Македонски Фармацевтски билтен

Macedonian Pharmaceutical Bulletin

волумен **60** (2) 2014 / volume **60** (2) 2014

Македонско фармацевтско друштво, ул. Маршал Тито 13б/8, Скопје, Македонија
Macedonian Pharmaceutical Association, Marshal Tito 13b/8, Skopje Macedonia

Macedonian pharmaceutical bulletin, **60** (2) 3 - 19 (2014)
ISSN 1409 - 8695
UDC: 615.246:616.34-002
616.34-002-085.246
Review paper

Probiotics, prebiotics, synbiotics in prevention and treatment of inflammatory bowel diseases

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Received: July 2014; Accepted: September 2014

Abstract

Probiotics, prebiotics, and synbiotics are functional components able to exert positive effects on human health. Numerous medical conditions lack effective and safe approaches for prevention or treatment, thus usage of probiotics, prebiotics, and synbiotics is an alternative. Further, the benefit related to the consumption of these compounds is associated with lower morbidity of chronic diseases and reduced health-care costs. Various types of mediums to deliver probiotics/synbiotics to the human GIT are used. Although capsules and tablets are frequently applied as delivery systems for probiotics, the major challenge of the commercial sector is to market new functional foods containing probiotics and/or prebiotics. Discovering of new probiotic/synbiotic functional foods is connected to the interest of the food industry to revitalize continuously through introduction of products with improved nutritional value and pleasant taste, but also with health benefit for the consumers. The review provides insights and new perspectives in respect to usage of functional components and foods in prevention and treatment of inflammatory bowel diseases (IBD) that are highly correlated with the modern lifestyle. The therapeutic and safety properties of probiotics and prebiotics, their role in pathogenesis of IBD, potential to prevent and treat these diseases as well as postulated mechanisms of action will be discussed, highlighting the main areas in which further research is an emergence.

Keywords: probiotic, prebiotic, synbiotic, functional foods, inflammatory bowel disease

Introduction

The primary role of human diet is to provide sufficient nutrients that supply energy to maintain physiologic processes and well-being. At this level all foods are functional and the intake of some bioactive ingredients like unsaturated fatty acids, fibers, vitamins and essential minerals is enabled by common diet. However, there is a new concept comprising specific features of the use of foods to promote optimal health and reduce the risk of diseases. Foods that contain compounds known to provide additional benefit beside the basic nutrition are classified as functional foods. Probiotics and prebiotics and both together termed synbiotics are bioactive components attracting much attention nowadays, although their use as fermented foods containing beneficial microbes, particularly lactic acid bacteria have been used by humans for thousands of years. Scientific evidence about the impact of beneficial microorganisms on the well-being of humans dated back 1990s (Ouwehand and Röytiö, 2015). Actually, the concept of functional food was introduced in Japan on the proposal of Ministry of Health and Welfare due to the

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escalation of healthcare costs and aiming improved quality of life of elderly people during the 1980s as foods for specific health use (FOSHU) (Ashwell, 2002). In general, functional foods include conventional foods, modified foods (fortified, enhanced, or enriched), medical foods, and foods for special dietary use (ADA, 2009). Economic growth increases the profit and expenditure improving the quality of life, but implicates diseases associated with the lifestyle. It is well known that the modern lifestyle, increased use of drug and drug toxicity and risks of adverse effects initiate the development of functional foods and pharmaceuticals as safe approach to improve the health. This leads to evolution of new era in healthcare system with the food industry gaining the research role similar as pharmaceutical industry.

Alternative application of probiotics, prebiotics and synbiotics in prevention and treatment of different diseases engendered the researchers to study the benefits and risks related to the consumption of these functional compounds. Various mechanisms of actions of probiotics and prebiotics contributing to improved health have been postulated (Fig. 1). Probiotics have demonstrated efficacy for a number of inflammatory conditions, including arthritis (So et al., 2008), necrotizing enterocolitis (Ezaki et al., 2008), atopic dermatitis and eczema (Kukkonen et al., 2007; Wickens et al., 2008), ulcerative colitis (UC) (Pronio et al., 2008; Fujimori et al., 2009) and Crohn's disease (CD) (Fujimori et al., 2007). Probiotics are known to reduce the symptoms of lactose intolerance (He et al., 2008), while their antihypertensive effects, anticholesterolemic and anticarcinogenic effects are subject of intensive research (Gill and Prasad, 2008; Lye et al., 2009). However, further research is essential as the data originating from the studies with experimental animals and clinical studies with relatively low number of subjects are insufficient to derive evident conclusions. Observed effects for one probiotic strain are specific and cannot be extrapolated to another strain of the same species or genus without confirmation in separate studies (Aureli et al., 2011). The standpoint of strain-specificity of properties of different probiotic species further complicates the evaluation of probiotic effects as well as their potential application in prophylaxis and treatment of diseases.

Development of new therapeutics, including probiotics and prebiotics, which can reduce intestinal inflammation and restore balance of the gastrointestinal microbiota

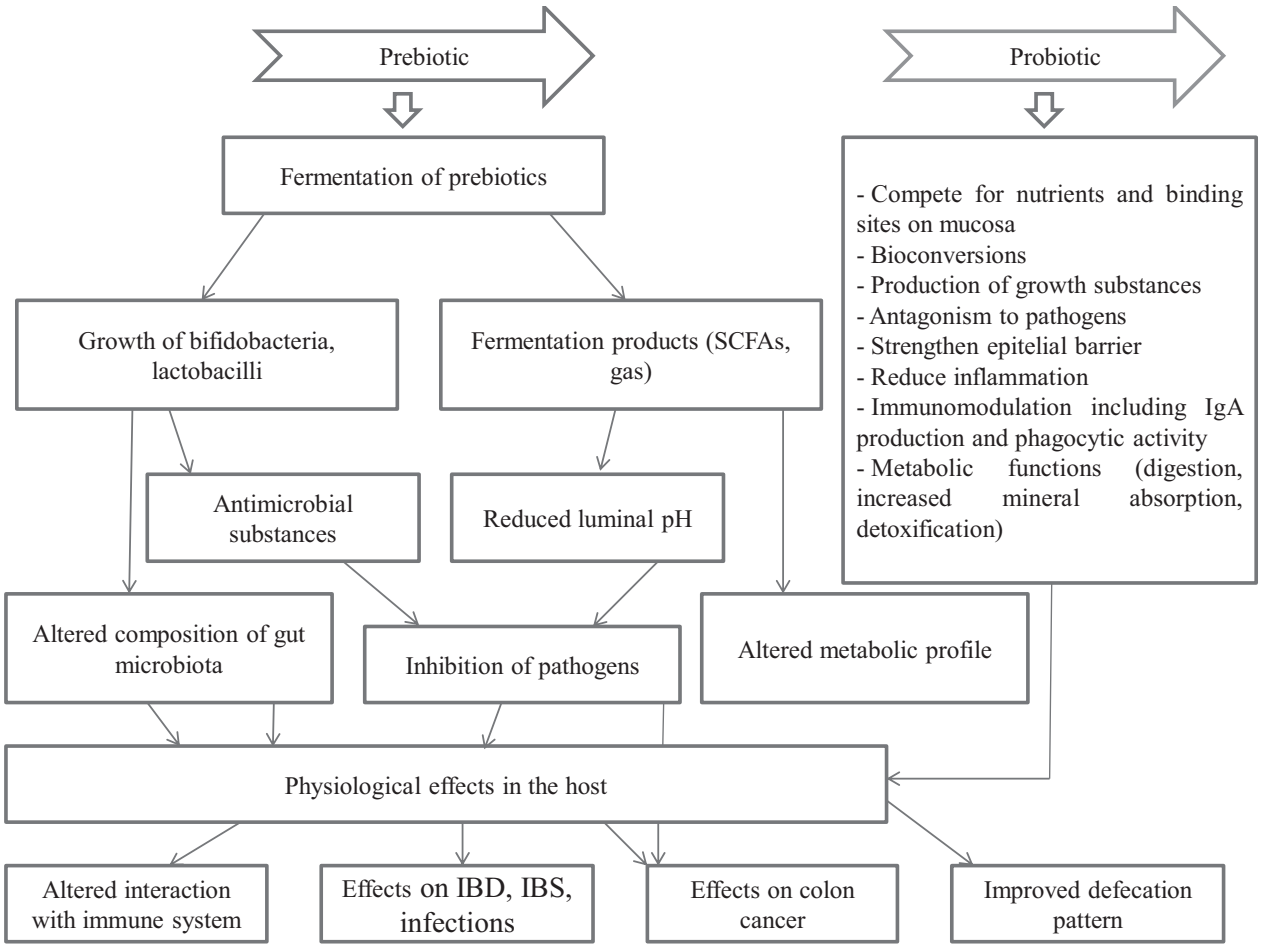

Fig. 1. Mechanisms of action of probiotics and prebiotics.

due to increased prevalence of IBD with the socioeconomic development in Western society (Looijer-van Langen and Dieleman, 2009; Prisciandaro et al., 2009; Shanahan, 2012). Interactions among the immune system, the gut microbiota and the host genotype are thought to underlie the pathogenesis of IBD. A large number of host susceptibility genes responsible to maintain the mucosa barrier function have been identified (Wapenaar et al., 2008). Genetic predisposition with a loss of antigen tolerance is characterized by alteration in the pattern of cytokine production by T cell subclasses leading to overlay aggressive T cell responses to a subset of commensal enteric bacteria and misbalance between beneficial and pathogenic enteric bacteria (Kaser and Blumberg, 2011; Adolph et al., 2013). This aggressive T cell response followed by immune activation, mucosal damage and permanent inflammation that usually develops in genetically susceptible individuals is considered the main factors involved in IBD etiology together with the environmental factors. Environmental factors responsible for the onset and reactivation of IBD includes lifestyle, diet, socio-economic conditions, and use of non-steroid anti-inflammatory drugs, psychological stress and the presence of caecal appendix (Strober et al., 2007; Neuman and Nanau, 2012). The current therapy strategies include administration of antibiotics, 5-aminosalicilates, non-steroid anti-inflammatory drugs like mesalazine, steroid drugs like cortisol and immune-suppressive agents, which reduce symptoms of IBD (abdominal pain, bleeding, diarrhea), but are often associated with severe side effects (Hörmannsperger and Haller, 2010). Although the precise etiology of IBD remains unknown, the major role of gut microbiota in development and persistence of IBD highlighted the importance of interactions between microbiota and host in health and diseases. In this respect, modification of intestinal microbiota composition by using probiotic organisms in order to restore tolerance to microbial antigens of the host's own microbiota is extensively explored. The induction of regulatory versus effector immune responses at the gut mucosa can be modulated by diet-induced changes in the composition of the gut microbiome. Therefore, modulation of gut microbiota composition with fermented milk products, probiotics and prebiotics may contribute to improved health, reduction of diseases or disease symptoms and support of established treatments (Ceapa et al., 2013) and is being researched as a promising prophylactic and therapeutic tool against gut inflammation. This new therapeutic approach is supported by the ability of the intestinal microbiota to regenerate itself completely (Guarner and Malagelada, 2003) and also by multifactorial mechanism of the disease. Main factors enrolled in pathogenesis of IBD and therapeutic strategies are schematically presented in Fig. 2.

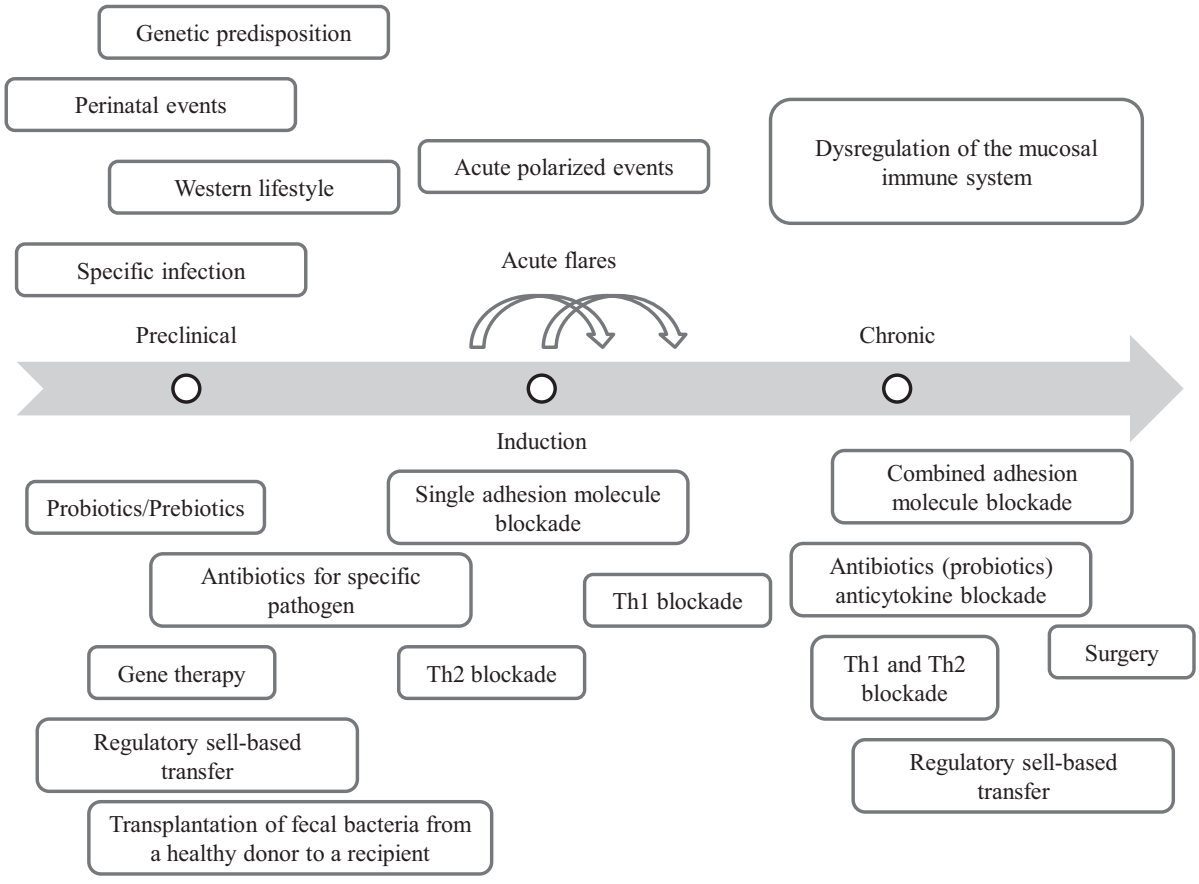

Fig. 2. Ethiopathogenesis and therapeutic strategies of IBD.

The concept and development of probiotic/synbiotic foods, evaluation of their characteristics as well as their safety aspects are discussed in this paper. Therapeutic relevance of probiotics, prebiotics and synbiotics in the context of IBD and evidences of their efficacy in a form of pharmaceuticals and functional foods are also discussed. Moreover, areas of research that are emerged to be further investigated are pointed.

Probiotics, prebiotics, synbiotics

Positive effects of lactic acid bacteria on human health were firstly recognized in the 19th century when the Russian Nobel Elie Metchnikoff established the concept of probiotics (Metchnikoff, 1907). Lactic acid bacteria can produce lactic acid from sugars by fermentation and the metabolism of these microorganisms was used for fermentation and preservation of food for centuries. Metchnikoff believed that the long life of the Bulgarian farmers due to the consumption of fermented dairy products. Almost in the same time, the French scientist Tissier has found the positive relationship between bifidobacteria present in the infant microflora fed by mother's milk and improved symptoms of diarrhea (Tissier, 1984). These scientifically confirmed data explained prescribing of yoghurt to treat diarrhea and other intestinal disorders by Hippocrates, as well as existence of some scriptures documented the usage of yoghurt in treatment of different illnesses (Lourens-Hattingh and Viljoen, 2001).

Probiotics are defined as live microorganisms that, when administered in adequate amounts, confer a health benefit to the host (FAO/WHO, 2001). In addition, it is assumed that probiotics improve microbial balance in the intestines. The level of viable probiotics needed to obtain a clinical effect is often quoted as $\geq 10^6$ cfu/ml in the small bowel and $\geq 10^8$ cfu/ml in the colon (Bertazzoni Minelli and Benini, 2008). *Lactobacillus* and *Bifidobacterium* strains are the most used as probiotics (Ranadheera et al., 2010). Lactic acid bacteria grow optimally at pH 5.5-5.8 and their growth is supported in the presence of the nutritional compounds like amino acids, fatty acids, peptides, nucleotide basis, vitamins and minerals (Hayek and Ibrahim, 2013). Some species are aero-tolerant and may utilize oxygen through the enzyme flavoprotein oxidase, while others are strictly anaerobic. Other microorganisms also known to possess probiotic properties include *Escherichia coli* Nissle, *Saccharomyces boulardii*, *Streptococcus thermophilus*, *Enterococcus francium*, *Propionibacterium*, *Pediococcus*, and *Leuconostoc*, but some strains of these microorganisms are known to be pathogenic (Senoc et al., 2005).

Probiotic organisms evaluated using *in vitro* and *in vivo* evaluation tests prove that they show: resistance to gastric acidity, bile acid resistance and resistance to intestinal enzymes, adherence to mucus or human epithelial cells and cell lines, antimicrobial activity against potentially pathogenic bacteria, ability to reduce pathogen adhesion to surfaces, bile salt hydrolase activity and resistance to spermicides if applied for vaginal use (Saarela et al., 2000; Petrushevska and Mladenovska, 2009). Production of pathogen-inhibitory substances, competition with pathogenic bacteria for epithelial adhesion sites, nutrient competition and production, degradation of toxins and toxin receptors and modulation of immune and non-specific host responses are several health protection mechanisms of probiotics able to exert within the gut (Prakash et al., 2011).

According to the most recent definition "A prebiotic is a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host well-being and health" (Gibson et al., 2004; Macfarlane et al., 2006). Several criteria have to be fulfilled when a substance is to be classified as prebiotic: safety, stability, organoleptic properties, resistance to digestion in the upper bowel and fermentability in the colon, as well as the ability to promote the growth of beneficial bacteria in the gut (Gibson, 2004). Carbohydrates as oligofructose, fructo-oligosaccharides (FOS), inulin, galacto-oligosaccharides (GOS), transgalacto-oligosaccharides, soybean-oligosaccharides, gluco-oligosaccharides, xylo-oligosaccharides, gentio-oligosaccharides, isomalto-oligosaccharides, lactulose and polysaccharides as starch and pectins are considered to be effective prebiotics (de Vrese and Schrezenmeir, 2008). However, majority of studies on prebiotics have focused on inulin-type fructans (inulin, FOS) and GOS which selectively stimulate the growth of bifidobacteria (Guarner, 2013) and have been linked to long-lasting safe commercial use (Macfarlane et al., 2008). These compounds are most often used as prebiotics and specified as bifidogenic oligosaccharides (Birkett and Francis, 2010). Bifidogenic oligosaccharides reaching the colon intact are fermented to short chain fatty acids (SCFAs) and followed by production of gases by colonic bacteria (Meyer and Stasse-Wolthuis, 2009). These products of carbohydrates fermentation, acetate, propionate and butyrate as the main SCFAs and lactate as important intermediate in the formation of SCFAs are thought to be beneficial to host health. Chemical conversion is one mechanism of the prebiotic action to generate a bifidogenic shift in the colonic microbiota. However, certain oligosaccharides are able to selectively prevent adhesion of pathogen bacterial species by mimicking binding sites (Saulnier et al., 2009) and others inhibit the expression of genes for some enteropathogens (Gilbreth et al., 2004).

Many examples have shown that prebiotics seem to be more effective when used together with a probiotic as a part of synbiotic combination. The term synbiotic refers to synergism where the prebiotic compound is selectively favored by the live probiotic compound. Maintaining viability of probiotic organisms during manufacture and storage of the products in order to ensure effective delivery of the cells after consumption has been subject of increased interest (Oliveira et al., 2009; Paseephol and Sherkat, 2009;

Oliveira et al., 2011b; Nazzaro et al., 2012). It is therefore important to select a combination of a specific substrate and a microorganism for a synbiotic product that can improve the beneficial effect compared to products containing a probiotic or a prebiotic alone (Capela et al., 2006; Huebner et al., 2007).

Dairy probiotic/synbiotic foods

Probiotic/synbiotic foods are a group of functional foods with growing market shares and increasing commercial interest due to proposed health benefits of probiotics and prebiotics. Some of these benefits are established and have been well documented, while the others have shown a promising potential in animal models, with human studies required to confirm these claims (Vasiljevic and Shah, 2008). Functional food products that contain probiotics, prebiotics or synbiotics are mainly classified as dairy products (yoghurt, cheese, ice-cream) (Anal and Singh, 2007) and non-dairy products (vegetables, fruits, grains) (Ranadheera et al., 2010). Dairy foods are the most commercialized functional products as these products are stored at refrigerated conditions and show inherent relationship with probiotic bacteria. In addition dairy matrix provides suitable environment supporting probiotic growth and viability and plays important role in delivering probiotic bacteria to humans (Phillips et al., 2006). Probiotic dairy products are recognized by the consumers as products that contain bioactive compounds beneficial for the general health and therefore they are easily accepted (Kiliç, 2013). Development of these products is a priority in design of functional foods being a challenge for both, scientific and applied research. Furthermore, development of dairy probiotic products requires detailed knowledge of both, products and consumer's attitude and expectations for palatable as well as healthy products (Granato et al., 2010). A successful functional food product development is a scientific approach that is complex and expensive including several criteria as consumer demands, technological conditions and regulatory legislative issues (Jousse, 2008). Consideration of several aspects of any functional food product as functional properties, sensory appeal, shelf-life, physicochemical stability, health claim approval and safety evaluation has to be taken into account, while consumer knowledge of the health benefits of functional compounds may positively affect the acceptance of specific innovative product. However, sensory profile of functional foods containing probiotics and/or prebiotics is still the most important marker of consumer's acceptance of these products. Therefore, even though the consumers are aware that functional product consumption will result in health advantages, the designers of functional foods should take notice to maintain the intrinsic properties of the conventional product. On the other hand, the legislation background of functional products concerns maintenance of nutritional value of conventional products, ensuring claims of no risks related to consumption of these products and prohibited use of labeling which may mislead the consumers (Cruz et al., 2010).

The most convenient dairy mediums for application of probiotics are fermented milks and cheeses as they improved the tolerance of probiotic bacteria to the GI conditions after consumption (Lourens-Hattingh and Viljoen, 2001). Although several factors need to be addressed for applying probiotics in dairy products such as physicochemical and sensory properties, health effects and regulation issues, the survival rate of the bacteria in the product gained much importance. Factors that affect the viability of probiotics during the manufacture and storage of fermented milks included acidity, pH, dissolved oxygen content, redox potential, hydrogen peroxide, starter cultures, additives and flavoring compounds which may be present in the products (Phillips et al., 2006; Saarela and Paquin, 2009). Additional factors as probiotic strain, availability of nutrients, growth promoters and inhibitors, inoculation size and incubation temperature, fermentation time, concentration of metabolites such as lactic and acetic acids, buffering capacity of the media and storage temperature, affect the survival rate of bacteria in yoghurt (Donkor et al., 2006; Donkor et al., 2007). The main factors for loss of probiotic viability involved metabolic products of organic acids which further decrease the pH reached at the end of yoghurt fermentation (Donkor et al., 2006). In order to compensate potential viability loss, inoculation level of probiotics can be increased, but this may negatively affect the quality characteristics of the product (appearance, texture, acidity, and flavor) (Aryana and Mc Grew, 2007). Probiotic inoculation at the end of fermentation is another possibility to achieve improved viability (Vasiljevic and Shah, 2008), while encapsulation of probiotics using different protective agents as alginate, chitosan, starch, pectin, whey proteins, or by adding prebiotics or cysteine into yoghurt are widely used (Capela et al., 2006; Kailasapathy, 2006; Oliveira et al., 2009; Paseephol and Sherkat, 2009; Sandoval-Castilla et al., 2010; Burgain et al., 2011; Cook et al., 2012).

Probiotic viability in yoghurt varied according to large number of strains used and different conditions for preparation and storage of the products. The addition of fruit in yoghurt may have variable effect on the viability of probiotics, since berries might have antimicrobial activities and may lead to further reduction of pH. The loss of viability of *L. acidophilus* was reduced when yoghurt was supplemented with mango and strawberries, while adding of mix berries and passion flower have not provide positive effects on viability compared to the plain yoghurt (Kailasapathy et al., 2008). Other supplements as whey proteins which improve yoghurt buffer capacity and cystein being nutrient for bacterial growth may positively affect the probiotic viability in yoghurt (Ranadheera et al., 2010). The growth and viability of *L. acidophilus*, *L. paracasei* subsp. *casei* and *B. bifidum* was significantly improved in the presence of cystein during fermentation and storage of yoghurts obtained from goat milk (Güler-Akin and Akin, 2007). Several stud-

ies have reported a positive effect of the prebiotics added to dairy products, both on the viability of probiotic bacteria and on the physicochemical attributes (Castro et al., 2009a; Castro et al., 2009b; Oliveira et al., 2009; Oliveira et al., 2011a; Oliveira et al., 2011b; Debon et al., 2012). However, positive effects of prebiotics on the probiotic viability are variable, since different probiotic strains preferentially use them as substrates. Prebiotics, Hi-maize and inulin improved the growth of *L. acidophilus* and *L. casei* in yoghurt as well as organic acids production, but comparison of the prebiotics used has shown more significant effect of inulin on probiotic viability and increased proteolytic activity in the presence of Hi-maize (Donkor et al., 2007). In the study of Capela and co-workers (Capela et al., 2006), fructooligosaccharide was found as the most efficient prebiotic, although Hi-maize and inulin positively affected the viability of *L. acidophilus*, *L. casei*, *L. rhamnosus* and *Bifidobacterium* sp. in yoghurt.

Cheese is an example of a food matrix that may be a valuable alternative to yoghurt and fermented milks as a vehicle in probiotic delivery contributing to high number of viable bacteria. Namely, cheese has higher pH and buffering capacity providing good probiotic viability and stability after consumption. More solid consistency and relatively higher fat content are important factors contributing to reduced loss of probiotic viability during GI transit (Bergamini et al., 2005). Since cheese was observed as advantageous in probiotic delivery, general consumption of cheese has increased (da Cruz et al., 2009). Prebiotics inulin and oligofructose improved the viability of both *L. acidophilus* and *B. animalis* subsp. *lactis*, while eucalyptus honey reduced their survival level in the petit-suisse cheese. It was concluded that the reason may be the low oligosaccharide content of honey. In addition, positive influence of inulin and oligofructose has not been demonstrated only in terms of probiotic viability, but also in improving sensory attributes and consumer acceptance (Cardarelli et al., 2008). Selection of appropriate food matrix to deliver probiotic bacteria is very important as different types of food products have shown variable effect on the probiotic viability, even same strain was used. For example, the viability of *L. paracasei* subsp. *paracasei* LBC 82 in Minas fresh cheese obtained using lactic acid for direct acidification increased from 6.61 to 8.82 log cfu/g during 21 days of storage at 5 °C (Buriti et al., 2005), while the viability of the same strain in chocolate mousse was slightly increased (7.36 to 7.66 log cfu/g) under the same storage conditions (Aragon-Alegro et al., 2007).

The pH of the probiotic ice cream that is near to seven and high total solids level including the fat and milk solids provide effective protection for the probiotic bacteria and together with low temperatures of storage are factors contributing frozen dairy desserts to gain popularity as suitable vehicles in probiotic delivery (Homayouni et al., 2012). However, freeze injury and oxygen toxicity may negatively affect the viability of probiotics incorporated into frozen dairy desserts (Kailasapathy and Sultana, 2003; Homayouni et al., 2008d). In the study of Akin and co-workers (Akin et al., 2007) sugar content was found to affect the probiotic viability, while improved viability of *L. acidophilus* and *B. lactis* was noticed in the presence of inulin in the ice cream. Although, adjusting the production and storage conditions of the frozen dairy products is one way to provide higher viability, microencapsulation is an effective approach to enhance the probiotic survival. The growth and survival of *L. acidophilus*, *L. casei*, *B. lactis* and *B. longum* have been studied in different sucrose concentrations (10, 15, 20 and 25%), oxygen scavengering components (0.05% L-cysteine and 0.05% L-ascorbate) and low temperatures (4 and 20 °C) during 30, 60 and 90 days in MRS broth in order to select suitable strains for manufacture of probiotic ice cream. Results have shown the strains *L. casei* (Lc 01) and *B. lactis* (Bb 12) as the most appropriate to be incorporated in ice cream as their resistance to simulated acidic and alkaline and ice cream conditions was higher compared to other studied strains (Homayouni et al., 2008b; Homayouni et al., 2008d). This is in accordance to the claim that different probiotic strains are showing variable resistance when studied in the same conditions.

Ayran is a Turkish traditional fermented non-alcoholic beverage manufactured by mixing of yoghurt with salt and water and application of starter cultures (Altay et al., 2013). The product is rich in vitamins and calcium and is easily digestible. Although ayran is one type of yoghurt which is suitable medium for growth and survival of probiotic bacteria, as a vehicle for probiotic delivery it is sparingly studied (Uysal-Pala et al., 2006; Ayar and Burucu, 2013). Therefore, our investigations were directed towards ayran as a matrix to incorporate the probiotic *Lactobacillus casei* 01 (Chr. Hansen, Hoersholm, Denmark) and the prebiotic Synergy 1 (oligofructose enriched inulin) (Orafti® Synergy 1, Orafti-Rue L. Maréchal, Tienen, Belgium) to prepare functional dairy food (Petreska Ivanovska et al., 2013). Namely, commercially available Ayran (Zdravje Radovo, Macedonia) was enriched with free probiotic, free synbiotic and microencapsulated synbiotic, respectively. For this purpose, microencapsulated synbiotic was previously manufactured using spray-drying method and subsequent freeze-drying (Petreska Ivanovska et al., 2012). The concentrations of biopolymers alginate and chitosan applied to encapsulate the synbiotic and cross-linking agent $CaCl_2$ were optimized using an experimental design to obtain microparticles with relatively high number of viable cells during microencapsulation and storage (Petreska Ivanovska et al., 2014a). Optimized synbiotic formulation effectively protected the probiotic in simulated conditions of the upper GIT (acid pH, bile salts) ensuring therapeutic level of viable cells able to reach and colonize the lower intestine. Moreover, favorable physicochemical properties of the microencapsulated synbiotic (positively charged particles with relatively low diameters) indicated adherence to the intestinal mucosa and controlled delivery of probiotic

viable cells in the colon.

Qualitative and quantitative examinations (total solids content, pH, titratable acidity, protein, fat and carbohydrate content, salt content) have confirmed that functional samples of ayran containing non-encapsulated probiotic and synbiotic and encapsulated synbiotic maintain the high quality criteria of the conventional product. During the storage of conventional ayran usually syneresis occurs due to compounds aggregation which cannot be prevented by common technological process. Supplementation of ayran by prebiotic or synbiotic increased the solids content and prevented syneresis improving the sensory performance of the prepared functional samples. Herewith, there are data supporting the usage of prebiotic inulin as a fat replacer in skimmed functional dairy products improving their sensory properties (Akin et al., 2007; Cruz et al., 2010). Evaluation of functional properties of the samples prepared in our investigations have shown relatively high level of viable cells indicating the probiotic *L. casei* 01 is therapeutically active during shelf-life of the product and able to produce lactic, acetic and propionic acids, positively associated with health. The most significant improvement of functional properties of ayran was observed when enriched with microencapsulated synbiotic.

Modern trends are shifting to design and manufacture of non-dairy probiotic/synbiotic food products and food supplements named pharmaceuticals and/or nutraceuticals. The growing interest in the development of non-dairy probiotic foods is linked to the need of commercially available products with decreased allergic potential and without cholesterol. However, problems like sensory performance and consumer acceptance are highly associated with non-dairy products. On the other hand, widespread use of probiotic foods versus probiotic supplementation by pharmaceutical dosage forms may be due to potential synergistic effect of food leading to increased functional efficacy of probiotics. These imply the buffering properties of foods during gastrointestinal transit of probiotic bacteria thus preventing the cell loss, providing nutrients for maintaining the activity and efficacy of the probiotic bacteria and consumer attitude toward use of probiotic food products instead tablets and capsules containing probiotics (de Vrese and Schrezenmeir, 2008; Ranadheera et al., 2010; Del Piano et al., 2011).

