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# Chitin: New Facets of Research

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J. E. Castle, J. P. Zikakis

*Summary.* Research on chitin as a marine resource is pointing to novel applications for this cellulose-like biopolymer. Discovery of nondegrading solvent systems has permitted the spinning of filaments, for example, for use as surgical sutures. New methods for preparing the bioactive alkyl glycosides of *N*-acetyl-D-glucosamine (the monomer unit of chitin) and a microcrystalline chitin have encouraged their use as promoters for growth of bifidobacteria and as an aid in digestion of high-lactose cheese whey by domestic animals. Chitin-protein complexes of several crustacean species show great variability in ratios of chitin to covalently bound protein and in residual protein in the "purified" chitins.

Chitin, poly-β-(1→4)-*N*-acetyl-D-glucosamine, is a cellulose-like biopolymer distributed widely in nature, especially in marine invertebrates, insects, fungi, and yeasts. Both chitin and chitosan (deacetylated chitin) are being processed industrially in million-kilogram quantities in Japan, but in much lesser amounts in this country (*1*). Chitin is a source of glucosamine, which serves as a potentiator for antibiotics, and chitosan is used in wastewater treatment. The broad field of chitin chemistry, structure, and applications has been thoroughly reviewed by Muzzarelli (*2*) and updated at a recent international conference on chitin and chitosan (*3*).

Chitin has an unusual combination of properties, including toughness, bioactivity, and biodegradability, which makes it an attractive specialty material. With these factors in mind, studies were instituted to promote chitin from crab and shrimp shells as a marine resource and to alleviate a growing waste-disposal problem in the shellfish food industry. Previous investigations of chitin have included basic biological and physiological studies, in vitro syntheses promoted by enzyme preparations (*4, 5*), and an examination of its digestibility (as crab meal) by calves (*6*), accelerating effects on wound healing (*7*), and sequestering of plutonium from aqueous systems (*8*).

The applications research (*9*) has largely been focused on chitosan because the free amino groups in this modified product contribute polycationic, chelating, and film-forming properties, along with ready solubility in dilute acetic acid. However, chitin itself is substantially lower in cost and, although intractable, appears amenable to modern manipulative technology. In this article, we outline approaches to chitin utilization via nondegradative solvent systems, microcrystalline chitin, and the monomeric alkyl *N*-acetyl-D-glucosamine (NAG) glycosides. We have also studied species variations in chitin among marine Arthropoda. The bioactivities of the chitin products open new avenues for future research, particularly in the biochemical, pharmaceutical, and nutritive supplement fields.

## Solvents and Filaments

Chitin, like cellulose, is insoluble in ordinary solvents; it is crystalline, and sometimes oriented in its natural state, suggesting fiber-forming potential. Strong acids, fluoroalcohols, and certain hydrotropic salt solutions are chitin solvents but degrade the chitin or are incon-

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Table 1. Optical rotations of chitins. The specific rotations given are for the portions of the chitins that could be dissolved in DMAc-LiCl. The portions ranged from 58 to 92 percent for the samples listed here.

| Chitin* | Rotation $[\alpha]_D$ | |
|---|---|---|
| | Initial | After 2 weeks |
| Horseshoe crab | −56° | −56° |
| Blue crab | +33° | −52° |
| Japanese crab | +23° | −22° |
| Pink shrimp | +75° | −54° |
| Brown shrimp | −36° | −36° |

*In order: *Limulus polyphemus*, *Callinectes sapidus*, *Chionectes opilio*, *Pendalis borealis*, and *Penaeus aztecus*.

venient to use. Hence, early in our program we gave attention to a search for nondegradative solvents. This led to an effective solvent system comprising dimethylacetamide containing 5 percent dissolved lithium chloride (DMAc-LiCl), which gave a syrupy chitin solution that could be extruded or "wet" spun into alcohol or acetone to yield continuous filaments. A key point in these manipulations was maintaining anhydrous conditions. Remarkably, the filaments could be cold-drawn and thus oriented to give added strength, as with some fibers from synthetic polymers (*10*).

Chitin promotes wound-healing activity and is nonallergenic and absorbable (*7*), two properties that prompted consideration of the monofilaments as surgical sutures. Spinning and evaluation tests were undertaken by a pharmaceutical firm, but are in abeyance because of solvent problems and competing research priorities. However, the resistance of chitin filaments to biodeterioration has been found to be adequate for use as a suture material (*11*), although these filaments are expected to biodegrade and be absorbed eventually. These promising results should encourage further investigations, particularly with dry spinning to minimize solvent problems and enhance drawing capability as desired for highest quality filaments.

