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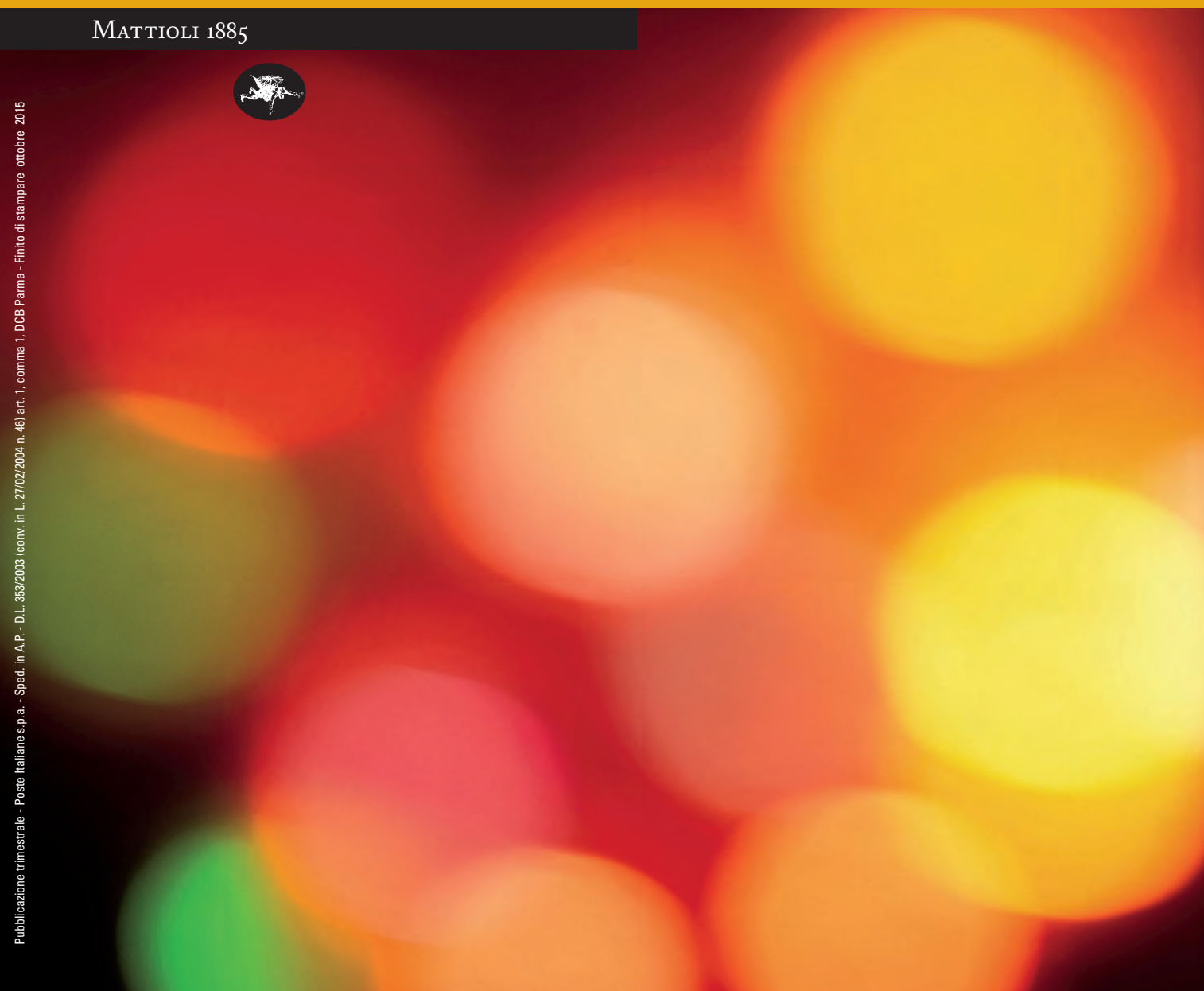

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PROGRESS IN NUTRITION

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Mattioli 1885
srl- Strada di Lodesana 649/sx
Loc. Vaio - 43036 Fidenza (Parma)
tel 0524/530383
fax 0524/82537
www.mattioli1885.com

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Review

Parmigiano Reggiano cheese: general and metabolic/nutritional aspects from tradition to recent evidences

L. Arsenio[1], S. Bernasconi[2], F. Cioni[3], M. Nocetti[4]

[1]Direttore Responsabile Progress in Nutrition, [2]Professore Ordinario di Pediatria - già Direttore Cliniche Pediatriche, Università di Modena-Reggio Emilia e Parma, [3]SSD Trattamento Intensivo del Diabete e delle sue complicanze, Azienda Ospedaliera Universitaria, Parma, [4]Responsabile Servizio Ricerca e Sviluppo, Consorzio Formaggio Parmigiano Reggiano

Summary. Parmigiano Reggiano cheese is the oldest italian Protected Designation of Origin product, in accordance with Regulation (EU) No. 1151/2012 of the European Parliament and of the Council of 21 November 2012 on quality schemes for agricultural products and foodstuffs. It is produced in a strictly delimited territory, which includes the provinces of Parma, Reggio Emilia, Modena, Mantua to the right of the River Po and Bologna to the left of the River Reno; also the milk must come exclusively from this strictly delimited territory. It is a hard-textured, semi-fat cheese cooked and matured slowly. Additives are not allowed. The cheese must be matured for a minimum of 12 months, with significant variations between the fresh product and the aged one, especially in terms of protein digestibility. Cheese proteins essentially consist of casein, with traces of lactoglobulin and lactalbumin: the protein intake is approx. 33.0%, and the biological value of this nutritious substance is high. Parmigiano Reggiano is a semi-fat cheese with a fat content (29.7%), lower than the protein content. Conjugated isomers of linoleic acid are found in Parmigiano Reggiano cheese in quantities of approx. 3 mg/g of lipids. The amount of vitamins present in Parmigiano Reggiano cheese, while varying in relation to aging, is sufficient to cover a high percentage of the daily vitamin requirement in children and adults. A 100 g serving of Parmigiano Reggiano cheese covers also the entire daily requirement of calcium and phosphorus for an adult person with an extremely favourable calcium/phosphorus ratio. Lactose content ranges from below limit of detection to 0.39 mg/100 gr of cheese, thus allowing to classify Parmigiano Reggiano cheese among the "lactose-free" cheeses, according to the European Commission for the formulation of infant milk criteria. The absence of lactose means that it can be administered to patients with lactase deficiency. The energy value is, in any case, high (402 kcal per 100 g), so Parmigiano Reggiano cheese has not to be considered as a supplement to a normal meal, but a food in its own right. Thanks to its composition and its richness in protein substrates, in calcium, in trace nutrients and bioactive peptides, together with its excellent lipid profile, Parmigiano Reggiano cheese can be considered a "naturally" functional food, ideal for use in every age of life and in several disorders such as diabetes, ostheoporosis and dyslipidaemias.

Key words: Parmigiano Reggiano cheese, metabolic/nutritional aspects, PDO-certified

Introduction

Parmigiano Reggiano cheese is the oldest Italian PDO-certified cheese, which saw the production of 3 million wheels in 2014. This is equivalent to around 120,000 tons (1). Its production is governed by strict specifications, the last update of which has been in force since 29 August 2011 (2) and takes place exclusively in cheese factories located in a strictly delimited territory, the so-called "typical area", which includes the provinces of Parma, Reggio Emilia, Modena, Mantua to the right of the River Po and Bologna to the left of the River Reno; also the milk must come exclusively from this strictly delimited territory. This area provides the elements that make the production of this typical cheese possible: soil composition, forage types, specially selected cows and a tradition handed down by expert personnel.

General aspects

Parmigiano Reggiano cheese is a semi-fat, hard-textured cheese cooked and matured slowly. It is produced using raw cows' milk from livestock whose diet consists of at least 50% forage mainly produced in farm's own land and typical area because it is only within this area that the typical mesophilic lactic acid bacteria are found.

Traditionally, production used to stop during the winter season (running from St Martin's Day on 11 November to St Joseph's Day on 19 March): today production lasts all year long and is divided into three batches (1st batch January-April, 2nd batch May-August, 3rd batch September-December) (2).

Sixteen litres of milk are needed to make 1 kg of Parmigiano Reggiano cheese and the milk used is that from the previous evening's milking, partially skimmed, plus that from the next morning's milking. The milk must not be subjected to heat treatments prior to the cheese-making process and the use of additives is prohibited. The milk is delivered to the cheese factory within two hours of the end of each milking and while being kept in the cowshed, the temperature must never drop below 18°C, in order to avoid damage to the mesophilic lactic acid bacteria. Coagulation is obtained through the use of calf rennet after the adding of autochthonous whey starter, which brings about the complete transformation of the lactose into lactic acid within the first 72 hours of the cheese's life; after a period in brine of about 20 days, the aging process begins (minimum 12 months) during which the proteins are partially hydrolysed with the release of proteins of low molecular weight responsible for the aroma and endowed with important nutritional properties.

A brief history of Parmigiano Reggiano cheese

There is some controversy regarding the specific place and time in which Parmigiano Reggiano cheese was first produced. According to reliable historical evidence, a cheese with the same granular structure, straw-colour and fragrant aroma first appeared on the scene about half way through the 11th century in the mid-valley of the River Enza, straddling the current border between the provinces of Parma and Reggio Emilia, after which it spread towards the plain. In actual fact, the Benedictine monks living in the Po Valley, along the Via Emilia road, which has connected Milan with the Adriatic coast ever since Roman times, made a valuable contribution to the creation of this cheese, inasmuch as they played a fundamental role in the reclamation of the land (which had become marshy after the fall of the Roman Empire), in the development of crops that were indispensable for cattle forage and in the breeding of livestock. Thanks to the availability of large quantities of milk and the need to store it in one way or another, for the winter period, during which the cattle did not produce it due to lack of grass, a number of hard-textured cheeses were developed, among which Parmigiano Reggiano, already mentioned (under the title "caseus parmesanus") in a document regulating a bequest dating back to 1254 and filed in the State Archives of Genoa. The most famous quotation is, however, that of Boccaccio's Decameron, written around 1350, in which Maso describes the marvels of Bengodi to the gullible Calandrino, saying "and on a mountain, all of grated Parmigiano Reggiano cheese, dwell folk that do nought else but make macaroni and ravioli…" (3-5).

Characteristics of Parmigiano Reggiano cheese

Parmigiano Reggiano cheese is produced in cylindrical wheels that have a slightly convex heel between 20 and 26 cm in height, flat faces featuring slightly raised edges and a diameter of between 35 and 45 cm. The average weight of a wheel is around 40 kg at 12 months. The rind is around 6 mm thick and has a natural straw-coloured exterior. The cheese itself, which is a light-straw colour, has a texture made of tiny structured granules that when fractured breaks into scale-like fragments.

The cheese must be matured for a minimum of 12 months, but is most frequently used around 24 months and longer: It is important to remember that the "Parmigiano Reggiano" Protected Designation of Origin is only extended to the grated product if it is obtained from whole PDO certified cheeses, on condition that the grating process is carried out within the area of production of the cheese and the packaging is carried out immediately after without any kind of treatment and without adding any substances designed to change the preservability and original organoleptic characteristics of the product (2).

Parmigiano Reggiano cheese safety

Parmigiano Reggiano cheese has been made in substantially the same way for eight centuries, while the production process in its current form has been detailed and regulated by the Consortium since 1956.

While there are concerns about the safety of some soft and semi-hard raw milk cheeses, specific independent epidemiological studies and indeed time itself have clearly established that hard cheeses such as Parmigiano Reggiano are safe and, after decades (centuries, even…) of being eaten worldwide, no cases of any adverse effects have been reported.

What makes Parmigiano Reggiano cheese safe is:

- the synergy between the antimicrobial enzyme systems in raw milk and mesophilic milk flora (non-starter lactic acid bacteria - NSLAB),
- the addition of a natural whey starter (a culture of thermophilic lactic acid bacteria – SLAB) to the milk vat (approximately 3% v/v), and
- the subsequent fermentation of sugar substrates that lead to a sudden drop (5.1) in the pH during the first hours of the cheese making process,
- the high (55°C) temperature used for cooking the curds and the long time (at least 3 hours) for which the cheese is heated,
- the brine salting, responsible for the gradual decrease of water activity down to 0.90 and salt content in relation to water totalling around 4.2%,
- the consistency and thickness of the rind, which naturally protects the cheese from external agents and
- the long maturation (at least 12, usually 24 and often more than 30 months).

Every single cheese factory guarantees the application of appropriate productive practices by its own HACCP plan managed according to Regulation (EC) No. 852/2004.

The use of predictive microbiology models to study and state safety and hygiene parameters of foods is recognized and accepted at international level. A number of different predictive mathematical models have been used showing that, even if milk is raw at the beginning of the production process, the process itself guarantees heat treatment equivalent to pasteurization.

In order to validate the theoretical data described above from an empirical standpoint and to verify the effective ability of the Parmigiano Reggiano cheese-making process to reduce the potential presence of food-borne pathogens, various challenge tests have been carried out in the last years showing that this particular production process can eradicate (5-7 log reductions) pathogenic microbes potentially contained in milk, such as Salmonella spp., *Listeria monocytogenes*, E*scherichia coli* O157:H7, *Staphylococcus aureus* enterotoxigenic and even *Mycobacterium avium paratuberculosis* (CFPR, data on file).

Nutritional aspects

Parmigiano Reggiano cheese is one of the best cheeses from a nutritional perspective as it has characteristics that are particularly interesting for the human diet (Tab. 1).

The high protein intake (on average 32.4%) that Parmigiano Reggiano cheese can supply to the human diet is undoubtedly an important characteristic, asso-

Table 1. Parmigiano Reggiano cheese composition (100 g)

Water	31.4 g
Protein	32.4 g
Free aminoacids on total protein[1]	23.3%
Energy[2]	402 kcal 1671 kJ
Fat	29.7 g
Satured fatty acids	19.6 g
Monounsaturated fatty acids	9.3 g
Polyunsatured fatty acids	0.8 g
Fat in dry matter	43.3 % d.m.
Carbohydrates	0 g
of which sugars	0 g
Lactose	<1 mg
Fiber	0 g
Salt[3]	1.6 g
Lactic acid	1.6 g
Calcium	1155 mg
Phosphorus	691 mg
Sodium	650 mg
Potassium	100 mg
Magnesium	43 mg
Iron	0.2 mg
Copper	0.83 mg
Zinc	4 mg
Cholesterol	83 mg
Vitamin A	430 µg
Thiamine (Vitamin B1)	0.03 mg
Riboflavin (Vitamin B2)	0.35 mg
Vitamin B6	0.06 mg
Vitamin B12	1.7 µg
Vitamin C	0 mg
Niacin (Vitamin PP/B3)	0.06 mg
Vitamin E	0.55 mg
Vitamin K	1.6 µg
Pantothenic Acid (Vitamin B5)	0.320 mg
Choline	40 mg
Biotin	23 µg

Values, expressed per 100 g, are obtained from random samples of PDO-certified Parmigiano Reggiano cheese by the Consortium, not attributable to a specific manufacturer, production lot or geographical area.

[1] The percentage of free amino acids is referred to Parmigiano Reggiano 24 months ripened.

[2] Energy value is evaluated by the conversion coefficients as reported in All. XIV, EU Reg. 1169/2011 (11)

[3] Salt: salt equivalent content is evaluated by the formula Salt = sodium x 2.5 as reported in EU Reg. 1169/2011, Ann. 1. (11)

ciated moreover with the high biological value of this protein (Tab. 2) that is characterized by a very high utilization coefficient, having an index of 93, versus 69 for beef, 62 for meat of veal, 50 for bread (6-9). The proteolysis that takes place during the aging process gives rise to lighter polypeptide chains and to an amount of free amino acids equivalent to 7.5% of the edible part, much higher than that found in other types of cheese. This characterises the product from a nutritional perspective because these compounds can be assimilated rapidly and absorbed without the need for any digestive processes, making this a "digestion facilitated" food (an hectogram of this cheese is digested in 40 minutes against more than three hours of an hectogram of beef), that is very useful in the two extremes of age (children and elderly) (6, 7) as in sports. So, Parmigiano Reggiano cheese simultaneously contains an amount of whole casein, peptides of various lengths and free amino acids. During digestion, these three protein components have different speeds of absorption: slow, accelerated and fast, respectively, enabling modulation of the absorption of the protein substrate and a better use of the same in the diet (9). The bioactive peptides, which are released

Table 2. Amino acid composition of Parmigiano Reggiano cheese (6)

Amino acid	mg/100 g of product
glutamic acid	6030
proline	3560
leucine*	2880
lysine*	2460
aspartic acid	2260
serine	1860
tyrosine	1750
phenylalanine*	1610
valine*	1360
isoleucine*	1280
threonine*	1100
methionine*	1030
alanine	940
histidine*	920
arginine	810
glycine	700
tryptophan*	320
cysteine	200

** Essential amino acids*

Table 3. Overview of the main bioactive peptides and their physiological role (6)

Bioactive peptides	Precursor protein	Bioactivity
casomorphins	α-β-casein	Opioid agonist
α-lactorphin	α-lactalbumin	Opioid agonist and ACE-inhibitory
β-lactorphin	β-lactalbumin	Opioid agonist and ACE-inhibitory
lactoferroxins	lactoferrins	Opioid antagonist
casoxin C	κ-casein	Opioid antagonist
casoxin D	αS1-casein	Opioid antagonist
casokinins	α-β-casein	ACE-inhibitory and immunoregulatory
lactokinins	α-lactalbumin β-lactoglobulin seroalbumin	ACE-inhibitory
immunopeptides	α-β-casein α-lactalbumin β-lactoglobulin	Immunoregulatory
lactoferricin B	lactoferrin	Immunoregulatory and antimicrobial
casocidin	αs2-casein	Antimicrobial
isracidin	αs1-casein	Antimicrobial
casoplatelins	κ-casein	Antithrombotic
peptide inhibitorof thrombin	κ-casein	Antithrombotic
peptide inhibitor of thrombin	lactoferrin	Antithrombotic
casein phosphopeptides	αs1-casein αs2-casein β-casein	Bonding and transport of minerals

by the digestion of milk proteins, can exert many regulating effects: the intake of nutrients and the transport of minerals (calcium) in the intestine (phosphopeptides), the transport of amino acids, of intestinal fluid, gastrointestinal motility and the secretion of hormones (insulin, somatostatin) (beta-casomorphins), immunostimulation (fragments of alphaS1 and betacasein), anti-hypertensive effects (casokinins) (Tab. 3) (6-10). Of particular interest, moreover, is the presence of substances, with activities similar to opioids (morphine-like substances) also known as exorphins, which have an analgesic and calming effect, thereby inducing a feeling of well-being (6).

The fat content (on average 29.7%), lower than the protein content (Parmigiano Reggiano is a semi-fat cheese), is extremely precious from a nutritional standpoint (Tab. 4). The modifications of the lipid component during the aging phase release an amount of free fatty acids and facilitate their absorption. The saturated fatty acids are, for the most part, made up of short- and medium-chain fatty acids (from C4 to C10), compounds which are easily absorbable and which supply energy very rapidly, since they follow different utilisation pathways from long-chain fatty acids (8, 9). Among the compounds present in the lipid fraction of Parmigiano Reggiano cheese, it is important to highlight certain antioxidant phospholipids

Table 4. Fat partition

	% of total fat
Saturated fats	65.88*
Monounsaturated fats	31.31*
Polyunsaturated fats	2.81*

** from INRAN (Istituto Nazionale di Ricerca per gli Alimenti e la Nutrizione - National Institute Research Institute for Food and Nutrition)*

(7) such as phosphatidylserine, sphingomyelin and its catabolite sphingosine, conjugated isomers of linoleic acid (CLA), to which a great many beneficial effects are attributed as carcinogenesis inhibitors, antioxidants and modulators of the metabolism and the immune system (10).

Among the vitamins belonging to the B group, B2, B6 and B12 are found in considerable quantities, while among the liposoluble vitamins, vitamin A remains in substantial quantities (8, 9) (Tab. 1). The amount of vitamins present in Parmigiano Reggiano cheese, while varying in relation to the cheese aging process, is sufficient to cover a high percentage of the daily vitamin requirement in children and adults (Tab. 5) (6, 8, 9): a 100 g serving of Parmigiano Reggiano cheese covers over 25% of the adult's vitamin B2 requirement, 54% of the daily vitamin A requirement, and 68% of the vitamin B12 requirement according Regulation EU 1169/2011 (Tab. 5) (11) that are quite similar to 2014 LARN SINU (12), except for some differences for specific population groups, as shown in Table 5.

As far as minerals are concerned (8, 9), Parmigiano Reggiano cheese has a very high calcium content (1155 mg/100 g), which is in the form of lactate and hence, highly available also due to the presence of caseinophosphopeptides (CPP), which are released in the course of proteolytic aging by the casein in the milk and which play a key role in the stimulation of the intestinal absorption of calcium, a primary action for keeping the bones healthy. A 100 g serving of Parmigiano Reggiano cheese covers over the 100% of an adult's calcium requirement and up to 90-100% of that recommended for teenaged and 90% recommended for elderly women (12, 13).

The calcium/phosphorus ratio is extremely favourable, being around 1.7, making it possible to balance the mineral content of other protein foods, which generally contain more phosphorus than calcium. The salt content, which is not negligible (on average 1625 mg), is, in any case, around intermediate levels, between the 860-870 mg of cheeses such as provolone and taleggio, and the 1800 mg of pecorino. The considerable presence of zinc is not to be underestimated: a 100 g serving of the product supplies approximately 40% of the zinc requirement with important antioxidant effects.

The carbohydrate content of Parmigiano Reggiano cheese has been evaluated through high resolution chromatography on a variety of samples differing in terms of area of production and aging, and a lactose content ranging from "below limit of detection" to 0.39 mg/100 g of cheese was found, values that make it possible to class Parmigiano Reggiano among the "lactose-free" cheeses, on the basis of the criteria laid down by the European Commission for the formulation of infant milk (14).

The chromatographic characterisation of the oligosaccharides (highlighted in Figs. 1 and 2) confirmed also the presence in Parmigiano Reggiano cheese of

Table 5. Contribution of a 100 g intake of Parmigiano Reggiano cheese towards the main daily reference intakes referred to the Regulation EU 1169-2011 data (11) and LARN SINU data (12)

Nutrient	Adult (EU 1169-2011)	Adult (LARN-SINU) Male-Female	Adolescent (LARN-SINU) Male-Female
Protein	65%	55%-65%	60%-65%
Calcium	144%	100%-100%	90%-90%
Phosphorus	99%	100%-100%	55%-55%
Magnesium	11%	18%-18%	18%-18%
Zinc	40%	30%-45%	30%-45%
Copper	85%	95%-95%	100%-95%
Vit. B2	25%	22%-30%	22%-30%
Vit. B6	4%	4%-4%	4%-4%
Vit. B12	68%	70%-70%	75%-75%
Vit. A	54%	85%-110%	110%-110%

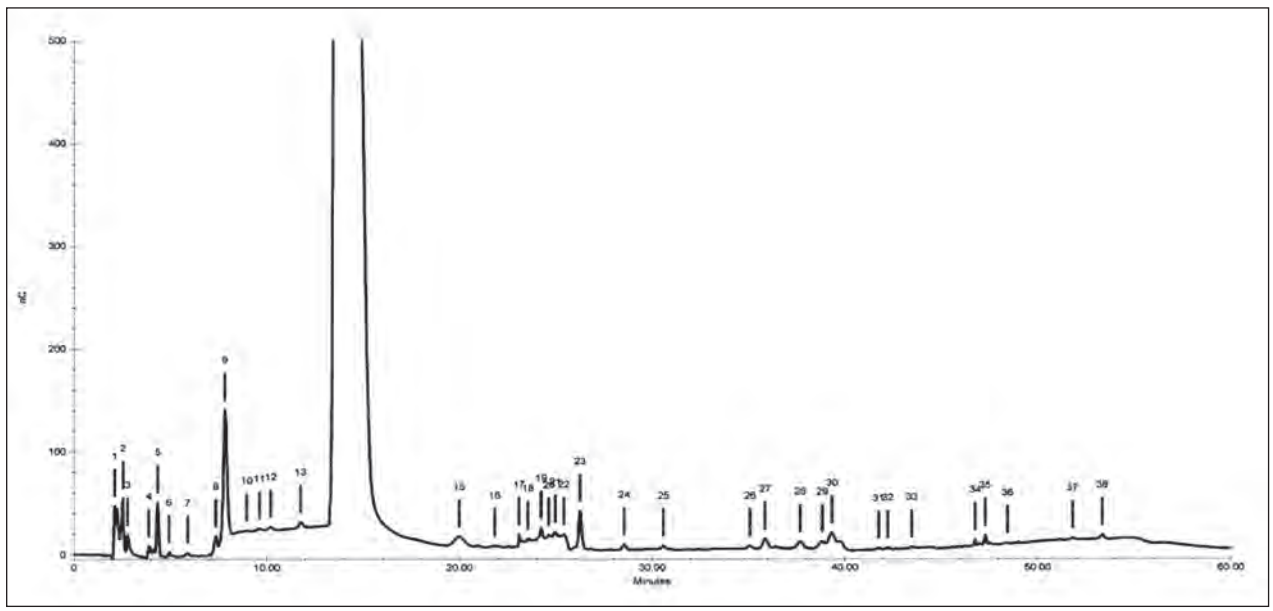

Figure 1. Chromatography of carbohydrates in cow's milk: the highest peak corresponds to the lactose whilst the other peaks to the oligosaccharides.

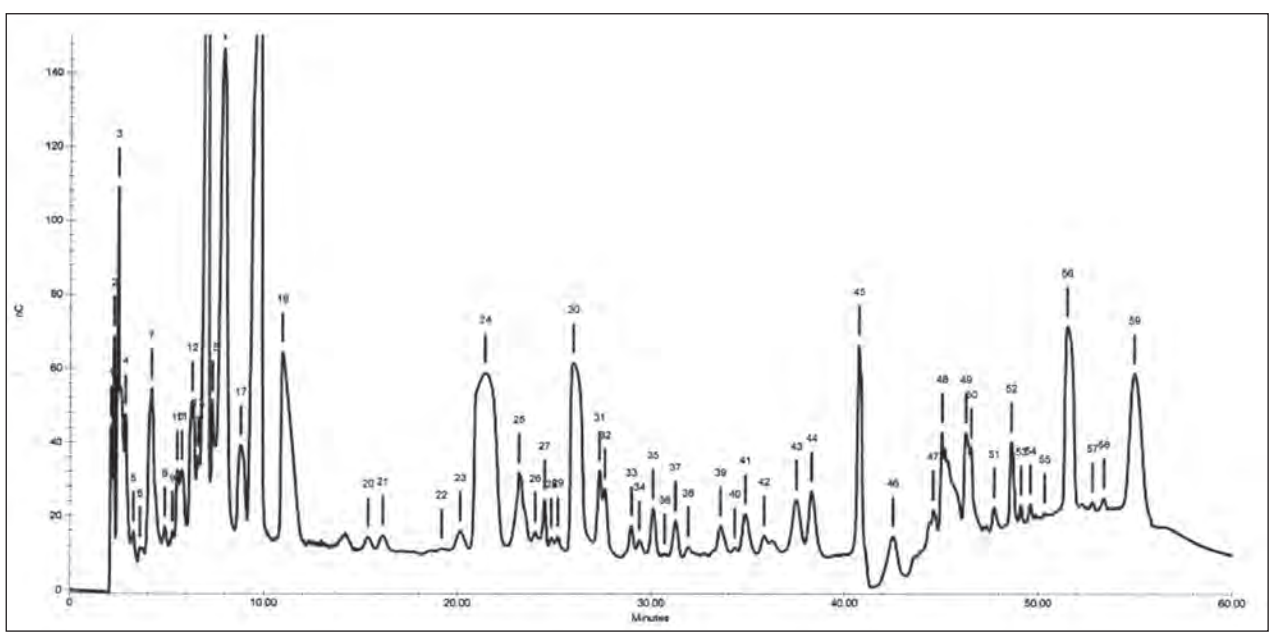

Figure 2. Chromatography of carbohydrates in Parmigiano Reggiano cheese: peak 21 corresponds to the lactose whilst the other peaks to the oligosaccharides.

numerous new oligosaccharide fractions, presumably responsible for the bifidogenic effect of the cheese, already confirmed some considerable time ago by *in vitro* studies, substantially different from those present in the base milk, so, probably, their presence in the finished product is attributable to the fermentation processes (14). This is the biochemical rationale behind the therapeutic use of Parmigiano Reggiano cheese in the treatment of enteritides (15), particularly viral forms (16), also in infants (17).

Use of Parmigiano Reggiano cheese in human nutrition

Parmigiano Reggiano cheese may be used as a topping, as an ingredient in the preparation of complex dishes, as a second course or as an ingredient in snacks (8, 9). When used as a topping in quantities of 10-15 g – the normal amount for a plate of pasta – it supplies approx. 4-5 g of protein, equivalent to the protein intake of 35 g from meat or fish. The amino acids contained in Parmigiano Reggiano cheese, moreover, "complement" the vegetable proteins which have a fairly unbalanced amino acid spectrum, because they are lacking in lysine, an essential amino acid that is particularly abundant in Parmigiano Reggiano cheese. Its use as a topping also improves the organoleptic characteristics and palatability of the dish stimulating the secretion of gastric juices and facilitating the digestive process. Parmigiano Reggiano cheese may be used in the form of slivers as a starter or dessert, or in association with other products. In this case, a portion of approximately 30 g supplies just over 120 kcal and 10-11 g of high biological value protein. The third possible use is to consume it as a meal. In this case, 50-60 g of the product supplies approximately 16 g of protein which is the same amount of protein contained in 100 g or more of fish or meat.

Given, therefore, that 50-60 g of Parmigiano Reggiano cheese is equivalent, from a nutritional standpoint, to 100 g of meat or fish, the quantity of cholesterol consumed using Parmigiano Reggiano cheese as a second course is around 40-45 mg, and therefore, less than that supplied by meat, the protein intake being equal.

The energy value is, in any case, high (402 kcal per 100 g), therefore, Parmigiano Reggiano cheese (like other cheeses) is not to be considered as a supplement to a normal meal, but a food in its own right, the daily intake of which should vary on the basis of the energy requirements of the person and a series of parameters such as, for example, age (usually a higher intake is recommended for infants/children and for the elderly), gender, weight, height, intensity and duration of physical exercise.

In any case, the huge volume of research conducted over the past ten years in the food sector has made it possible to radically correct the guidelines for a correct diet, expressly through the Food Pyramid. A comparison between the first Food Guide Pyramid formulated in the Seventies, and the latest version (18) has revealed an in-depth review of nutritional advice, among which - of particular importance - that relating to the role of milk and its derivatives. These foods have, in fact, gone up the pyramid, passing from the third to the fourth levels (foods not recommended, to be reduced) of the old Pyramid to the second of six levels (recommended foods, to be eaten every day) in the latest Pyramid (Fig. 3) (18).