Gut microbiota and inflammatory bowel diseases

The gastrointestinal microbiota is a complex ecosystem of approximately 300-500 bacterial species (Quigley and Quera, 2006). The fetal gut is sterile, and colonization with bacteria is initiated by contact between the child and its environment with the first feed (Palmer, 2007). The bacterial load of the bowel consists of "native" species that permanently colonize the intestine (Tannock, 2007) and transient bacteria that are continuously ingested from the external environment (Damaskos and Kolios, 2008). The stomach and proximal small intestine contain relatively small numbers of bacteria as 10^3 cfu/ml due to the normal intestinal motility and the antimicrobial effect of gastric acid and bile. Bacterial counts in the terminal ileum may be as high as 10^9 cfu/ml, with a predominance of Gram-negative bacteria and anaerobes. At the transition to the colon, bacterial counts arise to 10^{12} cfu/ml with anaerobic bacteria outnumbering aerobic bacteria by a factor of 100-1000:1 (Neish, 2009). Microbiota in the lower intestine comprised anaerobes such as *Bacterioides*, *Porphyromonas*, *Bifidobacterium*, *Lactobacillus* and *Clostridium*. The activity of the intestinal microbiota is mainly expressed in the distal part (Quigley, 2010; Aureli et al., 2011). These bacteria are involved in the maturation of the gut immune system, as it has been demonstrated in animals bred in a germ-free environment (Bauer et al., 2006), which exhibit crypt hyperplasia, lack of lymphoid follicle development and other structural changes (Damaskos and Kolios, 2008). The cells of the gastrointestinal immune system, responsible for the defensive responses against pathogens, are mostly located in the lymphatic structures of *lamina propria*. Gastrointestinal immune system is consisted of numerous follicular structures and Peyer's patches form part of the specific gut-associated lymphoid system (GALT), together with T lymphocytes, antigen presenting cells (APCs), and B lymphocytes. In the intestinal *lamina propria*, B cells are differentiated into plasma cells and secrete IgA antibodies that are released into the lumen through binding to a polymeric immunoglobulin receptor transporting them from the basolateral surface of intestinal epithelial cells to the apical surface. Secretory IgA are important elements of mucosal immunity which are resistant to proteolysis and do not activate the complement displaying protective function without pro-inflammatory actions. T lymphocytes as representatives of adaptive immunity are found as CD4+ helper T lymphocytes with their subsets (Th1, Th2, Th9, and Th17), or as CD8+ T cytotoxic cells and immunosuppressive regulatory T cells (Tregs). Adaptive immunity includes dendritic cells (DCs) which are APCs able to regulate immune responses to self and foreign antigens guiding the T cell responses. DCs can also pass through the layer of epithelial cells and capture antigens directly from the lumen. Their contact with antigens or inflammatory stimuli can induce the maturation of DCs accompanied by activation of T cells towards functional and phenotypic differentiation along the Th1/Th2 pathway. In the absence of inflammation, the DCs remain in immature state leading to either deletion of effector T cells or the generation of Tregs (Steinman et al., 2003). Mounting of an effective immune response implies discrimination of harmful antigens by the immune system and tolerance to host microbiota and dietary antigens in the intestines. In fact, mucosal barrier comprising the microbiota, the epithelial lining of the mucosal tissue and the mucus together represent an important defense system against luminal pathogens and immunogenic factors. The lumen containing the microbiota is separated from the GALT

by epithelial interface (Aureli et al., 2011). Intestinal microbiota has an important role in the diet, maintenance of the mucosal barrier integrity and development of mucosal immunity (Shanahan, 2002). Hence, disruption of the triad comprising secretory IgA, polymeric immunoglobulin receptor and microbes increases the risk of inflammatory disease of intestine (Kaetzel, 2014). The failure to control immune responses leads to breakdown of tolerance towards resident microbiota and inappropriate inflammatory response of the gut immune system to the constituents of the normal microbiota which may ultimately lead to inflammatory conditions such as IBD (Sartor, 2007). Clinical symptoms of IBD include abdominal pain, weakness, rectal bleeding, flushes, edemas, ulcerations, diarrhea, malnutrition, and weight loss (Reiff and Kelly, 2010). Ulcerative colitis is primary located in the colon and proximally to the rectum which becomes inflamed and ulcerated. UC is characterized by superficial mucosal inflammation of the colon, increased number of neutrophils in *lamina propria* and crypts and production of pro-inflammatory mediators such as interleukin 12 (IL-12) and tumor necrosis factor alpha (TNF-α) (Geier et al., 2007; Hutfless et al., 2007). Crohn's disease usually affects the lower ileum and colon, although any part of the GIT may be involved. The pathology of CD is characterized by transmural inflammation and aggregation of macrophages stimulating formation of epitheloid granulomas (Geier et al., 2007; Kozuch and Hanauer, 2008). Activation of T helper cell (Th1) responses associated with elevated levels of interferon gamma (IFN-γ) and TNF-α and tissue infiltrate of Th17 cells are characteristic immune pathogenic features in CD (Pene et al., 2008).

Probiotics, prebiotics and synbiotics have the capacity to reverse pathologic changes in gastrointestinal flora and immune tolerance decreasing pathogen attachment and prevent subsequent invasion of the mucosa by blocking binding sites and by up regulating antimicrobial substances in the GIT (Du Pont and Du Pont, 2011). The disproportion of the number of lactobacilli and bifidobacteria in favor of enterobacteria, coliforms and bacteroides in fecal samples of IBD patients (Parkes et al., 2008) also indicated the contribution of the intestinal microbiota to a healthy microbial community.

Probiotics and prebiotics in the management of inflammatory bowel diseases

The evidence for anti-inflammatory effects of probiotics comes from three types of researches: *in vitro* studies using cell lines, animal models and clinical studies.

The strain *L. fermentum* ACA-DC 179 possesses desirable probiotic properties, such as antimicrobial activity and immunomodulation *in vitro*. Moreover, this strain was successfully applied in an experimental *Salmonella enterica*-infection mouse model with elevated levels of the anti-inflammatory IL-10. Underlying mechanisms confirmed the potent anti-inflammatory potential of the applied strain, both *in vitro* and *in vivo* and have been correlated with improved barrier function and protection against colitis in animal studies (Zoumpopoulou et al., 2008). *L. paracasei* IBB2588 decreased the adhesion of *Salmonella enterica* towards Caco-2 cells, so that the pre-incubation of the probiotic provided significantly increased reduction of the pathogen adhesion compared to co-incubation of the probiotic and the pathogen (Jankowska et al., 2008). Strains *B. longum* Bar33 and *L. acidophilus* Bar13 competitively inhibited the binding of pathogens to Caco-2 cells and decreased the production of pro-inflammatory cytokine IL-8 in HT-29 cell type indicating immunomodulatory activity (Candela et al., 2008). *B. breve* and *L. lactis*, prebiotic GOS or a synbiotic combination demonstrated the ability to exert anti-inflammatory and some anti-proliferative effects in different *in vitro* models in the context of inflammatory diseases (Grimoud et al., 2010). *L. johnsonii* induced the production of anti-inflammatory cytokine transforming growth factor-β (TGF- β) in leukocyte-sensitized intestinal epithelial cell lines (Haller et al., 2010).

The *in vitro* results using multiple cell lines should be further evaluated in animal models since confirmation of the observed anti-inflammatory effects taking into account the factors of the environment is necessary to be evaluated. Studies in animal models provided critical insights into pathogenesis of IBD and enabled evaluation of different modalities to prevent or ameliorate inflammation which may be used as relevant evidence in the development of novel therapies for human IBD. Oral administration of probiotics *L. acidophilus*, *L. casei* and *B. lactis* have shown intestinal anti-inflammatory activity in the trinitrobenzen sulfonic acid (TNBS) model of rat colitis (Peran et al., 2007a). Reduction in the extent of induced colonic inflammation was observed in this study, and confirmed biochemically by decreased colonic myeloperoxidase (MPO) activity as well as production of TNF-α and inducible nitric oxide synthase (iNOS). However, better efficacy of the *L. fermentum* administration was noticed due to its ability to promote the production of SCFAs into the colon and reduced expression of cyclo-oxygenase-2 (COX-2). In other study of Peran and co-workers (Peran et al., 2007b) each probiotic strain used has shown its own anti-inflammatory properties. *B. lactis* treatment reduced the production of colonic TNF-α, iNOS and COX-2 expressions; *L. acidophilus* reduced the production of colonic leukotriene B_4 and iNOS expression, while *L. casei* decreased the expression of COX-2 in the colon. Biochemically, all the strains applied restored the decreased glutathione levels in the colon as a consequence of the inflammatory process accompanied by oxidative stress. Nishitani and co-workers (Nishitani et al., 2009) used *in vivo* and *in vitro* models to evaluate anti-inflammatory effects of the strain *Lactococcus lactis* subsp. *cremoris* FC. In the cell assessment, the treatment of a gut inflammation model resulted in significant down-regulation of IL-8 mRNA expression in Caco-2 cells and inhibition of NF-κB nucle-

ar translocation in RAW264.7 cells. Administration of the strain in C57BL/6 mice inflamed using dextrane sulfate sodium (DSS) significantly ameliorated histological parameters such as shortening of colon length and tissue inflammation. Intragastric administration of a *L. casei* BL23 with improved anti-oxidative potential using recombinant technology to obtain strain producing superoxide dismutase resulted in decreased oxidative stress and reduction in the extent of DSS-induced colitis in mice (Watterlot et al., 2010). Oral administration of *L. plantarum* K68 isolated from Taiwan fermented food *fu-tsai* attenuated DSS-induced ulcerative colitis in BALB/c mice exhibiting anti-inflammatory and immunomodulatory activities (Liu et al., 2011). As it was reported, the disease activity index and histological scores showed significant reduction of the severity of UC. Additionally, the strain has shown significant inhibition of the production of TNF-α and prostaglandin E_2 (PGE_2) in lipopolysaccharide (LPS)-induced murine macrophage RAW 264.7 cells and stimulation of production of IFN-γ in human peripheral blood mononuclear cells. Treatment with *B. bifidum* 17 partially protected mice from Th1-driven inflammation in a chemically induced colitis using relatively high dose of TNBS (120 mg/kg) (Philippe et al., 2011). However, the levels of pro-inflammatory cytokines IL-1β, IL-6, keratinocyte-derived chemokine and the inflammatory markers COX-2 and MPO activity as well as histological scores were significantly reduced. Spores from two distinct colony types of the strain *Bacilus subtilis* PB6 provided as a powder preparation to mice inflamed by 110 mg/kg TNBS prevented colitis followed by extremely low levels of pro-inflammatory mediators IL-12, TNF-α and IFN-γ and stimulation of production of anti-inflammatory IL-10. Macroscopic and histological assessment, blood inflammatory markers and determination of infiltration of mucosal neutrophils were based on blind protocol and compared with the effects of the drug prednisolone (Foligné et al., 2012). *L. plantarum* CLP-0611 isolated from kimchi ameliorated TNBS-induced colitis in mice inhibiting the expression of IL-1β and IL-6, TNF-α production and MPO activity, while inducing NF-kappaB (NF-κB), mitogen-activated protein kinase and polarizing M1 to M2-like macrophages (Jang et al., 2014). TNBS-induced colitis (10 mg/kg) in Wistar rats was significantly ameliorated after 21 days lasting oral administration of *L. casei* loaded whey protein-alginate microparticles suspended in milk as vehicle (Smilkov et al., 2014). Total damage score of the inflamed colon, colon weight/total weight ratio, histological evaluation and activity of MPO, indicated apparent reduction of the inflammation parameters, while treatment with non-encapsulated *L. casei* was effective in reducing inflammation to a lesser extent. This investigation showed the potential of microencapsulated *L. casei* to be used as adjuvant therapy in IBD when incorporated in food product. Our *in vivo* study comprising different extent of animal model of colitis (induced with 10 and 30 mg/kg TNBS) has shown the anti-inflammatory potential of prepared probiotic (*L. casei* 01) and synbiotic ayran (*L. casei* 01 and Synergy 1) (Petreska Ivanovska et al., 2014b). The most significant decrease in parameters of inflammation (body weight, colon weight/length ratio, macroscopic and microscopic ulceration, MPO activity) was observed administering the ayran containing synbiotic chitosan-Ca-alginate microparticles. Determination of bacterial translocation of lactobacilli in extra-intestinal tissues approved the safety issue of the probiotic strain used in the study. The potential of the microencapsulated synbiotic applied in ayran for target and prolonged delivery of active probiotic cells able to colonize the lower GIT should be emphasized, thus showing no concern of increased frequency of the applied treatment.

These studies illustrate different mechanisms of probiotic anti-inflammatory effects comprising inhibition of growth, epithelial adherence and mucosal uptake of enteropathogens, alteration of gut microbial diversity, reduction of mucosal permeability improving the intestinal barrier function and modulation of immune response developing Tregs and tolerogenic dendritic cells (DCs), all contributing to enhanced host-microbiota crosstalk (Reiff and Kelly, 2010).

Literature data summarize the potential use of probiotics in humans applied for remission of UC, pouchitis and CD. Probiotic combination VSL#3 containing eight lyophilized cultures (*L. acidophilus*, *L. bulgaricus*, *L. casei*, *L. plantarum*, *S. thermophilus*, *B. breve*, *B. infantis*, and *B. longum*) effectively prevented recurrence of chronic relapsing pouchitis in two double blind and placebo-controlled trials (Gionchetti et al., 2000; Mimura et al., 2004). The same preparation decreased the disease activity index increasing the number of mucosal regulatory T cells after surgery in patients undergoing ileal pouch anal anastomosis for UC (Pronio et al., 2008), while reduced inflammation in patients with UC up regulating intestinal mucosal alkaline sphingomyelinase was observed (Soo et al., 2008). Remission rate of 56% assessed through clinical colitis activity index, UC endoscopic score, inflammatory markers, erythrocyte sedimentation rate, C-reactive protein, serum cytokine profile and rectal tissue microbial determinations at beginning and at week eight of VSL#3 administration in pediatric patients with mild to moderate UC was found (Huynh et al., 2009). Synbiotic therapy experienced increased quality of life compared to probiotic and prebiotic treatment alone in a randomized controlled trial conducted with 100 UC patients divided in three groups, with no adverse effects observed (Fujimori et al., 2009). The results had been summarized on the basis of completed IBD questionnaires at the onset of the study, at the second week and at the forth week including the evaluation of blood variables. A randomized, controlled study consisted of 165 patients with CD who had achieved remission after treatment with steroids or salicylates has shown no significant differences in percentage of patients in remission between group administering *Saccharomyces boulardii* and placebo group (Bourreille et al., 2013). Another recent study indicated no

difference in colonic microflora between 8 patients with CD and 8 patients with UC who completed one month lasting synbiotic treatment followed by placebo the second month. Measurements of colonic microflora using terminal restriction fragment length polymorphism technique identified the probiotic bacteria in the stool samples, but overall alterations in the microflora spectrum were not found (Ahmed et al., 2013). While studies of animal models of intestinal inflammation enabled promising results, clinical trials are not apparently conclusive probably due to the differences among patients and heterogeneity of inflammatory diseases. Differences between results obtained from animal and human studies might due to differences in the composition of the microbiota in mice and humans, diet, metabolism or immune responses (Shanahan and Quigley, 2014). However, animal models are very helpful to study mechanisms of inflammation, interactions between genetic and environmental factors that increase susceptibility to IBD and host-microbes crosstalk. Moreover, opposing findings are further encourage to well-defined, randomized, double-blind clinical studies with large number of subjects in order to identify specific characteristics related to nature of intestinal inflammation and real role of probiotic/synbiotic treatment for IBD.

Application of prebiotics in the treatment of IBD is also beneficial approach due to findings indicating changes in the gut microbiota of CD patients and decreased number of bifidobacteria in fecal samples (Lindsay et al., 2006). Prebiotics such as inulin, FOS and barley are able to modify the micro-ecology in the lumen enhancing the growth of *Lactobacillus* and *Bifidobacterium* species and providing a substrate for the production of SCFAs by these bacteria. SCFAs are a subset of fatty acids that are produced by the gut microbiota during the fermentation of partially and non-digestible carbohydrates with the highest levels found in the proximal colon, and can be used locally by enterocytes or transported across the gut epithelium into the bloodstream (Tan et al., 2014). SCFAs and especially butyrate are preferred metabolic substrates of colonocytes and can stimulate various mucosal barrier functions. Additionally, immune function may also be modified by these prebiotics changing the dysbiosis towards rebalance and more tolerant response (Sartor, 2004; Guarner, 2007). SCFAs have been found to alter gut integrity and to possess anti-inflammatory, antimicrobial and anticarcinogenic effects, thus playing significant role in maintenance of gut and immune homeostasis (Tan et al., 2014).

Oligosaccharides isolated from goat milk (GMO) contributed to the recovery of damaged colonic mucosa in DSS-treated rats confirmed by decreased MPO activity, up regulation of expression of genes involved in intestinal function as mucine-3 and clinical scoring system (body weight, blood cell counts, presence of blood in the stools) (Lara-Villoslada et al., 2006). In addition, although insignificant, the concentration of SCFAs tended to be higher in rats receiving GMO diet compared to the control rats as well as colon glutathione content, both total and reduced. Intragastric lavage of FOS decreased the severity of the DSS-induced colitis in C57BL/6 mice leading to reduced damage in the distal colon (Winkler et al., 2007), while feeding of Sprague-Dowley rats by FOS reduced caecal inflammation with no effect on colon recovery (Moreau et al., 2003), which is probably due to the different animal models used as well the administration patterns. FOS has shown antagonistic effects in the intestines. Namely, FOS administered as a part of the diet stimulated the growth of lactobacilli and bifidobacteria (Bovee-Oudenhoven et al., 2003), enhanced SCFAs production in the lower intestine which was confirmed in clinical trials (Lewis et al., 2005) and inhibited colonization of pathogens, but FOS can also stimulate bacterial translocation, mucosal irritation and increased activity of MPO in caecum and colon (Bovee-Oudenhoven et al., 2003). Inconsistency in the data may be the result of fast bacterial fermentation of FOS and overproduction of organic acids that may damage the colonic mucosa. These processes are probably provoked by increased level of FOS in the caecum (Ten-Bruggencate et al., 2005). *B. infantis* with and without a combination of oligofructose and inulin ameliorated significantly the disease activity index and decreased colonic MPO activity in rats with DSS-induced colitis (Osman et al., 2006). Production of propionic, succinic and butyric acids was also significantly increased, while bacterial translocation to mesenteric lymph nodes and liver decreased significantly in all experimental groups, compared to the colitis control. Increased total concentration of fatty acids in rats fed with prebiotic mixture indicated the inhibitory effects of SCFAs on the inflammation. In the study of Cherbut and co-workers (Cherbut et al., 2003), increased concentrations of lactate and butyrate were determined and reduced damage of the rat colonic mucosa, contributing to the previous conclusion. Inhibition of expression of pro-inflammatory mediators TNF-α and IL-6 through IFN-γ-induced macrophages and stimulated production of IL-10 are postulated mechanisms of SCFAs anti-inflammatory effects (Park et al., 2007).

Clinical studies in pediatric patients with CD administered with combination of FOS, inulin and whey proteins have shown reduced severity of the disease associated with decreased sedimentation rate as a biochemical marker of inflammation (Hussey et al., 2003). Bifidogenic effect of FOS was observed analyzing the effect of FOS towards bifidobacteria counts and function of dendrites in mucosa of patients with moderately active CD (Lindsay et al., 2006). Consumption of 15 g FOS per day significantly reduced the disease activity index providing increased number of bifidobacteria and elevated IL-10 production in *lamina propria* as a modulator of inflammatory Th1 response. Potential anti-inflammatory effects of prebiotics indicated important contribution to increased power of the probiotic therapy as a synbiotic combination. Meanwhile, evidences from human studies are lacking and immunomodulato-

ry effects of prebiotics are yet to be researched profound.

Safety aspects of probiotic/synbiotic products

Usage of probiotic bacteria in health and disease which dates back for centuries is the best evidence for their safety. However, insufficient data, especially when new probiotic strain is a candidate to be placed on the market, constructing of randomized studies for safety evaluation of the probiotic strain is the essence. According to the review of Boyle and co-workers (Boyle et al., 2006) probiotics are characterized with generally safe profile, but should be used with caution in certain population groups such as pregnant women, neonates born prematurely or with immune deficiency. The working groups of WHO and FAO have proposed several criteria that should be considered in order one strain to be defined with the GRAS status (generally regarded as safe): resistance of the probiotic strain to antibiotics, assessment of metabolic properties of the strain (lactate production, bile salts deconjugation), monitoring of adverse effects in clinical studies, epidemiological studies of side effects incidence after approval for commercial use, identification of any substance secreted from the strain that may be toxic for mammals and determination of haemolytic potential of the strain (WHO/FAO, 2002; Petrushevska and Mladenovska, 2009). According to EFSA, qualified presumption of safety of microorganisms of food and feed (QPS) designation could be applied only to microorganisms which may be identified at the strain level and there is a history of apparent safe use (EFSA Scientific Colloquium Summary Report, 2004). Although the characterization of a specific strain is based on the absence of resistance to antibiotics and virulence factors (Barlow et al., 2007), this property is not a major safety issue. Namely, different microorganisms are inherently resistant to the activity of antibiotics and the real concern comes when it is accompanied by a horizontal transfer of genetic determinants (Jansen et al., 2006). Thus, the EU legislation recommends safety assessment for probiotics of food supplements to verify the absence of transferable resistance (Aureli et al., 2011).

Nevertheless, case reports of infections caused by probiotic supplementation containing *L. rhamnosus* GG (LGG) have been published. Liver abscess and pneumonia was developed in 74-years old diabetic woman after 4 months administration of LGG supplement (Rautio et al., 1999), while endocarditis was diagnosed after dental extraction in 67-years old man with mitral regurgitation who was taking capsules containing *L. rhamnosus* once daily (Mackay et al., 1999). In both cases the difference between the probiotic and infective *L. rhamnosus* using pulsed-field gel electrophoresis of chromosomal DNA restriction fragments or standard biochemical analysis was not found, thus the source of infection cannot be conclusively proven. Moreover, cases of probiotic bacteraemia and fungemia have been described in chronic diseases, immuno-compromised or debilitating subjects (Boyle et al., 2006). Patients with AIDS and Hodgkin have experienced bacteraemia and pulmonary embolism after probiotic supplementation with *L. acidophilus* (LeDoux et al., 2006). In patients suffering IBD and other diseases where the intestinal barrier may be compromised, there is a risk of translocation of live probiotic across the gut leading to systemic sepsis. Herewith, septicaemia was reported among premature infants with short bowel syndrome who were taking ¼ LGG capsule daily (De Groote et al., 2005). Increased mortality which was related rather to intestinal ischemia of unknown origin than sepsis was noticed among patients with severe acute pancreatitis administering a probiotic cocktail of 4 lactobacilli and 2 bifidobacteria through a naso-enteric tube (Besselink et al., 2008).

Some reports addressed safety use of probiotics in subjects at higher risk to develop adverse reactions to probiotic supplementation. Study of Kalliomaki and co-workers (Kalliomaki et al., 2001) have shown no side effects related to daily administration of capsules with LGG in 132 pregnant women who were supplemented 2 to 4 weeks before expected delivery. Immuno-compromised HIV-infected patients have not developed adverse effects as a result of consumption of LGG to treat diarrhea (Salminen et al., 2004). No increased trend in bacteraemia cases was found during increased consumption of probiotic products containing *Lactobacillus* sp. in surveys in Finland (years 1995-1999) and Sweden (years 1998-2004) comprising general population (Salminen et al., 2002; Sullivan and Nord, 2006).

Many prebiotics are constituents of the normal diet and in low concentrations are generally safe for the humans. Clinical studies have not found toxicity effects related to the consumption of prebiotics such as inulin and FOS (Carabin and Flamm, 1999). However, the safety issue of prebiotic administration implies examination of acceptable daily level since the gastrointestinal tolerance for prebiotics is limited. Namely, intestinal fermentation of prebiotics may exceed under increased intake of prebiotics (> 20 g/day) accompanied by changes in osmotic potential and induction of side effects such as increased gas production, flatulence, abdominal bloating and diarrhea (Tuohy et al., 2005). In order to avoid side effects while remaining beneficial effects, the administration level should be assessed according to targeted population group. School-age children tolerated FOS in the diet at level of 9 g per day (Carabin and Flamm, 1999), while infants tolerated an intake of 4-8 g prebiotic mix per day (Ziegler et al., 2007). IBD patients have demonstrated tolerance to 15-20 g FOS per day (Lindsay et al., 2006). According to Bouhnik and co-workwrs (Bouhnik et al., 2004) the relative increase in numbers of faecal bifidobacteria is more dependent on the baseline concentration of bifidobacteria than on the prebiotic dose consumed. In addition, Vanhoutte and co-workers (Vanhoutte et al., 2006) have found that prebiotic consumption was much effective in subjects with low intrinsic level of

bifidobacteria such as patients with GI disorders and formula fed infants. Taking this into account minimal effective bifidogenic dosage should be recommended.

Summary

Probiotics have been shown to affect intestinal microbiota, intestinal barrier function and immune responses representing alternative approach in prevention and treatment of IBD. The lack of complete knowledge of the molecular mechanisms involved in pathogenesis of IBD seems to make this issue more complex. In a search for an effective probiotic-based treatment for IBD, scientists have tried to elucidate the underlying mechanisms of probiotic action. The challenge ahead is to gain much understanding of the mechanisms of crosstalk interactions between microbiota and host tissues which will be a contribution to conceptualization of new and successful therapeutic approaches based on clear etiologic, pathogenic processes and predictable responses. Probiotics and prebiotics have been subjected to confer a health benefit in many animal models of intestinal inflammation and clinical studies, but large, rigorously designed and high quality human trials are necessary to be evaluated to investigate individual efficacy of any probiotic strain, dosage, duration of use, single or multi-strain formulation, and the concomitant use of probiotics, prebiotics and antibiotics. Furthermore, it is very important potential anti-inflammatory effects mediated by novel probiotic/synbiotic treatments to be highly superior compared to the risks and accepted by the population.

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Резиме

Пробиотици, пребиотици и синбиотици во превенција и третман на инфламаторни цревни заболувања

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Клучни зборови: пробиотик, пребиотик, синбиотик, функционална храна, инфламаторно цревно заболување

Пробиотиците, пребиотиците и синбиотиците претставуваат функционални состојки кои покажуваат поволни ефекти врз здравјето на луѓето. Интересот за пробиотиците, пребиотиците и синбиотиците произлегува од потенцијалната примена во превенцијата и третманот на голем број заболувања и медицински состојби за кои недостасува ефикасен и безбеден пристап. Придобивките од употребата на овие биоактивни состојки се состојат и во намалување на морбидитетот на хроничните заболувања и трошокот за здравствената грижа. За испорака на пробиотиците/синбиотиците во гастроинтестиналниот тракт се користат различни видови на медиуми. Главниот предизвик на комерцијалниот сектор е да овозможи присуство на нови функционални прехранбени производи со пробиотици и/или пребиотици на пазарот, и покрај тоа што предмет на интерес се и пробиотските капсули и таблети. Дизајнот на новите пробиотски/синбиотски прехранбени производи е резултат на интересот на прехранбената индустрија за континуиран напредок од аспект на подобрување на нутритивните и сензорните својства на производите, како и здравствените придобивки за потрошувачите. Целта на ова издание е да овозможи пошироко согледување и нови перспективи во употребата на функционалните компоненти и производи во превенција и третман на инфламаторните цревни заболувања (ИЦЗ) кои се тесно поврзани со современиот начин на живот. Дискусијата ги опфаќа терапевтските и безбедносните карактеристики на пробиотиците и пребиотиците, нивната улога во патогенезата на ИЦЗ, механизмите на дејство и значењето во превенцијата и третманот на овие заболувања, со истакнување на клучните фактори кои се неопходен предмет на понатамошни истражувања.

Macedonian pharmaceutical bulletin, **60** (2) 21 - 27 (2014)
ISSN 1409 - 8695
UDC: 616.833-097
Review paper

Glycoconjugates as target antigens in peripheral neuropathies

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Received: October 2014; Accepted: December 2014

Abstract

Identification and characterization of antigens present at the human peripheral nerve is a great challenge in the field of neuroimmunology. The latest investigations are focused on the understanding of the biology of glycoconjugates present at the peripheral nerve, and their immunological reactivity. Increased titers of antibodies that recognize carbohydrate determinants of glycoconjugates (glycolipids and glycoproteins) are associated with distinct neuropathic syndromes. There is considerable cross-reactivity among anti-ganglioside antibodies, resulting from shared oligosaccharide epitopes, possibly explaining the overlap in syndromes observed in many affected patients. Sera from patients with neuropathies (GBS, chronic inflammatory demielynating polyneuropathy - CIDP, multifocal motor neuropathy - MMN), cross-react with glycoproteins isolated from human peripheral nerve and from *Campylobacter jejuni* O:19. The frequency of occurrence of antibodies against these glycoproteins is different, depending of the type of neuropathy. Identification of the cross-reactive glycoproteins and possible additional auto antigens could be useful in laboratory evaluation of peripheral neuropathies and help to develop a more effective therapeutic approach.

Key words: glycoproteins, gangliosides, cross-reactivity, neuropathies, *Campylobacter jejuni*

Autoimmune neuropathies

Autoimmune neuropathies are described by presence of symptoms of weakness, sensory loss and autonomic dysfunctions as a consequence of immune mediated damage of peripheral nerve. Autoimmune neuropathies are heterogeneous in clinical presentation and mechanism of development (Asbury, 1994). From the clinical point of view, autoimmune neuropathies may have acute onset and monophasic course (Guillian-Barré syndrome - GBS and its variants), or chronic slow progressive or relapsing clinical course (chronic inflammatory demyelinating poliradiculoneuropathy - CIDP, and Multifocal motor neuropathy - MMN) with different pathological and electrophysiological features (Griffin and Sheikh, 2005).

Guillian-Barré syndrome (GBS) is an acute monophasic inflammatory neuropathy, which can be classified in several variants according to pathological changes and electrophysiological findings. The histologic features of the GBS support a classification into demyelinating and axonal forms. The subtypes of GBS are acute inflammatory demyelinating polyneuropathy (AIDP), acute motor axonal neuropathy (AMAN) and Miller Fisher syndrome (MFS). AIDP is characterized by primary demyelination, while AMAN is characterized by primary axonal degeneration of the peripheral nerves developed trough noninflammatory complement dependent mechanism mediated by antibodies (Asbury et al., 1969; McKhann et al., 1993, Grif-

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fin et al., 1996, Hafer-Macko et al., 1996). MFS is characterized by an acute onset of ataxia, areflexia and ophtalmoplegia (Fisher 1956). All of these GBS variants have CSF finding characterized by increased protein level and normal cell count (albumino-cytological dissociation) and may have a detectable spectrum of serum antiganglioside antibodies. The molecular mimicry is the basic immune mechanism involved in the onset of the disease. Antibodies induced by the primary infective agent recognize the peripheral nerve antigens and consequentially activate cell-mediated autoimmune response (Kuwabara et.al., 2002, Willison and Yuki, 2002, Yuki and Hartung, 2012).

Chronic inflammatory demielynating neuropathy (CIDP) is well defined demyelinating autoimmune polyneuropathy with relapse-remittent course of the disease and a good therapeutic response to corticosteroids. CIDP is accompanied with the presence of a number of different antibodies to the constituents of the peripheral nerve, which explains the good response of the plasma exchange in these patients. The presence of the antiganglioside antibodies in sera from patients with CIDP is also very important (Willison and Yuki N, 2002).

Multifocal Motor Neuropathy (MMN) is a chronic immune mediated neuropathy characterized by asymmetric, predominantly distal upper limb weakness without sensory impairment and by the presence of multifocal persistent partial conduction blocks on motor but not on sensory nerves (Nobile-Orazio, 2001). The muscle weakness related to individual motor nerve is associated with motor conduction block, at site distinct from common entrapment or compression syndromes (Ghosh et al., 2005; van der Meché et al., 1995; Willison and Yuki, 2002).

Peripheral nerve antigens in autoimmune neuropathies

Increased titer of antibodies that react with human peripheral nerve antigens are detected in patients with immune-mediated neuropathies (Quarles et al., 1990, Latov, 1994, O'Leary and Willison, 2000). Identification and characterization of auto-antigens present at the human peripheral nerve represents a great challenge in the field of neuroimmunology, especially in demyelinating disorders. Analysis of the molecular composition of human peripheral nerve showed presence of highly complex and organized structure including different glycoprotein and glycolipid molecules, which can be targeted by auto antibodies in inflammatory processes (Willison and Yuki, 2002).

There are a number of studies indicating that myelin-specific proteins present in peripheral nervous system are implicated in autoimmune neurological diseases including autoimmune peripheral neuropathies. These include myelin proteins myelin protein zero (P0), myelin -associated glycoprotein (MAG), peripheral myelin protein 2 (P2), peripheral myelin protein 22 (PMP22) and other myelin specific proteins that have an important role in myelin formation and in mediating autoimmune peripheral nerve disease (Quarles 1989, Hughes and Cornblath, 2005, Khalili-Shirazi et al., 1993, Quarles et al., 1990, Gabriel et al., 2000, Allen et al., 2005). Recent studies are focused on a wide range of proteins that play a crucial role in the formation of the nodes of Ranvier, localized on the nodal complex, including neurofascin, gliomedin and contactin, which are considered as potential antigens for participation in the pathological process of GBS and CIDP (Devaux et al., 2012, Devaux, 2012, Ng et al., 2012, Hughes and Willison, 2012).

Neural glycolipids, including galactocerebrosides and gangliosides (LM1, GM1, GD1A and GQ1b) are another important category of peripheral nerve antigens, which might participate in the immunological targeting that results in neural inflammation (Asbury, 1994, Latov, 1994, Willison and Yuki, 2002).

