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جامعة أبي بكر بلقايد
تلمسان الجزائر

Faculty of Nature and Life Science,
Geology and the Universe Science
Department of Biology
Laboratory of product natural

كلية علوم الطبيعة والحياة
وعلوم الأرض والكون
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Evaluation of Antioxidant Activity of Non-alcoholic Water Kefir and Its Metabolic Effects on *Wistar* Rats

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Prepared by:

Muneer Mohammed Saleh Alsayadi

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Committee in charge:

Mrs. MERZOUK Hafida	**Pr. U. A.B.B. Tlemcen**	**President**
Mrs. BELARBI Meriem	**Pr. U. A.B.B. Tlemcen**	**Supervisor**
Mrs. BENDAHMANE Malika	**Pr. U. Sidi Bel Abbes**	**Examiner**
Mr. ABDELOUAHED Djamel eddine	**Pr. U. A.B.B. Tlemcen**	**Examiner**
Mr. MOSSAOUI Abdellah	**Pr. U. Bachar**	**Examiner**
Mr. KHALED Meghit boumediene	**M.C.A. U. Sidi Bel Abbes**	**Examiner**

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اعوذ بالله من الشيطان الرجيم

بِسۡمِ ٱللَّهِ ٱلرَّحۡمَٰنِ ٱلرَّحِيمِ

In the Name of Allâh, the Most Beneficent, the Most Merciful.

Au nom d'Allah, le Tout Miséricordieux, le Très Miséricordieux.

﴿وَٱتَّقُواْ ٱللَّهَۖ وَيُعَلِّمُكُمُ ٱللَّهُۗ وَٱللَّهُ بِكُلِّ شَيۡءٍ عَلِيمٞ ٢٨٢﴾ سورة البقرة

﴿*so be afraid of Allâh; and Allâh teaches you. and Allâh is the All-Knower of each and everything*﴾

(282). Surat Al Baqarah

﴿*et craignez Allah. alors Allah vous enseigne et Allah est Omniscient*﴾. *(282) Surat Al Baqarah*

﴿هُوَ ٱلَّذِيٓ أَنزَلَ مِنَ ٱلسَّمَآءِ مَآءٗ لَّكُم مِّنۡهُ شَرَابٞ وَمِنۡهُ شَجَرٞ فِيهِ تُسِيمُونَ ١٠﴾

سورة النحل

﴿*He is who sends down water (rain) from the sky; from it You drink and from it (grows) the vegetation on which You send Your cattle to pasture.(10)*﴾ *surat al nahl*

﴿*C'est Lui qui, du ciel, a fait descendre de l'eau qui vous sert de boisson et grâce à la quelle poussent des plantes dont vous nourrissez vos troupeaux (10)*﴾. *surat al nahl*

صدق الله العظيم

Dedication

This thesis dedicated to….

The immaculate and pure souls of my father, my grandfathers, my grandmothers, my uncles Abdanaser, and Mosaed, and to the souls of all martyrs, (in the paradise of bliss, god willing).
my darling and expensive mother who I'm indebted for her in my life.
my dear brothers (Abdalazyz, Hafedh ,Saleh),
my uncles (Abdalwahed , Abdalkarim),
And (Sadeg, Salah)
my darling sisters, and for my dearest wife, and our children 'Gaida, hafedh, abdalaziz, abdlnaser, shahd, rfif' and to all of my family.
And for my classmate Yasser al gawfi and all my classmates.
And my brothers and friends (Foad T, Ahmad M., Ahmad A., Ali A, Akram, Malik, Radhwan , Matloob, Adnan, Gaid, Abdo Q, Hameed, Belal Ab, Saleh, Hameed, Ebrahim, Sadam, Hamzah, Arafat, Faris, Mohammed A, Najy, Belal Ah)
And for all of my friends everywhere.

Muneer Mohammed Saleh al sayadi

at 29/03/ 2014

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الملخص

كيفير الماء منتج تكافلي يصنع بتخمير محلول سكري بواسطة مزرعة من البكتيريا والخمائر مجتمعه في قالب من السكريات المتعددة تدعى (حبوب الكيفير). يعتقد انه يحتوي على الكثير من المواد الفعالة حيويا، و يستطيع المساهمة تحسين صحة الانسان. هدفنا في هذه الدراسة من خلال تعيين ظروف التخمر المثلى (الزمن، الاس الهيدروجيني، درجة الحرارة) الى إنتاج كيفير خالي من الكحول. علاوة على ذلك هدفنا الى تقييم التأثيرات المضادة للأكسدة والايضية لهذا المنتج. وتم فيها عزل وتعريف الاحياء المجهرية بناء على الاختبارات المورفولوجية و البيوكيميائية. ثم تم تقييم الفعالية المضادة للأكسدة علي مستوي المختبر بخمس طرق هي: الدي بي بي أتش، و تثبيط اكسدة الاسكوربات الذاتية، والقوة الاختزالية، و الهيدروكسيل الحر والسعة الكلية المضادة للأكسدة. وتم حث مرض السكري لدى فئران ويستار البالغة بالحقن داخل التجويف البطني(داخل الصفاق) بمادة الاستربتوزوتوسين، وأعطيت الكيفير لمدة خمسة اسابيع، وتم تقدير وزن الجسم ونسبة الجلوكوز ومستوى دهون الدم. كما تم تقدير دلائل الضغط التأكسدي وهي: المالونداياليديهيد، و الهيدروبيروكسيدات والبروتين كربونيل وفعالية الكاتالاز وفيتامين سي. تم التعرف على ثلاثة مجموعات رئيسية من الاحياء المجهرية في شراب الكيفير، شملت بكتيريا حمض اللاكتيك، وبكتيريا حمض الخليك، والخمائر. وأظهرت النتائج كميات هامة من المواد المغذية والمعادن في كيفير الماء، وأبدي كيفير الماء فعالية ممتازة ضد الأكسدة، كما بينت النتائج تحسنا معنويا في وزن الجسم ونسبة جلوكوز ودهون الدم والدلائل الحيوية للضغط التأكسدي لدي الفئران المعاملة مقارنة مع الفئران المريضة بالسكري والكنترول. تقود هذه النتائج الى إستنتاج أن كيفير الماء يمكن ان يكون منتج تكافلي واعد من الأحياء المجهرية النافعة، والمركبات الفعالة حيويا، ومضادات الاكسدة الطبيعية، ذو قدرة ممتازة على تحسين الصحة والمحافظة عليها. كما أن كيفير الماء لديه امكانية ممتازة كغذاء وظيفي مفيد لمرضى السكري، للسيطرة علي مستوى جلوكوز ودهون الدم وتحسين النظام الدفاعي في الجسم ضد الضغط التأكسدي.

الكلمات المفتاحية:

كيفير الماء الخالي من الكحول، مضاد ارتفاع السكر، مضاد زيادة الدهون، مضادات الاكسدة، المنتج التكافلي، فئران ويستار.

Abstract

Water Kefir is a symbiotic product made by sugar solution fermentation using a culture of bacteria and yeast held in a polysaccharide matrix called (kefir grains). It is believed to contain many bioactive substances, and it may contribute in human health improving. By optimize the fermentation conditions (Time, pH, Temperature), we tried to produce nonalcoholic kefir. Furthermore we aimed to investigate antioxidant and metabolic effects of this product. Microorganisms were isolated and identified on the basis of morphology and biochemical tests. We Also, determined the nutrients and minerals contents of this product. Antioxidant activity *in vitro* evaluated using five methods: DPPH, inhibition of ascorbate autoxidation, reducing power, hydroxyl radicals, and total antioxidant capacity. Diabetes was induced in adult *Wistar* rats by intraperitoneal injection of streptozotocin, and they were given kefir in drinking water for five weeks. Body weight, glucose and lipids levels were then measured. The parameters of oxidative stress, Malondialdehyde, catalase, protein carbonyl, hydroperoxides, vitamin C. were also examined. Three distinct microbial populations were identified in water kefir beverage including, lactic acid bacteria, Acetic acid bacteria, and yeasts.The results showed important amounts of nutrients and minerals in water kefir. Water kefir exhibited an excellent antioxidant activity. It also demonstrated significant improvement in body weight, glucose, lipids profiles, and oxidative stress biomarkers of treated rats comparing with diabetic and control ones. These results conduce to conclude that water kefir could be promisor symbiotic of beneficial microorganisms, bioactive compounds, and natural antioxidants with excellentcapability in health developingand maintenance. Water kefir can potentially be useful functional food for diabetics to control glucose lipids levels, and enhancing of defenses system against oxidative stress.

Key words: Nonalcoholic Water Kefir, anti-hyperglycemic, anti-hyperlipidimic, Antioxidant activity, Symbiotic, *Wistar* rats.

Résumé

Le kéfir eau est un produit symbiotique issu de la fermentation d'une solution sucrée à l'aide de bactéries et de levures maintenues dans une matrice de polysaccharides appelée (grains de kéfir). Il contient de nombreuses substances bioactives qui pourraient contribuer à l'amélioration de la santé humaine. En optimisant les conditions de fermentation (temps, pH, température) nous avons essayé de produire du kéfir non alcoolique. En outre, nous avons étudié les effets antioxydants et métaboliques de ce produit. Les micro-organismes ont été isolés et identifiés selon leur morphologie et par des tests biochimiques. Nous avons aussi déterminé les nutriments et les minéraux que renferme le produit. L'activité antioxydante in vitro a été évaluée par cinq méthodes : le DPPH , l'inhibition de l' auto-oxydation de l'ascorbate, le pouvoir réducteur des radicaux hydroxyles et la capacité antioxydante totale. Le diabète a été induit au préalable chez des rats Wistar adultes par une injection intra péritonéale de streptozotocine, et ils ont ensuite ingéré le kéfir eau pendant cinq semaines. Le poids corporel ainsi que le taux de glucose et de lipides ont été mesurés. Les paramètres du stress oxydatif, Le malondialdehyde, la catalase, les protéines carbonylées, les hydroperoxydes, la vitamine C ont été ensuite déterminés. Trois populations microbiennes distinctes ont été identifiées dans le breuvage du kéfir d'eau, à savoir : des bactéries lactiques, des bactéries acétiques et des levures. Les résultats ont montré d'importantes quantités de nutriments et de minéraux dans le kéfir eau. Ce dernier a montré une excellente capacité antioxydante .Il a également été démontré une amélioration significative du poids corporel, du taux glucose, des profils lipidiques et des bio marqueurs du stress oxydatif des rats traités en comparaison avec les rats diabétiques et les témoins. Les résultats obtenus nous mènent à déduire que le kéfir eau pourrait être un bon apport symbiotique de micro-organismes bénéfiques, de composés bioactifs et d'antioxydants naturels. Ainsi qu'un aliment fonctionnel utile pour les diabétiques pour le contrôle des taux de glucose et de lipides et pour renforcer le système de défense contre le stress oxydatif.

