	123	+	25.35	+	.2	.023	.063
14	-	115	+	19.12	+	.4	.068	.025
15	+	135	+	26.45	+	-	.000	.026
16	+	118	+	20.97	+	1.0	.000	.000
17	-	75	-	25.57	-	1.5	.042	.032
18	-	80	-	23.75	-	1.4	.061	.064
19	+	98	-	24.26	-	3.7	.127	.129
20	+	90	-	25.80	-	12.2	.171	.018
21	-	80	+	23.14	-	.3	.012	.000
22	-	95	+	21.31	-	1.2	.056	.128
23	+	115	+	22.67	-	5.1	.088	.021
24	+	120	+	18.05	-	2.8	.072	.000
25	-	75	-	26.28	+	-	.035	.040
26	-	85	-	19.68	+	-	.031	.042
27	+	75	-	24.84	+	-	.034	.010
28	+	100	-	20.57	+	1.9	.003	.036
29	-	75	+	25.29	+	2.3	.022	.029
30	-	90	+	20.69	+	1.1	.032	.042
31	+	80	+	24.19	+	1.2	.046	.006
32	+	90	+	21.83	+	.6	.043	.070

[a] 3% Fumaric acid.
[b] Temperature at the seventh/last extruder barrel head.
[c] 2% Calcium chloride.
[d] Moisture at the die.
[e] Units of trypsin inhibitor activity per mg.
[f] Absorption at 405nm, being proportional to antigenic activity.

units/mg commonly found in commercial heated soy flours. It seems that extrusion processing is capable of adequately reducing TI activity.

Antigens

The enzyme active soy flour had antigenic glycinin activity of 0.234 and antigenic ß-conglycinin activity of 0.233, as measured by ELISA. The ELISA results for the 32 extrusion products are shown in Table 6. The activity of the recognized antigens (glycinin and ß-conglycinin) remained relatively high in some extrusion products. Most of the extrusion products had markedly reduced antigenic activity of one or both of the antigens. Three treatments gave extrusion products with no detectable activity for one of the two antigens. Extrusion at high temperature, high moisture, high salt, high sulfur and low pH gave a product with no detectable antigenic activity for either antigen. This latter extrusion product had a low TI activity (1 TI unit/mg) and was chosen as the protein source for an in vivo study. The "antigen free" extrusion product was produced on a second occasion using the same set of processing conditions. However, it is noted that the first attempt at reproducing the "antigen free" extrusion product failed. A possible reason for this failure lies in the mixing of the acid, salt, sulfur and soy flour approximately 1 month prior to extrusion. It was found that mixtures which contained acid developed a gritty texture on storage, it may be that this change in some way affected the extrusion properties. Thus, it is recommended that only freshly mixed material be used in this type of extrusion processing. It should be noted that though extrusion

appears to have inactivated the known antigens it remains possible for there to be residual antigens not detected by the assay used. Also it is probable that modification of the proteins has produced new antigens; these may themselves be allergenic to the calf.

It seems that extrusion processing can inactivate the known soy antigens, but that the processing conditions required are quite specific. Further study would identify the effective range and optimal processing conditions. Also the desirability of using sulfur in extrusion processing has been questioned as its "possible" mode of action (fusion of the globular soy proteins) may decrease protein digestibility. An alternative to sulfur may be cystein, with its tendency to "open" globular proteins.

Calf health

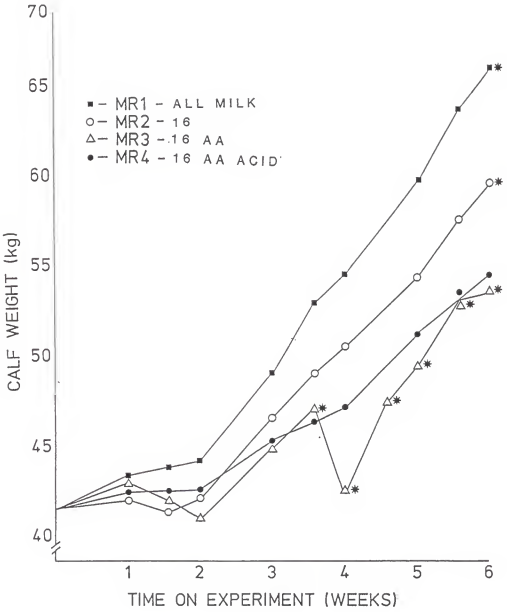
There were several problems with calf health during the in vivo trial. In the first treatment block the calf given MR3 developed a swollen right knee. This calf suffered some discomfort and refused to drink milk replacer on several occasions. During the second treatment block there was an outbreak of infectious scours, some calves developed a more severe infection than others, with the youngest calf (MR4) most severely affected. Also in the second treatment block one apparently healthy calf on MR3 died after 3 wks; necropsy examination failed to determine the cause of death. There was some refusal of soy based diets, though with the exception of the calf with the swollen knee these were minimal. Several soy-fed calves had considerable hair loss, while all soy-fed calves had some

browning of the hair coat. The milk fed calves developed shiny coats without hair loss.