Antibodies to a wide range of glycolipids including GM1, GD1A, GalNAc-GD1A GM1, GD3, GalC, LM1, SGPG ,GQ1b, GT1a and to peripheral nerve proteins have been reported in more than a 200 papers on GBS and other inflammatory neuropathies as a case studies and in larger series (Table 1). The epidemiological patterns of anti-ganglioside antibodies vary substantially between geograph-

Table 1. Peripheral nerve antigens associated with autoimmune neuropathies

Clinical syndrome	Antibody type	Antigen
Guillain-Barré syndrome AMAN AIDP MFS	IgG	GM1, GD1A, GalNAc-GD1A GM1, GalC, LM1, SGPG GQ1b, GT1a PO, P2, MAG, PMP 22, β-tubulin, Conexin 32, gliomedin, contactin, neurofascin
Chronic inflammatory demyelinating poliradiculoneuropathy (CIDP)	IgM	GM1, GD1a, GM2
	IgG, IgM	PO, P2, MAG, PMP 22, β-tubulin, Conexin 32, neurofascin
Multifocal motor neuropathy (MMN)	IgM	GM1, GA1, GD1b

ic regions, according to the prevalent subtypes of GBS and their relationship to preceding infections, such as *Campylobacter jejuni (C. jejuni)* infection (Azhary et al., 2010).

Increased titers of antibodies against GM1 ganglioside are found in patients with motor neuropathies. Anti-GM1 antibodies can be of different isotype, IgM antibodies are associated with MMN, whereas IgG antibodies are typical for acute onset neuropathies, or variants of GBS (Latov, 1990, Pestronk and Choski, 1997). Anti GM1 antibodies of IgG type are characteristic feature of AMAN, but a proportionof of patients with AIDP have also these antibodies (Willison and Yuki, 2002, Yuki and Hartung, 2012). Over 90 % of patients with MFS have elevated titers of anti-GQ1b antibodies, indicating the role of the anti-ganglioside antibodies in the disease (Yuki et al., 2000). Increased antibody titers against multiple gangliosides have been reported in multifocal acquired sensory and motor neuropathy, possibly implicating immune mechanisms in their pathogenesis. IgM anti-GM1 antibodies are associated with multifocal motor neuropathy (MMN). Up to 80 % of the patients with MMN may have anti-GM1 antibodies. The antibodies are most often polyclonal, but can also occur as IgM monoclonal gammopathies. Most of the antibodies bind to the Gal(beta1-3)GalNAc epitope, shared by asialo-GM1 and GD1, but some bind to GM1 alone, or to GM1 and GM2. This syndrome, MMN, has a stronger association with the occurrence of antiganglioside antibodies, especially against GM1, compared to GBS and CIDP (Cats et al., 2010, van der Meché et al., 1995, Willison and Yuki, 2002).

Autoantibodies, against myelin proteins P2, P0, Myelin associated glycoprotein, PMP22, β-tubulin and Conexin 32, mostly of IgG isotype, are detected in sera from patients with GBS and chronic demyelinating neuropathies, including CIDP (Khalili-Shirazi et al., 1993, Quarles et al., 1990, Gabriel et al., 1999, Allen et al., 2005). Also IgG antibodies directed to neurofascin, gliomedin, and contactin are also detected in sera from patients with GBS and CIDP and were associated with demyelination and conduction loss in experimental model (Devaux et al., 2012, Devaux, 2012, Ng et al., 2012, Hughes and Willison, 2012).

There is considerable cross reactivity among anti-glycoconjugate antibodies, resulting from shared oligosaccharide epitopes, possibly explaining the overlap in syndromes observed in many affected patients (Latov, 1994). Antiganglioside antibodies in sera from patients with GBS recognize galactosyl (β1-3) N-acetylgalactosamine (Gal(β1-3) GalNAc) epitope, a carbohydrate determinant shared by several gangliosides (GM1, GD1a, GD1b, GT1b and asialo ganglioside GA1), several peripheral nerve glycoproteins and several antigens present in bacterial cell wall (Thomas et al., 1989, Aspinall et al., 1994).

Molecular mimicry

The current investigations are based on hypothesis of ganglioside mimicry of *C. jejuni* lipopolysaccharides in GBS together with the pathogenic role of different antibodies which interfere with nerve conduction or induce damage of the nerves. There are experimental evidence based on research on animal models and human about the molecular mimicry between gangliosides and lipooligosaccharide (LOS) from *C. jejuni.* The bacteria isolated from GBS patients have LOS, which structure resembles GM1 or GD1a gangliosides, whereas isolates from patients with Miller Fisher syndrome, have LOS with structure similar to GQ1b (Yuki et al., 1993, Koga et al., 2005). Antibodies that cross-react with LOS from *C. jejuni* and with different gangliosides from peripheral nerves are detected in sera from patients with GBS following infection with *C. jejuni* (Wirguin et al., 1994).

Serotype of *C. jejuni* which is the most frequently associated with developing of GBS is Penner's serotype O:19, which has (LOS), with structure similar to the structure of oligosaccharides present in GM1 and asialo-GM-1gangliosides (Yuki et al., 1990, Walsh et al., 1991, Fujimoto et al., 1992). The Penner's O:19 serotype of *C. jejuni* contains LOS with GM1 - like oligosaccharides-determinants and is most commonly associated with pure motor GBS (Fujimoto et al., 1992, Yuki et al., 1995).

Although ganglioside mimicry associated with the production of anti-ganglioside antibodies accelerates the symptoms of GBS, the pathogenesis of this disease remains unclear. A number of patients with enteritis caused by *C. jejuni*, which contain LOS with structure similar to human gangliosides, do not develop neurological symptoms, indicating that only the presence of these structures is not sufficient to induce production of antiganglioside antibodies. The data that the most of *C. jejuni* infections caused by the serotypes containing ganglioside-like LOS, do not result in GBS, indicate that other host related and/or bacterium related factors contribute in the development of neurological symptoms after an infection with *C. jejuni,* but these factors still remain unknown (Willison and Yuki, 2002, Karlyshev et al., 2005). The possible factor could be the influence of other host related factors such as polymorphism in immune response genes, in addition to bacterium related factors, in the development of neurological symptoms after an infection with *C. jejuni*. This may either depend on differential expression of ganglioside mimics in *C. jejuni* LOS, due to phase variation in genes encoding glycosyltransferases, or to the influence of other immunomodulating factors (Ang et al., 2002).

The role of antibodies to the peripheral nerve myelin proteins and glycoproteins was not sufficiently investigated in GBS (Gabriel et al., 2000). Sera from patients with GBS showed immunological reactivity to 70 kDa, 50-60 kDa, 40, 35 and 30 kDa protein isolated from *C. jejuni* (Kaldor and Speed, 1984). GM1-positive sera from patients with GBS following infection with *C. jejuni* showed reactivity to a 63-kDa flagellar protein purified from *C. jejuni* (O:19) (Lange et al., 1999). It has also been shown that GM1 antibodies cross-react with Gal-GalNAc-bearing

glycoproteins from the peripheral nerve (Apostolski et al., 1994). Cross-reactive determinants were detected in glycoproteins from human peripheral nerve and *C. jejuni* O:19, recognized by peanut agglutinin (PNA) and by GM1 positive sera from patient with GBS associated with *C. jejuni* infection. Results from our research group showed positive cross-reactivity of the peptides from the human peripheral nerve and *C. jejuni* recognized by GM1-positive GBS serum associated with *C. jejuni* infection (Brezovska et al., 2011, Poceva Panovska et al., 2011). Results of our study revealed structural similarity in oligosaccharide portion and immunoreactivity of the glycoproteins isolated from peripheral nerve and *C. jejuni*, indicating that they are potentially cross-reactive determinants and may contribute to the development of GBS associated with antecedent *C. jejuni* infection (Poceva-Panovska et al., 2011).

There are literature data for the reactivity of anti-GM1 and asialo-GM1 antibodies from patients with MMN or chronic neuropathies with the LOS of *C. jejuni* (Wirguin et al., 1994). The possibility that *C. jejuni* may also be involved in the pathogenesis of MMN has supported by several reports of patients developing MMN and high titers of anti-GM1 antibodies after *C. jejuni* enteritis (White et al., 1996, Abbruzzese et al., 1997, Taylor et al., 1998, Terenghi, et al., 2002).

In our research we have shown the cross-reactivity of GM1 positive sera from patients with MMN and GM1-like protein antigens isolated from human peripheral nerve and from *C. jejuni* O:19. Higher IgG anti-GM1 frequency was found in patients with GBS (36.4%). In other groups, IgG anti-GM1 antibodies were present in patients with CIDP (6.7%) and in patients with MMN. Patients with MMN represented a group with the highest IgM anti-GM1 reactivity (45.8%). Higher frequency of the presence of anti-ganglioside and anti-glycoprotein antigens in patients with MMN compared to healthy controls and to patients with other neuropathies point out their pathogenic significance in the inducing and propagation of the nerve damage and development of neurological symptoms (Suturkova et al., 2013).

The cross-reactivity of antibodies from tested sera to human peripheral nerve antigens and antigens isolated from *C. jejuni*, could be explained by the concept of natural auto antibodies, reflecting the natural non-pathogenic autoimmunity or by the presence of antibodies generated from infections and natural exposure to pathogens, including *C. jejuni* (Avrameas, 1991, Panigrahi et al., 1992, Gabriel et al., 2000).

The increased titer of antibodies to human peripheral nerve antigens in patients with neuropathies, could be explained as a secondary reaction to the exposure of non-immunogenic nerve antigens, following demyelination and nerve cell damage (Gabriel et al., 2000). These autoantibodies may further contribute to the progression and deterioration of the disease.

The higher frequency of the presence of anti-ganglioside and anti-glycoprotein antigens in patients with GBS, CIDP and MMN, compared to healthy controls and to patients with other neuropathies point out their pathogenic significance in the inducing and propagation of the nerve damage and development of neurological symptoms. The cross-reactivity of these antibodies to *C. jejuni* antigens, indicate on the shared epitopes between human and bacterial glycoproteins, and their possible role in the induction of autoantibodies and peripheral neuropathies following infection with *C. jejuni*. Determination of the structure and localization of the cross-reactive PNA-binding glycoproteins will help in understanding the mechanisms that trigger myelin-related neurological diseases.

Conclusions

The cross-reactivity of anti-glycolipid and anti-glycoprotein antibodies to *C. jejuni* antigens, indicate on the shared epitopes between human and bacterial glycoproteins, and their possible role in the induction of autoantibodies and peripheral neuropathies following infection with *C. jejuni*. These cross-reactive glycoproteins, together with GM1 ganglioside are potentional antigens for autoantibodies, and may play a significant role in the development of autoimmune peripheral neuropathies. These findings provide a new concept in the antibody-antigen interactions based on carbohydrate epitopes structure. Studies using autoimmune neuropathy sera may continue to identify additional glycosphingolipids and glycoproteins that have yet to be identified as autoantigens, these will be aided by further development of purification and analysis methods. The findings will be useful in laboratory evaluation of peripheral neuropathies and help to develop a more effective therapeutic approach.

Resolving the mechanisms which regulate the B cells to produce anti glycoconjugate antibodies in patients with neuropathies, will give an opportunity to develop specific and effective strategy for targeted immunotherapy in order to prevent or limit the processes that result in autoimmune neuropathy. The other important issue is genetic factor from bacteria and host involved in the immune response of peripheral neuropathies. Still the research of the genetic factors is under intensive investigation. Maybe in the future testing of the polymorphism will be part of laboratory evaluation of peripheral neuropathies.

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Резиме

Гликонјугати како целни антигени во периферните невроаптии

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Клучни зборови: гликопротеини, ганглиозиди, вкрстена-реактивност невропатии, *Campylobacter jejuni*

Идентификацијата и карактеризацијата на автоантигените присутни во периферниот нервен систем на човекот, претставува голем предизвик во полето на клиничката невроимунологија. Најновите истражувања се насочени кон разбирањето на биологијата на гликоконјугатите присутни во периферните нерви и нивната имунолошка реактивност. Зголемен титар на антитела кои ја препознаваат јаглехидратната детерминанта на гликонјугатите (гликолипиди и гликопротеини) се поврзани со ралични невропатски синдроми. Утврдена е значителна вкрстена реактивност помеѓу

антиганглиозидните антитела, како резултат на постоење на заеднички епитопи, што претставува една од можните причини за постоење на заеднички симптоми кај пациентите со невропатии. Серуми од пациенти со невропатии (Guillain - Barré синдром, хронична инфламаторна демиелинизирачка полиневропатија, мултифокална моторна невроаптија) покажуваат имунолошка реактивност со протеини изолирани од хуман периферен нерв и од *Campylobacter jejuni* O:19. Фреквенцијата на појава на антителата насочени кон овие гликопротеини е различна во зависност од типот на невропатијата. Идентификацијата на овие вкрстено реактивни антигени, или други потенцијални антигени, може да помогне во разбирањето на етиологијата на периферните невропатии, како и во лабораториската евалуација на периферните невропатии и развивањето на поефикасни терапевтски пристапи.

Macedonian pharmaceutical bulletin, **60** (2) 29 - 37 (2014)
ISSN 1409 - 8695
UDC: 582.477.6-113.55(497.7)
582.477.6:547.56(497.7
Original scientific paper

Chemical characterization and radical scavenging activity of leaves of *Juniperus foetidisima*, *J. excelsa* and *J. communis* from Macedonian flora

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Received: October 2014; Accepted: December 2014

Abstract

Chemical characterization of three *Juniperus* species: *J. foetidisima* (JF), *J. excelsa* (JE) and *J. communis* (JC) from Macedonian flora enclosed determination of yield and essential oil composition of the oils obtained by hydro-distillation of dried leaves and determination of the content of total phenols and total flavonoids in dried plant material. GC/FID/MS analysis showed mainly monoterpene profile of the JC oil and combined monoterpene/sesquiterpene profile of JF and JE oils. Sesquiterpene cedrol was found as an important constituent of the JF and JE, thus the JF oil was characterized by three main components (α–pinene, limonene and cedrol, in amount up to 67.63%, 27.11% and 33.91%, respectively) and JE oil by four components (α–pinene, sabinene, *cis*-thujone and cedrol, in amount up to 33.83%, 29.49%, 26.20% and 24.44%, respectively). The JC oil was free of cedrol, but contained relatively large sesquiterpene fraction (sesquiterpene hydrocarbons and oxygen containing sesquiterpenes in amounts up to 28.64% and 13.57%, respectively). The JC oil was characterized by three monoterpene components (α–pinene, sabinene and terpinen-4-ol, presented up to 28.68%, 16.27% and 12.16%, respectively). The content of total phenols determined by Folin-Ciocalteu method ranged from 96.18-122.91 mg GAE/g dw (water extraction) while the content of total flavonoids ranged from 2.05-11.91 mg CE/g dw (ethanol extraction). Both water and ethanol extracts possessed radical scavenging activity against DPPH radical. Water extracts were more powerful with % of inhibition of DPPH ranging up to 64.52%, 67.40% and 78.23% for water extract (10 mg/ml) of JF, JE and JC, respectively. Obtained results showed correlation with the content of total phenols.

Keywords: *Juniperus communis, Juniperus excelsa, Juniperus foetidisima,* essential oil composition, GC/MS, total phenols, total flavonoids, DPPH.

Introduction

Juniperus is one of the major genera of Cupressaceae family consisting of approximately 70 species variable in size and shape, from tall trees to columnar or low spreading shrubs. The plants are evergreen with leave-like or scale-like leaves. Many of *Juniperus* species are known and used as medicinal or commercially valuable plants. The common juniper, *Juniperus communis* L. (Cupressaceae), is an evergreen shrub or small coniferous tree, wide spread through the cool temperate Northern Hemisphere. *Juniperus excelsa* Bieb. is a large shrub or tree, spread mainly

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throughout the eastern Mediterranean starting from north-eastern Greece and southern Bulgaria across Turkey to the Middle-East countries (Syria and Lebanon) and the Caucasus Mtn. It occurs in Iran, Pakistan and Oman as well (Khan et al., 2012). *Juniperus foetidissima* Willd. is a medium-size tree, spread mainly throughout the southeastern Europe and southwestern Asia, starting from south-eastern Albania and northern Greece, across Turkey, Syria and Lebanon to the northern Iran and southwestern Turkmenistan. It often occurs together with *J. excelsa* Bieb., but it could be distinguished by its thicker shoots and green leaves (Marcysiak et al., 2007).

The above-ground parts, especially leaves and berries of *Juniperus* species are rich in essential oil that has characteristic aromatic flavour and bitter taste. Due to its diuretic and gastrointestinal properties, common juniper (*J. communis*) is used as medicinal plant for centuries. Besides berry essential oil, other juniper essential oil can be obtained from leaves, wood and seeds of the plant, usually by hydrodistillation (Orav et al., 2010a; Chatzopoulou and Katsiotis, 1993; Kumar et al., 2007). The oils are used in the pharmaceutical and cosmetic industries, for food and beverages, as well as for the production of perfumes. *J. excelsa* is a medicinal plant that has been used in folk medicine to treat dysmenorrhe, cough, bronchitis and colds, jaundice and tuberculosis and to induce menses and expel fetus (Emami et al., 2011). It is known as a remedy for diarrhea, abdominal spasm, asthma, fever, gonorrhea, headache and leucorrhoea (Khan et al., 2012). Among limited biological and pharmacological properties studied *in vitro* such as cytotoxic (Topcu et al., 2005) and antispasmodic activity (Atas et al., 2012), the most investigated were the antioxidant (Atas et al., 2012; Emami et al., 2007; Moein et al., 2010; Emami et al., 2011a) and antimicrobial activity (Ehsani et al., 2012; Atas et al., 2012; Asili et al., 2008; Unlu et al., 2008). However, there are few investigations regarding the biological activities of *J. foetidissima* also, with reports on antifungal (Balaban et al., 2007), antimicrobial (Asili et al., 2010), cytotoxic (Sadaeghi-Aliabdi et al., 2009), anticholinesterase (Ozturk et al., 2010), fumigant (Tayoub et al., 2012), anti-inflamatory (Orhan et al., 2012; Lesjak et al., 2013) and antioxidant effects (Lesjak et al., 2013; Emami et al., 2007; Emami et al., 2011b). These activities are probably result to the complex chemical pattern of terpene and other components.

There is a lot of literature data concerning the chemical composition of different *Juniperus* species, predominantly reporting the essential oil composition of berries and leaves. Thus, for *J. communis* considerable variations in the leaf oil composition were observed depending on the plant origin, however the essential oils is characterized with- α-pinene, sabinene and myrcene, followed by *trans*-(E)-caryophyllene, muurolene, germacrene D and B and humulene (Orav et al., 2010a; Chatzopoulou and Katsiotis, 1993; Kumar et al., 2003, Ottavioli et al., 2009; Filipowicz et al., 2009; Shahmir et al., 2003; Orav et al., 2010b). The major oxygen containing terpenoids were terpinen-4-ol (Chatzopoulou and Katsiotis, 1993), rarely citronellol (Koukos and Papadopoulou, 1997) and terpenyl acetate (Angioni et al., 2003). The *J. excelsa* leaf essential oil is reach in cedrol, α-pinene and limonene (Adams, 1990a), rarely α-pinene (Topcu et al., 2005; Emami et al., 2010b) or cedrol (Topcu et al., 2005; Emami et al., 2010b). *J. foetidissima* contain essential oil in almost all parts of the plant, with variable composition. Sabinene, α-thujone, terpinen-4-ol and γ-terpinene have been reported as major components of the leaf essential oil of *J. foetidissima* from Greece (Adams, 1987; Adams, 1990b). The monoterpenes sabinene, α-pinene and limonene were predominant components of the essential oils of fruits and leaves of male and female plant of *J. foetidissima* from Iran (Asili et al., 2010). Turhish *J. foetidissima* contained β-thujone and cedrol as major components of the leaf essential oil, while sabinene was predominant component in the berry oil (Tunalier et al., 2002).

On the other hand, there is lack of information for the chemical composition and content of other secondary metabolites derived from the leaves of *Juniperus* species. Modnicki and Labedzka (2009) reported 2.40-3.43% of total phenol contents (TPC) estimated as pyrogallol in leaves of *J. communis*. Aditionally, Moein at al. (2014) found a relationship between antioxidant potentials and phenolic compounds in fruits of *J. excelsa*, pointing out that most active fraction posses the highest reducing power (IC_{50} 61.4 μg/ml) and highest phenolic content (82.9 mg/g) (Moein et al., 2014). Further, the leave extracts of some other *Juniperus* species such as *J. oxycedrus* exhibited very high contents of polyphenols (133.08 mgGAE/g dw) and flavonoids (61.52 mg CE/g dw) (Chaouche et al., 2014).

Juniperus species occur in Macedonian flora as an important floristic elements. *J. communis* is widely spread shrub throughout the whole territory of Republic of Macedonia (RM) (Micevski, 1998). The berries of this plant are extensively utilized in production of blended teas and other herbal medicinal products, in food industry and as a spice in production of alcoholic beverages. For years, the juniper berries and the juniper essential oil are exported from RM. Additionally, the juniper leaves are used in folk medicine for various purposes. *J. excelsa* medicinal properties are also known by people of RM as pain reliever, for curing cold, asthma, edema or skin diseases. *J. foetidissima* grows in southern parts of the country, but could be found in the valleys of the River Crn Drim and the River Treska in western and Karadzica Mtn. in central RM (Micevski, 1998). Shoots decoction of the plant is known in folk medicine for curing coughs and common cold. Up to now the chemistry of the leaves of these three *Juniperus* species from Macedonian flora is scientifically unknown and only one report on chemical composition, antioxidant and anti-inflammatory effects of Macedonian *J. foetidissima* was published (Lesjak et al., 2013). Therefore the aim of the present study was chemical characterization of the

leaves as well as assessment of radical scavenging activity of leaves extracts from *J. communis, J. excesla* and *J. foetidissima* from RM.

Material and methods

Plant material

Plant samples were collected in late autumn in 2010 and 2011. The terminal twigs of *J. communis* (JC) were collected from tree different localities in RM (Shara Mtn., Galicica Mtn. and Bistra Mtn.) (6 samples), terminal twigs with leaves of *J. excelsa* (JE) from two different localities (Velestovo and Dojran) (4 samples) and terminal plant twigs of *J. foetidissima* (JF) from four different localities in RM (Valandovo, Udovo, Veles and Velestovo) (8 samples). After air-drying in shadow the leaves were separated from stems, packed in paper bags and kept at dark and cool place until analysis.

Chemicals

All chemicals were purchased from Sigma-Aldrich (Missouri, USA), Merck (Darmstadt, Germany) and Alkaloid (Skopje, R. Macedonia).

Essential oil isolation

Essential oil isolation from plant leaves was made by steam distillation in special all-glass Clevenger type apparatus. For that purpose, 50 g of minced and dried leaves was distilled for 4 hours. After isolation, anhydrous sodium sulfate was added to remove residual water from the oil. Essential oil yield was expressed as %, calculated on dried weight (dw).

Extraction procedure

In order to determine the content of total flavonoids and total phenolic compounds, samples containing 1.0 g of powder dried plant material were processed. Two types of extracts were prepared, with 96% ethanol and water. The extraction procedure for sample preparation was performed with 10 ml of extractive solvent for 30 min in ultrasonic bath (50/60 Hz, 720 W). The extracts were filtered and the volume was made up to 10 ml. The obtained ethanol and water extracts were used for evaluation of radical scavenging activity as well.

GC/FID/MS analysis of essential oil

Essential oil samples were analyzed on Agilent 7890A Gas Chromatography system equipped with FID detector and Agilent 5975C Mass Quadrupole detector as well as capillary flow technology which enables simultaneous analysis of the samples on both detectors. For that purpose, HP-5ms capillary column (30 m x 0.25 mm, film thickness 0.25 μm) was used. Operating conditions were as follows: oven temperature at 60 °C (5 min), 1 °C/min to 80 °C (2 min) and 5 °C/min to 280 °C (5 min); helium as carrier gas at a flow rate of 1ml/min; injector temperature 260 °C and that of the FID 270 °C. 1μl of each sample was injected at split ratio 1:1. The mass spectrometry conditions were: ionization voltage 70 eV, ion source temperature 230 °C, transfer line temperature 280 °C and mass range from 50 - 500 Da. The MS was operated in scan mode. For GC/FID/MS analysis, the essential oil was dissolved in xylene to obtain 1 μl/ml oil solution.

Identification of the components present in essential oils was made by comparing mass spectra of components in essential oils with those from Nist, Wiley and Adams mass spectra libraries, by AMDIS (Automated Mass Spectral Deconvolution and Identification System) and by comparing literature and estimated Kovat's (retention) indices that were determined using mixture of homologous series of normal alkanes from C_9 to C_{25} in hexane, under the same above mentioned conditions.

The percentage ratio of essential oils components was computed by the normalization method of the GC/FID peak areas without any correction factors.

Determination of total phenolic content

The total phenolic content (TPC) of the leaves was determined with the Folin-Ciocalteu reagent according to a procedure described by Singleton et al. (1999) with slight modifications. To 1.0 ml of test sample (leaves extract), 0.5 ml (1:10 v/v diluted with distilled water) Folin-Ciocalteau reagent was added and stirred for 5 min at room temperature. After 5 min, 0.4 ml of 7.5% of sodium carbonate was added and made up to 10 ml with distilled water. These mixtures were incubated at room temperature in the dark for 2 hours. After incubation, absorbance of blue color was measured at 765 nm using a UV-Vis spectrophotometer (Agilent 8453 UV-Vis spectrophotometer, Agilent Technologies, USA). The total phenolic content was determined as mg of gallic acid equivalents per gram of dried weight of plant material (mg GAE/g dw) using an equation obtained from standard gallic acid calibration graph.

Determination of total flavonoid content

The total flavonoid content (TFC) was determined using the aluminum chloride assay described by Talari et al. (2012) with slight modification. To an aliquot of the test sample (1.0 ml of extract), 4.0 ml of distilled water and 0.3 ml of 5.0 % sodium nitrite were added and allowed to stand for 5 min. Later, 0.3 ml of 10.0 % aluminum chloride was added and the mixture was incubated for 6 min. 2.0 ml of 1 M sodium hydroxide was added and the volume was made up to 10.0 ml with distilled water. After incubation of 15 min, the mixture turned to pink and the absorbance was

measured at 510 nm using a UV-Vis spectrophotometer (Agilent 8453 UV-Vis spectrophotometer, Agilent Technologies, USA). The TFC was expressed in mg of catechin equivalents per gram of dried weight of plant material (mg CE/g dw) using an equation obtained from standard (+)-catechin calibration graph.

Free radical scavenging activity - DPPH assay

The scavenging activity of DPPH free radical of leaves extracts was done according to the method reported by Gyamfi et al. (1999) with minor modifications. 200 µl of different concentrations of tested samples (100, 50, 20 and 10 mg/ml for ethanol extracts and 10, 5, 2 and 1 mg/ml for water extracts) were placed in a cuvette and 4 ml of 100 µm methanolic solution of DPPH was added. Mixtures were shaken vigorously for 1 min and left to stand 10 min in the dark at ambient temperature. The reduction of the DPPH free radical was measured by reading the absorbance at 517 nm. Methanol was used as control.

The percentage of inhibition was calculated from the absorbance of the control (A_c) and the sample (A_s) using the following equation: Inhibition (%) = $[(A_c - A_s) / A_c]$ x 100

Results and discussion

Essential oil yield and chemical composition

The essential oil yield of *Juniperus* leaves was found in variable amounts in all investigated species, ranging from 0.50-1.38 %, 0.16-3.30 % and 0.75-0.90 % dw for JF, JE and JC, respectively (Table 1). GC/FID/MS analysis showed presence of four main classes of components in all investigated samples of oils: monoterpene hydrocarbons (MH), oxygen-containing monoterpenes (OM), sesquiterpene hydrocarbons (SH) and oxygen-containing sesquiterpenes (OS). In some samples diterpenes (D) were also determined in very small amounts as well as some non-terpene components (NT) (Table 1). The monoterpene hydrocarbons were the most abundant fraction in all investigated oils ranging from 39.97% to 53.39%, 40.96% to 42.80% and 38.22% to 83.21%, in the essential oils of JC, JE and JF, respectively. The second dominated fraction was variable for different oils, thus for JC it was sesquiterpene hydrocarbons (12.27-28.64%), while for other two species it was oxygen containing sesquiterpenes, presenting a broad range from 10.10% to 39.71% and from 0.67% to 25.28%, for essential oil of JF and JE, respectively (Table 1).

The two main components in JC essential oil were α-pinene (21.27-28.68%) and sabinene (2.29-16.27%). Additionally, limonene was presented up to 6.95% and terpinene-4-ol up to12.16%. The monoterpene α-pinene was extremely high in some samples of JE and JF (up to 33.83% and up to 67.61%, for JE and JF, respectively). Additionally, the percentage of limonene was high, ranging up to 6.14% and 27.11% in samples of JE and JF, respectively. Some samples of JE contained high percentage of *trans*-sabinyl acetate (up to 10.38%). In the same time, these oils were the only one containing thujones (*cis*-thujone up to 26.20% and *trans*-thujone up to 12.86%).

Concerning the presence of sesquiterpene components, obtained results could not be classified and generalized as deferent components were present in deferent oils in a very different amount, from traces to very high percentage. Interesting component was sesquiterpene cedrol, found in JF and JE from 9.60-33.90% and from 0.0-24.44%, respectively, while in JC it was not detected (Table 1). Other often identified sesqiterpene components were: *trans*-(E)-caryophyllene (0.22-3.305 and 0.81-3.45%, for JF and JC, respectively, not detected in JF), germacrene D (1.21-4.16% and 1.43-3.23% in JE and JC, respectively and only 0.2-0.5% in JF), δ-cadinene (0.2-1.5%, 0.31-3.95% and 2.05-7.98% for JF, JE and JC, respectively), α-cadinol (up to 2.1%, 1.31% and 6.05% for JF, JE and JC, respectively). For JC oils, γ-cadinene and *epi*-α-muurolol were characteristic, presented up to 2.26% and 4.13%, respectively.

In general, considering the maximum determined percentages of the constituents of the oils, JF oil was characterized by three main components (α–pinene, limonene and cedrol, presented up to 67.63%, 27.11% and 33.91%, respectively), JE oil by four components (α–pinene, sabinene, *cis*-thujone and cedrol, presented up to 33.83%, 29.49%, 26.20% and 24.44%, respectively) and JC oil by three components (α–pinene, sabinene and terpinen-4-ol, presented up to 28.68%, 16.27% and 12.16%, respectively).

Compared to literature data, similarity in the composition of the leave essential oil was found with the Greek *J. communis* where α-pinene (41.25%) and sabinene (17.4%) have been found as predominant constituents followed by smaller amounts of limonene (4.2%), terpinen-4-ol (2.7%), β-myrcene (2.6%) and β-pinene (2.0%) (Chatzopoulou and Katsiotis, 1993). Estonian *J. communis* leave oil was rich in α-pinene (33.3-45.6%) and sabinene (0.2-15.4%) while limonene, *trans*-(E)-caryophyllene, α-humulene and germacrene D were presented in smaller amounts (Orav et al., 2010a, 2010b). Filipowicz et al. (2009) have reported that populations of *J. communis* from Northern Poland have essential oils with different α-pinene/sabinene ratio. Iranian authors reported that juniper leaves essential oil was rich in sabinene (40.7%), followed by α-pinene (12.5%) and terpinen-4-ol (12.3%) (Shahmir et al., 2003). Asili et al. (2008), confirmed α-pinene as predominant component in the Iranian *J. communis* subsp. *hemisphaerica* LEO, while Ottavioli et al. (2009) for French *J. communis* subsp. *alpina* reported limonene (9.2-53.9%), β-phellandrene (3.7-25.2%), α-pinene (1.4-33.7%) and sabinene (0.1-33.6%) as major constituents. The leave essential oil from Indian *J. communis* contained predominantly sabinene (22.8%), β-pinene (10.7%), *trans*-sabinene hydrate (6.0%) and γ-cadinene (10.6%) (Kumar et al., 2007).

