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(Review Article)

PROBIOTICS AS FUNCTIONAL FOOD IN DAIRY PRODUCTS

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This review focuses on the role of microorganisms as probiotics which when administrated in adequate amount confer a benefit to human health such as: normalization of the intestinal microflora, ability to block the invasion of potential pathogens in the gut, amelioration of lactose intolerance, prevention of colon cancer, reduction of blood cholesterol levels and many others. Identification and differentiation of probiotics are also reviewed in this article.

Kcy words: dairy products, fermentation, functional food, probiotics.

1. INTRODUCTION

Modern consumers are increasingly interested in their personal health, and expect the food that they eat to be healthy or even capable of preventing illness. Fermented products such as yoghurt, lactic acid are produced by the starter culture bacteria to prevent the growth of undesirable microorganisms (Ray and Daeschel, 1992). It has been accepted that fermented products contribute in improving human health especially in foods containing probiotics or health promoting bacteria. Probiotics are nonpathogenic microorganisms that when ingested, exert a positive influence on the health or physiology of the host (Marteau *et al.,* 2001). The development of dairy products containing probiotic bacteria is, currently, an extremely important topic with industrial and commercial consequences.

Probiotics have well defined and proven clinical effects for the treatment and prevention of diseases of intestinal and extra intestinal origin. These effects have been extensively reviewed in this manuscript.

2. TERM OF DEFINITION

2.1. Functional food

It can be defined as any food that may provide a health benefit beyond the traditional nutrients it contains (Roy, 2005). As a marketing term, functional food was initiated in Japan in the late 1980s and was used to describe food fortified with ingredients capable of producing health benefits. Probiotic dairy products, in particular probiotic yoghurts and milks, are the most active area within the functional food market in the world. Organisms such as *Lactobacillus* and *Bifidobacterium* species are incorporated into these dairy products (Stanton *et al.*, 2001).

2.2. Probiotics

FAO/WHO (2002) has adopted the definition of probiotics as (Live microorganisms which when administrated in adequate amounts confer a health benefit on the host). The term probiotic, meaning (for life) is derived from the Greek language (Schrezenmeir and de Verse, 2001). In 1989, fuller defined a probiotic as (A live microbial feed supplement which beneficially affect the host animal by improving its intestinal microbial balance). There are a large number of probiotics currently used and available in dairy fermented foods, especially in yogurts (Grajek *et al.*, 2005).

2.3. Prebiotics

Are defined as non-digestible or low-digestible food ingredients that benefit the host organism by selectively stimulating the growth or activity of one or a limited number of probiotic bacteria in the colon (Zimmer and Gibson, 1998; Manning and Gibson, 2004). The term synbiotic is used when a product contains both probiotics and prebiotics. This term should be reserved for products in which the prebiotic compound selectively favors the probiotic (Schrezenmeir and de Verese, 2001). Bifedogenic factors fall under the concept of prebiotics, which can be defined as compounds usually of carbohydrate nature, that survive direct metabolism by the host and reach the large bowel or cecum, where they are preferentially metabolized by bifidobacteria as a source of energy. Fructo-oligosaccharides and lactulose are commercially available and are considered as bifidogenic compounds (Gomes and Malcata, 1999).

2.4. Fermentation

Fermentation, as defined by Gale (1948), is the process leading to the anaerobic breakdown of carbohydrates. Other major compounds than carbohydrates, such as organic acid, proteins and fats are fermentable in the broader view that fermentation is an energy-yielding, oxidation- reduction process (Kosikowski, 1977). Fermentation is one of the oldest food preservation which is a process dependent on the biological activity of microorganisms for the production of a range of metabolites which can suppress the growth and survival of undesirable microflora in foodstuffs. Fermentation process involves the oxidation of carbohydrates to generate a range of products which are principally organic acids, alcohol and carbon dioxide (Ray and Daeschel, 1992). Fermentation process may produce other compounds which may have positive health implications such as vitamins, antioxidants and bioactive peptides.

Lactic acid bacteria are primarily responsible for many of the microbial transformations found in the more common fermented food products. The most common member of the group which is exploited for dairy uses includes *Lactococci* for cheese manufacture, *Streptococcus salivarius* subspecies *thermophillus* for cheese and yogurt manufacture and various members of the *Lactobacillus* genus for a variety of dairy fermentation.