Mots-clés: kéfir d'eau non-alcoolique, anti-hyperglycémiant, anti-hyperlipidémiant, activité antioxydante, symbiotique, rats Wistar.

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Abbreviates

AA	Ascorbic Acid
AAS	Atomic absorption spectrophotometry
ADA	American Diabetes Association
ADP	Adenosine Di pehosphate
AGEs	Advanced glycation end-products
ATCC	American type culture collection
ATP	Adenosine Tri pehosphate
BHA	butylated hydroxyanisole
BHT	butylated hydroxytoluene
BMI	Body Mass Index
BSH	Bile salt hydrolase
CA	Cholic acid
CAT	catalase
CE	Cholesteryl ester
CFU	Colony forming unit
CVD	Cardio Vascular Disease
DCA	desoxycholic acid
DCCTRG	Diabetes and Complications Trial Research Group
DM	Diabetes Mellitus
DN	diabetic nephropathy
DNPH	2, 4- dinitrophenylhydrazine
DP	diabetic neuropathy
DPPH	2,2,-diphenyl-1-pricrylhydrozyl
DR	diabetic retinopathy
EASD	The European Association for the Study of Diabetes
EDTA	ethylenediamine-tetraacetic acid
EPS	Extracellular polysaccharides
FA	fatty acids
FAO	Food Agricalture Orgnisation
FFA	free fatty acids
FOS	Fructooligosaccharide
FPG	Fasting Plasma Glucose
FRAP	ferric reducing ability of plasma
GAD	autoantibodies to glutamic acid decarboxylase
GDM	Gestational diabetes mellitus
GSH-Px	glutathione peroxidase
HbA1c	Glycated Haemoglobin
HDL	High-density lipoprotein
HDL-C	High-density lipoprotein cholesterol
HMG-COA	reductase : 3-hydroxy-3- methyle-glutaryl coenzyme A reductase.
HO_2	hydroperoxyl radicals.
HOBr	hypobromous acid
HOCl	hypochlorous acid,
IAAs	insulin autoantibodies
IBS	Irritable bowel syndrome

ICAs	include islet cell autoantibodies
IDDM	insulin dependent diabetes mellitus
IDF	The International Diabetes Federation
IFG	Impaired Fasting Glucose Tolerance
IGT	Impaired Glucose Tolerance
L	Lactobacillus
LAB	lactic acid bacteria
Lc	*Lactococcus*
LDL	Low-density lipoprotein
LDL-C	Low-density lipoprotein cholesterol
LPDE	low-density polyethylene film
LPS	lipopolysaccharides
Lu	*Leuconostoc*
MDA	Malondialdehyde
MODY	maturity-onset diabetes of the young
MOPET	methallized oriented polyster film
MRS	Mann, Rogosa, Sharpe agar
MW	molecular weight
NADH	Nicotinamide Adenosine Di pehosphate Hydrogen
NDDG	National Diabetes Data Group.
NDIC	The National Diabetes Information Clearinghouse
NIDDM	Non-insulin dependent diabetes mellitus
NO_2•	Nitric oxide and nitrogen dioxide
OD	Optical density
OGTT	Oral Glucose Tolerance Test
$ONOO^-$	peroxynitrite
OPET	Oriented polyster film
ORAC	oxygen radical scavenging capacity
OxS	oxidative stress
PAI-1	plasminogen activator inhibitor-1
PARP	poly ADN-ribose polymerase
PG	Plasma Glucose
PG	propylgallate
PHA-P	phytohaemagglutinin- P
PTP1B	bprotein-tyrosine phosphatase 1B
PUFAs	polyunsaturated fatty acids
RBC	Red blood cell
RCS	reactive chlorine species
RNS	reactive nitrogen species
RO	alkoxyl
RO_2•	peroxyl
ROS	reactive oxygen species
Rpm	rotation per minute
RSM	reconstituted skim milk
SCFA	Short-chain fatty acids
SOD	superoxide dismutase
STZ	Streptozotocin.

TAC	total antioxidant capacity
TBA	thiobarbituric cid
TBHQ	*tert*butylhydroquinone
TC	Total cholesterol
TCA	Trichloroacetic acid
TEAC	trolox equivalent antioxidant capacity
TG	Triglycerides
TGF-β	transforming growth factor
TRAP	total radical-trapping antioxidant parameter
WHO	World Health Organization
WK	Water Kefir

1. INTRODUCTION

The aim and importance of functional foods and nutraceuticals is at the confluence of two major factors in our live: food and health. The link between diet and disease has now been quite widely accepted, not only at the institutional level by organizations, but also by a large portion of the populace. In recent years, there appears to have been a growing desire by individuals to play a greater role in their own health and well-being rather than rely strictly on conventional medical practice. As a result, there has been a burgeoning market for a wide range of dietary supplements and nutraceutical products that are perceived by the consuming public to be beneficial in the maintenance of their health and in the prevention of disease.

Traditionally, the healthiness of food has been linked to a nutritionally healthy diet recommended by nutrition specialists and the role of diet as a whole has been emphasized instead of emphasising individual food items. Lately, new kinds of foods, so-called *functional foods*, have been developed and launched. They provide a novel approach to the idea of healthy eating by linking a single component with a certain health effect in a single product (Lähteenmäki, 2003).

Functional foods" is essentially a marketers 'or an analysts' term and globally is not recognized in law or defined in the dictionary. For the purposes of this report, the definition of functional foods as proposed by Health Canada will be used. In 1998, it was proposed that a functional food was "similar in appearance to a conventional food, consumed as part of the usual diet, with demonstrated physiological benefits, and/or to reduce the risk of chronic disease beyond basic nutritional functions (Canada, 2000). These foods are now officially recognized as foods for specified health use or FOSHU.

Functional foods have also been defined as products that have been modified or enriched with naturally occurring substances with specific physiologically preventative and/or health-enhancing effects (Poulsen, 1999).

Dairy products form the major part of functional foods, functional dairy products with a proven health benefit. The most common functional dairy products are those with probiotic bacteria, quite frequently enriched with prebiotic carbohydrates (Mattila-Sandholm and Saarela, 2003).

Prebiotics are non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon. Prebiotic foods are food products that contain a prebiotic ingredient in an adequate matrix and in sufficient concentration, so that after their ingestion, the postulated effect is obtained, and is beyond that of usual nutrient suppliers. Synbiotics are mixtures of pro- and prebiotics that beneficially affect the host by improving the survival and implantation of selected live microbial strains in the gastrointestinal tract (De vrese and schrezenmeir, 2001).

As a result of the accumulative knowledge about the probiotics and prebiotics, and in addition to the advantages of symbiotics specifically, also because of a growing consumer awareness and demand for foods with therapeutic benefits, in this time, Kefir come to sight to be the opportune product which is possess the capacity to be symbiotic product, in the same time kefir is gaining in popularity and commercial production of kefir products has greatly increased over the past few years.

Kefir is produced in large amounts, particularly in Russia. It is also well-known in Sweden, Norway, Finland, Hungary, Poland, Germany, Greece, Austria, Brazil, Argentina, Taiwan, Portugal, Turkey, France, and Iran. In addition to that it is as a new product for wide-scale introduction into the United States, and it is also sold in Japan (Iconomopoulou and Psarianos, 2004; Farnworth, 2005).

Currently, kefir is recognized as a complex probiotic, or symbiotis, and is under study for many potential in-vivo and in-vitro positive health effects.

Kefir is at the same time a functional food and a probiotic, and there is growing evidence that this unique fermented milk product may indeed be helpful in many disease or infection conditions (Farnworth, 2008).

Kefir can be considered a probiotic resource, because it is hold a variety of health claims (immunomodelatory, anti-neoplastic and pro-digestive effects), in addition to its nutritional value (Yang et al., 2008).

Kefir is reported to possess antitumor (Furukawa et al., 1990), antioxidant (Can et al., 2012; Sirirat and Jelena, 2010; Liu et al., 2005a), antimicrobial (Ulusoy et al., 2007), hypocholesterolemic (Cenesiz S. et al., 2008), anti-diabetic (Young-In et al., 2006), and immunity enhancing activities (Thoreux and Schmucker, 2001).

The diverse types of yeasts contribute to the symbitotic relationship through producing compounds that act as growth stimulants for the bacteria (Adriana and Socaciu, 2008). Non-lactose fermenting yeasts make up the majority of the fungal species within kefir grains (Simova et al., 2002).

Water kefir is the results of fermentation of sugar solution by kefir grain which contains of lactic acid, acetic acid and yeasts, which produce important molecules such as polypeptide, polysaccharide, organic acid, and other compounds. Thus and according to the previous findings, water kefir can provide benefit microorganisms and bioactive molecules, and help in health improving.

Accordingly, and to introduce a new refreshing and beneficial drink to food's table or drink's list of Arabic and Muslim peoples in Arabic countries, and for offers alternative kefir for the Muslims in the other world's countries, this study was designed.

In this work, we analyzed kefir grain to determine its chemical composition, in addition to isolation and identification of its microorganisms, and determined their distribution in kefir grain, and studying their contribution in organoliptic and therapeutic properties of kefir.

Moreover, we produced kefir with modification in production procedures and conditions to avoid or minimize the production of alcohol during production processes. Also for stopping the production of alcohol we tried to determine the microorganisms that responsible on alcohol forming and alcoholic fermentation conditions, for modifying it or even isolate these microorganisms from the starter of kefir, or inhibit its growth bay addition inhibitors to fermentation medium, for obtains a new kefir grains or (Islamic kefir grains) which don't produce alcohol, and consequently producing Islamic kefir.

In the same time, we investigated the antioxidant activity of water kefir as the first study in this subject by several methods *invitro*.

We also studied the therapeutic and metabolic effects of water kefir on wistar rats, that had been investigated through studying, antihyperglycemic, andanti-hyperlipidimic effects of water kefir on streptozotocin-induced diabetic *wistar* rats, the effects of water kefir in oxidative stress of streptozotocin-induced diabetic wistar rats were also determined.

