CHAPTER 2

REVIEW OF LITERATURE

2.1 Milk and milk-derived products:

Milk is the secretion of the mammary gland, containing approximately 5% lactose, 3.1% protein, 4% lipid and 0.7% minerals. The components of milk provide critical nutritive elements, immunological protection, and biologically active substances to both neonates and adults. It is not surprising, therefore, that the nutritional value of milk is high. The concept of bovine milk as a biologically active fluid is not new [58], but the identification of factors within bovine milk that may be relevant to improving human health, and the potential development of bovine milk-containing preparations into products with proven health-promoting properties, certainly is.

Milk is not only consumed as a raw material but it is transformed in a variety of products to preserve its nutrients. Among all the dairy products, milk fermentation and cheese making are the oldest methods used to extend the shelf-life of milk, and they have been practiced by human beings for thousands of years [59]. Recently, numerous scientific works [60-62] have demonstrated and confirmed that the consumption of fermented milk and cheeses manifests health beneficial effects that go beyond the nutritional value. Indeed, fermented milk consumption has been associated with reduction of serum cholesterol [63], antihypertensive [64] and osteo-protective [65] effects. The mechanisms of action responsible of these properties have been investigated and have been attributed to the numerous bioactive peptides contained in milk and/or released during milk processing. It is not surprising that in recent years intense research interest has been focused on identifying biologically active components within bovine milk and milk-derived products, and characterising the way by which mammalian physiological
function is modulated by these components. Not surprisingly, a significant proportion of this research has sought to characterise the potential of bovine milk, milk products or milk components to influence some of the most important body physiological functions, such as blood pressure [66], the immune system [67], and the resistance to the infections [68]. For example, there is now a substantial body of evidence to suggest that the major components of bovine milk, as well as several constituents or even yogurt and cheese, can regulate blood pressure in humans [69, 70]. The most significant advances in this field have been made over the last five to ten years, and this review will focus primarily on the recent advances and current knowledge in this rapidly expanding field. Moreover, particular attention is given to the milk-derived bioactive peptides responsible of some important health properties.

2.2  Bioactive peptides:

2.2.1  Definition:

Accordingly to a widely shared definition [71], a bioactive dietary substance is "a food component that can affect biological processes or substrates and, hence, have an impact on body function or condition and ultimately health". In addition, dietary substances should give a measurable biological effect in the range of doses it is usually assumed in the food and this bioactivity should be measured at a physiologically realistic level [72]. Following this definition, milk-derived bioactive peptides are milk components able to influence some physiological functions, finally acting on body health condition. Moreover, among the numerous bioactive substances studied up to now, increasing interest is focused on milk-derived bioactive peptides because at present, bovine milk, cheese and dairy products seem to be extremely important sources of bioactive peptides derived from food.
2.2.2 Mechanisms of production of bioactive peptides:

Milk-derived bioactive peptides, and more generally food bioactive peptides, are usually composed of 2-20 amino acids and become active only when they are released from the precursor protein where they are encrypted. Different mechanisms can release the encrypted bioactive peptides from the precursor proteins as seen in Photo 2.1 [73]:

1. In vivo, during gastrointestinal digestion through the action of digestive enzymes or of the microbial enzymes of the intestinal flora;

2. During milk processing (e. g. milk fermentation, cheese production) through the action of microbial enzymes expressed by the microorganisms used as starter;

3. During milk processing through the action of a single purified enzyme or a combination of selected enzymes;

**Photo 2.1 Scheme of the mechanisms by which bioactive peptides can be released from the precursor proteins by microbial fermentation and/or gastrointestinal digestion**
2.2.2.1 Bioactive peptide release during gastrointestinal digestion through the action of digestive enzymes or microbial enzymes of the intestinal flora

Bioactive peptides may be released in vivo during gastrointestinal digestion. These bioactive peptides are mostly the result of the degradation of casein with several proteases such as pepsin, trypsin or chymotrypsin. At present, despite some experimental works on the stimulation of gastrointestinal digestion of eggs and meat proteins [74, 75], the production of milk-derived bioactive peptides in vivo during digestion remain unclear. While the peptide products resulting from milk proteins digestion with site-specific pancreatic proteases, such as trypsin or chymotrypsin are well investigated [76, 77], there are only few papers regarding this primary step of human digestion of milk proteins [78]. Microbial proteolysis can be a potential source of bioactive peptides during milk processing [79].

2.2.2.2 Bioactive peptide release during milk processing through the action of microbial enzymes

Many industrially utilized dairy starter cultures are highly proteolytic. Bioactive peptides can, thus, be generated by the starter and non-starter bacteria used in the manufacture of fermented dairy products. The proteolytic system of lactic acid bacteria (LAB), e.g. *Lactococcus lactis, Lactobacillus helveticus* and *L. delb. bulgaricus*, is already well characterized. Rapid progress has been made in recent years to elucidate the biochemical and genetic characterization of these enzymes. Many recent articles and book chapters have reviewed the release of various bioactive peptides from milk proteins through microbial proteolysis [80, 81 and 82]. In addition, a number of studies have demonstrated that hydrolysis of milk proteins by digestive and/or microbial enzymes may produce peptides with immunomodulatory activities [83].
2.2.2.3 Bioactive peptide release during milk processing through the action of a single purified enzyme or a combination of selected enzymes

The most common way to produce bioactive peptides is through enzymatic hydrolysis of whole protein molecules. ACE-inhibitory peptides and calcium-binding phosphopeptides, for example, are most commonly produced by trypsin [84-86]. Moreover, ACE-inhibitory peptides have recently been identified in the tryptic hydrolysates of bovine αs2-casein [87] and in bovine, ovine and caprine k-casein macropeptides [88]. Other digestive enzymes and different enzyme combinations of proteinases - including alcalase, chymotrypsin, pepsin and thermolysin as well as enzymes from bacterial and fungal sources - have also been utilized to generate bioactive peptides from various proteins [89, 90].

Proteolytic enzymes isolated from LAB have been successfully employed to release bioactive peptides from milk proteins. David and colleagues in 1991 [91] reported that casein hydrolyzed by the cell wall-associated proteinase from _L. helveticus_ CP790 showed antihypertensive activity in spontaneously hypertensive rats. Several ACE-inhibitory peptides and one antihypertensive peptide were isolated from the hydrolysate. Kunio et al., 2000 [92] hydrolyzed casein using the same proteinase and identified a β-casein-derived antihypertensive peptide, the fragment β-CN (169-175), whose amino acidic sequence is KVLPVPQ. In a recent study, Julius and colleagues in 2004 [93] measured the ACE-inhibitory activity of casein hydrolysates upon treatment with nine different commercially available proteolytic enzymes. Among these enzymes, a protease isolated from _Aspergillus oryzae_ showed the highest ACE-inhibitory activity in vitro per peptide.