Lin (2003) confirmed the wide range beneficial effects of probiotics which include the inhibition of *Helicobacter pylori* and possible enhancement of calcium absorption. While the properties of the probitics to be recognized as function food components were mentioned by Ouwehand *et al.* (1999).

Bezkorovainy (2001) investigated the survival of bifidobacteria and lactobacillus during their passage through the human gut, when administrated in fermented milk products, and the important aspect of this issue is to examine the various determinants and factors that allow for probiotic passage through the gut and enhancement of their metabolic activity. The most promising approach for enhancing the role of endogenous probiotic organisms in the gut is the use of prebiotics, which are simple, naturally occurring or synthetic sugars that are normally indigestible in the human gut but they are used by certain colonic bacteria, especially bifidobacteria, as a carbon source for growth and metabolism. Finally, he concluded that, perhaps probiotics in combination with prebiotics may become an important means of preventing and treating disease.

3. Application of probiotics

Hamilton-Miller (2004) on his review confirmed that today, probiotics are familiar to the public as the components of bioyoghurt and dietary supplements, are widely available, and extensively purchased. In 1997, Europeans spent the equivalent of almost $900 million on probiotic yoghurt and milk, and the market is growing rapidly.

The review also suggested that three problems common in the elderly, namely: undernutrition, constipation, and the decline in efficiency of immune system, may all be beneficially affected by appropriate probiotics organisms. Gill and Guarner (2004) showed that bacteria used as starter culture in yogurt (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* subspecies *bulgaricus*) improve lactose digestion. The benefit is due to the presence of microbial β- galactosidase (lactase) in the bacteria. A large number of human studies in which consumption of fresh yogurt (with live yogurt culture) was compared with consumption of a pasteurized product (with heat killed bacteria), demonstrated better lactose digestion and absorption in subjects that consumed yogurt with life cultures as well as the reduction of gastrointestinal symptoms. Two *Lactobacillus brevis* strains ATCC 8287 and ATCC 14869 were evaluated for their applicability as putative probiotics in dairy products by Ronka *et al.*, (2003) and they found that although the above strains were not suitable for fermenting yogurt, but they had no negative effects on yogurt taste, outlook, preservation. Their study also showed that *L.brevis* ATCC 8287, being able to survive in the gastrointestinal tract and was shown to strongly inhibit *Bacillus cereus* and to some extent also the growth of *Staphylococcus aureus* and other harmful microorganisms, while Koga *et al.*, (1998) showed the ability of these bacteria to be antagonistic toward *Vibrio cholerae*.

Ryan *et al.* (1996) indicated that Lacticin 3147 is a broad spectrum bacteriocin produced by a *Lactococcus lactis* strain that was originally isolated from an Irish Kefir grain has a very broad spectrum of action that includes all Gram-positive bacteria tested including food pathogens such as *Listeria monocytogenes* and *Staphylococcus aureus* and food spoilage microorganisms such as *Clostridium tyrobutyricum*.The activity of Lacticin 3147 suggested that this bacteriocin has potential in a wide range of applications spanning food safety to use in the treatment or prevention of human and animal infection. *Lactobacillus acidophilus* NCFM is a probiotic strain available in conventional foods (milk, yogurt, and Toddler formula) and dietary supplements. Its commercial availability in the United States since the mid-1970s is predicated on its safety, its amenability to commercial manipulation, and its biochemical and physiological attributes presumed to be important to human probiotic functionality. Sanders and Klaenhammer (2001), on their review about this strain concluded that antagonistic activity was produced by NCFM against foodborne disease agents *Staphylococcus aureus*, *Salmonella typhimurium*, enteropathogenic *Escherichia coli*, and *Clostridium perfringens* . Also they showed that *in vitro* NCFM can adhere to human cells, but the fate of NCFM inside the human intestine has not been assessed. For lactose intolerance they indicated that studies showed that not all uses of NCFM resulted in an improved digestion of lactose. However, given in adequately high levels, some symptom relief and improved digestion of lactose may occur in lactose maldigesters when they consume NCFM. Presumably, these effects result from the bacterium ability to metabolize lactose during digestion and transit it through the gastrointestinal tract.