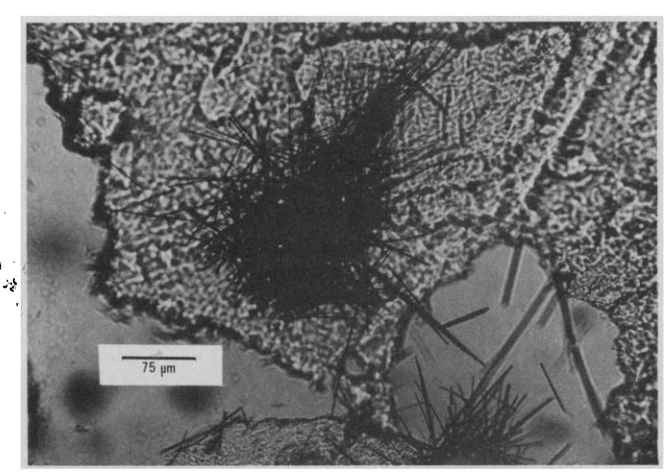


Fig. 1. Microcrystalline chitin prepared by partially hydrolyzing chitin in a mixture of 2-propanol and phosphoric acid, separating, and shearing in an aqueous dispersion at 20,500 rev/min. For this photograph, a sample of the aqueous dispersion was stirred in an excess of acetone. A small sample was evaporated on a microscope slide and stained in place with safranin (immersion oil was used with phase contrast).

## Chiroptical Properties of Chitin

The discovery of an inert solvent for chitin also provided the opportunity to employ optical activity as an additional parameter in measuring physical properties (*12*). The chiral structure of chitin arises from both the asymmetric carbon atoms of the NAG units and an indicated helical conformation of the polymer molecules. Supplemental evidence for this picture is the slow change in direction of the specific optical rotation of certain chitins in solution (*13*) (Table 1).

The horseshoe crab (*Limulus polyphemus*) has an uncalcified carapace and thus its chitin can be isolated under mild alkaline conditions. This chitin is levorotatory and is probably typical of native chitin. Some of the other chitins whose isolation requires harsher treatments with acid or higher temperatures are dextrorotatory. In solution, such chitins are visualized as having a random coil structure, with the positive rotation arising from the asymmetric carbons in the myriad of NAG units. On standing in solution, single-stranded helices with neutral or slightly positive rotation are believed to form. Subsequently, multistrand left-hand helices may form with strong negative rotations. These postulates seem plausible in view of the reported helical nature of chitin in the solid state (*14*). Confirmation of these views by circular dichroism and optical rotatory dispersion was prevented by the opacity of the DMAc-LiCl solvent system below 200 nanometers.

Although this striking variation in the optical rotations of the several chitins is believed to be related primarily to severity of the chemical treatments, species variation also may be a factor. However, determinations of optical activity should be useful in the quality control of the polymer, particularly when biological evaluations are planned (*15*).

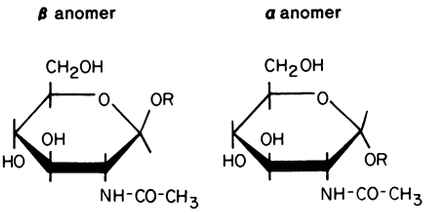


Fig. 2. Alkyl *N*-acetylglucosamine (NAG) glycosides (ring hydrogens omitted for clarity).

## Microcrystalline Chitin

In one of the novel cellulose technologies, partial hydrolysis and high shear are used to produce lower molecular weight microcrystalline celluloses (*16*). These products have been developed for use in low-calorie diet foods and as binders in the manufacture of pills and tablets. They also find application as thickening agents in cosmetic preparations (*17*).

Microcrystalline chitin (MCC) has been prepared previously as a thixotropic aqueous dispersion (*18*), with cited advantages of stability and freeze-thaw resistance. We developed a redispersible chitin powder through a modified procedure involving mild hydrolysis with phosphoric acid in 2-propanol, quenching with water, filtering, and shearing an aqueous dispersion at 20,500 revolutions per minute in a blender; the sheared product was then filtered and finally freeze-dried (*19*). A friable, easily dispersible chitin was obtained in this way. The molecular weight had been reduced from the original 800,000 to about 75,000 and some of the acetyl groups were removed (Fig. 1).