This study is dividing to tow main parts, the theatrical part, this part also divided to four divisions , Kefir, which including its history, definition, chemical and microbial structure, production, and water kefir, its structure, water kefir (definition, preparation, water kefir, microorganisms, and the health benefits of water kefir). Diabetes mellitus section contained of definition, classification, diagnosis, Signs and symptoms, its complication and prevention. Oxidative stress comprising, definition, free radicals, generations, classification, reactive, effects on diabetes, antioxidant (types, sources, action, effects of diabetes). And finally the Metabolic and Therapeutic effects of kefir as probiotic which are, productivity of bioactive components, anti-diabetic, anti-hyperlipidimic, antimicrobial, antioxidative, effects on lactose tolerance and digestion, anti-inflammation and anti-allergic, antitumor, and Stimulate the Immune System. The second part is the experimental part, dividing to four sections, materials and methods, results, discussion, and conclusion.

2. LITERATURE REVIEW

2.1. Kefir

2.1.1. Kefir history and Origin

The production of fermented milks was known to the ancient Greeks and Romans. Historically, kefir grains were considered a gift from Allah among the Muslim people of the northern Caucasian mountains (Lopitz-Otsoa A. *et al.*, 2006). The Greek historian Herodotus (485–425 BC) reported that a refreshing drink produced from mare's milk was popular among the Ghets tribes. According to Caucasian legend, Mahomet (ca. 570–632) gave Mahomet's grains (also known as mushrooms) together with the secret kefir recipe to the inhabitants of the region (Koroleva, 1991).

Because of its ancient and apparently mysterious origin, kefir was known in antiquity as the "Drink of the Prophet [Mohammad]" and the culture used to prepare it as the "Grains of the Prophet Mohammad"; it was believed that the Prophet of Islam, Mohammad, was given the original kefir grains by the Angel Gabriel to be given to his followers, thus introducing kefir to the Orthodox Christians living in the mountainous regions of modern day Georgia (Rosell, 1932; Margulis, 1996). Thus, kefir is one of the oldest fermented milk beverages, and the technology of production and the use of a specific starter culture have developed through the ages. Its origin can be traced back to the Caucasus region where it has been produced by a traditional method in bags made from animal hides, or in oak barrels or earthenware pots. As these containers were used continuously (i.e. after some kefir being consumed, a new batch of fresh milk was added), the micro-organisms tended to form a thin layer and later clusters on the surfaces of the containers. This process of microbial film formation was helped by the warm conditions of the Caucasus (Wszolek *et al.*, 2006).

The first fermented foods may have been produced by accident. However, fermentation of foods such as milk became a widespread method of preservation before refrigeration was introduced or preservation procedures such as canning and pasteurization were developed and used to extend shelf life. It would appear from the oral tradition of kefir that fermentation of milk in skin bags as a way of preserving milk led to the production of the first kefir grains and started the long tradition of producing kefir.

Kefir has been produced using milk from cows, ewes, goats, and buffalo and has been sold in Europe under a variety of names including *kephir*, *kiaphur*, *kefyr*, *képhir*, *kéfer*, *knapon*, *kepi*, and *kippe* (Kemp, 1984). Leben is another fermented milk, which originated in the Middle East countries. It is a concentrated yogurtlike or kefirlike product. The related fermented milks are known under different names, e.g., labneh or lebneh from Lebanon or other Arabian countries; tan from Armenia; torba and tubem from Turkey; gioddu from Italy; and matzun from Russia (Batish and Grover, 2004).

Traditional kefir was made in skin bags. The milk was poured in daily and a natural fermentation took place. It was customary to hang the bag near the door, and everyone who came in or out had to push or kick the bag in order to mix the liquid (Koroleva, 1988; IDF, 1991).

2.1.2. Kefir definition

Kefir had defined in different sources according to its characteristics' contains' microorganisms and the manner of its production. We will adduce the more common definitions.

1) Kefir defined as the thick flowing cream and slightly effervescent fermented milk that is obtained from cow's, goat's, or sheep's milk. It contains 0.8 to 1 percent lactic acid, 0.3 to 0.8 percent ethanol, carbon dioxide, and other flavor products, such as acetaldehyde and acetoacetate. The alcohol and carbon dioxide, together with small amounts of diacetyl, acetaldehyde, and acetone, contribute to its characteristic refreshing taste. Kefir is produced in large amounts, particularly in Russia, where it accounts for 70 percent of the total amount of fermented milk consumed. In many countries, the popularity of kefir is only slightly less than that of yogurt. It is also well known in Sweden, Norway, Finland, Hungary, Poland, Germany, Greece, Austria, Brazil, and Israel. Fermented dairy products comprise an important segment of the dairy industry. Kefir is being investigated as a new product for wide-scale introduction into the United States, and it is also sold in Japan, although consumption is lower (Iconomopoulou and Psarianos, 2004). This definition cites some properties of kefir and the milk used in kefir production, its flavor composition and its distribution in the world.

And there are other definitions combined with organoleptic characteristics of kefir which are: 2) Kefir is a viscous pour able liquid, with a smooth, slightly foamy body and whitish color. It is yeasty, acidic, mildly alcoholic, refreshing, and slightly effervescent. The name kefir is derived from the Turkish term kef (which means “pleasant taste.”)or from kefy in the Caucusus region implying pleasant taste (Vikram, 2004). 3) Kefir is a refreshing fermented milk beverage that has an exotic sour and slightly alcoholic flavor (Rimada and Abraham, 2001). 4) Kefir is an acidic and mildly alcoholic fermented dairy product that is believed to contain many functional substances, and it has been postulated that the longevity of Bulgarian peasants is partially due to their frequent consumption of this fermented milk (Gilliland, 1990)

5) (Kemp, 1984) Defined kefir as a beverage produced by the action of lactic acid bacteria (LAB), yeasts, and acetic acid bacteria on milk. This complex mixture of microorganisms produces a distinctive fermented milk product with unique properties.

6) Kefir also defined as a stirred beverage made from milk fermented with a complex mixture of bacteria, including various species of lactobacilli, lactococci, leuconostocs, and aceterobacteria and yeasts (both lactose-fermenting and nonlactose-fermenting). Kefir differs from yogurt and other fermented milks in that kefir grains (small clusters of microorganisms held together in a polysaccharide matrix) or mother cultures from grains are added to milk and cause fermentation (Halle´ *et al.*, 1994). These two last definitions the first focused on the microorganisms and the production of kefir, while the next definition gives more detail of bacteria and explain the different between kefir and yogurt.

The FAO/WHO (2001) have proposed a definition of kefir based on the microbial composition of both kefir grains (the starter culture used to produce kefir) and the final kefir product (Table 2-1) (Farnworth, 2005).

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Table 2-1 Codex Alimentarius description of kefir

Definition	
Starter culture prepared from grains, *lactobacillus kefiri,* and species of the genera *leuconostoc, lactococcus and Acetobacter* growing in a strong specific relationship. Kefir grains constitute both lactose-fermenting yeasts (*Kluuyveromyce marxianus)* and non-lactose-fermenting yeast (*Saccharomyces unisporus, Saccharomyces cerevisiae and Saccharomyces exiguous)*	
Composition Milk protein (% W/W)	min. 2.8
Milk fat (% m/m)	< 10
Titratable acidity expressed as % of lactic acid (% m/m)	Min. 0.6
Ethanol (% vol.w)	Not stated
Sum of specific microorganisms constituting the starter culture (cuf/g in total)	Min. 10^{7}
Yeasts (cuf/g)	Min. 10^{4}

From Codex standard for fermented milks CODEX STAN 243-2003

2.1.3. Kefir manufacture

Kefir is manufacturing in several methods including four principle steps: 1) Inoculation (with kefir grains or culture), 2) Incubation, 3) separation of starter, 4) Maturation or ripening. The main differences between all processes are represented with the type of used milk, the state of milk (pasteurized, or not), the rate of kefir grains (3-10%), slightly temperature, time of fermentation and ripening process. Here are details of some methods.

The prepared milk is heated in the heat exchanger to 70°C, and is then homogenized and pasteurized at 85°C for 30 min. Pasteurized milk is cooled to the fermentation temperature of 20 to 25°C (usually 22°C). The milk is seeded with kefir cultures 2 to 10 percent (w/v) and fermented in the ripening vessel for 18 to 24 h until the pH reaches to 4.6 to 4.4. After incubation, the kefir grains are removed via sieving. The grains can be reused in subsequent starter culture preparation or be washed well with cold water and added to a new production batch. With each subsequent incubation, the size of the kefir grains increases slightly. The kefir is cooled to 7 to 12°C and filled either into glass bottles or plastic beakers. It can be used for consumption or left to post ripen at 7°C for 24 to 48 h. The closure of the bottles permits carbon dioxide to escape, and a significant amount of the alcohol is lost (Iconomopoulou and Psarianos, 2004). Traditional kefir has to be prepared from starter cultures produced from kefir grains, kefir grains are added

into pasteurized milk, then incubated with stirring periodically at 24-26°C, until the pH is decreased to 4.6. After incubation kefir grains are removed by sieving to reuse as kefir starter culture (Güzel-Seydim *et al.*, 2000).

The traditional artisanal production involves milk inoculation with a variable amount of grains and fermentation for a period between 18-24 h at 20-25 °C. At the end of the fermentation process the grains are sieved and can be used for a new fermentation or kept (1-7 days) in fresh milk, while the kefir beverage is stored at 4 °C, ready for consumption

(Otles and Cagindi, 2003; Beshkova *et al.*, 2002; Farnworth and Mainville, 2008). summarize the traditional method and industrial method of kefir production in schema in (Figure 2-1).

In another process, a kefir starter is first obtained from the grains for fermentation. Here, freeze-dried kefir grains are rehydrated in a sterilized 0.9% sodium chloride solution at 20°C for 5 hr. The grains are then washed with sterile water and added in a 1:30 ratio to skim milk that has been heated to 95°C for 30 min and cooled to 25°C. After incubation for a day, the grains are sieved and the process of fermentation in skim milk is repeated two more times. After the final fermentation, the grains are sieved and the skim milk without the grains is then added as the kefir culture at the rate of 5% to milk that has been heated to 85°C for 30 min and adjusted to 22°C. Fermentation ensues for 12 hr (pH 4.5 to 4.6) followed by ripening for 1 to 3 days at 8 to 10°C. During ripening, yeast fermentation occurs (Vikram, 2004).