Timmerman *et al.* (2004) carried out a literature review to make comparison of functionality and efficacy between three groups of probiotis, monostrain (containing one strain of a certain species), multistrain (containing more than one strain of the same species), and multispecies (containing strains that belong to one or preferentially more genera) They concluded that multispecies probiotics were superior in treating antibiotic-associated diarrhea in children, improvement of mortality broilers, better protection against *Salmonella typhimurium* infection in mice, and many other cases. Finally they emphasized that strains used in multistrain and multispecies probiotics should be compatible or, preferably, synergistic and their use should be encouraged.

Elisabeth *et al.* (2005) showed that during co-culture of *Lactobacillus* (five strains) or *Lactococcus* (two strains) with *Bacillus cereus* in milk, organic acids and other potentially antimicrobial metabolites are produced. Lactic acid was produced at very different rates by the lactic acid bacteria and the final concentrations varied much, however, the crucial point of rapid pH reduction during the initial hours of fermentation coincides with lactic acid production. Moderate amounts of acetic acid were produced during fermentation and the final concentrations were much smaller compared to lactic acid. According to their experiments, production of diacetyl, carbon dioxide and ethanol was considered too small to contribute to inhibition of *B. cereus*. Their conclusion was the strains that produced lactic acid fastest inhibited *B. cereus* best. The inhibititing characteristics of lactic acid bacteria on shiga toxin-producing *Escherichia coli* (STEC) O157:H7 (Three strains, clinically isolated) were investigated by Ogawaa *et al.* (2001). Their data suggested that the bactericidal effect of *Lactobacillus* on STEC depends on its lactic acid production and pH reductive effect.

During the past decade, oxidative stress and antioxidative potency have been revealed as the key points in molecular regulation of cellular stress responses (Demple *et al.,* 1999). Oxidative stress occurs when abnormally high levels of reactive oxygen species (ROS) are generated, resulting in DNA, protein and lipid damage. Two antioxidative strains tentatively identified as *Lactobacillus fermentum*, E-3 and E-18, were isolated from intestinal microflora of a healthy Estonian child and studied by Kullisaar *et al.* (2002). They examined the resistance of the above mentioned two strains to the different unhealthy milieu of ROS, and they compared the survival of E-3 and E-18 both with the non- antioxidative strain of *L.fermentum* E-338-1-1(isolated from another healthy Estonian child) and with the antioxidative strains *Salmonella typhimurium*. Their major findings were as follows: the strains E-3 and E-18 (compared with the non-antioxidative strain E338-1-1) survived longer in 0.4 mM hydrogen peroxide milieu, possessed the ability to multiply in a medium containing abundant superoxide radicals, and had increased resistance to hydroxyl radicals. In the presence of highly damaging hydroxyl radicals, their antioxidative lactobacilli survived for 34 minutes. This is quite impressive fact considering the data that a highly ROS resistant *S.typhimurium* was able to survive in the presence of hydroxyl radicals only twice longer than their antioxidative lactobacilli strains. Finally their opinion that such significant antimicrobial activity combined with antioxidative properties may serve as defensive principles in the intestinal microbial ecosystem and overcome exo-and endogenous oxidative stress.

Gill and Guarner (2004) reported that the balance between bifidobacteria and clostridia appears to be the key factor in determining predisposition to allergies. Lower incidence of allergies in breast-fed infants compared with formula-fed infants, is associated with higher counts of bifidobacteria in their flora; formula-fed infants are known to have more clostridia. Several epidimemiological and experimental studies have indicated that stimulation of the immune system by certain microbes or microbial products may be effective in the prevention and management of allergic diseases. Bezkorovainy (2001) admitted that the large number of bifidobacteria flora was observed in breast-fed infants, who show a greater resistance to various infectious diseases than did bottle-fed infants.

The results of the study carried out by Psomas *et al.* (2003) indicated that yeast strains *Saccharomyces cerevisica* KK1 (isolated from infant feces) and *S. cerevisica* 832 (isolated from the traditional Greek Feta cheese), along with *S. boulardii* (isolated from the commercial yeast product Ultra Levure) , were able to assimilate cholesterol ($\geq$ 83.4 %) *in vitro* after 24 hours of incubation at 37 C°. Moreover, the strain *Isaatchenkia orientalis* KK5.Y.1° (isolated from infant feces),exhibited comparable to the above mentioned yeast strains ability to assimilate cholesterol but the ability was developed after 48 hours of growth at 37°C.