2.2.3 Mechanisms of action of bioactive peptides:

It has been already demonstrated that milk-derived peptides show biological effects and are able to influence some specific body function. At present, the bioactivities
described for milk-derived peptides includes opiate [94], antithrombotic [95], antihypertensive [96], immunomodulating [97], antioxidative [98], antimicrobial [99], anticancer [100], mineral carrying [101] and growth-promoting properties [102]. In the Table 2.1, a brief summary of bioactive peptides from milk proteins is given.

Bioactive milk peptides could express their function in the intestinal tract [103-107] or inside the body after being absorbed. This signifies that milk-derived bioactive peptides have to be resistant to gastrointestinal, brush border intracellular and serum peptidases [108]. For this reason, scientific works aiming to evaluate the bioavailability of bioactive peptides in vivo are gaining of importance [109, 110].

Table 2.1 Bioactive peptides from milk proteins

<table>
<thead>
<tr>
<th>Bioactive peptide</th>
<th>Precursor protein</th>
<th>Bioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casomorphins</td>
<td>α-CN, β-CN</td>
<td>Opioid agonist</td>
</tr>
<tr>
<td>α-lactorphin β-lactorphin</td>
<td>α-LA β-LG</td>
<td>Opioid agonist</td>
</tr>
<tr>
<td>Lactoferoxins</td>
<td>LF</td>
<td>Opioid agonist</td>
</tr>
<tr>
<td>Casoxins</td>
<td>K-CN</td>
<td>Opioid antagonist</td>
</tr>
<tr>
<td>Casokinins</td>
<td>α-CN, β-CN</td>
<td>Opioid antagonist</td>
</tr>
<tr>
<td>Lactokinins</td>
<td>α-LA, β-LG</td>
<td>ACE-inhibitory</td>
</tr>
<tr>
<td>Immunopeptides</td>
<td>α-CN, β-CN</td>
<td>ACE-inhibitory</td>
</tr>
<tr>
<td>Lactoferricin</td>
<td>LF</td>
<td>Immunomodulatory</td>
</tr>
<tr>
<td>Casoplatelins</td>
<td></td>
<td>Antimicrobial</td>
</tr>
</tbody>
</table>
2.2.4 Bioactive peptide based commercial dairy products:

It is now well documented that bioactive peptides can be generated during milk fermentation with the starter cultures traditionally employed by the dairy industry. As a result, peptides with various bioactivities can be found in the end-products, such as various cheese varieties and fermented milks. These traditional dairy products may, under certain conditions, carry specific health effects when ingested as part of the daily diet. Table 2.2 below lists a number of studies which have established the occurrence of peptides in various fermented milk products. An increasing number of ingredients containing specific bioactive peptides based on casein or whey protein hydrolysates have been launched on the market within the past few years or are currently under development by international food companies.

Table 2.2 Studies which have established the occurrence of peptides in various fermented milk products

<table>
<thead>
<tr>
<th>Product</th>
<th>Example of identified peptide</th>
<th>Bioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cheese type Parmigiano-Reggiano</td>
<td>β-CN (8-16), β-CN (58-77), αs2-CN(83-33)</td>
<td>Phosphopeptides, precursor of β-casomorphin</td>
</tr>
<tr>
<td>Cheddar</td>
<td>αs1-CN fragments</td>
<td>Several</td>
</tr>
</tbody>
</table>

Phosphopeptides | K-CN, Transferrin
α-CN, β-CN | Antitrombotic
Mineral binding,
Anticariogenic
<table>
<thead>
<tr>
<th>Variety</th>
<th>β-CN Peptides</th>
<th>Active Peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Italian varieties:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mozzarella, Crescenza,</td>
<td>β-CN (58-72)</td>
<td>phosphopeptides</td>
</tr>
<tr>
<td>Gogonzola, Italico Gouda</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Festivo</td>
<td>αs1-CN (1-9), β-CN (60-68)</td>
<td>ACE-inhibitory</td>
</tr>
<tr>
<td></td>
<td>αs1-CN (1-9),</td>
<td></td>
</tr>
<tr>
<td></td>
<td>αs1-CN (1-7), αs1-CN (1-6)</td>
<td>ACE-inhibitory</td>
</tr>
<tr>
<td>Emmental</td>
<td>αs1-CN fragments</td>
<td>Immunostimulatory, antimicrobial</td>
</tr>
<tr>
<td></td>
<td>β-CN fragments</td>
<td></td>
</tr>
<tr>
<td>Manchengo</td>
<td>Ovine αs1-CN, αs2-CN, β-CN fragments</td>
<td>ACE-inhibitory</td>
</tr>
<tr>
<td>Fermented milks</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sour milk</td>
<td>β-CN (74-76), β-CN (84-86), κ-CN (108-111)</td>
<td>Antihypertensive</td>
</tr>
<tr>
<td>Yogurt</td>
<td>Active peptides not identified</td>
<td>Weak ACE-inhibitory</td>
</tr>
<tr>
<td>Dahi</td>
<td>SKVYP</td>
<td>ACE-inhibitory</td>
</tr>
</tbody>
</table>
2.3 Digestion of bioactive peptides:

Some bioactive peptides can express their activity directly on the gastrointestinal tract but the majority of them have to reach their target site inside the body. They have to remain stable during the digestion process and cross the gastrointestinal barrier maintaining their biological activities. It is thus important to know the physiology of digestion of proteins and peptides in the gastrointestinal tract, more specifically the human GI system, to understand the mechanisms determining the bioavailability of bioactive peptides in vivo [148]. In humans, the most important sites for the digestion of proteins and peptides are the stomach and the small intestine. The stomach is the portion of the GI tract that is located between the cardiac and pylorus valves (Photo 2.2). It can be divided in different regions which differ for the structure and functionality of the glands distributed in the gastric mucosa.

Photo 2.2 The anatomic structure of the human stomach
Regardless of the mechanisms of absorption, the bioactive peptides that enter the enterocyte undergo the action of the peptidases of the cytosol or the cellular organelles. Indeed, the lysosome contains a massive array of enzymes, estimated over 60 in number, which are capable of degrading any biological macromolecule, including peptides and proteins. The release of ACE-inhibitory peptides upon gastrointestinal digestion of milk proteins or protein fragments, as well as the resistance to digestion of known ACE-inhibitory sequences has been tested in several in vitro studies where the gastrointestinal process was mimicked by the sequential hydrolysis with pepsin and pancreatic enzymes (trypsin, chymotrypsin, carboxy and aminopeptidases). These studies showed that gastrointestinal digestion is an essential factor in determining ACE-inhibitory activity [149]. The conditions of the simulated gastrointestinal digestion (enzyme preparation, temperature, pH and incubation time) greatly influence the degree of proteolysis and the resultant ACE-inhibitory activity. The action of brush-border peptidases, the recognition by intestinal peptide transporters and the subsequent susceptibility to plasma peptidases also determine the physiological effect [150].