The question of the relationship between Parmigiano Reggiano cheese and health forms part of the larger context of the connection with milk, dairy products and metabolic and atherosclerotic diseases, which over recent years have been subjected to a thorough review, with the discarding of preconceived and obsolete ideas. According to recent research, not only are these foods not atherogenic, as was believed in the past, but they actually seem to play a protective role. In 494 young people between the ages of 15-18 years, the cardiometabolic risk score was significantly lower in those who drank more milk (19). In a French population, a higher intake of milk and its derivatives, cheese and calcium was associated with a lower incidence at 9 years of metabolic syndrome and fasting hyperglycaemia/Type 2 diabetes: all 3 factors were associated with lower arterial diastolic pressure and a lower BMI; a higher consumption of cheese and calcium was associated with a lower increase in waist circumference and lower triglyceridemia; calcium with a lower arterial systolic pressure and lower triglyceridemia (20). Analogous results as regards dairy products and type 2 diabetes were found in a Chinese population (21). A study, presented at the recent European Congress of EASD 2014, on 26,930 Swedes between the ages of 45 and 74 years (60% women), confirms a protective role towards diabetes: 20% of the participants with high consumption of whole milk and dairy products had a 23% lower risk of Type 2 diabetes and cream, consumed daily in quantities of 30 ml, reduces this risk by 15%, while whole yoghurt reduces it by 20% (at least 180 ml/day). On the other hand, no association was observed between the consumption of light products and the risk of Type 2 diabetes. In conclusion, all

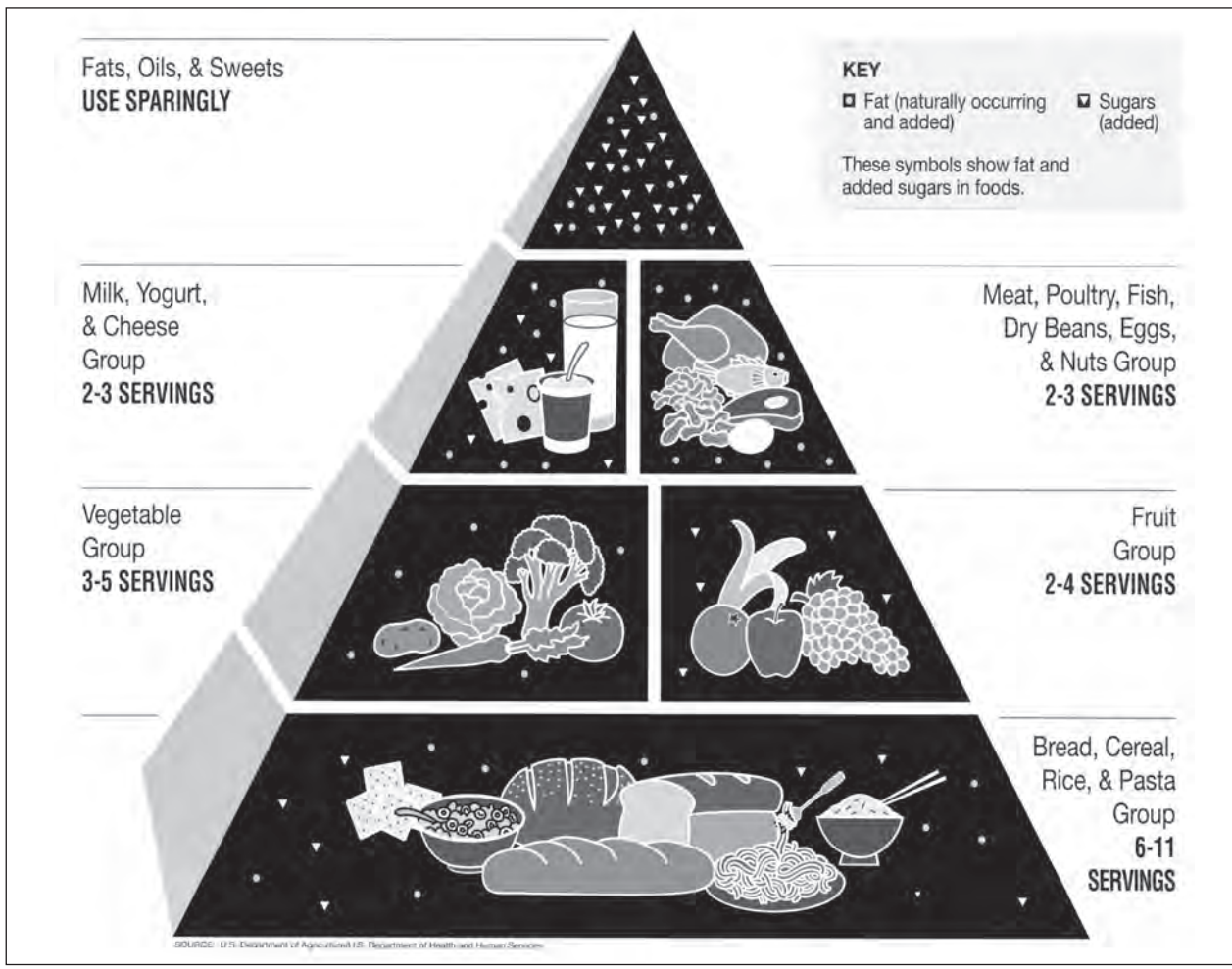

Figure 3. USDA Food Guide Piramid (18)

types of meat irrespective of their fat content, increased the risk of Type 2 diabetes, and indeed lean meats increased it more (24% against 9% in meats with a higher fat content) (22). Other research has confirmed that the consumption of milk and its derivatives is inversely associated with the risk of obesity (23, 24). The mechanism by means of which milk and its derivatives allegedly combat obesity is not very clear and has led to the formulation of a variety of hypotheses, probably due to the fact that it is difficult to identify a single factor, given the great nutritional richness of these foodstuffs. The first factor taken into consideration was the calcium content. The mechanism is allegedly linked to an alteration in the intestinal absorption of fats, with the formation of fatty acid and calcium soaps (25-28). In fact, diets enriched with milk and cheese, are alleged to have brought about a reduced increase in total and LDL blood cholesterol, notwithstanding the high content of saturated fats (identical in all 3 cases and equivalent to 60% of total lipids), due to an increase in the faecal excretion of fats (29). Another hypothesis concerns hormonal interference (oestrogens, cortisol) with calcium (30). Other studies have suggested stimulation of thermogenesis in order to uncouple oxidative phosphorylation (31). Moreover, the protein component of milk was found to be inversely related with adiposity (32-36) and, particularly the amino acids in milk are allegedly responsible for modifications in incretin secretion leading to a reduction in appetite and with an insulin response that is markedly greater with respect to the glycemic curve (37-40), to such an extent that the effect is comparable to that of sulpho-

nylureas (41). Other researchers, on the other hand, have reassessed the dangerousness of fats of animal origin, confirming that it is not saturated fats but trans fats (i.e. those of fast foods, margarine, crisps, crackers and biscuits) that have an oxidative effect, fuelling inflammatory and degenerative processes, increasing the risk of heart disease, increasing LDL and decreasing HDL cholesterol (42). The fermented products of milk contain bioactive peptides derived from the native proteins through a microbial action during the digestive process, with anti-hypertensive properties (43). All this has led researchers to conjecture that the "French paradox" might be a phenomenon due, not only to the moderate intake by the French population during meals of red wine, as traditionally believed, but also to the abundant intake of milk and its derivatives (44). Last but not least, there is thought to be an inverse relationship between diabetes and the intake of cheese and fermented products (45, 46).

The rich content of milk enzymes in Parmigiano Reggiano cheese, due to its area of production and to the specific processing techniques employed, which ensure an optimal temperatures and exclude the use of antibacterial substances, have led researchers to a better understanding of its positive effects on health. In fact, the relationship between our body and the gut microbiota represents a new scientific frontier, shifting the attention of researchers to relationships not yet fully explained, but undoubtedly important, between metabolic diseases and gut microbiota (47-50). The human intestine has been colonised by thousands of species of bacteria during the coevolution of humans and microbes; there are 3.3 million microbial genes compared to only 23,000 human genes. The majority of these bacterial genes encode enzymes and structural proteins with the power to influence immune system function, modify the epigenome of mammals and change the regulation of the metabolism. It is this very alteration in the gut microbiota that is thought to lead to chronic and immunological diseases, cancer of the colon, gastric ulcers, cardiovascular diseases, intestinal diseases and especially obesity (51, 52). The products of the intestinal microbes, such as butyrate, can induce positive metabolic effects by boosting mitochondrial activity, preventing metabolic endotoxemia and activating intestinal gluconeogenesis through different methods of gene expression and hormonal regulation (53). Some of the recent research studies are particularly interesting: the weekly consumption of three portions of yoghurt brings about an increase in weight which is more than halved compared to the consumption of a half portion (54); the microbiota plays a role in childhood obesity (55). A microbiota transplant from the intestine of monozygotic twins, one brother being thin and the other obese, brings about different metabolic consequences in the receivers of the bacterial flora of the thin twins, which protects against obesity (56); a family of bacteria (Christensenellaceae), which has been identified very recently, is much more present in thin people and rare in obese people and the administration to a group of mice of colonies of bacteria from this family, showed that the mice treated in this way acquired less weight in comparison to the mice in the control group which were subjected to the same diet but not treated (57).

The use of Parmigiano Reggiano cheese in special diets

Thanks to its excellent nutritional qualities and especially to its digestibility and high protein and calcium content, Parmigiano Reggiano cheese appears to be a valuable food for all ages but particularly during the age of development (6), pregnancy, old age as well as during convalescence subsequent to debilitating clinical conditions (8, 9). Its almost total absence of carbohydrates, its richness in highly digestible protein substrates, in trace elements, trace nutrients and biofunctional peptides, together with its excellent lipid profile, make it a "naturally" functional food in the real sense of the term (7), ideal for use in numerous metabolic disorders such as obesity, diabetes, hypertension (taking care to monitor the daily intake of NaCl) and dyslipidaemias, disorders for which ever increasing evidence confirms the beneficial effect of the regular consumption of dairy products, also in terms of reduced incidence of cardiovascular complications (58-64).

As regards nutrition applied to sport, Parmigiano Reggiano cheese can be an interesting resource, both during the training phase and during the post-exercise recovery phase, as an important "recovery meal" i.e. consisting of that mixture of nutrients which, thanks

to their properties, can rapidly activate muscle recovery mechanisms. In the hours after fairly heavy physical exercise there is a "metabolic window" thanks to which the intake of carbohydrates with a high glycaemic index (GI) and proteins makes it possible to improve the glycogen re-synthesis process and facilitate protein synthesis. Although the best proteins are found in the whey, the casein can also stimulate protein synthesis and for a very long time (1 hour for the whey against 5-7 hours for the casein). If then, in addition to whole proteins and to peptones or peptides, recovery meals also contain FAAs (Free Amino Acids, as per aged Parmigiano Reggiano cheese), the effect on protein synthesis is particularly accentuated. Even more so if BCAAs (Branched Chain Amino Acids) are also available: these have the special property of being absorbed directly by the muscles and used, both for the construction of new proteins as well as a source of energy. Supplementation with BCAAs and essential amino acids has been under investigation for some time now. Their effects have been studied first and foremost in the post-exercise phase in which maximum priority is given to the stimulus of the mechanisms responsible for repairing the damaged muscle cells and restoring the integrity of the muscle protein chains. A substantial volume of scientific papers have demonstrated how an intake of essential amino acids, BCAAs, and leucine in particular, in the initial post-exercise phases (within one hour) can improve and substantially quicken muscle recovery processes, reducing protein degradation phenomena and increasing protein synthesis.

The considerable presence of these amino acids in Parmigiano Reggiano cheese makes this product ideal as a snack for post-exercise recovery, as well as being an ideal food for consumption during normal meals as a contribution to the overall protein requirement needed for the type of physical energy involved.

Another interesting fact is that, along with post-exercise metabolic recovery, the body also has to restore its sodium/water balance, given the losses it has suffered during exercise, both through sweating and even through breathing. It is important to remember that sweat causes the loss of large quantities of sodium. Therefore, imagining a recovery meal that provides water, sodium, carbohydrates and protein (particularly essential AAs), the combination of water, bread (or other high GI carbohydrates) and Parmigiano Reggiano cheese (proteins + essential AAs + sodium) can offer a simple and appetising solution. In general, the use of dairy products in sport is supported by recent clinical observations (58) and – with particular reference to Parmigiano Reggiano cheese – by field studies conducted on various types of sport, including those practised in extreme environmental conditions, such as mountain sports (65, 66).

As regards allergies to egg proteins, often associated in early infancy with cow's milk protein allergies, it is important to remember that people who are allergic to eggs can usually consume milk and its derivatives, provided that they do not contain lysozyme (67-71). From this perspective, the consumption of Parmigiano Reggiano cheese cheese is not a problem as it is totally free from lysozyme, the egg protein which is, on the other hand, present in numerous other cheeses, having been used in place of formaldehyde over the course of recent years as an anti-fermentation agent (72). A recent study conducted on a paediatric population of 70 children allergic to cow's milk proteins demonstrated that 58% of the patients were tolerant to the consumption of aged Parmigiano Reggiano cheese (24-40 months), probably thanks to the digestion of the casein, which takes place during the cheese's aging process. The absence of β-lactoglobulin-specific IgE appears to be an efficient tolerance marker (73). On this basis, Berni Canani et al. suggested the use of Parmigiano Reggiano cheese as an effective immunonutrient, i.e. as a food with the power to modulate the immune system function, either directly or by exerting an action on the composition and function of the gut microbiota (74): it is, in fact, possible to stimulate a more rapid acquisition of immunological tolerance in children allergic to cow's milk protein by administering extensive hydrolysate of casein containing the probiotic *L. rhamnosus* GG (LGG) (75). This effect seems induced by the combination of a direct immunomodulating action exerted by peptides deriving from the beta-casein and the action of the LGG (76), which the same Authors have demonstrated to be able to regulate the composition and functions of the microbiota in children affected by cow's milk allergy and regulate directly some immunological mechanisms involved in the pathogenesis of this condition (75). At the same time, other groups have dem-

onstrated the likelihood that a high number of people affected by IgE-mediated cow's milk allergy can tolerate foods containing cow's milk proteins hydrolysed through various techniques (77). Moreover, it has been hypothesised that these strategies might facilitate the acquisition of immunological tolerance in patients with cow's milk allergy (78). One of these foods is Parmigiano Reggiano cheese, which in the course of its aging process is subject to extensive hydrolysis of the cow's milk proteins with degradation of its caseins and production of a high quantity of peptides and free amino acids (79). At the same time, in samples aged for longer periods, there is an appreciable quantity of *L. rhamnosus* (80). A prospective, multicentre case-control study lasting 12 months is currently underway. Its aim is to evaluate the efficacy of a regular Parmigiano Reggiano cheese intake as a nutritional strategy for stimulating the acquisition of immunological tolerance in children allergic to cow's milk (81).

As in the pediatric field, the studies conducted at the University of Modena by professor O. Olivi in the '70s on the utility, for the treatment of enteric problems in neonates, of a not yet identified "bifidogenic factor" existing in Parmigiano Reggiano cheese, have been continued also in recent years (16). Since it is well-known that, as Parmigiano Reggiano cheese ages, proteolytic processes take place, due to which a variety of peptides with positive biofunctional activities are formed, it has been hypothesised that these peptides might exert an action on the intestine, modulating the composition of the commensal microbiota. Prebiotics are food supplements that stimulate growth and the bifidobacterium and lactobacillus metabolism: although some hydrolysed proteins have been proven to be bifidogenic, most of the prebiotics used are fibres and oligo- and polysaccharide carbohydrates, while the prebiotic/bifidogenic potential of the peptides contained in Parmigiano Reggiano cheese has not yet been explored. Recently, it was suggested that the development of bifidobacterium and lactobacillus stimulated by these prebiotics might have positive effects on the treatment of paediatric enteropathies: this theory is currently under study both *in vitro* and *in vivo* (82) with the aim of investigating the previously documented nutritional aspects of Parmigiano Reggiano cheese, providing scientific evidence in terms of the bifidogenic and/or prebiotic effect of the peptides that form from the hydrolysis of the milk proteins during the aging of Parmigiano Reggiano cheese.

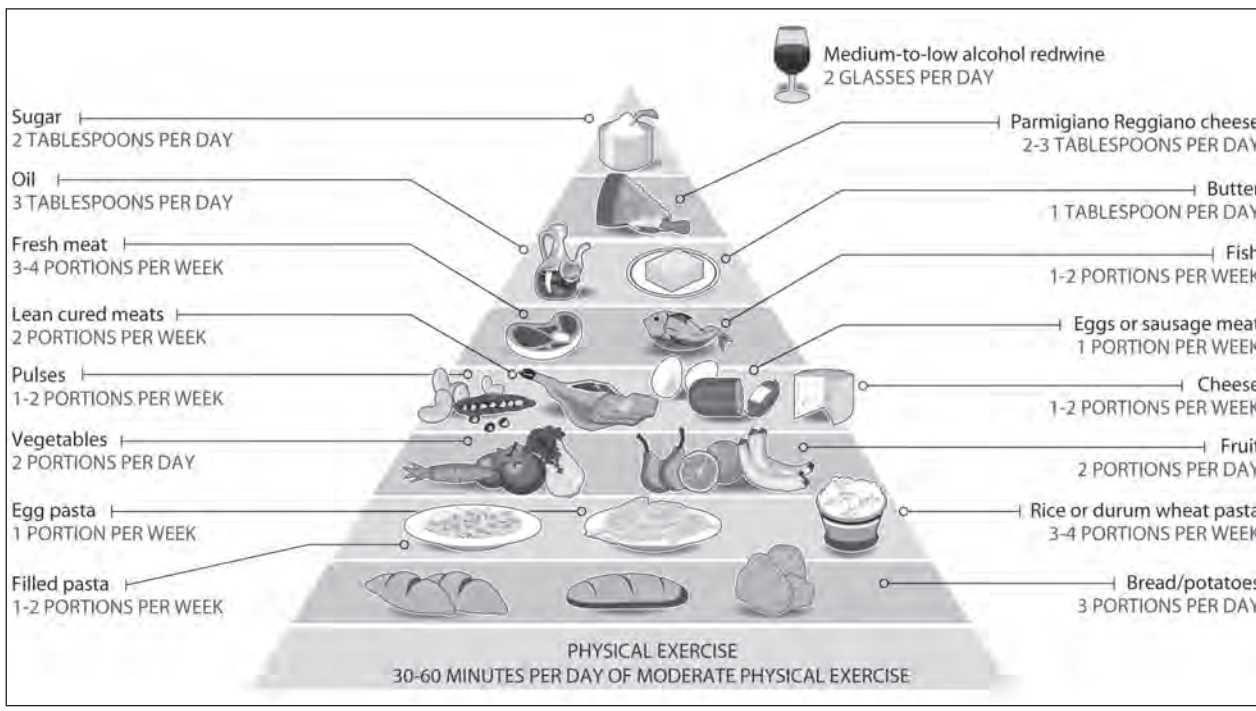

Figure 4. The Pyramid diet (83)

The absence of lactose means that it can be administered to patients with lactase deficiency, for whom it could be used as an element to "re-educate" them to consume milk or dairy products.

Conclusions

Nutrition should not be considered as a mere replenishment of nutrients because it also involves psycho-emotional, socio-cultural, relational and symbolic aspects. This multi-disciplinary approach to nutritional science seems to be the most effective in promoting healthy lifestyles, possibly also thanks to the use of educational/information models that are well-known in literature, such as the classic food pyramids, for which versions deeply-rooted in the local culinary tradition of the various Italian regions have recently been proposed (Fig. 4) (83).

Parmigiano Reggiano cheese is perhaps the most famous PDO product in the world and unquestionably one of the most important ingredients of the Italian culinary tradition; but although it has eight centuries of history behind it, it is nonetheless totally modern from a nutritional standpoint, able to fit into the most recent dietary models, also in the light of ever newer evidence capable of clarifying scientifically why Parmigiano Reggiano cheese has always been considered a "functional" food, even before this term was actually coined.

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Correspondence:
Dr. Federico Cioni
E-mail: federicocioni@mattioli1885.com

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Review

Structured lipids: methods of production, commercial products and nutraceutical characteristics

Wissam Zam

Department of Analytical and Food Chemistry, Faculty of Pharmacy, Al-Andalus University for Medical Sciences, Al-Quadmous, Tartous, Syrian Arab Republic

Summary. Structured lipids (SLs) are generally defined as triacylglycerols (TAGs) that have been modified to change the fatty acid composition and/or their positional distribution in glycerol molecules by chemical and/or enzymatic reactions and/or by genetic engineering processes. They are designed for obtaining TAGs with improved functional properties (i.e. fats with specific physical properties for food applications) and/or for medical and nutritional applications, especially to meet for the growing need for healthier foods and to prevent obesity, cancer and cardiovascular disease cardiovascular disease. Production methods of SLs and commercial products examples are discussed in this review. Moreover, nutritional and medical uses of SLs and their effect on human health are also reviewed in this paper.

Key words: Structured lipids, methods of production, commercial products and nutraceutical characteristics.

Introduction

Lipids have long been recognized for the richness they impart to foods as well as their satiety value in the diet. Lipid is an important component of the diet, because it provides both energy and essential fatty acids (EFAs). It is the most concentrated energy source in the diet, with an average energy value of 9 kcal/g compared to 4 kcal/g for carbohydrates and proteins. They serve several important biological functions including: 1) acting as structural components of all membranes; 2) serving as storage form and transport medium of metabolic fuel; 3) serving as a protective cover on the surface of several organisms; and 4) being involved as cell-surface components concerned with cell recognition, species specificity and tissue immunity (1, 2). The role of dietary lipids in health and disease -notably coronary heart disease, obesity, hyperlipidemia, diabeties and cancer- is one of the most active areas of research in modern food science, nutrition, and biochemistry (3, 4).

A high-fat diet poses at least two risks to one's health. First, fats produce a relatively large amount of energy when metabolized, nine calories per gram, compared with four calories per gram for carbohydrates and proteins (5). Second, saturated fats and trans fatty acids are believed to be responsible for an increase in LDL cholesterol levels and decrease of HDL cholesterol levels which, in turn, have been implicated with an increased risk for heart disease (6, 7).

The guidelines for a healthy diet issued in various countries recommend to lower the diet fat content to 20-35% of total energy content (5). A reduction of energy intake through a reduction of dietary fat intake is easier said than done because fat contributes strongly to the sensory characteristics of our food such as taste, appearance and texture. New developments in food technology now allow the partial replacement of dietary fat with substitutes called structured lipids (SLs), which combine unique characteristics of component fatty acids such as melting behavior, digestion, absorption, and metabolism to enhance their use as

functional lipids and as nutraceuticals of much lower energetic value and many health benefits.

Structured lipids are generally defined as triacylglycerols (TAGs) that have been modified by the incorporation of new fatty acids, restructured to change the positions of fatty acids, or synthesized to yield novel TAGs aiming at obtaining some desirable properties (Figure 1) (8, 9). Various fatty acids, including different classes of saturated, monounsaturated, and n-3 and n-6 polyunsaturated fatty acids (PUFAs) or their mixtures may be used in this process, depending on the desired metabolic effect (10). Lipids can be restructured to meet essential fatty acid requirements or to incorporate specific fatty acids of interest. SLs may offer the most efficient means of delivering target fatty acids for nutritive or therapeutic purposes as well as to alleviate specific disease and metabolic conditions. Structured lipids can also be produced to obtain TAG with modified physical and/or chemical features, including melting point, iodine and saponification values. They can be produced via inter-esterification reactions of fats, oils, or mixtures thereof, either chemically or enzymatically (11-14).

Much attention is being paid to SLs due to their potential biological functions and nutritional perspectives. The aim of this review is to focus on the component fatty acids, production strategies, medical and food applications and future prospects for research and development in this field.

Methods of SLs production

Chemical or enzymatic reactions. SLs can provide medium-chain fatty acids (MCFA) as a quick energy source and long-chain fatty acids (LCFA) as essential fatty acids to hospital patients (15). Basic strategies for developing structured lipids are essentially based on one of the following approaches:

- replacement of glycerol moiety of triacylglycerols with alternative alcohols such as carbohydrates, sugar alcohols or polyols such as sucrose fatty acid esters;
- replacement of long-chain fatty acids with alternative acids such as short-, medium and long-chain fatty acids esterified to glycerol.

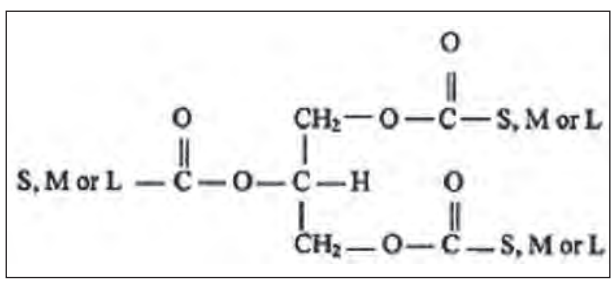

Figure 1. General structure of structured lipids.
S, M, and L is for short-chain, medium-chain, and long-chain fatty acids, respectively.

To produce SLs, chemical or enzymatic reactions such as direct esterification, acidolysis, alcoholysis, or interesterification can be used depending on the types of substrates available.

Chemical interesterification is a random reaction conducted at relatively high temperature and producing complete randomization of the fatty acid moieties in the triacylglycerol backbones (16). Chemical interesterification seems to be attractive due to the low cost and large scale application. However, under the perspective of producing lipids with very specific compositions aiming at functional and medical applications, enzymatic interesterification is far more interesting (17). With this respect, the enzymatic interesterification has the advantage of allowing a greater control of the positional distribution of fatty acid moieties in the final product due to both selectivity and regiospecificity of lipases (16, 18).

Many factors can influence the synthesis of SLs such as the type of lipase and the lipase/substrate ratio (19), the reaction medium (19), substrate concentrations, content of water (20), temperature (19), and operational mode (21-23).

Lipases occur widely in nature and are active at oil/water interface in heterogenous reaction system. They catalyze the hydrolysis of triacylglycerols into monoacylglycerols, diacylglycerols, free fatty acids and glycerol, under macroaqueous conditions (24). In addition to acylglycerol ester hydrolysis, lipases can also catalyze a wide variety of esterification, transesterification, and polyesterification reactions (24). The set of transesterification reactions includes acidolysis, interesterification, and alcoholysis (24, 25).

Most lipases have their substrate selectivity according to chain length, unsaturation, and positional distribution (26, 27). Many different types of lipases

have been investigated for the enzymatic modification of oils and fats. Commercial lipases are available from microbial, plant, and animal sources. Among those, microbial lipases are the most attractive ones and their utilization has been described extensively (28). Lipases are enzymes that preferentially catalyze the hydrolysis and synthesis of esters and TAG. Some lipases exhibit substrate selectivity. Lipase from *Penicillium camembertii* U-150 can hydrolyze mono- and diacylglycerols but not TAG (29). TAG with lower molecular weight fatty acids were hydrolyzed more easily with lipase from *Penicillium caseicolum* than those with higher molecular weight fatty acids (30). Lipase from *Geotrichum candidum* has shown preference to the unsaturated substrates with a double bond at the 9-position (31). When cis- and trans- form of 18:1 in l-elaidate-2,3-dioleate were compared for lipolysis, lipase from *Geotrichum candidum* preferentially hydrolyzed the cis-form to free fatty acid (32).

Among the currently available methods for modifying lipids, lipase-catalyzed reactions are better than conventional chemical methods since lipases mimic natural pathways, which concern mild reaction conditions, high catalytic efficiency, and the inherent selectivity of natural biocatalysts (33, 34). Typical applications of lipase-catalyzed interesterification reactions include the production of cocoa butter substitutes, human milk fat substitutes, partial acylglycerols, modified fish oil products, margarines, structured lipids, and several lipid products (35, 36).

Genetic engineering. Genetic modification of oilseed crops to improve quality, pest and disease resistance and yield has expanded in recent years to include modification of the fatty acid composition of oils for food use.

The main method of fatty acid profile modification is the cloning and transfer of a gene from one plant species into another species to produce the desired levels of specific fatty acids. As well, naturally occurring enzymes can be modified or new ones can be introduced to modify the fatty acid profile of the oilseed (37). Genes from bacterial, animal and yeast sources have also been incorporated into oilseeds for fatty acid modification (38).

Genetic codes are available to introduce double bonds, elongate carbon chains, synthesize eicosapentaenate, and produce fatty acid isomers not normally found in common sources of edible oils. Plant engineers are now trying to incorporate the principles used in chemical and enzymatic synthesis of "tailor-made" structured lipids into their genetic engineering techniques.

Since oleic acid (18:1) appears to have a similar effect on cholesterol as linoleic acid (18:2 n-6) and is not as susceptible to oxidation, researchers increased the ratio of monounsaturated fatty acids (MUFAs) to PUFAs in soybean and canola oil by modifying the activity of a microsomal membrane-bound oleate desaturase (39). Trans fatty acids are produced during the hydrogenation process used by food companies and their presence become a major health concern for consumers. Several companies are actively pursuing the development of seed oils that contain levels of saturated fatty acids high enough to permit the elimination of the need for hydrogenation, and, subsequently, the production of trans fatty acids (40). Cloning and characterizing genes for a family of thioesterases was the 1st step toward the goal of incorporating MCFAs into oil seed crops that naturally do not contain such fatty acids. A gene from the California bay tree that produces MCFAs in its seeds was incorporated into canola plants. The transgenic canola now accumulates up to 65% more lauric acid in their seed TAGs (41). The sn-2 acyltransferase has a high degree of specificity for an unsaturated fatty acid; therefore, most of the oleic acid found in these TAGs is at the sn-2 position. This oil was marketed as Laurical® (Table 1).

Commercial products examples of structured lipids

Caprenin. Caprenin is a common name for caprocaprylobehenin, a structured lipid containing C8:0, C10:0, and C22:0 fatty acids esterified to glycerol moiety (Figure 2) (42). It is manufactured by Procter & Gamble's (Cincinnati, Ohio, U.S.A.) from coconut, palm kernel, and rapeseed oils by a chemical transesterification process. The MCFAs are obtained from the coconut oil and the LCFAs from rapeseed oil. Because behenic acid is only partially absorbed and capric and caprylic acids are more readily metabolized than other longer chain fatty acids, caprenin provides only 5 kcal/g (43, 44).

Procter & Gamble filed a Generally Recognized as Safe (GRAS) affirmation petition to the U.S. Food and Drug Administration (FDA) for use of caprenin in soft candies such as candy bars, and in confectionery coatings for nuts, fruits, cookies, and so on. Caprenin has a bland taste, is liquid or semisolid at room temperature, and is fairly stable to heat. It can be used as a cocoa butter substitute. Caprenin, in combination with polydextrose, was commercially available briefly in reduced-calorie and reduced- fat chocolate bars (45). Swift et al. (46) showed that Caprenin fed as an SL diet to male subjects for 6 days did not alter plasma cholesterol concentration but decreased HDL-chol by 14%. However, the medium chain triacylglycerol (MCT) diet raised plasma TAGs by 42% and reduced HDL-chol by 15%.

Salatrim/Benefat. Salatrim (an acronym derived from short and long acyl triglyceride molecule) is the generic name for a family of structured triglycerides comprised of a mixture containing at least one short chain fatty acid (primarily C2:0, C3:0, or C4:0 fatty acids) and at least one long chain fatty acid (predominantly C18:0, stearic acid) randomly attached to the glycerol backbone (Figure 3) (47).

Salatrim was developed by the Nabisco Foods Group (Hanover, N.J., U.S.A.) but now marketed as Benefat® by Cultor Food Science (Ardsley, N.Y., U.S.A.). Benefat is produced by base-catalyzed interesterification of highly hydrogenated vegetable oils with TAGs of acetic and/or propionic and/or butyric acids (48).