Furthermore, the above mentioned yeast isolates could tolerate low pH levels, gastric juice and bile concentrations typically found in the gastrointestinal tract of humans. Duggan *et al.* (2002) indicated that the non-pathogenic yeast *S.boulardii*, has been used in both animal studies and clinical trials as probiotic food.

Wollowski *et al.* (2001) mentioned that in the beginning of the twentieth century, the Russian Nobel prize winner Elie Metchnikoff observed high life expectancy in Bulgarian persons who ate large amounts of fermented milk products. One hundred years later, the consumption of fermented milk products is still associated with several types of human health benefits. In addition to favorable effects against diseases caused by an imbalance of the gut microflora, several experimental observations have indicated a potential protective effect of lactic acid bacteria against the development of colon tumors. According to their study they concluded that colon cancer is due to somatic mutations occurring during lifetime of an individual, could be retarded or prevented by preventing these mutations. Lactic acid bacteria and prebiotics have been shown to deactivate genotoxic carcinogens. In model system *in vitro* they have shown to prevent mutation, DNA damage has prevented and chemopreventive system may be stimulated *in vivo* in colon tissues. From a mechanistic point of view, lactic acid bacteria offer potential as chemoprotective agents.

According to the opinion of Marteau *et al.* (2001) that the proven medical indications of probiotics in dairy products for gastrointestinal disturbances are to replace milk with yogurt in subjects with lactose intolerance and use fermented milk containing *Lactobacillus rhamnosus* GG to shorten the duration of the diarrhea during rotavirus enteritis in children.

The possibility of improving nutritional benefits of a traditional semi-hard goat cheese by adding probiotic species such as *Bifidobacterium lactis* and *Lactobacillus acidophilus* was assessed by Gomes and Malcata (1998). Their results showed that the starter composed of *Bifidobacterium lactis* and *L. acidophilus* could be used for the successful manufacture of a goat cheese with good flavor and texture characteristics. Survival of the probiotic species was dependent on the physiochemical characteristics of the cheese, but final numbers were still above the recommended threshold for probiotic effect.

Otes and Cagindi (2003) indicated that kefir grains are the mixture of beneficial bacteria and yeast with a polysaccharide matrix and kefir which is made from the fermentation of milk by kefir grains and kefir cultures used for the treatment or control of several diseases for many years in Russia. Kefir as probiotic, kefir consumption, kefir process, its chemical and nutritional composition and its health benefits are all reviewed in the same article.

A study to evaluate the viability of the probiotic strains *Lactobacillus casei* subspecies *rhamnosus* and *Bifidobacterium infantis* in starter milk was done by Maldonado *et al.* (2003) using time and temperature as variables, as well as to evaluate the viability of these micro-organisms over other bacteria normally found in milk. Their results showed that the survival count of the above probiotic bacteria is suitable for achieving positive health results at the time that they are prepared or after they have remained in the fridge for one hour.

Lee and Salminen (1995) indicated that traditional probiotic dairy strains of lactic acid bacteria have long history of safe use. There is considerable interest in extending the range of foods incorporating probiotic organisms from dairy foods to infant formulae, baby foods, fruit juice based products, and cereal based products and pharmaceuticals.

A study by Vinderola *et al.* (2000) showed that Argentinian Fresco Cheese can be used as an adequate carrier of probiotic bacteria (Bifidobacteria, *Lactobacillus acidophilus, Lactobacillus casie*).A combination of the above bacteria showed satisfactory viable counts (higher than 10^6 cfu/g) up to 60 days of refrigerated storage, and when the probiotic cultures were added to the cheese, they demonstrated an excellent ability to remain viable up to 3 hours in a cheese homogenate at pH 3.

Murooka *et al.* (2005) indicated that *Propionibacterium freudenreichii* is a commercially important bacterium that is

Table (1): The probiotic bacteria and their effect on health improvement of human body.