This new type of MCC has limited solubility in the DMAc-LiCl system, perhaps because about 0.4 to 0.8 percent phosphorus is introduced during its preparation. The phosphoric acid may be held in the form of a phosphate salt by the several free amine groups since exhaustive water extraction and attempted elution with aqueous hydrochloric acid reduced the phosphorus content to 0.1 percent (*20*). This material has been tested in animal feed, referred to later. Since chitin, like glucosamine itself, yields pyrazines on pyrolysis (*21*), and since pyra-

zines are responsible in part for the "roasted" aroma of certain cooked foods (*22*), the MCC is being tried as a variant to improve aroma in diet foods (*23*).

### Bifidobacteria and Alkyl Glycosides of *N*-Acetyl-D-glucosamine

One of the fascinating discoveries neglected for many years is the promoting effect of alkyl NAG glycosides on the growth of bifidobacteria (*24, 25*). Colostrum and human milk contain a so-called bifidus factor that provides an NAG moiety and promotes the growth of bifidobacteria. In turn, these predominating bacteria block other types of microorganisms and generate the lactase required for digestion of milk lactose (*26*). Only limited amounts of the factor are present in cow's milk; hence some infants who are fed cow's milk may have trouble with indigestion, colic, and resistance to infection. Many animals and certain groups of humans, including some of the elderly, have similar lactose intolerances.

An improved method was developed to provide a ready supply of alkyl NAG glycosides for study. An alcohol and NAG were reacted with an ion-exchange catalyst and the water formed was continuously removed (*27*). However, because of the chiral nature of the glucosamine products, a mixture of the α and β anomers was produced (Fig. 2), of which the β form is the desired bioactive material. Accordingly, a method was worked out for partially separating the mixture by fractional crystallization from an ethanol-ethyl acetate system (*28*). This yielded a concentrate containing up to 70 percent of the β anomer. The inactive material was equilibrated by treatment with a small amount of sodium hydroxide in ethanol solution and fed back into the process.

It should be noted that the NAG moiety is a constituent of several important biopolymers, such as heparin, the hyaluronic acid of vitreous humor and synovial fluid, as well as the bifidus factor of human milk. Hence, it might be considered as a dietary supplement in stress conditions, as in aging. In view of the potential importance of the alkyl NAG glucosides, a renewed investigation of microbiological syntheses of these compounds might be advantageous (*29*).

Table 2. Growth rates of chicks fed microcrystalline chitin (MCC) and whey (*44*). The diet for group 1 consisted of 100 percent basal ration; for group 2, it was 98 percent basal ration and 2 percent MCC; for group 3, it was 80 percent basal ration and 20 percent whey; for group 4 it was 78 percent basal ration, 2 percent MCC, and 20 percent whey. Each group contained ten 4-day-old chicks. The whey in each diet was equivalent to 13.6 percent lactose.

| Time fed (days) | Average weight of chicks (g) | | | |
|---|---|---|---|---|
| | Group 1 | Group 2 | Group 3 | Group 4 |
| 0 | 50 ± 1.2 | 49 ± 2.0 | 50 ± 2.2 | 51 ± 1.9 |
| 4 | 69 ± 2.6 | 66 ± 2.4 | 76 ± 3.0 | 78 ± 2.8 |
| 8 | 109 ± 4.9 | 116 ± 6.1 | 133 ± 5.6 | 133 ± 4.8 |
| 10 | 154 ± 7.1 | 156 ± 9.1 | 184 ± 6.1 | 190 ± 7.2 |
| 18 | 289 ± 16.2 | 292 ± 13.1 | 304 ± 14.6 | 360 ± 12.7 |
| 25 | 501 ± 24.6 | 523 ± 22.3 | 541 ± 20.4 | 615 ± 19.4 |
| 33 | 677 ± 26.3 | 586 ± 27.1 | 635 ± 24.3 | 815 ± 25.2 |
| 40 | 1087 ± 40.2 | 783 ± 31.0 | 1033 ± 32.6 | 1222 ± 38.9 |
| 46 | 1378 ± 82.1 | 1077 ± 37.9 | 1178 ± 39.7 | 1556 ± 58.8 |

### Chitin and Whey

With the above background, it was considered that one might formulate a digestible and highly nutritious animal feed if the alkyl NAG glycosides or one of the chitinous products could be used in conjunction with high lactose cheese whey. Whey is a by-product of the cheese manufacturing industry and is produced in multibillion-kilogram quantities, giving rise to serious waste disposal problems. Dried whey contains about 13 percent high-quality protein and nearly 70 percent lactose (*30*). This high content of lactose is the reason for its underutilization, since the prevalence of lactose malabsorption and intolerance ranges from 70 to 90 percent in human populations studied all over the world (*31–34*). A similar incidence of lactose intolerance exists in most animal species and frequently leads to diarrhea.