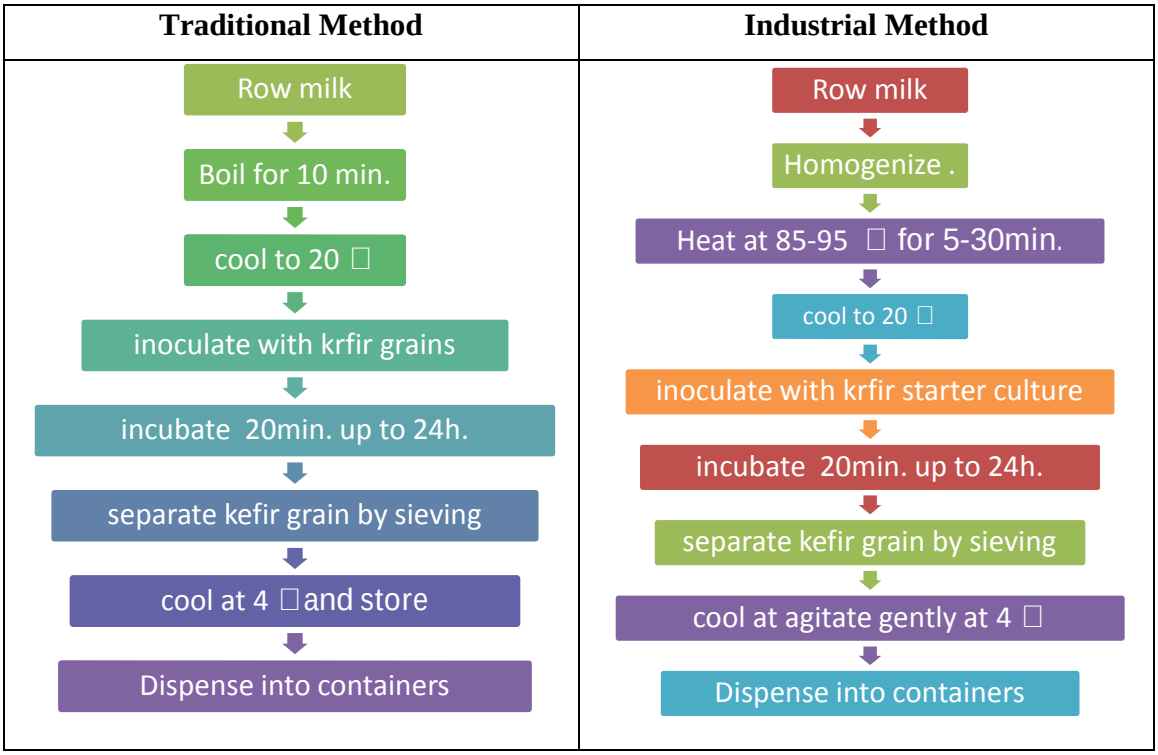


Figure 2-1 Tradition and modren manufacturing process for kefir (Otles and Cagindi, 2003)

Agitation during fermentation also influences kefir microbial composition, favoring the development of homofermentative *lactococci* and yeast (Farnworth and Mainville, 2008; Rattray and O'Connell, 2011; Tamime, 2006). Incubation at temperatures above 30 °C stimulates the growth of thermophilic LAB, while being a disadvantage for yeast growth and mesophilic LAB (Rattray and O'Connell, 2011). Kefir grains can ferment any fresh mammalian milk, but they can also be used to ferment soy, rice, or almond milks (Lopitz-Otsoa A. *et al.*, 2006). Kefir has been produced using milk from cows, Sheep, goats, and buffalo, milk whey, soy milk, soy whey, rice milk (Cais-Sokolinska *et al.*, 2008; Farnworth, 2008 ; KESENKAŞ *et al.*, 2011; Monajjemi *et al.*, 2012; Sirirat and Jelena, 2010). Recently kefir can be made from other medias of fermentation as Carbohydrates solutions (Bergmann *et al.*, 2010; Harta *et al.*, 2004), and cheese whey, and a lactose-rich waste which is negligible cost (Papapostolou *et al.*, 2008), Peanut-Milk (Bensmira and Jiang, 2011), this kefir which based on carbohydrates is called sugary kefir or water kefir.

2.1.4. Kefir grains

Kefir grains are the most important components of kefir manufacture. The concept of kefir grains is believed to have originated in the accidental discovery of kefir many hundreds of years ago in the Caucasus region. These grains vary in size from a wheat grain to the size of a walnut (Figure 2.2). They are of whitish to yellowish color and gelatinous and irregular in shape and with a rough surface (Vikram, 2004). Kefir grains range in size from 0.3 to 2.0 cm or more in diameter, and are characterized by forming an irregular, folded or uneven surface; the grains resemble cauliflower florets in shape and color. They are elastic and white or slightly yellow in color, and have a characteristic smell. Kefir grains have a specific structure and biological function (Tamime, 2006).

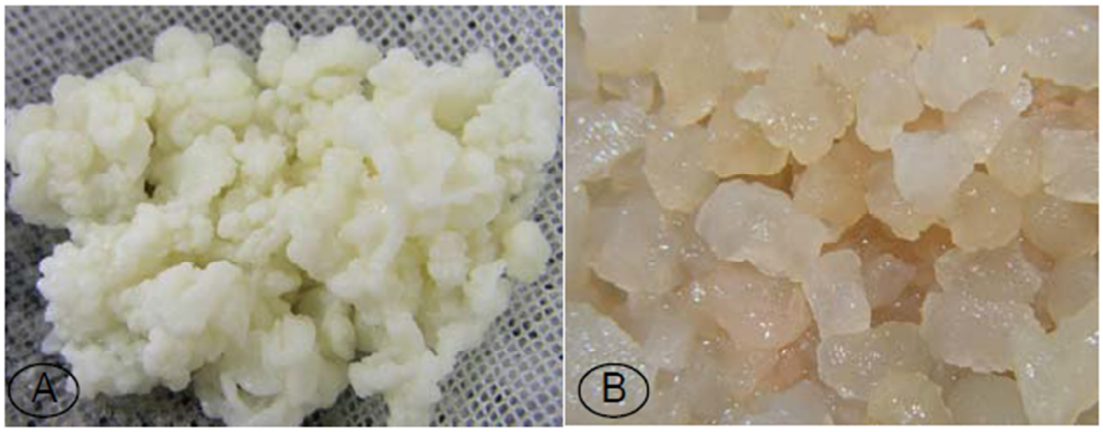


Figure 2-2 Morphological structure of A) Milk kefir grains, B) Water kefir grains

Kefir grains are whitish-yellow clumps. The diameter of each kefir grain is 3 to 20 mm, and together they resemble small cauliflower florets. The kefir grains contain symbiotic, plural lactic acids bacteria and plural yeasts. Using the sparingly water-soluble, but highly swell able polysaccharide kefiran and acid-coagulated casein, each cell is bound to the others .“ kefir grains. ” They are white, irregular, gelatinous, cauliflower - like structures of variable size ranging from 0.3 to 3.5 cm in diameter. They are composed of proteins (Iconomopoulou and Psarianos, 2004) and polysaccharides in which the complex mirobiota is enclosed. During fermentation, grains increase in size and number, and this is how new biomass is obtained; grains are generally recovered from the fermented milk to be reused (Mozzi *et al.*, 2010).

Kefir grains consisting of yeasts, bacteria, and polysaccharides are used for kefir production (Tamime and Marshall, 1997). The yeasts include *Saccharomyces kefir* and *Torula spp.* or Candida kefir and bacteria include *Lb. kefir*, *leuconostocs*, *lactococci*, and various others. Takizawa *et al.* (1998), isolated 120 strains of lactobacilli from kefir grains; the most prominent was *Lb. kefirogranum.* The grains require proper care and should be held using routine sanitary practices.

2.1.4.1. *Chemical Composition of kefir grains*

Kefir grains are a curd-like material, which are filtered-off after each use and reused for inoculation of the next batch (Chandan *et al.*, 2006). The grains are made of a conglomerate of microbial cells and their metabolites, coagulated milk proteins and carbohydrates. Kefir grains generally contains of moisture, proteins, carbohydrates, lipids, and minerals, the concentration of these components is differs according the source of kefir grains and the type and structure of milk or the media (soy, sugar solution, rise, fruit juice, cocoa,…..) of production and propagation of kefir grains. Table 2-2 show the chemical composition of kefir grains from different sources.

Table 2-2 Chemical Composition of kefir grains

	Chemical Composition							
Units	Water	Carbohydrates	Proteins	Fats	Ash	Dry M	NPSN[a]	References
g %		25-50	30	-		10–16	-	(Libudzisz and Piatkiewicz, 1990)
g%	90	-	3.2	0.3	0.7		5.8	(Ottogalli *et al.*, 1973)b ; (Bottazzi *et al.*, 1994)[c]
g %	83	9_10	4.5	-	-	-	-	(Abraham and De Antoni, 1999) ; (Garrote *et al.*, 2001)d
by dry weight		24%	13%	-	-	-	-	(Halle´ *et al.*, 1994)
g %	82.6-83.5	6.34-7.76	4.61-5.43	1.35-1.69	-	-	-	(Anine and Trevor, 2003)
%		46[e] 24[f]	13	-	-	12_14		(La Riviere and Kooiman, 1967)
%	84.4	4.7-8.7	6.6-9.6	-	-	-	-	(Liu *et al.*, 2002)[g]
%	86.3		4.5	0.03	1.2	13,7		(Liutkevičius and Šarkinas, 2004)

a non-protein soluble nitrogen, **b** kefir grains originating from Russia, Yugoslavia and Bulgaria, **c** grains originating from Sweden, **d** grains obtained from households in Argentina, **e** insoluble in cold water , **f** soluble in cold, **g** the structure is for milk and soy milk kefir grains

2.1.4.2. *Kefir grains production, propagation and maintenance*

Modern kefir grain production is based on continuous cultivation in milk, resulting in biomass increases of 5–7% per day (Libudzisz and Piatkiewicz, 1990). (Anine and Trevor, 2003), found the highest grain increase occurred when activated grains were cultivated with agitation at 25°C in low-fat milk containing tryptose and with the fermented milk replaced daily. Kefir grains are propagated at room temperature by daily transfer in tap water-sugar solution (60 g/litre) (Pidoux, 1989). The effects of different parameters including percentage of fat in milk, skim milk proportion, incubation temperature, incubation time, tryptose, glucose, CO concentration and amount of milk on the percentage yield 2 of kefir grain were investigated, this investigation found that, skim milk and low fat milk were the best medium for kefir grain production, and the Optimum condition for maximum kefir grain production were: Fat 0, Glucose 0.01%, Skim milk 10%, Temperature 37°C, Time 36 hr, Tryptose 0%, CO2 10%, Skim milk 1.5 ml (Habibi *et al.*, 2011).

