DISCUSSION
Pulses are used extensively in India as a regular part of the dietary regime of the entire population. However, it is probably the only source of protein in majority of individuals living at the poverty line (Aykroyd, 1964; Parpia, 1971). Therefore, it is important that the pulses should be wholesome and free from pathogenic organisms so that this population which is extremely vulnerable to infectious diseases, particularly to diarrhoeal conditions, due to lowerer nutritional status and extremely poor sanitary conditions, is protected. Fortunately, the cooking procedures in this country are such that most of the natural microflora is destroyed during cooking. The post cooking storage conditions, though extremely poor, are not of a major consequence if consumed immediately. With development in society, the storage of left overs and their subsequent consumption will definitely pose public health problems if the food poisoning organisms could grow in these pulses and produce toxins. Pulses are consumed either in whole, split or dehusked form. This provides a variety because the same pulse in whole, split or dehusked form has different texture and flavor. Survey of the natural microflora of these three forms of pulses showed that the bacterial load in whole pulses was extremely low and it ranged from 30 - 40 CFU/g. No coliforms were detected in any of the pulses sampled. The fungal load was the most predominant flora and this was not surprising because of the method in which the pulses are
grown, harvested and handled before marketing. Similarly, the nature of the contaminating organisms is an indication of their environmental niches. The low bacterial count may be related to the waxy nature of the coating which probably restricts the adherence of bacteria to pulses. On the other hand, fungal spores and mycelia, because of their rough nature and high load in the environment, are capable of sticking to the surfaces. Extremely limited information is available in literature on whole pulses though the microflora of cereals has been widely studied. Kent - Jones and Amos (1967) reported a bacterial load ranging between 13,000 and 1,260,000 CFU/g in different varieties of Wheat. Ramakrishnan (1979) reported bacterial counts ranging from $0.19 \times 10^4$ CFU/g to $37.5 \times 10^4$ CFU/g in various pulses.

The survey of split and dehusked pulses showed a different trend. The bacterial count increased from 30 CFU/g to $1 \times 10^4$ CFU/g and in most cases it was similar to the fungal count. These differences reflect the role of additional handling by man during the manufacture of split and dehusked pulses. The contaminants in these processed pulses belonged to the environment or human beings. Bacilli which constituted the predominant bacterial flora belong to the former category. *S. faecalis*, *S. albus*, Moraxella, Acinetobacter species are examples of organisms of human and water origin.

The lack of coliforms in dehusked pulses, though
they get extensive water treatment is an indication that either the water supply is extremely good or the contaminating organisms are destroyed during process of drying which follows soaking and removal of husk. These results are similar to the findings of Ramakrishnan (1979) who reported the presence of *S. faecalis*, *P. acidilactici*, *Sacillus* species and *L. mesenteroides* in Bengal gram dal, Besan, Soydal, Urd Split, Rice, Maize, Wheat, Maida and Suji. The counts ranged between $0.19 - 37.5 \times 10^4$ CFU/g, the minimum counts being of split Bengal gram and split Urd while a high count was seen in Maida. This author also did not detect any coliforms. Reports by Amos (1931) showed the presence of *Aflavobacterium*, *Achromobacter* species and *Streptococcus lactis* in Wheat flour.

Tuite and Christensen (1955) have reported the presence of *Alternaria*, *Cladosporium* and *Fusarum* in barley. Christensen and Cohen (1950) report that *Aspergillus* species represented the predominant microflora. No specific studies with pulses are available in the literature. Our findings show the isolation of fungal organisms which were similar to those isolated from wheat though the counts were higher.

The fungal load in whole and split pulses was qualitatively and quantitatively the same and was represented by saprophytic fungi. *Aspergillus flavus*, the known producer of aflatoxin, was also isolated in several
cases though the production of aflatoxin was not tested. The pulses do not offer any danger because of their though surface coating and low moisture content. But there are no reports of the production of mycotoxins in pulses. Hence the natural microflora in pulses does not pose any health hazard due to the nature of storage, pre-cooking treatments and cooking methods. Pulses, however, are used in a number of fermented products and these organisms under adverse circumstances could grow and produce metabolic products which may be hazardous to man.