In tests with rats (*35*), propyl NAG glycoside and whey supplements in standard feeds gave encouraging results. After 8 weeks, rats on a diet containing 30 percent whey and 1.2 percent propyl NAG glycoside had higher body weights than rats on the same diet without the propyl NAG glycoside supplement. The latter group, receiving whey but no propyl NAG glycoside, developed severe diarrhea and eventually died from dehydration and malnutrition.

In studies with a limited number of chickens we used the MCC described above, and the results were promising. It appeared that a whey and MCC combination could replace up to 20 percent of the normal feed without an apparent adverse effect on weight gains (Table 2).

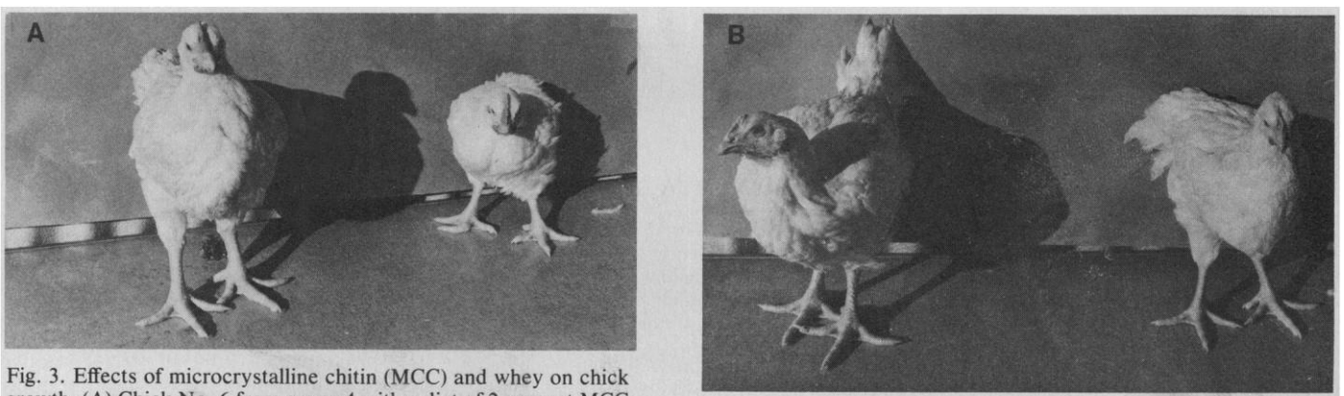


Fig. 3. Effects of microcrystalline chitin (MCC) and whey on chick growth. (A) Chick No. 6 from group 4 with a diet of 2 percent MCC and 20 percent dry whey (see Table 2) in a commercial broiler starter diet; chick No. 4 from group 3 with 20 percent whey, but without MCC. Note the crooked toe in chick No. 4. (B) Chick No. 6 from group 4 and chick No. 3 from group 1 control receiving 100 percent of the commercial broiler starter diet and neither whey nor MCC.

At 46 days, chicks receiving both MCC and whey were significantly heavier ($P < .05$) than the controls. Birds in group 2 (MCC control) and group 3 (whey control) had lower weights than group 1 (positive control). This suggests that when either MCC or whey was added to the chick diet alone, weight gain was depressed. This effect was overcome when both MCC and whey were added to the diet (group 4). Chickens in group 3 developed severe diarrhea, their feathers appeared drier, and they had more broken body feathers. Furthermore, 20 percent of birds in group 3 developed crooked toes outward (Fig. 3), a deformity reported (*36*) in chickens fed a high lactose diet. A similar condition is caused by riboflavin deficiency, but the toes curve inwardly. However, it should be pointed out that the curved toe condition is not uncommon in commercially raised broilers. For the duration of the experiment, group 2 had three mortalities and group 1 had one mortality.