Considering leaves essential oils of *J. excelsa*, many authors reported higher amounts of α-pinene and cedrol. In

Table 1. Essential oil yields (%) and chemical composition of leaves essential oils of *Juniperus foetidissima* (JF), *Juniperus excelsa* (JE) and *Juniperus communis* (JC) from R. Macedonia (%)

Components	RI	RIE	JF min	JF max	JE min	JE max	JC min	JC max
Tricyclene	921	930.4	tr	0.12	-	-	-	0.04
α-Thujene	931	933.8	-	-	0.84	1.42	-	2.43
α-Pinene	932	937.2	9.62	67.63	1.76	33.83	21.27	28.68
Camphene	946	945.5	0.21	1.01	-	0.47	-	0.91
Sabinene	969	962.8	-	-	0.34	29.49	2.29	16.27
β-Pinene	974	964.1	0.21	1.42	-	-	-	2.28
β-Myrcene	988	976.4	0.62	1.94	0.46	2.78	0.55	2.12
Δ^3-Carene	1008	989.8	0.41	3.42	-	-	-	3.15
α-Terpinene	1018	998.3	-	-	-	1.87	-	0.12
p-Cymene	1020	1001.8	0.21	0.32	0.19	0.88	tr	5.78
Limonene	1024	1006.7	10.0	27.11	1.75	6.14	-	6.95
γ-Terpinene	1054	1033.4	0.12	0.53	tr	3.53	-	0.23
α-Terpinolene	1086	1062.5	0.33	0.73	-	1.02	-	0.21
cis-Thujone	1101	1079.2	-	-	-	26.20	-	-
trans-Thujone	1112	1090.9	-	-	-	12.86	-	-
trans-Pinocarveol	1135	1117.1	-	-	-	-	-	0.47
trans-Sabinol	1140	1120.3	-	-	-	1.74	-	1.16
Camphor	1141	1123.7	0.12	0.22	-	-	-	-
Borneol	1165	1150.0	tr	0.12	-	-	-	0.28
Terpinen-4-ol	1174	1161.8	-	0.11	-	5.87	0.55	12.16
p-Cymene-8-ol	1179	1171.1	-	0.31	-	-	-	0.24
α-Terpineol	1186	1175.1	-	tr	-	-	-	0.37
Bornyl acetete	1284	1263.4	-	0.11	-	-	-	0.78
trans-Sabinyl acetate	1289	1268.5	-	-	-	10.38	-	0.13
δ-Elemene	1335	1306.0	-	0.32	-	0.12	-	0.14
α-Cubebene	1345	1318.6	-	0.21	tr	0.17	-	0.12
α-Copaene	1374	1345.4	tr	0.21	0.05	0.28	-	0.67
β-Bourbonene	1387	1354.5	-	0.11	0.06	0.25	-	0.32
β-Elemene	1389	1362.2	-	-	-	-	1.37	4.17
Sibirene	1400	1380.1	-	-	-	-	0.31	2.31
β-Funebrene	1413	1381.3	-	4.32	-	-	-	-
trans-Caryophyllene	1418	1386.9	tr	tr	0.22	3.30	0.81	3.45
β-Cedrene	1419	1387.7	0.54	2.72	-	-	-	-
β-Copaene	1430	1396.1	-	-	0.05	1.19	-	0.20
γ-Elemene	1434	1399.7	-	-	-	2.17	0.58	2.95
α-Humulene	1452	1421.8	tr	0.24	tr	0.45	0.07	2.89
γ-Muurolene	1478	1444.6	-	0.24	0.06	0.92	-	-
Germacrene D	1484	1449.9	0.27	0.54	1.21	4.16	1.43	3.23
β-Selinene	1489	1455.1	-	-	-	-	0.11	0.64
α-Selinene	1498	1463.2	-	-	-	-	0.71	1.07

Components	RI	RIE	JF min	JF max	JE min	JE max	JC min	JC max
α-Muurolene	1500	1468.3	-	-	0.21	1.83	0.45	1.32
γ-Cadinene	1513	1482.1	-	-	0.11	1.92	0.60	2.26
δ-Cadinene	1522	1490.3	0.21	1.51	0.31	3.95	2.05	7.98
trans-Cadina-1,4-diene	1533	1538.8	-	0.52	-	0.51	-	0.31
α-Cadinene	1537	1504.1	-	-	-	0.17	-	0.50
Germacrene B	1559	1525.6	-	-	-	1.91	-	0.49
Spatulenol	1577	1546.0	-	-	-	-	-	1.67
Caryophyllene oxide	1581	1552.6	0.11	0.42	-	-	-	1.73
Cedrol	1600	1576.2	9.62	33.91	-	24.44	-	-
Humulene epoxide II	1608	1578.2	-	-	-	-	0.71	1.17
1,10-di-*epi*-Cubenol	1618	1582.8	-	-	-	0.08	-	tr
1-*epi*-Cubenol	1627	1595.2	0.12	0.82	0.05	0.93	0.39	2.31
epi-α-Muurolol	1640	1609.7	-	0.21	-	-	1.07	4.13
α-Muurolol	1645	1613.4	-	0.41	-	0.02	-	0.44
α-Cadinol	1653	1621.9	-	2.11	-	1.31	1.67	6.05
Manool oxide	1987	1962.4	-	0.12	-	-	-	0.27
Abietatriene	2054	2024.7	-	tr	tr	0.08	-	0.62
Abietadiene	2080	2051.9	tr	0.12	tr	0.47	-	0.12
4-*epi*-Abietal	2298	2264.5	-	0.13	tr	0.21	-	0.04
Total (%)			93.23	96.55	82.69	95.61	86.07	96.31
Monoterpene hydrocarbons (MH)			38.27	83.25	40.96	42.80	39.97	53.39
Oxygen containing monoterpenes (OM)			0.55	1.75	0.34	44.97	3.89	12.16
Sesquiterpene hydrocarbons (SH)			2.32	15.45	6.91	16.11	12.27	28.64
Oxygen containing sesquiterpenes (OS)			10.15	37.94	0.67	25.28	5.98	13.57
Diterpenes (D)			tr	0.37	tr	0.76	-	1.05
Non-terpene components (NT)			0.3	0.12	-	-	-	0.04
Essential oil yield (%, dw)			0.50	1.38	0.16	3.30	0.75	0.90

RI - Retention index - literature data (Adams, 2007); RIE - Retention index experimentally determined with reference to a homologous series of *n*-alkanes on HP-5ms column (AMDIS); (-) - not found, tr - traces < 0.02

some earlier papers Adams reported that the oil from wild growing *J. excelsa* from Greece contained cedrol (28.1%), α-pinene (22.5%) and limonene (22.7%) as predominant constituents (Adams, 1990). Also, Turkish authors found α-pinene (29.7%) and cedrol (25.3%) (Topcu et al., 2005) as dominant for this species. Moreover, Iranian researchers confirmed these two components as predominant in the leaf essential oil from wild growing *J. excelsa* from Iran (α-pinene 32.34% and cedrol 13.06%) (Emami et al., 2011a). Recently, Adams et al., revealed presence of moderate geographical variations in the volatile leaves oil of *J. excelsa,* comparing the samples from Greece, Bulgaria, Turkey and Cyprus (Adams et al., 2013) where cedrol was found to be the most abundant constituent of the oils, ranging from 11.3 to 35.8%. These data are in correlation with our findings for some samples of *J. excelsa,* but some other samples from Republic of Macedonia possess essential oil with deferent oil composition characterized with larger percentages of sabinene and thujone (*cis* + *trans*) (29.49% and 39.06%, respectively).

According to literature data leaf essential oil of *J. foetidissima* was characterized by monterpene hydrocarbons as predominated constituents of the oil. For the leaves essential oil of *J. foetidissima* from Greece, Adams reported sabinene (19.6%), α-thujone (18.6%), terpinen-4-ol (17.6%) and γ-terpinene (6.5%) as major components (Adams, 1990b). At the same time, the minor compounds of this essential oil were α-terpinene (4.3%), β-thujone (3.5%), cedrol (3.2%), myrcene (2.7%) and α-pinene (2.6%). Iranian researchers found that the major components of the essential oils of fruits, leaves of male and leaves of female plant of *J. foetidissima* were sabinene (37.1, 19.9 and 16.8%), α-pinene (29.9, 22.2 and 18.6%) and limonene (11.8, 20.9 and 13.6%), respectively (Asili et al., 2010). Considering essential oil composition of *J. foetidissima* from Balkans, only one article was published by Lesjak et al. (2013) who reported sabinene (39.9%), γ-terpinene (10.1%) and terpinen-4-ol (17.0%) as major monoterpenes and germacrene D (0.7%) and γ-cadinene (2.9%) as major sesquiterpene. Our findings showed higher percentage of α–pinene (67.63%), followed by larger amounts of cedrol (up to 33.91%) and limonene (27.11%).

The contents of total phenols and total flavonoids and DPPH radical scavenging activity

The obtained values for total phenols content determined with the Folin-Ciocalteu reagent (TPC-FC) as well as total flavonoid content determined spectrophotometrycally using $AlCl_3$ as chelating agent (TFC-$AlCl_3$) are presented in Table 2. In all investigated samples of *Juniperus* leaves the content of TPC-FC was almost twice higher in water extracts (96.18-122.91 mg GAE/g dw) compared to ethanol extracts (47.72-64.42 mg GAE/g dw). On the contrary, the contents of TFC-$AlCl_3$ were slightly higher in ethanol extracts (2.05-11.91 mg CE/g dw) compared to water extracts (1.95-10.25 mg CE/g dw). Leaves extract of JF and JE, both water and ethanol contained much higher content of TFC-$AlCl_3$ than JC extracts.

Leaves extracts of investigated *Juniperus* species (JF, JE and JC), both water and ethanol extracts, showed radical scavenging capacity expressed as % of inhibition of the DPPH radical that had broad range depending on the extract concentration. In both cases more concentrated extracts have shown better results and water extracts have demonstrated stronger scavenging activity (Table 3). Thus, the highest radical scavenging activity against DPPH radi-

Table 2. The content of total phenols (TPC - FC, mg GAE/g dw) and total flavonoids (TFC - $AlCl_3$, mg CE/g dw) in leaves of *Juniperus foetidissima* (JF), *Juniperus excelsa* (JE) and *Juniperus communis* (JC)

Specimen	TPC-FC water extract	TPC-FC ethanol extract	TFC-FC water extract	TFC-$AlCl_3$ ethanol extract
JF	118.87 ± 0.68	60.45 ± 1.62	10.25 ± 1.16	11.91 ± 1.24
JE	122.91 ± 0.35	64.42 ± 1.34	8.85 ± 0.94	8.92 ± 1.84
JC	96.18 ± 1.22	47.72 ± 0.86	1.95 ± 0.04	2.05 ± 0.52

(n=3)

Table 3. Radical scavenging activity against DPPH radical expressed as % of inhibition for leaves extracts of *Juniperus foetidissima* (JF), *Juniperus excelsa* (JE) and *Juniperus communis* (JC)

Sample	% of inhibition of DPPH radical		
	JF	JE	JC
Ethanol extracts (mg/ml)			
100	18.32 - 20.12	48.73 - 48.82	74.4 - 76.7
50	17.25 - 18.36	39.32 - 42.12	55.7 - 70.7
20	5.61- 6.64	33.03 - 34.32	27.3 - 42.8
10	0.0	14.42 - 16.24	11.2 - 23.1
Water extracts (mg/ml)			
10	60.56 - 64.52	59.74 - 67.4	60.56 - 78.23
5	45.40 - 48.25	40.72 - 72.6	41.95 - 45.40
2	18.23 - 20.19	15.04 - 43.4	19.86 - 20.19
1	10.42 - 12.32	8.54 - 25.71	11.19 - 12.31

cal showed water extract of JF, JE and JC leaves in concentration 10 mg/ml, with % of inhibition of DPPH ranging up to 64.52%, 67.40% and 78.23%, respectively.

Tavares et al. (2009) evaluated the possibility of application of *Juniperus* leaves from species naturally occurring in Portugal (*J. phoenicea* subsp. *phoenicea*, *J. turbinata*, *J. oxycedrus* subsp. *oxycedrus*, *J. oxycedrus* subsp. *badia* and *J. navicularis*) against some diseases in which oxidative reactions play a crucial role. They found that all species exhibited minimum polyphenol and flavonoid contents in March/April and July and therefore a reduced antioxidant activity while the maximum concentrations of these compounds were detected in November/December, when they demonstrated higher antioxidant capacity (Taveras et al., 2009).

The antioxidant activity of leaves and fruits of 11 different conifer taxons growing wild in Iran were evaluated by Emami et al. (2007). Methanol extract of leaves and fruits were prepared and antioxidant activity of each extracts was measured using two different tests (the ferric thiocyanate method and thiobarbituric acid test, TBA). Results indicated that the methanol extracts of leaves, of male and female, and fruits of all these species possessed antioxidant activity when tested with both methods. The antioxidant capacity was then compared with those of α-tocopherol (a natural antioxidant) and butylated hydroxytoluene (BHT, a synthetic antioxidant). Methanol extract of *Juniperus* species, especially *J. excelsa, J. excelsa* ssp. *polycarpos, J. oblonga* and *J. foetidisima,* demonstrated antioxidant activity comparable to the BHT and even higher than α-tocopherol (Emami et al., 2007).

A correlation was found between the primary antioxidant activity and the total phenolic contents in different *Juniperus* species (*J. communis* var. *communis* (Jcc), *J. communis* var. *saxatilis* Pall. (Jcs), *J. drupacea* Labill. (Jd), *J. oxycedrus* subsp. *oxycedrus* (Joo) and *J. oxycedrus* subsp. *macrocarpa* (Sibth. & Sm.) Ball. (Jom)) from Turkey by Taviano et al. (2011). Both in DPPH and TBA test, Jom resulted the most active (IC_{50} = 0.034 ± 0.002 mg/ml and 0.287 ± 0.166 μg/ml, respectively). Different extracts of leaves, ripe fruits, and unripe fruits of *Juniperus* species, including *J. communis*, were studied for antioxidant activity by the ferrous ion-chelating, superoxide radical scavenging and ferric-reducing antioxidant power (FRAP) assays. It was found that all investigated *Juniperus* samples possessed antioxidant activity, but the leaves extracts usually had higher antioxidant activity (Orhan et al., 2011).

Conclusion

Chemical characterization of three *Juniperus* species, *J. foetidisima* (JF), *J. excelsa* (JE) and *J. communis* (JC) from Macedonian flora enclosed determination of yield and essential oil composition of the oils obtained by hydrodistillation of dried leaves and determination of the content of total phenols and total flavonoids in dried plant material. With GC/FID/MS analysis the essential oil profile of all investigated species was characterized dominantly with monoterpene hydrocarbon fraction. Sesquiterpene cedrol was found as an important constituent of the JF and JE oils, however the essential oil from JF was additionally characterized by two other main components (α–pinene and limonene), while the JE oil by three components (α–pinene, sabinene and *cis*-thujone). The essential oil of JC was free of cedrol, despite the fact that the fraction of sesquiterpene components of this oil was relatively high, but it consisted of many components presented in lower percentages and this oil was characterized mainly by three monoterpene components (α–pinene, sabinene and terpinen-4-ol).

The content of total phenols determined by Folin-Ciocalteu method ranged from 96.18-122.91 mg GAE/g dw (water extraction) while the content of total flavonoids ranged from 2.05-11.91 mg CE/g dw (ethanol extraction). Both water and ethanol extracts possessed radical scavenging activity against DPPH radical but water extract were more powerful with % of inhibition of DPPH ranging up to 64.52%, 67.40% and 78.23% for water extract (10 mg/ml) of JF, JE and JC, respectively. Obtained results showed correlation with the content of total phenols as the water extracts contained higher amounts of total phenols and exhibited better antioxidant activity.

The leaves of *Juniperus* species from Macedonian flora (*J. foetidisima, J. excelsa* and *J. communis*) can be taken in further consideration as a plant source for isolation of essential oil as well for extraction of phenolic compounds with promising antioxidant activity. Further research is needed to evaluate the chemical and biological potential of this raw plant material.

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Резиме

Хемиска карактеризација и инхибиторна радикалска активност на листови од *Juniperus foetidisima*, *J. excelsa* и *J. communis* од Македонска флора

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Клучни зборови: *Juniperus communis, Juniperus excelsa, Juniperus foetidosima,*состав на етерично масло, GC/MS, вкупни феноли, вкупни флавоноиди, DPPH.

Хемиската карактеризација на три *Juniperus* вида, *J. foetidisima* (JF), *J. excelsa* (JE) и *J. communis* (JC) од Македонската флора, вклучува определување на содржина и состав на етерични масла добиени со дестилација со водена пареа на суви листови, како и определување на содржина на вкупни феноли и вкупни флавоноиди во сувиот материјал. Со GC/FID/MS анализата утврден е монотерпенски профил на маслото од JC и монотерпенско/сесквитерпенски профил на маслата од JF и JE. Сесквитерпенот цедрол е идентификуван како важен конституент на маслата од JF и JE, при што маслото од JF се карактеризира со три главни компоненти (α–пинен, лимонен и цедрол, во количини што се движат до 67,63%, 27,11% и 33,91%, соодветно), додека маслото од JE се карактеризира со четири главни компоненти (α–пинен, сабинен, *cis*-тујон и цедрол, присутни во количини до 33,83%, 29,49%, 26,20% и 24,44%, соодветно). Маслото од JC не содржи цедрол, иако содржи релативно висок удел на сесквитерпени (сесквитерпенски јаглеводороди и сесквитерпени со кислорд во количини што се движат до 28,64% и до 13,57%, соодветно). Ова масло се карактеризира главно со три монотерпенски компоненти (α–пинен, сабинен и терпинен-4-ол, застапени до 28,68%, до 16,27% и до 12,16%, соодветно). Содржината на вкупните феноли определена со метод по Folin-Ciocalteu се движи од 96,18 до122,91 mg GAE/g (водена екстракција), а содржината на вкупни флавоноиди од 2,05 до 11,91 mg CE/g (етанолна екстракција). Двата екстракти (водениот и етанолниот) поседуваат инхибирачка активност во однос на DPPH радикалот. Водените екстракти се помоќни бидејќи во концентрација од 10 mg/ml покажуваат % на инхибиција на DPPH радикалот кој се движи до 64.52%, до 67.40% и до 78.23%, за JF, JE и JC, соодветно. Добиените резултати покажуваат корелација со содржината на вкупните феноли.

Macedonian pharmaceutical bulletin, **60** (2) 39 - 50 (2014)
ISSN 1409 - 8695
UDC: 613.26:635.655]:615.225.2
Original scientific paper

Formulation of synbiotic soy-based food product with antihypertensive potential

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Received: September 2014; Accepted: December 2014

Abstract

The specific aims of this study were to select the favorable prebiotic for *L casei*-01 as well as the suitable inoculum of the probiotic for fermented soy with ACE-inhibitory potential. For that purpose the metabolic activity of *L casei*-01 in soymilk supplemented with (1.5% *w/v*) prebiotics Fructooligosaccharide (FOS) or oligofructose enriched inulin (Synergy 1) was assessed. The evaluated parameters were: pH, viable cell counts, proteolysis, organic acid production and inhibition of angiotensine converting enzyme activity (IACE). The cell growth of *L casei*-01 reached the recommended therapeutic level of 9.58 ± 0.035 log cfu mL^{-1} for low inoculum samples (0.005 and 0.01%w/v) and 11.543 ± 0.13 log cfu mL^{-1} for high inoculum samples (0.075 and 0.1%w/v) regardless of the prebiotic used. The lower pH during fermentation, faster cell growth and superior proteolysis in Synergy 1 samples indicated better utilization of that prebiotic vs. FOS. The hydrolysis depended on the prebiotic used, showing higher values in Synergy 1 samples. The faster proteolysis was confirmed by SDS-PAG electrophoresis. The Mw of polypeptides in the synbiotic end-products were lower than 30kD. The observed values for inhibition of ACE activity were app. 71, 74, 77 and 78% for inoculum rates of 0.005, 0.01, 0.075 and 0.1% w/v, respectively.

Based on the results obtained in our study, the prebiotic Synergy 1 (1.5% w/v) and *L. casei*-01 at inoculum of 0.01% w/v for low dose and 0.075% w/v for high dose were considered more favorable for the production of synbiotic soy drink with antihypertensive potential.

Key words: *L. casei*, Synergy 1, FOS, soy beverage, ACE inhibition

Introduction

Awareness of complex relationship between food and health has challenged researches to design functional food, food that not only provide basic nutrition but also has health benefit effects (Gibson and Rastall, 2004). High prevalence of hypertension, one of the major risk factors for coronary heart disease, indicates an urgent need for prevention and choice of therapy. From the standpoint of preventive medicine, soybean-based foods are of great interest because of the evidence that consumption of soy proteins 25 g per day can lower the risk of cardiovascular dis-

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ease, an indication approved by the U. S. Food and Drug Administration (1999).

Many improvements have been implemented in the processing and methods of preparation of soymilk to improve functionality and usability of soy proteins, among which fermentation of soymilk with probiotic bacteria (Favaro Trindade et al., 2001; Lopez-Fandino et al., 2006).

Soymilk-derived bioactive peptides during fermentation have many beneficial properties, including prevention or delay in the onset of hypertension (Wang and Mejia, 2005; Liu et al., 2011). Well-known mechanism of action of these peptides is based on the inhibition of angiotensin-I converting enzyme (ACE) (Vermeirssen et al., 2004). ACE inhibitory activity of milk products can be enhanced by using the highly proteolytic lactic acid bacteria (LAB). According to Donkor et al. (2005) the use of probiotic strains such as *L. paracasei*, as a part of starter culture in fermented soymilk resulted in a considerable increase in ACE inhibitory activity *in vitro* compared with the control produced by yogurt culture only (Donkor et al., 2005).

Probiotics differ in their ability to ferment the bioactive fibers. For the production of fermented milk with hypotensive and/or ACE-inhibitory activity, various LAB species have been used, including probiotic *Lactobacillus paracasei* (Fuglsang et al., 2003; Fitzgerald et al., 2006).

Nowadays, to improve therapeutic effects, dairy foods usually contain probiotics along with prebiotics. Inulin and fructooligosaccharide (FOS) are the premium prebiotics used as safe ingredient supplements to fermented milk (Oliveira et al., 2012), with protective effect on the lactic acid bacteria by stimulating their survival and activity of the end-product during storage (Donkor et al., 2006). Furthermore, they enhance the proteolytic activity and ACE-inhibition activity of lactobacilli in fermented soymilk (Yeo and Liong., 2010).

The mixture of probiotics and prebiotics is termed a synbiotic (Gibson and Roberfroid., 1995). The ability of microorganisms to utilize prebiotics is strain- and substrate-specific. Because of that, one of the essentials for good synbiotic formulation is a proper choice of pro- and pre mixture.

The synbiotic mixture of probiotics and prebiotics as well as the composition of a delivery matrix are responsible for variations in the amounts of organic acids, bioactive peptides production and bacterial count in the end-product of fermented functional food. The proteolytic activity should not destroy the product, but it should be able to produce the bioactive peptides in sufficient amount and desired activities. Moreover, it is important for probiotic bacteria to survive the food processing and storage in amount above therapeutic level ranging from 10^6 to over 10^7 or 10^8 cfu mL^{-1} (Kurmann and Rasic., 1991; Lourens-Hattingh and Viljeon., 2001).

The aim of the study was to prepare fermented soymilk supplemented with prebiotic, in which probiotic viability would be increased, and thus its proteolytic and/or ACE inhibitory activity enhanced. The effects of prebiotics, FOS and oligofructose-enriched inulin and different inoculum rates of *L. casei-01*, on the metabolic activity and bioactivity of probiotic-fermented soymilk was evaluated *in vitro* by determination of pH, organic acid production, proteolytic activity and ACE inhibition. In addition, peptide profile of the soy beverage was evaluated.

Material and methods

Fermentation of soymilk with pro- and pre-biotic

Two batches of 5 glass flasks, each containing 200 mL commercial (cholesterol, lactose and gluten free) soymilk (Valsoia Original – VALSOIA SpA, Italy) were supplemented with 1.5 % *w/v* prebiotic. Batch 1 was supplemented with oligofructose-enriched inulin (Synergy-1, Orafti® Synergy 1, Orafti-Rue L. Maréchal, Tienen, Belgium) and batch 2 with fructooligosaccharide (FOS, Sigma Aldrich Chemie Gmbh, Germany). The mixtures were heat treated in a water bath at 90 °C for 30 min, then cooled to 37 °C and aseptically inoculated with *L. casei*-01, commercial name for *Lactobacillus paracasei sp paracasei* (FD-DVS/*Lactobacillus casei*-01 nu-trish, Chr. Hansen, Hoersholm, Denmark), with different inoculum rates: 0.005% *w/v* (SN1 and FS1), 0.01% *w/v* (SN2 and FS2), 0.075% *w/v* (SN3 and FS3), 0.1% *w/v* (SN4 and FS4) and 1% *w/v* (SN5 and FS5). SN represents samples supplemented with Synergy 1 and FS samples with FOS. All batches were incubated at 37 °C until the required pH of 4.5 was reached; then the fermentation was terminated by cooling at 4 °C. At predetermined time points and at the end of fermentation the aliquots of 30 mL were taken from each bottle for determination of cell count, pH, proteolysis and organic acid production. The end-products were analyzed for ACE-inhibitory activity.

pH measurements

Changes in pH were monitored during fermentation of soymilk at 0, 6, 12, 24, 36, 48, 54 h and at the end of fermentation using pH meter (Mettler Toledo Five Easy™ FE 20, Switzerland).

Enumeration of viable cells

Serial dilutions of the samples made in saline water (0.9 % *w/v* NaCl, Alkaloid, Skopje, Republic of Macedonia) were spread onto MRS agar plates (de Man, Rogosa Sharpe agar, Oxoid, Basingstore, UK) and incubated for 48 h at 37 °C in anaerobic conditions. All dilutions were plated in triplicate. Enumeration was performed counting the plates with 25-250 colonies. Mean numbers from two different dilutions were used, and results were expressed as $\log_{10}$ colony forming units per milliliter of fermented milk ($\log_{10}$ cfu mL^{-1}).

Determination of proteolytic activity

Proteolytic activity was determined as the difference between the free amino (NH_3) groups in the fermented and unfermented (untreated) soymilk. The *o*-phthaldialdehyde (OPA) method of Church et al. (1983) was used, with some modifications as reported by Donkor et al. (2005). Briefly, 3 mL of the sample with 3 mL 0.75N TCA (CCl_3COOH) were vortexed for 1 min, centrifuged at 12 000 rpm, 15 min and afterwards the supernatants were filtered through 0.45 μm membrane filter. The OPA reagent was prepared by mixing 40 mg of OPA (*o*-phthaldialdehyde, P1378, Sigma Aldrich Chemie Gmbh, Germany) freshly dissolved in 1 mL methanol (CH_3OH, Merck, KGaA Damstadt, Germany), 25 mL of 100 mM sodium tetraborate decahydrate ($Na_2B_4O_7{\times}10H_2O$, Alkaloid, Skopje, Republic of Macedonia), 2.5 mL 20% *w/w* sodium dodecyl sulfate (SDS, $NaC_{12}H_{25}SO_4$ Merck, KGaA Damstadt, Germany), 100 μL β–mercaptoethanol (C_2H_6OS, Merck, KGaA Damstadt, Germany) and deionized water to final volume of 50 mL. 150 μL of filtered sample (supernatant) was mixed with 3 mL of OPA reagent and after 2 minutes at room temperature (20 °C) the absorbance was measured at 313 nm by a UV/Vis Spectrophotometer-Agilent 8453 (USA). The proteolytic activity was expressed as absorbance of free amino groups measured at 313 nm as a difference in absorbance between fermented and non-fermented samples.

Analysis of peptide profile by SDS-PAG electrophoresis

To determine the proteolytic pattern of soy beverage**,** samples were centrifuged at 4 000 rpm for 20 min (Rotofix 32A Hettich, Hettich Lab technology, Fohrenstrase 12, D-78532 Tuttlingen, Germany). Then, the supernatants were separated, centrifuged again at 12 000 rpm for 20 min and filtered through 0.45 μm membrane filter (MILLEX®-HP, Merck Millipore Ltd., Ireland). The protein transition to low molecular peptides was analyzed with horizontal (4-22 %) gradient gel sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAG electrophoresis) (Gorg et al., 1985). When electrophoresis was completed, the protein bands were stained with Coomassie Blue R-250 (Sigma –Aldrich) in methanol (CH_3OH, Merck, Germany) / water/acetic acid ($C_2H_4O_2$, Alkaloid, Skopje, Republic of Macedonia) and then distained in the same solvent. The fractions were identified by using low molecular mass standard mixture. The molecular weights (Mw) of different proteins were as follow**:** phosphorylase b, 94 kDa; albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor 20.1 kDa and α-lactalbumin 14.4 kDa (Phatmacia LKB, Biotechnoligy AB, Uppsala Sweden); and high molecular weight proteins as follows: myosin, 212 kDa; α_2 –macroglobulin, 170 kDa; β-galactosidase, 116 kDa; transferrin, 76 kDa; and glutamic dehydrogenase 53 kDa.

Determination of produced organic acids in fermented soymilk

The amount of short chain organic acids was determined by high-performance liquid chromatography using a HPLC system apparatus equipped with an ultraviolet detector (Agilent Technologies 1200, Palo Alto, USA). The method used by Wang et al. (2003) was applied with certain modifications. In brief, to determine the concentrations of lactic and acetic acid, 2 mL of 0.5 M H_2SO_4 were added to a 2-mL aliquot of the sample, thoroughly mixed for 30 s and centrifuged (12 000 rpm for 15 min). The obtained supernatants were filtered through a 0.45 μm membrane filter (MILLEX®-HP, Merck Millipore Ltd., Ireland). Samples were loaded onto a thermostatically controlled reverse phase column (Discovery HS C 18, 250 mm x 4.6 mm, 5 μm, Supelco Park, Bellefonte, PA, USA) set at 40 °C and eluted with 0.005 M H_2SO_4 (Alkaloid, Skopje, Republic of Macedonia) at flow rate of 1 mL min^{-1}. According to the method applied, the detection wavelength was 210 nm, while identification of lactic and acetic acids was done using their respective standards.

Determination of ACE-inhibitory activity (IACE)

For the IACE (angiotensin-converting enzyme inhibition) assay the following chemicals were used: the substrate N-[3-(2-furil)-acryloil]-L-phenylalanine-glycyl-glycine (FAPGG, $C_{20}H_{21}N_3O_6$, No 7131 Mw 399.4 g mol^{-1}; Sigma-Aldrich, Co. St Louis. Mo. USA), ACE control E (pure ACE from porcine kidney, Trinity Biotech, USA), and Tris buffer (2 hydroxymethyl-1,3-propanediol, No 108382, Mw 121,14 g mol^{-1}; Merck, KGaA Damstadt, Germany).

Whey samples from the fermented soymilk were prepared by the following procedure: the end-products were centrifuged (4 000 rpm for 20 min at 4 °C) and the supernatants were adjusted to pH 8, centrifuged again at 12 000 rpm for 20 min (Biofuge Fresco Heraeus Instruments, USA) and filtered through 0.45 μm membrane filter (MILLEX®-HP, Merck Millipore Ltd., Ireland).

Whey samples were analyzed by ACE inhibition assay using the method introduced by Tomovska et al. (2011), with slight modifications. Pure ACE from porcine kidney (ACE control E) for ACE solution was used (Vermeirssen et al., 2002). The reaction mixture consisted of a substrate (0.8 mmol L^{-1} FAPGG in 50 mmol L^{-1} Tris buffer, pH 8.2, chlorides 300 mmol L^{-1}), ACE solution (ACE control E - freshly reconstituted with 1 mL deionized water according to the distributor), and sample (supernatant of fermented soymilk) in ratio 10 : 1 : 1. The reaction mixture was stirred by flipping the cuvette, which was then placed at 37°C. During incubation, the absorbance at 340 nm (with deionized water as a blank) was recorded for 45 min at exactly 5 min intervals. The absorbance was measured by the UltroSpec 6300pro UV/Visible Spectrophotometer (GE Healthcare, Fisher Scientific, UK Ltd). ACE activity was

expressed as the slope of the decrease in absorbance at 340 (ρA) over a linear interval of 45 min. The percentage of ACE inhibition (%IACE) was calculated from the ratio of the slope in the presence of inhibitor (sample) to the slope obtained in the absence of inhibitor (deionized water instead) using the following equation (Shalaby et al., 2006):

$$\text{ACE inhibition (\%IACE)} = [1 - (\rho A\, C - D\, /\rho A\, A - B)] \times 100$$

where A contained substrate solution, ACE solution and deionized water; B, substrate blank contained only substrate solution without ACE solution; C, sample (inhibitor) contained substrate solution, ACE solution and sample; D, sample blank contained substrate solution, sample and deionized water instead of ACE solution.

Statistical analysis

The results obtained are presented as means±SD. Differences were determined by using multiple comparison tests: the ANOVA analyses and Tukey honest significant difference (HSD) test. Correlation analyses between parameters were also made (Statgraph for Windows 3.0). In all tests, a probability level of $p<0.05$ was used as a significant difference.

Results and discussion

Changes of pH and viability of L. casei-01 during fermentation of prebiotic soymilk

pH changes, viable cell counts of *L. casei*-01 and its metabolic activity were examined to evaluate the fermentation patterns of the prebiotics added.

The pH changes during fermentation at 37°C for all samples are shown in Fig. 1.