Agents	Treatment	Mode of action	Condition	Reference
S. thermophilus *L. delbrueckii* Supspecies *bulgaricus*	Improve lactose digestion	The presence of microbial lactase in the bacteria	*In vivo*	Gill and Guarner (2004)
L. brevis strain ATCC 8287	Inhibit *B.Cereus* *Staph. Aureus* *V.cholera*	Antagonistic activity	*In vitro*	Ronka *et al.* (2003) Koga *et al.* (1998)
Lactococcus lactis	Very broad spectrum against G+ bacteria	Produce Lacticin 3147 as a broad spectrum bacteriocin	*In vivo*	Ryan *et al.* (1996)
L. acidophilus NCFM	Inhibited food borne disease agent	Antagonistic activity	*In vitro, in animal studies, and in humans.*	Sanders and Klaenhammer (2001)
Lactobacillus (five strains) Lactococcus (two strains)	Inhibition of *B. cereus*	Production of potentially antimicrobial metabolites	*In vitro*	Elisabeth *et al.* (2005).
Lactic acid bacteria	Inhibition of shiga toxin- producing *E.coli*(STEC) 0157:H7	Lactic acid production and pH reductive effect.	*In vitro*	Ogawaa *et al.* (2001)
L. fermentum E-3 and E-18	Antimicrobial activity and antioxidative activity	antioxidative enzymes	*In vitro*	Kullisaar *et al.* (2002)
Bifidobacteria	Management of allergic disease.	Stimulation of the immune system by the bacteria or by their products.	*In vivo*	Gill and Guarner (2006)
Yeast strains *S. cerevisica* KKI and 832 *S. boulardii*	Ability to assimilate cholesterol	Cholesterol was assimilated into the yeast cells	*In vitro*	Psomas *et al.* (2003)
B. lactis *L. acidophilus*	Improving nutritional value of a traditional semi-hard goat cheese	Adding probiotic species to the cheese	*In vivo*	Gomes and Malcata (1998)
Complex mixture of bacteria and yeasts as well as probiotic bacteria found in Kefir	Antimicrobial activity against a wide variety of G+ and G- bacteria and against some fungi	Fermentation of milk with kefir grains and mother cultures prepared from grains.	*In vitro*	Otes and Cagindi (2003)
Ingestion of viable probiotics or prebiotics	Anticarcinogenic effect	These factors lead to a reduced load of genotoxic agents in the gut and to an increased production of agents that deactivate toxic components	Shown experimentally in animals with use of the rat colon carcinogen	Wollowski *et al.* (2001)
L. rhamnosus GG	Shorten the duration of the diarrhea during rotavirus enteritis in children	The effect was more pronounced on rotavirus specific immunoglobulin A response	*In vivo*	Marteau *et al.* (2001)

used in the production of cheese, cobalamin(vitamin B_{12}) and propionic acid. Also they mentioned that metabolic engineering using genetically improved strains will make the fermentation process more economical and also enhance the quality of the products. Finally they reported on their review the advancement of genetic engineering in *Propionibacterium freudenreichii* in recent years, covering the molecular aspects of the formation of tetrapyrrole compounds and vitamin B_{12}.

A review by Roy (2005) aimed to address the technological factors involved in the use of bifidobacteria as probiotics, and according to his conclusion that the functional cheese may be more effective than yoghurt-like product to deliver probiotic bacteria to the intestinal tract. It is possible to include bifidobacteria in the traditional production of cheddar cheese and fresh cheeses. It is also possible to produce Swiss-type cheese after slight modification. Bifidobacteria do not deteriorate the product, enhance the development of flavors in fresh cheeses and give to the products a functional character.

The production of yoghurt with probiotic bacteria isolated from eight infants living in Amman, Jordan was investigated by Haddadin *et al.* (2004), three promising isolates were identified as *Lactobacillus gasseri, Lactobacillus casei,* and *Bifidobacterium infatis.*Their results showed that yoghurt containing counts of $\geq 1.0*10^8$ cfu ml^{-1} of the individual probiotics and high counts of the traditional species from yoghurt were produced, and storage trials at 4°C showed that the viability of the probiotic cultures was retained over 15 days.