The role of the dietary regime on the intestinal microflora has been studied by a number of workers (Nath et al., 1948; Dubos et al., 1965; Riely et al., 1966; Gorbach et al., 1967; Spack et al., 1970; Conn et al., 1970; Crowther et al., 1973; Draser et al., 1974; Alcantara et al., 1976). Most of them have reported similar microflora between vegetarians and nonvegetarians. Our findings are in agreement with those workers and those of Aron (1971) who reported no qualitative or quantitative difference in the intestinal microflora of vegetarian and nonvegetarian populations. The anaerobic organisms which have not been studied as thoroughly may differ in the two groups particularly because of the influence of meat in creating redox potentials which are more conducive to the growth of anaerobes.

Similarly, the effect of starch, fibre and lipids on the intestinal microflora as well as the metabolic
patterns of these organisms, particularly with respect to the transformation of bile salts may be critical in developing certain pathological conditions. The role of fibers in reducing the cholesterol level is now well established. Similarly, the predominance of certain types of cancers such as the cancer of colon in Western populations and cancer of the stomach in Japanese population has been linked to the diet and the activities of the intestinal microflora. Therefore, further studies with the anaerobic bacteria should be carried out to correlate and establish the effect of dietary regimes on the metabolic patterns of the intestinal microflora.

In order to determine the suitability of the pulses as substrates for microbial growth, aqueous slurries of pulses were tested. Three out of the four organisms used in this study grew very well in the pulses. *Micrococcus luteus* showed limited multiplication in pulses indicating that the pulses either lack certain growth factors or have specific inhibitors for this organisms. The growth pattern of *K. pneumoniae, E. coli* and *S. aureus* in pulses was similar to that in nutrient broth. The nature or concentration of the pulse did not have a significant effect on the growth of these organisms. Similarly, the incubation period beyond 16 h was not critical as maximum count was achieved before this incubation period. No similar studies are available in the literature for comparison.
The growth of the four selected intestinal isolates in mixed cultures did not follow the same pattern as in pure cultures. *M. luteus* and *K. pneumoniae* were almost completely inhibited and no increase in viable count was noticed during the 36 h incubation period. In contrast, *K. pneumoniae* in pure cultures showed an increase of 3 log cycles within 16 h of growth while *M. luteus* showed an increase of 1 or 2 log cycles. The pattern of growth of these two organisms was similar in 2% corn starch and nutrient broth. However, the growth behaviour of *E. coli* and *S. aureus* was different in nutrient broth and corn starch. An increase of about 2 log cycles was noticed in nutrient broth. While corn starch, *E. coli* showed no increase in viable count. *S. aureus* showed an initial increase of one followed by decline of the same magnitude on further incubation (36 h). The growth behaviour of organisms in mixed population is extremely unpredictable and depends on the nature of microorganisms, substrate and a number of other environmental parameters. No specific information is available on the growth of these 4 organisms in corn, but changes in the microbial population in fermented cereals and pulses have been reported by Ramakrishnan (1979) who showed an increase in microbial population by 2 - 3 log cycles during fermentation.

A major barrier in the consumption of pulses in developed countries is related to the production of flatus. A number of workers including (Burr, 1963, 1967; Murphy, 1964a, b; 1966; Callowly, 1964, 1966; Weyers et al, 1965; Richards
et al, 1968; Rackis et al, 1970; Fleming (1981), have studied the influence of the chemical composition of pulses and the nature of the microorganisms on the production of flatus.

The production of gas by intestinal microflora was influenced by the nature of the pulse, the incubation time and the test organism. It follows therefore, that the production of flatus in vivo is a net result of interaction of nature of pulse, microflora and environmental influences including competition from other organisms as well as the status of the flatus factors after the pre-digestion in the upper gastro-intestinal tract. The initial incubation period is quite critical and the differences between pulses are obvious only at these stages. Longer incubation periods showed a decrease in the differences in gas production by all pulses. Therefore, the individuals evacuation pattern is also important and conditions like intestinal hurry or slow peristaltic movement and constipation could play a role in flatus production. The in vitro studies on pure cultures and individual pulse may have little relevance to the in vivo situation which is much more complex.