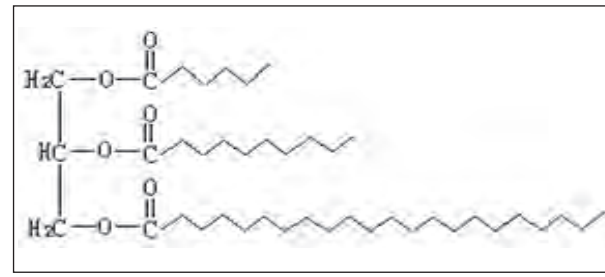

Figure 2. Caprenin chemical structure.

Benefat is a low-calorie fat like Caprenin, with a caloric availability of 5 kcal/ g. Stearic acid is poorly or only 50% absorbed (49), whereas acetyl and propionyl groups in Benefat are easily hydrolyzed by lipases in the stomach and upper intestine and readily converted to carbon dioxide (50). Nabisco filed a Generally Recognized as Safe (GRAS) affirmation petition to the U.S. Food and Drug Administration (FDA) in 1994 for use of Benefat in baking chips, chocolate-flavored coatings, baked and dairy products, dressings, dips,

Table 1. Commercial Sls containing polyunsaturated fatty acids and their applications.

Brand name	Fatty acid composition	Application
Betapol	C16:0 (45%)	Infant food formulation
Impact	Interesterification with high lauric acid oil and high linoleic acid oil	Pharmaceuticals for patients suffering from trauma or surgery, sepsis or cancer
Laurical	C12:0 (40%) and unsaturated fat (C18:1, C18:2 and C18:3)	Medical nutrition and confectionery coating, coffee whiteners, whipped toppings and filling fats
Neobee	C8:0, C10:0 and LCFA (n–6 and n–3)	Nutritional or medical beverages
Structolipid	LCT (63%) and MCT (37%) – caprylic (27%), capric (10%), palmitic (7%), oleic (13%), linoleic (33%) and α-linoleic acid (5%)	Intravenous fat emulsion as a rapid source of energy for patients and parenteral nutrition
Captex	C8:0, C10:0, C18:2	Captex diet resulted in increased heat production and altered energy metabolism in obese Zucker rats. It also improved absorption of 18:2 n-6 when administered to cystic fibrosis patients.

LCFA: Long chain fatty acid, LCT: Long chain triacylglycerol, MCT: Medium chain triacylglycerol.

and sauces, or as a cocoa butter substitute in foods. The consistency of Benefat varies from liquid to semisolid, depending on the fatty acid composition and the number of short chain fatty acids (SCFAs) attached to the glycerol molecule.

Olestra/Olean®. Olestra is an acylated sucrose polyester with six to eight fatty acids obtained from vegetable oil (e.g., soybean, corn, sunflower) as shown in figure 4. It is prepared by interesterifying sucrose and edible oil methyl esters in the presence of an alkali catalyst, at 100-140°C (51). Sucrose polyester (SPE) development dates back to the year 1880, when a derivative of sucrose was prepared by acetylation to sucrose octaacetate. In 1952 the concept of sucrose fatty acid polyester (SPE) production was initiated for use in detergents. The other concept was to come up with a fatlike molecule that would significantly reduce fat calories by preventing their hydrolysis and absorption. This led to the discovery of a non-digestible and non-absorbable fatlike molecule called sucrose fatty acid polyester, now known as olestra, by Mattson and Volpenhein while working on the absorption of fats by infants (52).

Olestra has the organoleptic, and thermal properties of fat. Is not hydrolyzed by gastric or pancreatic enzymes because the large size and number of the nonpolar fatty acids, thus it is nondigestible, hence noncaloric; it is also nontoxic, yet nutritional concerns potentially exist (53). Its functionality is dependent on the chain length and unsaturation of the esterified fats, as with normal lipids (54). Olestra made from highly unsaturated fatty acids is liquid at room temperature; olestra made from highly saturated fatty acids is solid (55). Because of its unique properties, olestra can serve as a zero-calorie replacement (up to 100%) for conventional fat in a variety of foods. It can be exchanged for fats in products such as ice cream, margarine, cheese, and baked goods, and it can be blended with vegetable oil (56). Olestra's configuration also makes it possible for the substance to be exposed to high temperatures, such as frying.

Neobee. Neobee is another caloric reduced fat, it is composed of capric and caprylic acids and produced by Stepan Company (Maywood, N.J., U.S.A.). This class of specialty lipids includes different products. For example, Neobee 1053 and Neobee M-5 contain both capric and caprylic acids, while Neobee 1095 is made up of only capric acid (57). Neobee 1095 is a solid product. Therefore, this product may be suitable in certain applications which require solid fats. Neobee 1814 is an MCT derivative made by interesterification of MCT with butter oil (58); it contains half of the long-chain saturated fatty acids found in conventional butter oil and is suitable to replace butter oil in a variety of applications. Neobee 1814 may serve as a flavor carrier and functions as a textural component for low-fat food products (57).

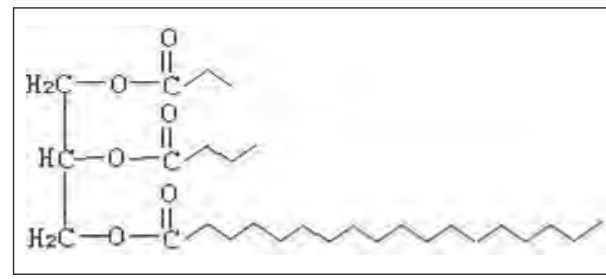

Figure 3. Salatrim chemical structure.

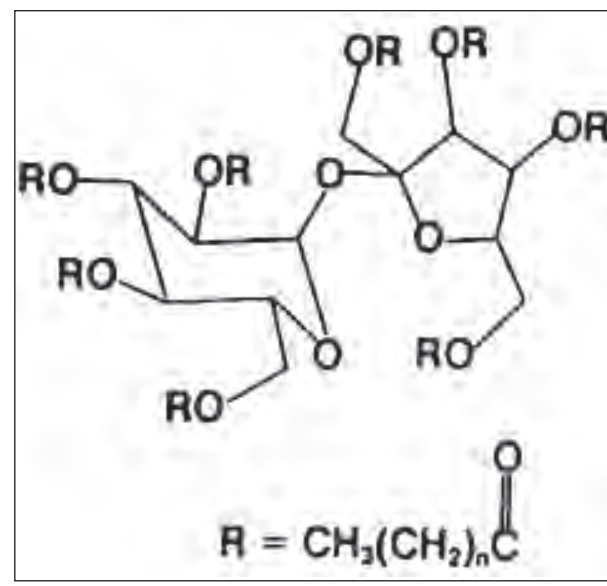

Figure 4. Olestra chemical structure.

Structured lipid containing polyunsaturated fatty acids (PUFA). Nowadays, the most familiar types of low-calorie lipids are triacylglycerols with short- and long-chain acyl residues (SLCTs), triacylglycerols with medium- and long-chain acyl residues (MLCTs) and diacylglycerols (DAGs) (59). To act as an ideal lipid substitute, the products should contain

unsaturated fatty acids, especially essential fatty acids, and have no harmful effects. SLs containing n–3 highly unsaturated fatty acids were produced with immobilized sn–1,3 specific and non-specific lipases as biocatalysts. Highly unsaturated fatty acids, such as eicosapentaenoic (EPA, 20:5 n–3), docosahexaenoic (DHA, 22:6 n–3), linolenic (18:3 n–3) and gamma linolenic (18:3 n–6) acids, are important in foods, nutrition, and pharmaceutical applications (60). SLs containing these fatty acids and medium-chain fatty acids may be desirable as 'nutraceuticals' for supplementation in infant formula or as food supplement for adults to enhance overall health (61).

For the most part, the position of the highly unsaturated fatty acid in the glycerol moiety is key to their functionality in foods and absorption when consumed. Perhaps, these designer lipids may replace conventional fats and oils in certain specialty applications because of their structure-health (nutraceutical or medical lipids) and structure-function (functional lipids) attributes. In most cases, insertion of the desired highly unsaturated fatty acid at the sn–2 position will provide maximum nutritional benefits (60). Specific structured lipids were designed with PUFA residues at the sn-2 position and MCFA residues at the sn-1,3 positions. In this form, the PUFA residues are protected against oxidation by the two saturated MCFA residues. Hamam et al. (62) showed that the presence of palmitate in the sn-2 position of the TAG, in infant formula instead of conventional fats, improved digestibility of the fat and absorption of other important nutrients such as calcium. In a study conducted by Decker (63), saturated fatty acids at the sn-2 position have been found to be beneficial in terms of providing increased caloric intake through infant formula and enteral supplements.

An SL made by reacting tripalmitin with unsaturated fatty acids using an sn-1,3 specific lipase closely mimicked the fatty acid distribution of human milk was commercially developed for application in infant formulas under the trade name Betapol (Loders Croklaan, Glen Ellyn, Ill., U.S.A.) (64).

Structured lipids (SL) enriched with omega 6 PUFA were synthesized from coconut oil triglycerides by employing enzymatic acidolysis with free fatty acids obtained from safflower oil (65).

Structured triacylglycerols (ST) enriched in eicosapentaenoic acid (EPA) in position 2 of the triacylglycerol (TAG) backbone were synthesized by acidolysis of a commercially available EPA-rich oil and caprylic acid, catalyzed by the 1,3-specific immobilized lipase lipozyme IM (66).

Table 1 summarize some commercial SLs containing polyunsaturated fatty acids and their food and medical applications (67).

Despite the health benefits of SLs containing polyunsaturated fatty acids, they are highly prone to oxidative deterioration and thus require adequate protection to deter their oxidation (68). Some studies have shown that the rate of autoxidation and melting properties of TAGs can be affected by the position of unsaturated fatty acids on the glycerol molecule (69). TAGs having unsaturated fatty acids at the 2-position of glycerol are more stable toward oxidation than those linked at the 1- and 3-positions (70).

Further research are conducted in order to optimise the SLs' stabilisation and storage by use of appropriate antioxidants and packaging technologies.

In Nagachinta and Akoh study (71), Maillard reaction products, obtained from heated whey protein isolates and corn syrup solids solution, were used as encapsulants for microencapsulation of 2 enzymatically synthesized SLs for infant formula applications. The encapsulated SL powders had low peroxide and thiobarbituric acid-reactive substances values.

Nutraceutical characteristics of SLs

Functional SLs

The interesterification and genetic engineering processes have been used in the production of structured lipids with specific physical properties such as having a desired melting point, slow rancidification, and also for the production of functional structured lipids possessing specific compositions and nutritional properties. Table 2 summarize the potential uses of functional structured lipids.

Margarine fats. Chemical and enzymatic interesterification has been specially employed in the formulation of margarines and shortenings with no trans FAs while still maintaining physical properties, taste

and stability. The vegetable oils including corn, palm, peanut, cottonseed, canola, and sunflower oils can be randomly interesterified with fully hydrogenated soybean oil or fully hydrogenated cottonseed hard fats to produce desirable fat compositions for margarines and shortenings (72).

Cocoa butter equivalents. Due to high cost and fluctuations in the supply and demand of cocoa butter, cocoa butter equivalent (CBE) with a TAGs composition similar to cocoa butter is used as an alternative source. Recently, vegetable oils such as Mahua, Kokum and mango fats, palm oil, tea seed oil, and olive oil have been used to prepare CBE through enzymatic catalyzed interesterification until a similar composition of cocoa butter is obtained. The triacylglycerol composition of oils was redesigned so that properties such as the melting point, solid fat content and fat crystal network microstructures of the structured oil and cocoa butter were very much similar (73).

Frying oils. Genetic engineering process had been used for the production of modified oils that have a lot of benefits which include high oxidative stability, zero trans-fat and low saturated FAs, non-hydrogenated, high oleic content, liquid at room temperature, and excellent taste and flavor (74). Recently, genetically modified soybean oil has been introduced that eliminates the need for hydrogenation to be used in bakery goods and for frying. The oil also has a healthier FA composition. High-oleic sunflower oil having better oxidative stability in deep frying applications and extended shelf life compared to traditional sunflower oil has been developed using selective breeding and mutagenesis (75). Other example includes canola oil seed mutants with low linolenic/high oleic acid content (76).

Breast milk fat substitute. Lipids are the major source of energy in human milk or infant formulas. Hence, modification of fats and oils for infant formulas in order to obtain not only the correct fatty acid (FA) composition but also the same positional distribution as in human milk fat (HMF) via interesterification had been widely investigated. Christensen and Holmer (77) prepared a HMF analogue using a Rhizomucor miehei lipase-catalyzed modification of butter oil. Unilever produced a milk fat substitute named Betapol for infant formulas (64). Also, Yang et al. (78) modified lard by lipase to produce HMF substitutes.

Health benefits of SLs

One of the earliest uses of SL was in enteral and parenteral nutrition followed by its application in a range of clinical settings including prevention of thrombosis, improved nitrogen balance, and enhanced

Table 2. Potential uses of functional structured lipids.

Potential uses	SL related to food application	References
Margarine, butter, spreads, shortening, dressings, dips, and sauces	Benefat, Neobee and Olestra	48, 56, 58
Cocoa butter equivalents	Caprenin and Benefat	45, 50
Confectioneries and soft candies	Caprenin and Laurical	41, 45
Baking chips, baked goods	Benefat and Olestra	48, 56
Snack foods	Caprenin, Captex	45
Low caloric food	Caprenin, Benefat, Neobee	43, 49, 57
Frying oil	Genetically modified soybean oil, high-oleic sunflower oil and canola oil seed mutants with low linolenic/high oleic acid content.	75, 76
Infant food formulas	SLs containing EFAs and MCFAs such as Betapol	61, 64
Dairy products	Benefat	50

immune function (Table 3). Low-calorie structured lipids (SLs) are mainly designed for special nutritional applications, especially to meet the growing need for healthier foods and to prevent obesity (79, 80).

Data from several short-term investigations suggest that SLs are well tolerated and rapidly oxidized and cleared from the plasma (81-83).

Enteral and parenteral nutrition. The advantages of enterally fed SLs may well relate to differences in absorption and processing. Structure TAGs that contain MCFA may provide a vehicle for rapid hydrolysis and absorption, due to their smaller molecular size and greater water solubility in comparison to long-chain TAGs (84).

The TAGs in total parenteral nutrition (TPN) are normally administered as an emulsion. These emulsions are suspected of suppressing the immune function because pneumonia and wound infection often occur in patients treated with TPN. Kruimel et al. (85) attempted to explain this phenomenon, the results indicated that physical mixtures caused higher peak levels and faster production of oxygen radicals, compared to SLs. Chambrier et al. (86) conducted a similar study comparing the effect of physical mixtures and SL on postoperative patients. They did not see the hepatic function disturbances in patients given the SL, which are often observed with TPN.

Structured lipids synthesized from fish oil and MCFA were administered to patients undergoing surgery for upper gastrointestinal malignancies. This diet was compared to a control diet that differed only in its fat source. The SL diet was tolerated significantly better, led to improved hepatic and renal function, and reduced the number of infections per patient (87).

In a recent study, a novel SL was designed and synthesized based on lipase-catalyzed interesterification of camellia oil fatty acid methyl esters and triacetin. The SL product contains relatively high amounts of unsaturated fatty acids and has a lower risk of safety problems (88). Triacetin was found to be metabolically beneficial in hypermetabolic states, it improves protein utilization and structural components of the small and large bowel and reduces the development of intestinal mucosal atrophy associated with conventional parenteral nutrition in burn injury (89).

The use of fish oil emulsions in patients undergoing stent implantation resulted in lower incidences of atrial fibrillation and the length of intensive therapy unit ITU and hospital stay is reduced, compared to the therapy with soybean oil-based emulsions (90).

SLs containing MCFAs and n-3 PUFAs could be a therapeutic or medical lipid source, and may be useful in enteral and parenteral nutrition. These SLs provided an efficient way to supplement n-3 PUFAs and to provide energy from the MCFAs, which were the preferred substrate for oxidative metabolism (91). No signs of central nervous system toxicity were noted in patients given the SL, and there was no tendency to ketosis (92). Additionally, SLs were safe and efficient when provided to patients on home parental nutrition on a long-term basis because they may be associated with possible reduction in liver dysfunction (93).

Immune function. The essential constituents of structured lipids in terms of their effects on the immune system are fatty acids, which are composed of the hydrocarbon chain of various lengths. Fatty acids used in structured lipids can affect the immune system via several mechanisms. The first mechanism involves incorporation of lipids into the structure of the cell membranes and thus affecting their fluidity, permeability of ion channels and functions of membranous receptors. The second mechanism is associated with penetration of fatty acids to the cell where they can affect the production of eicosanoids, resolvins, cytokines, pathways responsible for signal transduction into the cell, and expression of genes. Moreover, fatty acids can alter cell apoptosis and production of reactive oxygen species (94).

Table 3. The main applications of SLs in human health

The main application of SLs in human health	References
Lipids in enteral and parenteral nutrition	79-88
Enhanced immune function	89-95
Improved nitrogen balance	96-100
Prevention of thrombosis	101-104
Reduced cholesterol and triacylglycerols	60, 105-107
Decreased cancer risk	98, 108-113
Reduced-calorie triacylglycerols	114-120
Absorption of structured lipids	121, 122

Studies reporting on novel emulsions based on olive and fish oils, structured lipids or mixed-type emulsions in which various lipid species replace conventional long-chain triglycerides indicate that these lipids are generally well tolerated. While long-chain triglycerides may promote inflammation due to conversion of n-6 polyunsaturated fatty acids into arachidonic acid-derived eicosanoids, structured lipids and olive oil emulsions appear more immune-neutral (95, 96). The structured lipid diet named Impact, containing low levels of linoleic acid, resulted in decreased length of hospital stay compared to other enteral formulae. Bower et al (97) also demonstrated a decrease length of hospital stay and infection rate when using diets with low level of linoleic acid an added fish oil.

Fish oil-based emulsions contain mainly long-chain n-3 polyunsaturated fatty acids. They have inhibitory effects on signal transduction and expression of genes involved in the inflammation, they also modify significantly the cytokine profile and increase the EPA levels in serum (98). Moreover, its use was demonstrated to enhance the production of DHA and EPA metabolites without affecting the production of AA, whose products show pro-inflammatory effects (99). Importantly, recent investigations indicate beneficial effects of parenteral fish oil on relevant clinical outcome measures.

Leukocyte-activating effects of medium-chain triglycerides in experimental studies await further characterization in vivo, although the recent data indicate that MCTs are not indifferent to the functioning of the immune system (100).

Nitrogen balance. Patients with sepsis and trauma are characterized by hyper-metabolism, insulin resistance and protein catabolism. Fat emulsions containing medium chain triglycerides have been suggested to be beneficial for these patients since medium chain fatty acids are a more readily available source of energy when compared to long chain fatty acids. Lindgren et al. (101) show a better nitrogen balance by the infusion of a structured lipid emulsion comprising medium chain fatty acids (MCFA) and long chain fatty acids (LCFA) compared to a pure long chain triglyceride (LCT) emulsion during short term infusion over three days in ICU patients. The amelioration in nitrogen balance in the SLs group was despite the lack of effect on respiratory quotient or energy expenditure. The mechanism behind the improved n-balance by infusion of a structured triglyceride comprising MCFA, compared both to pure LCT emulsions and to physical mixtures of MCT and LCT, is not obvious. It has been suggested that this occurs as a result of a more favorable energy metabolism.

In the study of Teo et al. (102), the effects of enteral feeding with SL composed of MCT and fish oil were compared with sunflower oil on energy metabolism in burned rats. A decrease in total energy expenditure (7%) and improved nitrogen balance were obtained in the SL group, suggesting that SL reduced the net protein catabolic effects of burn injury. A similar study by Mendez et al. (103) compared the effects of a structured lipid (made from fish oil and MCFAs) with a physical mix of fish oil and MCTs and found that the SL resulted in improved nitrogen balance in animals, probably because of the modified absorption rates of SL.

More studies in humans and animals indicate that the use of SLs in catabolic subjects improves nitrogen balance and preserves the function of the hepatic reticuloendothelial system (104, 105).

Thrombosis. Thrombosis is the formation of blood clots. Blood clotting involves the clumping together of platelets into large aggregates and is triggered when endothelial cells lining the artery walls are damaged. If the platelet membranes are rich in long-chain n-3 PUFAs, formation of certain eicosanoids such as prostacyclin I3 and thromboxane A3 is promoted. These do not trigger platelet aggregation as much as the corresponding eicosanoids, prostacyclin I2 and thromboxane A2, that are formed from n-6 PUFA. Therefore, long-chain n-3 PUFAs may help to reduce the tendency for blood to clot (106).

Mori et al. (107) suggested that n-3 fatty acid intake from fish consumption in conjunction with a low-fat diet was most beneficial in terms of reducing cardiovascular disease. Studies indicate that the n-3 fatty acids, especially EPA and DHA, may be effective in reducing the clinical risk of cardiovascular disease by favorably altering lipid and hemostatic factors such as bleeding time and platelet aggregation (108). EPA incorporating into the atheromatous plaque decreases the number of foam cells and T lymphocytes, reduces

the inflammatory process and increases the stability of platelets (109).

Cholesterol and triacylglycerols concentrations. Long-term feeding studies with an SL containing MCFAs and fish oil fatty acids showed that SL modified plasma fatty acid composition, reflecting dietary intake and induced systemic metabolic changes that persisted after the diet was discontinued (110). When SL (emulsion of MCT + fish oil composed of 50% MCT, 40% fish oil, and 10% canola oil) and soybean oil were provided to rats enterally, TAG and cholesterol levels in liver were lowered in the SL group (111).

Rats were fed a diet containing coconut oil, coconut oil-sunflower oil blend (1:0.7 w/ w) or structured lipid enriched with omega 6 PUFA at 10% levels for a period of 60 days. The SL lowered serum cholesterol levels by 10.3 and 10.5% respectively in comparison with those fed coconut oil and blended oil. Similarly the liver cholesterol levels were also decreased by 35.9 and 26.6% respectively in animals fed structured lipids when compared to those fed on coconut oil or the blended oil. Most of the decrease observed in serum cholesterol levels of animals fed structured lipids was found in LDL fraction. The triglyceride levels in serum showed a decrease by 17.5 and 17.4% while in the liver it was reduced by 45.8 and 23.5% in the structured lipids fed animals as compared to those fed coconut oil or blended oil respectively (65).

SL containing caprylic and n-3 polyunsaturated fatty acids was synthesized and this enzymatically produced SL vs soybean oil (20% of diet weight) were fed to female mice for 21 days. The result showed that the concentration of total cholesterol, LDL cholesterol, and triacylglycerol were significantly decreased in SL-fed group (112).

Tumor and cancer risk.

In contrast to the tumor promoting effects of diets high in fat, some FAs of chain length 8-C or higher have been found to have cytolytic activity, which can be directed against tumor cells in some situations, and represent a novel type of antitumor agent. In a study by Burton (113), caprylic acid showed oncolytic effects in liver of mice and rats.

Many studies have shown that n-3 fatty acids can decrease the number and size of tumors and increase the time elapsed before the appearance of tumors (114). Reddy and Maruyama (115) showed that diets containing high levels of fish oils were effective in destroying some cancer cells, but it is not known whether such results are reproducible with humans, or what potential side effects exist (116).

Medium chain fatty acids possess a nutritional advantages compared with other fatty acids in that they are non-tumor-producing forms of fat (117). Ling et al. (118) demonstrated that tumor growth in mice was decreased when they were fed with a SL made from fish oil and MCTs.

Diet and calorie intake. Salatrim, Neobee and Caprenin are widely known as the low-calorie fats, whereas Olestra is known as a zero calorie fat. Reduced calorie SLs are designed by taking advantage of either limited absorption of long-chain saturates or the low caloric value of SCFAs. In humans, SCFAs contribute to 3% of total energy expenditure and these are more easily absorbed in the stomach and provide fewer calories than MCFAs and LCFAs (119). Thus, acetic, propionic, and butyric acids have caloric values of 3.5, 5.0, and 6.0 kcal/g, respectively.

Despite containing saturated fatty acids, MCT are utilized by the human body more readily than triacylglycerols containing other fatty acids. Their digestion process omits the lymphatic system and they enrich the cardiovascular system without hydrolysis or re-esterification. Therefore, MCT do not accumulate in the fatty tissue and do not form a reserve fat and, unlike other triacylglycerols, they have lower caloric values. Thus, MCT are used as a source of easily available energy and a low-calorie product (120).

Although MCTs provide fewer calories than absorbable long chain triacylglycerols (LCTs), MCTs need to be used with LCTs to provide a balanced nutrition in enteral and parenteral products (121, 122). In many medical foods, a mixture of MCTs and LCTs is used to provide both rapidly metabolized and slowly metabolized fuel as well as EFAs. Clinical nutritionists have taken advantage of the simpler digestion of MCTs to nourish individuals who cannot utilize LCTs owing to fat malabsorption. Thus, patients with certain diseases (Crohn's disease, cystic fibrosis, colitis, enteri-

tis, etc.) have shown improvement when MCTs are included in their diet (123).

For example, Akoh and Yee (124) interesterified tristearin with tricaprin (C10:0) or tricaprylin (C8:0) with sn-1,3-specific immobilized lipase to produce a low calorie SL.

Another group of researchers synthesized an SL from natural vegetable oils so it would contain EFAs and natural antioxidants (125). The synthesized product delivered 5.36 kcal/g and had an improved plastic nature, which increases the potential food applications for such a product, especially since it is a trans-free solid fat. After producing the SL, it was fed to rats and compared to a control group fed sunflower oil. No differences were observed in the amount of food consumed, which indicates that the palatability and taste of the SL was very similar to the native sunflower oil (125).

Absorption of structured lipids. In the absence or deficiency of pancreatic lipase, previous studies have indicated that a large fraction of MCT can be absorbed as triacylglycerol, whereas LCT are not absorbed. However, structured triacylglycerols containing LCFAs in the sn-2 position and MCFAs in the primary positions have improved metabolic benefits and have potential advantages for providing polyunsaturated fatty acids. The presence of MCFAs in dietary fatty acid as well as the triacylglycerol structure may influence the absorption and lymphatic transport of fatty acids (126). Swails et al. (127) demonstrated that diets containing an SL composed of MCFAs and linoleic acid led to improved absorption of EFAs in patients with cystic fibrosis.

Finally, the differences in the health effects of structured lipids are largely dependent on the composition of lipid mixtures, particularly the content of MUFA, n-6 or n-3 PUFA. Table 4 gives the suggested levels of some of these fatty acids in SLs intended for clinical applications (123).

Critics attribute a variety of gastrointestinal complaints to the consumption of olestra. Symptoms cited include bloating, diarrhea, cramps, loose stools, and urgency of defecation (38, 48). In addition, olestra is lipophilic, non-digestible and non-absorbable, so it has the potential to interfere with the absorption of other components of the diet, especially lipophilic ones, eaten at the same time as olestra. Among these biochemicals are fat-soluble vitamins (A, D, E, and K) and carotenoids, such as beta-carotene, lycopene, lutein, and zeaxanthin (128, 129). However, the effects can be offset by adding specified amounts of the vitamins to olestra foods.

The concentration of vitamins A, D, E, and K required for supplementation in olestra-containing foods are 0.34 X RDA (Recommended Dietary Allowance) for vitamin A/10g olestra, 0.3 X RDA for vitamin D/10g olestra, 0.94 X RDA for vitamin E/10g olestra, and 1.0 X RDA for vitamin K/10g olestra (130).

As a result, the Food and Drug Administration (FDA) requires that food containing olestra be labeled with the statement: "This Product Contains Olestra. Olestra may cause abdominal cramping and loose stools. Olestra inhibits the absorption of some vitamins and other nutrients. Vitamins A, D, E, and K have been added".

Conclusion

Public concerns about obesity, cancer and cardiovascular disease have increased our interest in minimizing the consumption of saturated fats and trans fats. These concerns have been a driving force in the lipid industry to develop fat-based ingredients that retain the physical, functional and sensory features of traditional lipids and provide specific nutritional properties and health benefits.

Food chemists have developed a number of synthetic fats using new processing technologies, along with the creative use of newly discovered functional properties of triglycerides. Chemical and enzymatic interesterification lead to the development of structured lipids which can be useful for diabetics, people who are trying to lose weight, and others concerned about maintaining a healthy diet. At the same time, this is not to ignore that while FDA had approved the use of different SLs, EFSA (European Food Safety Authority) restricted the use of some SLs such as Olestra because of the potential health risks for some people who may be allergic to such products and may develop other health problems by using them.

Research on structured lipids remains an interesting area that holds great promise for the future and has certainly not come to an end. Food chemists will continue to search for new products with which to aug-

Table 4. levels and function of different fatty acids in SLs intended for clinical applications

Fatty acid	Levels and function	References
n-3	2–5% to enhance immune function, reduce blood clotting, lower serum triacylglycerols, and reduce risk of coronary heart disease.	93, 102, 103, 107
n-6	3–4% to satisfy essential fatty acid requirement in the diet.	118
n-9	monounsaturated fatty acid (18:1n-9) for the balance of long chain fatty acid.	118
SCFA and MCFA	30–65% for quick energy and rapid absorption, especially for immature neonates, hospitalized patients, and individuals with lipid malabsorption disorders. SCFAs affect gastrointestinal function by stimulating pancreatic enzyme secretion and increasing sodium and water absorption in the gut. The TAGs, containing MCFA, are applied in the nutrition of infants as well as in the clinical nutrition of patients with digestion or nutrient absorption disorders, since their digestion requires negligible amounts of bile salts and pancreatic lipase.	106, 108, 114
LCFA	They are mixed with bile salts and lecithin to form micelles, which are absorbed through the wall of the intestine. They are very slowly oxidized to release energy. LCFAs need to be used with MCFAs to provide a balanced nutrition in enteral and parenteral products. In many medical foods, a mixture of MCFAs and LCFAs is used to provide both rapidly metabolized and slowly metabolized fuel as well as EFAs.	116,117

SCFA: Short chain fatty acid, MCFA: Medium chain fatty acid, LCFA: Long chain fatty acid, TAGs: Triacylglycerols.

ment and improve peoples' diets. Designing SLs with specific fatty acids at specific locations of the TAG for use in medicine needs more studies. For example, it may be desirable to develop a SL for patients with cystic fibrosis that contains PUFA (e.g., EPA or DHA) at the sn-2 position, and MCFA at the sn-1, 3 positions.

Further research is also needed to stabilize the modified fats containing PUFAs during storage by incorporation of appropriate antioxidants and adequate packaging technologies. Moreover further research should focus on the various esterification processes, the metabolism and medicinal importance and economic feasibility of large-scale production of SLs.