There is an increasing need to develop in vitro gastrointestinal digestion models that could mimic the human digestion processes. In vitro methods therefore offer an appealing alternative to human and animal studies. They can be simple, rapid, and low in cost and may provide insights not achievable in whole animal studies. In fact, in the last years new in vitro gastrointestinal digestion models incorporating the multi-phase nature of the digestive processes, to mimic the passage the food into the stomach and then into the gut, have been developed or adapted for assessing digestibility of food allergens [151], but a potential application on the study of physiology of the digestion of bioactive peptides could be feasible. Such models have to be sufficiently refined to allow the process of digestion to be followed in some detail and have to be validated against in vivo data. Ideally, an in vitro model should offer the advantages of rapid representative sampling at any time point, testing the whole food matrix (or diet) instead of the isolated protein precursor of the bioactive peptide and be capable of handling solid foods which
cannot easily be tested in vivo. Moreover, in vitro digestion models should consider three main stages: (i) processing in the mouth, (ii) processing in the stomach (cumulative to the mouth) and (iii) processing in the duodenum (cumulative of mouth and stomach).

2.4 Bioactive peptide absorption:

After digestion, di- and tri-peptides can be easily absorbed in the intestine, but it is not clear if larger bioactive peptides can be absorbed from the intestine and reach the target organs. Some bioactive peptides, in particular C-terminal proline containing peptides, are resistant to proteolysis [152], suggesting that this class of peptides have a better chance to be absorbed in their active form.

2.4.1 Physiology of the absorption of proteins and peptides:

Approximately 90% of the absorption in the gastrointestinal tract occurs in the small intestinal region. The specialized epithelial barriers of the gastrointestinal tract separate fluid-filled compartments from each other. They restrict and regulate the flux of substances in both directions. In general, the transfer of all substances, from H+ ions to the largest proteins, across these barriers can occur via paracellular or transcellular routes (Photo 2.3).

**Photo 2.3 Different pathways for intestinal absorption of a compound.**
The intestinal absorption of a compound can occur via several pathways: (a) transcellular passive permeability; (b) carrier-mediated transport; (c) paracellular passive permeability, and (d) transcytosis. However, there are also mechanisms that can prevent absorption: (e) intestinal absorption can be limited by P-gp, which is an ATP-dependent efflux transporter; and (f) metabolic enzymes in the cells might metabolize the bioactive peptide.

The transcellular route requires the transport of the solute across two morphologically and functionally different cell membranes (e.g. the apical and the basolateral membrane), by either active or passive processes. The extent of simple passive diffusion of substances across the membranes depends on their size, charge and lipophilicity and could be facilitated by a carrier system and has been observed for most smaller inorganic and organic solutes [153].

2.4.2 Physical and chemical characteristics of potentially absorbable bioactive peptides:

To exert physiological effects after oral ingestion, it is of crucial importance that milk-derived bioactive peptides remain active during gastrointestinal digestion and absorption and reach the circulation. The bioavailability of peptides depends on a variety of structural and chemical properties, i.e. resistance to proteases, charge, molecular weight, hydrogen bonding potential, hydrophobicity and the presence of specific residues [154]. Indeed, proline- and hydroxyproline-containing peptides are relatively resistant to degradation by digestive enzymes. Furthermore, tripeptides containing the C-terminal proline-proline are reported to be resistant to proline-specific peptidases [155] and have been shown to be stable under simulated gastrointestinal digestion conditions.

As already explained before, peptides consisting of two or three amino acids can be absorbed intact from the intestinal lumen into the blood circulation via different mechanisms for intestinal transport. The presence of the milk-derived ACE-inhibitory
peptide IPP was recently demonstrated in measurable amounts in the circulation of volunteers that consumed a drink enriched in IPP and VPP [156]. Other characteristics contribute to the resistance to hydrolysis. For example, when isolated, some casein-derived peptides tend to be highly negatively charged and phosphorylated, making them resistant to further proteolysis. Thus, some of the bioactive peptides could be absorbed across the intestinal mucosa to enter the circulation or be retained in the lumen and pass into the colon. The latter is likely based on evidence that ingested casein-derived phosphopeptides can be isolated from rat feces.

2.4.2.1 The absorption of bioactive peptides derived from milk proteins:

For some bioactive tripeptides the intestinal absorption has been already demonstrated. For example, VPP was detected in the abdominal aorta of SHR 6 hours after its administration in sour milk, which strongly suggests that it is trans-epithelially transported [157]; more recently the absorption was observed also in humans. Paracellular transport, through the intercellular junctions, was suggested as the main mechanism, since the transport via the short-peptide carrier, PepT1, led to a quick hydrolysis of the internalized peptide. In the case of larger sequences, the susceptibility to brush border peptidases is the primary factor that decides the transport rate. For example, the heptapeptide lactokinins (ALPMHIR) was transported intact, although in concentrations too low to exert an ACE-inhibitory activity, which suggests cleavage by aminopeptidases.

2.5 Bioactivities of milk and fermented milk peptides:

Milk-derived bioactive peptides are potential modulators of various regulatory processes in the body, and they can express hormone-like activities. Moreover, the primary sequence of some specific bovine proteins, as caseins, contains overlapping regions, partially protected from proteolytic breakdown, that manifest multifunctional properties and influence different biological functions [111]. In particular, ACE-
inhibitory and immunomodulatory properties seem to be associated, possibly because both are correlated to the presence of short chain peptides such as VPP and IPP formed during milk fermentation with selected bacterial strains [112].

2.5.1 ACE-inhibition:

The inhibition of the Angiotensin-I-Converting Enzyme (ACE) is a key point in the treatment of the hypertension. ACE is carboxypeptidase and catalyzes the cleavage of dipeptides [113]. ACE is responsible for the conversion of angiotensin I, a decapeptide generated by the action of rennin on the substrate angiotensinogen, to the vasoconstrictor octapeptide angiotensin II. Angiotensin II directly acts on blood vessels increasing blood pressure, but it also stimulates the release of aldosterone from the adrenal cortex. Aldosterone increases the reabsorption of sodium and water and the secretion of potassium by the kidney, so the overall effect is an increased blood pressure. Examples of ACE-inhibitory peptides derived from milk is given in Table 2.3

Table 2.3 Some examples of ACE-inhibitory peptides derived from milk

<table>
<thead>
<tr>
<th>Peptide sequence</th>
<th>Fragment</th>
<th>IC_{50} (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VAP</td>
<td>\alpha_{s1}-CN (25-27)</td>
<td>2</td>
</tr>
<tr>
<td>FFVAP</td>
<td>\alpha_{s1}-CN (23-27) \alpha_{s1}-</td>
<td>6</td>
</tr>
<tr>
<td>FFVAPPFPEVFGK</td>
<td>CN (23-34) \alpha_{s1}-CN</td>
<td>77</td>
</tr>
<tr>
<td>FPEVFGK</td>
<td>(28-34) \alpha_{s1}-CN</td>
<td>140</td>
</tr>
<tr>
<td>FGK</td>
<td>(32-24)</td>
<td>160</td>
</tr>
<tr>
<td>YKVLPQL</td>
<td>\alpha_{s1}-CN (104-109) \alpha_{s1}-</td>
<td>22</td>
</tr>
</tbody>
</table>
2.5.2 Immunomodulation:

The immune response can be influenced by various factors. Numerous reports demonstrate that milk bioactive peptides can interact with the immune system at different levels [114].