Calf growth

The growth of calves on the four milk replacers is shown in Figure 7. Young calves fed milk replacer frequently have a quadratic growth pattern, such was the case in this experiment. In the early phase (2-3 wks) calves have an immature digestive system, small energy reserve and protein pool, and are vulnerable to infection. The response of calves to milk replacer in the first 2-3 wks is therefore of great importance. During the early phase in this experiment (the first 2 wks) the MR1 group gained the most weight, the MR2 and MR4 groups had small weight gains, while the MR3 group had a small weight loss. In the later phase (early phase to 6 wks) calves appear to maximize their usage of a milk replacer, presumably due to improved gut function. In this phase, performance differences between milk replacers tend to be less pronounced, but are particularly important to operations involving prolonged periods of liquid nutrition, i.e., veal producers. In the second phase the MR1 group had a slightly higher growth rate than MR2, this in turn being higher than the MR3 and MR4 groups. The fact that calves on MR3 had an inferior growth rate to calves on MR2 suggests that supplemental lysine and methionine had a detrimental effect on calf performance. A detrimental response to supplemental amino acids is not unique and may be due to an amino acid imbalance (5). Calf growth on

Figure 7. Growth of calves fed milk replacer with all milk protein (MR1), or with 70% of the protein from an extruded soy flour (MR2); extruded soy flour with supplemental amino acids (MR3); extruded soy flour, acidified and with supplemental amino acids (MR4). Values are the mean of two calves per treatment.


70
65
60
55
50
45
40
CALF WEIGHT (kg)
■ – MR1 – ALL MILK
○ – MR2 – 16
△ – MR3 – 16 AA
● – MR4 – 16 AA ACID
1
2
3
4
5
6
TIME ON EXPERIMENT (WEEKS)

MR3 and MR4 was similar, except during the second week. At this time the MR4 group was able to maintain its weight while both MR2 and MR3 groups suffered a weight loss.

Fecal scores

Treatment means of the twice daily fecal scores are shown in Figure 8. Because of the variability of the data and the small number of calves involved no clear treatment differences could be seen. One observation of note was an apparent reversal of the trend to an increase in fecal score in the second week by the MR4 group, possibly associated with the selective bacteriostatic effect of the lower pH.

Nitrogen balance

Nitrogen retention by calves at 2, 4 and 6 wks is shown in Table 7. There were five occasions when a nitrogen balance could not be obtained at the designated period (2, 4 or 6 wks). This was usually due to a calf refusing part of its feed, either immediately before or during the nitrogen balance period, disturbing the approximate steady state needed for this test. Where possible, nitrogen retention was monitored the week following the loss of a planned period. The first nitrogen balance period (2 wks) for the second block of treatments coincided with an outbreak of infectious scours. The three calves monitored at this time had a marked decrease in nitrogen retained compared to the first treatment block. Because of the

Figure 8. Fecal score (1=normal, 4=watery) of calves fed milk replacers with all milk protein (MR1), or with 70% of the protein from an extruded soy flour (MR2); extruded soy flour with supplemental amino acids (MR3); extruded soy flour, acidified and with supplemental amino acids (MR4). Values are the mean of two calves per treatment. *Data from one calf only.

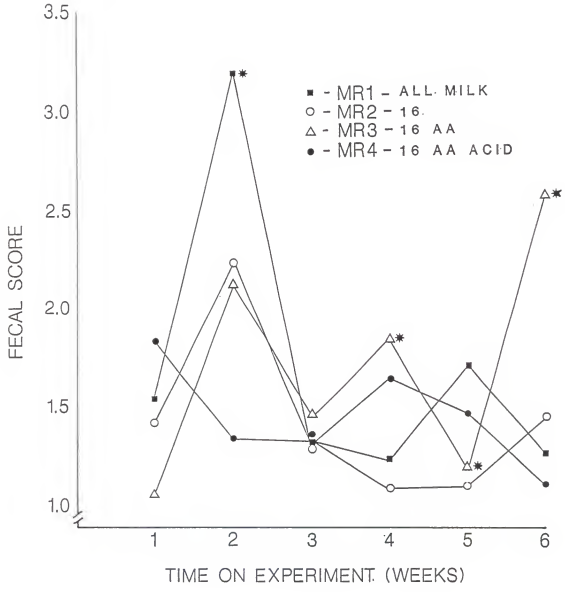

3.5
3.0
2.5
2.0
1.5
1.0
FECAL SCORE
■ - MR1 - ALL MILK
○ - MR2 - 16
△ - MR3 - 16 AA
● - MR4 - 16 AA ACID
1
2
3
4
5
6
TIME ON EXPERIMENT (WEEKS)

TABLE 7. Percent dietary nitrogen retained by calves fed milk replacer.