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Correspondence:
Dr. Wissam Zam
Department of Analytical and Food Chemistry, Faculty of Pharmacy, Al-Andalus University for Medical Sciences, Al-Quadmous, Tartous, Syrian Arab Republic
Phone number: +963932724703
E-mail: ws.sarah2005@gmail.com

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Original article

Efficacy and tolerability of a novel food supplement (Turbofer®) containing microencapsulated iron in liposomal form, in female iron deficiency anaemia

A. Pleşea-Condratovici, C. Pleşea-Condratovici, N. Rosoga, N. Nedelcu
Lower Danube, University of Medicine, Galati, Romania

Summary. *Introduction:* According to the WHO, iron deficiency and related iron deficiency anaemia (IDA) are the most prevalent nutritional disorders worldwide. Anaemia occurs in all age racial and ethnic groups. The goal of treatment of anaemia is to increase the amount of oxygen that blood can carry by raising the red blood cell count and/or Haemoglobin level and to treat the underlying cause of the anaemia. *Objectives:* To evaluate the efficacy of a novel food supplement, Turbofer®, formulated with microencapsulated iron in liposomal form, on the capacity to raise Haemoglobin and Haematocrit levels, the most important indicators of IDA in the study population. Tolerability was also evaluated. *Materials and methods:* 30 subjects, post-menopausal females, were enrolled in an open multi-center study. The subjects were administered Turbofer®, twice daily, independently by food intake, for eight weeks. The subjects included suffered from iron deficiency anaemia (Haemoglobin < 11,5 g/dl) and had already been treated with other iron supplements or drugs experiencing side effects. Laboratory tests were performed at visit 1 (baseline) and visit 2 (eight weeks after beginning treatment) together with registration of side effects (signs and symptoms) caused by previous and actual iron treatments by means of Gastrointestinal Sign and Symptoms Score. *Results:* The mean level of Haemoglobin at the beginning of the study was 10.65 ± 0.59 and 12.77 ± 1.08 at the end of the study. This increase is highly significant ($p<0.00001$). Also the value of Haematocrit showed a highly significant rise ($p<0.00001$) from 33.32 ± 2.78 to 38.95 ± 2.92 in the population study. There were no dropouts and Turbofer® was very well tolerated by all subjects, with a marked decrease of the gastrointestinal symptoms compared to previous iron treatments. *Conclusion:* 8 weeks of Turbofer® daily supplementation significantly rise the Haemoglobin and the Haematocrit level. Microencapsulated iron in liposomal form improves iron absorption leading to high bioavailability: as a result, Haemoglobin increase quickly and Turbofer® treatment does not cause the stomach upset and constipation associated with the use of other iron tablets, that is often responsible for the poor compliance.

Key words: iron deficiency anaemia, iron, Haemoglobin, Haematocrit

Introduction

The World Health Organization (WHO) considers iron deficiency the number one nutritional disorder in the world. As many as 80% of the world's population may be iron deficient, while 30% may have iron deficiency anaemia (IDA) (1, 2).

Anaemia, defined as a low blood Haemoglobin concentration, has been shown to be a public health problem that affects low-, middle- and high-income countries and has significant adverse health consequences, as well as adverse impacts on social and economic development (3).

Although the most reliable indicator of anaemia at the population level is blood Haemoglobin concentration, measurements of this concentration alone do not determine the cause of anaemia. Anaemia may result from a number of causes, with the most significant contributor being iron deficiency. Approximately 30%- 50% of cases of anaemia are considered to be due to iron deficiency (1-3).

Even though anaemia occurs in all ages, racial, and ethnic groups, women of childbearing age, pregnant women, preterm and low birth weight infants, older infants and toddlers, and teenage girls are at greatest risk of developing iron deficiency because they have the greatest need for iron (4-7).

The goal of treatment of IDA is to increase the amount of iron providing oral iron supplements to restore normal storage levels of iron, ferritin and to replenish Haemoglobin deficits. Consequently, the amount of oxygen that blood can carry increases by raising the red blood cell count and/or Haemoglobin level. Moreover, it is important to treat the underlying cause of the anaemia.

Tolerance of oral iron treatments is generally low and the frequency and severity of side effects, especially gastrointestinal (GI) (i.e., nausea, vomiting, dyspepsia, constipation, diarrhea, dark colored stools, abdominal distress) often leads to poor compliance (4).

Turbofer® is formulated with microencapsulated iron pyrophosphate in liposomal form, a water dispersible micronized source of iron that has been microencapsulated to enhance iron absorption and to reduce both GI side effects and undesirable organoleptic attributes. Turbofer® contains 14 mg iron in liposomal form, 80 mg vitamin C, 2.5 mcg vitamin B_{12} and 200 mcg folic acid in sticks with orodispersable granulate which directly dissolves in the mouth without the need for water and therefore suitable also for those who have trouble swallowing

An open multicentre clinical study has been conducted to demonstrate efficacy and tolerability of Turbofer®.

The study has been carried out in accordance with the ICH Topic E6 (R1)(CPMP/ICH/135/95) Guideline for Good Clinical Practice and the principles enunciated in the Declaration of Helsinki and the approval by an Institutional Ethics Committee.

Methods

30 subjects, post-menopausal female, 45-65 years of age, were enrolled according to the following inclusion criteria:

- iron deficiency anaemia (Haemoglobin <11.5 g/dl);
- subjects able to provide written informed consent;
- subjects already treated with other iron supplements or drugs that experienced side effects (not including allergy) related to iron administration.

Subjects were instructed to take the food supplement, twice daily, once in the morning and once in the evening, independently by food intake as Turbofer® has no interaction with food.

The treatment lasted eight weeks, with a week washout period from other possible treatments containing iron. Out of 30 patients, 20 remembered the previous iron therapy as in Table 1.

During the 2 visits, baseline (Visit 1) and end of study (Visit 2), eight weeks after the beginning of the treatment, laboratory tests were performed together

Table 1. Previous treatment of Iron

Patients	Iron salt
12	Iron Sulphate
5	Iron Gluconate
2	Iron Saccharate
1	Iron Polymaltose

with registration of side effects (signs and symptoms) caused by previous (Visit 1) and Turbofer® (Visit 2) iron treatments by means of Gastrointestinal Sign and Symptoms Score.

Results

Efficacy

The results of the study have been analyzed by descriptive statistics (means, standard deviations and percentages) using the Student t-test for paired samples with a significance value of p <0.05:

- **Haemoglobin**: almost all subjects show a highly relevant Haemoglobin increase (Fig. 1).
  The mean level at the beginning of the study was 10.65 ± 0.59 g/dl and 12.77 ± 1.08 g/dl at the end of the study, after 8 weeks of Turbofer® supplementation: this increase was highly significant (p<0.00001) and higher than the planned level of significance.
- **Haematocrit**: almost all subjects show a relevant Haematocrit increase (Fig. 2).
  The mean level % at the beginning of the study was 33.32 ± 2.78 and 38.95 ± 2.92 at the end of the study, after 8 weeks of Turbofer® supplementation: this increase was highly significant (p<0.00001) and higher than the planned level of significance.

Tolerability

Turbofer® was very well tolerated by all subjects: no patient discontinued the treatment due to side effects.

The following outcomes were registered:

- Turbofer® significantly (p<0.05) reduces the occurrence of the following symptoms: nausea, vomiting, bloating, abdominal cramps, early satiety, acid eructation/ heartburn, sickness, loss of appetite, retrosternal discomfort, epigastric or upper abdominal pain, constipation (Fig. 3).
  9 out of the 12 symptoms reported with previ-

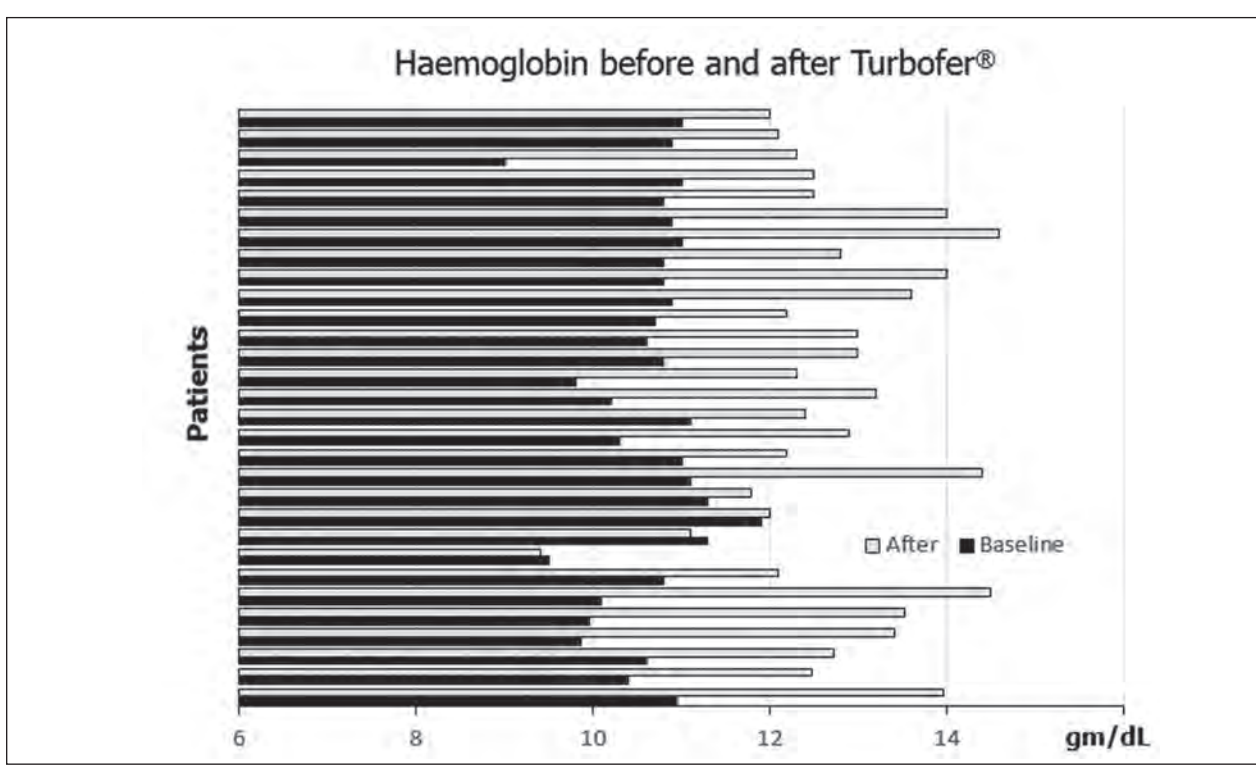

Figure 1. Haemoglobin measurements per patient at Baseline and After 8 weeks of Turbofer® supplementation (g/dL)

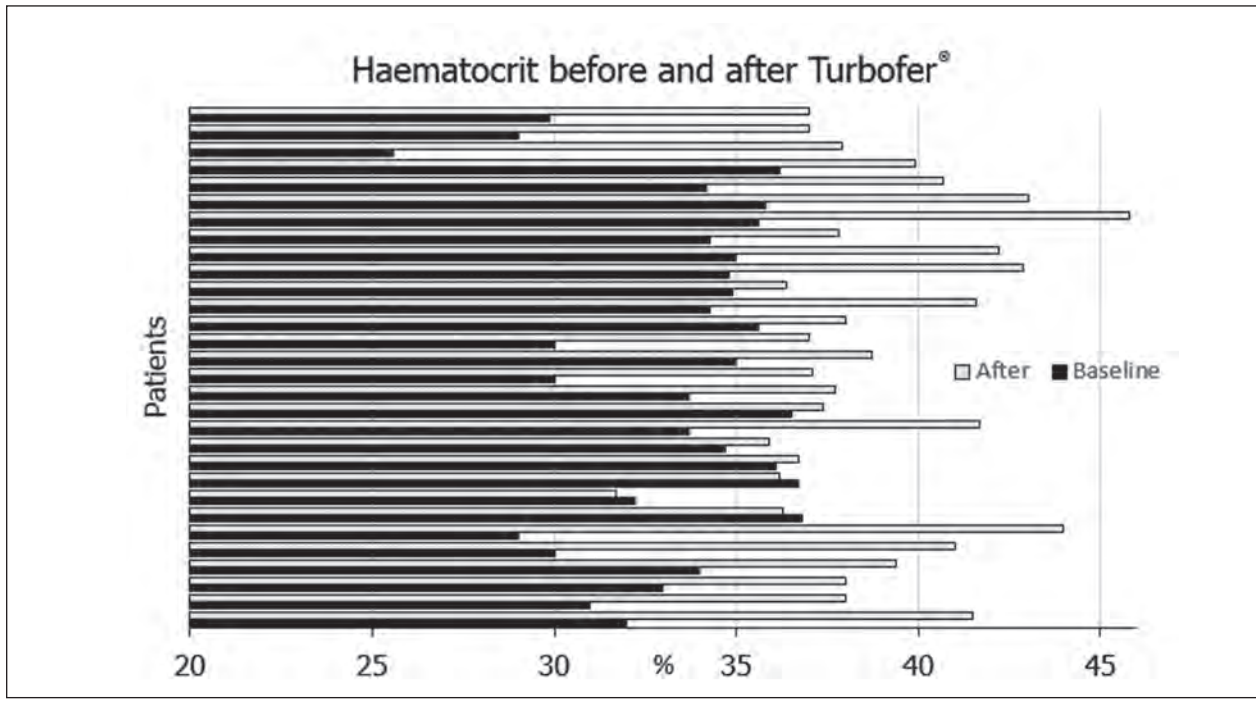

Figure 2. Haematocrit measurements per patient at Baseline and After 8 weeks of Turbofer® supplementation (%)

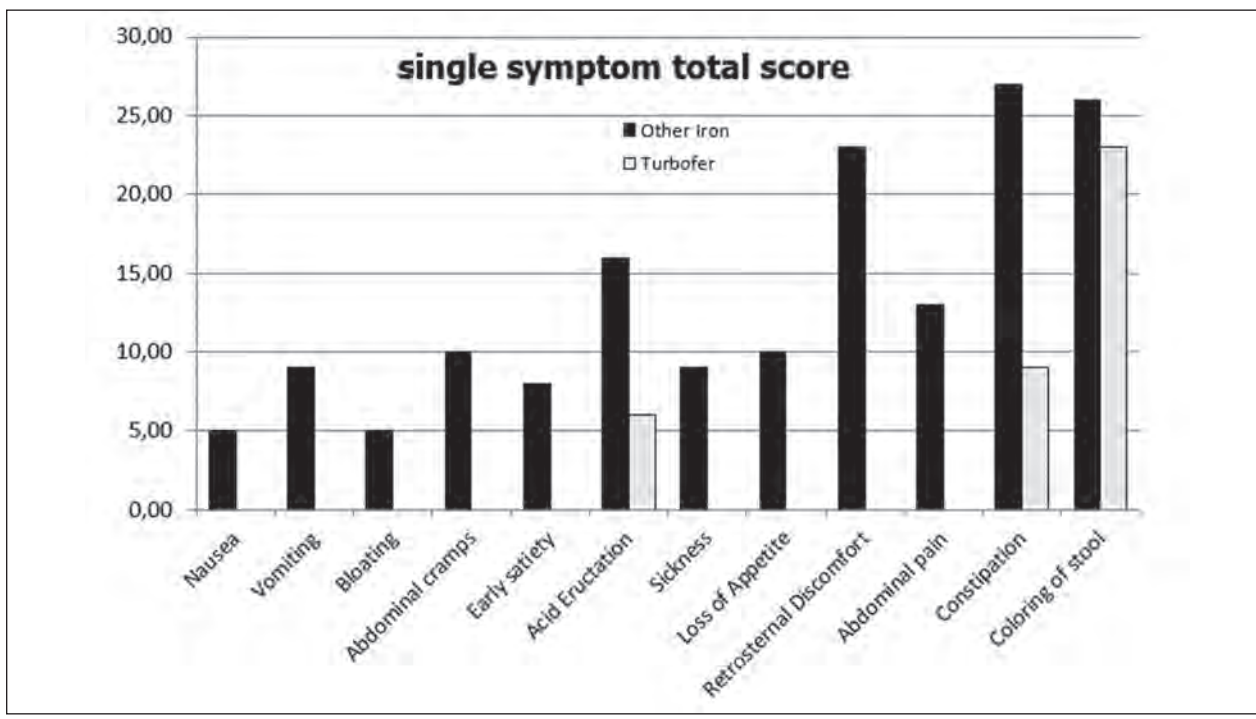

Figure 3. Outcomes for all single side effect total score from previous iron treatment to the end of 8 weeks of Turbofer® supplementation

ous iron therapy were not reported with Turbofer® supplementation.

- The Signs and Symptoms mean Average Total Score was 6.19 with previous iron treatment. After 8 weeks of Turbofer® the Signs and Symptoms mean Average Total Score was 1.46 (Fig. 4), significatively reduced.
- Ten patients reported "epigastric pain" with previous iron treatment, pain that did not occur with Turbofer® (Fig. 5). Noteworthy, epigastric pain is the most frequent side effect usually related to other iron treatments, leading to poor compliance and to discontinue treatment.

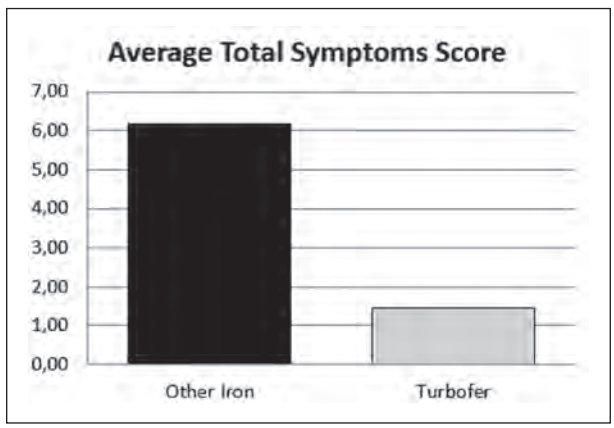

Figure 4. Signs and Symptoms Average Total Score per patient. From previous iron treatment to the end of 8 weeks of Turbofer® supplementation

The most frequent side effect recorded with Turbofer® was stool coloring that has been evaluated as mild in a 5-points Likert scale. This side effect has no impact on bowel function or on treatment efficacy.

Conclusion

In this study 8 weeks of Turbofer® daily supplementation significantly rises the Haemoglobin and the Haematocrit level in female iron deficiency anaemia.

Microencapsulated iron in liposomal form improves iron absorption leading to high bioavailability: as a result, Haemoglobin (a measure for circulating iron) increases significantly and quickly.

Turbofer® significantly reduces the occurrence of the gastrointestinal signs and symptoms caused by other iron treatments and registered at baseline. Noteworthy, epigastric pain, which is the side effect more

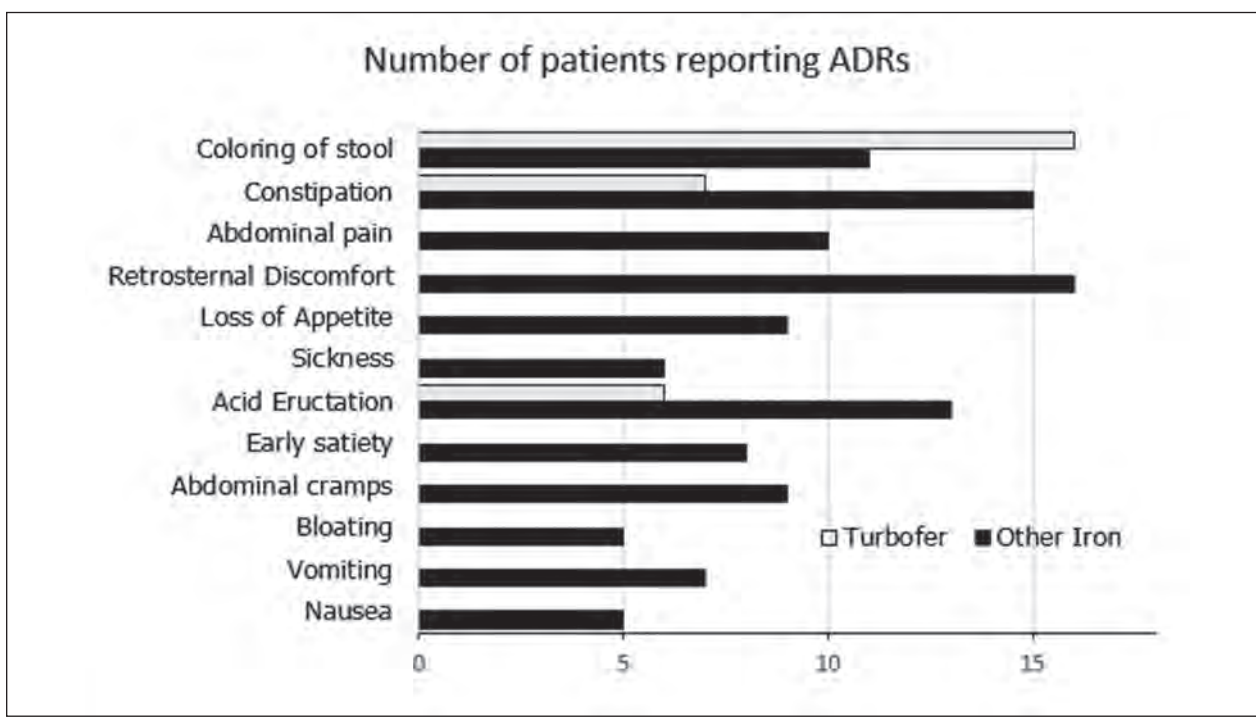

Figure 5. Number of patients per side effects at Baseline (previous iron treatment) and After 8 weeks of Turbofer® supplementation

frequent related to other iron treatments, disappeared during Turbofer® daily supplementation. The only side effect registered, coloring of stools, had no impact on bowel function.

Due to the Turbofer® high safety profile, no drop-out for treatment related reason was registered.

It is important to underline the fact that problems of adherence limit the effectiveness of iron supplementation programs with reports suggesting adherence rates of 40-60% in the treatment and prevention of iron deficiency anaemia (8).

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Correspondence:
E-mail: fer.pht@gmail.com

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Original Article

Effect of feeding whole soybean and linseed on milk and Parmigiano Reggiano cheese lipid fraction

Attilio Luigi Mordenti, Nico Brogna, Flavia Merendi, Giorgia Canestrari, Massimo Dall'Olio, Giacomo Biagi, Andrea Formigoni

Dipartimento di Scienze Mediche Veterinarie (DIMEVET), Ozzano Emilia (BO), Italy

Summary. Aim of this study was to assess the effects of feeding whole soybean flakes and whole extruded linseed to dairy cows on milk lipid fraction and Parmigiano Reggiano cheese produced from it; specifically, PUFA and CLA contents were evaluated. For 14 weeks, a herd of 145 cows received a diet with a daily supplementation of 1 kg of whole soybean flakes and 0.3 kg of whole extruded linseed. In the pre-trial period, cows received an isoproteic ration devoid of both seeds. From 20 cows selected from the herd, individual milk, bulk morning milk and vat milk samples of the entire herd were taken every 14 days. After 24 months of aging, cheese samples were taken from twelve cheese wheels, obtained from milk produced at 0, 4, 8, 10, 12 and 14 weeks on the same days as bulk milk and vat milk samples were made. Fatty acid composition of the lipid fraction was determined in all milk and cheese samples. Feeding whole extruded linseed and soybean flakes has determined a decrease in short-chain fatty acids content and an increase in total CLA concentration of the milk. Bulk milk, vat milk and aged cheese analysis samples confirmed a decrease of short-chain fatty acids content in milk and revealed a decrease in n-6/n-3 fatty acid and in saturated/unsaturated fatty acid ratios. In conclusion, feeding a ration including whole soybean flakes and extruded linseed can improve the nutritional characteristics of milk and of Parmigiano Reggiano cheese, particularly with respect to their lipid fractions.

Key words: Dairy cows, milk quality, cheese quality, polyunsaturated fatty acids (PUFA), conjugated linoleic acids (CLA)

Introduction

According to the World Health Organization, cardiovascular diseases today represent the greatest threat to human health and will continue to do so in the upcoming years. Standing alongside them in terms of severity and dissemination are various forms of cancer, whose incidence is progressively increasing (1). It is by now widely acknowledged that the combined actions aimed at changing people's lifestyles and diet are effective as part of the strategy to prevent the aforesaid pathologies, in particular those affecting the cardiovascular system. In this context, a foremost role in nutrition is played by lipids, understood both as a source of energy and polyunsaturated fatty acids (PUFA) and a balanced source of PUFA of the n-6 and n-3 series (2).

The fatty acid (FA) content and balance of dietary lipids are qualifying elements of the nutritional properties and health properties of food. As regards milk and dairy products in particular, an adequate presence of long-chain polyunsaturated fatty acids (LCPUFA) and the balance between the n-6 and n-3 series represent characterizing elements of great importance (3).
The factors capable of modulating milk fat quality have been addressed by numerous publications (4-9) and are associated with breed, lactation phase and

diet. Milk lipid fraction of ruminants, it is well known that saturated fatty acids (SFA), with different chain lengths, prevail over PUFA in the balance among the FA of triglycerides.

Among the LCPUFA, conjugated linoleic acids (CLA) are a series of positional isomers of linoleic acid (C18:2), which have biological activities that are beneficial for health. The properties of CLA include (10-14) anti-carcinogenic, anti-atherogenic, anti-adipogenic, immunomodulating and anti-diabetic effects. However, the biological activity of CLA that has been most thoroughly investigated is that tied to the protection against the onset of some types of tumors, such as breast and colon cancer, as well as stomach and skin cancer.

It has been demonstrated that the inclusion of naturally CLA-enriched butter in rat diets is capable to reduce the incidence of experimentally induced tumors by about 50% during the development of the mammary gland, thus providing the same results as obtained with administration of synthetic CLA (15). Naturally CLA-enriched butter also contains a higher level of vaccenic acid (C18:1 trans11), which represents another intermediary of ruminal biohydrogenation; since vaccenic acid can be metabolized to CLA through the action of the enzyme Δ9-desaturase, it represents an additional source of CLA for the body (16).

CLA are synthesized into rumen as intermediate products of the process of hydrogenation of LCPUFA, and in particular of linoleic acid (17); as noted, CLA are also produced in the udder and in tissues by desaturation of vaccenic acid due to Δ9-desaturase (18).

In consideration of the continuing ban on using animal meals (fish meal in this specific case) in the diet of ruminants that are a source of food for humans, the dietary enrichment of milk in LCPUFA, and CLA in particular, can be pursued by:

- feeding fresh forages and/or oilseed crops, that are raw materials rich in linoleic and α-linolenic acid and precursors of both LCPUFA and CLA;
- administering microencapsulated feed supplements, with rumen-protected inert material, consisting of substrates rich in PUFA (linseed oil, fish oil, algae, etc.) in order to prevent them from undergoing the alterations otherwise induced by the ruminal bacterial microflora and thus make them available for intestinal absorption (19, 20).

The awareness that, in human nutrition, diets with a generally low n-6/n-3 ratio are to be preferred, has placed zootechnical products obtained from grazing ruminants (4, 8, 21) in a favorable light, as these animals receive diets rich in fresh forages containing good amounts of CLA precursors (3, 21, 22). However, a challenge arises in intensive (or conventional) farming, where animals are fed cereal-rich diets and only rarely significant amounts of fresh forages. Under these conditions, dietary intake of n-3 precursors is significantly reduced, whilst the n-6 FA content increases (23). Given the objective difficulty of reducing dietary intakes of n-6 FA (in any case, sources of corn with a different linoleic acid concentration (24) and of sunflower with a high oleic content (25) are already available on the market), it is evident that the desired decrease in n-6/n-3 ratio can be achieved by increasing n-3; among other things, this would make the characteristics of the milk obtained in intensive farming more similar to those of milk from grass-fed cows.

Aim of this study was to evaluate the possibility of increasing of n-3 PUFA and CLA contents in milk, and thus in the Parmigiano-Reggiano cheese produced from it, by supplementing cows diet with raw materials (whole soybean flakes and whole extruded linseed) containing α-linolenic acid and other CLA precursors, within the limits imposed by Parmigiano Reggiano PDO (Protected Designation of Origin) cheese production regulations (26).

Materials and Methods

The University of Bologna Scientific Ethics Committee on Animal Experimentation Examined and approved the experimental protocol (no. 13825-X/10 All.: 67).

Animals and diet

This study was conducted at the Caretti family dairy farm (S. Giovanni in Persiceto, Bologna), situated within the Parmigiano Reggiano Production Area; milk is produced in accordance with the Parmigiano Reggiano cheese production regulations (26), and is processed in their cheese-making facility (Caseificio S. Angelo – Registration no. 3552).

During the pre-trial period (4 weeks), 145 cows of the Italian Friesian breed had been milked there, in a loose housing barn and divided equally among four boxes; 20 of the lactating cows were selected for milk sampling (5 cows per box) based on age and order of lactation (Table 1).

Prior to the start-up of the trial, representative samples of the batches of forages that would be used during the study were taken with the aid of a core sampler.

The entire herd was fed using the total mixed ration (TMR) technique, with unifeed provided *ad libitum*. Diet was based using high quality forages (mainly alfalfa hays) supplemented with concentrate produced on the farm, based on: corn, barley, sorghum, bran, sugar-beet pulp, soybean meal, minerals and vitamins.

TMR and concentrate compositions, optimized using CPM software (CPM-Dairy V3 Program), are shown respectively in Tables 2 and 3. During the trial period (14 weeks), cows were fed a TMR with the same protein composition as normally used, supplemented with 1 kg/head/d of whole soybean flakes and 0.3 kg/head/d of whole extruded linseed (Table 2).

Table 1. Selected animals features at the beginning of the trial (average ± s.d.)

Animals	n	20
Delivery	n	3.14 ± 2.01
Days of lactation	n	71.48 ± 39.66
Body Condition Score	pts	2.80 ± 0.14
Milk yield	kg/head/d	39.74 ± 6.81
Fat	%	3.39 ± 1.07
Protein	%	3.31 ± 0.29
Casein	%	2.60 ± 0.24
Lactose	%	5.05 ± 0.13
Urea	mg/dl	24.63 ± 3.65

Measured parameters

The average daily feed intake (DMI) for each of the four boxes was recorded weekly, as a difference between TMR delivered and refusal before the next TMR was supplied; samples of the TMR were collected, immediately after unloading, for subsequent analytical determinations to be performed in the Department of Veterinary Medical Sciences laboratories.

Every month, individual milk production of the 20 selected cows was measured and evening milk samples were collected on a biweekly basis, for the determination of fat, protein, casein, lactose and urea content, as well as the FA composition.

Every two weeks, a bulk milk sample, from each box, was taken in the morning upon delivery of milk to cheese-making facility and a vat milk sample, from four vats: these samples were analyzed to determine fat, protein, casein, lactose and urea content and FA composition.

During the trial, to evaluate cheese yield, the amount of milk processed in four different cheese vats was recorded weekly. Weights of the "twin cheese wheels" obtained after 24 hours of processing were subsequently recorded; moreover, samples of vat milk and of cream naturally skimmed were collected on a weekly basis. Data concerning bulk milk, vat milk and cream samples were recorded throughout the trial period.

After 2 years of aging, on a monthly basis, two of the twelve twin cheese wheels obtained from four

Table 2. Composition of total mixed ration technique

Period		Pre-trial	Experimental
Alfalfa hay	kg/head/d	10.00	10.00
Alfalfa hay (1° cut)	kg/head/d	5.00	5.00
Whole soybean flakes	kg/head/d	-	1.00
Whole extruded linseed	kg/head/d	-	0.30
Concentrate	kg/head/d	12.30	11.00
Water	kg/head/d	6.00	6.00

Table 3. Composition of concentrates

Period		Pre-trial	Experimental
Corn	%	40.00	40.00
Sorghum	%	20.00	20.00
Barley	%	6.90	14.90
Sugar beet pulps	%	11.00	11.00
Soft wheat bran	%	11.00	11.00
Soybean meal	%	8.00	-
Sodium bicarbonate	%	1.00	1.00
Sodium chloride	%	0.90	0.90
Calcium carbonate	%	0.70	0.70
Magnesium oxide	%	0.30	0.30
Trace elements and vitamins	%	0.20	0.20

experimental vats were sampled for determining fatty acid profile of lipid fraction of the aged cheese.