Temperature increase up to 40 °C when whey was fermented with kefir grains negatively affected the biomass growth with simultaneous increment of EPS in culturing media that could be explained as extraction. The kefiran synthesis by kefir starter was suppressed as well, pointing on lower temperature optimum for the primary kefiran producers (Enikeev, 2012). Kefir grains replicated in soy milk have been reported to be smaller in size compared to grains replicated in cows' milk (Liu *et al.*, 2002).

It has been recommended that in a commercial operation using grains to produce kefir, grains should be kept viable through daily transfers and should only be replaced if their ability to ferment milk becomes impaired (Koroleva, 1982).

The results of Chen *et al.* (2006), indicated significant loss in viability of microorganisms in kefir after freeze-drying. Addition of 10% galactose or 10% sucrose as lyoprotectants significantly increased the survival rates of both lactic acid bacteria and yeasts ($p<0.05$). The 4°C rehydration temperature showed the best viabilities for yeasts, however, viability was not significantly affected by rehydration media ($p>0.05$).

Garrote *et al.* (1997), showed that storage of kefir grains at−80 or −20°C for 120 days did not change their fermentation properties compared to grains that had not been stored; however, grains stored a − 4°C did not produce acceptable kefir after thawing. And Liu *et al.* (1999), recommended storage of lyphilized kefir grains at −20°C and viability of LAB retained stable and resulted in kefir with viable population similar to those obtained from non-stored grains. Generally low temperature storage appears to be the best way to maintain kefir grains for long periods, kefir grains could be preserved by frozen storage without milk at -18°C, refrigerated storage at 4°C, air drying at room temperature for three weeks in a dessicator or freeze drying but air drying is not recommended for commercial application due to unacceptable color and flavor of grain (Witthuhn *et al.*, 2005a).

The grains grown in the process of kefir production can be dried at room temperature and kept at cold temperature (4°C). When kefir grains are washed with clean, cold water and dried on cloth or paper for two days at room temperature, they can then be stored in a dry, cool place for well over a year and still stay active. For longer preservation, they can also be freeze-dried. Upon sterilization, freeze-dried kefir grains are to be supplemented with yeast preparation showing the lowest survival in the course of freeze drying. A mixture of 20 percent sucrose solution and starch appears to be the most efficient dispersing agent to protect yeast in the freezedrying process (Iconomopoulou and Psarianos, 2004).

It is important to conserve kefir grains in appropriate containers that not permit pollution and permeating of harmful substances to kefir grains and effect on their viability. (Witthuhn *et al.*, 2005b), recommend low-density polyethylene film (LPDE), oriented polyster film (OPET) and methallized oriented polyster film (MOPET), for retaining the activity of kefir grains for extended storage period.

2.1.5. Microorganisms of kefir and kefir grains

The isolation and identification of microorganisms in kefir grains and kefir depends on the choice of suitable growth media, and more recently, sophisticated methods of identification have been used. It should be noted here that the identity of several of the microorganisms found in kefir has been revised over time as more definitive methods of

identification have been used. In some cases, the nomenclature assigned to various bacterial species has also changed (Farnworth and Mainville, 2008).

The symbiosis found in the kefir grain microorganism population allows the grains to maintain uniformity so that throughout the year the microbiological profile of kefir grains and the kefir drink remain stable in spite of variations in milk quality and the presence of antibiotics and other inhibiting substances (Koroleva, 1982).

Kefir grains contain polysaccharides and milk residue embedded with bacteria *Lb. kefir, Lb. kefirogranum*, and species of *leuconostocs, lactococci,* and *lactobacilli.* Along with bacteria, the grains contain yeasts including *Saccharomyces kefir, Candida kefir*, and *Torula* species (Chandan *et al.*, 2006). Zubillaga *et al.* (2001), described kefir grains as a symbiotic association between lactic bacteria, acetic bacteria, and yeasts among other microorganisms.

Kefir grains a starter culture for kefir production, contain a complex microbial symbiotic mixture of lactic acid bacteria (LAB) (*Lactobacillus, Lactococcus, Leuconostoc,* and *Streptococcus spp.*), acetic acid bacteria (*Acetobacter*) and yeasts (*Kluyveromyces, Sa charomyces* and *Torula*) included in a polysaccharide ,protein matrix (Irigoyen et al., 2005; Farnworth, 2005).

The microorganism profile of the final product does not necessarily parallel that of the grains because of conditions (pH and other) during the fermentation process. Also, the location of the microorganisms in the grains may be a factor. Yeasts are generally found in the interior of the grains, while lactococci are found on the exterior. Therefore, the numbers of yeasts found in the final product are lower than those counted in the grains themselves, while lactococci are numerous in the final drink. The complex microbiological composition of kefir grains produces kefir. Therefore, unlike the situation with yogurt, the drink kefir cannot be used as a starter to produce more kefir (Farnworth and Mainville, 2008).

Kefir grains are mostly composed by proteins and polysaccharides and enclose a complex microflora. Lactic acid bacteria (LAB) and yeasts exist in a complex symbiotic relationship (Papapostolou *et al.*, 2008). Kefir grains have a complex microbiological composition, and they consist of a blend of lactic acid bacteria ~ 83–90%, yeasts ~ 10–

17%, acetic acid bacteria and possibly a mould. According to Polish Standards, the microscopic observation of the grains should consist of 80% lactobacilli, 12% yeasts and 8% lactococci (Tamime, 2006). The account of microorganisms in kefir grains was, 1.5-2.1 $\times 10^8$ lactic acid bacteria, 1.8-5.6 $\times 10^7$ yeast, 2.5-2.6$\times 10^5$ Acetic acid (Garrote *et al.*, 2001). The geometric means of the microbial counts, expressed in CFU g-1 of grains, were of 1.4 $\times$ 10^8, 3.9 $\times$ 104 and 1.1 $\times$ 10^7 respectively for the lactobacilli, the lactic acid streptococci and the yeasts (Ninane *et al.*, 2005). The amount of microorganisms was in Portugese kefir grains 109 cfu/ml lactobacilli,108 cfu/ml lactococci, and 106 - 109 cfu/ml yeast (Ferreira *et al.*, 2010), in irish kefir grains 106 cfu/ml, lactococci 109 cfu/ml 106 cfu/ml (Rea *et al.*, 1996). The microbial enumeration of Brazilian kefir grains during fermentation at 24h was 7.54 log10 c.f.u./ml for Edwards modified/Lactic acid bacteria, 7.34 log10 c.f.u./ml for Nutrient Agar/Lactic acid bacteria, 10.41 $\log_{10}$ c.f.u./ml for LUSM/Lactic acid bacteria, 12.41 $\log_{10}$ c.f.u./ml for MRS/Lactic acid bacteria, 7.71 $\log_{10}$ c.f.u./ml for M17/Lactic acid bacteria, 7.72 $\log_{10}$ c.f.u./ml for 135 medium/Acetic acid bacteria, and 8.11 $\log_{10}$ c.f.u./ml for YEPG/Yeast (Magalhães KT. *et al.*, 2011b). Table 2-3 show microorganisms of kefir and kefir grains.

Table 2-3 Microorganisms of kefir and kefir grains

Microorganisms	**Reference**
Yeasts	
Kluyveromyces lactis	(Bergmann *et al.*, 2010; Gerrit *et al.*, 2004 ; Magalhaes *et al.*, 2010b; Ottogalli *et al.*, 1973)
Candida kefir	(Bergmann *et al.*, 2010; Gerrit *et al.*, 2004 ; Knut, 2004 ; Vikram, 2004).
Saccharomyces cerevisiae	(Bergmann *et al.*, 2010; Chen *et al.*, 2008; Garrote *et al.*, 2001; Gerrit *et al.*, 2004 ; Jianzhong *et al.*, 2009; Knut, 2004 ; Magalhaes *et al.*, 2010b; Simova *et al.*, 2002; Wang *et al.*, 2008a).
Saccharomyces delbrueckii	(Gerrit *et al.*, 2004 ; Knut, 2004)
Saccharomyces florentinus	(Leroi and Courcoux, 1996)
Candida pseudotropicalis	(Chen *et al.*, 2008; Vikram, 2004; Wang *et al.*, 2008a)
Candida tenuis	(Ottogalli *et al.*, 1973)
Kluyveromyces marxianus subsp. marxianus	(Bergmann *et al.*, 2010; Chen *et al.*, 2008; Garrote *et al.*, 2001; Gerrit *et al.*, 2004 ; Jianzhong *et al.*, 2009; Knut, 2004 ; Magalhaes *et al.*, 2010b; Simova *et al.*, 2002; Wang *et al.*, 2008a; Koktas *et al.*, 2012; Witthuhn *et al.*, 2004).
Saccharomyces kefir	(Koktas *et al.*, 2012)
Saccharomyces turicensis	(Chen *et al.*, 2008; Vikram, 2004; Wang *et al.*, 2008a)
Torula spp.	(Vikram, 2004)