2.5.2.1 Immunomodulatory peptides derived from milk:

Immunomodulatory milk peptides act on the immune system and cell proliferation responses thus influencing downstream immunological responses and cellular functions. Indeed, in 1981 Cinquina and colleagues in 2003 [115] discovered that a tryptic hydrolysate of human milk possessed in vitro immunostimulatory activity (more specifically, stimulation of phagocytosis of sheep red blood cells and production of hemolytic antibodies against the same cells). In the following years, a number of potentially immunoregulatory peptides were identified encrypted in bovine caseins and whey proteins, which can manifest different effects (Table 2.4). Some casein-derived peptides (residues 54-59 of human β-casein and residues 194-199 of α_s1-casein) can stimulate phagocytosis of sheep red blood cells by murine peritoneal macrophages [116], exert a protective effect against *Klebsiella pneumoniae* [117] or modulate proliferative responses and immunoglobulin production in mouse spleen cell cultures (fragment 1-28 of bovine β-casein, [118].

More recently, lactoferricin B, obtained by hydrolysis of lactoferricin by pepsin, was found to promote phagocytic activity of human neutrophils [119]. Others fragments (fragment 18-20 of -casein, fragment 90-96 of α_s1-casein) can either stimulate or inhibit
lymphocyte proliferation depending upon the concentration used, while some whey-derived peptides can affect cytokine production from leucocytes [120].

**Table 2.4 Immunomodulatory peptides derived from milk proteins**

<table>
<thead>
<tr>
<th>Protein sequence</th>
<th>Fragment</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine αs1-CN</td>
<td>αs1-CN (1-23)</td>
<td>Stimulation of phagocytosis and immune responses against bacterial infections</td>
</tr>
<tr>
<td>Bovine αs1-CN</td>
<td>αs1-CN (23-34)</td>
<td>Stimulation of phagocytosis and immune responses against bacterial infections</td>
</tr>
<tr>
<td>Bovine αs1-CN</td>
<td>αs1-CN (90-96)</td>
<td>Stimulation effect on lymphocytes proliferation, NK activity and neutrophil locomotion</td>
</tr>
<tr>
<td>Bovine αs1-CN</td>
<td>αs1-CN (90-95)</td>
<td>Stimulation effect on lymphocytes proliferation, NK activity and neutrophil locomotion</td>
</tr>
<tr>
<td>Bovine αs1-CN</td>
<td>αs1-CN (194-199)</td>
<td>Stimulation of phagocytosis and immune responses against bacterial infections</td>
</tr>
</tbody>
</table>
Immunomodulatory milk-derived peptides may contribute to the overall immune response and may ameliorate immune system function. Weinrichter et al., 2001 [121] suggested that casein derived peptides are involved in the stimulation of the newborn's immune system. It cannot be excluded that the immunostimulating activities may also have a direct effect on the resistance to bacterial and viral infection of adult humans.

**2.5.2.2 Microorganisms for the production of fermented milk with immunomodulatory activity**

Also in the case of immunomodulatory peptides, milk fermentation contributes to the generation of fermented milk with potential immunological activity (Table 2.5). Amitha and colleagues in 1997 [122] demonstrated that milk fermented with *L. helveticus* modulates lymphocyte proliferation in vitro.

**Table 2.5 List of microorganisms producing immunomodulatory activity from fermented milk**

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Protein source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. helveticus</em> 5089</td>
<td>Caseins</td>
</tr>
<tr>
<td><em>L. helveticus</em> R389</td>
<td>Milk</td>
</tr>
</tbody>
</table>
Fermented milks with immunomodulatory properties are not produced exclusively by *L. helveticus*. Milk fermented by *L. paracasei* [123] shown to produce peptides from β-lactoglobulin that stimulate IL10 production and depress lymphocyte proliferation. Additionally, *L. casei* was used to produce a casein hydrolysate that suppresses human T cell activation, modulating IL2 expression [124, 125 and 126]. The immunomodulatory activity is independent from the presence of living microorganisms, as evidenced by Perdigon [127] and by Vinderola [128] who reported that the supernatant of fermented milk cultured with *L. casei, L. acidophilus* and *L. helveticus* strains increased the immune response independently from the presence of lactobacilli. This result was obtained also by De Simone [129] that tested the INF-α production of human peripheral blood lymphocytes in response to filtered yoghurt devoid of microorganisms. More recently LeBlanc examined the antibody production following *E. coli* O157:H7 infection following the administration of a cell- free supernatant from *L. helveticus* fermented milk and found that the increased antibody production is not related to viable microorganism
Microorganisms other than bacteria, as a cell-free extract obtained from the yeast *S. cerevisiae* can be used for milk fermentation, producing a milk hydrolysate with potential apoptosis-inducing effect in human leukemia HL-60 cells, as observed by Rudolf *et al.*, 1990 [131].

In addition, as already demonstrated for milk proteins [132, 133], the bioactive peptides present in yoghurt actually decreased cell proliferation with IEC-6 or Caco-2 cells, which may explain, at least partially, why consumption of yoghurt has been associated with a reduced incidence of colon cancer [134]. The molecular mechanism by which the previous mentioned microorganisms enhance the immune system is not yet clear but the previously discussed reports strongly support the fact that immunomodulatory peptides released in fermented milk contribute to the immunoenhancing and antitumor properties of dairy products. It should be stressed that the extreme difficulty to establish how immunomodulatory peptides and fermented milks influence the immune function is strictly linked to the immune system complexity. This system comprises a complex interplay between different cell populations and molecules. Thus, when the immunomodulatory activity of a bioactive peptide is assessed in vitro, the single experimental result could demonstrate the specific involvement of a particular milk-derived peptide in an immune mechanism but this result is not conclusive in determining if this peptide its effects would be significant for the whole immune system.

### 2.5.2.3 Examples of immunomodulatory peptides derived from milk proteins:

At present, most attention on immunomodulatory peptides has been focused on lactoferricin, a pepsin-derived peptide from lactoferrin and on glycol-macropeptide, a k-casein-derived peptide (β-CN (amino acid sequences 106-169)) present in appreciable amounts in some whey protein concentrates and whey protein isolates. Particular attention has been given to the fragment β-LA (amino acid sequences 18-20) (a tri-peptide named YGG) and to the long fragment β-CN (amino acid sequences 193-209).
because they have been chosen as model peptides to study the immunomodulatory activity and the absorption mechanism of bioactive peptides derived from milk proteins [135].