Chemical analyses

TMR samples were analyzed to determine dry matter, nitrogen, ether extract and starch content in accordance with AOAC guidelines (27; method 930.15 for dry matter, method 954.01 for nitrogen, method 920.39 for ether extract, and method 920.40 for starch). Fiber fraction was determined using the method described by Van Soest et al. (28), whereas the method of Licitra et al. (29) was used to determine soluble nitrogen.

With regard to analytic determinations performed on milk samples, fat, protein, casein, lactose and urea contents were quantified using a Milko Scan (Foss Electric, Hillerod, Denmark). FA composition was determined by gas chromatography (GC), using the method described by Christie (30), on extracted lipids [method described by Folch et al. (31) and methylated lipids (method ISO 15884)].

GC was performed with a Fisons HRGC MEGA2 series 8560 gas chromatograph with autosampler and Fisons Chrom-Card software; the chromatograph is equipped with a 100-meter-long Varian, CP-SIL 88 WCOT Fused Silica capillary column with an internal diameter of 0.25 mm and internal film thickness of 0.2 μm. The initial column temperature was 45°C for 8 min, followed by an increase of 12°C/min, isotherm at 173°C for 47 min, increase of 4°C/min and final isotherm at 220°C for 30 min; injector temperature 250°C and (FID) detector temperature 270°C. The pressure of the carrier gas (helium) was 215 kPa and 1 microliter of sample was injected with a split ratio of 50:1. The individual FA were expressed as a percentage of total FA.

Statistical analysis

All data obtained were subjected to one-way ANOVA for repeated-measures, with time as the main effect; Dunnett test was used for post hoc analysis. For all individual data, the individual cow was used as experimental unit, the box for feed consumption (n=4); as regards cheese-making data, bulk milk samples (n=4), milk vat samples (n=4), cream and cheese samples (n=4) represented the experimental unit. Differences where $P \leq 0.05$ were considered statistically significant relative to the pre-trial period. Statistica 10.0 software (StatSoft Italia, Vigonza (PD), Italy) was used for the analysis.

Results

Zootechnical and production parameters

Characteristics of the rations: chemical composition of the TMR sampled weekly is shown in Table 4, whereas Table 5 shows the FA content of the pre-trial diet and the experimental one.

Feed intake and individual production: DMI was not influenced by inclusion of whole soybean flakes and whole extruded linseed (24.1 ± 0.9 vs 24.2 ± 1.1 kg d.m./d. in pre-trial and trial periods, respectively).

Milk production of selected cows (Table 6) became significantly lower during trial period as the stage of lactation advanced.

Individual and bulk milk samples quality: the fat content of both individual and bulk milk samples was significantly lower in the period of supplementation, as were urea concentrations, which were lower than in the pre-trial phase (Table 6 and 7); this could be due to the lower solubility of provided proteins.

Table 4. Analytical composition of diets (average ± s.d.)

Period		Pre-trial	Experimental
Analysis	n.	4	14
Dry matter	%	83.45 ± 2.95	80.27 ± 3.16
Protein	%	14.62 ± 0.68	14.40 ± 0.82
Ether extract	%	2.81 ± 0.13	3.71 ± 0.23
Soluble protein	%	3.65 ± 0.31	3.48 ± 0.44
NDIP	%	3.66 ± 0.82	3.41 ± 0.71
ADIP	%	0.83 ± 0.28	0.98 ± 0.22
Ash	%	6.98 ± 0.51	7.84 ± 0.43
NDF	%	38.01 ± 3.03	39.67 ± 2.99
ADF	%	23.51 ± 1.48	22.46 ± 1.78
ADL	%	4.07 ± 0.45	4.29 ± 0.39
Starch	%	23.45 ± 1.89	24.39 ± 2.02
Ca	%	0.73 ± 0.07	0.75 ± 0.08
P	%	0.41 ± 0.05	0.38 ± 0.09

Table 5. Fatty acids composition of diets (% of total fatty acids)

Period	Pre-trial	Experimental
Analysis, n	2	2
C12:0	0.5	0.3
C14:0	1.1	0.7
C16:0	18.1	14.1
C16:1	0.9	0.5
C18:0	2.4	3.2
C18:1trans11	0.1	0.1
C18:1cis	13.6	17.0
C18:2	44.5	39.8
C18:3	15.5	22.2
Others	3.4	2.2

Table 6. Milk yield and quality of the selected animals

Period		Pre-trial	Experimental	Pooled SEM	ANOVA P
Samples, n	-	40	160	-	-
Milk yield	kg/d	39.49	35.44	1.321	≤0.001
Fat	%	3.58	3.48	0.022	≤0.05
Protein	%	3.26	3.27	0.012	n.s
Casein	%	2.55	2.57	0.009	n.s.
Lactose	%	5.04	5.03	0.005	n.s.
Urea	mg/dl	25.79	24.12	0.315	≤0.01

Table 7. Analytical composition of bulk milk

Period		Pre-trial	Experimental	Pooled SEM	ANOVA P
Samples, n		8	32	-	-
Fat	%	3.67	3.60	0.021	≤0.05
Protein	%	3.27	3.22	0.029	n.s.
Casein	%	2.48	2.47	0.029	n.s.
Lactose	%	4.96	4.95	0.018	n.s.
Urea	mg/dl	24.23	23.00	0.713	≤0.01

Cheese-making parameters

Vat milk quality and cheese yields: milk fat content in cheese vat decreased in the period of whole soybean and linseed supplementation (Table 8), whereas no significant differences were observed for protein and casein concentrations. Results in terms of cheese yield are similar to those obtained in other trials we conducted on the same farm (32, 33); it is worth pointing out a significant decrease of cheese yield in experimental period, which may be attributed to the lower protein and fat contents of vat milk.

GC determination of fatty acids in milk and cheese

Individual and bulk milk samples: a comparison between the FA concentrations of individual samples in trial period versus pre-trial period (Table 9) shows that the content of short-chain SFA was significantly reduced (P≤0.01). In contrast, α-linolenic acid

Table 8. Analytical composition of vat milk and cheese yield

Period		Pre-trial	Experimental	Pooled SEM	ANOVA P
Samples, n		8	32	-	-
Fat	%	2.68	2.60	0.021	≤0.05
Protein	%	3.28	3.26	0.029	n.s.
Casein	%	2.54	2.51	0.029	n.s.
Lactose	%	5.03	5.00	0.018	n.s.
Urea	mg/dl	25.33	23.45	0.713	≤0.001
Cheese yield	kg/100kg	8.15	7.80	0.087	≤0.001

Table 9. Fatty acids composition (% of total fatty acids) of selected cows

Period	Pre-trial	Experimental	Pooled SEM	ANOVA P
Samples, n	40	160	-	-
C12:0	3.98	4.04	0.404	n.s.
C14:0	12.06	12.90	0.953	n.s.
C16:0	33.07	33.89	1.800	n.s.
C18:0	9.76	9.25	1.225	n.s.
C18:1 trans 11	0.47	0.72	0.194	n.s.
C18:1 n-9	19.78	19.14	2.155	n.s.
C18:2 n-6	2.64	2.53	0.207	n.s.
C18:3 n-3	0.67	0.80	0.060	≤0.05
CLA tot	0.33	0.49	0.054	≤0.05
Short chain	7.01	5.65	0.411	≤0.01
Medium chain	22.24	23.21	1.854	n.s.
Long chain	70.75	71.14	1.878	n.s.
Saturated	75.52	75.86	2.612	n.s.
Unsaturated	27.54	27.62	2.271	n.s.
Monounsaturated	23.50	23.39	2.148	n.s.
Polyunsaturated	1.40	1.71	0.091	≤0.01
n-6/n-3	0.08	0.06	0.011	≤0.01

and cis-9, trans-11 CLAs concentrations significantly increased (P≤0.05). Moreover, n-6/n-3 ratio underwent a significant decrease (P≤0.01) compared to pre-trial period. *Cream samples*: dietary treatment had no effect on FA composition of cream (results omitted), that were in line with what reported by Verardo et al. (34).

Milk vat samples: vat milk analysis showed some differences compared to pre-trial period (Table 11); it is worth highlighting, in particular, the reduction (P≤0.05) in linoleic acid content and increase in CLA concentration (P≤0.05), confirming what was observed in individual and bulk milk samples.

Cheese samples after 24 months of aging: cheese lipid fraction analysis (Table 12) revealed a significant decrease in short- and medium-chain SFA (P≤0.05 and P≤0.001, respectively), as well as an increase in unsaturated fatty acids (UFA) (P≤0.01), monounsaturated in particular (P≤0.001).

A significant decrease linoleic and α-linolenic acid concentrations was also observed (P≤0.001); the content of these PUFA in aged cheeses was lower than that in cheese obtained in pre-trial period. With regard to total CLA, significant differences in favor of the trial period were maintained (P<0.05).

The trend in both SFA/UFA and n-6/n-3 FA ratios in aged cheese was particularly interesting: in fact, a statistically significant decrease (P≤0.001) in both ratios was observed.

Discussion

Zootechnical and production parameters

Feed intake and individual production: soybean and linseed supplementation did not negatively impact feed intake, contrary to what was observed by other authors (35), who observed a lower DMI in dairy cows receiving three different amounts (78, 142 and 209 g/kg d.m. of the diet) of whole crushed linseed. DMI recorded during trial is to be considered normal for production levels and type of unifeed fed. Forages were finely chopped in order to limit the possibility for cows

Table 10. Fatty acids composition (% of total fatty acids) of bulk milk

Period	Pre-trial	Experimental	Pooled SEM	ANOVA P
Samples, n	8	32	-	-
C12:0	4.17	4.63	0.710	n.s.
C14:0	13.64	14.98	1.726	n.s.
C16:0	35.29	31.58	2.617	≤0.05
C18:0	9.18	9.99	1.542	n.s.
C18:1 trans 11	0.48	0.75	0.083	n.s.
C18:1 n-9	22.45	22.58	3.582	n.s.
C18:2 n-6	2.65	2.32	0.199	n.s.
C18:3 n-3	0.73	0.73	0.068	n.s.
CLA tot	0.37	0.50	0.056	≤0.05
Short chain	5.69	6.49	0.532	n.s.
Medium chain	20.92	22.77	2.733	n.s.
Long chain	73.39	70.74	2.969	n.s.
Saturated	71.08	70.91	3.593	n.s.
Unsaturated	28.92	29.09	3.523	n.s.
Monounsaturated	25.02	25.37	0.310	n.s.
Polyunsaturated	3.90	3.73	0.532	n.s.
n-6/n-3	3.01	2.62	0.152	n.s.

Table 11. Fatty acids composition (% of total fatty acids) of the vat milk

Period	Pre-trial	Experimental	Pooled SEM	ANOVA P
Samples, n	8	32	-	-
C12:0	3.77	4.46	0.458	n.s.
C14:0	12.99	14.99	1.303	n.s.
C16:0	35.26	33.68	2.084	n.s.
C18:0	9.86	9.74	1.276	n.s.
C18:1 trans 11	0.51	0.73	0.177	n.s.
C18:1 n-9	22.16	21.23	2.750	n.s.
C18:2 n-6	2.79	2.26	0.237	≤0.05
C18:3 n-3	0.79	0.73	0.061	n.s.
CLA tot	0.34	0.46	0.050	≤0.05
Short chain	5.99	6.44	0.485	n.s.
Medium chain	19.90	22.20	1.947	n.s.
Long chain	74.11	71.37	2.091	n.s.
Saturated	71.01	72.19	2.572	n.s.
Unsaturated	28.99	27.81	2.572	n.s.
Monounsaturated	24.88	24.14	2.487	n.s.
Polyunsaturated	4.10	3.67	0.299	n.s.
n-6/n-3	2.84	2.62	0.173	n.s.

to select among unifeed and also to increase DMI and could have effect in reducing rates of biohydrogenation associated with UFA, thereby increasing the transit time of feed.

Quality of individual, bulk and vat milk samples: the decrease in lipid content in individual (P≤0.01), bulk (P≤0.05) and vat (P≤0.05) milk samples is in line with what has been reported by other authors (20, 36), who observed a decrease in milk fat in cows fed 2.0 kg of extruded soybean and DHA-based rumen-protected product, respectively. In general, many studies (37; 10, 38, 39) have found a reduction in lipid content of cow's milk when feed supplements containing significant amounts of PUFA are used, and this is attributable precisely to the synthesis of CLA, the trans-10, cis-12 isomer in particular.

The decrease of urea levels in milk we observed could be attributed to the use of lots of hays with particularly high levels of degradable fiber and hence of energy for cellulosolytic bacteria growth, which, as is well known, use ammonia as their principal source of nitrogen: the farm uses hot-air forage dryers (55-60°C) in order to obtain dehydrated forages of excellent quality; data obtained are wholly comparable to the ones we obtained in ours previous trials (40).

GC determination of fatty acids in milk and cheese

Individual, bulk and vat milk samples: Tables 9, 10 and 11 show the FA composition of individual, bulk and VAT milk samples respectively. The decrease of short-chain FA content is in agreement with what was observed by Cavalieri et al. (41), who had fed dairy cows with whole linseed (11.5% of d.m. of unifeed). With regard to the increased concentration of cis-9, trans-11 CLA, similar results were obtained by Moallem (42) consequently the administration of higher amounts (700 g/head/d) of extruded linseed (700 g/head/d) to dairy cows.

Cheese samples after 24 months of aging: the lipid fraction analysis of cheeses (Table 12) revealed a signi-

Table 12. Fatty acids composition (% of total fatty acids) of the Parmigiano Reggiano cheese

Period	Pre-trial	Experimental	Pooled SEM	ANOVA P
Samples, n	2	12	-	-
C12:0	4.37	3.95	0.028	≤0.001
C14:0	12.39	12.08	0.042	≤0.01
C16:0	27.90	29.93	0.048	≤0.001
C18:0	9.08	7.51	0.028	≤0.001
C18:1 trans 11	0.93	1.14	0.033	n.s.
C18:1 n-9	16.23	16.34	0.067	≤0.01
C18:2 n-6	2.76	2.06	0.012	≤0.001
C18:3 n-3	0.65	0.67	0.003	n.s.
CLA tot	0.26	0.38	0.021	≤0.05
Short chain	3.73	3.58	0.032	≤0.05
Medium chain	20.95	20.11	0.081	≤0.001
Long chain	75.32	76.32	0.111	≤0.01
Saturated	61.07	60.18	0.102	≤0.01
Unsaturated	38.93	39.82	0.102	≤0.01
Monounsaturated	35.44	36.95	0.107	≤0.001
Polyunsaturated	3.48	2.87	0.047	≤0.01
n-6/n-3	3.39	3.38	0.029	≤0.001

ficant decrease, compared to pre-trial period, in short- (P≤0.05) and medium-chain (P≤0.001) fatty acids; on the whole, the medium-chain fatty acids content was comparable with that reported by Prandini et al. (43), who evaluated the acidic profile of numerous samples of Grana Padano cheese lipid fractions, produced in different seasons of the year. PUFA content (linoleic and α-linolenic acid) in aged cheeses was lower (P≤0.001) than observed in cheeses produced in pre-trial period. As a possible justification for this unexpected finding, it must be remembered that, in Parmigiano Reggiano production, the long aging (more than 24 months) induces an extremely reducing environment within the cheese, which may be cause saturation phenomena involving UFA. This would confirm how much production technology can influence FA composition of the cheese (44).

A significant increase was observed in total CLA content, likely with what was affirmed by other authors (45) who assessed the effects resulting from the administration of 300 g/head/d of extruded linseed meal; moreover, these FA concentrations are completely in line with what has been reported in the literature for various types of aged PDO cheeses (8).

The trend in both the SFA/ UFA and n-6/n-3 FA ratios in aged cheese (Table 12) was particularly interesting: there was a statistically significant decrease (P≤0.001) in both ratios, and this trend was maintained throughout the trial period. Similar findings were reported by other authors who used whole linseed (46).

Conclusions

The results of this study show that adding whole soybean flakes and whole extruded linseed to the diet of dairy cows may significantly modify the lipid fraction of milk.

In particular, supplementing the diet with soybean and linseed determined a decrease in the milk fat (individual and bulk) and short-chain FA content, an increase n-3 FA relative to n-6 FA content and a significant increase in the total CLA concentration.

The increase in total CLA concentration and their precursors in milk seems to confirm that feeding a diet containing whole soybean flakes and whole extruded linseed leads to an increase in CLA concentration as a result of high dietary amounts of linoleic and α-linolenic acid.

Taking into consideration the entire production chain of Parmigiano Reggiano PDO cheese, from the bulk milk, through natural skimming, to the vat milk and, finally to the aged cheese, it may be affirmed that aging substantially influenced cheese FA composition, which differed from milk it was obtained from. The experimental diet caused about an overall decrease of short- and medium-chain SFA contents, an increase in total CLA amount and a significant improvement in n-6/n-3 and SFA/UFA ratios, thus bringing milk and cheese closer to meet nutritional requirements of wholesomeness increasingly demanded by consumers. In conclusion, these results enable us to affirm that it is possible to modulate milk lipid fraction intended for cheese production by means of a targeted and appropriate diet, whilst remaining within the limits on the use of raw materials imposed by Parmigiano Reggiano PDO cheese production regulations (26).

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Correspondence:
Attilio Luigi Mordenti
Via Tolara di sopra, 50 - 40064
Ozzano Emilia (BO)
Tel. 0512097381 Fax. 0512097373
E-mail: attilio.mordenti@unibo.it

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Original Article

Effects of yogurt dietary supplementation on the intestinal ecosystem of a population of Emperor tamarins (*Saguinus imperator*)

Carlo Pinna[1], Eleonora Nannoni[1], Giulia Rigoni[1], Monica Grandi[1], Carla Giuditta Vecchiato[1], Caterina Spiezio[2], Camillo Sandri[2], Giacomo Biagi[1]

[1] Department of Veterinary Medical Sciences, University of Bologna, Italy; [2] Research and Conservation Department and Veterinary Department, Parco Natura Viva, Bussolengo, Italy.

Summary. Providing hidden food is a method of nutritional and environmental enrichment for captive animals and yogurt is sometimes used with this purpose for non-human primates. Objective of the present study was to evaluate the effect of feeding fresh yogurt on the intestinal ecosystem of Emperor tamarins (*Saguinus imperator*). A population of nine adult/juvenile emperor tamarins received during the whole trial a diet mainly consisting of different fruits. During the first 30 d, the diet did not contain any yogurt; during the following 28 d, every two days, a total of 300 g of fresh fruit yogurt was provided to the animals. A fresh fecal sample was collected from each animal the day before administration of yogurt started (Day 0) and again after 21 and 28 days for chemical and bacterial determinations. Throughout the study, all tamarins remained in good health and no clinical signs of intestinal discomfort were observed. During yogurt supplementation, fecal pH, moisture and ammonia resulted unchanged respect to the beginning of the study. Similarly, fecal volatile fatty acids were not affected by the yogurt intake. On the contrary, fecal spermine concentration resulted significantly decreased at Day 28 respect to Day 0 (4.4 *vs*. 30.1 nmol/g of feces; $P < 0.05$). Furthermore, the consumption of yogurt resulted in reduced fecal concentrations of coliforms, enterococci and lactobacilli on Rogosa Agar (respectively, -1.9, -1.5 and -2.8 log CFU/g of feces; $P < 0.05$). Results from the present study showed that emperor tamarins can tolerate high amounts of yogurt in their diet without showing any signs of lactose malabsorption (for example, soft feces or diarrhea). On the other hand, yogurt ingestion failed to exert any major influence on the animals' intestinal microbiota.

Key words: Intestinal ecosystem, lactose, *Saguinus imperator*, tamarins, yogurt.

Introduction

During the last decades, yogurt and similar fermented milk products have gained popularity in human nutrition for their beneficial properties and, today, they are considered as functional foods. In fact, yogurt, besides being an excellent source of calcium and vitamins, can exert probiotic effects as it contains live lactic acid bacteria (LAB). Yogurt LAB are in general represented by cultures of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* (1) that, if found alive in adequate numbers, might improve consumer's intestinal health acting as probiotic strains (2). Lactose is the disaccharide that is found in milk and can be absorbed only after hydrolysis to glucose and galactose by β-galactosidase (lactase), an enzyme produced in the intestinal mucosa of young mammals. However, lactase production strongly decreases after weaning in mammals and it is believed that only some human populations retain the ability to digest lactose during adulthood (3). Before fermentation, yogurt contains about 6% of lactose which is only partially (20-30%) hydrolyzed by bacteria during the fermentation process (4). As such, yogurt ingestion by individuals lacking lactase might lead to lactose maldigestion and intolerance. At the same time, incomplete or absent digestion of lactose may provide a natural

prebiotic contributing to improved balance of the intestinal microbiota (5).

Environmental enrichment has become a fundamental part of management of captive animals, in order to enhance their physical and psychological well-being. Environmental enrichment has been defined as "an improvement in the biological functioning of captive animals resulting from modifications to their environment" (6) and involves the practice of increasing the physical, social and temporal complexity of captive environments (7). More recently, the Enrichment Working Group of the Behavior and Husbandry Advisory Group, a scientific advisory group of the American Zoo and Aquarium Association, defined enrichment as "a dynamic process in which changes to structures and husbandry practices are made with the goal of increasing behavioral choices available to animals and drawing out their species-appropriate behaviors and abilities, thus enhancing animal welfare" (8). Providing hidden food is one of the methods that can be used for environmental enrichment. Furthermore, a feeding enrichment program might include foods that are not always available and are occasionally added to a stable, nutritionally complete diet. For example, yogurt is sometimes used with this purpose for non-human primates. Nevertheless, at present, little is known about the effect of feeding food containing lactose to adult monkeys and apes and about the ability of these animals to tolerate lactose in their diet.

The objective of the present study was to evaluate the effect of feeding fresh yogurt on the intestinal ecosystem of a population of emperor tamarins (*Saguinus imperator*).

Materials and methods

The Ethical Committee of the University of Bologna reviewed and approved the experimental protocol.

The present study was conducted at Parco Natura Viva, Bussolengo (Italy) and involved a population of eleven emperor tamarins.

The family group, comprised of a single breeding adult male and female, along with the independent (seven juvenile subjects) and dependent (two infants) offspring of the breeding pair, was housed in a facility consisting of an indoor area connected (via guillotine doorways) to an outdoor enclosure. The indoor-outdoor enclosure measured more than 60 m^2 (the outdoor being 27 m^2) and was approximately 8 m high. The enclosure allowed and promoted a full range of naturalistic behaviors, social interactions, and locomotion patterns. The primary furnishing for these animals' housing were natural tree branches that were arranged to provide a network of pathways by which the animals can move. Branches are particularly important because tamarins use these as the normal substrate for scent marking. Food and water were made available on a feeding platform and in bowls placed high in the cage in a location that prevented contamination by urine and feces. Prior to the study, adult and juvenile animals received a diet mainly consisting of different fruits (about 250 g of fruit per animal per day, divided in two meals: 150 g in the morning and 100 g in the afternoon). Moreover, animals received each day live larvae of *Tenebrio molitor* or, alternatively, *Zophobas moiro* as a source of dietary protein (about a table spoon of larvae for each animal) and gum arabic (about a table spoon for each animal). Both live larvae (placed in paper cups) and gum arabic (dispensed into holes of a wooden structure in order to simulate the presence of tree exudates) were hidden in order to encourage search for food by the tamarins. Once or twice a week, animals received other foodstuffs including a mineral-vitamin supplement for primates, carbohydrates (noodles, rice or legumes) and protein (eggs, turkey meat, fresh cheese or yogurt) sources and vegetables (carrots, tomatoes, cucumbers and more).

During the whole study, tamarins kept receiving the same diet that has already been described but, during the first 30 d, the diet did not contain any yogurt, fresh cheese or other lactose-containing ingredient. During the following 28 d, every two days, a total of 300 g of fresh fruit yogurt was provided to the animals within 20 paper cups (each paper cup contained approximately 15 g of yogurt). Once the yogurt had been dispensed, paper cups were sealed with paper tape and placed all over the outdoor enclosure. Paper cups were removed after 24 h. Fresh fruit yogurt was a commercial product (containing 21% strawberries and cultures of *Streptococcus thermophilus* and *Lactobacillus bulgaricus*) that was provided by the seller every 10 d (during which it was stored at 0-4°C) in a 5 kg container (in total, 3 containers were used throughout the study). At the opening, a sample of yogurt from each container was collected and stored at 4°C for bacterial (lactobacilli and streptococci) determination.

A fresh fecal sample was collected from each adult and juvenile animal the day before administration of yogurt started (Day 0) and again after 21 and 28 days. Individual fecal samples were collected in sterile containers and frozen at -18°C within 20 min after excretion for chemical (pH, moisture, ammonia, volatile fatty acids, biogenic amines) and bacterial determinations. No fecal samples were collected from infant tamarins.

Chemical and microbiological analysis

Fecal moisture was determined according to AOAC standard method (9). Fecal ammonia was measured using a commercial kit (Urea/BUN – Color, BioSystems S.A., Barcelona, Spain). Volatile fatty acids (VFA) in feces were analyzed by gas chromatography (10). For the determination of biogenic amines, samples were diluted 1:5 with perchloric acid (0.3 M); biogenic amines were later separated by high performance liquid chromatography and quantified through fluorimetry, according to the method proposed by Stefanelli et al. (11).

The day after collection, yogurt samples were serially diluted with Wilkins-Chalgren Anaerobe Broth (WCAB, Oxoid LTD, Basingstoke, Hampshire, UK), added with L-cysteine HCl (0.5 g/L) and plated in triplicate onto selective media: MRS Agar (Oxoid LTD) for lactobacilli and ST Agar (12) for streptococci. Average bacterial counts were 5.72 and 5.46 log CFU/ml of yogurt on MRS Agar and ST Agar, respectively.

Within 10 days from collection, individual fecal samples were homogenized, serially diluted with anaerobe half-strength WCAB and plated in triplicate onto selective media: MacConkey Agar (Merck, Darmstadt, Germany) for coliforms, OPSP Agar (Oxoid LTD) for *C. perfringens*, LAMVAB Agar (13) and Rogosa Agar (Oxoid LTD) for lactobacilli, and Azide Maltose Agar (Biolife, Milano, Italy; added with triphenyl tetrazolium chloride at 10 ml/L) for enterococci. MacConkey Agar and Azide Maltose Agar plates were incubated aerobically at 37°C for 24 and 48 h, respectively; all other media were incubated anaerobically at 37°C for 48 h.

The Fluorescence In Situ Hybridization technique was used to determine counts of bifidobacteria. For this purpose, a ready-to-use commercial kit (BioVisible BV, Groningen, The Netherlands) containing specific FITC-labeled probes for the enumeration of *Bifidobacterium* spp. was used. The slides were evaluated with a Nikon Eclipse E-600 epifluorescence microscope, equipped with an FITC specific filter.

Statistical analysis

Data were analyzed by repeated measurements ANOVA with time as the main effect; each animal formed an experimental unit. Differences among means of groups were analyzed using the Student-Newman-Keuls test. Differences were considered statistically significant at $P < 0.05$.

Results

Throughout the study, all tamarins remained in good health and no clinical signs of intestinal discomfort were observed (for example, soft feces or diarrhea). Yogurt was always completely consumed by tamarins and no leftovers were found when paper cups were removed from the animals' cage.

Chemical analyses of fecal samples are presented in Table 1 (pH, moisture, ammonia and VFA) and Table 2 (biogenic amines). After 21 and 28 d, fecal pH, moisture and ammonia were not different than before yogurt administration. Similarly, fecal VFA were not influenced by treatment. Among biogenic amines, fecal spermine was significantly lower at 28 d than at trial start (-85%; $P < 0.05$).

Bacterial populations in fecal samples are reported in Figure 1. After 28 d, compared with fecal bacterial populations before yogurt administration started, fecal counts of coliforms, enterococci and lactobacilli on Rogosa Agar were significantly lower (-1.9, -1.5 and -2.8 log CFU/g of feces; $P < 0.05$).

Discussion

For 28 d, each adult/juvenile tamarin received, every two days, about 30-35 g of fresh yogurt. In the present study, lactose content of yogurt was not determined; still, based on literature, lactose concentration in yogurt varies from 25 to 40 g per kg of yogurt (4, 14) which means that, every two days, each monkey presumably ingested an amount of lactose comprised between 0.8 and 1.4 g of lactose. Human beings are considered to be the only

Table 1. pH, moisture and concentration of ammonia and volatile fatty acids (VFA)[1] in feces of emperor tamarins after 0, 21 and 28 d of inclusion of fresh yogurt in their diet.

Item	Day 0	Day 21	Day 28	ANOVA, P value	Pooled SEM
pH	7.42[ab]	7.94[b]	6.78[a]	0.024	0.27
Moisture, g/100 g of feces	81.6[ab]	85.4[b]	79.0[a]	0.041	1.62
Ammonia, µmol/g of feces	119	111	108	0.135	3.81
VFA, µmol/g of feces					
Acetic acid	73.4	66.0	71.7	0.732	6.92
Propionic acid	14.5	10.1	13.7	0.210	1.78
n-Butyric acid	7.41	5.97	6.04	0.720	1.40
iso-Butyric acid	0.83	0.78	0.97	0.888	0.36
Total VFA	95.4	82.6	92.4	0.633	9.57

[1]Values are means of nine animals; [a,b]Within the same row, means without a common letter differ (P < 0.05)

Table 2. Concentration of biogenic amines (nmol/g of feces)[1] in feces of emperor tamarins after 0, 21 and 28 d of inclusion of fresh yogurt in their diet.

Item	Day 0	Day 21	Day 28	ANOVA, P value	Pooled SEM
Putrescine	107	79	132	0.576	34.6
Cadaverine	72.0	19.1	77.6	0.131	21.2
Spermidine	52.8	50.3	42.0	0.400	5.75
Spermine	30.1[a]	16.4[ab]	4.4[b]	0.047	6.60
Total biogenic amines	262	165	256	0.424	56.9

[1]Values are means of nine animals; [a,b]Within the same row, means without a common letter differ (P < 0.05)

mammals that show persistence of lactase in adulthood, whereas in other mammal species lactase activity falls prior to adulthood (15). Nevertheless, to our knowledge, little is known about lactase activity in adult nonhuman primates and, in particular, in tamarins. In human medicine, the lactose load is one method of diagnosis of lactose intolerance and is usually performed in adult patients with amounts of lactose up to 12 g which account for 0.02% of body weight of an adult human being (16). Considering that the average body weight of an adult tamarin is around 300-400 g, in the present study, monkeys were eating a considerable amount of lactose (between 0.3 and 0.5% of their body weight) without showing any clinical signs of malabsorption. In this respect, it has been shown in humans that fermented milk products such as yogurt can improve lactose digestion due to the presence of LAB producing β-galactosidase (17). After 28 d of yogurt supplementation, fecal moisture was significantly lower than at 21 d. Conversely, no difference was observed between fecal moisture detected during the supplementation period and the beginning of the trial. Based on these results, it is not possible to link the variations in fecal moisture content with lactose ingestion.

The ingestion of yogurt, a foodstuff containing a combination of viable LAB and lactose (a potential synbiotic, according to the definition given by Schrezenmeir and De Vrese (18)), was expected to reduce bacterial proteolysis, thus reducing fecal ammonia concentrations and pH. The latter effect was expected also as the consequence of lactose fermentation by LAB with production of lactic acid and VFA (19). However, in the present study, ingestion of yogurt did not influence fecal pH and ammonia concentrations. In fact, after 28 d of yogurt administration, fecal pH, moisture and ammonia were not different from values obtained at the end of the adaptation phase (Day 0), during which animals had received for 30 d a diet not containing yogurt or any other source of lactose.