Candida friedrichii	(Angulo *et al.*, 1993)
Candida maris	(Simova *et al.*, 2002)
Candida inconspicua	(Bergmann *et al.*, 2010; Simova *et al.*, 2002)
Pichia fermentans	(Chen *et al.*, 2008; Wang *et al.*, 2008a)
Candida lambica,	(Pidoux, 1989)
Candida valida	(Pidoux, 1989)
Kloeckera apiculata	(Pidoux, 1989)
Torulaspora pretoriensis	(Pidoux, 1989)
Zygosaccharomyces florentinus	(Pidoux, 1989)
Kazachstania unispora,	(Jianzhong *et al.*, 2009)
Kazachstania exigua	(Jianzhong *et al.*, 2009)
Kazachstania aerobia	(Magalhaes *et al.*, 2010b)
Lachancea meyersii	(Magalhaes *et al.*, 2010b)
Candida colliculosa, Toruspola delbruechii, Candida magnoliae, Kloekera sp., Candida famata	(Bergmann *et al.*, 2010)
Bacteria	
Acetobacter aceti	(Jianzhong *et al.*, 2009; Vikram, 2004)
Acetobacter pasteurianus	(Magalhaes *et al.*, 2010b; Ottogalli *et al.*, 1973)
Acetobacter lovaniensis	(Magalhaes *et al.*, 2010b)
Lactobacillus casei	(Jianzhong *et al.*, 2009; Pidoux, 1989; Vikram, 2004)
Lactobacillus helveticus	(Jianzhong *et al.*, 2009; Koktas *et al.*, 2012; Simova *et al.*, 2002; Vikram, 2004)
Lactobacillus kefir	(Bergmann *et al.*, 2010; Chen *et al.*, 2008; Garrote *et al.*, 2001; Gerrit *et al.*, 2004 ; Jianzhong *et al.*, 2009; Knut, 2004 ; Magalhaes *et al.*, 2010b; Simova *et al.*, 2002; Wang *et al.*, 2008a; Koktas *et al.*, 2012; Witthuhn *et al.*, 2004; Mainville *et al.*, 2006; Ottogalli *et al.*, 1973; Takizawa *et al.*, 1998).
Lactobacillus kefirogranum	(Takizawa *et al.*, 1998; Vikram, 2004)
Lactobacillus kefironafaciens	(Bergmann *et al.*, 2010; Chen *et al.*, 2008; Garrote *et al.*, 2001; Gerrit *et al.*, 2004 ; Jianzhong *et al.*, 2009; Knut, 2004 ; Magalhaes *et al.*, 2010b; Simova *et al.*, 2002; Wang *et al.*, 2008a; Koktas *et al.*, 2012; Witthuhn *et al.*, 2004; Mainville *et al.*, 2006; Ottogalli *et al.*, 1973; Takizawa *et al.*, 1998).
Lactobacillus delbrueckii subsp. bulgaricus	(Simova *et al.*, 2002; Witthuhn *et al.*, 2004)
Lactobacillus hilgardii	(Leroi and Courcoux, 1996; Pidoux, 1989; Pidoux *et al.*, 1990)
Lactobacillus casei ssp. rhamnosus	(Pidoux, 1989)
Lactococcus lactis subsp. Lactis	(Bergmann *et al.*, 2010; Chen *et al.*, 2008; Garrote *et al.*, 2001; Gerrit *et al.*, 2004 ; Jianzhong *et al.*, 2009; Knut, 2004 ; Magalhaes *et al.*, 2010b; Simova *et al.*, 2002; Wang *et al.*, 2008a; Koktas *et al.*, 2012; Witthuhn *et al.*, 2004; Mainville *et al.*, 2006; Ottogalli *et al.*, 1973; Takizawa *et al.*, 1998; Vikram, 2004))
Lactobacillus lactis cremoris	(Bergmann *et al.*, 2010)
Leuconostoc mesenteroides subsp. Dextranicum	(Bergmann *et al.*, 2010; Garrote *et al.*, 2001; Pidoux, 1989; Vikram, 2004)
Leuconostoc mesenteroides subsp. Cremoris	(Chen *et al.*, 2008; Jianzhong *et al.*, 2009; Vikram, 2004; Wang *et al.*, 2008a; Witthuhn *et al.*, 2004)
Leuconostoc lactis,	(Jianzhong *et al.*, 2009; Knut, 2004)

Leucon. Mesenteroides	(Garrote *et al.*, 2001; Jianzhong *et al.*, 2009; Knut, 2004 ; Mainville *et al.*, 2006)
Leuconostoc citreum	(Magalhaes *et al.*, 2010b)
Lb. Fermentum	(Knut, 2004 ; Ottogalli *et al.*, 1973)
Lb. acidophilus	(Koktas *et al.*, 2012; Ottogalli *et al.*, 1973)
Lb. casei(4)	(Jianzhong *et al.*, 2009; Knut, 2004 ; Magalhaes *et al.*, 2010b; Ottogalli *et al.*, 1973)
Lb. paracasei subsp. paracasei ,tolerance	(Magalhaes *et al.*, 2010b; Pidoux *et al.*, 1990; Simova *et al.*, 2002)
Lb. brevis,	(Angulo *et al.*, 1993; Knut, 2004 ; Ottogalli *et al.*, 1973; Simova *et al.*, 2002)
Lb. Buchneri and barabuchneri	(Knut, 2004 ; Magalhaes *et al.*, 2010b)
Acetic acid bacteria (Acetobacter pasteurianus)	(Angulo *et al.*, 1993; Bergmann *et al.*, 2010; Garrote *et al.*, 2001; Knut, 2004 ; Ottogalli *et al.*, 1973)
Pseudomonas sp.	(Chen *et al.*, 2008; Jianzhong *et al.*, 2009)
Streptococcus thermophilus,	(Angulo *et al.*, 1993; Koktas *et al.*, 2012; Simova *et al.*, 2002)
Streptococcus lactis	(Pidoux, 1989)
Streptococcus cremoris	(Pidoux, 1989)
Lactobacillus rhamnosus	(Angulo *et al.*, 1993)
Bifidobacterium bifidum	(Koktas *et al.*, 2012)
Lactobacillus plantarum	(Garrote *et al.*, 2001; Pidoux, 1989)
Lactobacillus parakefir	(Garrote *et al.*, 2001; Takizawa *et al.*, 1998)
Weissella viridescens	(Angulo *et al.*, 1993)

2.1.6. Nutritional and chemical structure of kefir

Kefir as a probiotic product contains various compounds, the types and concentrations of kefir ingredients are varied, money factors effect on the variety of kefir structure, the principal responsible on the final kefir structure can be restricting in the characteristics of kefir grains or cultures and fermentation media, manufacturing method including fermentation conditions (Temperature, period, pH,…), concentration of inoculates, ripening and storing time. Several studies accomplished to determine the chemical structure of kefir, most of these studies focused on the compounds that responsible of the organoleptic properties of kefir like CO_2, alcohol, lactic and acetic acid, and aroma compounds in addition to bioactive compounds such as polysaccharides and polypeptides. Bakhshandeh *et al.*, (2011), evaluated flavor and aroma compounds cresent in kefir and found that levels of acetaldehyde, diacetyl, acetoin and ethanol were increased with raising the temperature and decreased during cold storage from the first day until the 14th day. pH was decreased and acidity was increased in all samples during cold storage.

The major products formed during fermentation are lactic acid, CO_2 and alcohol. Diacetyl and acetaldehyde, which are aromatic compounds, are present in kefir (Zourari and Anifantakis, 1988). Diacetyl is produced by *Str. lactis subsp*. diacetylactis and *Leuconostoc sp*. (Libudzisz and Piatkiewicz, 1990).

During fermentation various flavouring compounds are produced by starter cultures. Acetaldehyde is produced by *L. delbrueckii subsp. bulgaricus,* diacetyl by *S. thermophilus, L. helveticus and L. lactis subsp*. *lactis*, acetone by *L. delbrueckii subsp. bulgaricus* and *L. helveticus*, ethanol by *S. cerevesiae (Beshkova et al., 2002)*. The chemical and nutritional composition of kefir are shown in Table 2-4.

Table 2-4 Chemical and nutritional composition of kefir

Components	**Quantity**	**Description**	**References**
Water %	87.53-90.63	Strawberry Kefir	(Ergönül, 2007)
Dray matter%	11.3-11.7 9.3-13.39 8.24-19.9 5.3-5.56	1or 5% KG in cow's milk Poland kefir Turkish kefir(altered fat Kefir of isolated kefir starter	(Irigoyen *et al.*, 2005) (Sady *et al.*, 2007) (Ertekin and Guzel-Seydim, 2010; Assadi *et al.*, 2000)
Proteins%	3.10-4.72 1.36-166	polish kefir Kefir of isolated kefir starter	(Sady *et al*., 2007; Assadi *et al*., 2000)
Carbohydrates Lactose% EPS(µg/mL) Lactose%	 2.94-3.75 18.23-115.25 35.87-118.19 4.78-5.06 3.7 1.1-1.46	 1% or 5% (w/w) kefir grains. with entrapped starter culture with kefir grains.goat milk kefir(18-24h) Norwegian kefir (8 week) Kefir of isolated kefir starter	 (Irigoyen *et al.*, 2005; Chen *et al.*, 2009) (Purnomo and Muslimin, 2012; Grønnevik *et al.*, 2011) (Assadi *et al.*, 2000)
Fats%	3.23-3.59 2.2-2.6 1.11-2.55 1.4-1.95	1% or 5% (w/w) kefir grains. Strawberry Kefir Poland kefir Kefir of isolated kefir starter	(Assadi *et al.*, 2000; Ergönül, 2007; Sady *et al.*, 2007)
Ash%	0.75-0.86	Kefir of isolated kefir starter	(Assadi *et al.*, 2000)
Lactic acid%	3.44-7.22 4.79-7.56 2400±50mg/kg 7.0-9.2g/L	with entrapped starter culture with kefir grains. Norwegian kefir (8 week) Turkish kefir(altered fat)	(Chen *et al.*, 2009; Grønnevik *et al.*, 2011) (Ertekin and Guzel-Seydim, 2010)
Acetic Acid	750±50 mg/kg 6100-8200 µg.g^{-1}fat	Norwegian kefir (8 week) ewe's milk(1-14 days)	(Grønnevik *et al.*, 2011; REGUŁA, 2007)
Ethanol%	0.00-0.65 0.05-0.67 0.82-0.94 860 mg/kg 151.46-323.89mg/L 0.1-0.53 298-1863.3 µg/g	with entrapped starter culture with kefir grains goat milk kefir(18-24h) Norwegian kefir (8 week) Turkish kefir(altered fat) Kefir of isolated kefir starter Sacco and Christian Hansen starter	(Chen *et al.*, 2009; Purnomo and Muslimin, 2012; Grønnevik *et al.*, 2011; Assadi *et al.*, 2000; Bakhshandeh *et al.*, 2011; Ertekin and Guzel-Seydim, 2010)
CO2	2.5±1 mg/kg	Norwegian kefir (8 week)	(Grønnevik *et al.*, 2011)
Acetaldehyde	0.4-6±1 mg/kg	Norwegian kefir (8 week)	(Grønnevik *et al.*, 2011; Cais-