The economics of this development remain to be resolved when judged exclusively on the relatively low protein content of the whey solids. However, such factors as protein efficiency, caloric value, limited fat production, and improved organoleptic characteristics of meat remain to be quantified and evaluated economically. At present, microbiological tests are under way to define the optimum type and amount of chitinous product to use with whey. When this information is available, more extensive animal tests should be carried out (*37*).

## The Family of Chitins

Throughout the program it became evident that there were substantial variations in solubility, molecular weight, acetyl values, and specific rotation among chitin samples as a result of species source as well as methods of isolation. From work reported on in this field, including significant work on insects (*2, 14, 38*), it would appear that chitin is not a single polymeric entity, but rather represents a family of closely related products derived from natural chitin-protein complexes (*39, 40*). Such chitin-protein complexes are the rule, although a closely related product, called chitan, has been reported to be completely free of protein (*41*). Chitins also vary in nature in the degree of deacetylation, that is, in the number of free amine groups, and such features are reflected in the isolated chitins (*2*, p. 30; *14*). Accordingly, a systematic comparison of chitins prepared by consistent methods from several species was undertaken.

The EDTA (ethylenediaminetetraacetic acid) technique (*2*) was tested and chosen for the decalcification of the shells, since it minimizes both chain hydrolysis and deacetylation. Treatment of shell particles 850 micrometers or smaller with a neutral, phosphate-buffered Formalin solution saturated with disodium EDTA was routinely completed within 12 hours at room temperature (*42*). This treatment was also effective in simultaneously removing physically admixed and salt-bound protein. The naturally calcium-free samples from the horseshoe crab were not treated with EDTA in some instances but instead were extracted successively with warm water and 0.16*N* $Na_2SO_4$ to remove the physically associated protein. All crustacean as well as horseshoe crab samples were then extracted with 7*M* urea to remove hydrogen-bonded protein, leaving the covalently bound chitin mucopolysaccharides. These covalently bound fractions were extracted successively with sodium hydroxide, first under relatively mild conditions (0.01*N* NaOH, 25°C, 5 hours, then 1*N* NaOH, 50°C, 6 hours) and then more stringently (1.0*N* NaOH, 100°C, 48 hours). The amount of residual protein remaining with the chitin after the final alkali treatment was determined by amino acid analysis (*43*).

The protein fractionation data (Table 3) reveal that the strongly covalently bound protein varies from 25.0 percent for the red crab to 45.9 percent for the brine shrimp. The protein residues remaining with the chitin after the most drastic alkali treatment, although less than 0.5 percent, are significant because they may particularly affect physical and biological properties.

To carry this comparison a step further, the chitin content of the several shell materials, the total protein, and the covalently bound protein have been compared, and the ratios of chitin to covalently bound protein have been determined (Table 4). The protein content of the dry shell material ranged from 12.3 percent for the red crab to 73.4 percent for the horseshoe crab. Covalently bound protein varied with species from 3.1 to 27.9 percent of the dry shell. The ratios of chitin to covalently bound protein in the shells of the different species were strikingly different: 2.8 to 1 for the blue crab, 3.2 to 1 for the stone crab, 9.0 to 1 for the red crab, 1.7 to 1 for the brine shrimp, and 0.9 to 1 for the horseshoe crab.

It is also evident from the data overall that each species has its own characteristic protein binding matrix. Apparently there is no simple relation between chitin content and the amount of covalently bound protein in the chitin-protein complex. Although the ratio of chitin to total protein in a few crustacean and other arthropod exoskeletons has been deter-

Table 3. Fractionation of chitin-protein complexes.

| Organism | Total protein (%)* | Physical association (%)† | | Covalently bound protein (%)† | | | |
|---|---|---|---|---|---|---|---|
| | | EDTA 20°C, 12 hours | 7*M* urea 20°C, 48 hours | 0.1*N* NaOH 20°C, 5 hours | 1*N* NaOH 50°C, 6 hours | 1*N* NaOH 100°C, 48 hours | Residual in chitin |
| Blue crab | 16.4 | 31.4 | 36.9 | 9.1 | 22.1 | 0.2 | 0.3 |
| Stone crab | 15.4 | 22.4 | 40.6 | 1.7 | 33.1 | 1.8 | .4 |
| Red crab‡ | 12.3 | 58.4 | 16.6 | 2.0 | 16.0 | 6.9 | .1 |
| Brine shrimp | 34.9 | 30.0 | 24.1 | 12.5 | 28.2 | 4.7 | .5 |
| Horseshoe crab | 73.4 | 15.6§ | 46.4 | 2.8 | 32.7 | 2.4 | . .1 |

*Total protein in dry shell was determined independently by extraction with 1*N* NaOH for 48 hours. †Percent of total shell protein. ‡This species of Atlantic red crab (*Geryon quirquedons*) is different from the Pacific species in Table 1 (*Chionectes opilio*). §Water extraction (50°C, 6 hours) 15.0 percent, then 0.16*N* $Na_2SO_4$ (20°C, 72 hours) 0.6 percent, prior to urea treatment.