In the present study, fecal concentrations of VFA were not influenced by yogurt ingestion. Among VFA, acetic acid accounted for more than 75% of total VFA and, throughout the study, the acetic to propionic acids ratio was comprised between 5:1 and 6:1. This high ra-

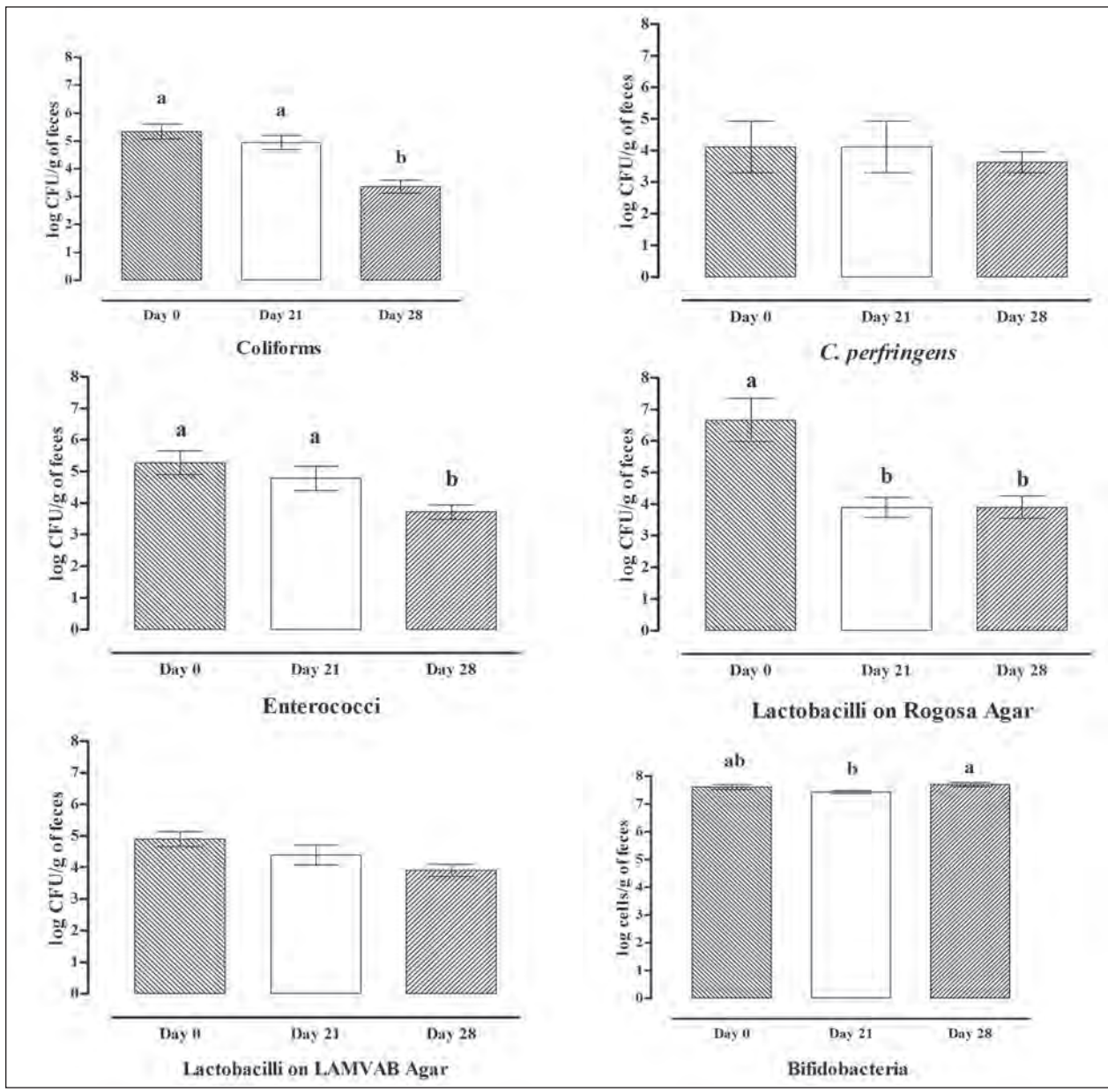

Figure 1. Bacterial populations in feces from emperor tamarins after 0, 21 and 28 d of inclusion of fresh yogurt in their diet.
[a,b]Within the same graph, columns without a common letter differ (P < 0.05).

tio may be explained by the fact that tamarins consumed a diet containing high amounts of fruits. In fact, bacterial fermentation of soluble fiber (such as pectins) that is found in fruits leads in general to the production of acetic acid, as already observed in humans (20), rats (21) and dogs (22). On the contrary, fermentation of lactose by LAB usually results in increased concentrations of lactic acid (23) which is later converted to propionic acid by lactate utilizers (24).

Consumption of yogurt for 28 d resulted in reduced fecal spermine, but did not influence concentrations of other biogenic amines. Since biogenic amines are produced during protein fermentation, reduction of spermine may be interpreted as a sign of reduced intestinal bacterial proteolysis.

In general, findings from the present study regarding the effect of yogurt supplementation on fecal bacterial metabolites seem to highlight that the presence in

the tamarins' diet of large amounts of soluble highly fermentable fiber from fruits may overwhelm the influence of yogurt on the animals' intestinal ecosystem. Moreover, it is known that concentrations of bacterial metabolites that are absorbed by the intestinal mucosa decrease while digesta move along the intestine. As such, feces might not reflect the changes in the concentration of ammonia, VFA and biogenic amines that yogurt might have induced in the hindgut (25).

In other animal species, the consumption of yogurt or similar fermented dairy products resulted in changes of metabolic activities of the intestinal microbiota, as reported by some authors. In a study by Djouzi et al. (26), human flora-associated rats fed for six weeks a diet containing 30% of fermented milk (in presence of a strain of *L. casei* and yogurt starters) showed a significant increase of fecal acetate, propionate and butyrate. In another study (27), the consumption of 125 g/d of fresh yogurt for one month by healthy infants, did not affect fecal pH, water content, and concentrations of VFA but resulted in decreased concentrations of branched-chain fatty acids, the latter being considered a marker of proteolytic fermentation.

There is enough evidence that the utilization of probiotic bacteria and prebiotic substances represents an effective strategy to modulate the gastrointestinal ecosystem of humans (28, 29) and mono-gastric animals (30), increasing the abundance of beneficial bacteria and reducing the presence of undesired microbes. In the present study, after 28 d of yogurt supplementation, compared with counts at trial start, fecal coliforms were significantly lower whereas *C. perfringens* counts were not affected. Coliform bacteria, including *Escherichia coli*, are microorganisms that are commonly found in the intestine of humans and animals where, usually, they are not harmful; however, coliforms are undesired microbes as they include pathogens. In a study with human volunteers conducted by Chen et al. (31), the ingestion of yogurt increased the counts of anaerobic bacteria, suppressed aerobic bacteria and significantly elevated the bifidus to coliform ratio. Furthermore, the elevated bifidus to coliform ratio gradually diminished after yogurt consumption was discontinued. Beneficial effects deriving from yogurt consumption on fecal microflora composition were reported by other authors, who observed increased concentrations of LAB (32), enterococci and lactobacilli (27) and bifidobacteria (33, 34). In the present study, compared with values at trial start, fecal counts of enterococci and lactobacilli grown on Rogosa agar showed a significant reduction at 28 d. This result is surprising considering the fact that fresh yogurt used in the present study was a source of viable cells of *Streptococcus thermophilus* and *Lactobacillus bulgaricus*. Furthermore, after 28 d of yogurt administration, fecal counts of bifidobacteria were slightly higher than at 21 d but unchanged respect to the beginning of the trial. Nowadays, modern molecular identification methods have been developed to achieve a better understanding of the composition of intestinal microbiota of animals; however, Azide Maltose Agar (also known as KF Streptococcal Agar) is still used for the selective isolation and enumeration of enterococci and fecal streptococci (35), whereas Rogosa (1) and LAMVAB (36) agar are still proposed for the enumeration of lactobacilli.

Conclusions

Results from the present study showed that adult and juvenile emperor tamarins can tolerate very high amounts of yogurt in their diet without showing any signs of lactose malabsorption. However, ingestion of yogurt, despite resulting in decreased fecal concentrations of spermine and coliform bacteria, failed to exert any major influence on the animals' intestinal microbiota.

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Correspondence:
Prof. Giacomo Biagi
Department of Veterinary Medical Sciences,
Alma Mater Studiorum, University of Bologna,
Via Tolara di Sopra 50, 40064 Ozzano dell'Emilia (BO).
Tel: +39 051 2097379 - Fax: +39 051 2097373
E-mail: giacomo.biagi@unibo.it

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Original article

Protective effect of kaempferol, a flavonoid compound, on oxidative mitochondrial damage in streptozotocin-induced diabetic rats

Govindasamy Chandramohan, Khalid S. Al-Numair, Chinnadurai Veeramani, Mohammed A. Alsaif, A. Madi Almajwal

Department of Community Health Sciences, College of Applied Medical Sciences, King Saud University, Saudi Arabia

Summary. The present study was designed to investigate the effect of kaempferol on oxidative mitochondrial damage in liver of streptozotocin (STZ)-induced diabetic rats. Diabetes was induced into adult male albino rats of the Wistar strain, by intraperitoneal administration of STZ (40 mg/kg body weight (BW)). Kaempferol (100 mg/kg BW) or glibenclamide (600 μg/kg BW) was administered orally once daily for 45 days. Diabetic rats showed a significant elevation of mitochondrial thiobarbituric acid reactive substances (TBARS) levels in liver as compared to control rats. The level of enzymatic (superoxide dismutase (SOD), glutathione peroxidase (GPx)) and non-enzymatic (reduced glutathione (GSH)) antioxidants were decreased significantly in liver mitochondria of STZ-induced diabetic rats as compared to control rats. Administration of kaempferol or glibenclamide resulted in significant decrease in TBARS and significant increase in SOD, GPx and GSH when compared to diabetic control rats. The activities of mitochondrial enzymes such as isocitrate dehydrogenase (ICDH), alpha-ketoglutarate dehydrogenase (α-KGDH), succinate dehydrogenase (SDH), and malate dehydrogenase (MDH) decreased significantly in STZ-induced diabetic rats. In addition, the activities of mitochondrial respiratory chain enzymes such as NADH dehydrogenase and Cytochrome c-oxidase also decreased significantly in STZ-induced diabetic rats as compared to control rats. Administration of kaempferol or glibenclamide resulted in significant reversal of these enzymes' activities to near normal when compared to diabetic control rats. Thus, obtained results indicate that administration of kaempferol attenuates the mitochondrial damage in STZ-induced diabetic rats.

Key words: albino Wistar rats, streptozotocin, diabetes, mitochondrial damage, kaempferol

Introduction

Diabetes mellitus (DM) is characterized by hyperglycemia due to defective insulin action, insulin secretion or both. The World Health Organization reports that the number of diabetics is expected to increase to 366 million or more by 2030 from 171 million in 2000 (1). Hyperglycaemia is associated with the generation of reactive oxygen species (ROS) and consequent oxidative damage in the liver, kidneys, heart, and pancreas (2). Implication of oxidative stress in the pathogenesis of diabetes is suggested not only by oxygen free radical generation due to non-enzymatic protein glycosylation, auto-oxidation of glucose, impaired glutathione metabolism, alteration in antioxidant enzymes and decreased level ascorbic acid (3, 4). Increased oxidative stress plays an important pathogenic role in the development and progression of diabetes and its complications (5).

Mitochondria are dynamic organelles that not only produce ATP for cellular function, but also participate in a number of intracellular processes such as cell division, the initiation of mitochondrial signaling pathways, modulation of cytosolic metabolic pathways and ultimately determination of cell life or death. In addition, mitochondria are a continuous source of su-

peroxide anions ($O_2^{\cdot -}$) and their ROS products during cell injury (6-8). Hyperglycemia-induced ROS generation within mitochondria plays a major role in the development of diabetic complications (9). Mitochondria are one of the most important cell organelles in diabetes research because of its crucial role as a regulator of energy balance (10). Various NAD/NADP-linked enzymes are intricately involved in the maintenance of the reduced redox state in mitochondria in order to provide the reducing power to generate adenosine triphosphate (ATP) via oxidative phosphorylation (11). DM is associated with mitochondrial dysfunction that may result in increased ROS generation and impaired bioenergetics (12).

Medicinal plant drug discovery continues to provide new and important leads against various pharmacological targets including diabetes (13). Bioflavonoids are currently considered as promising natural substances to develop a modern therapy for diabetes (14). Kaempferol (Fig. 1), a flavonoid, naturally occurs in a variety of fruits, vegetables, wine, and tea. It can be isolated from tea, broccoli, witch-hazel, propslis, grapefruit, and other plants (15). The medicinal properties of kaempferol include antioxidant, anti-inflammatory and anticancer activity (16-18). Several studies have shown that intake of foods containing kaempferol is associated with reductions in mortality, the incidence of myocardial infarction, and the incidence of cerebrovascular disease, as well as with a slightly reduced risk of coronary heart disease (19-21). Previously, in an *in vitro* study, it was shown that kaempferol ameliorates hyperglycemia by improving insulin-stimulated glucose uptake in adipocytes (22). Kaempferol also performs a beneficial role in diabetes by preventing oxidative damage in pancreatic β cells (23). Our previous *in vivo* study found that the administration of kaempferol is having good antihyperglycemic and hypolipidemic activities on STZ-induced diabetic rats (14).

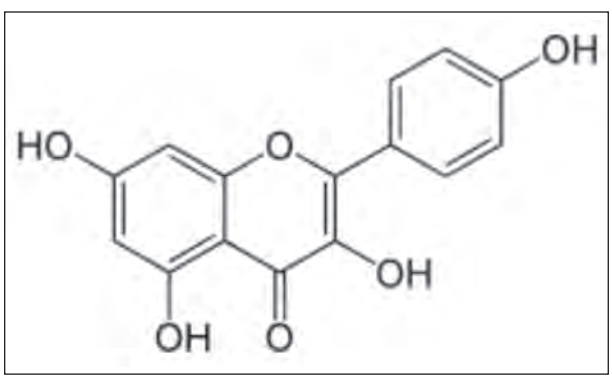

Figure 1. Chemical structure of kaempferol

So far no study has been conducted on the effect of kaempferol on oxidative mitochondrial damage in STZ-induced diabetic rats. Hence, in the present study we have sought to examine the effects of kaempferol on oxidative mitochondrial damage in liver of STZ-induced diabetic rats.

Materials and Methods

Drugs and chemicals

STZ and kaempferol were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of analytical grade.

Experimental animals

Male albino rats of Wistar strain of body weight (BW) ranging from 180 to 200 g were procured from Central Animal House, King Saud University, and they were maintained in an air-conditioned room (25 ± 1°C) with a 12-h light/12-h dark cycle. The animals were fed *ad libitum* with normal laboratory pellet diet used in the study and procedures involving animals and their care were accordance with the Policy of Research Centre, King Saud University.

Experimental induction of diabetes

The animals were made diabetic by a single intraperitoneal injection of streptozotocin (STZ, 40 mg/kg BW, between 8:00 AM to 9:00 AM) in a freshly prepared citrate buffer (0.1 M, pH 4.5) after an overnight fast. STZ injected animals were given 20% glucose solution for 24 h to prevent initial drug-induced hypoglycaemic mortality. Diabetes was confirmed by measuring the fasting plasma glucose concentration 96 h after induction. Albino rats with a plasma glucose level above 220 mg/dL were considered diabetic and used in this experiment.

Experimental design

The animals were randomly divided into five groups consisting of six animals each. Kaempferol (100 mg/kg BW) or glibenclamide (600 µg/kg BW) was dissolved in 5% DMSO and administered by intubation (p.o.) once a day, between 9 a.m. and 10 a.m., for 45 days. In our previous study, we have chosen 50, 100 and 200 mg/kg doses of kaempferol for tested the glucose lowering action (14). Of the three doses of kaempferol (50, 100 and 200 mg/kg BW), 100 mg gave the maximum improvement in plasma glucose and insulin (14). Hence, the active dose of 100 mg was used in this study.

Group I: Control rats (5% DMSO alone)

Group II: Control rats + kaempferol (100 mg/kg BW)

Group III: Diabetic control

Group IV: Diabetic rats + kaempferol (100 mg/kg BW)

Group V: Diabetic rats + glibenclamide (600 µg/kg BW)

After 45 days administration of kaempferol and glibenclamide, the rats were fasted for 12 h, anesthetized by ketamine (24 mg/kg BW via intramuscular injection) and sacrificed by decapitation. The liver was dissected out immediately and stored for mitochondrial isolation.

Biochemical assays

The mitochondrial fraction of the liver tissue was isolated by the standard method of Johnson and Lardy (24). The concentration of thiobarbituric acid reactive substances (TBARS) in the liver mitochondria fraction was estimated by the method of Niehaus and Samuelsson (25). The activity of superoxide dismutase (SOD) and glutathione peroxidase (GPx) in the liver mitochondrial fraction were assayed by the method of Kakkar et al. (26) and Rotruck et al. (27) respectively. Reduced glutathione (GSH) was estimated by the method of Ellman (28).

The activity of isocitrate dehydrogenase (ICDH), α-ketoglutarate dehydrogenase (α-KGDH), succinate dehydrogenase (SDH) and malate dehydrogenase (MDH) were assayed in the mitochondria fraction of the liver by the method of Bell and Baron (29), Reed and Mukherjee (30), Slater and Borner (31) and Mehler et al. (32) respectively. NADH-dehydrogenase was assayed by the method of Minakami et al. (33) and cytochrome-c-oxidase was assayed by the method of Pearl et al. (34).

Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) using SPSS software package 9.05. Results were expressed as mean ± standard deviation (S.D) from six rats in each group. P values < 0.05 were considered as significant.

Results and Discussion

Diabetes is a complex metabolic disorder with characteristic modulation of glucose metabolism leading to excessive ROS production and generation of various diabetic complications such as nephropathy, neuropathy, cardiopathy and even hepatopathy. The liver is intimately involved in the pathogenesis of diabetes, where hepatic insulin resistance is regarded as a key contributing element to high fasting blood glucose (35) and ketone body formation, and thus to the development of diabetic complications. In insulin dependent diabetes mellitus (IDDM) various agents like interleukin-1 beta, interferon gamma, tumor necrosis factor alpha, alloxan and streptozotocin could operate by forming free radicals that could attack the mitochondrial genome (36). The structural damage of tissues or complications in diabetes mellitus may be due to oxidative stress. Oxidative stress may play an important role in the development of mitochondrial damage associated diabetic complications (37). The superoxide anion radical is generated by liver mitochondria under both physiological and pathological conditions. Table 1 depicts the levels of TBARS, activities of enzymic antioxidants (SOD and GPx) and the levels of non-enzymic antioxidant (GSH) in the liver mitochondria of normal and STZ-induced diabetic rats. In our study mitochondrial levels of TBARS in liver significantly increased in STZ-induced diabetic rats. It indicates that increased level of mitochondrial TBARS in diabetic rats cause lipid peroxidation, which

Table 1. Effect of kaempferol on TBARS and the activities of enzymatic antioxidants and level of GSH in mitochondrial fraction of the liver of STZ-diabetic rats

Groups	TBARS (mmol/mg protein)	SOD (Ua/mg protein)	GPx (Ub/mg protein)	GSH (μg/mg protein)
Control	1.76 ± 0.10[a]	2.85 ± 0.14[a]	7.92 ± 0.68[a]	13.56 ± 1.10[a]
Control + kaempferol (100 mg/kg BW)	1.81 ± 0.14[a]	2.83 ± 0.21[a]	8.05 ± 0.76[a]	13.48 ± 1.02[a]
Diabetic control	3.02 ± 0.28[b]	1.55 ± 0.10[b]	4.78 ± 0.29[b]	8.01 ± 0.64[b]
Diabetic + kaempferol (100 mg/kg BW)	1.62 ± 0.12[c]	2.43 ± 0.22[c]	6.45 ± 0.55[c]	11.93 ± 0.98[c]
Diabetic + glibenclamide (600 μg/kg BW)	1.68 ± 0.15[c,d]	2.80 ± 0.19[a]	6.87 ± 0.44[c]	12.07 ± 1.17[c]

Values are means ± S.D for six rats in each group.
Values not sharing a common superscript differ significantly at p< 0.05 (DMRT).
U[a]- Enzyme concentration required for 50% inhibition of NBT reduction/min.
U[b]- μmol of reduced glutathione consumed/min.

may alter the structure integrity of mitochondrial membranes resulting in mitochondrial dysfunctions. When kaempferol was administered in diabetic rats the level of TBARS significantly reverted to that of near normal rats. Kaempferol has been shown to possess antioxidant and anti-inflammatory effects (16, 17). Thus antioxidative action of kaempferol may have an excellent ability to scavenge hydroxyl and peroxyl radicals.

Increased production of oxygen free radicals in diabetes is suggested by protein glycosylation and auto-oxidation of glucose and decreased availability of enzymatic and non-enzymatic antioxidants (38). SOD catalyzes the conversion of superoxide anion to hydrogen peroxide (H_2O_2). H_2O_2 is cleared from the system by the activity of catalase and glutathione peroxidase. In present study the activities of SOD and GPx were decreased significantly in the liver mitochondria of diabetic rats, which is probably due to an increased generation of accumulation of ROS and severe oxidative stress by STZ. GSH is a major defence mechanism against oxidative stress within mitochondria. DM has been found to profoundly alter mitochondria, including the selective depletion of mitochondrial GSH (39). In our study the level of GSH significantly decreased in liver mitochondria of STZ-induced diabetic rats. It has been suggested that depletion of mitochondrial GSH resulted in depletion of other antioxidants and enhanced the susceptibility of cells to further damage. This condition may aggravate the overproduction of mitochondrial ROS. Administration of kaempferol and glibenclamide to diabetic rats significantly reversed these enzymatic and non-enzymatic antioxidants towards normalcy. Some antioxidants are suggested to have beneficial effects in the treatment of oxidative stress-associated diseases including diabetes (40, 41). Kaempferol has good antioxidant and anti-inflammatory effects (16, 17). The beneficial effects of kaempferol may also be attributed to improved antioxidant activity in tissues, which potentially reduces the membrane lipid peroxides (42).

Mitochondria are important subcellular organelles involved in energy production and are susceptible to oxidative stress. The mitochondrial enzymes (ICDH, α-KGDH, SDH and MDH) catalyze the oxidation of several substrates through the tricarboxylic acid (TCA) cycle, yielding reduced equivalents, which are channelled through the respiratory chain for the synthesis of ATP by oxidative phosphorylation. STZ-elicited ROS leads to oxidative insult, which may be the reason for increased susceptibility of mitochondrial proteins to oxidative damage. Therefore, it is highly probable that STZ-associated elevations in ROS and lipid peroxides might have the effect of inactivating mitochondrial proteins, which would diminish mitochondrial function and ultimately lead to some of the toxic effects that have been observed in the mitochondrion of tissues of diabetic rats. Figure 2 and 3 represents the activities of TCA cycle enzymes and respiratory chain enzymes in the liver mitochondria of normal and STZ-induced diabetic rats. In the present study the activities of mitochondrial enzymes such as ICDH, α-KGDH, SDH, and MDH were decreased significantly in STZ-induced diabetic rats. A decreased activity of

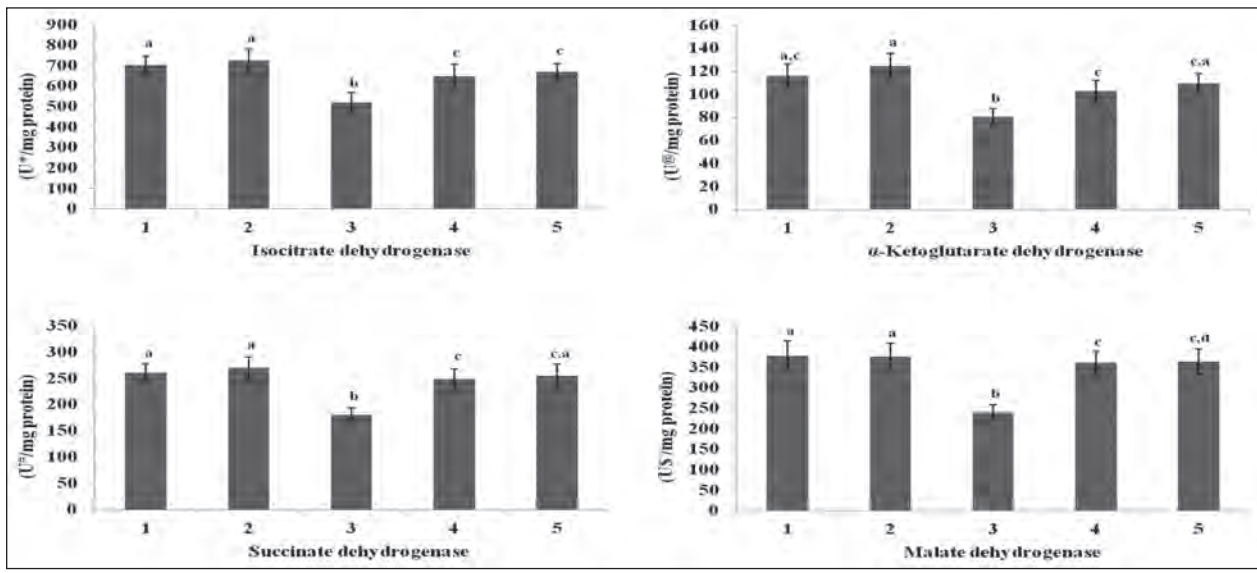

Figure 2. Effect of kaempferol on the activities of mitochondrial respiratory chain enzymes in the liver of STZ-diabetic rats.
Group 1: Control
Group 2: Control + kaempferol (100 mg/kg BW)
Group 3: Diabetic control
Group 4: Diabetic control + kaempferol (100 mg/kg BW)
Group 4: Diabetic control + glibenclamide (600 mg/kg BW)
Values are means ± S.D. for six rats in each groups.
Values not sharing a common superscript differ significantly at $p < 0.05$ (DMRT).
U* – nmol of α-ketoglutarate formed/h; U@ – nmol of ferrocyanide formed/h; U# – nmol of succinate oxidized/min; U$ – nmol of NADH oxidized/min.

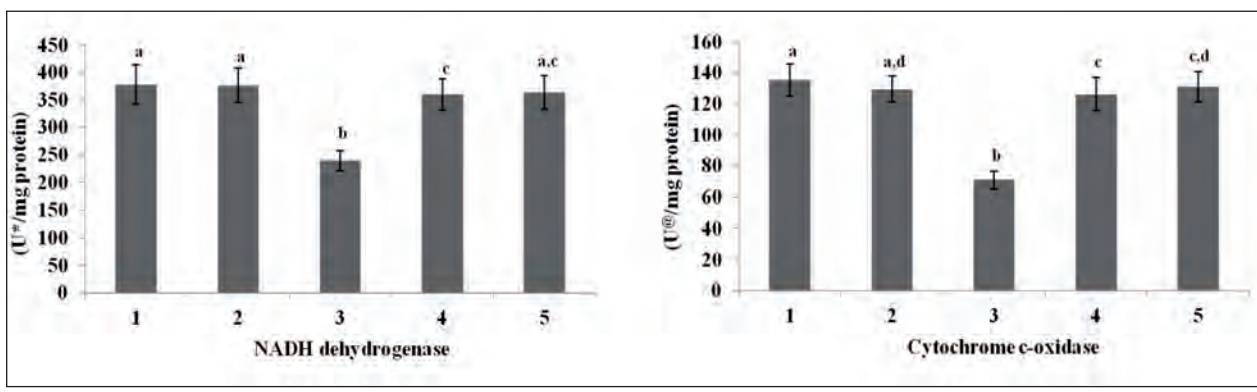

Figure 3. Effect of kaempferol on the activities of mitochondrial tricarboxylic acid cycle enzymes in the liver of STZ-diabetic rats.
Group 1: Control
Group 2: Control + kaempferol (100 mg/kg BW)
Group 3: Diabetic control
Group 4: Diabetic control + kaempferol (100 mg/kg BW)
Group 4: Diabetic control + glibenclamide (600 mg/kg BW)
Values are means ± S.D for six rats in each group.
Values not sharing a common superscript differ significantly at $p < 0.05$ (DMRT).
U* – nmol of NADH oxidized/min; U@ – change in OD x 10^{-2}/min

mitochondrial TCA cycle enzymes has observed in the STZ-diabetic rats (43). Inhibition of these enzymes by ROS may affect the mitochondrial substrate oxidation, resulting in reduced oxidation of substrates, reduced rate of transfer of reducing equivalents to molecular oxygen and depletion of cellular energy (44). Oral administration of kaempferol and glibenclamide to diabetic rats the activities of TCA cycle enzymes significantly reversed to near normal rats. Thus results indicate that kaempferol may improve the mitochondrial antioxidant defence system, and minimizing the mitochondrial damage associated with diabetes complications.

Cytochrome c oxidase and NADH dehydrogenase are present in the inner mitochondrial membrane and are involved in the synthesis of high-energy compound ATP. NADH-dehydrogenase constitute complex I of the electron transport chain, which passes electron from NADH to coenzyme Q. Cytochrome c-oxidase donates electrons directly to molecular oxygen and constitutes complex IV. In the present study, the activity of these enzymes significantly decreased in STZ-induced diabetic rats, which might be due to depletion of reducing equivalents like NADH and NADPH, which are utilized for the formation of reduced glutathione to counter oxidative damage of mitochondrial components. Administration of kaempferol and glibenclamide significantly increased these enzymes activities in STZ-induced diabetic rats due to its free radical scavenging activity.

In conclusion, the results suggest that kaempferol could maintain liver mitochondrial function in STZ-induced diabetic rats. The possible mechanisms for the observed preventive effects of kaempferol could be due to scavenging of free radicals and improving antioxidant status. This study may be beneficial to prevent mitochondrial damage associated with diabetes complications. Further detailed investigation is necessary to understand kaempferol's mechanism of action and establish its therapeutic potential in the treatment of diabetes and diabetic complications.

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Correspondence:
Dr. G. Chandramohan, Ph.D
Assistant Professor
Department of Community Health Sciences
College of Applied Medical Sciences
King Saud University, P.O. Box 10219,
Riyadh 11433 - Saudi Arabia
Fax: +9664678394; Off: +9664697434;
Mobile: +966508393530
E-mail: gcmohanphd@yahoo.com

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Original article

Investigation of the wound healing potential of *Onosma hispidum* root extract in rabbit models

Asad Ali, Fatma Hussain, Muhammad Shahid

Department of Chemistry and Biochemistry, Faculty of Sciences, University of Agriculture, Faisalabad-38040, Pakistan.