2.5.2.3.1 YGG peptide with immunomodulatory activity:

The peptide YGG (Tyr-Gly-Gly) represents an interesting example of cryptic peptide with putative immunomodulating effects, as it can originate from at least two different sources. First, it is the product of the hydrolysis of Leu-enkephalin and Met-enkephalin and thus it is an endogenous peptide. In addition, it can be considered as a potential nutraceutical, because it is also encrypted in milk proteins and can be released during the digestion of bovine milk, in particular from β-lactalbumin (fragment amino acid sequences 18-20) [136]. It is known that Met-enkephalins, the YGG endogenous progenitor, can enhance human T cell proliferation and IL2 production in vitro in the absence of mitogens, possibly through the activation of opioid receptors present on the cell surface [137]. The enhancement of human peripheral blood lymphocytes proliferation and protein synthesis in vitro was obtained also with YGG administration in presence of ConA [138, 139]. In addition, it was observed that YGG can affect INF-α and IL2 secretion in murine splenocytes stimulated with suboptimal concentration of ConA in serum- free medium [140].

Stimulatory effects on cell proliferation were observed also in leukocytes obtained from mice administrated in vivo with either Met-enkephalin or YGG, suggesting that Met-enkephalin effects on the immune cells are mediated by YGG [141]. More recently, the immunomodulatory effect of YGG was confirmed in vivo by the observation that the peptide administration modulated the delayed-type hypersensitivity responses to tuberculin derivatives in hairless guinea pigs [142]. It is noteworthy to observe that YGG seems to have a biphasic effect on the parameters studied so far, as it showed an enhancing effect at low doses and an inhibitory effect at higher doses [143, 144]. It
should be noted that YGG is contained several times in the primary structure of bovine β-casein and α-lactalbumin and it could be released during milk fermentation or gastrointestinal digestion from the precursor proteins. In addition, it is a tripeptide and, as already demonstrated for other milk-derived bioactive peptides [145], it can be assumed that it can pass across the intestine by a carrier-mediated peptide transport system in quantitatively significant amounts and, hence, may reach peripheral target sites.

2.5.2.4.2 β-CN (193-209) peptide with immunomodulatory activity:

The β-CN (193-209) peptide is released from the C-terminal end of β-casein by hydrolysis with pepsin-chymosin. It is a 17 residues long peptide with the amino acid sequence Tyr-Gln-Glu-Pro-Val-Leu-Gly-Pro-Val-Arg-Gly-Pro-Phe-Pro-Ile-Ile-Val. This peptide was isolated and identified from yoghurt and fermented milks as well as several types of cheese including Feta and Camembert [146]. This peptide displays immunomodulatory properties and shows mitogenic activity on primed lymph node cells and unprimed rat spleen cells, it manifests chemotactive activity on L14 lymphoblastoid cell line and enhances phagocytosis in rat macrophages. In addition, a smaller fragment of β-CN (193-209), corresponding to the amino acid sequence Gly-Pro-Val-Arg-Gly-Pro-Phe-Pro-Ile-Ile, displayed ACE-inhibitory activity, further supporting the concept that ACE-inhibitors may also act as immunomodulatory peptides by acting as bradykinin-potentiating peptides [147]. Interestingly, the presence of 4 proline residues within the sequence can protect the long peptide β-CN (193-209) from the action of peptidases. So it could be possible that this peptide can cross the intestinal barrier in an intact bioactive form.

2.6 Prebiotics and Probiotics:

According to Gibson and Roberfroid, prebiotics are defined as a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon [158]. Recently, some
researches have been conducted to manipulate beneficial bacteria in gastrointestinal tract. Folkenberg et al., 2006 [159] suggested that the use of prebiotics is a promising approach for enhancing the role of endogenous beneficial organisms in the gut. They can be used as potential alternatives to growth promoting antibiotics. Several reports have shown that supplementing a diet with oligofructose (OF) improved growth in nursery pigs and in weaned pigs while other reports by Vinderola et al., 2005 [160] did not find growth effect in young pigs. The reasons for the different results are not clear yet. It may be due to the different chemical structure (degree of polymerization, DP) and compositions of the OF used. Ganji et al., 2004 [161] reported that the site in the gut of pigs where the fermentation of OF occurs depend on the molecular structure of the non-digestible carbohydrates. Bifidobacteria may preferentially utilize non-digestible oligosaccharides with a lower DP, whereas bacteroides degrade preferentially oligosaccharides with a higher DP. Thus, it was hypothesized that OF with a low DP is (DP= less than 10), may be more beneficial than FOS with a high DP (DP=10~60) for the development of bifidobacteria in the large intestine of young pigs. It appears that the places of fermentation in the small intestine and large intestine will determine the conditions in these parts of the GIT.

Probiotics are live microbial feed supplements which beneficially affect the host animal by improving its microbial balance. Probiotics have been reported to increase feed intake, growth, immune responses, the numbers of lactobacilli and decrease the numbers of E. coli [162].

2.7 Fermentations and microorganisms:

Fermenting fruits and vegetables can bring many benefits to people. They play an important role in providing food safety, enhancing health and improving the nutrition and social well-being of millions of people around the world. Lactic acid bacteria are the most important bacteria in desirable food fermentations, being responsible for the
fermentation of sour dough bread, sorghum beer, all fermented milks, and most "pickled" (fermented) vegetables. *Lactobacillus acidophilus, Lb. bulgaricus, Lb. plantarum, Lb. pentoaceticus, Lb. brevis* and *Lb. thermophilus* are examples of lactic acid-producing bacteria involved in food fermentations. Some of the species are homo-fermentative, because they produce lactic acid only, while others are hetero-fermentative and produce lactic acid plus other volatile compounds and small amounts of alcohol.

*L. mesenteroides* is a bacterium associated with the sauerkraut and pickle fermentations. This organism initiates the desirable lactic acid fermentation in these products. *Leuconostoc mesenteroides* produces carbon dioxide and acids which rapidly lower the pH and inhibits the development of undesirable microorganisms. The carbon dioxide produced replaces the oxygen, making the environment anaerobic and suitable for the growth of subsequent species of lactobacillus. Several other bacteria, for instance *Leuconostoc citrovorum, Streptococcus lactis* and *Brevibacterium* species are important in the fermentation of dairy products. Most lactic acid bacteria work best at temperatures of 18 to 22ºC and tolerate high salt concentrations. The salt tolerance gives them an advantage over other less tolerant species and allows the lactic acid fermenters to begin metabolism, which produces acid that further inhibits the growth of non-desirable organisms. In general, bacteria require a fairly high water activity (0.9 or higher) to survive.