The initial pH of the *L. casei*-01 and prebiotic supplemented soymilk, at time point 0, for all samples was 6.80 ± 0.01. Upon fermentation at 37°C for 30 to 62 h, depending on the inoculum rate (Table 1), it reduced by app. 34%, reaching a value of 4.50. Among the samples fermented with different inoculum rates, both Synergy 1 and FOS supplemented, difference in pH decline rate was observed. Significant difference ($p<0.05$) in pH decline rate was observed within the first 6 h of fermentation between the samples supplemented with low (0.005 and 0.01% *w/v*) and high (0.075, 0.1 and 1% *w/v*) inoculum rates. The high inoculum samples showed a continuous decrease in pH from the very beginning of fermentation, while in the low inoculum samples, a delay of 6 h was observed. Namely, six hours after inoculation, the pH value in the high inoculum samples decreased to

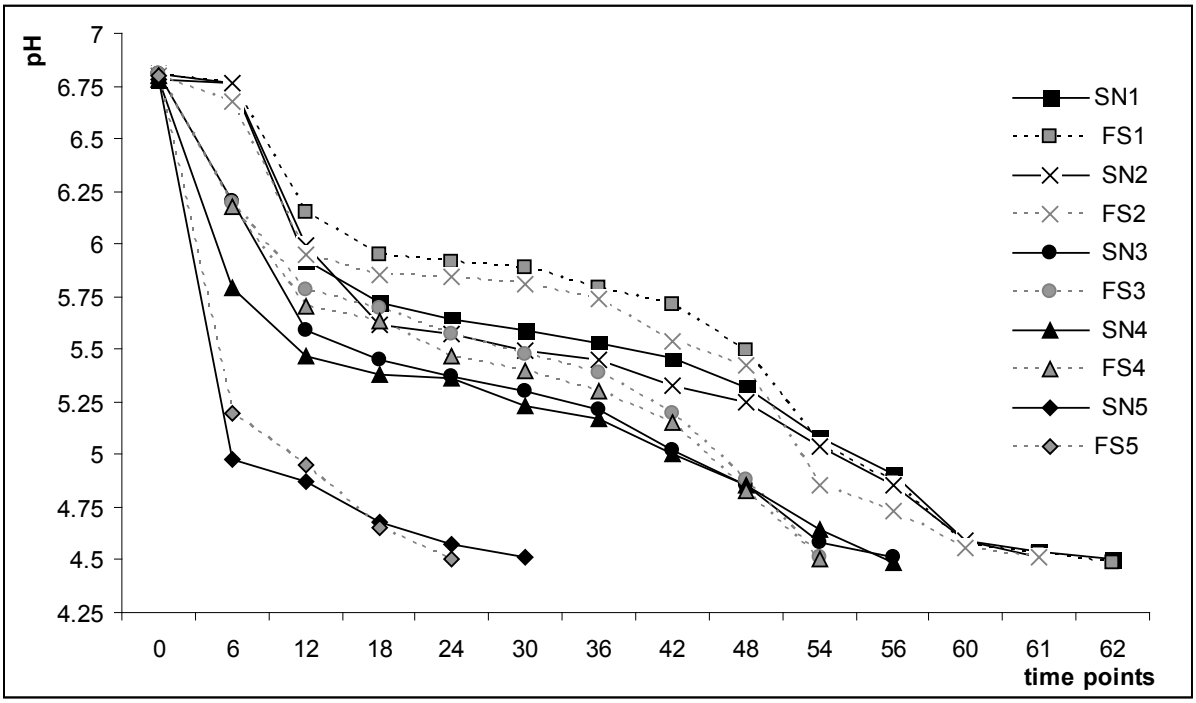

Fig. 1. pH changes during fermentation of Synergy 1 (SN) vs. FOS (FS) -supplemented soymilk by *L casei-01*; Time points (hours). (Inoculum rate: 0.005%w/v, SN1 FS1; 0.01%w/v, SN2 FS2; 0.075%w/v SN3 FS3; 0.1%w/v, SN4 FS4; 1%w/v SN5 FS5).

Table 1. Viability and proteolytic activity of *L. casei*-01 in end-products

L. casei-01 Inoculum rate % w/v	Cell count (log_{10} cfu mL^{-1}) Synergy 1	FOS	Proteolytic activity (absorbance at 313 nm) Synergy 1	FOS
0.005*	9.59±0.03[a]	9.54±0.09[a]	0.54±0.02[a]	0.31±0.01[d]
0.01*	9.63±0.18[a]	9.58±0.16[a]	0.59±0.03[a]	0.35±0.07[d]
0.075[#]	11.49±0.13[b]	11.45±0.06[b]	1.11±0.04[b]	0.60±0.07[a]
0.1[#]	11.69±0.36[b]	11.56±0.21[b]	1.13±0.03[b]	0.67±0.06[a]
1[&]	10.51±0.34[c]	10.25±0.28[c]	3.09±0.14[c]	N.D.

End-product was obtained after 62 h (*), 56 h (#) and 30 h (&) of fermentation (pH values 4.5). Results are expressed as means±SD; values are means of duplicates from three separate runs. Means with different lowercase superscript letters are significantly different ($p<0.05$). N.D. – not determined.

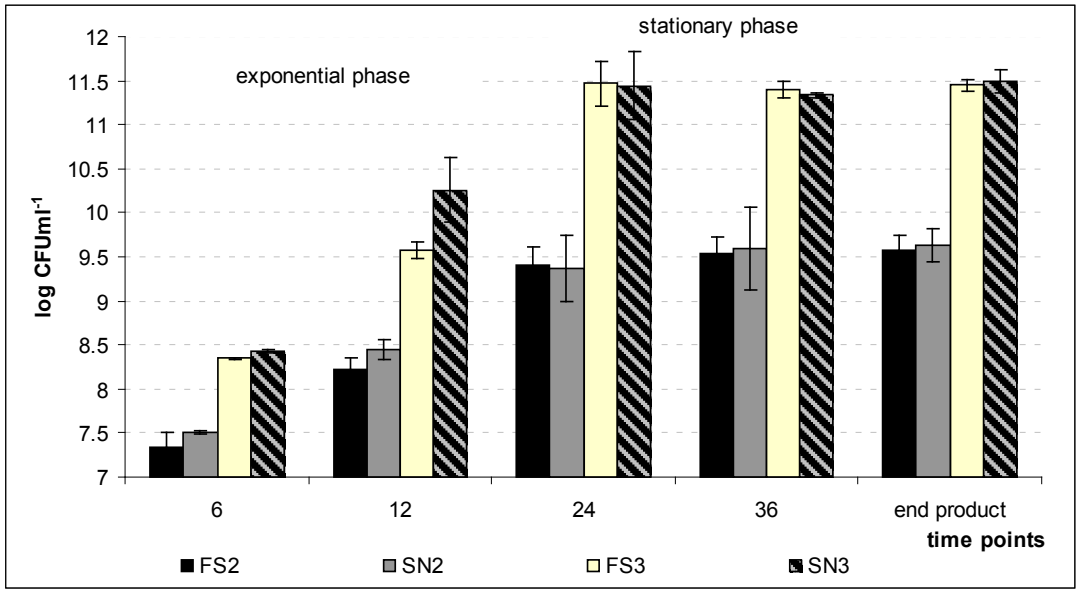

Fig. 2. Growth rate of *L casei*-01 in soymilk supplemented with Synergy 1 vs. FOS, during fermentation at 37 °C. (Viable cell counts, log_{10} cfu ml^{-1}; time points, hours).

5.80±0.58 (app. 15%), while in low inoculum supplemented samples, non-significant decrease in pH was observed, 6.74±0.04 (≈ 1 %), compared to the initial value (Fig. 1). Regarding the prebiotic used, significant difference ($p<0.05$) was observed between the low inoculum samples (SN vs. FS) after 12 h of fermentation, when pH decline in Synergy 1 supplemented samples showed steeper and longer slope. For example, the pH value of the SN1 samples after 12 h of fermentation was 5.92±0.01 *vs.* 6.15±0.02 in the FS1 samples. Consequently, throughout the fermentation, pH values in Synergy 1 supplemented samples were significantly lower than in the FOS enriched samples. Few hours before termination of the fermentation (pH 4.5), the sharper decline (slope) of pH occurred in FOS samples, with the end pH value of 4.5 occurring app. 60 min faster than in Synergy 1 enriched samples. A similar decline pattern, with higher rate, was observed for high inoculated samples, probably because of the higher initial biomass of the probiotic (Fig. 1).

The viable cell counts of *L casei*-01 in all end-products are summarized in Table 1.

At this stage, terminated fermentation (pH 4.5), significant difference in cell counts ($p<0.05$) was observed between the batches with low (0.005 and 0.01% *w/v*) and high inoculum (0.075, 0.1 and 1 % *w/v*) rates. Obtained values within batches were similar regardless the prebiotics used. As shown in Table 1, in high inoculum batches, the viable cell counts were significantly higher ($p<0.05$) in both SN and FS samples. Exceptions were the samples inoculated with 1% *w/v L. casei*-01, where the cell count was lower for 1 log in comparison with the other high inoculum rate samples.

Therefore, cell growth during fermentation was followed in inoculum of 0.01% *w/v*, representing the low inoculum samples (SN2 and FS2), and 0.075% *w/v*, representing the high inoculum samples (SN3 and FS3). The cell counts were determined at 6, 12, 24 and 36 h and at the end of fermentation (Fig. 2). Time points were cho-

sen based on changes in pH values observed during fermentation.

The growth rate of *L casei*-01 in SN *vs.* FS samples was significantly higher during the exponential (log) phase of growth. The exponential phase of *L. casei*-01 associated with FOS lasted few hours longer regardless of the inoculum rate. Despite the variations observed in log phase, there was no significant difference in viable cell counts of *L. casei*-01 associated with FOS *vs*. Synergy 1 in the stationary phase of growth and at the end of fermentation.

Growth rate and viability of probiotics in beverage depends on the strain used, the initial inoculation, incubation temperature, fermentation time, availability of nutrients, and growth promoters (prebiotics). The ability of microorganisms to utilize prebiotics is strain- and substrate-specific (Shah, 2001; Pan et al., 2009). As for the degradation mechanism of inulin-type fructans, it has been reported that *L. paracasei spp paracasei* degrades inulin type fructans with different degree of polymerization (DP) (Makras et al., 2005). Furthermore, Goh et al. (2007) reported that *L. paracasei* 1195 has an extracellular enzyme that is cell wall associated and responsible for the degradation of large fractions of FOS and inulin. The results of sugar degradation analysis published by Tsujikawa et al. (2013), clearly indicated that *L. paracasei DSM 20020* was capable of degrading the highly polymerized inulin into fructose and sucrose, whereas *L. paracasei JCM 8130^T* failed to do so.

L. casei-01 showed a considerable growth with both prebiotics. Still, variations due to different prebiotics and inoculum rates were observed. Based on literature data, we assume that the variations in changes of pH and viable *L. casei*-01 counts, shown during fermentation, are due to the differences in chain lengths of the prebiotics used. The utilization of inulin-type fructans is strain specific. Both prebiotics were inulin-type fructans, but with different DP. Namely, the DP of FOS varies from 2 to 60 with an average DP of >10, while the commercial Synergy 1 mixture is composed of oligofructose (DP 2-8; $DP_{av} = 4$) and long-chain inulin fraction (DP 10-60; $DP_{av} = 25$), known as HP inulin (Roberfroid, 2007).

Our results correspond to those published by Perrin et al. (2002), who showed that shorter chains were the first to be consumed by probiotic bacteria. Furthermore, Aryana et al. (2007) reported lower pH in a medium with *L. casei* and short chain length oligofructose P95 ($DP_{av} = 5$), compared to long and medium chain inulins. Therefore, the faster growth rate during exponential phase of growth in SN samples compared to FS samples was due to better utilization of short chain oligofructose from Synergy 1 mixture. This was followed by utilization of inulin, but with slower rate than FOS, which resulted in longer fermentation time to determined pH of app. 4.5 in SN samples. However, there was no significant difference in cell counts between the end-products with Synergy 1 and FOS, with the corresponding inoculums.

Changes of pH and viability of L. casei-01 during storage of prebiotic fortified soymilk

The acidity and cell viability counts were determined after storage at 4 °C for two and four weeks. The pH values declined insignificantly with time, while for cell counts a significant change was observed, depending on the prebiotic used. Namely, during two weeks of storage at 4 °C, the cell counts in SN samples were stable. However, after 4 weeks, they significantly decreased. For example, in samples with inoculum rate of 0.01% *w/v* (SN2) and 0.075% *w/v* (SN3), they decreased to 9.10±0.14 and 10.45±0.36 $\log_{10}$ cfu mL^{-1}, respectively. The results are in agreement with the results obtained by Donkor et al. (2008), who reported a general decline in viable cell counts during storage at 4 °C from 21 to 28 days for *L. acidophilus LAFTI L10, B. lactis LAFTI B94* and *L. casei LAFTI L26*. The viable cell counts of *L. casei*-01 in FS samples declined significantly after 2 weeks of storage. Namely, in the end-product with the inoculum rate of 0.075% *w/v* (FS3), the viable cells decreased from 11.45±0.06 $\log_{10}$ cfu mL^{-1} to 9.44±0.06 $\log_{10}$ cfu mL^{-1}.

Nevertheless, the obtained values were above the recommended minimum for probiotic beverages, which characterize the product as functional food. This is in agreement with studies reporting that high chain prebiotics maintain the viability of probiotics at level above the recommended minimum, up to 4 weeks of storage at 4°C (Aryana et al., 2007).

Peptide profile

Probiotics, including *L. casei*-01, have a complex proteolytic system that enables them to hydrolyze soy proteins and grow in soymilk (Donkor et al., 2007). During hydrolysis the peptides with various biological effects are produced (Gibbs et al., 2004)

Protein patterns in fermented soy products have raised an increased interest because of their health benefit effects, especially in cardiovascular diseases since soy proteins were approved by the Food and Drug Administration in 1999 (FDA 1999). But, not all probiotic/synbiotic soy products are equal. Some variations are based on the probiotics and/or prebiotics used (Erdman et al., 2013). Results, obtained by SDS-PAGE analysis of soymilk protein profile during fermentation, confirmed the proteolytic activity of *L. casei*-01 as well as the influence of associated prebiotics, Synergy 1 and FOS, on the proteolytic rate (Fig. 3).

Approximately 90% of soy proteins are constituted of two major globulins, β-conglycinin (7S globulin α', α, β subunits; Mw ≥ 44 kDa) and glycinin (11S globulin acidic and basic subunits; Mw < 40 kDa) (Gianazza et al., 2003). SDS-PAGE protein profile of synbiotic soymilk demonstrated that these proteins were gradually degraded during fermentation (Fig. 3). At Lane 2 (FS3 sample, 12 hours of fermentation), the Mw of observed polypeptide bands were 14 kDa and 20 kDa (corresponding to Mw of glycinin basic

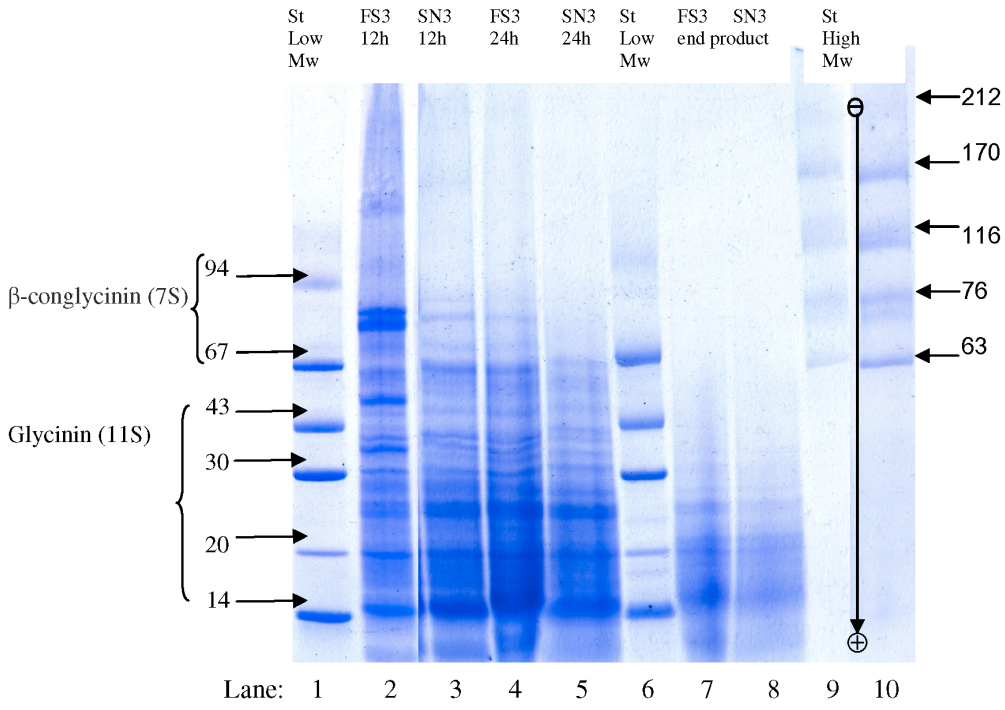

Fig. 3. SDS-PAG electrophoretic profiles of proteins in synbiotic soymilk during fermentation: Lane 1 and 6=Low Mw Standard; Lane 9, 10, = High Mw Standard; Lane 2 = FN3, time point 12 h; Lane 3 = SN3, time point 12 h; Lane 4 = FS3, time point 24 h; Lane 5 = SN3, time point 24 h; Lane 7 = FS3 end-product; Lane 8 = SN3 end-product.

subunit), 28-30 kDa and 38-40 kDa (corresponding to Mw of glycinin acidic subunits) and 48-76 kDa (corresponding to Mw of β-conglycinin). At Lane 3 (SN3 sample, 12 h of fermentation), dominant bands were with Mw corresponding to glycinin subunits. The bands of β-conglycinin were less intensive, but still existed. Lane 4 (FS3 sample, 24 hours of fermentation) resembled Lane 3. Lane 5 (SN3 sample, 24 h of fermentation) demonstrated complete degradation of α',α, subunits of β-conglycinin.

As shown in Lane 7 (FS3 sample, end-product) and Lane 8 (SN3 sample, end-product), the α', α, β subunits of protein β-conglycinin (7S) and the acidic chains of glycinin (11S globulin) were completely hydrolyzed. Observed polypeptide bands were with lower Mw (25, 20 and 14 kDa). The bands of basic glycinin of the end-products were less intensive compared to bands of samples obtained at 12 and 24 h of fermentation, indicating incomplete degradation. The bands in Lane 8 were less intensive than in Lane 7 indicating higher proteolysis.

As a consequence of delay in the growth rate of probiotic and pH drop, there was postponed soy proteins degradation in FS-enriched samples of soymilk.

Proteolytic activity

As it was confirmed by SDS-PAGE, there was an extended proteolysis of the soymilk proteins during fermentation. The result of protein hydrolysis is an increase in the amount of free amino groups. The OPA-method, based on spectrophotometric detection of released NH_3 groups upon hydrolysis, provided a direct measurement of the proteolytic activity. The extent of proteolysis in the end-products is shown in Table 1. The free amino acid content increased along with the enlargement of the inoculum rate. The values obtained for SN samples were significantly higher than those obtained for FS samples, indicating the higher proteolytic activity of *L. casei*-01 in association with the prebiotic Synergy 1. This was confirmed by the observation that the values for proteolysis in FS samples at inoculum rate of 0.075% *w/v* (FS3) were similar to those of inoculum rate of 0.01% *w/v* in SN samples (SN2).

The pH in SN samples was significantly lower than in FS samples throughout the fermentation, resulting in a significantly faster and higher hydrolysis and amount of generated free amino groups in the end-products. Higher proteolysis in SN samples is consistent with the findings of Ng et al. (2008) who claimed that the proteolytic activity of probiotics is growth-associated. In addition, De Giori et al. (1985) reported that by decreasing pH of the growth medium, the proteolytic activity is increased, mainly due to the alteration of active structure and hydrogen-ion equilibrium at the active site of the proteolytic enzymes upon pH changes.

Optimization of inoculum rate in synbiotic soymilk product

Evaluating the results obtained for pH change, viable cell counts and proteolytic profile during fermentation of soymilk with *L. casei*-01 in association with FOS and Synergy 1, respectively, we observed delay in pH change, biomass yield and proteolysis in FS samples in function of time as well as lower proteolytic activity in FS end-products. Moreover, the results for pH change during fermentation and proteolytic activity of *L. casei*-01 with inoculum of 0.075% *w/v* in association with FOS (FS3) were similar with the results obtained for significantly lower inoculum size, 0.01% *w/v*, of *L. casei*-01 in association with Synergy 1 (SN4). In addition, although there was no difference in viable cell counts between FS and SN end-products significantly lower cell counts were observed in FS samples after 2 weeks of storage at 4 °C, while SN samples were stable, showing a significant decrease after 4 weeks storage at 4 °C. Based on these findings, Synergy 1 protects the viability of *L. casei*-01 more efficiently than FOS, being more favorable for formulation of functional soymilk beverage.

Therefore, to optimize the inoculum rate of *L. casei*-01 to ferment the soymilk fortified by Synergy 1, the proteolytic activity in function of time was also determined. The sample with inoculum rate of 0.005% *w/v* (SN1) was excluded due to the low proteolytic activity, as well as all FOS fortified samples (Fig. 4).

The decreased pH during fermentation correlated with the increased proteolytic activity (e.g., r = -0.885, p=0.0007 and - 0.902, p =0.0009, for SN2 and SN3, respectively).

In order to further optimize the inoculum of *L. casei*-01 for soymilk fermentation fortified by Synergy 1, the production of organic acids (lactic and acetic acids) in function of time was followed in the same synbiotic samples (SN2, SN3, SN4 and SN5).

The results obtained by HPLC showed an increased production of organic acids during fermentation. The concentration of lactic acid was higher than that of acetic acid in all batches (Fig. 5). Similar results were reported by Donkor et al. (2005), who made soy yogurt by fermenting the commercial soymilk using *L. acidophilus, B. lactis* and *L. casei*.

Results have shown that the probiotic inoculum of 0.075% *w/v* used to ferment the soymilk produced lactic and acetic acid in higher quantity compared to the inoculum of 0.01% *w/v*. Using an inoculum of 0.1% *w/v*, the production of lactic acid was not significantly changed (Fig 5a), while the production of acetic acid was increased (Fig. 5b). Acetic acid is an undesirable end-product in fermented soymilk due to its vinegary flavor and unpleasant sensory properties; therefore, the high production of lactic acid over acetic acid is desirable (Donkor et al., 2007; Donkor and Shah, 2008).

This indicates that the inoculum of 0.075% *w/v* can be considered optimal for preparation of the fermented soymilk beverage.

ACE-inhibitory activity

Proteolytic activity is an important attribute for production of peptides that could act as ACE inhibitors. The

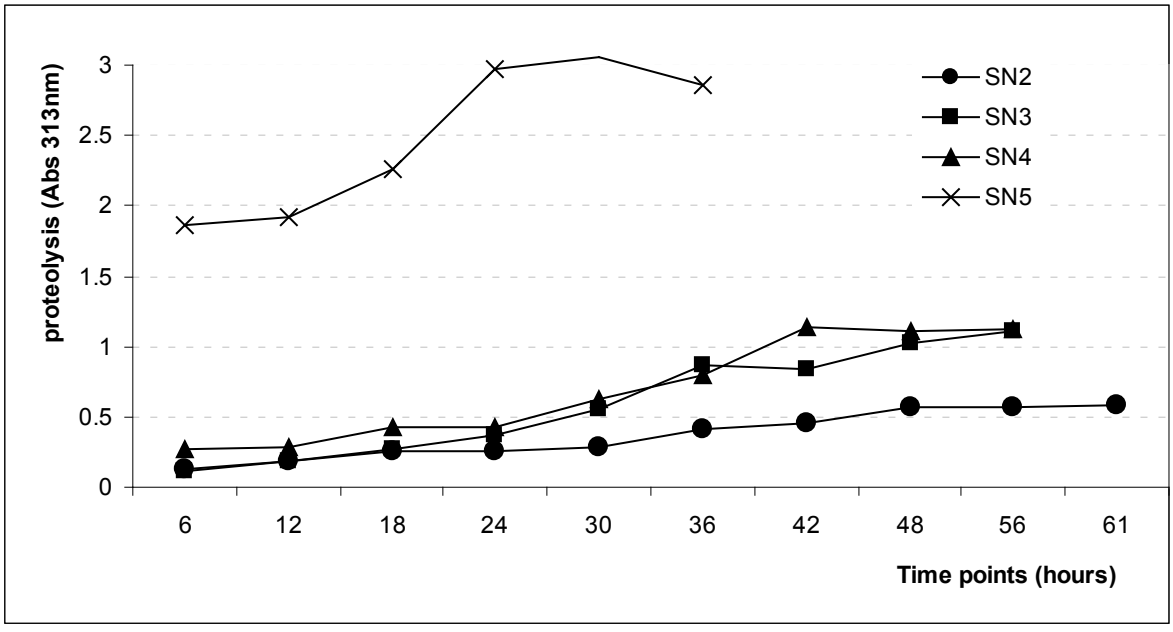

Figure 4. Absorbance *vs.* time curves for proteolysis during fermentation of Synergy 1-supplemented soymilk by *L. casei*-01 at different inoculum rates.

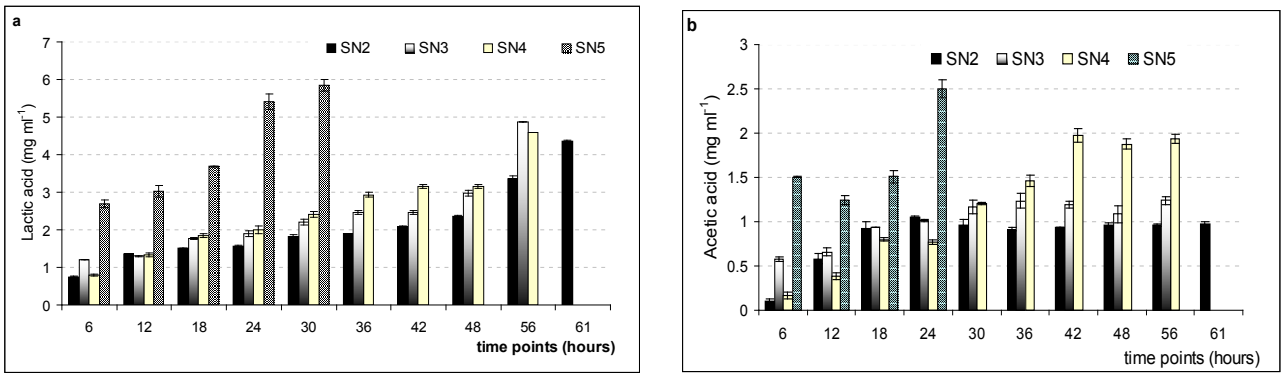

Fig. 5. Production of lactic acid (a) and acetic acid (b) in *L. casei*-01 and Synergy 1 fortified soymilk during fermentation with different inoculum rates.

development of fermented soymilk containing higher concentrations of released bioactive ACE inhibitors and viable probiotic may deliver health benefits of these functional compounds more efficiently (Donkor et al., 2005). For that purpose, the potential antihypertensive effect of the fermented soymilk beverage *in vitro*, based on percentage inhibition of the ACE activity was evaluated.

The %IACE of fermented Synergy 1-fortified end-products was evaluated in relation to different inoculums of *L. casei*-01 (SN1, SN2, SN3 and SN4 samples). Curves obtained by spectrophotometric measurements showed a nice linear decrease of absorbance. Sample without fermented soymilk showing 100% of ACE activity resulted in steeper slope than samples with fermented soymilk. Whole interval from 5 to 45 min was used for calculating the slope.

For the inoculum of 0.005 (SN1), 0.01 (SN2), 0.075 (SN3) and (SN4) 0.1% *w/v L. casei*-01, the %IACE was calculated as 71, 74, 77.8 and 78.4%, respectively (Fig. 6).

There was a significant increase in values obtained by increasing the inoculum from 0.005 to 0.075% *w/v*. Values between high inoculum batches were similar. Namely, increasing the inoculum to 0.1% *w/v* the %IACE did not change, indicating the inoculum of 0.075% *w/v* optimal for fermented synbiotic soymilk with antihypertensive potential.

Conclusion

This study showed that prebiotics FOS and Synergy 1 had different effect on the metabolic activity of *L. casei*-01 in soymilk. Both prebiotics provided high viability of *L. casei*-01 to above therapeutic minimum of 6 log cfu_{10} ml^{-1} after preparation and within 4 weeks of storage at 4 °C. But, faster cell growth during fermentation and higher viability during storage was observed in SN samples. Faster and higher proteolytic activity in samples fortified by Synergy 1 also indicated this prebiotic more favorable for preparation optimal formulation of functional soymilk beverage with *L. casei*-01 as probiotic. Protein profile of samples showed polypeptides with Mw lower than 25 kDa.

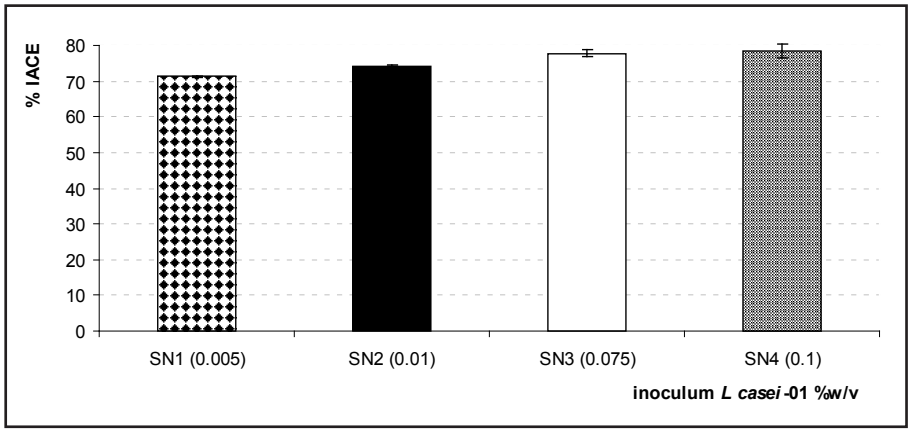

Fig. 6. %IACE in fermented 1.5% *w/v* Synergy 1-fortified soymilk end-products at different inoculum rate of *L. casei*-01.

The end products exerted appreciable *in vitro* IACE activity. Based on organic acid production and %IACE values in Synergy 1 fortified samples, the *L. casei*-01 inoculum rate of 0.01 for low dose and 0.075% *w/v* for high dose synbiotic beverage were chosen for further *in vivo* studies needed to assess the real antihypertensive potential of our synbiotic product. The development of fermented low-fat synbiotic soymilk with antihypertensive potential and high viable count of probiotic may deliver health benefits to target population with cardiovascular disease risk factors.

Acknowledgments

This research was financially supported by the Ministry of Education and Science of the Republic of Macedonia (Project No. 13-3583/1). The authors would like to express gratitude to Orafti Beneo Group and to IMCD (UK) for the donation of Orafti® Synergy 1 (L. Maréchal, Tienen, Belgium), and sodium alginate (Protanal 10/60 LS, FMC BioPolymer, USA), respectively.

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Резиме

Синбиотски прехранбен производ базиран на соја – формулација и определување на антихипертензивниот потенцијал

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Клучни зборови: *L. casei*, олигофруктоза-збогатен инулин (Sinergy 1), фруктоолигосахарид (FOS), сојин напиток, АКЕ-инхибиција

Специфични цели на оваа студија беа да се избере пребиотик кој ќе обезбеди поголема виталност на пробиотикот *L casei*-01 и соодветен инокулум на пробиотикот за подготовка на ферментиран напиток од соја со потенцијал да го инхибира ангиотензин-конвертирачкиот ензим (АКЕ). За овие цели беше подготвен напиток од сојино млеко збогатен со 1.5% *м/в* фруктоолигосахарид или олигофруктоза-збогатен инулин во кој беа додавани различни инокулуми на *L casei*-01 (0.005-0.1% *м/в*). Метаболичката активноста на пробиотикот беше следена преку pH, број на витални клетки, протеолитичка активност, создавање на органски киселини, додека терапевтскиот потенцијал на финалниот напиток преку инхибиција на АКЕ. Во текот на ферментацијата, виталноста на *L casei*-01 го достигна препорачаното терапевтско ниво, 9,58±0,035 log cfu/mL за примероците со низок инокулум (0,005 и 0,01% *м/в*) и 11,54±0,13 log cfu/mL за примероците со висок инокулум (0,075 и 0,1% *м/в*), без разлика на користениот пребиотик. За време на ферментацијата, пониска pH, побрз клеточен раст и поизразена протеолитичка активност беше забележана со користењето на олигофруктоза-збогатен инулин што упатува на подобра искористливост на овој пребиотик

споредено со фруктоолигосахаридот. Хидролитичката активност беше зависна од користениот пребиотик, односно побрза со примената на олигофруктоза-збогатениот инулин, што беше потврдено со SDS-PAGE. Молекуларната маса на полипептидите, добиени со хидролиза на протеините, во синбиотскиот финален продукт беше пониска од 30kD. Инхибицијата на активноста на АКЕ изнесуваше 71, 74, 77 и 78% за инокулуми од 0,005, 0,01, 0,075 и 0,1% *м/в*, соодветно. Врз основа на овие резултати може да се заклучи дека комбинацијата на олигофруктоза-збогатен инулин (1.5% *м/в*) и ниско-дозен инокулум од 0,01% *м/в* или високо-дозен инокулум од 0,075% *м/в* на *L. casei*-01 е оптимална за подготовка на сојин напиток со задоволителен антихипертензивен потенцијал®.

Macedonian pharmaceutical bulletin, **60** (2) 51 - 56 (2014)
ISSN 1409 - 8695
UDC: 616.314-77:546.831
Original scientific paper

Evaluation of zirconia bonding to veneering porcelain

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Received: August 2014; Accepted: October 2014

Abstract

Zirconium dioxide as core ceramic material for dental crowns and bridges, possess high strength, chemical stability and superior aesthetics after veneering. Veneering ceramic is considered to be the weakest part of all-ceramic restorations. The adhesion between the core and veneering porcelain is based on the manner in which the connection occurs in metal-ceramic structures. Standard procedures for connecting zirconia to hard dental tissues and veneering materials do not achieve the required strength of bonding.

The aim of the paper is to investigate different surface treatments of the zirconium dioxide ceramic core and find the best, for achieving highest adhesive bonding values to veneering porcelain. The study was primarily designed to investigate the bonding strength of the veneering porcelain to zirconia with in vitro Macro shear bond strength test. The specimens with different surface treatment of the zirconia were divided in five groups of twelve according to the treatment of zirconium surface and results showed highest bonding values for specimens treated with Rocatec system.