Summary. *Onosma hispidum* Wall. (Boraginaceae) has historically been used to treat a wide assortment of diseases. Present study was conducted to investigate the wound healing activity of *Onosma hispidum* root extracts in animal models. Excision wound were inflicted upon four groups of five rabbits each. Group 1 assigned as vehicle (negative control) was treated with petroleum jelly. Group 2 and 3 were treated with *O. hispidum* MeOH and n-hexane extracts. Group 4 (positive control) was accorded standard drug polyfax. Healing was assessed by the percentage of wound contraction and histopathological analysis. Animal models treated with n-hexane and MeOH extracts ointments of *O. hispidum* had significantly ($p < 0.05$) positive healing effects as compared to the negative controls. Significant reduction (54-56%) in terms of percentage wound contractions were observed in plant treated groups as compared to the vehicle control. *O. hispidum* treatment resulted in partially developed dermal layer with stratified squmaous, stratum corneum and stratum germinatium development. Although major bioactive principles involved are yet to be determined, this investigation reveals the potential of *O. hispidum* for use as a natural wound curing agent.

Key words: *Onosma hispidum,* excision wounds, petroleum jelly, wound contraction, histopathology

Introduction

Wound is defined as the loss of breaking cellular and functional continuity of the living tissues. Wounds offer psychosocial issues and their management is frequently encountered with different problems such as drug resistance and toxicity (1).

The modern medicine faces the challenge to pursuit dynamic and low cost therapeutic approaches for wound healing. In this context, plants and their metabolites being great sources of novel biomolecules offer potent treatment options. Today a substantial number of drugs are developed from plants that are active against numerous diseases. About 450 plant species having wound curing properties have been identified (2-8). Majority of these are either the active ingredient or their subsequent modifications. Multiple phytochemicals concentrated and blended in optimal concentrations, are expected to be available in future to optimize wound healing as more curative properties of the key constituents are unveiled (9-11).

The genus *Onosma* L. (Boraginaceae) includes about 150 species distributed worldwide. The plants of this genus possess anticancer, antioxidant, antimicrobial, antipyretic, antidiabetic, antitussive and spasmolytic activities and traditionally used in rheumatism, bladder pain, kidney irritation, palpitation of heart. While roots are used as astringent, demulcent and diuretic agent and to treat hypertension, fever, pain and inflammatory disorders (12-14). *Onosma hispidum* Wall. possess antiseptic, antipyretic, antibacterial, anthelmintic, hypoglycemic and cholinesterase inhibitory activities. Its role in wound healing, foot ulcer, optical diseases, bronchitis and itch is well recognized (15-20).

O. hispidum Wall. is reported to be the source of ratanjot, a red dye yielding root, commonly used for col-

oring food stuffs, oils and medicinal preparations. Owing to its color, it has also been used as an adulterant in spices like chilli powder and food preparations (1).

Wounds are enormous socioeconomic encumber. Development of economical, effective agent with fewer side effects that could preclude hospitalization rate is the prerequisite. Pharmacological objectives of wound healing implicates the assessment of therapeutic options opted to endorse healing. In folklore medicine, *Onosma hispidum* has been used as a wound healer for thousands of years; however, there is a paucity of scientific data in support. With multiple medicinal mechanisms, the natural products demand further endorsement. Herein, we intend to investigate the wound healing activities of MeOH and *n*-hexane root extracts of *O. hispidum* in excision wound-induced rabbit models, so as to provide scientific evidence for the traditional application.

Materials and methods

Plant material and preparation of extract

Onosma hispidum roots were authenticated by the Department of Botany, University of Agriculture, Faisalabad, Pakistan. Shade-dried, powdered material was homogenized with methanol (MeOH) and *n*-hexane. The final yields of MeOH (5.7%) and *n*-hexane (3.6%) extracts were prepared in 0.5% w/v carboxymethylcellulose.

Animal models

Male rabbits (*Oryctolagus cuniculus*) were housed in the metal cages (three per cage) with free access to standard feed, tap water *ad libitum*. The study protocol was approved by the Bioethical Committee, University of Agriculture, Faisalabad, Pakistan.

Determination of Wound Healing Activity

Animals were divided into the four groups (five per group): Group 1 (negative control; vehicle): treated with petroleum jelly without *O. hispidum* extracts; Group 2: treated with petroleum jelly ointment containing *O. hispidum* MeOH extract; Group 3: treated with petroleum jelly ointment containing *O. hispidum n*-hexane extract; Group 4 (positive control): treated with standard drug polyfax (GlaxoSmithKline). Animals were anaesthetized prior to and during excision wound induction. The plant extracts and the standard reference drug (polyfax) were applied once daily till complete healing of the wound. Specimens of skin were isolated from the healed skin of each group for the microanatomy (20-22). Data was analyzed by t-test with significance level set at $P<0.05$ (SPSS; version 12.0, 2003 ® SPSS Inc., Chicago, IL, USA).

Results

The effect of two different preparations, MeOH and *n*-hexane extracts, from *O. hispidum* roots on wound curing were investigated under the established conditions. The measurements of the progression of wound healing induced by the extracts, reference drug, negative and vehicle groups are shown in figure 1. MeOH extracted ointment had highest curing potential after 3 days applications. However, *n*-hexane ointment dominated MeOH in terms of percent wound contraction and it showed competitive potency after 7 and 9 days as compared to the standard ointment. The groups treated with *n*-hexane and MeOH extracts ointments had sig-

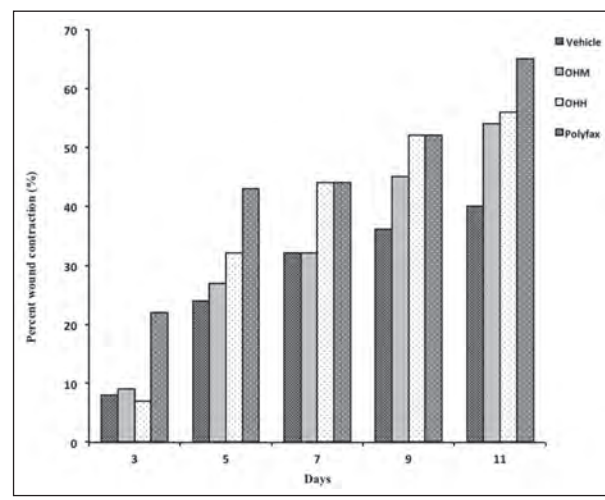

Figure 1. Comparative percent wound contraction of control and treatment groups. Values are averaged percent wound contraction. Vehicle: negative control; OHM: group given *O. hispidum* methanol extract; OHH: group given *O. hispidum* n-hexane extract; Polyfax: group given polyfax skin ointment

nificantly ($p < 0.05$) positive healing impact as compared to the negative controls. At the end of experimental period, measurements of the progression of wound healing induced by the extracts, reference drug and negative groups were done on per diem bases. Wound healing is a natural physiological process. Hence, for untreated animals, 40% contraction was observed. Whereas, in MeOH and *n*-hexane treated models, it was 54 and 56% respectively. Such a remarkable wound restoration by plant extracts were comparable with the results of reference drug (65%).

Wound histology was performed to verify the wound healing activity of the *O. hispidum*. Wound repairing involves regeneration of dermal and epidermal tissue. Therefore, in the first group; epidermis which is composed of stratified squamous epithelium is thin and consists of four layers of stratum corneum and stratum germinatium. The dermal layer which is composed of dense singular connective tissue is partially developed. Papillary layer (subepithelial layer) of dermis has very thin projection in the dermal connective tissue (fig. 2a). In second group (methanol extract treated), proliferation of cells of epidermal layer is quite prominent. Layer of stratified squmaous, stratum corneum and stratum germinatium are developed. Subepithelial layer is prominent. Dermal layer of dense connective tissue is normal in thickness (fig. 2b). In third (*n*-hexane extract treated) group, there is high proliferation of cells of epidermal layer. Layer of stratified squmaous epithelium is fully grown. Layers of stratum corneum and stratum germinatium are fully developed. Subepithelial layer is quite prominent. Dermal layer of dense connective tis-

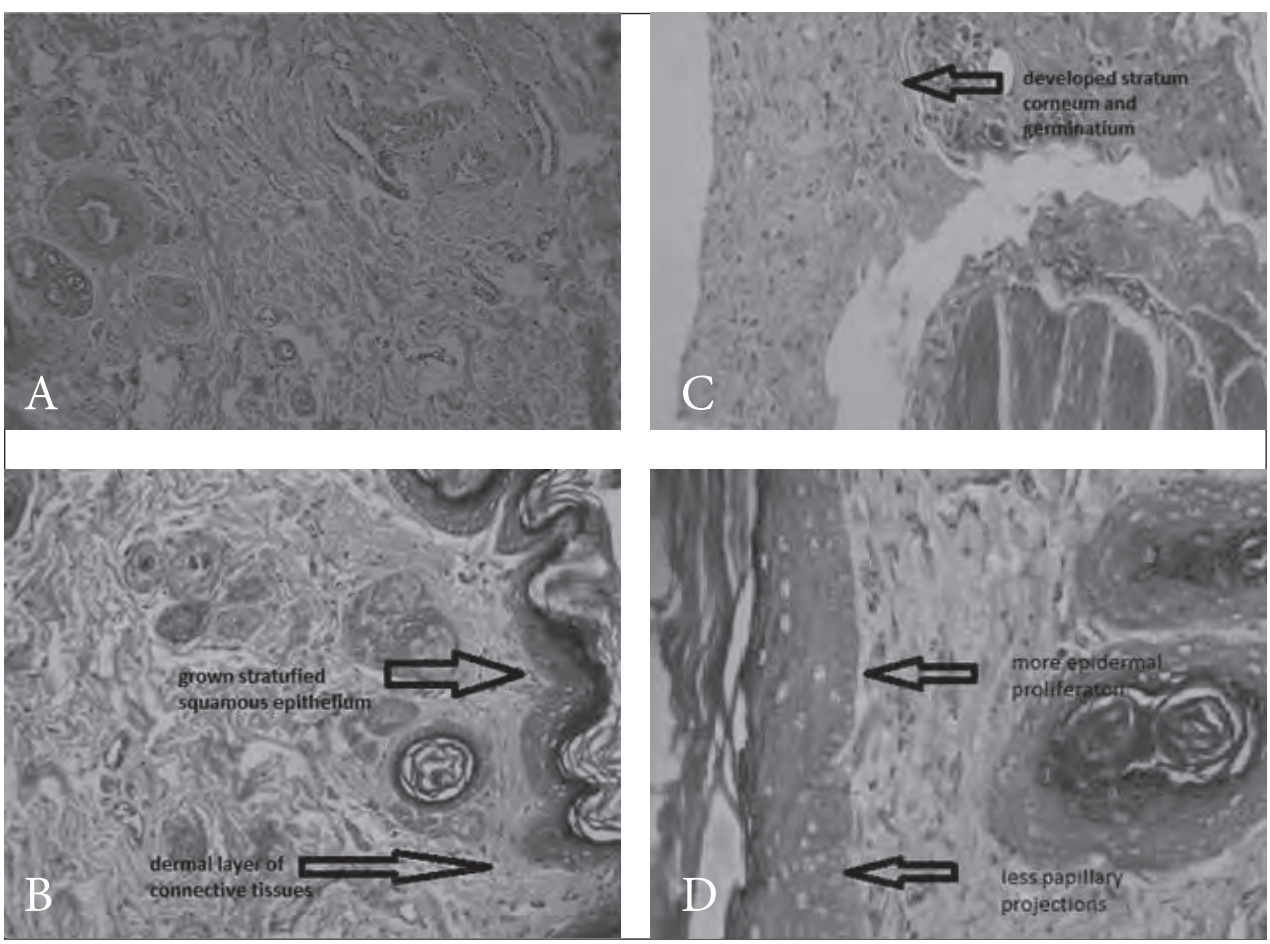

Figure 2. Microscopic view of wound healing. a- wound treated with petroleum jelly with partially developed dermal layer; b & c- methanol and n-hexane extracts treated wounds with stratified squmaous, stratum corneum and stratum germinatium development and dermal layer of connective tissues; d- Polyfax treated wound with epidermal proliferation and fewer papillary projections

sue is fully grown (fig. 2c). While in fourth group (Polyfax treated), proliferation of epidermal layer is relatively more as compared to the rabbits of the control group. Papillary projections into the dermal layer are less as that of control group (fig. 2d). Microanatomy revealed that all the extract treated models showed almost similar regeneration patterns in dermal and epidermal layers as compared to those treated with reference drug.

Discussion

The search for natural remedies has drawn attention to plants. Flavonoids, alkaloids, saponins and phenolic compounds are active constituents that facilitate wound closure. Plants of Boraginaceae family contain naphthoquinones, shikonin and alkannin that contribute wound curing properties. Naphthoquinones are major chemical constituent of *O. hispidum* roots (19).

Present study explored the wound healing activity of *O. hispidum* by *in vivo* models. Wound healing is a complex phenomenon involves various phases e.g. coagulation, inflammation, collagenation, wound contraction and epithelization. Both the extracts showed remedial potency. Current inferences are in accordance with that of previous studies. It was observed previously that *O. hispidum* root methanol extract exhibited significant increase in mean percentage wound contraction and tensile strength in excision and incision wound models in both normal and diabetic rats (20). Moyer et al. (23) stated that *O. hispidum* might provoke faster maturation of granulation tissues. Perhaps it is able to promote the granulation tissue, myofiroblast proliferation and contraction for the faster closure of wound. These pharmaceutical properties of *O. hispidum* may engross experiential wound healing potential. They illustrated that the faster wound contraction rate from the *O. hispidum* treatment may be due to antioxidant, antimicrobial, and antiseptic properties of the plant extract and to stimulation of interleukin-8, an inflammatory *a*-chemokine that affects the function and recruitment of various inflammatory cells, fibroblasts, and keratinocytes. *O. hispidum* may increase the gap junctional intracellular communication in cultured fibroblasts and induce a more rapid maturation of granulation tissue. Contrary to that, negligible restorative effects of *Onosma dichroanthum* roots extracts on the burn wound in animal models were documented (24). Previously, some species of Boraginaceae have shown accelerative effects on granuloma tissues proliferation owing to the presence of naphthoquinone derivatives (25, 26).

Survey of literature has revealed that several Boraginaceae plants *viz. Alkanna tinctoria, Cordia dichotoma, Onosma argentatum, Symphytum x uplandicum, Arnebia densiflora, Heliotropium indicum, Symphytum officinale, Echium amaenum, Helichrysum graveolens* have demonstrated their potent role in wound healing (27-35).

The exact mechanism of current healing by plant extracts is uncertain. Though, contribution of phytoconstituents in wound curing can be narrated in terms of their antimicrobial, antioxidant, mitogenic activities, as free radical scavengers and angiogenesis enhancers. These chemicals especially, naphthoquinones not only affect wound restoration phases positively but also constrain the factors that may dwindle the curative process (10). The antioxidant activity of plants extract may be ascribed to biologically active polyphenolic bioflavonoids that facilitate wound closure. *O. hispidum* extracts demonstrated considerable wound healing activity. Although, which phytochemical(s) of the extracts are responsible for this effect, is not investigated in detail yet (19, 36).

In conclusion, it can be stated that *O. hispidum* roots exhibited promising wound healing properties and data obtained may contribute towards validation of its traditional use for the healing of wounds.

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Correspondence:
Fatma Hussain,
Department of Chemistry and Biochemistry,
Faculty of Sciences, University of Agriculture,
Faisalabad-38040, Pakistan.
E-mail: fatmauaf@yahoo.com

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Original Article

The effect of orange juice against to H_2O_2 stress in *Saccharomyces cerevisiae*

Abdullah Aslan[1], Muhammed Ismail Can[2]

[1]Firat University, Faculty of Science, Department of Biology, Elazig-Turkey; [2]Aksaray University, Department of Biotechnology and Molecular Biology, Aksaray-Turkey

Summary. In this study, seven groups were composed. i: Control group, ii: H_2O_2 group, iii: 5 mM H_2O_2 + orange juice (OJ) group, iv: 10 mM H_2O_2 + OJ group, v: 15 mM H_2O_2 + OJ group vi: 20 mM H_2O_2 + OJ group, vii: 25 mM H_2O_2 + OJ group. After sterilization, fruit juice (25%) and H_2O_2 were inserted different concentration to *Saccharomyces cerevisiae* (*S. cerevisiae*) cultures and the cultures were developed at 37°C for 1h, 3h, 5h and 24 hours (overnight). *S. cerevisiae* cell growth was determined by spectrophotometer, total protein alteration was identified by SDS-PAGE electrophoresis and calculated with biuret method. With respect to our studies results; cell growth rised in fruit juice groups to which OJ was taken in proportion to the positive control (H_2O_2) group at different growing times (1, 3, 5 and 24 hours) ($p<0,05$). As a result orange fruit juices has a protective role for decrease the oxidative damage and increased cell growing and stimulating protein synthesis in *S. cerevisiae*.

Key words: *S. cerevisiae*, orange juice, oxidative damage, protein synthesis, SDS-PAGE

Introduction

Saccharomyces cerevisiae is essential yeast that has been used for a lot of working (1). H_2O_2 is a reactive oxygen species (ROS) in organism, being perennially generated intracellularly as a production of the metabolism in aerobic organisms and otherwise extracellularly during contagion in expert organisms (1-3). The inge-stion of H_2O_2 by *Saccharomyces cerevisiae* is to modify the synthesis of fatty acid and total protein in plasma membrane (1). ROS can oxidate nucleic acid, protein, fat and carbohydrates. for example, the oxidative injury to proteins give rise to breakdown of amino acid chains decreasing the biologic activity. Under normal physiological conditions, oxidative injury are forestalled by antioxidant defenses. then again, under abnormal conditions, antioxidant defense system is deficient and give rise to oxidative injury in cell. According to a study it has been observed that the ingestion of H_2O_2 at lower dose, caused fatal stress in *Saccharomyces cerevisiae* and lead to negative effect on the synthesis of essential proteins (1, 3-5). inherent antimicrobials can be used with different novel protection technologies to simplify the changing of traditional approaches in food conservation. (6). In the last decade, new species of fruit juice products, including strawberry, pomegranate, cherry, grapefruit, lemon juice, orange juice etc. have come into the consumption (7, 8). Fruit and vegetable juices are important for the healthy lives of people at every age. Low sodium, cholesterol, fat; rich polyphenol, flavonoids and vitamin C play key roles in the healthy lives of people (9). The almond very important for human health according to its fatty acid and protein contents (10, 11). OJ is one of the most consumed fruit juices in the world and it has a nice color, aroma and scent. In addition, OJ is also the source of carotenoid, vitamin C and important phenolic compounds. Thanks to this rich content, it has a significant antioxidant potential. A living being that is rich in these compounds is thus more resistant to free radicals

and is stronger against oxidative damage in comparison with his/her peers (12).

In this study we studied the effect of OJ on the proportion of the cell growing, total protein and cell growth that the induced with H_2O_2 opposite to oxidative stress growing at 37°C temperature of adding to OJ in *S. cerevisiae* culture.

Material and Methods

Research groups and growth conditions

Seven groups were composed. i: Control group, ii: H_2O_2 group, iii: 5 mM H_2O_2 + OJ group, iv: 10 mM H_2O_2 + OJ group, v: 15 mM H_2O_2 + OJ group, vi: 20 mM H_2O_2 + OJ group, vii: 25 mM H_2O_2 + OJ group. After sterilization, fruit juice (25%) and H_2O_2 were inserted different concentration to *Saccharomyces cerevisiae* (*S. cerevisiae*) cultures and the cultures were developed at 37°C for 1h, 3h, 5h and 24 hours (overnight). *S. cerevisiae* cell growth was determined by spectrophotometer, total protein alteration was identified by SDS-PAGE electrophoresis and calculated with biuret method for the developed and reproduce of yeast, YEPD (for 50 mL 1,5 g yeast extract, 1 g trypton, 1,5 g glucose) in addition, for the developed and reproduce of *S. cerevisiae*, orange fruit juices was added and improved. After sterilization, yeasts were cultured into media and the samples were incubated for 1h, 3h, 5h, 24 h (overnight, h: hour) at 37°C (3, 13).

Orange juice extract and H_2O_2 Chemical

Fruit (From center county of Elazığ city) was squashed in water and added in to *S. cerevisiae* media cultures and added 25% (v/v) ratio in at the reproducing for 37°C. H_2O_2 was inserted in H_2O_2 and OJ + H_2O_2 groups.

Cell Intensity measurements

In these measurements, culture samples that were developed at 37°C for 1, 3, 5 hours and overnight (24 hours) have been analyzed. The measurement has been carried out using a spectrophotometer at 600 nm (OD_{600}).

SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Elecrophoresis)

SDS-PAGE was carried out using BIO-RAD Mini-PROTEAN® 3 Cell gel electrophoresis system. The samples of *Saccharomyces cerevisiae* cultures were prepared for SDS-PAGE after which they were loaded to sample loading wells to be subject to electrical current and after this process the gels were dyed, their images were taken and the intergroup protein bandings were used as data in the study (14).

Protein density measurements

The measurement has been carried out using a spectrophotometer at 540 nm (OD_{540}) according to biuret method. BSA protein standards at different concentrations were obtained using BSA protein. Accordingly, the total protein amount in *Saccharomyces cerevisiae* groups corresponding to this standard value was calculated (Fig. 4).

Statistical analysis

For statistical analysis SPSS 20.0 software was used. The comparison between experimental groups and the control group was made using one way ANOVA and Post Hoc Hochberg tests. Statistically important differentiation among groups have been stated as $p<0.05$ and the statistically non-significant differences have been specified as $p>0.05$. Standard deviations were point out as ±.

Results and Discussion

We believe that the results obtained from this study will make significant contributions to the current literature. When the results in Table 1 and Figure 1 are examined, it is observed that OJ has significant effects on *Saccharomyces cerevisiae* development. It is observed that orange juice preserves its live cell amount despite the increasing hydrogen peroxide concentrations. A difference is observed between the yeast development amounts for 1h in comparison with the control ($p<0.05$). It is observed that OJ protects the cell almost as much as the

Table 1. *Saccharomyces cerevisiae* cell growth in orange juices

OD_{600} 37 °C	1h	3h	5h	Overnight
Control	1,78 ± 0.002[a]	1,60 ± 0.002[e]	1,83 ± 0.002[c]	1,59 ± 0.002[d]
H_2O_2	1,74 ± 0.002[b]	1,51 ± 0.002[f]	1,88 ± 0.002[b]	1,99 ± 0.002[a]
50 µl H_2O_2 + orange juice	1,66 ± 0.002[d]	1,84 ± 0.002[b]	1,84 ± 0.002[c]	1,89 ± 0.002[c]
100 µl H_2O_2 + orange juice	1,70 ± 0.002[c]	1,93 ± 0.002[a]	1,90 ± 0.002[a]	2,00 ± 0.002[a]
150 µl H_2O_2 + orange juice	1,73 ± 0.002[b]	1,68 ± 0.002[d]	1,79 ± 0.002[d]	2,01 ± 0.002[a]
200 µl H_2O_2 + orange juice	1,54 ± 0.002[e]	1,72 ± 0.002[c]	1,74 ± 0.002[e]	1,94 ± 0.002[b]
250 µl H_2O_2 + orange juice	1,74 ± 0.002[b]	1,68 ± 0.002[d]	1,67 ± 0.002[f]	1,98 ± 0.002[a]

[a,b,c,d,e,f]among the groups which bearing of different letter are significant (p<0.05). Anova Post Hoc Hochberg Test.

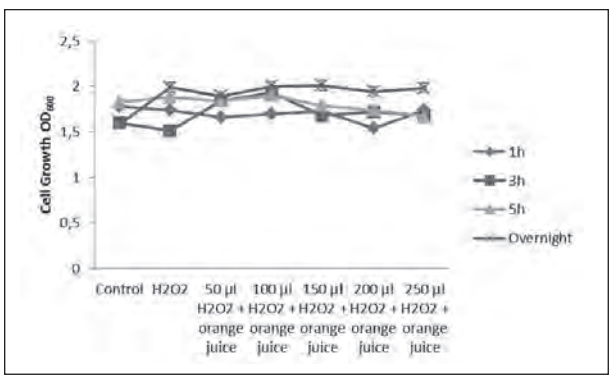

Figure 1. The growing of *Saccharomyces cerevisiae* in orange fruit juices at different hours.

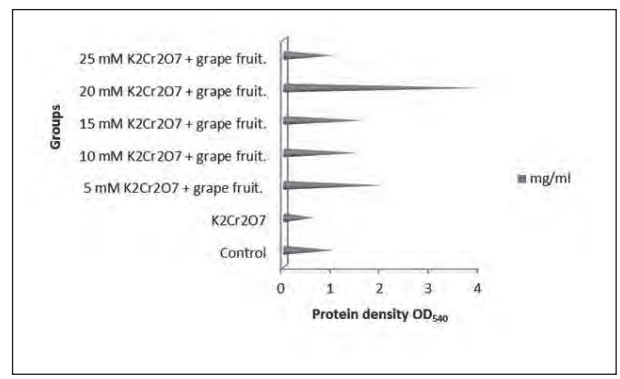

Figure 2. Protein density at between groups

control against hydrogen peroxide which is the largest radical source in the 250 µl H_2O_2 + OJ group. When 3h values are examined; it is observed that OJ has increased yeast development in the 100 µl H_2O_2 + OJ group despite the adverse effects of the hydrogen peroxide radical in comparison with the control ($p<0.05$). When the 5h values are examined; it is again observed that OJ has increased yeast development at a maximum level in the 100 µl H_2O_2 + OJ group despite the adverse effects of the hydrogen peroxide radical in comparison with the control ($p<0.05$). When the overnight (24 h) values are examined; it is observed that orange juice has increased yeast development in the 150 µl H_2O_2 + OJ group despite the adverse effects of the hydrogen peroxide radical in comparison with the control; in addition it can also be observed that yeast development has increased at a statistically significant level in all other groups in comparison with the control ($p<0.05$) (Table 1). Stinco et al (2015) have put forth that OJ activates the antioxidant defense system against free radicals thereby making a positive impact on yeast development (12). Aslan et al (2014a) have indicated that pomegranate juice is protective against oxidative damage in *Saccharomyces cerevisiae* (1). Again Aslan et al (2015) have put forth as a result of the study carried out with different fruit juices and their mixtures that different fruit juices and their mixtures are protective against oxidative damage in *Saccharomyces cerevisiae* and that they increase yeast development (7). Tserennadmid et al (2011) have put forth that apple juice has a protective role for development in yeasts (15). Krivoruchko and Nielsen (2015) have stated that resveratrol and flavonoids play protective roles against oxidative damage in bacteria and yeasts (16). When the SDS-PAGE results are examined; it is observed that protein band density in-

Table 2. Biuret protein density

OD_{600} 37°C	Mg/ml
Control	1
H_2O_2	0.5
50 µl H_2O_2 + orange juice	2
100 µl H_2O_2 + orange juice	3
150 µl H_2O_2 + orange juice	1
200 µl H_2O_2 + orange juice	1
250 µl H_2O_2 + orange juice	1

crease in supernatant and pellet gel images is greater in groups to which OJ is administered in comparison with the control (Figure 3a and 3b). Aslan et al (2014b) have put forth that pomegranate juice has a protective effect in *Saccharomyces cerevisiae* against oxidative damaged caused by the administration of hydrogen peroxide and that protein band density increase is greater in pomegranate administered groups in comparison with hydrogen peroxide administered groups (3). When the biuret results in Figure 2 and Table 2 are examined; greater protein amount has been measured in OJ (100 µl H_2O_2 + OJ and 50 µl H_2O_2 + OJ) administered groups in comparison with control and H_2O_2 groups (Table 2, Figure 2). On the other hand there are a lot of study in vivo on rat about fruit and vegetable mechanism. For example these, Tuzcu et al (2012) have stated that tomato powder is protective in rats against colorectale cancer (17). Aslan et al (2014c) have stated that the milk thistle extract is protective against lung damage in rats (18). Sahin et al (2010) have stated that EGCG increases antioxidant defense in rats (19). According to these results, OJ has a positive effect on *Saccharomyces cerevisae* cell growth and reduced the oxidative damage effect.

Conclusion

When these results are examined; we can stated that OJ is quite effective against the hydrogen peroxide induced oxidative damage in *Saccharomyces cerevisiae*, that it protects cell development and even increases cell development; thus encouraging protein synthesis in yeast cells.

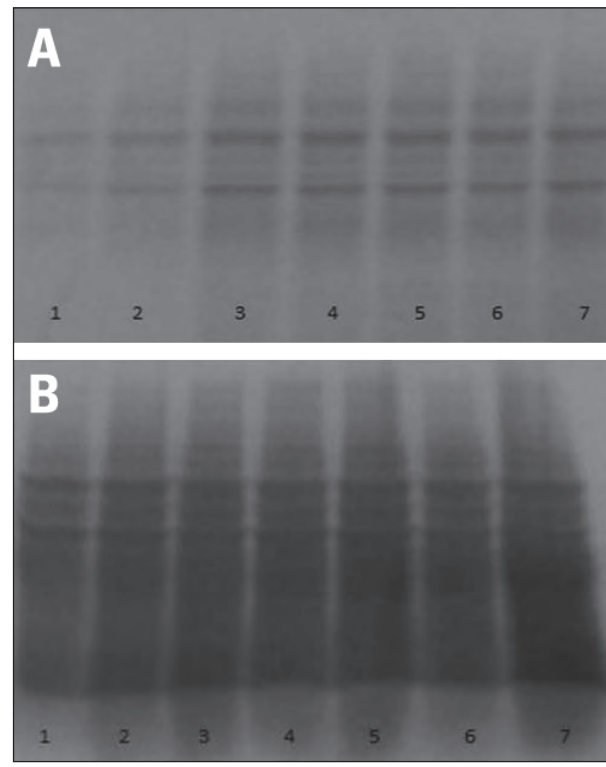

Figure 3. A) SDS-PAGE pelet total protein bands profiles for development at 37°C. Lanes 1: Control; 2: H_2O_2; 3: 50 µl H_2O_2 + orange juice; 4: 100 µl H_2O_2 + orange juice; 5: 150 µl H_2O_2 + orange juice; 6: 200 µl H_2O_2 + orange juice; 7: 250 µl H_2O_2 + orange juice. B) SDS-PAGE supernatant total protein bands profiles for development at 37°C. Lanes 1: Control; 2: H_2O_2; 3: 50 µl H_2O_2 + orange juice; 4: 100 µl H_2O_2 + orange juice; 5: 150 µl H_2O_2 + orange juice; 6: 200 µl H_2O_2 + orange juice; 7: 250 µl H_2O_2 + orange juice

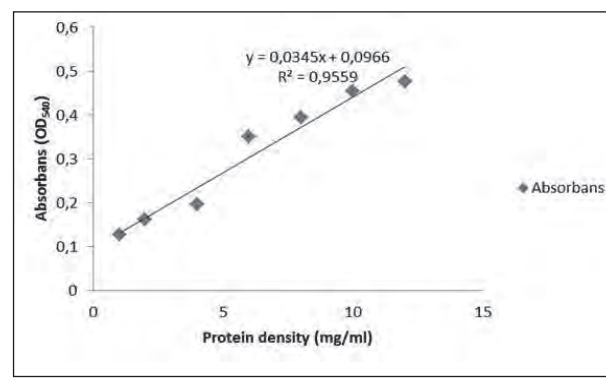

Figure 4. Biuret BSA (bovine serum albumin) standart graph

In the light of these findings, we hope that our study will encourage other studies to try OJ in animal experiments and that in this regard OJ will be consumed more by people based on the positive results that will be obtained.