The advantages of the use of starter cultures against spontaneous fermentation are well known and widely spread especially for dairy and meat products, but are not often used in the vegetable fermentations. The spontaneous fermentation of sauerkraut can result in the formation of biogenic amines. The utilisation of starter cultures enables producers to make food products with a standard quality in a shorter time. Selection of starter culture, however, should not be only done considering the lactic acid production of the strains but also their activity for biogenic amine synthesis. Several authors have investigated histamine concentration in commercial sauerkraut samples. Canzi et al.,
analysed 50 samples and detected histamine in the range of 9-130 mg/kg. Furthermore the histamine levels increased after fermentation for 10 weeks. Red beet is a well-known vegetable which is a considerable source of vitamins C and B, and minerals such as K, Fe, P and Mg. In addition, red beet contains natural pigments (betalains) which have several biological activities, for example modification of blood pressure and antitumor effect as per Apostolids et al., 2006 [164]. Although numerous studies have been carried out on cabbage, olive and pickle fermentation, little is known on the lactic fermentation of other vegetables. Usually, lacto fermented vegetables are pasteurized and there is no information on the behaviour of lactobacilli during storage of unpasteurized fermented vegetables. With fermentation of beetroot by appropriately selected lactobacilli a juice could be produced which combines benefits of betalains and lactobacilli.

2.8 Probiotics and their role in the human health:

The gastrointestinal (GI) microflora plays an important role in the health status of people and animals. The GI tract represents a much larger contact area with the environment, compared to the 2 m² skin surface of our body [165]. The mucosal surface of the small intestine is increased by forming circular folds, intestinal villi and the formation of microvilli in the enterocyte resorptive luminal membrane. The resulting surface of the GI system is calculated to be 150-200 m² [166], therefore it provides enough space for the interactions related to the digestion and for adhesion to the mucosal wall. It is estimated, that about 300-400 different cultivable species belonging to more than 190 genera are present in the colon of healthy adults. Among the known colonic microbial flora only a few major groups like 'main flora' dominate at levels around 10¹⁰ - 10¹¹/g, all of which are strict anaerobes such as Bacteroides, Eubacterium, Bifidobacterium and Peptostreptococcus [167]. Facultative aerobes are considered to belong to the subdominant flora, constituting Enterobacteriaceae, lactobacilli and streptococci. Minor groups of pathogenic and opportunistic organisms, the so-called 'residual flora' are
always present in low numbers. Bacteria present in the 'normal' intestinal flora may exert beneficial effect and are able to degrade certain food components, produce certain B vitamins, stimulate the immune system and produce digestive and protective enzymes. The normal flora also takes part in the metabolism of some potentially carcinogenic substances and may play a role in drug efficacy. In the last few decades there is an increasing interest for influencing the composition of the gut microflora by foods or food ingredients. The goal of these attempts is to induce the number and the activities of those microorganisms which possess health promoting properties, such as *Lactobacillus* and *Bifidobacterium* species [168]. The health promoting effect of *lactobacilli* was first hypothesized at the beginning of last century. In the last four decades there have been growing attempts to improve the health status of the indigenous intestinal flora by live microbial adjuncts, "probiotics." Although a number of definitions for probiotics have been proposed, an appropriate one was suggested by Makino *et al.*, 2006 [169], according to which probiotics are defined as "mono- or mixed cultures of live microorganisms which, when applied to animal or people, beneficially affect the host by improving the properties of the indigenous microflora". This definition does not restrict 'probiotic' activities to the intestinal microflora, but also to the other sites of the body and it might consist of more than one bacterial species. A new definition for probiotics may better characterize both the specific strains and components used for probiotic purposes. Perdigon and coworkers in 2003 [170] proposed that "probiotics are microbial cell preparations or components of microbial cells that have beneficial effect on the health and well-being of the host". The probiotics do not have to be viable as non-viable forms of probiotics have also been shown to exert health promoting effect.

### 2.8.1 Pathogens and the intestine:

Interactions between the host cells and the pathogenic bacteria initiate infectious diseases. Several enterovirulent bacteria by different physiopathological mechanisms are able to increase the volume of water in stools, resulting from the imbalance between the
processes of intestinal absorption and secretion of water [172]. Important feature of *Salmonella* and other genera is the flagella which confer motility to the bacterium and so contribute to the colonization of pathogen. The filament of members of the genus *Salmonella* is a multimer of a single protein, the flagellin. Comparison of the amino acid sequences of *Salmonella* flagellins led to the definition of 8 regions of different variability. The flagellins play an important role in holding the flagellum together.

### 2.8.1.1 *Salmonella* infection in human:

Attachment of pathogen bacteria to intestinal epithelial cells is the first step of bacterial pathogenicity. It requires specialized factors encoded by the bacteria which directly bind to host cell receptors [173]. Attachment of pathogenic bacteria to intestinal epithelial cell surfaces can lead to colonization, cell damages, internalization, intracellular proliferation and disturbances of regulatory cell mechanisms. The invasive bacteria cross the epithelial membrane, proliferate and promote cell death and exfoliation. Due to these effects the mucosal surface is reduced and is characterized by a large number of immature enterocytes. It was shown that some *Salmonella* spp. is sensitive against the organic acids produced by the *Lactobacilli* and it produces other metabolites with anti-*Salmonella* properties, for example *Lb. acidophilus* LB secretes a compound into the growth medium with a broad inhibitory spectrum. Wellman and coworkers in 2003 [174] performed one of the most convincing animal studies. Ninety percent of conventional mice fed with *Lb. rhamnosus* HN001 survived the single dose *Salmonella* challenge while only 7% of control mice survived. It was shown that leucocyte phagocytosis responses significantly increased and *Salmonella* translocation decreased in the visceral tissue after administering of probiotics. Similar mechanism was observed for *E. coli* and *Shigella sp.*

### 2.8.1.2 *Salmonella enterica* serotype *enteritidis*:

*Salmonella enterica* serotype *enteritidis* (*Salmonella enteritidis, S. enteritidis*) is a
facultative anaerobe Gram negative rode-shaped bacterium and it belongs to the family *Enterobacteriaceae*, trivially known as "enteric bacteria". Some species are ubiquitous. Other species are specifically adapted to a particular host. In humans, *Salmonella* are the cause of two diseases called salmonellosis: (i) enteric fever (typhoid), resulting from bacterial invasion of the bloodstream, and (ii) acute gastroenteritis, resulting from foodborne infection. Similar mechanism was observed for *E. coli* and *Shigella sp.*

### 2.8.2 Therapeutic effects of probiotics:

Several clinical studies [177,178] have investigated the application of probiotics, especially lactobacilli and bifidobacteria, as dietary supplements for the prevention and treatments of several gastrointestinal diseases.