	14.5±1 mg/dl 2.89-7.28mg/L 6.0 mg/l 0.2-1.0					Sheep kefir 23°C and 26°C Turkish kefir(altered fat whey-based kefir beverages Sacco and Christian Hansen starter	Sokolinska *et al.*, 2008) (Ertekin and Guzel-Seydim, 2010) (Magalhães *et al.*, 2011a) (Bakhshandeh *et al.*, 2011)
ethyl acetate	9.7–11.5 mg/l					whey-based kefir beverages	(Magalhães *et al.*, 2011a)
Acetoin	0.050 -0.396 µg/g					Sacco and Christian Hansen starter	(Bakhshandeh *et al.*, 2011)
diacetyl	0.032-0.168 µg/g					Sacco and Christian Hansen starter	(Bakhshandeh *et al.*, 2011)
Minerals(µ/g) Zn Fe Mn Cu Na K Mg Ca	PMK 3.15 3.9 1.94 0.48 206 712 225 281	70%pmk 3.07 3.07 1.6 0.61 281 1063 187 630	wmk 5.86 1.16 0.21 0.78 586 1529 97.7 1018			PMK: peanut milk kefir 70%PMK :70% peanut milk kefir WMK : whole-milk kefir	(Bensmira and Jiang, 2011)
Titratable acid (%)	0.25-0.95 0.25-1.50 0.51-0.76 48.2,41.4°SH 31.33-33.33SH 75-87%					with entrapped starter culture with kefir grains. goat milk kefir(18-24h) Sheep kefir 23°C and 26°C Strawberry Kefir Sacco and Christian Hansen starter	(Chen *et al.*, 2009; Purnomo and Muslimin, 2012) (Cais-Sokolinska *et al.*, 2008) (Ergönül, 2007; Bakhshandeh *et al.*, 2011)
pH	4.4- 4.7 4.5-5.78 3.41-4.19 4.36-4.77 4.41-4.52 4.59,4.61 3.82-4.39 4.26-4.40					1% or 5% (w/w) kefir grains. with entrapped starter culture with kefir grains. goat milk kefir(18-24h) Norwegian kefir (8 week) Sheep kefir 23°C and 26°C Strawberry Kefir Turkish kefir(altered fat)	(Irigoyen *et al.*, 2005; Chen *et al.*, 2009) (Purnomo and Muslimin, 2012; Grønnevik *et al.*, 2011) (Cais-Sokolinska *et al.*, 2008; Ergönül, 2007) (Ertekin and Guzel-Seydim, 2010)
Viscosity (mPa s)	179-425					1% or 5% (w/w) kefir grains.	(Irigoyen *et al.*, 2005)
FFA(µg Eq×cm^{-3})	10.68,10.34					Sheep Kefir 23°C and 26°C	(Cais-Sokolinska *et al.*, 2008)
Riboflavin	0.07-0.100					Kefir of isolated kefir starter	(Assadi *et al.*, 2000)
amino acids Tryptophane Phenylala+tyros Leucine Isolucine Threonine Methionine+cys Lysine Valine Aspartate Glutamate Serine Histidine Glycine Arginine	MK 0.05 0.35 0.34 0.21 0.17 0.12 0.27 0.22	PMK 3.05 1.3 1.04 1.52 1.84 4.78 8.78 2.22 0.96 1.73 4.72 1.52	70% pmk 3.28 1.49 1.17 2.03 1.99 4.25 8.72 2.21 1.05 1.41 3.68	wmk 3.38 1.56 1.37 2.84 2.06 2.51 7.35 1.92 0.95 0.59 1.00 1.06	MK: milk kefir PMK: peanut milk kefir 70%PMK :70% peanut milk kefir WMK: whole-milk kefir		(Otles and Cagindi, 2003) (Bensmira and Jiang, 2011)

Alanine		1.59	1.34	1.5		
Tyrosine		0.37	1.6	0.13		
Cysteine		0.38	0.28	0.7		
Methionine		2.24	0.49	1.48		
Phenylalanine		2.37	2.06	3.42		
proline			2.67			

Kneifel and Mayer, (1991), reported that the vitamins in kefir made using grains and milk from different species of mammals increased by > 20% as follows: thiamine (B1) only in ewe's milk kefir; pyridoxine (B6) in kefir made with ewe's, Goat's and mare's milk; folic acid in all kefir products except mare's milk kefir; and the orotic acid content was reduced in all the kefir products throughout the fermentation stage. Khamnaeva *et al*., (2000), reported that application of mechanical vibration and the injection of atmospheric oxygen into the growth medium during the fermentation of kefir with grains caused an increase in biosynthesis of riboflavin (B2) and ascorbic acid when compared with the control sample.

The existance yeast in kefir fermentation is to produce ethanol and carbon dioxide, and the grains usually contain either lactose and non-lactose fermenting yeast (Angulo *et al.*, 1993; Koroleva, 1988).

Kefir also contains enzymes, such as proteinases and peptidases, produced by the microorganisms within kefir grains. These enzymes and their products, are necessary for proper metabolism and growth of the microorganisms, and are also assumed to be bioactive components related to kefir's beneficial effects (Abraham and De Antoni, 1999; Adriana and Socaciu, 2008).

2.1.7. Water kefir

2.1.7.1. *Definition, properties, and history of water kefir*

Water kefir is hazy and gluey beverage with a smooth, streamlined, fizzing texture and blond to yellowish color, it is acidic and yeasty, slightly alcoholic and refreshing taste with feeble sweetness. It is home-produced drink made by adding of kefir grain to sugar solution in water and incubating this mixture at 20-25 °C for at least 12 h, and then separation of kefir grain to other production. Pieces of fresh or dried fruit can be added for flavor and removed in the end of fermentation period. It is a fermented beverage that is made by adding water kefir grains, which are polysaccharide grains that serve as the inoculum, to a mixture of water, sugar mostly sucrose, dried figs, and possibly other ingredients such as lemon, depending on the recipe (Gulitz *et al.*, 2013; Gulitz *et al.*, 2011). Kefir d'aqua, sugary kefir, or water kefir, is generally a homemade fermented beverage based on a sucrose solution with or without fruit extracts (Rodrigues *et al.*, 2005). This beverage is mainly consumed in Mexico and Brazil. In Brazil, the beverage is only homemade, and there are no reports regarding the microbiological and chemical compositions either of the grains or of the beverage (Magalhaes *et al.*, 2010b). Water kefir is available worldwide but it is still unknown what the real origin of the water kefir grains is. Once it has been postulated that the polysaccharide grains originate from the leaves of the *Opuntia* cactus fig plant (Lutz, 1899). The origin of water kefir is unknown. First description of similar grains called (ginger beer plant) was made by Ward in 1892 (Ward, 1892). Until today various synonyms are known, thus this symbiosis is also called California bees, African bees, Ale nuts, Balm of Gilead, Japanese Beer Seeds or Sugary kefir grains (Pidoux *et al.*, 1988; Kebler, 1921).

Water Kefir is vary from milk kefir according the substrate and kefir grain, color, shape, microbial composition at the strains level. There are no sufficient scientific literatures specified for water kefir, its origin, microbial and chemical structure, and the standard method of preparation. The few findings that have been obtained about water kefir focused on the microflora and chemical structure of sugar kefir drink and , sugary Brazilian kefir, brown sugar kefir ,sugary kefir grain(gingerbeer plant)in French, tibico (sugary kefir) and water kefir in Germany. These studies and that of milk kefir and kefir

grains indicated that both kefir grains contain the same common groups of microorganisms (lactic acid, acetic acid bacteria, and yeasts) (Chen *et al.*, 2009; Franzetti *et al.*, 1998; Galli *et al.*, 1995; Garrote *et al.*, 2001; Gulitz *et al.*, 2011; Magalhaes *et al.*, 2010b; Pidoux, 1989; Waldherr *et al.*, 2010).

2.1.7.2. *Water kefir grains*

Water kefir is the results of fermentation of sugar solution by kefir grain which contains of lactic acid, acetic acid and yeasts, called water kefir grains or sugary kefir grains. Sugary kefir grains are very similar to milk kefir grains in terms of their structure, associated microorganisms and products formed during the fermentation process (Pidoux *et al.*, 1990). Kefir grains were passed from generation to generation among the tribes of Caucasus being considered a source of family wealth (Lopitz-Otsoa A. *et al.*, 2006). Sugary kefir grains are small transparent mucilaginous masses that consist of a polysaccharide gel containing embedded lactic acid bacteria (Pidoux *et al.*) and yeasts. In contrast to milk kefir grains, which are made up of a complex of heteropolysaccharides namely kefiran, sugary kefir grains mainly consist of dextran (α 1–6 linked glucose polymer) (Waldherr *et al.*, 2010). Kefir consists of a gelatinous and irregular grains formed by a consortium of yeasts and lactic acid bacteria embedded in a resilient polysaccharide matrix named kefiran (Rodrigues *et al.*, 2005). Kefiran produced by *L. kefiranofaciens* is a branched, water-soluble polysaccharide, containing equal amounts of D-glucose and D-galactose. The production of this polysaccharide is stimulated when *L. kefiranofaciens* grows in co-culture with *S. cerevisiae* (Cheirsilp *et al.*, 2003). (Zubillaga *et al.*, 2001) described kefir grains as a symbiotic association between lactic bacteria, acetic bacteria, and yeasts among other microorganisms. The morphological properties of water kefir grains are illustrated in (Figure 2-3).

Figure 2-3 Water kefir and water kefir grains

2.1.7.3. *Microbial populations of water kefir*

Microbial and chemical properties may change depending on the culture media used and the country where the grain is produced. The microbial populations of water kefir include LAB, such as *Lactobacillus paracasei, Lactobacillus hilgardii, Leuconostoc mesenteroides and Streptococcus lactis, acetic acid bacteria (AAB) such as Acetobacter, Bacillus,* and a small proportion of yeasts, such as *Zygosaccharomyces, Candida, and Kloekera* (Pidoux, 1989; Pidoux *et al.*, 1990).

Recent studies, indicated that bacteria, such as *Lactobacillus paracasei, Lactobacillus kefiri, Lactobacillus parabuchneri and Acetobacter lovaniensis* as well as yeast, such as *Saccharomyces cerevisiae and Kluyveromyces lactis*, were the predominant microorganisms present in the beverage (Magalhaes *et al.*, 2010b; Miguel *et al.*, 2011c).