Differences in organisms are quite independent of incubation periods. E. coli produced much lesser amount of gas as compared to K. pneumoniae when the total gas production was compared. Gas production per unit cell was more in E. coli as compared to K. pneumoniae. In general,
the viable count was one log cycle lower in *E. coli* as compared to *K. pneumoniae*. Similarly, the percentage increase in gas production in *E. coli* was lower as compared to *K. pneumoniae* for most of the pulses both at 16 and 24 h of incubation. The differences were much greater after 16 h incubation indicating that there were differences in total capacity which is related to genetic makeup of the organism which is responsible for the differences between the two organism as far as gas production is concerned.

Studies with total fecal inoculum showed that growth was lower by about 2 - 3 log cycles, as compared to *E. coli* and *K. pneumoniae*. This is a reflection of the interactions in mixed cultures. Similar observations were made when mixed cultures were studied in nutrient broth and corn starch. The gas production, however, did not show a corresponding decrease and the values for total gas production were similar to *E. coli* in pure culture.

The presence of pulses in nutrient broth did not materially effect the growth of the test organisms. The physiological and metabolic patterns as determined by the intracellular content of proteins and the activity of citric acid enzymes was affected. No major differences were observed between pulses but in general, the activity of malate dehydrogenase and glucose-6-phosphatase activity of *E. coli* increased, the succinate dehydrogenase activity decreased, while its isocitrate dehydrogenase activity...
was not much affected by the presence of pulses. The intracellular content of protein also increased with the presence of pulses.

The increase in enzyme activity, which appears to be specific could be due to the presence of specific stimulators or inhibitors in the pulses. These substances are probably of low molecular weight because they must get into the cell to cause stimulation/inhibition of the enzyme. No studies are available in literature on presence of specific inhibitors of TCA cycle but it is well known that legumes contain several anti enzymes and haemagglutinins which interfere in functioning of the cell. The specificities of increase, decrease or no action are probably related to the sensitivity of certain enzymes or presence or absence of affectors in the pulse.

Bile salts play an important physiological role in the digestion of food. Our studies have shown that there is no major difference in growth patterns of microorganisms in presence of bile salts. This is understandable as the test organisms used were gram negative and bile salts do not inhibit the growth of gram negative organisms.

The biochemical changes produced in the pulses by the growth of microorganisms were monitored by inoculating the test organism in the slurries made of pulses. The changes in acid degree value, fatty acids and amino acid
composition and changes in the electrophoretic and chromatographic patterns were used to establish changes in the lipid, protein and carbohydrate constituents of pulses.

In contrast to the resistance of dry pulse to microbial attack, the pulse slurries were very easily degraded by microorganisms. Proteolytic and lipolytic changes were brought about by the organisms used in this study. In general, the electrophoretic studies showed good separation in uninoculated and unincubated controls. Inoculations with the test organisms produced a great deal of degradation and no clear cut pattern was observed. There was smearing and disappearance of bands. This observation was in accordance with the report of Sathe et al (1981) where the authors showed the disappearance of bands and smearing of protein in the electrophoresis of a fermented Phaseolus vulgaris (Rajmah) and rice preparation though clear bands were observed before fermentation. None of the patterns provided any unique feature which could be used either to identify the nature of the pulse or the nature of the organism.

The nutritional value of food at least in part is based on the content and nature of amino acid and vitamin in food because a number of these are indispensable and have to be supplied to the body through outside sources. Legumes have fairly good protein but lack in methionine, an essential amino acid. Several attempts have been made
to improve the amino acid content of legumes by genetic manipulations and plant breeding. Several workers (Bandemer et al, 1963; Brassani, 1968; Hackler et al, 1973; Wang et al, 1978; Zamora et al, 1978; Agnes et al, 1979, Ahmed et al, 1979) have reported a fermentation route to improve the nutritional quality. These authors claimed an increase in the amino acid and vitamin contents of legumes and cereals by microbial growth. In this study, the amino acid profile of various pulses after the growth of the natural microflora or with E. coli and K. pneumoniae was determined. The amino acid content of all pulses increased between 2 - 40% by the growth of microorganisms. Agnes et al (1979) showed some what lower increase of limiting amino acids in chickpeas and cowpeas by fermentation. Wang et al (1978) used Saccharomyces cerevisiae and Candida tropicalis for improving the amino acid balance of corn meal. Our studies showed that the amino acids present in lower concentrations showed the greatest increases. There was no difference in the amino acid profiles of whole pulses and in their water soluble fractions. Glutamic acid, Aspartic acid was present in higest amount followed by Leucine, Arginine and others. These findings are similar to those reported by Pant et al (1969) and Hang et al (1980) who determined the amino acid contents of mung, pea and red kidney beans. Sathe et al (1981) reported the amino acid content of Phaseolus vulgaris and Sahastrabudhe et al (1981) reported the same order of amino acid content of pulses,
methionine being present in the lowest amount. The observation were similar to this study.