Table 4. Percentages of chitin and protein content of dry shell.

| Organism | Chitin | Total protein | Covalently bound protein* | Ratio of chitin to bound protein |
|---|---|---|---|---|
| Blue crab | 14.9 | 16.4 | 5.3 | 2.8 to 1 |
| Stone crab | 18.1 | 15.4 | 5.7 | 3.2 to 1 |
| Red crab† | 27.6 | 12.3 | 3.1 | 9.0 to 1 |
| Brine shrimp | 27.2 | 34.9 | 16.0 | 1.7 to 1 |
| Horseshoe crab | 26.4 | 73.4 | 27.9 | 0.9 to 1 |

*Total covalently bound protein (Table 3) as percent of total dry shell. †*Geryon quirquedons*.

mined (*39, 40*), the ratio of chitin to covalently bound protein has not. This finding also lends further support to the existence of species variation.

## Perspective

Chitin, commonly found as the tough polymer matrix of crab and shrimp shells, has a special combination of biological activity and physical properties that has stimulated renewed attention to its potential utility. As an example, the edibility of chitin and its promotion of the growth of selected digestive microorganisms leads to improved digestion of milk lactose. These factors may permit incorporation of waste cheese whey in animal feeds. Solution of chitin in nondegrading solvents, and the spinning and drawing of strong filaments could open the way to new absorbable suture materials. The filaments appear to be body-acceptable and slowly biodegradable. A better understanding of the chemistry of chitin and its conversion to a series of functional derivatives have pointed to development of other high-value applications in food technology and pharmacology. Chitin itself should probably be considered as a family of chitins because its properties vary with source and method of preparation. Despite complexities of its collection, isolation, and variability, it appears that chitin will find a place among commercial natural polymers.

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19. A slurry of 150 grams of chitin in 475 milliliters of 85 percent $H_3PO_4$ and 1000 milliliters of 2-propanol was stirred while at the boiling temperature for 2 hours. The mixture was quenched with 2 liters of water, allowed to stand for 15 minutes, and centrifuged. The solid chitin was washed twice with hot water and then with acetone, redispersed in 10 percent of its weight of water, sheared for 15 minutes, and freeze-dried. The dry cake was ground to pass a 40-mesh (425 μm) screen; the yield was 130 grams (87 percent).
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42. Fresh samples of blue crab (*Callinectes sapidus*), stone crab (*Menippe mercenaria*), red crab (*Geryon quirquedons*), and horseshoe crab (*Limulus polyhemus*) were trapped locally or obtained through commercial sources. Brine shrimp (*Artemia salina*) were obtained from a commercial producer already freeze-dried. Shell materials for analysis were stripped from the stored specimens, scraped free of loose tissue, cleaned of adventitious protein by sonication in a lauryl phosphate detergent solution, and washed in deionized water at room temperature. The shell samples were dried in air and ground to 20 mesh (850 micrometers) in a Wiley laboratory mill for further analytical and purification treatments.
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44. Each of the four test groups contained five male and five female 4-day-old white broiler cross Ross-Arbor Acre chicks which had been debeaked, vaccinated, and assigned randomly to the cages. For the first 4 days prior to the test they were maintained on a commercial broiler starter diet free of dairy products. Water and diet for each group were freely available. All diets were isonitrogenous, isocaloric, and fortified with equal amounts of vitamins and trace mineral supplements. For the first 3 weeks, the chicks were maintained in heat-controlled brooder batteries and thereafter transferred to wire cages. Group 3 birds, receiving only whey, developed severe diarrhea. Chicks in group 4, fed both whey and MCC supplement, were initially diarrhetic but gradually became normal. The adjustment period apparently is needed to develop the bifidobacteria capable of handling the large amount of lactose in the whey. Statistical analysis included analysis of variance and Dunkans multiple range test. The analyses were all part of the Delibr/ Naovmain computer program, University of Delaware Computing Center.


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