**Photo 2.4 Invasion of *S. enteritidis* 857 to Caco-2 cells (A: Intact microvilli of Caco-2 cells; B and C: Rearrangements of cytoskeleton with the formation of membrane ruffles; D: *S. enteritidis* 857 are present in the vacuoles)**
2.8.2.1 Acute gastroenteritis:

Rotavirus is one of the most common causes of acute childhood diarrhoea worldwide. After invasion in the small intestinal epithelium the rotavirus replicates and causes the partial disruption of the intestinal mucosa with the loss of microvilli and decrease in the villus /crypt ratio. The most often studied gastrointestinal condition treated by probiotics is acute infantile diarrhoea.

2.8.2.2 Inflammatory bowel disease:

Several lines of observational and experimental evidence implicate the normal flora in the pathogenesis of Crohn's disease and ulcerative colitis [179]. Crohn's disease is a chronic and idiopathic inflammation of the gastrointestinal tract with characteristic patchy transmural lesions containing granulomas. The outbreak of Crohn's disease is thought to require genetic predisposition, immunological disturbance and the influence of intraluminal triggering agent(s), e.g. bacteria or viruses.
2.9 Allergic diseases:

Atopic dermatitis is a common, complex, chronically relapsing skin disorder of infancy and childhood. The prevalence of atopic diseases has been progressively increasing in Western societies. The regulatory role of probiotics in human allergic disease was first emphasised in the demonstration of a suppressive effect on lymphocyte proliferation and IL-4 generation in vitro [180]. The preventive potential of probiotics in atopic disease has been shown in a double blind, placebo controlled study by Schaer et al., 2003 [181]. Administration of probiotics to pregnant women and postnatally to infants for six months at high risk of atopic diseases succeeded in reducing the prevalence of atopic eczema to half compared with that in infants receiving placebo.

2.10 Lactic acid bacteria and the immune system:

The intestinal epithelium with optimal intestinal flora serves as the first line of defence against the invading pathogenic microorganisms, antigens and harmful components from the gut lumen. In addition the mucosal surface of the intestine is essential for the assimilation of antigens. Proteases of the intestinal bacteria degrade the antigenic structure, an important step in the introduction of unresponsiveness to dietary antigens. Specialised antigen transport mechanisms take place in different intestinal lymphoid compartments: mesenteric lymph nodes, Peyer's patches, isolated lymph follicles, isolated T lymphocytes in the epithelium and the lamina propria, as well as at secretary sites [175]. The secretary IgA antibodies in the gut are part of the common mucosal immune system, which includes the respiratory tract, salivary and mammary glands. The hallmark of an inflammatory response is the generation of proinflammatory cytokines including interleukin-1, interleukin-2, tumor necrosis factor-α (TNF-α) and interferon-γ. There are several reports indicating that proinflammatory cytokines may be the primary mediators of inflammation in clinical conditions characterized by impaired gut
barrier functions. It has in fact been demonstrated by Zisu et al., 2005 [176] that probiotics participate in the exclusion of pathogens. They can help to stabilize the gut microbial environment by producing antimicrobial substances and binding pathogens thereby preventing the generation of inflammatory mediators produced by intraluminal bacteria (Photo 2.6). Attachment of probiotic lactobacilli to cell surface receptors of enterocytes also initiates signalling events that result in the synthesis of cytokines.

2.10.1 Role of cytokines in the immune response:

Inflammation, the response of tissue to injury, is characterized in the acute phase by increased blood flow and vascular permeability along with the accumulation of fluid, leukocytes and inflammatory mediators, such as cytokines. IL-4, IL-5, IL-6, IL-7, IL-13 are the cytokines mediating humoral responses and IL-1, IL-2, IL-3, IL-4, IL-7, IL-9, IL-10, IL-12, interferons, transforming growth factor-β, TNF-α and-β mediate cellular responses.

2.11 Interactions between epithelial cells and intestinal microflora:

The interface between a mammalian host and microflora in the lumen is the mucous gel layer and the underlying cell coat (glycocalix) which consists of glycoconjugates on the apical surface of the epithelium. The intestinal microflora can influence the expression of epithelial glycoconjugates which serve as receptor for attachments of pathogenic microorganisms. There are several studies related to the adhesion mechanisms of pathogenic bacteria by fimbrae (pilus) or flagella, but little is known about the adhesion mechanisms of non-pathogenic bacteria such as lactic acid bacteria. Cesena et al., 2001 [182] suggested that lectin-like components in surface-layered proteins of lactobacilli play an important role in the adhesion to receptors such as glycoproteins on the surface of intestinal epithelial cells.
2.12 Casein Phosphopeptide and its uses:

CPP are a large group of peptides that have a phosphoseryl residue in common. Phosphopeptides are formed either from casein by proteolytic enzymes during fermentation or in the gastrointestinal tract. CPP increase calcium absorption by forming a hydrophobic complex with calcium, thus preventing the formation of insoluble calcium phosphates. In vitro studies have shown the effects of CPP on calcium absorption by inhibiting the precipitation of calcium in the intestine. Casein phosphopeptides (CPP) are a tryptic hydrolysate of bovine casein, which enhances calcium absorption by increasing calcium solubility in vitro according to Drago et al., 1997 [183]. However, reports on the effect of dietary CPP on calcium absorption in vivo are controversial. Most of the reports have failed to show any enhancing effect of CPP on calcium absorption and retention in vivo. In contrast, Chung et al., 2002 [184] reported that CPP administration was associated with better absorption of co-ingested calcium by postmenopausal women with low basal absorptive performance. It was demonstrated that calcium bound to phosphopeptides could be absorbed from the digestive tract and promote bone calcification in rachide children. The purpose of this study was to evaluate the calcium and phosphorus availability from Cabound casein phosphopeptides (CaCPP) by testing the effect of long-term feeding on the bone loss in aged ovary ectomized rats.

In vitro studies demonstrated that CPP can prevent the precipitation of calcium ions as insoluble salts such as calcium phosphate. This suggested the possibility that CPP enhance the amount of soluble calcium in the intestinal lumen, thereby increasing the mineral availability for absorption in the small intestine. Experiments performed on intestinal preparations (everted sacs, loops) provided evidence supporting this possibility [185]. However, in vivo investigations performed on whole animals designed to ascertain a role of CPP in both absorption and bioavailability of calcium, generated some controversial results. In fact, studies on growing pigs, as well as on weaning and adult (female) rats, showed that diets supplemented with CPP influenced neither calcium
absorption nor bone mineralization. On the contrary, rats fed a CPP-supplemented soybean protein diet had significantly greater calcium absorption than controls fed soybean alone. Moreover, the bioavailability of calcium appeared to be increased by CPP-enriched infant formula in rat pups, and the presence of CPP in the diet prevented mineral density decline in old ovary ectomized female rats. Finally, CPP were shown to enhance calcium absorption in both rachitic and normal chicks [186]. Interestingly, CPP also induced Ca\textsuperscript{2+} uptake by boar spermatozoa, facilitating sperm penetration into pig oocytes; the effect was reduced by dephosphorylation of CPP.