The lipolysis was monitored by determining the acid degree value (ADV) which is a measure of the amount of free fatty acids. These studies have established that the test organisms were extremely lipolytic and the lipids of pulses despite their low content were extremely vulnerable to microbial attack. No major differences between E. coli and K. pneumoniae were observed except for minor differences in profiles. But there were differences in the initial stages of incubation, where, in the same pulse there was a lag period before the lipolysis started but the maximum final ADV was approximately the same. There have been no studies on storage deterioration in lipids of pulses stored in dry forms but it is available in cereals. Pomeranz et al (1966) showed a reduction of 20% lipids in stored damp wheat. Baker et al (1957) reported the increase of free fatty acids in stored wheat.

The comparison of the increases in acid degree values showed that split Urd was most vulnerable followed by White gram, Split Mung, Whole Masur and Whole Urd. It appears that the nature of the lipids in the pulses play a role in their susceptibility to microbial attack. Differences between split and whole Urd were surprising because the major constituents in lipids of the two pulses should be the same. Therefore, the lower ADV in
in whole Urd must be related to the presence of inhibitors or antioxidants in the husk of the whole Urd. In the manufacture of dehusked, split pulse, soaking treatment is also given. It is possible that during this treatment either the structural integrity of the pulse is altered to make it more vulnerable, or the native lipases in the pulse may be activated which is responsible for higher ADV values in the split pulse as compared to the whole pulse.

Since extensive lipolysis was also seen in uninoculated samples, it appears that there are native lipases in pulses which are activated by the addition of water. The contribution of bacteria to lipolysis may be insignificant because the bacterial load in uninoculated samples was low. However, mold as part of the natural microflora may contribute to lipolytic changes as fungi are known potent producers of lipases (Dawson et al. 1958; Kates et al., 1965; Beare and Kates 1967, Dawson and Hauser, 1967; Saito and Sato, 1968). No attempts were made to isolate either natural lipases of pulses or microbial lipases and this represents an important area for future research (Furig and Proulx 1969 and Raybin et al. 1972.)

The changes in fatty acid profiles by the growth of the microorganisms was studied using gas chromatography. The changes show two generalization a) decrease in level of palmitic acid and stearic acid and b) increase in the level of oleic and arachidic acids. The nature of the
pulse had a quantitative role only and the patterns were more related to the test organism. The decrease in the amount of certain fatty acids may be due to specific utilization of the fatty acid by the test organism or their release on lipolysis so that they are lost during the isolation procedure of phospholipids. The increases are probably a reflection of the contribution of the microorganisms to the total phospholipid fraction. A number of studies are available in literature giving similar fatty acid composition of pulses. Hilditch(1947), Meara(1957), Korytnyk et al (1963) reported the presence of linolenic, linoleic, palmitic, oleic and stearic acids in kidney and lima beans. Takayama et al (1965) determined the fatty acid contents of 7 types of beans and found that palmitic acid was present in the highest amount. Our study gives similar results.

The studies to monitor the saccharolytic changes showed that the two test organisms used were not extremely saccharolytic and the saccharification of pulses and cereals prior to inoculation did not considerably effect the final availability of reducing sugars.

The studies of biochemical parameters are a clear indication of the vulnerability of proteins, lipids and carbohydrates of pulses to microbial attack. It appears that proteins and lipids are easily degraded as compared to starches but the picture could be different if the test organisms were different. Though a number of
traditional fermented cereal and pulse based products are available, yet no major effort has been made to determine what kind of biochemical changes are produced by microorganisms in fermented products. The data from this study show extensive changes, therefore a detailed study of fermented products should add to scientific knowledge about the effect of microorganisms on the individual fractions of pulses and/or cereals.