Key words: surface treatment, zirconia, bonding, adhesion, shear bond, veneering porcelain

Introduction

The need for higher biocompatibility and better dental aesthetics led to development of all-ceramic prosthodontic systems which are increasingly applied, although their clinical performances and success are not yet proofed (Aboushelib et al., 2005). All-ceramic dental restorations have ceramic core instead of metal alloy and veneering ceramic porcelain in their composition (Guazzato et. al., 2004)

Zirconium dioxide as core ceramic material for dental crowns and bridges, possess high strength, chemical stability and white color with opportunity for superior aesthetics after veneering (Anusavice, 2003). The finer granulation of ceramic powder and tetragonal-monocyclic transformation plays important role in the strength of zirconium as material for all-ceramic substructure. Veneering porcelain for zirconia dioxide ceramics possess the same strength as the materials for veneering of the metal core, but clinical practice showed that the prosthetic structures made of veneered zirconia often damaged and fractured in the mouth (Fischer et al., 2008). Damage occurs during the mastication processes as shearing off the fragments of porcelain mass and serious fractures of the zirconium base (Stawarczyk et al., 2011). Veneering ceramic is considered to be the weakest part of all-ceramic restorations and several factors influence, such as: thermal expansion mismatch, overload

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at the premature contacts, ceramic strength and its adhesive bonding abilities (Estevam et al., 2010).

Different thermal expansion coefficients (CTE) of the bonded materials play important role in forming residual stress in the materials (O¨zcan, 2003). The stress which is formed during cooling process of the ceramic is so called "*transient"* stress, while the stress which occurs at the room temperature after cooling is called "*residual stress*". Both stresses can be compressive or tensile, and if we know that porcelain is not tolerant to tensile stress, it should be set at lower compressive stress (Gostemeyer et.al., 2010). This means that the substructure core material must be with higher contraction than porcelain during cooling process (Sui et al, 2013). In ideal cases the difference between two materials CTE should be less than 10%, but there is no ideal thermal cycle for metal-ceramic and all-ceramic restorations (Mainjot et al., 2012). Some *degree of compatibility* is possible due to the expansion coefficients of the two materials, porcelain firing temperature, the resistance of the core at high temperature, the release of internal stress of the core and ceramic, the thickness ratio of the two materials and the adhesive bond between them (Kelly et al., 1990).

Mechanisms for bonding zirconia to veneering porcelain are not sufficiently clarified, but it is known that the strength of the adhesion in all-ceramic system is weaker than the one in metal-ceramic systems (Aksoy et al., 2006). The adhesion between the core and veneering porcelain is based on the manner in which the connection occurs in metal-ceramic structures (Motoaki et al., 2011).

Zirconia is the solid material with inactive surface, resistant to most classical methods of bonding. Standard procedures for connecting to hard dental tissues and veneering materials do not achieve the required strength of bonding (Farga-Niñoles et al., 2013). Zirconia is basically metal and its connection with porcelain is chemical, while the residual thermal stress occurring during cooling has a great influence on the strength of the connection (Aboushelib et al., 2009).

The strength of the zirconia-porcelain bonding depends on several factors, as follows: zirconia surface, residual thermal stress, CTE, defects in materials, defects at the bonding interface, "wetting" and ceramic strength (Kosmač et al., 2008). Process of "wetting" actually represents the creation of an intimate contact between the two elements without the formation of cavities, and is very important when applying adhesive structures.

Great strength and density of sintered zirconia and the lack of glassy matrix make it resistant to most conventional surface treatments. Unlike silicate feltsphatic porcelain whose surface properly treated and roughened accomplishes good bonding, unreactive surface of zirconia is an issue due to poor adhesion with other elements (Shimizu et al., 1993). Examination in which the relationship is examined by SEM showed that there is a mutual diffusion of Zr and Si of SiO_2 from porcelain, presented on Fig.1. This phenomenon explains the effects of adhesion and bonding strength between both ceramics and adhesives fractures along connected part, suggesting that the relationship between porcelain and zirconia can be classified as *"strong"* (Song et al., 2013).

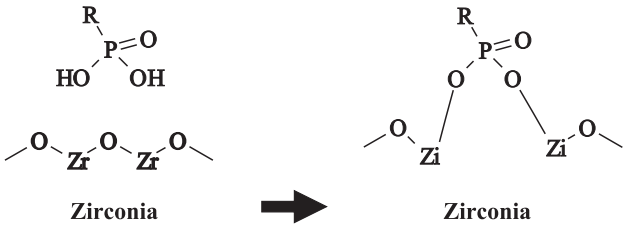

Fig. 1 Cohesive adhesion with oxide materials- chemical chelation with hydrogen bonds.

Several different surface treatment are suggested for improvement of the bonding procedures, such as: mechanical surface roughening, tribochemistry and silanisation, application of the liners, thermal spray, fusion with glassy balls, chloro-silane treatment with steam, selective infiltration etching which creates inter-granular porosity, complex phosphate primer that react with hydroxyl groups, corrosion with hot solutions, laser treatments (Nd: YAG, CO_2) and others (Sato et al., 2008; Al-Wahadni and Martin, 1998).

The aim of the study was to investigate different surface treatments of the zirconium dioxide ceramic and find the best, for achieving highest adhesive bonding values.

Material and methods

Materials

Sixty square-shaped specimens (6 mm x 10 mm x 10 mm) were made of zirconium dioxide ceramic blocks (DeguDent Cercon base 47**,** Dentsply International Inc, Hanau-Wolfgang, Germany), cut with diamond saw and then sintered in the oven (Cercon heat) at 1350 °C for 6 hours. Afterwards the samples were placed in an acrylic polymer material to form the bonding substrate for veneering porcelain stubs. The veneering ceramic (Cercon Ceram kiss, Degudent, Hanau, Germany) was built up to the final dimension (thickness of 3 mm) according to the firing program of the manufacturer as seen on Fig. 2.

The specimens with different surface treatment of the zirconia were divided in five groups of twelve:

1. Group A are control group without treatment-specimens were cleaned in an ultrasonic bath and porcelain was sintered in the oven according manufacturer;
2. Group A1 are specimens cleaned in an ultrasonic bath and treated with SiC-discs (P220, P500 and P1200 according ISO 6344-1: 1998);

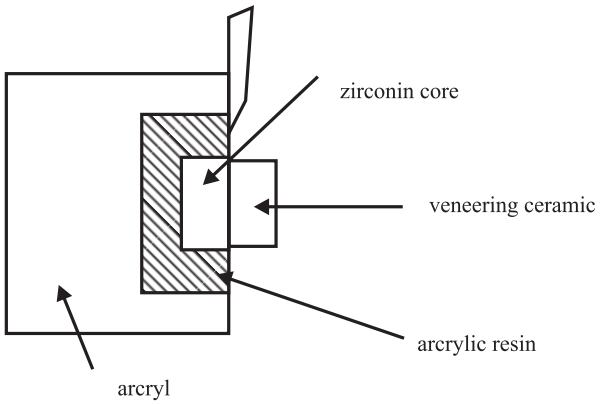

Fig. 2 Schematic view of the shear bond strength test.

3. Group A2 are specimens with surface treatment – air particle abrasion with alumina oxide (Al_2O_3) with diameter of 110 μm for 15 seconds with a sand blasting device at air pressure 15 bars, at the 10 mm distance from zirconia surface;
4. Group A3 are specimens with surface treatment – liner (Cercon Ceram Kiss Liner, Degudent Hanau, Germany) applied with brush on ultrasonically cleaned zirconia surface in thin layer and sintered according to the manufacturer.
5. Group A4 are specimens with surface treatment – tribochemical air abrasion with Rocatec system (3M ESPE, USA) and silane primer. Rocatec plus silica powder was used for air particle abrasion for 15 seconds with a sand blasting device at air pressure 2,8 bars, at the 10 mm distance from zirconia surface.

Testing design

The present study was primarily designed to investigate the bonding strength of the veneering porcelain to zirconia with in vitro Macro shear bond strength test. The experiment had been done with universal testing machine Shimadzu Autograph AGS-X in the Laboratory for calibration of the force and moment of the force at the Faculty for Mechanical Engineering in Skopje according to standards (ISO 29022: 2013 Dentistry–Adhesion-Notched- edge shear bond strength Test). This test has special bonding matrices in which the specimens are laid and notched-edged blade on cross-head is cutting the bonding interface as presented in Fig. 3.

Each specimen from every group was mounted in the testing jig and force was applied to the specimens, so that shear load directly adjacent the bonding interface as seen on Fig. 4. The cross-head was moving down the notched-edge blade with speed of 0, 75 ± 0, 30 mm / min. The cutting edge was in position as close as possible to the bonding surfaces at the distance of about 0, 5 mm to prevent movement from the loading. Testing machine was connected with computer software during the testing and automatically displayed load failure force in N (Newtons).

Calculation of the shear bond strength

The average shear bond strength was calculated with formula:

$$\text{Shear stress (MPa)} = \text{Load (N)} / \text{Area (mm}^2\text{)},$$

where load (N) is applied shear force and area is bonded

Fig. 3. Zirconia veneered specimens in acrylic molds.

interface area (mm^2). Fracture of the veneering porcelain could be easily seen, and the fracture surface and type were examined and classified in three different groups: Adhesive – failure at the connection between ceramic and porcelain; Cohesive – failure inside the porcelain and Combined – mix of the previous.

Fig. 4. Shear bond notched edge test with specimen in the bonding clamp.

Results

The statistical analysis was carried out and the results showed significant differences for the shear bond strength between measured data in all tested groups of specimens at the significant level of 0,05. Group A4 showed highest values of the shear stress, group A2 and A1 showed similar values, while lowest value of the shear stress showed group A3, and the results are presented on Table 1.

Broken specimens were examined under the SEM (scanning electronic microscope) and most of them showed mixed cohesive/adhesive failures and very small amount of porcelain mass left attached to the zirconia surface. Specimens from group A3 showed fracture surfaces covered with liner or veneer material.

Discussion

Bonding means connecting and establishing stable adhesive contact between two materials. There are several different theories about the adhesion between base metal and veneering porcelain in metal-ceramic restorations. Primary connection is chemical, mechanical, adhesive, Van der Waals's forces, compression and interzonal material (Dündar et al., 2007). According to ISO standards metal-ceramic restorations should possess minimum 25 MPa bonding strength, and several different studies showed values ranging 54-71 Mpa (Scherrer et al., 2010). There is not standard value for zirconia-veneering porcelain bonding strength, but the results of our investigation showed high bonding values with all different surface treatments.

Brittle all-ceramic restorations don't have standardized test for measuring of the bond strength, as metal-ceramic. Different testing methods had been used such as macro and micro shear bond strength test, three-point, four-point bending tests and micro-tensile test (Van Meerbeek et al., 2010). They all have certain disadvantages, but shear bond strength (SBS) test is used in this study because of easy preparation of the specimens and simple test protocol (Sadighpour et al., 2006). High standard deviations, influence from the shape and stress force are its main disadvantages. Very important thing for the examination is providing stabile and strong fixation and connection between zirconia and acrylic resin. Adjusting of the acrylic base with grinding can cause some damage to the bonded area, experience and skill of the operator is also very important (Scherrer et al., 2009). Fabrications of the specimens is very important because initial fractures that lead to failure may start from present pores and defects, surface polishing etc. However in order to minimize technical mistakes one person did the fabrication of the specimens and testing experiment. Most of the in vitro tests are static and held in dry environment which lead to faster delamination of the material (Betamar et al., 2007)

Zirconia surface is very compact and inert to most convenient treatment, but some materials for improving of the bonding are used in the experiment. They are different liners like paste applied on the surface in thin layer after activating with sandblasting (Isgro et al., 2003). Liners are material that should ensure physical bond and compensate inadequate CTE. They are products similar to opaque, and they can provide surface roughness, modify shade

Table1. Mean shear bond strength values for different surface treatments

Specimen group	Surface treatment	Shear bond strength values Mpa
A	Without treatment	30,03 ± 5,70
A1	Treated with sic-discs	21,30 ± 4,02
A2	Air Particle Abrasion (Al_2o_3)/110μm	26,30 ± 2,50
A3	Liner	20,30 ± 6,30
A4	Rocatec system	34,21 ± 5,49

and improve bonding to the porcelain. Surface abrasion or roughening (grinding, airborneparticle abrasion, rotary abrasion using diamond burs) establishes adhesion with micro-mechanical retention. There is a general consensus that airborne particle abrasion with 50-110 μm alumina particles at 0.25 MPa is effective in roughening and cleaning the bonding surface of zirconia. However, the effect of those treatments on the mechanical properties of Y-TZP materials is controversial and both positive and negative results have been described in the literature (Sato et al., 2008). Zirconium dioxide ceramic used in the study is material where manufacturer recommend surface treatments, if done correctly according to their instructions.

Treatment with Rocatec system (3M ESPE, USA) for silanization forms chemical bonding due to application of the mechanical energy, and the procedure is in three stages (cleaning, blasting and silanization). Blasting of the surface was made with aluminum oxide particle size 110 μm modified with silica (SiO2) and it was covered with silane liquid ethanol solution ES PE Sil (3M ESPE, USA) afterwards. Particles of the silicon oxide penetrate 15 μm and fuse to the surface. Silane solution has two molecules at the edges with different polarity and alchoxy group on the left side (RO) 3Si- allows chemical bonding on the surface. Our investigation showed that this treatment although quite expensive and complicated, can improve bonding between zirconia and veneering ceramic.

Conclusion

With the limitation of this in vitro study next conclusion were drawn:

1. There were significant differences in the shear bond strength values for all different groups of specimens;
2. Group A3 (treatment with liner) showed lowest value of the shear bond strength;
3. Group A4 (treatment with Rocatec) showed highest value of the shear bond strength;
4. Surface analysis showed that fracture originated from the porcelain, and thin layer of porcelain and liner remained on zirconia surface.
5. There must be effort to improve strength properties of the veneering ceramics for zirconium pointed towards increasing of the bonding strength between two materials

The main dilemma still remains over whether it is necessary or not to do the sanding of the surface, and how any treatment is going to impact on the strength and stability of the core material during the years of exploitation.

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Резиме

Евалуација на поврзувањето на цирконијата со порцеланските маси за фасетирање

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Клучни зборови: површински третман, цирконија, бондирање, атхезија, смолкнување, фасетен поцелан

Циркониум диоксид како материјал за основа на денталните коронки и мостови, поседува голема сила, хемиска стабилност и супериорна естетика после фасетирањето. Фасетната керамика пак се смета за најслабиот дел од целосно керамичките реставрации. Поврзувањето помеѓу основата и фасетниот порцелан се базира на поврзувањето кое се јавува и кај метал-керамичките конструкции. Стандардните процедури на поврзување на цирконијата со тврдите забни ткива и фасетните материјали не ја даваат потребната сила на бондирање.

Целта на студијата е да се истражат различни површински третмани на цирконијата за да се одредат оние со кои се постигнуваат највисоки вредности на поврзувањето. Студијата ги испитува силите на поврзувањето на фасетниот порцелан и цирконијата со Макро тестот на силата на смолкнување. Примероците беа поделени во пет групи по дванаесет, според различниот површински третман, а резултатите добиени после тестирањето покажаа највисоки вредности на силата на поврзување кај примероците третирани со Рокатек системот.

Macedonian pharmaceutical bulletin, **60** (2) 57 - 65 (2014)
ISSN 1409 - 8695
UDC: 615.454.1:638.135]:615.256.5
Original scientific paper

Bioinspired bioartifical polymer hybrid composites for propolis vaginal delivery II: formulation and characterization

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Received: October 2014; Accepted: December 2014

Abstract

In our previous work Box-Behnken experimental design was applied for formulation optimization of the thermoreversible mucoadhesive in situ vaginal hydrogels with propolis and optimized batches were identified. Optimized batches of bioartificial polymer hybrid composites (chitosan, Lutrol® F-127 and Lutrol® F-68 mixture)) (CP1, CP2, CP3) were prepared using so-called cold method. Formulation P3 (chitosan free) was prepared in order to evaluate the effect of chitosan on the physico-chemical and biopharmaceutical properties of the polymer hybrid composites (gels).

The pH values of the gels were 4-4.5. The gelation temperature for all formulations was in a range of 29-33 °C. Total flavonoids content was above 95%. Increase in concentration of Lutrol® F-127 and Lutrol® F-68/Lutrol® F-127 ratio lead to a higher viscosity values and slower gel erosion/dissolution. The presence of chitosan increased gel viscosity and hence slow-down erossion/dissoluiton. Propolis release rate was the highest in P3 which released propolis within 5 h, corresponding to time of complete erosion. The same correlation between erosion process and drug release rate was observed in CP1-CP3, where prolonged propolis release for more than 10 h was achieved. Microbiological quality was in accordance with the requirements of Ph. Eur. 7. All formulations demonstrated adequate stability at 5 ± 3 °C during 6 months. Based on overall results it can be anticipated that bioartificial blended bioinspired polymer hybrid composites for propolis vaginal delivery could represent intelligent delivery systems with physicochemical and biopharmaceutical properties in favor or efficacious and safe therapy of vaginal infections.

Key words: thermoreversible, mucoadhesive, vaginal gels, propolis, physicochemical and biopharmaceutical characterization

Introduction

Propolis (bee glue) exhibits well-known and documented biological and pharmacological properties as antimicrobial and antifungal and most of them antiviral agent. In this context, it is believed that most of the pharmacological effects of this composite biocomplex are due to its flavonoid content, even though the other components also contribute to its biological activity (Casaroto and Lara, 2010; Ramos and Miranda, 2007). A large number of investigations has confirmed the antifungal properties of propolis, especially its prominent activity against Can-

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dida albicans (Fearnley, 2001; Khalil, 2006; Kujumgiev et al., 1999; Marcucci, 1995; Sawaya et al., 2002), as well as its clinical efficacy in the treatment of vaginal infections. It is thought that propolis has local anesthetic and immunostimulatory effects which are also important in the treatment of vaginal infections (Pochinkova, 1995).

The gels as vaginal drug delivery systems are most commonly used dosage forms amongst the patients, due to their advantages such as easy administration and uniform spreading over the surface, allowing more intimate contact with the vaginal mucosa, precise dosing and the absence of discomfort after their administration (Das Neves and Bahia, 2006; Neves et al., 2009). However, despite these advantages, conventional gels demonstrate relatively fast drug release due to their dilution with vaginal fluids and dissolution. Another disadvantage is the possibility of liquefaction at room temperature; therefore they can leak out of the vagina. To avoid these problems, there is a need for formulation of vaginal gels with local antimicrobial activity which can provide uniform distribution over the surface of the vaginal mucosa, along with prolonged residence time and controlled drug release.

In the recent years, thermosensitive in situ-forming gel systems have been receiving a great deal of interest as vaginal delivery systems for efficient local treatment of various diseases. These environmentally sensitive systems can be easily administered due to their liquid state at room temperature, whereas their gelification in response to environmental changes offers significant advantages such as precise dosing, *in vitro* and *in vivo* stability and slow clearance from local sites due to mucoadhesive properties of the polymers used, therefore allowing prolonged drug release.

Poloxamers are poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) copolymers (PEO-PPO-PEO), known under the trade name of Lutrol®, which form micelles at low concentration and clear thermoreversible gels at high concentrations (Alexandridis and Hatton, 1995). Poloxamers differ in their physical and surface properties due to the differences in the chemical structure. Lutrol® F-127 (LF-127) is a hydrophilic PEO-PPO-PEO tri-block copolymer (Escobar-Chávez et al., 2006) which has low toxicity, good dissolving capacity and it considered as a good vehicle for numerous active substances (Kolsure and Rajkapoor, 2012). Because of its thermoreversible gelification properties and low toxicity, LF-127 can be applied in various drug delivery systems. Lutrol® F-68 (LF-68) contains 81% of PEO units in its composition, which makes this poloxamer easily soluble in water (Fussneger, 1999). At higher concentrations, the water solutions of LF-68 exhibit non-Newtonian behavior and thermosensitivity when the concentration exceeds 20%. However, LF-68 is not used alone as gel-forming agent. Usually, it is combined with LF-127 in order to modify the thermorheological properties of the gels. The addition of LF-68 leads to an increase in the gelaton temperature (*Tg*), most probably due to the formation of mixed micelles and offers the advantage of adjusting the *Tg* value to the desired one, by varying the amount of LF-68 up to 20%. (Fussneger, 1999). Notwithstanding the wide application of thermosensitive poloxamer gels in formulation of different drug delivery systems, their main drawback is the fast erosion due to the low mechanical strength. Therefore, prolonged retention time and controlled drug release from poloxamer gels can be achieved by the combination with other polymers, such as chitosan (CTS). The addition of CTS causes the formation of more dense poloxamer network, therefore resulting in increased mechanical strength of the gel matrix, as well as influencing the drug diffusion and slowing its release (Gratieri et al., 2010; Varshosaz et al., 2008).

As it was already rationalized (Simonoska Crcarevska et al., 2013b) by bioartificial blending bioinspired polymer hybrid composites for propolis vaginal delivery could represent intelligent delivery systems with physico-chemical and biopharmaceutical properties in favor or efficacious and safe therapy of vaginal infections. In our previous work, response surface, Box-Behnken, experimental design was applied for formulation optimization of the thermoreversible mucoadhesive in situ vaginal hydrogels with propolis and optimized batches were identified (Simonoska Crcarevska et al., 2013b). The main objective of this work was to characterize and evaluate physicochemical and biopharmaceutical properties of previously identified formulations.

Materials and methods

Materials

Chitosan (CTS, low viscous, 95% deacetylation) was supplied from Sigma-Aldrich, USA. Poloxamers (Lutrol® F-127 and Lutrol® F-68) were obtained from BASF, Ludwigshaften, Germany. 20% propylene-glycolic extract of propolis (PGEP) was kindly donated from Galafarm, Macedonia. All other reagents and solvents were of analytical grade and used as received.

Methods

Preparation of polymer hybrid composites

Optimized batches of bioartificial polymer hybrid composites (CTS, LF-127 and LF-68 mixture)) (thermo reversible mucoadhesive in situ gels) (CP1, CP2, CP3) were prepared according to the previously described procedure using so-called cold method with minor modification (Simonoska Crcarevska et al., 2013b). Detailed composition of the formulations is presented in Table 1.

Formulation P3 was prepared in order to evaluate the effect of CTS on the physicochemical and biopharmaceutical properties of the polymer hybrid composites (gels).

Table 1. Composition of designed polymer hybrid composites (thermo reversible mucoadhesive in situ gels)

Formulation code	CTS (%)	LF-127 (%)	LF-68/LF-127 mass ratio	PGEP (%)
CP1	1.5	16.39	0.06	3.0
CP2	1.5	17.36	0.09	3.0
CP3	1.5	18.24	0.12	3.0
P3	/	18.24	0.12	3.0

Preparation of simulated vaginal fluid (SVF)

Human vaginal fluid comes from several sources such as uterus, cervix and sometimes menstrual secretions and sperm. Due to the limited quantity of human vaginal fluid (approximately 0.5–0.75 mL) and its rapid degradation after collection from its source, a simulated vaginal fluid (SVF) was developed (Aka-Any-Grah et al., 2010). SVF was prepared as follows: NaCl (3.51 g), KOH (1.4 g), $Ca(OH)_2$ (0.22 g), bovine serum albumin (0.018 g), lactic acid (2.00 g), acetic acid (1.00 g), glycerol (0.16 g), urea (0.4 g) and glucose (5.00 g) were added to 900 ml distilled water and stirred mechanically until complete dissolution. The pH of the SVF was then adjusted to 4.5 using 0.1M HCl, and the final volume was adjusted to 1 L.

Characterization of polymer hybrid composites (thermo-reversible mucoadhesive in situ gels)

Visual characterization

Visual characterization of color, appearance, odor, texture and phase separation on designed and prepared formulations were carried out.

pH determination

Adequacy of designed polymer hybrid composites (thermo reversible mucoadhesive in situ gels) for vaginal use were evaluated by determination of their pH value (pH meter, Metler Toledo 340, Germany). pH evaluation was carried out in triplicate.

Determination of of the sol-gel transition temperature

The sol-gel transition temperature (*Tg*) of the designed formulations was measured by tube inversion method (Ur-Rehman et al., 2010) with minor modifications (Simonoska Crcarevska et al., 2013b). Briefly, 2 ml of prepared formulations were transferred into a glass test tube sealed with a parafilm and put in horizontal shaker water bath (50 strokes/ min). The temperature of the water bath was gradually increased (1 °C/ min) and the temperature at which the solution in the sample containing tube stopped flowing upon inverting the tube was recorded. Similarly, the temperature was decreased and the temperature, at which the gel started flowing, was recorded. The average of both temperatures was calculated as the critical *Tg*.

Considering the possibility of change in the *Tg* of the formulations after their dilution with vaginal fluid (Aka-Any-Grah et al., 2010), the *Tg* of each formulation was also investigated after the addition of 0.75 µl of SVF.

Determination of propolis content

Propolis content in the prepared formulations was determined by validated spectrophotometric method (395 nm, Lambda 16, Perkin Elmer, USA) where quantification of the total flavonoid content was done using chrysin as external reference standard. Briefly, 1g of prepared formulations was dissolved in 100 ml methanolic solution of acetic acid (0.5% v/v). 2 ml of prepared solution and exactly 1 ml of aluminum chloride water solution (2% w/w) were transferred in glass volumetric flask and methanolic solution of acetic acid (0.5%, v/v) was added to a total volume of 10ml. In parallel a compensatory solution was prepared on the same way without aluminum chloride. In the same manner external reference standard of chrysin was prepared (0.02% w/v). Three replicates were carried out to estimate the inherent variability of the determination and the total flavonoids content was expressed in mg of chrysin equivalents per gram of prepared formulations.

Viscosity determination

The viscosity (mPa*s) of the prepared polymer hybrid composites (thermo-reversible mucoadhesive in situ gels) was determined using cone/plate viscometer (Myr V2-L, 4.6/MOTv2, Viscotech, Spain). A sample solution (0.5 ml) was applied to the lower plate of the viscometer and viscosity was determined at at 25 ± 0.5 °C and 32 ± 0.5 °C using spindle 52 at a shear rate ranging from 5 to 400 rpm. All samples were analyzed in triplicate.

Erosion/dissolution and in vitro propolis release studies

Thermo-reversible mucoadhesive in situ gels erosion/ dissolution profile and the *in vitro* propolis release from the designed formulations were obtained simultaneously. Briefly, 2 g of each formulation were transferred in glass tubes, weighted and placed in horizontal shaker water bath (40 rpm) previously thermostated at 32 °C. After the gelation, 10 ml of SVF pre-equilibrated at 32 °C were added. At pre-determined time intervals (1, 2, 3, 4, 5, 6, 7, 8 and 12 h) the total volume of liquid medium was removed, and the weight of eroded/dissolved gel was calculated from the change in the weight (glass tube with gel) at the beginning of the experiment and at each time interval.

Afterwards the release medium was completely replaced by 10 ml of fresh medium at predetermined time intervals. The concentration of propolis in the release medium was determined spectrophotometrically as it was previously described. All experiments were performed as triplicates.

In vitro propolis release modeling and release profile comparison were performed with DDSolver 1.0 program (menu-driven add-in program for Microsoft Excel) (Simonoska Crcarevska et al., 2013a; Zhang et al., 2010).

Microbiological quality

The microbiological quality of the prepared formulations was evaluated according to the Ph.Eur.7 method 2.6.12 (Microbiological examination of non-sterile products (total viable aerobic count)) and method 2.6.13 (test for specified microorganisms)). The acceptance criteria for microbiological quality of vaginal preparations stated in the Ph.Eur.7 (5.1.4 - Microbiological quality of non-sterile pharmaceutical preparations and substances for pharmaceutical use) are based on the total aerobic microbial count (TAMC) and total yeast and mold count (TYMC).

Stability studies

Prepared formulations were sealed in glass vials and stored under controlled conditions (25 ± 2 °C and 5 ± 3 °C). Periodical testing of different parameters (organoleptic properties, pH value, propolis content, microbiological quality) during 6 months was performed.

Results and discussion

In our previous work (Simonoska Crcarevska et al., 2013b) response surface Box-Benken experimental design was used for development and optimization of propolis vaginal delivery system (polymer hybrid composites (thermo-reversible mucoadhesive in situ gels)) with desired gelling properties. Key formulation factors influencing *Tg* were determined and optimization of formulation was carried out. Optimized batches with *Tg* of 32 °C (CP1-CP3) were prepared and characterized accordingly.

Characterization of polymer hybrid composites (thermo-reversible mucoadhesive in situ gels)

The pH values of the prepared propolis vaginal delivery system were in the range of 4 to 4.5 pointing to suitability of formulations for vaginal use (Table 2).

The gelation temperature was determined for all prepared formulations (CP1-CP3, P3) before and after their dilution with 0.75 μL SVF (Table 2).

Obtained results showed an increase of ~ 2 °C in *Tg* of the formulations diluted with SVF. These differences could be explained by the presence of co-solutes, i.e. ions and electrolytes in the SVF which probably interacted with poloxamers. Considering the strong relation between the gelation and micellization processes, the observed increase of *Tg* value is most likely related to the increased critical micelle concentration or critical micelle temperature. The results of our study also showed that the *Tg* of the P3 formulation prepared without chitosan was slightly higher compared to other formulations (CP1-CP3). Similar findings were reported by Gratieri et al. (Gratieri et al., 2010), indicating that CTS has an effect on the crosslinking and the packing of polymer chains, thus resulting in a denser network which gelled at lower temperature.

Total flavonoids content in the prepared formulations (CP1-CP3) was above 95% (Table 2).

The efficacy of gels intended for local treatment of vaginal infections depends to a great extent on the rheological characteristics of the system. Namely, improved therapeutic efficacy could be achieved by prolonged residence time at the site of action (vaginal mucosa) as well as controlled drug release (propolis) from hybrid polymer matrix. Rheological characteristics are influenced by various factors such as the composition of formulations (polymers used as gel-forming agents), temperature, vaginal pH value, amount of vaginal fluid etc. Comparing viscosity values measured at 25 and 32 °C it can be noticed that prepared polymer hybrid composites (thermo-reversible mucoadhesive in situ gels) were characterized by higher viscosity at 32 °C as it was expected due to thermosensitive properties of used poloxamers. Obatined results pointed that by increasing the concentrations of LF-127 and LF-68/LF-127 ratio, higher viscosity values were observed. Formulation P3 was characterized by lower viscosity compared to CP1-CP3 (Table 2).

Erosion/dissolution and in vitro propolis release studies

Fig. 1 presents the effect of formulation variables (LF-127 and LF-68/LF-127 ratio (CP1-CP3)), as well as CTS

Table 2. Gelation temperature (*Tg*) (mean ± SD) of the designed polymer hybrid composites (thermo reversible mucoadhesive in situ gels) (n=3)

Formulation code	pH values ± SD	*Tg* (°C) ± SD		Total flavonoids		Viscosity (mPa*s ± SD)	
		without SVF	with 0.75 μl SVF	mg/g ± SD	% of the declared content ± SD	25 ± 0.5 °C	37 ± 0.5 °C
CP1	4.36 ± 0.03	29 ± 0.03	31 ± 0.05	2.46 ± 0.01	95.17 ± 0.67	1180 ± 0.2	65220 ± 0.5
CP2	4.06 ± 0.02	29 ± 0.02	31 ± 0.01	2.59 ± 0.02	99.87 ± 0.75	1340 ± 0.3	167930 ± 0.3
CP3	4.13 ± 0.04	29 ± 0.03	31 ± 0.05	2.55 ± 0.02	98.55 ± 0.79	1470 ± 0.1	219600 ± 0.3
P3	4.50 ± 0.05	32 ± 0.02	33 ± 0.04	2.58 ± 0.02	99.15 ± 0.74	930 ± 0.1	33700 ± 0.8

(P3 compared to CP3) on the gel erosion/dissolution vs time. Erosion/dissolution of formulation P3 (CTS free) was completed in 4.5 h, while CP1-CP3 showed decrease in the rate of gel erosion, 5.5-8.5 h accordingly. Results obtained for P3 (CTS free) were in accordance with the literature data, which suggest that gels consisted only by poloxamers dissociate rapidly in an aqueous environment (Chung et al., 2009). The observed results for CP1-CP3 pointed that increase in concentration of LF-127 and LF-68/LF-127 ratio lead to a higher viscosity of the system, thereby causing the formation of more dense gel network which erodes at a slower rate. The presence of CTS retarded the gel erosion/dissolution due to its incorporation into the gel skeleton most likely resulting with increased mechanical strength of the gel network (Varshosaz et al., 2008).

The results of *in vitro* propolis release study are presented in Fig. 2. It can be seen that propolis release follows gel erosion/dissolution processes. Propolis release rate was the highest in P3 formulation (CTS free), which released propolis within 5 h, corresponding to time of complete erosion. Furthermore, the same correlation between erosion process and drug release rate was observed in formulations CP1-CP3, where prolonged propolis release for more than 10 h was achieved, due to the effect of CTS.

The continuous swelling of poloxamers accounts for achieving prolonged drug release when used as gelling agents. Additionally, drug diffusion occurs through the extramicellar aqueous channels formed during matrix erosion/dissolution. Hence, the decrease in the rate of propolis release with the increase in LF-127 concentration could be attributed to the increase of number and size of the micelles formed at higher polymer concentration (Radivojša et al., 2013). This could cause a greater extent of polymer chains entanglement in the aqueous phase of the gel structure and slower dissolution rate. Additionally, higher poloxamer concentration could cause shorter intermicellar distance, leading to a larger number of cross-links between neighboring micelles and larger number of micelles per volume, along with subsequently slower rate of dissolution of the incorporated drug (Ibrahim et al., 2012).