A study was carried out by Mortazavian *et al.* (2007) to identify the best refrigerated storage temperature on the viability of *Lactobacillus acidophilus and Bifidobacterium lactis* BB-12 in ABY probiotic yoghurt. Their results demonstrated that a refrigeration temperature of 2°C led to the highest viability of *L.acidophilus* throughout the 20 days of refrigerated storage, whereas the highest viability for *B lactis* throughout the 20 days of storage time occurred when yoghurt was kept at 8°C.

4. IDENTIFICATION AND IFFERENTIATION OF PROBIOTICS

The number and type of lactobacillus strains in probiotic feed or food products for sale in Europe comparing with the information stated on the product labels was accomplished by Coeuret *et al.* (2004). Lactobacilli were recovered using a recently validated method, developed as part of the European community project SMT4 CT98-2235 for the official control of probiotic microorganisms used as feed additives. Polymerase chain reaction (PCR)-based methods were used to assess the accuracy of labeling with regard to genus and species, and pulsed-field gel electrophoresis (PFGE) was used to identify strains.

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The use of Polymers Chain Reaction – Denaturating Gradient Gel Electrophoresis (PCR-DGGE) technique in identifying the microorganisms present in commercial probiotic yoghurt and lyophilized products was evaluated by Fasoli *et al.* (2003). Identification was achieved comparing the PCR-DGGE patterns obtained from the analysed products with the ladder bands. Bands from members of the same species showed the same migration distance in denaturating gel, hence supporting the identificative value of the method. Finally they suggested that PCR-DGGE turned out to be an appropriate culture-independent approach for a rapid detection of the predominant species in mixed probiotic cultures.

The application and the evaluation of the culture- independent analysis of probiotic products by denaturating gradient gel electrophoresis in a collection of 10 probiotic products, including four dairy products were carried out by Temmerman *et al.* (2003). The culture- independent approach involved extraction of total bacterial DNA directly from the product, PCR amplification of the V3 regions of the 16S ribosomal DNA, and separation of the amplicons on a denaturating gradient gel. Digital capturing and processing of denaturating gradient gel electrophoresis (DGGE) band patterns allowed direct identification of the amplicons at the species level. Their study clearly demonstrated that DGGE is a fast, reliable, and reproducible culture-independent approach for analysis propiotic products and that it has greater detection and identification potential than conventional culture-dependent analysis. A potential drawback of the DGGE approach is that no information concerning the level of bacterial viability in probiotic products is obtained, implying that culture-dependent analysis may still add valuable information The major advantages of the DNA-based typing method lie in their discriminatory power and in their universal applicability (Holzapfel *et al.*, 2001).

Ward and Roy (2005) showed that the probiotic effect of Bifidobacterium explains popularity of these bacteria in different commercial products. A right identification of these microorganisms is important for the producers of probiotic products. To help microbiologists, researchers and producers to do the right identification and characterization of bacteria including Bifidobacterium some molecular methods have been developed. A review of these methods was described in their paper.

Conclusion

The potential benefits of the use of probiotics therapy in dairy products promise to be almost limitless. The review of the literature shows that many health common problems could be retarded or prevented by consuming dairy products in combination with probiotics microorganisms and this can be clearly shown by Table 1. Modern molecular typing techniques such as PFGE and PCR will help to solve the complexity of microorganism identification to provide the transparency required by the consumer, responsible scientists, industry and legislative.

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(مقالة مرجعية)

البكتريا الداعمة للحيوية كأغذية وظيفية في المنتجات اللبنية

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تلقي الورقة العلمية الضوء على مدى فائدة الأحياء الدقيقة على صحة الإنسان عند وجودها في منتجات الألبان بالكمية الكافية وخاصة فيما يخص التوازن الميكروبي في الجهاز الهضمي ومنع الأحياء المجهرية الضارة من مهاجمة هذا الجهاز وتأثيرها الايجابي للأشخاص المصابين بصعوبة هضم سكر اللاكتوز مع دراسة احتمالية الفائدة من هذه الأحياء في منع أمراض السرطان وتقليل الكوليسترول في الدم.

إضافة إلى ذلك فإن هذه الورقة قد تطرقت إلى أهم الطرق الحديثة المستعملة في التعرف والتفريق بين أنواع الأحياء المجهرية المستعملة في هذا المجال.

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