MILK REPLACER	TREATMENT BLOCK	WEEK 2	3	4	5	6
MR1	1	51.65	-	67.15	-	57.35
	2	20.65	-	66.14	67.63	-
	Mean	36.15	-	66.65	67.63*	57.35*
MR2	1	7.02	-	48.20	-	-
	2	-14.63	-	43.00	49.66	-
	Mean	-3.81	-	45.60	49.66*	-
MR3	1	0.90	-	-	52.46	51.16
	2	-	43.48	-	-	-
	Mean	-0.90*	43.84*	-	52.46*	51.16*
MR4	1	24.81	-	34.01	-	35.19
	2	6.09	-	49.52	-	50.53
	Mean	15.45	-	41.77	-	42.86

*One observation only.

problems experienced, little significance can be attached to the nitrogen balance data.

All treatment groups showed an increase in nitrogen retained after the second week. The all milk protein group (MR1) had superior nitrogen retention to the soy protein groups at all time periods. Among soy treatment groups MR4 had superior nitrogen retention at 2 wks. This is supportive of the fecal scores and growth of calves at this age in

suggesting an advantage to the very young calf from acidification of the milk replacer. Subsequent to 2 wks there was no major difference in nitrogen retention by calves fed soy based milk replacers.

SUMMARY

Thermoplastic extrusion was studied as a means of processing soy flour for use in milk replacer for calves. This method of processing generally reduced trypsin inhibitor to a low level. Extrusion of soy flour with sulfur, low pH, high salt, high moisture and at high temperature gave a product low in antimetabolites. Milk replacers based on this extruded soy failed to support calf performance equal to that on milk protein based milk replacer, particularly in calves <2 wks of age. Mild acidification of the soy milk replacer appeared beneficial to calves <2 weeks of age. Methionine and lysine supplementation of soy milk replacer was not of benefit and may have been detrimental to calf performance. The nitrogen retained by soy fed calves (in experiments 1 and 2) was lower than with milk fed calves, particularly for calves <3 weeks of age. This suggests that digestibility may be a problem with intensive use of soy proteins in milk replacers. Thermoplastic extrusion can modify soy flour to the extent of inactivating the recognized antimetabolites. Further work may develop a more digestible product, suitable for use with young calves. Also worth investigating is the potential benefit to the young calf from acidification of milk replacer.

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APPENDIX

TABLE I. Thermoplastic extrusion of soy flour - Processing parameters.

Extrusion Product	Flow at the die kg dry matter min^{-1}	Volume change* $m^3 kg^{-1}$
1	1.195	1.08
2	1.176	1.29
3	1.470	1.60
4	1.362	1.74
5	1.421	.98
6	1.263	1.05
7	1.481	2.06
8	1.420	1.67
9	1.424	3.03
10	1.388	5.13
11	1.499	2.37
12	1.385	2.98
13	1.343	2.84
14	1.390	3.80
15	1.323	1.76
16	1.198	2.38
17	1.336	1.13
18	1.137	1.22
19	1.199	1.00
20	1.185	.74
21	1.316	1.50
22	1.295	1.40
23	1.400	1.27
24	1.409	1.03
25	1.351	2.20
26	1.336	3.81
27	1.335	1.51
28	1.330	1.79
29	1.352	1.79
30	1.238	2.26
31	1.398	2.12
32	1.335	1.66

*Calculated as the volume (m^3) of the product per kg dry matter minus the volume (m^3) of raw material per kg dry matter.

SOYBEAN PRODUCTS AS A PROTEIN SOURCE IN MILK REPLACER FOR PRERUMINANT CALVES.

by

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An ABSTRACT OF A MASTER'S THESIS.

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Experiment 1. Metabolic, physiological and immunological parameters were monitored in preruminant calves fed one of four milk replacers. Protein sources for the milk replacers were all milk (AM) or 25% milk protein and 75% protein from either soy protein concentrate (SPC), commercial soy flour (CSF) or experimental soy flour (ESF). The experimental soy flour had trypsin inhibitor activity of <1 unit/mg. Using the nitrogen retained at 3 and 6 wks, the presence of abnormal intestinal mucosa, growth rates and the development of circulatory immunoglobulins specific to soy proteins, the three soy-based milk replacers were inferior to the AM milk replacer. The soy-based diets induced a marked antibody-mediated allergic response in the calves, with no major difference in this response between soy products. There was no cell-mediated immune response to any of the soy products. Calves showed improved performance with age, particularly on the soy-based diets. Milk replacers SPC and ESF gave comparable calf performance, both being superior to CSF.

Experiment 2. Enzyme active soy flour was used as the starting material for thermoplastic extrusion processing. Five processing conditions were varied in a 5 X 2 factorial design resulting in 32 products. All extrusion products had >90% reduction in trypsin inhibitor (TI) activity. The extrusion products had a wide range of antigenic (glycinin and ß-conglycinin) activities, these were not closely related. Only one product had no detectable activity for both glycinin and ß-conglycinin. This "antigen free" extrusion product had very low TI activity (1 unit/mg) and was used as the major protein source (70%) in three experimental milk replacers. The effects of amino acid supplementation and acidification of milk replacers based on the extruded soy were also studied. Metabolic and growth response

of calves were used to compare the experimental milk replacers with an all milk protein control. The extruded soy was inferior to milk as a protein source for milk replacers. AA supplementation of soy was detrimental to calf performance. Acidification of soy based milk replacer had some benefit in the young (<3 wks old) calf, but was not beneficial later.