Even though the chosen polymers differ in terms of chemical structure, both CTS and poloxamers have hydrophobic regions in their chains (D-glucose residues in chitosan and polypropyloxyethylene blocks in poloxamers). When the temperature is increased, water molecules structured around the hydrophobic regions of polymer chains in a sol state become disarranged. As a result, newly revealed hydrophobic regions attract each other to form bonds, whereas hydrophilic parts reorganize to maximize their contact with the aqueous medium. The resulting structures are micelles, which continue to grow in size and number at higher temperatures, leading to higher viscosity of the gel structure and consequently, slower rate of drug release is achieved (Varshosaz et al., 2008).

Drug release kinetics of propolis from the prepared formulations was determined by analyzing the dissolution data using various mathematical models. The kinetics constants and correlation coefficients for propolis release are shown in Table 3. Taking into account the values of correlation coefficients (R), it can be concluded that Korsmeyer-Peppas model is the best one to describe the propolis release from the prepared formulations. According to the obtained values of release rate constant (K), it can be claimed that the drug dissolution rate decreases with the increase of LF-127 and LF-68 concentrations, as expected. Predominance of diffusion in release mechanisam was observed for CP1-CP3, while P3 (without CTS) demonstrated release mechanism most likely controlled by diffusion and gel erosion/dissolution.

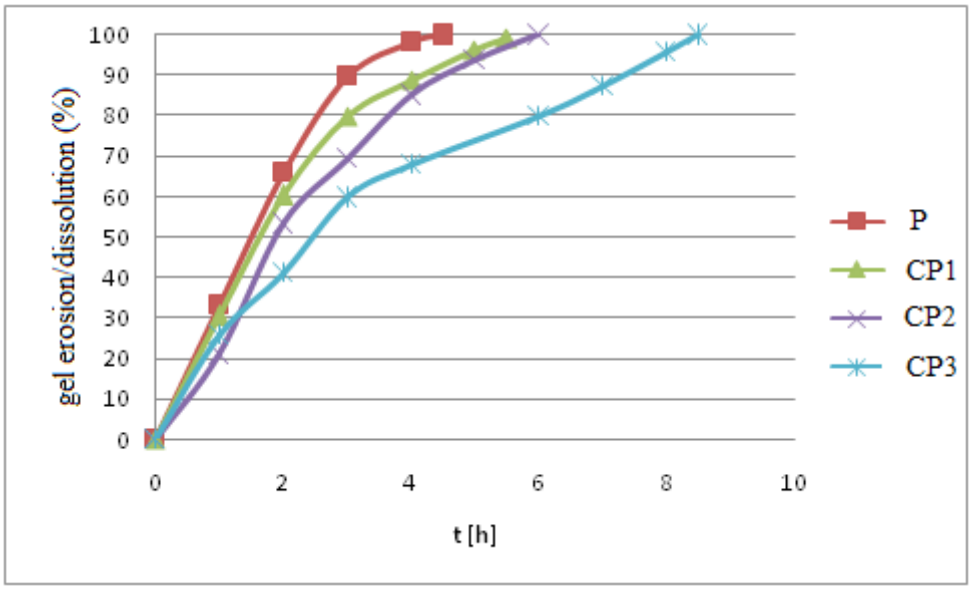

Fig. 1. Gel erosion/dissolution of designed polymer hybrid composites (thermo reversible mucoadhesive in situ gels).

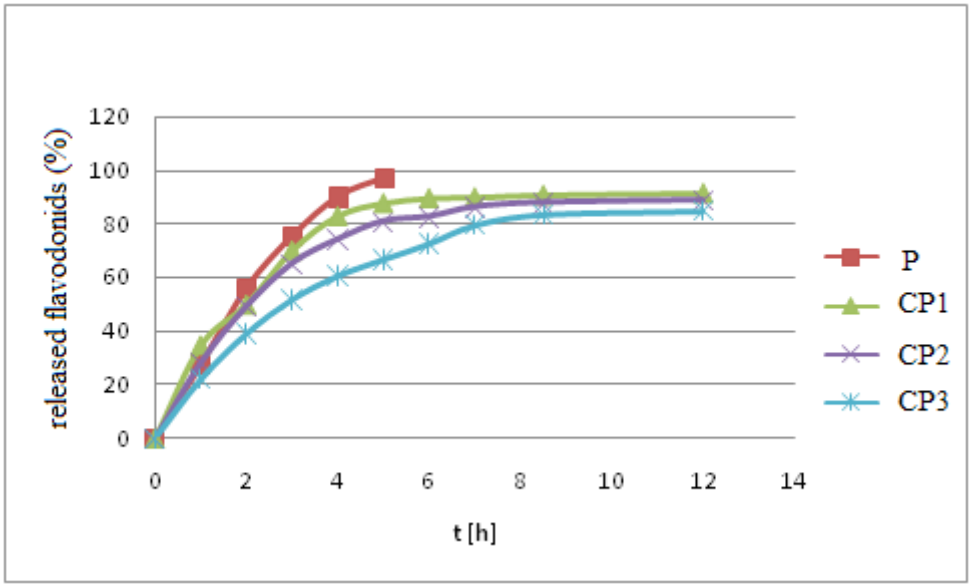

Fig. 2. *In vitro* release profiles of propolis from designed polymer hybrid composites (thermo reversible mucoadhesive in situ gels).

Table 3. Kinetics constants and coefficients for propolis release from the prepared formulations

Formulation code	First order		Higuchi		Korsmeyer-Peppas			Peppas-Sahlin		
	K (1/h)	R	K ($h^{-0.5}$)	R	n	K (h^{-n})	R	k1	k2	R
CP1	0.356	0.9890	33.475	0.9232	0.565	35.165	0.9913	51.756	-6.939	0.9807
CP2	0.367	0.9831	31.638	0.9456	0.613	30.445	0.9888	45.993	-5.449	0.9833
CP3	0.194	0.9811	28.019	0.9800	0.542	27.545	0.9951	33.266	-1.991	0.9856
P3	0.446	0.9869	42.264	0.9809	0.793	29.625	0.9906	25.012	9.175	0.9927

Microbiological quality

The obtained results related to microbiological quality were in accordance with the requirements stated in Ph.Eur. 7.

Stability studies

The results of the stability studies of the prepared formulations stored at 25 ± 2 °C and 5 ± 3 °C during 6 months are presented in Table 4 and 5, respectively.

Formulations CP1 and CP2 demonstrated adequate stability during 6 months of storage at 25 ± 2 °C without any significant changes in their organoleptic properties, pH values, propolis content, viscosity and microbiological quality. CP3 formulation showed adequate stability during 5 months of storage 25 ± 2 °C, but after 6 months changes in its organoleptic properties were observed, i.e. viscous liquid was formed. On the other hand, all formulations demonstrated adequate stability at 5 ± 3 °C during 6 months.

Conclusion

To summarize in this work by bioartifical blending of natural and synthetic polymers thermo reversible mucoadhesive in situ gels were prepared and characterized**.** Addition of bio/mucoadhesive macromolecules (chitosan) to the poloxamers (Lutrol® F-127 and Lutrol® F-68) based vaginal delivery system resulted with increased viscosity, decreased gel erosion/dissolution rate thus improving the sustained release of propolis. Chitosan not only helped to circumvent draw backs of poloxamer gels alone like fast erosion/ dissolution, but also did not adversely affect its thermosensitive behavior. Based on the results obtained it can be concluded that chitosan-poloxamer based systems would enable prolonged residence time allowing prolonged drug release at the desired site of action and hence resulting with better therapeutic efficacy.

Table 4. Results from stability studies of the prepared formulations stored at 25 ± 2 °C during 6 months period

Parameter	Formulation code	Time of evaluation (month) 0	1	2	3	4	5	6
Organoleptic properties	CP1	Dense homogenous liquid with yellow-brownish color and propolis smell						
	CP2							Non-homogeneous liquid with a yellow-brownish color and propolis smell
	CP3	Dense homogenous liquid with yellow-brownish color and propolis smell						
pH value	CP1	4.36 ± 0.2	4.25 ± 0.15	4.31 ± 0.1	4.2 ± 0.13	4.22 ± 0.08	4.18 ± 0.1	4.09 ± 0.11
	CP2	4.06 ± 0.1	3.97 ± 0.12	4.00 ± 0.13	3.95 ± 0.09	3.91 ± 0.1	3.85 ± 0.08	3.79 ± 0.1
	CP3	4.13 ± 0.14	4.00 ± 0.11	4.09 ± 0.1	3.98 ± 0.12	3.94 ± 0.07	3.93 ± 0.1	4.1 ± 0.13
Total flavonoid (% of declared content)	CP1	95.17 ± 1.1	94.83 ± 0.9	98.99 ± 1.07	95.98 ± 1.5	95.64 ± 1.34	95.98 ± 1.46	94.52 ± 0.94
	CP2	99.87 ± 1.5	99.88 ± 1.84	103.63 ± 0.6	101.35 ± 1.2	99.65 ± 1.74	99.77 ± 1.36	102.56 ± 0.8
	CP3	98.55 ± 1.3	98.3 ± 0.5	106.99 ± 2.3	103.78 ± 1.82	97.14 ± 1.12	94.17 ± 1.9	98.19 ± 0.96
Viscosity (mPa*s)	CP1	1180 ± 0.2	/	/	/	/	/	1190 ± 0.4
	CP2	1340 ± 0.3	/	/	/	/	/	1380 ± 0.9
	CP3	1470 ± 0.1	/	/	/	/	/	/
Microbiological quality criteria compliance	CP1	Yes	/	/	/	/	/	Yes
	CP2	Yes	/	/	/	/	/	Yes
	CP3	Yes	/	/	/	/	/	Yes

Table 5. Results from stability studies of the prepared formulations stored at 5 ± 3 °C during 6 months period

Parameter	Formulation code	Time of evaluation (month) 0	1	2	3	4	5	6
Organoleptic properties	CP1	Dense homogenous liquid with yellow-brownish color and propolis smell						
	CP2							
	CP3							
pH value	CP1	4.36 ± 0.2	4.02 ± 0.1	3.99 ± 0.14	3.97 ± 0.1	3.82 ± 0.3	3.90 ± 0.16	3.78 ± 0.2
	CP2	4.06 ± 0.1	4.30 ± 0.9	4.25 ± 0.5	4.20 ± 0.4	4.19 ± 0.1	4.23 ± 0.09	4.09 ± 0.18
	CP3	4.13 ± 0.14	4.09 ± 0.56	4.01 ± 0.28	3.98 ± 0.35	3.95 ± 0.2	3.97 ± 0.25	3.86 ± 0.3
Total flavonoid (% of declared content)	CP1	95.17 ± 1.1	98.84 ± 0.7	96.52 ± 1.22	94.98 ± 2.31	94.21 ± 0.8	96.14 ± 1.98	95.75 ± 2.21
	CP2	99.87 ± 1.5	103.08 ± 1.82	99.61 ± 1.93	99.23 ± 1.85	98.84 ± 1.32	101.54 ± 2.23	102.38 ± 1.3
	CP3	98.55 ± 1.3	107.72 ± 1.31	94.21 ± 2.15	98.84 ± 1.96	92.28 ± 1.64	103.47 ± 1.57	98.07 ± 1.84
Viscosity (mPa*s)	CP1	1180 ± 0.2	1180 ± 0.8	/	/	/	/	1190 ± 0.4
	CP2	1340 ± 0.3	1340 ± 0.7	/	/	/	/	1380 ± 0.9
	CP3	1470 ± 0.1	1470 ± 0.5	/	/	/	/	1560 ± 0.8
Microbiological quality criteria compliance	CP1	Yes	Yes	/	/	/	/	Yes
	CP2	Yes	Yes	/	/	/	/	Yes
	CP3	Yes	Yes	/	/	/	/	Yes

Reference

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Резиме

Биоинспирирани биовештачки полимерни хибридни композити со контролирано ослободување на прополис во вагина II: формулација и карактеризација

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Клучни зборови: термореверзибилни, мукоатхезивн, вагинални гели, прополис, физичко-хемиска и биофармацевтска карактеризација

Во нашиот претходен труд со примена на Box-Behnken експериментален дизајн беше направена оптимизација на термореверзибилни мукоатхезивни *in situ* вагинални хидрогели со прополис. Оптимизираните формулации на биовештачки полимерни хибридни композити (смеса на цитозан, Lutrol® F-127 и Lutrol® F-68) (CP1, CP2, CP3) беа подготвени со примена на т.н. ладен метод. Формулацијата P3 која не содржи цитозан, беше подготвена со цел да се евалуира влијанието на цитозанот врз физичко-хемиските и биофармацевтските особини на полимерните хибридни композити (гели). Подготвените гели се карактеризираа со pH од 4-4,5. Температурата на гелирање кај сите формулации беше во опсег од 29-33 ^{o}C. Содржината на вкупните флавоноиди беше поголема од 95%. Зголемувањето на концентрацијата на Lutrol® F-127 и односот на Lutrol® F-68/Lutrol® F-127 резултираше со поголеми вредности на вискозитетот и побавна ерозија/дисолуција на гелите. Присуството на цитозанот во формулацијата резултираше со зголемување на вискозитетот на гелот и негова побавна ерозија/дисолуција. Брзината на ослободување на прополисот беше најголема кај формулацијата P3 кај која целата количина на прополис беше ослободена за 5 часа, што кореспондираше со времето на комплетна ерозија на гелот. Слична корелација меѓу процесот на ерозија и брзината на ослободување на прополисот беше забележана и кај формулациите CP1-CP3, кај кои беше постигнато продолжено ослободување на прополисот во период поголем од 10 часа. Микробиолошкиот квалитет на подготвените формулации беше во согласност со барањата на Ph. Eur. 7. Сите формулации беа стабилни на 5 ± 3 °C во тек на 6 месеци. Врз база на севкупните резултати може да се заклучи дека биовештачките блендирани биоинспирарни полимерни хибридни композити со контролирано ослободување на прополис може да преставуваат интелегентни вагинални системи со физичко-хемиски и биофармацевтски особини кои ќе овозможат ефикасен и безбеден третман на вагиналните инфекции.

Macedonian pharmaceutical bulletin, **60** (2) 67 - 73 (2014)
ISSN 1409 - 8695
UDC: 614.2
Professional paper

Redefinition of the notion of Universal Access to the health care

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Received: September 2014; Accepted: December 2014

Abstract

In the last 10 years we are experiencing hidden debate where decision makers do not want to opt for the "unpopular" decisions, which need to be taken if we need a sustainable health systems on a long run. Lessons from the 2008 crisis have proven that policy decisions driven by external global forces that are beyond our controlwere inconsistent and reasonable damaging also on a mid term run. Instead of addressing the core of the problem, in the attempt to reply to the old/new challenges, governments were "fanning the fire". It becomes obvious that spending more money in uncoordinated way will not solve the problem. Reducing the cost by cutting the fiscal budgets, would further "squeeze" the capacity of the economies and reduce the demand, which has to be driver to the solution and not the problem. Consequently in a high market developed economies, cutting the health budgets will only temporarily "make up" the state budgets, creating structural financial instability of the Funds both private and State. In this lose-lose situation, with existing misbalances, contracting budgets, increasing demand and sensitive market players responses, there is a high time for redefinition of the Universal access to the health care systems and global policy responses which will on long term create balanced and sustainable growth of health markets.

Keywords: pharmacoeconomics, drug costs, new common model, health technology assessment

Introduction

Continuous loss of competitiveness in the European Union (EU) countries caused primarily because of the declining productivity levels, resulted in a recession or stagnation of Gross Domestic Product (GDP) driven further by the low demand levels. Policy makers' responses to the structural problems of the economies were rather confusing. Most of them opted to cut the budgets and reduce the deficits,instead of implementing countercyclical policies of spending more money in order to increase the demand in the economies and protect households' budgets. The element of decision in the contracting economies is very simple, states must increase the spending'sas answer to the financial problems instead of creating a cost cutting measures.While doing so, Governments must introduce so called expenditure switching policies of the state budgets including the health transfers, and to do this in a very controlled way. This will result in the partial solution of the existing fiscal disbalances but most important, it will put the large state funds into function as main drivers to the economy. This new policies will on the mid term essentially result with the stimulating mechanisms of increasing demand in the economies, including the health segment.

The fact that prevention and primary care have been mostly affected areas with the cuts on average of 13% (WHO Policy paper report 12, 2014) leaves no room for optimism that the gap will be covered in the next 5-10 years. With average 3.3% fall of the European GDP and the unemployment levels rising to 11%, (The World Bank indicators 2015, http://data.worldbank.org/indicator/NY.GDP.PCAP.CD/countries/EU), fiscal pressure on the governments forced them to reallocate public resources and spending's. More than half of the EU countries have reduced the health budgets in the attempt to limit the costs and finance other areas. The feedback of this new policies

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was with limited effects, simply because in attempt to address the problems, Governments were not dealing with the reasons. They have only been trying to balance the results. However, health systems generally need more, not fewer, resources in an economic crisis and there is good evidence underlining the importance of countercyclical public spending, especially on social and health sectors.

Ensuring that levels of public funding for the health system are adequate, public revenue flows are then predictable and revenue will be raised in a way that does not unfairly burden households. These are essential elements to promoting financial protection, equitable access to effective health services and equity in financing. Making this system sustainable requires that the Governments implement change to the way health care is funded and delivered, which limits the services provided by the state – creating new losers as well as winners. Although Governments may be pleased by this changed position, on the long run they will face with more problems than ever. Reason is simple:external global forces often beyond the control of individual states are driving the crisis. Governments don't want to opt for less control of the processes and to give up part of their economic sovereignty. In this new environment, pharmaceutical companies may experience price pressure, at the same time benefiting from the major shift in the policy implementation by trading price for access to markets.

Rationing of the New Model/some numbers

Recently published paper on the European policy framework and strategy for the 21st century called "Health 2020", surprisingly just confirms the old policies of investing in health through a life-course approach and by empowering people. In fact there is no change in the policies where the same ideas remain in the core of the health policies in the developed world for the last 30 years. Seems that there is no flexibility in the redefinition of some of the elements of the global systems that need to take the existing challenges to a whole new level. However, today more than ever before there is political willingness to address waste in the health systems and to possible reduce input costs without undermining performance and the gap between revenue and expenditures. The major challenge is how to transfer the rise of the expenditures and burden the taxpayers and ask them to pay more money when it does not make sense because of their budgetary limits. This is when the so-calledpolicy of efficiency gains will take place. This process might be painful but the facts of how the health problems have been addressed and results produced cannot be satisfactory. Something will need to change.

Lets address the facts. The demographic transformation in the countries requires an effective life-course strategy that gives priority to new approaches for promoting health and preventing disease. Healthy and active ageing is a policy priority and a major research priority. On the other side scarcity of resources is further deepening the gap between the developed countries and the remaining world. This leaves us with the question of why global health systems are unsustainable and have to change? Healthcare in 2013 consumes 12% of global GDP, and is estimated a $5.3 trillion industry (WHO annual report, 2014). The dynamics of the process shows that spending's is growing at average of 3% per annum, which will double the expenditure in less than 20 years. By 2080, 50% of world GDP will be spend on health costs (McKinzey&Company Quarter-

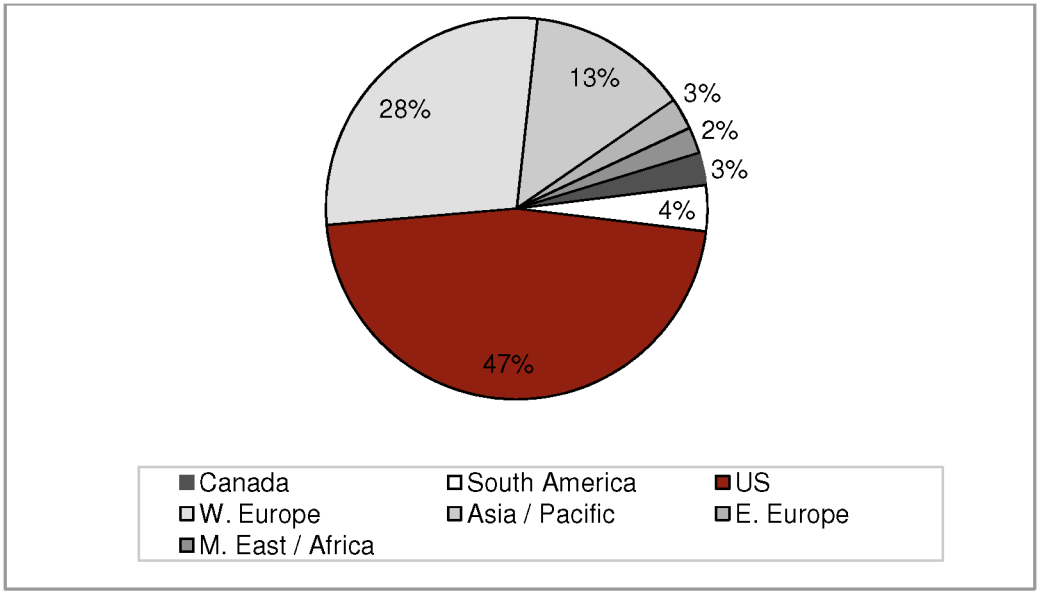
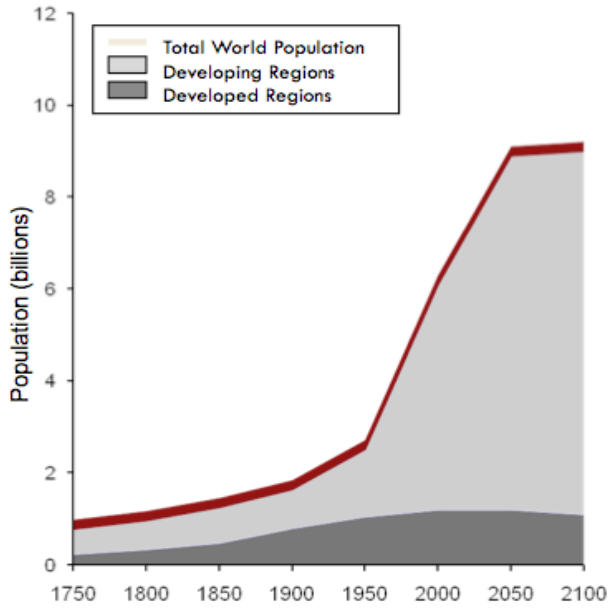


Fig. 1. Global health spending.

ly, 2008). This is not a surprise if we know that in the last 15 years health expenditures in the OECD countries have doubled, reaching average 13% share of the GDP (OECD, WHO annual reports 2014- Growth rate, average growth in spending in OECD countries 1995-2015). Inevitably fundamental global forces will change the way systems work. Finding a right balance between global trends, emergence of a new Common Model and implications need to be positioned in the focus of all debates. Results will answer to the following questions: *(i)* What is driving the evolution of health care globally? *(ii)* How are health systems reacting to this? *(iii)* What does this mean for Governments & the Pharma industry?

Existing disbalances in the world health care markets cannot be sustainable in the next period of 20 years. Numbers show that the consumption of the health expenditures is dominated by the USA, which with 5% of world population spends over 45% of total healthcare (Espicom, Industry report 2014). Furthermore, top 20 countries account for 90% of spend, which leaves to 84% of world population shares 11% of spending, but suffers 95% of diseases (Fig. 1).

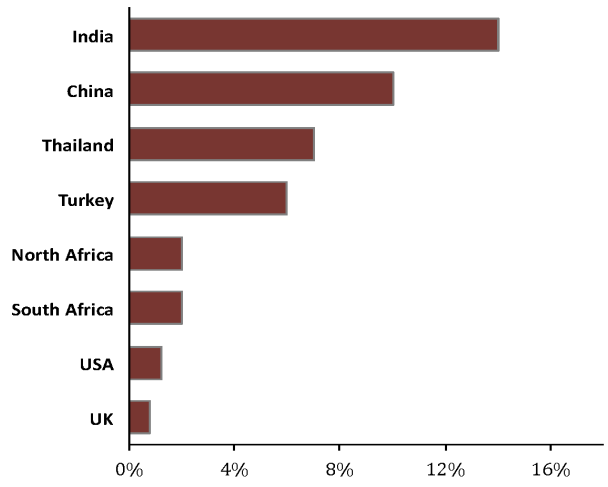
What becomes evident from the numbers is that population growth and wealth trends will in the future drive healthcare demand towards the developing countries (Fig. 2).



Source: Population Reference Bureau, U.S. Census Bureau 2014

Fig. 2. Population trends 1750-2100.

Growth in importance of developing/emerging markets will produce inequality to be unjustifiable, challenging the decision makers to meet basic health needs, of a growing "middle class" with developed world diseases, expectations, and money. This will produce need for redefinition of the global health policies,whereby balanced sustainable good health practices throughout the life-course will lead towards increasing healthy life expectancy. Result will produce important economic, societal and individual benefits. Strong evidence indicates that these cost-effective policy pathways can directly enhance population health and well-being. Creating better conditions for health, improving health literacy and making the healthier choice the easier choice, remains the driving factor, while attempting to address this burning problem of the increasing health expenditures in the developing countries. With population shifting to developing world and increasing wealth demands, health expenditure will rooftop creating continuous pressure to Governments to act (Fig. 3). Major reforms will reduce the reliance on market forces in which uncontrolled costs and unpropotioned relative performance is not sustainable. Both elements of the realignment to demographic shift and balancing of funding and expenditure will mark the future health policies.



Source: World Bank, U.S. Census Bureau 2014

Fig. 3. Compound Annual Growth Rate Consumer earning > $10K Purchasing Power Parity.

Universal access to the health care

While in Europe, most Governments are not yet ready to admit the "social contract" is under challenge, rationing of universal provision is inevitable. Universal access to the healthcare as a human right with the basic premises of equality of health outcomes, consumer choice and patient safetyis no longer affordable. Reason for this relies on the necessity to address widening inequalities in parallel to the pressures to increase healthcare spending and the new diseases caused by lifestyle and "medicalisation" as well as the new technologies to keep people alive and active. Finally, the pressure to increase the costs is coming from the increasing number of economically inactive people with high health needs. This is conflicting the possibili-

ty frontier and putting limit to the public funding of healthcare, based on the belief in "small" government and limits to taxation.

One of the major characteristics of the healthcare systems in the last 10 years is that they have moved progressively towards increased rationing, as other interventions have failed. Combining government leadership, supportive environments and approaches that promote a sense of control and empowerment can lead to success only after certain level of strengthening of social behavioral research is provided as growing evidence to underpin such developments. Health promotion programs based on principles of engagement and empowerment offer real benefits. However, options are limited to the implementation of the changes to improve efficiency, but control demand. While patient co-payment can be expected to rise, there is no political decision/agreement on how much the patient can bear. Governments need to shift spending to where it can deliver greatest health gain, but it's not just a rational decision. To achieve this goal both cost saving potentials and Quality of adjusted life years (QUALY)analysis has to be done throughout the major health lifecycle. Inevitably Governments should focus on spending's/investments that have the biggest impact on the health of the nation. In doing so, rationing of the services with implementation of clear mechanisms must take a lead to the health spending's. Governments should focus on the easiest and least contentious first, postponing the decisions for improving of the reference-pricing list, which is not an easy task. In parallel this balancing policy has to limit the scope of "Core Services" paid through taxation, releasing the budget pressure. Decisions are partly rational, but also driven by societal preferences, political readiness and partisan pressure.

The most difficult issue will be the content of "core provision" – and this choice is arbitrary. There is no known piece of work that tells you what the threshold for Health technology assessment (HTA) should be, and yet, something has to be done. Broad scope of participants, stakeholders, requires not one, but series of consecutive actions to bridge the existing gap between needs and capacities. Developed markets will need to decrease the affordability gap as defined by the HTA by squeezing the expenditures for the services that are not cost effective. In doing so, priority conditions have to be in the focus of the actions. As example, they should only in limited way address the local epidemiology problems. While doing so this group of countries has to give a high attention to the services which reduce overall burden on the health system and country, such as mental health, rehabilitation, prevention, screening, education, generics. Finally, developed countries must keep the main focus of financing and policy structuring to the services considered to be essential for basic health needs. Among others primarily to the care of the elderly, sanitation, nutrition, control of infectious diseases, accident & emergency care. (Fig. 4)

In parallel to these priority elements in the developed countries, developing markets will be forced to increase budgets allocated to health and to move from covering of

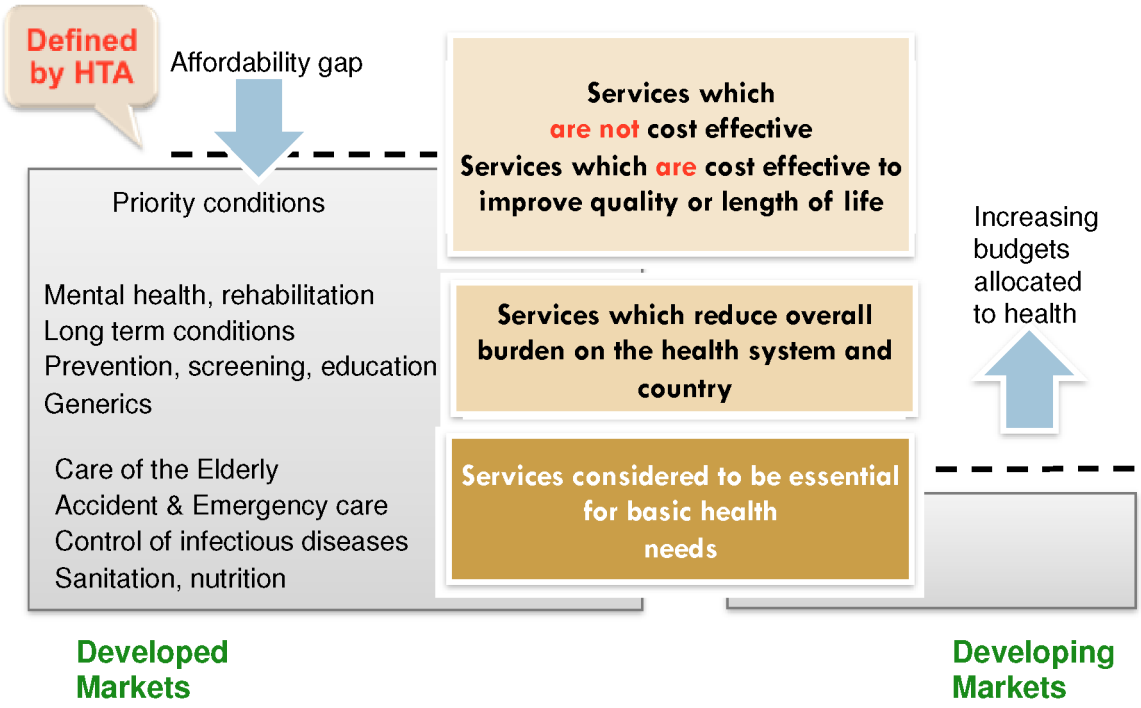


Fig. 4. Approach to the new health rationing.

the essential needs, towards prevention services and education, process which is opposite to the one in the developed countries. Balancing of those 2 approaches will result in balancing of the spending's, priorities and health markets. Eventually all health systems will converge largely into the same system structure.

One way street

Government can proceed to the new Common model by design, or by default. This course of actions cannot be reversed due to the fact that health market conditions have been changed and time is valuable category. In their intention to control the processes Governments will have to implement a list of measures and policy decisions that will in the core of the substance involve use of pharmacoeconomics so that they will avoid any voluntary and subjective choice. Reason for this is even stronger considering the "narrowband" pharmaceutical producers are experiencing in the last 10 years. Finally, transparent but also favorable policy for all interested groups (producers, health workers, patients) must be packed into sustainable Model which on a long term will produce win-win situation for all involved participants.

Definition of the new Model requires list of actions all partners in the Deal will perform (Fig. 5). The role of the Governments will remain more or less without changes but with higher focus on the core service elements. Prioritization of the Governments actions will be given to the emergency care, public health, prevention, mental health, disadvantaged groups which will be financed by taxation or compulsory contributions. On the other side payers will face with managed distribution of the "core service" funds and have market freedoms for non-core funding. Providers of the services and products that are represented by mix of public and private operators in this new Model will focus more on the regional planning activities which will also address the disbalances in the developments and presence. While doing so, they will be financed by tariff based payments but will also have to experience increasing use of capitated/risk share. Fourth partner in this New deal/Model are suppliers which will continue to face with regulated prices and pressures to cut the cost and increase the effectiveness. Moreover suppliers will need to further adapt to the rules of centralised procurement with strict evaluation of cost effectiveness ratios. Naturally, they will benefit from the new growing markets and economies of scales. Finally, in the core of the Model are citizens, segment that will need to accept a more controlled process. Tax or compulsory contributions will not change as core elements for financing of the health needs. The new role will be mostly in the areas of limited co-payments where citizens will experience control of demand keeping gate for expensive acute care services. Of course self-payment/insurance will remain for the non-core services.