Tamime et al., 1985 [187] stated that CPP were generally viewed as agents capable of maintaining intestinal calcium in its "soluble" form, thus facilitating the mineral flux through the membranes. However, the presence or absence of substances in the diet such as phosphate or phytate, that are capable of forming insoluble calcium salts or complexes, was not accurately assessed. This may be the basis for the conflicting results in in vivo studies. No determination was made of the direct interactions of CPP with the plasma membrane (particularly that of intestinal cells), which might affect calcium flux through the same membrane, regardless of any calcium-solubilizing action. The present work was designed to explore the possibility of a direct CPP influence on calcium uptake, using as a study model the human intestinal tumor cell line, HT-29, which tends to undergo an enterocytically oriented differentiation in culture. Calcium uptake was monitored as a rise in free cytosolic calcium concentration due to calcium ion movement through the plasma membrane.

CPP has always been a widely studied peptide group in dentistry [188]. CPP also has been researched in the areas of sports medicine, anti-hypertensive medicine, remineralisation, and immune-enhancement and immune modulation [189]. The concept of food based nutrition has been practiced and advocated in India from the time immemorial and this has again gained momentum in the recent past due to social trends such as globalization, booming economy, growing purchase power of Indian middle
class. CPP has the potential of becoming a food based nutritional item and boosting the immune system of humans. Virtual problem associated with any food based item is that it has less shelf life, chances of infection by micro organism become quite high and dissipation rate also increases considerably. If CPP is isolated from the fermented milk, then it reduces the dissipation rate, increases shelf life and the risk involved with the micro organisms decreases considerably [190]. Various parameters like viscosity, titratable acidity and pH are important to be studied and standardized before commencing the work. Viscosity of fermented milk that is prepared in a domestic environment will be lower than the fermented milk which is produced in a commercial firm.

Milk fermented by lactic acid bacteria (LAB) have previously been shown to enhance both specific and nonspecific immune responses. Though most related studies focus on the administration of live bacteria, there is a lack of recognition of the possible Immunomodulatory role of the bioactive peptides or other compounds released in the culture medium during fermentation with LAB. Indeed, many beneficial effects have been attributed to bioactive peptides derived from milk, including opiate activity, antimicrobial activity, antihypertension, antithrombotic activity, and immunomodulation. Cell-free supernatants have been used to study the possible role of bioactive compounds released during milk fermentation. Hugenholtz et al., 1999 [191] reported that cell-free supernatants of Lactobacillus helveticus - fermented casein-enriched medium modulated lymphocyte proliferation in vitro. In parallel, De Vin in the year 2005 [192] used cultured macrophages to demonstrate that cell-free supernatants of L. helveticus-fermented milks exhibit higher interleukin-6 (IL-6) production than with lipopolysaccharide alone. More recently, peptide fractions of cell-free supernatants of L. helveticus-fermented milks have been shown to significantly reduce fibro sarcoma in vivo. However, cell-free supernatants of L. helveticus-fermented milks have not yet been implicated in the prevention or attenuation of bacterial infections in vivo.
2.13 Anti-genotoxicity of CPP:

2.13.1 Classification of radioprotective agent:

Radioprotective agents can be classified as:

(i) chemical radioprotectors,

(ii) adaptogens, and

(iii) absorbents

The first group constitutes mainly sulf-hydryl compounds and other antioxidants. Adaptogens act as stimulators of radioresistance. These are natural protectors that offer chemical protection under low levels of ionizing radiations. They are generally extracted from the cells of plants and animals and have least toxicity. They can influence the regulatory system of exposed organisms, mobilize the endogenous background of radioresistance immunity, and intensify the overall nonspecific resistance of an organism. Absorbents protect organisms from internal radiation and chemicals.

These include drugs which prevent the incorporation of radioiodine by the thyroid gland and the absorption of radionuclides like 137Cs, 90Sr and 239Pu. Post-irradiation radioprotectors are important when an accidental exposure occurs during operation of equipments with radiation source or intentional exposures during war and such unnatural calamities. This area of radiation biology is a very slowly developing area since it is rather difficult to get such effective protectors.

2.13.2 Milk and fermented milk as an anti-genotoxic agent:

In the beginning of the 20th century, the Russian Nobel prizewinner Élie Metchnikoff observed high life expectancy in Bulgarian persons who ate large amounts
of fermented-milk products. One hundred years later, the consumption of fermented-milk products is still associated with several types of human health benefits [193]. In addition to the favorable effects against diseases caused by an imbalance of the gut microflora, several experimental observations have indicated a potential protective effect of lactic acid bacteria (LAB) against the development of colon tumors. Colon cancer is the second to third most frequent type of cancer in Western industrialized countries. Within the complex gut microflora, which consists of above $10^{11}$ CFU living bacteria/g colon content, LAB belong to those bacteria with such beneficial effects. LAB plays an important role in retarding colon carcinogenesis by possibly influencing metabolic, immunologic, and protective functions in the colon. Concentrations of LAB may increase in the colon after the consumption of foods containing probiotics; however, probiotic ingestion also increases the number and metabolic activity of LAB in the colon of humans and animals [194]. In animals, LAB ingestion was shown to prevent carcinogen-induced preneoplastic lesions and tumors.

A reduced activity of pro-carcinogenic enzymes in humans also was shown as a consequence of probiotic intake [195]. However, in humans, there is no evidence available on whether probiotics and prebiotics can prevent the initiation of colon cancer. Epidemiologic studies are contradictory; some studies could not find an association between the consumption of fermented-milk products and the risk of colon cancer whereas other studies showed a lower incidence of colon cancer in persons consuming fermented-milk products or yogurt [196]. In one case-control study, yogurt was the only milk product inversely related to the formation of large adenomas [197]. Therefore, the hypothesis that LAB may reduce the risk of developing colon tumors in humans is based mainly on experimental data. Within this context, it is postulated that the protective effects of probiotics and prebiotics can be effectively used in the near future.
2.13.3 Enzymes and their anti-genotoxic mechanism:

Oxidase and catalase enzymes have the potential to be useful as anti-genotoxic agents. Catalase enzyme prevents the nuclear degeneration and thus indirectly preventing the formation of micro nucleus. Alander et al., 1999 [166] have established the anti-genotoxic role of catalase enzyme through a series of test and have also stated that the possible mechanism by which the catalase enzyme carries out its role is by preventing nuclear degeneration of the cell which has been exposed to a genotoxic agent. Work carried out by Perdigon et al., 2003 [170] confirmed the anti-genotoxic role played by oxidase and they state that the possible mechanism of action by which oxidase enzyme is able to bring about the anti-genotoxic role is by stimulating a cellular level resistance which eventually leads to the anti-genotoxicity.