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Correspondence:
Dr. Abdullah Aslan
Tel: +90 (424) 2370000-3819; Fax: +90 (424) 2330062
Firat University, Faculty of Science, Department of Biology, Elazığ-Turkey
Email: aaslan@firat.edu.tr

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Original article

Antihypoxic, nephroprotective and antioxidant properties of hydro-alcoholic extract of loquat flowers

Seyed Fazel Nabavi[1], Seyed Mohammad Nabavi[1], Akbar Hajizadeh Moghaddam[2], Claire Hellio[3], Mohammad Ali Ebrahimzadeh[4]

[1]Applied Biotechnology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran
[2]Department of Biology, University of Mazandaran, Babolsar, Iran
[3]LEMAR, Institut Universitaire Européen de la Mer, Université de Bretagne Occidentale, Technopôle Brest Iroise – rue Dumont d'Urville, 29280, Plouzané, France
[4]Faculty of Pharmacy, Mazandaran University of Medical sciences, Sari, Iran

Summary. *Objectives:* Loquat is well known Chinese medicinal herb with numerous traditional usages. The fact of the promising effect of loquat on different diseases is approved by a plethora of recent studies. *Methods:* In the present study antihypoxic, antioxidant and nephroprotective activity of hydro-alcoholic extract of *Eriobotrya* japonica flower was an investigated employing different in vivo and in vitro assay system. The chemical analysis of *Eriobotrya* japonica flowers, using atomic absorption spectroscopy, highlighted the presence of eight elements. Gallic acid, rutin and quercetin were determined by HPLC/DAD. *Results:* Results indicated that the hydro-alcoholic extract has a protective effect against hypoxia induced lethality in mice. Extract at doses of 200 and 400 mg/kg/day for 10 consecutive days, i.p. gave nephroprotection by changing blood urea nitrogen (BUN), serum urea and creatinine levels. Extract showed good antioxidant and antihemolytic activity in all studied models. Present study demonstrates hydro-alcoholic extract of *Eriobotrya japonica* as a plant with high biological properties. *Conclusion:* In conclusion, loquat flowers are potential sources of antioxidant, anti-hypoxic and nephroprotective agents.

Key words: Free radical, *Eriobotrya japonica,* gentamicin, rutin

Introduction

Loquat, *Eriobotrya japonica* (Lindley), is a member of the Rosaceae family and its edible fruit is used. From ancient time, Japanese farmers cultivate loquat tree and so it has been become an important edible fruit and has considerable economic importance at a regional level in Iran. Its fruit is called "biwa", "Japanese medlar" or "Japanese plum". They also exist in northern India, the Mediterranean region, England, Madagascar and North, Central and South America (1). Loquat is often used in herbal remedies and traditional medicine for treatment of cough and asthma (1). In Iran it is called "azgil-e-zhaponi" and used as edible fruit in jellies and jams and has some uses in Iranian traditional medicine. Different parts of loquat tree have been used for their anti-inflammatory effect and in treatment of cough, tumors, liver problems, chronic bronchitis (1, 2), nephropathy, diabetes, as a tissue factor inhibitor and inhibitor of Nuclear Factor kappa B, p38 mitogen-activated protein kinase, and ERK (2). Oleanolic acid, ursolic acid and amygdalin has been reported from the flower of *Eriobotrya japonica* Lindl (3). Within this study, antioxidant, antihemolytic, antihypoxic and nephroprotective effects of *E. japonica* flowers hydro-alcoholic extract were assessed.

Methods

Chemicals

Ferrozine, Linoleic acid, Trichloroacetic acid, 1, 1-Diphenyl-2-picryl hydrazyl, Potassium ferricyanide, Sodium nitrite, Sodium fluoride, and Hydrogen perox-

ide were purchased from Sigma Chemicals Co. (USA). Gallic acid, Quercetin, Butylated hydroxyanisole, Vitamin C, Sulfanilamide, N-(1-naphthyl) ethylenediamine dihydrochloride, EDTA and Ferric chloride were purchased from Merck (Germany) and Gentamicin was purchased from Daru-pakhsh Co (Iran). All other chemicals were of analytical grade or purer.

Sample preparation

Loquat flowers were collected from Panbeh Chuleh village at December 2009, near the Caspian Sea, in Mazandaran, Iran. Loquat flowers were transported to the laboratory and kept at < 4°C within 24 h prior to sample preparation.

Determination of metal content

Instrumentation and analytical procedures

Dried and ground samples were ash-dried overnight at 400-420°C in a Vitreosil crucible. Two grams of ash were dissolved in a 1:3 mixture of hydrochloric and nitric acids diluted to 50 mL with distilled water and used for analysis by means of an atomic absorption spectrometer Perkin Elmer AAS 100 (Wellesley, MA).

Preparation of extract

Plant powder was extracted by percolation method using ethanol/distilled water (70/30) for 24 h at room temperature (4). Extract was filtered and concentrated under reduced pressure at 40°C using a rotary evaporator.

Determination of total phenolic compounds and flavonoid content

Total phenolic compound contents were determined by the Folin-Ciocalteau method (5). The extract sample (0.5 mL) was mixed with 2.5 mL of 0.2 N Folin-Ciocalteau reagent for 5 min and 2.0 mL of 75 g/L sodium carbonate were then added. The absorbance of reaction was measured at 760 nm after 2 h of incubation at room temperature. Result was expressed as gallic acid equivalents. Total flavonoids content was estimated as previously described (6). Briefly, 0.5 mL solution of extract in methanol was separately mixed with 1.5 mL of methanol, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1 M potassium acetate, and 2.8 mL of distilled water and left at room temperature for 30 minutes. The absorbance of the reaction mixture was measured at 415 nm. Total flavonoid content was calculated as quercetin from a calibration curve.

Assay of putative biological active components

Gallic acid

A Knauer series liquid chromatography system comprising degasser, pump, auto- sampler, thermostatted column compartment, and diode array detector was used. The column used was a C_{18} reversed phase Kingsorb 5 mm (250×4.6 mm). Mobile phase eventually adopted for this study was methanol/ water/ orthophosphoric acid (20/ 79.9/ 0.1) and the flow rate was 1.0 mL/min. Absorption wavelength was selected at 210 nm. The column was operated at 30°C. The sample injection volume was 20 µL (7).

Quercetin and Rutin

Chromatographic analysis was carried out by the column used was a C_{18} reversed phase Kingsorb (250 × 4.6 mm) packed with 5µm diameter particles. The mobile phase was methanol/ acetonitrile/ water (40/15/ 45) containing 1% acetic acid. This mobile phase was filtered through a 0.45 µm membrane filter (Millipore), then deaerated ultrasonically prior to use. Quercetin was quantified by DAD following HPLC separation 368 nm for quercetin and 257 for rutin, respectively. Flow rate and injection volume were 1.0 mL/min and l0 µL, respectively (8).

Free radical scavenging activity

The stable 1, 1-diphenyl-2-picryl hydrazyl radical (DPPH) was used for determination of free radical-scavenging activity of the sample (9). Different concentrations of sample were added, at an equal volume, to ethanolic solution of DPPH (100 µM). After 15 min at room temperature, the absorbance was recorded at 517 nm. Vitamin C, BHA and quercetin were used as standard controls.

Metal chelating activity

Briefly, the sample (0.2-3.2 mg/mL) was added to a solution of 2 mM $FeCl_2$ (0.05 mL). The reaction was

initiated by the addition of 5 mM ferrozine (0.2 mL), the mixture was shaken vigorously and left standing at room temperature for 10 min. Absorbance of the solution was then measured spectrophotometrically at 562 nm (10).

Assay of nitric oxide-scavenging activity

For the experiment, sodium nitroprusside (10 mM), in phosphate-buffered saline, was mixed with different concentrations of sample dissolved in water and incubated at room temperature for 150 min. The same reaction mixture, without extract, but with an equivalent amount of water, served as control. After the incubation period, 0.5 mL of Griess reagent was added. The absorbance of the chromophore formed was read at 546 nm. Quercetin was used as positive control (11, 12).

Scavenging of hydrogen peroxide

Briefly, a solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). Sample (0.1-1 mg/ mL) in distilled water (1.4 mL) was added to a hydrogen peroxide solution (0.6 mL, 40 mM). The absorbance of sample at 230 nm was determined after ten minutes against a blank solution containing phosphate buffer without hydrogen peroxide (13).

Antihemolytic activity of extract

Antihemolytic activity of extract against H_2O_2 induced hemolysis

Briefly, Erythrocytes from male rat blood were separated by centrifugation and washed with phosphate buffer (pH 7.4). Erythrocytes were then diluted with phosphate buffered saline to give 4% suspension. 1g of samples/mL of saline buffer was added to 2 mL of erythrocyte suspension and the volume was made up to 5 mL with saline buffer. The mixture was incubated for 5 min at room temperature and then 0.5 mL of H_2O_2 solution in saline buffer was added to induce the oxidative degradation of the membrane lipids. The concentration of H_2O_2 in the reaction mixture was adjusted to bring about 90% hemolysis of blood cells after 240 min. After incubation the reaction mixture was centrifuged at 1500 rpm for 10 min and the extent of hemolysis was determined by measuring the absorbance at 540 nm corresponding to hemoglobin liberation (14).

Animal

The study was performed on male NMRI mice of approximately the same age-group and body weight (2-3 weeks; 20-25 g), housed in ventilated animal rooms at a temperature of 24 ± 2 ˚C with a 12 h light/dark cycle and 60 ± 5 % humidity. All experiments were performed according to the norms of the ethical committee (DM42/2004-A).

Maximum non-fatal dose

Different doses of extract were injected into separate groups of seven. After 48 h, the highest dose that did not induce mortality was considered the maximum non-fatal dose (15).

Anti hypoxic activity

Haemic hypoxia

Twenty four mice were divided into four groups each containing six mice. Control group was treated with 0.9% (w/v) saline solution. Thirty minutes after i.p. administration of extract 125, 250 and 500 mg/kg, $NaNO_2$ (360 mg/kg) was applied i.p. to each mouse and antihypoxic activity was estimated as the latent time of evidence of hypoxia in minutes (15).

Circulatory hypoxia

Twenty four mice were divided into four groups each containing six mice. Groups were treated with 0.9% (w/v) saline solution. Thirty minutes after i.p. administration of extract 125, 250 and 500 mg/kg, NaF (150 mg/kg) was applied i.p. to each mouse and the antihypoxic activity was estimated in minutes as the latent time of evidence of hypoxia (15).

Nephroprotective assay

Experimental protocol

Animals were randomly divided into four groups of 10 animals each. Group I was kept as normal control receiving isotonic saline (0.5 mL, i.p.) for 8 consecu-

Table 1. Amount of trace elements in the plants by AAS Analysis (μg/g)

Sample	Yield %	Cr	Fe	Cu	Zn	Ni	Mn	Pb	Cd
E. japonica	35	ND	2.02	2.24	13.4	4	5.18	ND	ND

Note: Values are averages of three independent measurements having a precision of ± 1%.

Table 2. Amount of phytochemicals the plants by HPLC-DAD (mg/g of extract)

Sample	Rutin	Quercetin	Gallic acid
E. japonica	*56.0 ± 2.18*	*1.40 ± 0.06*	*2.41 ± 0.10*

tive days, and animals of groups II, III and IV were administered gentamicin, manufactured by Daru-Pakhsh Co., Iran (100 mg/kg/day, i.p.) for 8 consecutive days, which is well known to produce significant nephrotoxicity in mice. Injections of gentamicin were made daily at 08:00 hours to minimize the circadian variation in nephrotoxicity (16). Animal of Group II and III received extract (200 and 400 mg/kg/day, i.p.) and group IV received isotonic saline (0.5 mL, i.p.) for 10 consecutive days. After the last application, animal were anesthetized with ketamine (60 mg/kg) and xylazine (5 mg/kg) given intraperitoneally. Blood samples were collected via retro-orbital puncture in plain plastic tubes, left to stand at 48 °C for 1 hour, and centrifuged (900 g for 15 min at 5°C) to separate serum.

Biochemical analysis

Blood urea nitrogen (BUN), Ceratinine (Cr) and serum urea concentration was assessed as markers of nephrotoxicity. BUN, Cr and serum urea were determined spectrophotometrically from serum samples using commercially available kits (Sigma).

Statistical analysis

The values are presented as Mean ± SD. Differences between group means were estimated using a one-way ANOVA followed by Duncan's multiple range test. Results were considered statistically significant when $p<0.05$.

Results and Discussion

Table 1 presents the elemental analysis in ash of *E. japonica* flower by AAS technique. The concentration of various elements analyzed in the present work decreases in the order: Zn>Mn> Ni> Cu> Fe. Cells need to trace elements and their deficiencies may cause different diseases (17). Tuzcu et al. (18) report nephroprotective activity of zinc picolinate on cisplatin-induced renal injury. Therapeutic effect of copper in anemia, kinky hair syndrome and anti-inflammatory activity has been proved (17). It is well known that manganese compounds have antioxidant role. The daily requirement for an adult man is 10-15, 12-15 and 2-3 mg/d for Fe, Zn and Cu, respectively (17).

The total phenolic content of *E. japonica* was 115.23 ± 5.41 mg gallic acid equivalent/g of extract powder. Also, total flavonoid content of *E. japonica* was 30.77 ± 1.44 mg quercetin equivalent/g of extract powder. The amount of rutin, quercetin and gallic acid determined by HPLC/DAD were showed in table 2. IC_{50} for DPPH radical-scavenging activity was 145.7± 6.11. The IC_{50} values for vitamin C, quercetin and BHA were 5.05 ± 0.1, 5.28 ± 0.2 and 53.96 ± 3.1 μg/mL, respectively. DPPH is well known stable nitrogen-centered free radical. Any substances with hydrogen or electron donating activity can change it color can be considered as radical scavengers (19). Phenol and flavonoid contents of this plant, especially high rutin content, seem to have a crucial role in its good DPPH-scavenging activity.

In metal chelating model, extract showed weak activity with IC_{50} = 822.8 ± 32 μg/mL. EDTA showed better activity (IC_{50} = 18 μg/mL). Chelation therapy reduces iron-related complications in man and can improve life quality and overall survival in some diseases such as thalassemia, cancer, HIV or Wilson's disease (20). Also iron chelators showed that have antimalarial activity through the mechanism of preventing iron from essential metabolic pathways of the intra-erythrocytic plasmodium (21). In other hand many studies have demonstrated that iron chelators show anticancer activity (22). A growing body of evidences show potent transition metals chelating ability of phytochemicals with scarce side effect (23-25). Ferrozine can quantitatively

form complexes with Fe^{2+} monitored by forming red color. Chelator agent prevent from ferrozine-iron complex formation. In this assay, both extract and EDTA interfered with the formation of ferrous and ferrozine complex, suggesting that it has chelating activity and captures ferrous ion before ferrozine.

Excessive NO production has direct in numerous disease states including renal failure, burn, cancer and neurodegenerative disease and so, elimination of the excess NO could have beneficial effects (26). The *E. japonica* flower extract showed potent nitric oxide-scavenging activity (IC_{50} =76.5 ± 2.9 μg/mL vs. quercetin with IC_{50} = 20 ± 0.01 μg/mL). It is may correlate with high flavonoid compound especially rutin and quercetin. Nitric oxide scavenger compounds challenge with oxygen, and reduce nitrite ions formation and also can restrict nitric oxide mediated damages (26).

In another models, *E. japonica* extract showed potent H_2O_2 scavenging activity (IC_{50} was 320.7 ± 16.03 μg/mL). The IC_{50} values for vitamin C and BHA were 21.4 ± 1.1 and 52 ± 2.6 μg/mL, respectively. Although hydrogen peroxide is not very toxic, But it can give rise to hydroxyl radicals formation in the cell and causes cytotoxicity. Thus, elimination of excessive H_2O_2 is important throughout cells. *E. japonica* activity is originated from presenting flavonoid compounds in the extract specially rutin.

Tested extract showed good activity in hemoglobin-induced linoleic acid system (figure1). Cell membrane is rich in unsaturated fatty acids that are main target of free radical attacks (6, 27-29). Erythrocytes

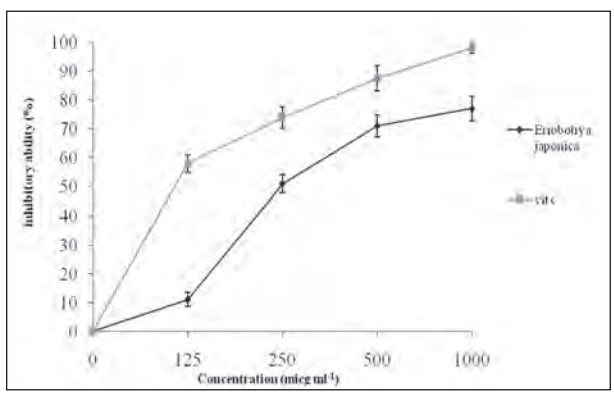

Figure 1. Antioxidant activities of *Eriobotrya japonica* against linoleic acid peroxidation induced by hemoglobin. Each value is expressed as mean 3 standard deviation positive control (Vitamin C).

membranes lipids have been known as prime targets lipid peroxidation (30, 31). Superoxide indirectly initiates lipid peroxidation because superoxide anion acts as a precursor of singlet oxygen and hydroxyl radical (32, 33). Lipid peroxidation caused by hydroxyl radical (34). They act by hydrogen atoms robbing from the membrane lipids (35, 36). Potent lipid peroxidation inhibitory activity of extract correlates with high amount of rutin and quercetin.

E. japonica extract showed potent antihemolytic activity (IC_{50} was 258.2 ± versus. 235± 9.1 μg/mL for vitamin C). Good antihemolytic effect of hydro-alcoholic extract of loquat flowers maybe result of high rutin content.

The maximum non-fatal dose of extract was 4 g/kg. A statistically significant antihypoxic activity of the extract (doses 500 mg/kg) was established in the experimental model of haemic and circulatory hypoxia in mice ($P<0.001$ vs. control). The effect was found to be dose-dependent in a range of 125-500 mg/kg for haemic and circulatory hypoxia (Table 3). Previous studies demonstrated that intraperitoneal administration of sodium fluoride increases histamine in blood and decreases the oxygen carrying potential (15). Results of present study may be leaning on other literature data that phytochemicals such as flavonoids increase cerebral blood flow and show antihypoxic effect. The mechanism of antihypoxic effects can be due in part to the antioxidant effects of phytochemicals such as rutin (15).

The results show that administration of gentamicin at dose of 100 mg/kg/day for 8 consecutive days brought a significant increase in BUN, serum creatinine and urea and extract in 200 and 400 mg/kg/day has recovery effects (Table 4). Many studies show that hydroxyl radicals play an important role in production of gentamicin-induced nephrotoxicity. Fenton's reac-

Table 3. Anti-hypoxic activity of E. japonica in the different tests

Group	Doses (mg/kg)	sodium nitrite test (min)	sodium fluoride test (min)
Control		9.50 ± 0.30	9.36 ± 0.32
E. japonica	125	10.74 ± 0.604 *	10.64 ± 0.45**
E. japonica	250	13.00 ± 0.87 **	12.23 ± 0.16****
E. japonica	500	16.68± 1.50 ****	14.51 ± 0.56****

*Each group represents the Mean ± SD (n = 10). *P >0.05, ** P< 0.05, *** P< 0.01 and****P<0.001 vs. control*

Table 4. Effect of extract on serum creatinine, serum urea and blood urea nitrogen levels in gentamicin-induced renotoxic mice.

Groups	Serum creatinine μmol/l	Serum urea mg/dl	Blood urea nitrogen mg/dl
Gentamicin control (100 mg/kg, i.p.)	34.88 ± 4.27	119.56 ± 2.63	54.99 ± 1.22
Normal	24.05 ± 3.12	59.683 ± 2.30	27.45 ± 1.07
Extract-treated (200 mg/kg, i.p.)	35.45 ± 1.36 [a]	107.00 ± 4.25 [c]	50.00 ± 1.98 [c]
Extract-treated (400 mg/kg, i.p.)	28.94 ± 0.57 [b]	70.91 ± 3.48 [b]	33.13 ± 1.62 [b]

Values are Mean ± SD (n = 10). Data for normal animals are considered as base-line data; there was no significant base-line difference between the groups. [a] P<0.01 versus control group; [b] P<0.001 versus control group; [c] P>0.05 versus control group

tion induced oxidative injuries and aminoglycoside-iron complexes formation that have been suggested to be the major mechanisms in the progression of gentamicin-induced nephrotoxicity (16). Previously it has been reported that gentamicin in vitro model enhances the hydrogen peroxide production by renal cortical mitochondria and that iron ion chelators and scavengers of hydroxyl radicals ameliorate renal damage induced by gentamicin (16). Many studies show that oxidative and nitrosative stress play an important role in the ensuing renal injuries (37) especially in renal injuries induced by aminoglycoside antibiotics. On the other hand it has been reported that nitric oxide scavenger could be better than inducible nitric oxide synthase inhibitors as a curative intervention (37). In present study we show that the extract has good antioxidant activity especially nitric oxide scavenging activity. BUN and serum creatinine and urea levels were augmented indicating glomerular injury (38). However, the combined intraperitoneally administration of *E. japonica* with gentamicin to mice resulted in significant reduction in the elevated levels of BUN and serum creatinine and urea. These results could be in accord with several other researches, which reported that, polyphenolic compound (39), partially prevented the increase in BUN and serum creatinine and urea levels induced by gentamicin.

Conclusion

In present study, phytochemical constituents and pharmacological activities of flowers extract of loquat have been reported. Potent pharmacological activity of *Eriobotrya japonica may* correlate with high flavonoid content specially rutin in the extract. These results can be useful as a starting point of view for further applications of this plant or its constituents in pharmaceutical preparations after performing clinical researches.

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Correspondence:
Seyed Fazel Nabavi,
Tel/fax: +98 21 88617712,
P.O. Box 19395-5487,
E-mail: Nabavisf@gmail.com

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Original Article

Yeast hydrolysate as a functional anti-obesity ingredient: appetite suppressive effects of yeast hydrolysate in food-deprived mice

Ki Bae Hong[1], Eun Young Jung[2*], Jae Hwan Kim[3], Un Jae Chang[4], Hyung Joo Suh[5]*

[1]Department of Public Health Science, Graduate School, Korea University, Seoul 136-713, Korea; [2]Department of Home Economic Education, Jeonju University, Jeonju 560-759, Korea; [3]NeoCremar Co. Ltd., Sungnam 462-806, Korea; [4]Department of Food and Nutrition, Dongduk Women's University, Seoul 136-714, Korea; [5]Department of Food and Nutrition, Korea University, Seoul 136-713, Korea

Summary. The aim of this study was to investigate the appetite suppression effects of yeast hydrolysate (YH). Male ICR mice (8 weeks old) were randomly divided into three groups (n=6) as follows: Saline group, which was treated with saline (control); YH0.5 group, which was treated with YH 0.5 g/kg BW; and YH1 group, which was treated with YH 1 g/kg BW. At the beginning of the experiment, the mice were intraperitoneally (IP) injected with either saline or YH. The results showed that YH caused a significant attenuation of food intake in fasted mice ($p<0.05$) and significantly lowered serum ghrelin levels (YH0.5, 2002.22 pg/mL; YH1, 2337.65 pg/mL vs. Saline, 3363.61 pg/mL, $p<0.05$). This study indicates that the appetite suppression effects of YH is likely explained by the attenuation of ghrelin.

Key words: Yeast hydrolysate, ghrelin, appetite, obesity

Introduction

Yeast naturally rich in minerals, vitamins, amino acids and proteins, plays a fundamental role in human food and nutrition. Yeast extract has high potential as a source of biologically active molecules and functional food ingredients (1). The small proportion of yeast extract (mainly containing peptides below 10 kD) and yeast hydrolysate (YH), which could be related to satiety, has been industrially purified from *Saccharomyces cerevisiae* by protein hydrolysis (2). Recently, YH has formed a large and growing dietary market as a useful anti-obesity supplement (3). It was reported that YH could suppress weight gain in obese animal models and assist in weight loss in obese humans (3-5). To elucidate the mechanisms of YH in fighting obesity, a distribution of neurotransmitters was investigated in the hypothalamus of rats treated with YH using histochemical methods (6, 7). YH was found to alter appetite-related neurotransmitters in the central nervous system (CNS). Although the exact mechanisms for the anti-obesity effects of YH are not fully understood, these studies support the idea that YH might induce weight loss through appetite control via the CNS. To confirm the hypothesis that YH suppresses appetite, we observed food intake by animal subjects for several hours after YH treatment in food-deprived mice. We also investigated whether YH treatment might affect serum appetite-related hormone levels.

Materials and methods

Preparation of yeast hydrolysate (YH)

Saccharomyces cerevisiae IFO 2346 was incubated in medium containing 2% molasses, 0.6% $(NH_4)_2SO_4$, 0.1% $MgSO_4 \cdot 7H_2O$, 0.2% KH_2PO_4, 0.03% K_2HPO_4,

*Ki Bae Hong and Eun Young Jung contributed equally to this study.

and 0.1% NaCl for 3 days at 30°C. After incubation, the culture was centrifuged at 10,000 × *g* for 20 min. The cells were suspended with 20 mM phosphate buffer (pH 7.0) and hydrolysed with bromelain at 30°C for 4 hr. The hydrolysate was subsequently centrifuged at 10,000×*g* for 20 min. The supernatant was then passed through a 10 kDa molecular weight screening membrane (Sartocon cassette, Sartorius, Germany). The fractions with peptides smaller than 10 kDa were freeze-dried.

Appetite suppression study

The experimental protocol was reviewed and approved by the Korea University Animal Care Committee. Male ICR mice were obtained at 8 weeks of age from Daehan Biolink (Cheongju, Korea). The mice were randomly divided into three groups (n=6) as follows: Saline group, which was treated with saline (control); YH0.5 group, which was treated with YH 0.5 g/kg BW; and YH1 group, which was treated with YH 1 g/kg BW. Food was withdrawn for 12 hr before the experiment. At the beginning of the experiment mice were intraperitoneally (IP) injected with either saline or YH. Venous blood was collected 30 min post-injection from the tail vein. Serum ghrelin and leptin levels were measured by enzyme immunoassay using a Bio-Plex Pro Mouse Diabetes kit (Bio-Rad Co., CA, USA). Pre-weighed food jars were then introduced into the cages and a sheet of clean white paper was placed under each cage to collect any spillage. The amounts of food consumed after 1, 2, and 4 hr post-injection were determined. The experiment began at approximately 10:00 a.m. so that all weighing was done during the light period of the day-night cycle (the lights were on between 8.00 a.m. and 6.00 p.m.).

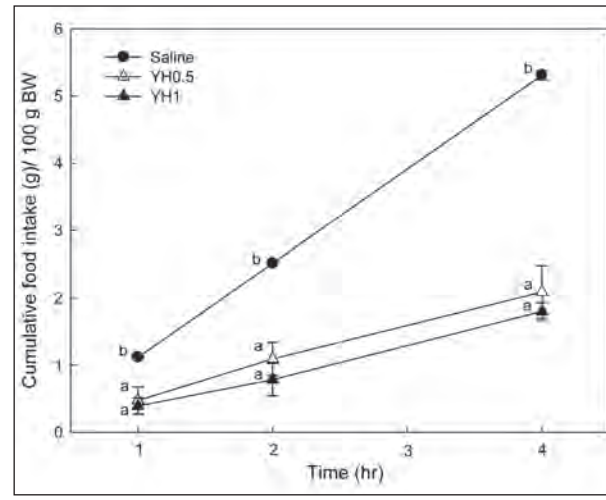

Figure 1. Cumulative food intake of ICR mice for 4 hr following intraperitoneal injection of yeast hydrolysate (YH) after 12 hr of food deprivation. The values are the means±SD for 6 mice. Means with different superscript letters are significantly different at $p<0.05$ according to Tukey's multiple range tests. Saline, group treated with saline (control); YH0.5, group treated with YH0.5 g/kg BW; YH1, group treated with YH 1 g/kg BW.

Statistical analyses

All statistical analyses were performed with the Statistical Package for Social Sciences ver. 12.0 (SPSS, IL, USA). The differences between groups were statistically evaluated by one-way analysis of variance (ANOVA) and Tukey's multiple tests. All data were two-sided with a 5% significance level and were reported as mean ± standard deviation (SD).

Results and discussion

Figure 1 shows the cumulative food intake during a 4 hr period after YH treatment following 12 hr of

Table 1. Serum leptin and ghrelin levels of ICR mice following the intraperitoneal injection (IP) of yeast hydrolysate after 12 hr of food deprivation

Parameters	Serum hormone level (pg/mL)		
	Saline	YH0.5	YH1
Leptin	371.50±28.33	402.64±21.23	476.66±42.05^{NS}
Ghrelin	3363.60±394.85^{b}	2002.22±724.58^{a}	2337.65±269.21^{a}

The values are the means±SD for 6 mice. Means with different superscript letters are significantly different at p<0.05 according to Tukey's multiple range tests. Saline, group treated with saline (control); YH0.5, group treated with YH 0.5 g/kg BW; YH1, group treated with YH 1 g/kg BW. NS, not significant.

food deprivation. YH caused a significant attenuation of food intake in fasted mice; when compared to the vehicle control (Saline group), YH significantly reduced food intake at all time points ($p<0.05$). However, dose-dependent results were not observed; there was no significant difference between YH0.5 and YH1. Serum leptin and ghrelin levels were measured at 30 min after YH injection (Table 1). YH tended to evaluate serum leptin level without a significant difference. YH significantly lowered serum ghrelin levels (YH0.5, 2002.22 pg/mL; YH1; 2337.65 pg/mL vs. Saline, 3363.61 pg/mL, $p<0.05$). However, there were no dose-dependent differences between YH0.5 and YH1 groups in terms of serum appetite-related hormone levels.

The results of this study on food intake agreed with a recent clinical study showing that the reduction of food intake in YH group was significantly greater than in the vehicle group (5). Faipoux et al. (2) investigated the suppression effects of yeast protein on food intake. They found that rats fed a high yeast protein load reduced their next meal and daily food intake more than rats fed any other well-balanced, amino acid, high protein load or wheat starch diet. They also reported on a preliminary study of gastric emptying in rats receiving yeast protein loads showing that yeast protein was emptied more rapidly through the pylorus than total milk protein during a meal, which may induce satiety. They concluded that yeast proteins enhance satiety more than other proteins. Catiau et al. (1) reported the satiating potential of yeast extract, which is rich in hydrolysed yeast proteins, particularly in terms of food intake. They suggested that the yeast extract had the satiety activity that could be attributable to hormone secretion, which would imply a decrease in food intake and a decrease in weight. As shown in Table 1, YH significantly inhibited the ghrelin secretion relative to that of the vehicle. The ghrelin is produced mainly by the stomach before the meal and acts on the hypothalamus to trigger the sensation of hunger (8). The satietogenic mechanism of YH could also be related to the reduction in ghrelin, which is known as an orexigenic hormone that stimulates appetite.

In conclusion, this study indicates that the appetite suppression effects of YH is likely to be explained by the ghrelin attenuation; it was believed that YH would diminish the sensation of hunger by reducing the secretion of orexigenic factors such as ghrelin that send satiety signals to the brain, terminating food intake in the short term. These observations suggest that YH may be useful in controlling food intake. However, additional investigations are required to determine the chemical identity of the bioactive functional constituents of YH on appetite suppression (2-6).

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Correspondence:
Hyung Joo Suh
Department of Food and Nutrition, Korea University,
Seoul 136-713, Republic of Korea.
Tel. 82. 2. 3290.5639
Fax 82. 2. 940. 2850
E-mail: suh1960@korea.ac.kr

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