The process that according to many signals has already started but needs to be with higher level of determination

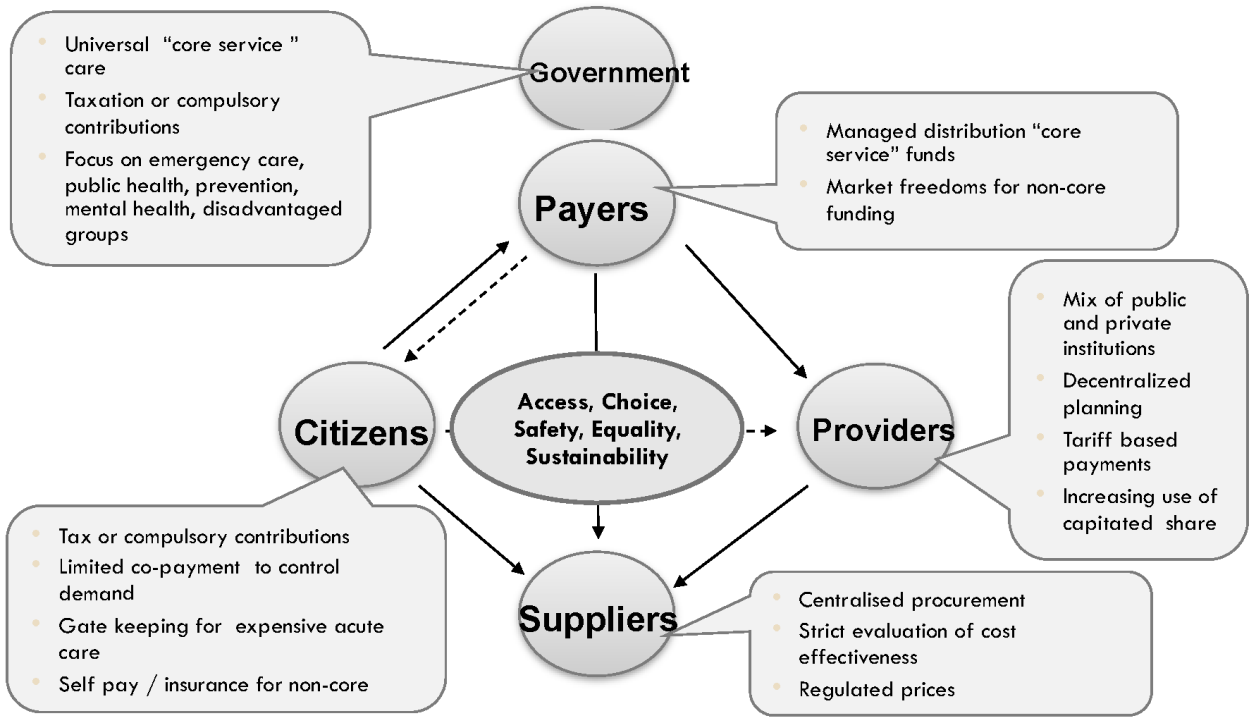


Fig. 5. New Common Model.

by the actors, is positioning the Government in the driver seat. It is therefore of no surprise that reaction to these new developments can be increase of the monopsony behaviour. This is the price that needs to be paid as counterweight to the economic and rational actions they will have to perform. Among others, Government will have to increase the budgets and switch the expenditures with higher focus towards preventive and primary care, to involve pharmacoeconomic studies in the assessments and address the demands from the innovative drug companies.

Conclusions

Increasing costs, stagnating demand, existing disbalances and prevention of the households and state budgets, by default create a need for introduction of a new common Model for financing of health care. The core of this Model is the redefinition of the notion of universal access to the health care, service prioritization and budget reallocations. Decisions will require more profound use of pharmacoeconomic studies that will focus on what really drives treatment decisions and what new data would be viewed as clinically meaningful for prescribers and value-creating for economic stakeholders. This requires series of actions on a global world level, which must not be exhaustive ones or limited, but rather a coordinated list of performances on a global scale basis. Process which will address all actors in the field of policy definitions and practice will start by initiation of a detailed analysis, including assessment of the critical drivers of behavior for each stakeholder (prescriber, payer, and patient behavior). The substance of this action is to deliver the global targets by inclusion and not exclusion of the major stakeholders. In this way they will be assertive but also creative elements of the process. Therefore stakeholder perception and its reflection in understanding of how competitors are perceived by each stakeholder against the most critical factors (financing, sustainability, covering, scope of services, budgets, tax policies etc.), which can help identify unmet needs, it is of utmost importance. This may request further detailed comparison of competitor labels and clinical data to informthe decision makers which efficacy and safety endpoints matter most. Branded results of this action point will then be mapped against the competitor label and clinical data so to enable more granular understanding of real-world data on treatment decisions and outcomes.

Final outcome of this process that will consume some years before it is fully addressed and results are evident, will be a balancing act between the producers, services providers, decision makers, payers and consumers. All parties involved will therefore experience a full package of actions based on a clear pharmacoeconomic studies and HTA reports. The sooner this process starts with full dynamics the faster results will come, preventing the negative developments we have experienced in the last 10 years.

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Резиме

Редефинирање на значењето на Универзален пристап кон здравствените услуги

Рубин Зарески

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Клучни зборови: фармакоекономија, трошоци на лекови, нов заеднички модел, проценка на здравствените технологии

Во последните 10 години се случува скриена дебата во која носителите на одлуки не сакаат да се определат за „непопуларни“ одлуки, кои што треба да се донесат доколку ни треба долгорочен и одржлив здравствен систем. Лекциите од кризата во 2008 година докажаа дека политичките одлуки управувани од страна на развиените држави беа недоследни и исто така штетни до одреден степен на среден рок. Наместо да се зафатат со решавање на суштината на проблемот, во обидот да се одговори на старо-новите предизвици, владите делуваа ациклично и дополнително го усложнија проблемот. Станува очигледно дека трошењето пари на некоординиран начин нема да го реши проблемот. Намалувањето на трошоците преку кратење на фискалните буџети уште повеќе ќе ја зголеми неликвидноста на економиите и ќе ја намали побарувачката, што во суштина треба да води кон решение, а не да креира дополнителни проблеми. Како резултат на овие мерки, кратењето на здравствените буџети кај високоразвиените пазарни економии само привремено ќе ги „нашминка“ државните буџети, создавајќи структурна финансиска нестабилност и на приватните и на државните фондови. Во ваква ситуација во која секој губи, со постоечки дисбаланси, договорни буџети, зголемена побарувачка и барања на значајните пазарни играчи, крајно време е да се редефинира универзалниот пристап кон здравствените услуги и да се дефинираат глобалните политички ставови кои ќе создадат долгорочен избалансиран и одржлив раст на здравствените пазари.

Macedonian pharmaceutical bulletin, **60** (2) 75 - 82 (2014)
ISSN 1409 - 8695
UDC: 658.86/.87:661.12
Case study

Implementation of Supply Chain Management (SCM) in pharmaceutical company, general principles and case study

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Received: October 2014; Accepted: December 2014

Abstract

Supply Chain Management (SCM) in pharmaceutical industry is defined as a "responsible SCM" and its implementation is according to the principles of: business ethics, rights of labor and principles of healthy and safe working environment. Pharmaceutical companies with implemented "responsible SCM" have to use management systems to facilitate continuous improvement in accordance with their working principles. The main purpose of this management system is to ensure the consistency, reliability and continuous improvement of all workflows within an organization.The analyzed case describes the project of European generic pharmaceutical company, which intends to implement best practice SCM operations for five European manufacturing sites and European logistics organizations (active ingredients supply, distribution centers, affiliate customers and third party manufacturers). The main objectives of the project were the creation of the future improved To-Be situation through implementation of new SCM models to the existing To-Day situation.

Keywords: Supply chain management (SCM), responsible SCM, implementation, evaluation

Introduction for SCM

Supply Chain Management (SCM) is defined as systematic and strategic coordination of the traditional business functions and their tactical coordination within the company itself, systematic and strategic coordination of the traditional business functions and their tactical coordination in cooperation with all business partners of the respective company, with one end point long-term improvement of performance of the company (Mentzer et al., 2001). Generally, SCM is a combination of different disciplines such as logistics, transport and distribution as part of the operational management system, marketing, part of the raw material's orders and procurement of information technology. The ultimate objective of SCM is to create coordination between all the previously mentioned operations in order to improve performance of a particular company (Peltz, 2008).

Unlike other industries in the pharmaceutical industry SCM is upgraded and implemented as a "responsible SCM". The term of responsibility is due to the fact that the implementation of SCM in the pharmaceutical companies is according with principles of: business ethics; the rights of labor force and principles of healthy and safe working environment (PSCI guidance, 2011).The pharmaceutical industry in the last two decades is faced by continuous changes in terms of testing and sales a new products. The major changes are mandated by the health authorities in the countries were companies produce or sale. Changes that are commonly encountered are: a permanent pressure on prices and tendency of the health authorities for their constant reduction, shortening the defined period of

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patent protection of original drugs and constant pressure from generic or biosimilar companies. Aimed in reducing their expenses, many pharmaceutical companies decide to merge their research and development process. The implementation of SCM systems in pharmaceutical companies is intended towards rationalization the process of planning, production, distribution and storage of the final product before it comes to its final customer/user. The process of rationalization, through the introduction of standard SCM modules and systems in the pharmaceutical companies, on the other hand allows decreasing the cost of the final product, thus increasing its competitiveness compared to other similar products produced by the competitive pharmaceutical companies (Rees, 2011).

The main purpose of the SCM is to ensure the consistency, reliability and continuous improvement of all workflows within an organization. Pharmaceutical companies with implemented responsible SCM should use management systems to facilitate continuous improvement in accordance with their working principles. By this process they ensure market competitiveness, able to meet the specific requirements of certain countries in terms of the area of business ethics, receive respectable relations with representatives of labor/trade unions, believe that these principles are best implemented through continuous improvement approach in everyday work activities, which indirectly leads to increased competitiveness (Cailet, 2011).

The cycle of this management system represented in Fig. 1 contains four separate activities: plan, do, check and action (PSCI guidance, 2011; ISO 14001, 2004).

Implementation of SCM in pharmaceutical companies usually result with transition from market focused strategy to strategy focused on product; emphasis on the possibility of producing a type of derivative products on one place; centralization of production activities in a manufacturing location; closing and reducing the number of unnecessary manufacturing sites; defining the distribution centers and defining the mode of transport of the products/services to selected distribution centers; defining selection's criteria of potential collaborators/companies that are subject to constant change and improvement in accordance with the requirements of the market and in order to maintain the competitive advantage of the company itself (PSCI guidance, 2011).

Major challenges that SCM program in the pharmaceutical companies could face in the future are: long time necessary for the development and approval of new pharmaceutical products, need for expansion of the product line and shorter life cycle of the product due to the aggressive approach of generic and non brand manufacturers, continued market pressure for new and innovative pharmaceutical products, continuous increasing need for quality and increasing demands by regulators, more common situations in which there is no possibility of negotiation, overloaded global supply chains: more production facilities, distribution and sales channels and markets and unpredictable business process: the need for continuous technical and scientific advancement and improvement (Rees, 2011).

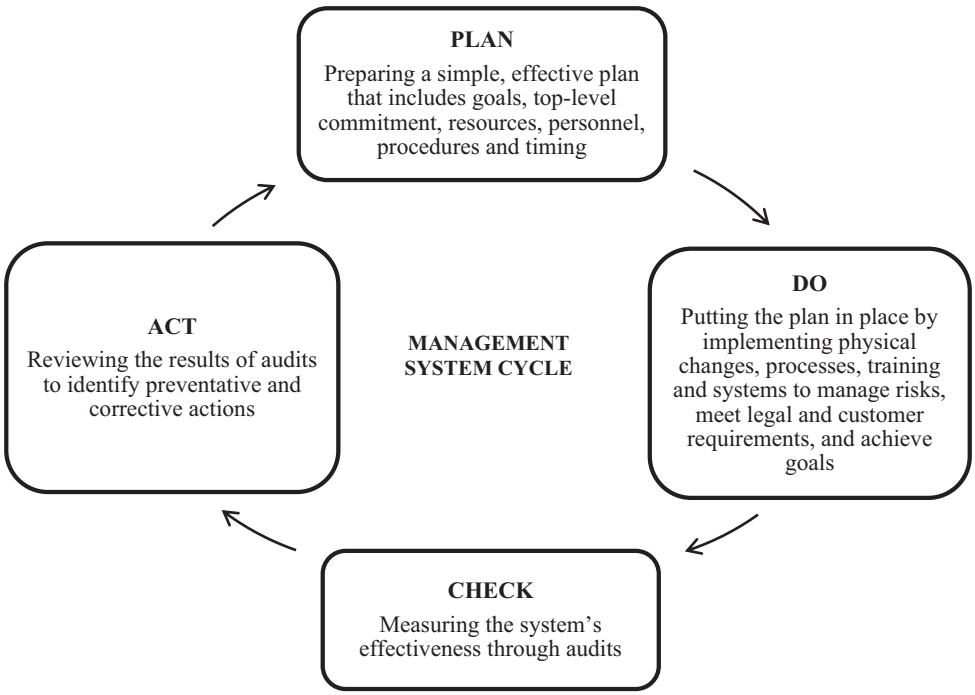

Fig. 1. Pharmaceutical industry principals for Responsible Supply Chain management (adapted from http://www.pharmaceuticalsupplychain.org/downloads/psci_guidance.pdf, 2011).

Case study of implementation of SCM in pharmaceutical company

In this case is describing the project of European generic pharmaceutical company, which intends to implement best practice SCM operations for five European manufacturing sites and European logistics organizations (supplier of the active component, distribution centers, affiliate customers and third party manufacturers). This project scope includes SCM planning process, supporting the production planning and detailed scheduling within the pharmaceutical plants as well as the network planning across the company's supply chain to optimally match supply and demand.

The planning process is implemented based on Systems Applications and Products (SAP) in data processing R/3 4.6C and Advanced Planner and Optimizer (APO) 3.1operating system.

The case study focuses on the implementation of the Advanced Planning System (APS) components of SAP APO Production planning (PP)/Detailed scheduling (DS) system as a model to support the production planning and detailed scheduling in the manufacturing plants and SAP APO Supply Network Planning (SNP) to model the supply network planning of the supply chain.

The main results and benefits of the project will be highlighted as well as the major hurdles encountered in the implementation of the SAP APO PP/DS and SNP solution.

Project's objectives

The main objectives of the project were the creation of future To-Be improve situation across implementation of new models in existing To-Day situation. The need to improve the To-Day situation arose because of no insight into the overall database within the company; not effective and efficient implementation of the manufacturing process; unstable planning process and poor utilization of all available resources; absence of master planning; major ongoing IT costs; no effective and efficient execution of administrative tasks; problems with distribution of raw materials to production locations/final product to end users; recognized and continuous dissatisfaction with end users.

To-Day situation

The start of the project is described like a To-Day situation. The following list highlights some key aspects of this To-Day situation in analysed company: There are several key aspects of this To-Day situation in analysed company. The first one addresses to 4 SAP R/3 systems (two running R/3 PP, Process Industry (PI), one Business Planning and Control System (BPCS) and one R/2 system. Data integration between the systems was low, data structures not harmonized. The same product existed with several material numbers in different systems. Information sharing as well as synergies out of a common system were not achievable. The second key aspect is related with the fact that there was no available centralized supply network planning systems, resulting in no central visibility of the supply chain constraints and problems. The next aspect stems from the product planning and detailed scheduling of the manufacturing processes which were performed in various standalone systems and spreadsheets, interfaced with local Enterprise Resource Planning Systems (ERP). This resulted in massive manual planning effort and sub optimal capac-

Table 1. Evaluation of existing To-Day situation

Planning (35% importance/contribution)	
Parametars	Score
Time for development of the production cycle	2
Total cycle time	2
Time for total money flow	2
Amount of product and service	3
Average score	*2.25*
Utilization of resources (15% importance/contribution)	
Parametars	Score
Interest collaborators for building partnership relations	3
Time of supply	2
Reservations for suppliers	2
Get prices from suppliers	2
Average score	*2.25*
Production (30% importance/contribution)	
Parametars	Score
Cost of production	2
Capacity utilization	2
Effective layout of the main planning	2
Cycle duration of the production process	2
Level Bearings	3
Average score	*2.2*
Distribution (10% importance/contribution)	
Parametars	Score
Delivery time	2
Number of existing deliveries	3
Degree of response to urgent deliveries	2
Total cost of delivery	3
Average score	*2.5*
Customers satisfaction (10% importance/contribution)	
Parametars	Score
Flexible to meet customer requirements partial	3
Levelat which the client perceived value of the product	3
Average score	*3.0*

ity utilisation. There was no central statistical forecasting system in To-Day environment. In addition the Key Performance Indicators (KPI) was not consistently defined and did not support common targets. At the end the business processes were rather complex, without uniquely defined responsibilities for core planning tasks like materials planning, detailed scheduling and master planning.

Evaluation of To-Day situation

The evaluation of this To-Day situation was made by scores received from the management team on the basis on analyzed data of planning process, utilization of available resources, manufacturing, distribution and customer satisfaction. The percentages of importance/contribution of the analyzed data in the final score evaluation were defined on the meeting of company top-management and were as follows: planning (35%), resource utilization (15%), production process (30%), and distribution process and customer satisfaction (10%). The evaluation is presented in Table 1. Total score of the current To-Day situation was 2.34 points (Eq. 1).

$$2.25 \cdot 0.35 + 2.25 \cdot 0.15 + 2.2 \cdot 0.3 + 2.5 \cdot 0.1 + 3.0 \cdot 0.1 = \underline{\mathbf{2.34\ points}} \quad \text{Eq. 1}$$

Description of To-Be vision

To-Be vision was designed to achieve target goals of harmonized processes, data, systems and organizational units in To-Day situation. In To-Be situation 6 ERP sys-

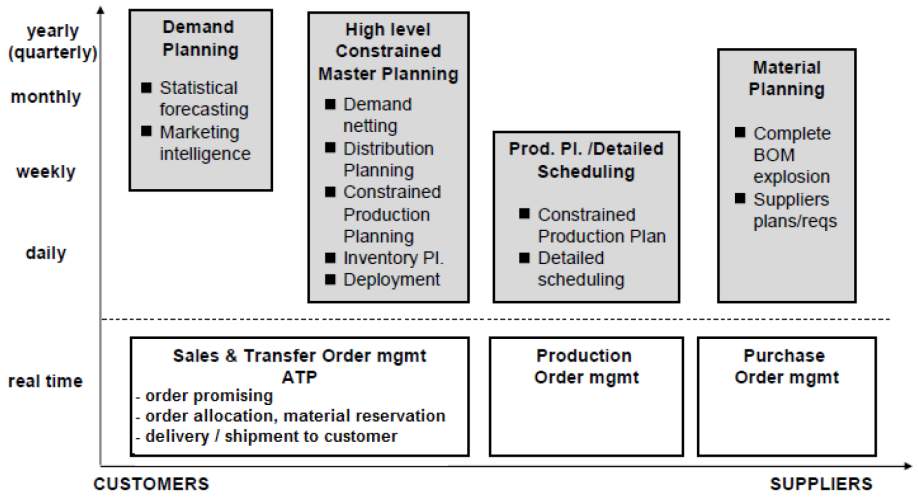

Fig. 2. Simple map of the whole process of planning and implementation of plan (adapted from Caillet, 2008).

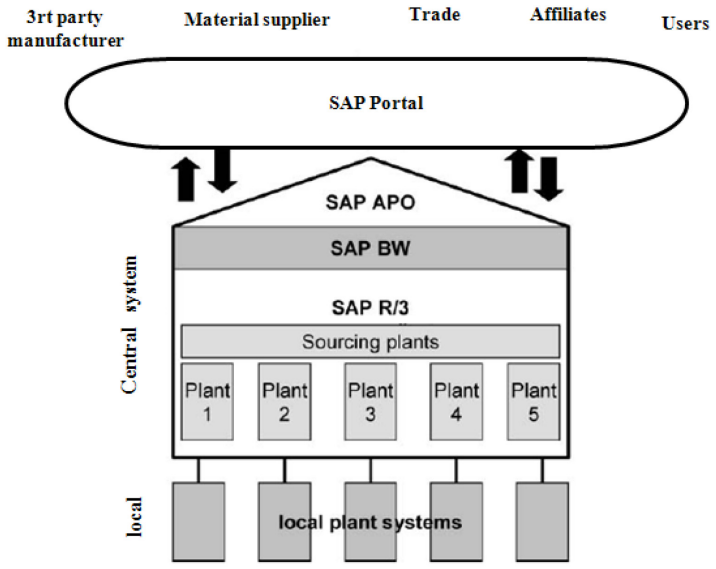

Fig. 3. Schematic presentation of the implemented IT systems. Legend: SAP - Systems Applications and Products, APO - Advanced Planner and Optimizer, BW - Business Warehouse (Caillet, T., 2008. SCM in Pharmaceutical Company).

tems are integrated into a central SAP R/3 system. The level of the entire company was setup a central Advanced Professional Solution System (APS), which represented the entire supply chain system. The major changes in To-Be vision are: change the entire organization from local, function-oriented thinking, to a common European company, sharing the same targets and commitment in true collaboration between the business functions and the supporting IT function; building of an European team to support that challenging vision on both IT and business side; base the project founding on expected benefits, proven by a business case performed before the implementation started; buy-in of all involved stakeholders right from the beginning to propagate the new vision and to support its implementation; setup a collaborative forecasting; visibility of the demand and the supply through the complete network of the supply chain based on one global, constrained master plan; one common detailed scheduling system used by all plants, customized to support local specificities and process inherent constraints; installation of a common European reporting and controlling process, supported by common KPIs; integration of suppliers into the master planning process; implementation of Vendor Managed Inventory (VMI) processes for the major affiliates and customers and transportation planning the vehicle scheduling done by the third party logistics providers. Simplification of these process is presented in Fig. 2.

Table 2. Planning process covered by Enterpise Resource Planning Systems (ERP) and Advanced Professional Solution System (APS) moduls

Enterpise Resource Planning Systems	(APS) moduls
Demand planning (DP)	SAP APO DP
Master planning (MP)	SAP APO SNP
Detailed scheduling (DS)	SAP APO PP/DS
Materials requirements planning (MRP)	SAP R/3 MRP
Production order management (POM)	SAP R/3 PP-PI
Inventory management (IM)	SAP R/3 IM-WM
Procurement direct materilas(PDM)	SAP R/3 MM
Master data management (MDM)	SAP R/3 MM
Supply chain management (SCC)	SAP R/3 Co and SAP BW

Legend: SAP - Systems Applications and Products, APO - Advanced Planner and Optimizer, SNP - Supply Network Planning, PP-DS - Production plainning/Detailed sheduling, MM – Material management, BW - Business Warehouse

Table 3. Evaluation of new To-Be situation

Planning (35% importance/contribution)	
Parametars	Score
Time for development of the production cycle	4
Total cycle time	3
Time for total money flow	4
Amount of product and service	4
Average score	*3.75*
Utilization of resources (15% importance/contribution)	
Parametars	Score
Interest collaborators for building partnership relations	3.5
Time of supply	3
Reservations for suppliers	3
Get prices from suppliers	2.5
Average score	*3*
Production (30% importance/contribution)	
Parametars	Score
Cost of production	3
Capacity utilization	4
Effective layout of the main planning	3
Cycle duration of the production process	3
Level Bearings	4
Average score	*3.4*
Distribution (10% importance/contribution)	
Parametars	Score
Delivery time	3
Number of existing deliveries	3
Degree of response to urgent deliveries	3
Total cost of delivery	3
Average score	*3*
Customers satisfaction (10% importance/contribution)	
Parametars	Score
Flexible to meet customer requirements partial	3.5
Levelat which the client perceived value of the product	3.5
Average score	*3.5*

Implementation of IT systems in the desired To-Be vision is shown in Figure 3. The figure visualizes the new IT system landscape supporting the To-Be vision of this project. The IT system landscape is based on SAP R/3 4.6C, SAP APO 3.1, the Standard Core Interface (CIF) to integrate R/3 and APO, SAP Business Warehouse (BW) and SAP Enterprise Portals (EP).The central SAP R/3 system covers functionalities provided by the following modules: PP/PI; Materials Management (MM); Sales and Distribution (SD); Quality Management (QM, for batch management, quality inspection lots only); Controlling (CO, for product costing and budget planning), Warehouse Management (WM).

After all relevant functionalities were migrated from the old (local) ERP systems of the plants to the new central ERP system, the remaining local non-ERP systems had to be interfaced to the central environment. These are

mostly execution control systems, Laboratory Information Management System (LIMS), Material Handling System (MHS) and WH system.

The central R/3 system provides the integration basis for the APO system. From APO, the modules Demand Planning (DM), Supply Network Planning (SNP) and Production Planning/Detailed Scheduling (PP/DS) are used. The process coverage of the APO modules is shown in Fig.3. SAP BW is the foundation of a common reporting and performance measurement system. SAP EP is used to integrate customers into the demand planning process and to enable customers to access sales orders and delivery confirmations. The planning process introduced in Fig. 3 were mapped to the following ERP and APS modules and are presented in Table 2.

Evaluation of new To-Be situation

Evaluation of a new To-Be situation is made by the same parameters: planning, resource utilization, production, distribution and customer satisfaction (one year after implementation of SCM and creation of new To-Be model) with the same percentage of importance/contribution to the final score evaluation of all measured parameters, reference evaluation of the initial To-Day situation. The evaluation is presented in Table 3.Total score of the new To-Day situation is 3.43 points (Eq. 2).

$$3.75\cdot0.35+3\cdot0.15+3.4\cdot0.3+3\cdot0.1+3.5\cdot0.1=\mathbf{3.43\ points} \quad \text{Eq. 2}$$

Discussion

The process which will be evaluated at the implementation of SCM activities depends of company top management assessment, reference the main weaknesses of the current organizational and operational set and which processes are most deserving for the current non reasonable profitability of the company. Selection the KPIs for each process, such as planning, utilization of resources, production, distribution and customer satisfaction is done by a free estimate of the top management of each company. Results without percentage of importance/contribution and with included a percentage of importance /contribution are presented in Table 4 and Table 5, respectively.

The data mentioned above indicated that the implementation of SCM processes allows the highest improvement in the final score in the area of planning, improvement of 1.5 points or 0.52% points with included percentage of importance/contribution. If we know that the plan-

Table 4 Results without percentage of importance/contribution

Pharmaceutical company without SCM (*To-Day)*		Pharmaceutical company with SCM *(To-Be)*	
Analyze the process through predefined KPI	*Score*	Analyze the process through predefined KPI	*Score*
Planning	2.25	Planning	3.75
Utilization of resources	2.25	Utilization of resources	3.0
Production	2.2	Production	3.4
Distribution	2.5	Distribution	3.0
Customer satisfaction	3.0	Customer satisfaction	3.5
Final score (To-Day)	*12.2*	*Final score (To-Be)*	*16.6*

Table 5. Results with included percentage of importance/contribution

Pharmaceutical company without SCM (*To-Day)*		Pharmaceutical company with SCM *(To-Be)*	
Analyze the process through hpredefined KPI	*Score*	Analyze the process through predefined KPI	*Score*
Planning	0.79	Planning	1.31
Utilization of resources	0.34	Utilization of resources	0.45
Production	0.66	Production	1.02
Distribution	0.25	Distribution	0.3
Customer satisfaction	0.3	Customer satisfaction	0.35
Final score (To-Day)	*2.34*	*Final score (To-Day)*	*3.43*

ning process was given weight of 35% as a factor of improving the company performance, we can conclude that implementation of the SCM processes allows improvement in the most important segment of the functioning of the analyzed pharmaceutical company. The results presented in Table 5 show that the least improvement was registered under the item customer satisfaction. This is due to the fact that the presented data were driven from the assessment of the activities after one year of the implementation of the SCM, while the relevant literature data for evaluation of SCM activities indicate that the assessment of the SCM could be obtained only after two years of its implementation. Further more the customers like end users need the longest period of time to note the improvement of the quality of performance of a particular company. The analysis shows that the implementation of SCM processes in the analyzed pharmaceutical company results with improvement of the final score for 1.09 percentage points with included percentage of importance/contribution. The operating profit of the company has increased for 3.1%. This improvement was achieved only one year after the implementation of defined SCM processes.

Conclusions

The main benefits envisioned in the business case prior to the APS implementation were achieved. First the visibility and problem solving capabilities of the entire organization were improved by the use of a common data basis and a common visualization tool, allowing better and faster decisions; system based finite capacity scheduling and fast simulation capabilities improved the plan stability and resource utilization significantly; collaborative demand planning with the customers allow for a proactive stabilization of the demand as changes in the demand by the customers are compared with a constrained demand from the previous master planning. By reducing the order to cash cycle, as well as pushing for more collaboration with the affiliates through a VMI process, the inventory levels were reduced and significantly reduced the IT maintenance costs by consolidating the system landscape.

Standardization of the master data enable the visibility and interchangeability of information faster across the supply chain. The overall administrative workload for tasks performed previously manually or based on wrong information was reduced significantly.

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Резиме

Менаџирање со ланец на набавки во фармацевтска компанија (СЦМ), основни принципи и приказ на случај

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Клучни зборови: Менаџирање со ланец на набавки (SCM), одговорен SCM, имплементација, евалуација

Supply Chain Management (Менаџирање со ланец на набавки) во фармацевтската индустрија се дефинира како „одговорен SCM“ и неговата имплементација е согласно принципите за бизнис етика, правата на работната сила и принципите на здрава и безбедна работна средина. Фармацевтските компании со имплементиран „одговорен

SCM“ треба да користат менаџмент системи со кои ќе се олесни континуираното подобрување во согласност со нивните работни принципи. Основна цел на овој менаџмент систем е да се обезбеди конзистентност, сигурност и континуирано подобрување на сите работни процеси во рамките на една организација.

Во презентираниот случај се објаснува проектот на Европска фармацевтска генеричка компанија, која има намера да имплементира најдобра пракса на SCM операции за пет Европски производни места и Европските логистички организации (снабдувачот на активната компонента, дистрибутивните центри, филијалите со нивните потрошувачи и други производители). Главна цел на проектната активност е создавање на идна посакувана и подобра To-Be ситуација преку имплементација на нови и дефинирани SCM процесни активности кај постоечката To-Day ситуација.

INSTRUCTIONS FOR AUTHORS

Macedonian Pharmaceutical Bulletin is an official publication f the Macedonian Pharmaceutical Association. The journal publishes original scientific papers, short communications, reviews, mini-reviews and professional papers from all fields of pharmacy and corresponding scientific fields of interest for pharmacy (pharmaceutical and medicinal chemistry, immunology and imunochemistry, molecular biology, pharmaceutical analyses, drug quality control, pharmaceutical technology, pharmacoinformatics, pharmacoeconomics, biopharmacy, pharmacology, applied botany, pharmacognosy, toxicology, clinical pharmacy, food and nutrition, physical pharmacy, organical synthesis, social pharmacy, history of pharmacy *etc*.)

The *Macedonian Pharmaceutical Bulletin*, also, publishes and other contributions (recommendations and announcements, reports of meetings, important events and dates, book reviews, various rubrics).

Types of paper

Original scientific papers (full length manuscripts) should contain own unpublished results of completed original scientific research.

Short communications also should contain completed but briefly presented results of original scientific research. The article should be prepared as described for full length manuscripts, except for the following: the number of pages should not exceed 10 (including 2 illustrations, figures or tables). An Abstract should be included as well as a full reference list.

Reviews and mini-reviews are written at the invitation of the Editorial Board. "Mini-reviews" of a topic are especially welcome.

They should be surveys of the investigations and knowledge of several authors in a given research area, the competency of the authors of the reviews being assured by their own published results.

Professional papers report on useful practical results which are not original but help the results of the original scientific research to be adopted into practical use. Professional papers might be based on the elaborating of theoretical data.

Language

Original scientific papers, short communications, reviews and mini-reviews should be written in good English (American or British usage is accepted, but not a mixture of these), while professional papers and all other contributions may be submitted in Macedonian.

Submission declaration

Submission of an article implies that the work described has not been published previously (except in the form of an abstract or as part of a published lecture or academic thesis), that it is not under consideration for publication elsewhere, that its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and that, if accepted, it will not be published elsewhere in the same form, in English or in any other language.

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EC Directive 86/609/EEC for animal experiments http://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm;

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Define abbreviations that are not standard in this field in a footnote to be placed on the first page of the article. Such abbreviations that are unavoidable in the abstract must be defined at their first mention there, as well as in the footnote. Ensure consistency of abbreviations throughout the article.

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Follow internationally accepted rules and conventions: use the international system of units (SI). If other units are mentioned, please give their equivalent in SI.

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Indicate each footnote in a table with a superscript lowercase letter.

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Examples: "as demonstrated (Allan, 1996a, 1996b, 1999; Allan and Jones, 1995). Kramer et al. (2000) have recently shown...."

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Reference to a journal publication:

Van der Geer, J., Hanraads, J.A.J., Lupton, R.A., 2000. The art of writing a scientific article. J. Sci. Commun. 163, 51–59.

Reference to a book:

Strunk Jr., W., White, E.B., 1979. The Elements of Style, third ed. Macmillan, New York.

Reference to a chapter in an edited book:

Mettam, G.R., Adams, L.B., 1999. How to prepare an electronic version of your article, in: Jones, B.S., Smith, R.Z. (Eds.), Introduction to the Electronic Age. E-Publishing Inc., New York, pp. 281–304.

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Journal names should be abbreviated according to

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