More recently, also *Bifidobacterium psychraerophilum*/*crudilactis* was found in water kefir via culture-dependent and culture-independent techniques (Gulitz *et al.*, 2013). In other study, brown sugar was used as substratum for kefir growth, the microbial strains that found included, *Leuconostoc* ssp., *Lactobacillus lactis cremoris, Chyseomonas luteola, Acetobacter sp., Sacharomyces cerevisae, Candida colliculosa, Toruspola delbruechii, Candida inconspicua, Candida magnoliae, Kloekera* sp., *Candida famata, Kluyveromices lactis, and Kluyveromices marxianus, Candida kefir* (Bergmann *et al.*, 2010). Leroi and Courcoux, ((1996) also isolated less common strains from kefir kept in

sugary water: *Lactobacillus hilgardü* and *Saccharomyces florentinus*. The yeast and LAB strains present in sugary kefir grains are in symbiosis. Leroi and Pidoux (1993) indicated that this symbiotic relationship is confirmed by the fact that the LAB strains in the kefir are stimulated to grow by the release of glucose and fructose by the yeasts present in the kefir. Furthermore, among the microorganisms found in the sugary kefir grains, Lactobacillus higardii has been reported to be very important for grain formation (Waldherr *et al.*, 2010). Other studies had reported some strains of bacteria and yeast that present in water kefir are summarized above in Table (2-5).

2.1.7.4. *Preparation of water kefir*

Different methods can be used in the industrial process of kefir production, but all based on the same principle. Similar to milk kefir, water kefir is also a consortium of different microorganisms which is used for preparing a homemade fermented beverage. Grains are plunged in a sucrose solution (8%) supplemented with dried or fresh fruits, best figs (Reib, 1990) and fermented at room temperature for two or three days. The water kefir grains are used to initiate the fermentation. The grains show irregular dimensions ranging from few millimeters to centimeters, the consistency is inelastic and fragile (Franzetti *et al.*, 1998). The resulting beverage is fizzy and cloudy, low acid, somewhat sweet and slightly alcoholic, depending on how long it was fermented. Mainly, the consortium of water kefir is comprised of 108 lactobacilli, 10^6-10^8 acetic acid bacteria and 10^6-10^7 yeasts per gram granules (Gulitz *et al.*, 2011). Kefir grains are propagated at room temperature by daily transfer in tap water-sugar solution (60 g/litre) (Pidoux, 1989).

The use of commercial cultures can standardize the commercial production of kefir, if the selection of species and strains of yeasts and bacteria is carried out accurately and carefully, thus allowing for the production of a “kefir type” beverage with acceptable flavour and good conservation properties (Beshkova D *et al.*, 2002; Carneiro, 2010). The commercial beverage may have a commercial life period of up to 28 days, while it is recommended that kefir produced with grains be consumed between 3-12 days. However, the “kefir-type” beverage may not present the same therapeutic and probiotic properties present in traditional kefir (Rattray and O’Connell, 2011).

2.1.7.5. *Healthy benefits of water kefir*

As one of the most complex consumed probiotics, kefir has been associated with many health benefits. Many studies have demonstrated that water kefir has money properties that contribute beneficially in health improvement and maintenance. Since sugary kefir is a popular health promoting beverage, the microorganisms in its grains might relate to its health benefits. *Leu. mesenteroides,* isolated from both brown sugar and milk samples in this study, was found to produce bacteriocin against several strains of *Listeria monocytogenes* (Wang, 1993). Yeast and lactic acid bacteria co-exist in a symbiotic association and are responsible for an acid–alcoholic fermentation. This mixed culture is able to utilize lactose and therefore whey could be used as a raw material for kefir production (Iconomopoulou and Psarianos, 2004; Koutinas *et al.*, 2005). Recently, an oligosaccharide isolated from water kefir fermentation, and named aqueous kefir carbohydrate (Öner Z *et al.*, 2010), it is different from the milky bacteria-encapsulated polysaccharide kefiran, AK seems to be an oligosaccharide isolated from an aqueous fraction of kefir grains (Moreira *et al.*, 2008; Piermaria *et al.*, 2008).

Silva *et al.*, (2009) indicated that sugary kefir grains fermented with brown sugar show the greatest antimicrobial activity and inhibit *Candida albicans, Salmonella typhi, Shigella sonnei, Staphylococcus aureus* and *Escherichia coli* when compared to kefir fermented using molasses and demerara sugar. Liu *et al.*, (2005a), found that the inhibitory effect upon linoleic acid peroxidation of milk-kefir and soymilk-kefir were significantly greater than those of milk and soymilk after being incubated for 32 h. Kefiran from kefir grains was shown to have the property of retarding tumor growth in vivo when administered orally (Shiomi *et al.*, 1982).

2.2. Diabetes mellitus

2.2.1. Introduction

DM is an important health problem and it has been recognized as a major risk factor for the development of complications in target organs, including retinopathy, neuropathy, nephropathy and cardiovascular disease, the comprehension of the mechanisms involved in the association among diabetes is the subject of many research groups (IDF, 2009).

The major cause of death among diabetic patients, cardiovascular disease, is two to four times more common in diabetic than in nondiabetic populations. In addition, peripheral vascular disease and neuropathy can cause ischemia and, in some cases, gangrene of the lower limbs leading to amputation (Packer, 2000a). As a very common chronic disease, diabetes is becoming the third 'killer' along with cancer, cardiovascular and cerebrovascular diseases because of its high prevalence, morbidity and mortality (Li *et al.*, 2004).

Diabetes has reached epidemic proportion and has become one of the most challenging health problems of the 21st century. The number of people with diabetes in the year 2010 is estimated to be 285 million, representing 7% of the adult world population and by the year 2030, an estimated 439 million individuals worldwide will have this disorder with the most marked increase projected for the population greater than 65 years of age (Unwin *et al.*, 2010).

The International Diabetes Federation (IDF) estimated in 2011 that 366 million adults, aged 20–79 years, of the world's 7 billion population have diabetes (IDF, 2011). This gives a comparative prevalence of 8.5%. Since more than 90% of the global cases of diabetes are type 2, it is evident that the epidemic is mainly due to the escalation of the causes of type 2 diabetes (WHO., 1997; WHO., 2003).

Several prospective studies have documented that obesity is probably the most powerful predictor of the development of type 2 diabetes. However, not every obese subject develops diabetes, that is obesity alone is not sufficient to cause type 2 diabetes; there are other factors that considerably modify the effect of obesity on diabetes risk. It is probable that the genetic susceptibility to diabetes is a necessary prerequisite for diabetes. This was demonstrated in the Pima Indians in whom the incidence increases more steeply

with body mass index (BMI, kg/m2) in thosewhose parents have diabetes than in these who do not. Vice versa, in non-obese people the incidence of type 2 diabetes is low in the middle-aged even in populations such as the Pima Indians where the overall risk of the disease is very high (Barnett and Kumar, 2009).

2.2.2. Definition Of Diabetes

Diabetes mellitus (DM) is a group of metabolic disorders characterized by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism. The hyperglycemia results from defects in insulin secretion, insulin action or both. The chronic hyperglycemia of diabetes is associated with specific chronic complications resulting in damage to or failure of various organs, notably the eyes, kidneys, nerves, heart and blood vessels (Scobie, 2007; WHO., 1999). The word diabetes means 'to run through' or 'a siphon' in Greek and the condition has been recognized since the time of the ancient Egyptians. Mellitus (from the Latin and Greek roots for 'honey') was later added to the name of this disorder when it became appreciated that diabetic urine tasted sweet (Scobie, 2007).

It is a chronic disease with long-term macrovascular and microvascular complications, including diabetic nephropathy, neuropathy, and retinopathy. It is a leading cause of death, disability, and blindness in the United States for persons 20−74 years of age (EDPRG, 2004; Harris *et al.*, 1987; Zhang *et al.*, 2008).

Diabetes mellitus is a chronic, hereditary disease characterized by an abnormally elevated level of blood glucose (hyperglycemia) and by the excretion of the excess glucose in the urine (glycosuria). The basic defect appears to be an absolute or relative lack of insulin or decrease in insulin receptors on the membrane of the target cells, which lead to abnormalitiesin carbohydrate (glucose) metabolism as well as in the metabolism of protein and fat (Anderson *et al.*, 1982).

The cut-off points for plasma glucose levels which are indicative of diabetes diagnosis are based on certain thresholds above which the incidence of diabetic vascular complications begin to increase (ADA., 1997; Balkau, 2000; Balkau *et al.*, 2004).

2.2.3. Diagnosis Of Diabetes

The clinical diagnosis of diabetes is often suggested by the presence of hyperglycemic symptoms and glycosuria, sometimes with drowsiness or coma. Diabetes mellitus (DM) should be suspected if a venous plasma glucose level drawn irrespective of when food was last eaten (a "random" sample of blood glucose) is "unequivocally elevated" (i.e., ≥ 200 mg/dL), particularly in a patient who manifests the classic signs and symptoms of chronic hyperglycemia (polydipsia, polyuria, blurred vision, headaches, rapid weight loss, sometimes accompanied by nausea and vomiting). To confirm the diagnosis, the patient should fast overnight (10-16 hours), and the blood glucose measurement should be repeated. Values of less than 110 mg/dL are considered normal. Values greater than 140 mg/dL are indicative of DM. Glycosylated hemoglobin should be measured to determine the extent of hyperglycemia over the past 4 to 8 weeks. Values of fasting blood glucose between 111 and 140 mg/dL are designated impaired fasting glucose tolerance (IGT), and further testing should be performed to determine whether these individuals will eventually develop overt diabetes mellitus. Although the oral glucose tolerance test (OGTT) is contraindicated for patients who clearly have diabetes mellitus, it is used for patients with fasting blood glucose in the IGT range (between 115 and 140 mg/dL). In the OGTT, a nonpregnant patient who has fasted overnight drinks 75 g glucose in an aqueous solution. Blood samples are drawn before the oral glucose load and at 30, 60, 90, and 120 minutes thereafter. If any one of the 30-, 60-, and 90-minute samples and the 120-minute sample are greater than 200 mg/dL, overt DM is indicated. The diagnosis of IGT and the more severe form of glucose intolerance (DM) is based on blood glucose levels because no more specific characteristic for the disorder exists. The distinction between IGT and DM is clouded by the fact that a patient's blood glucose level may vary significantly with serial testing over time under the same conditions of diet and activity. The renal tubular transport maximum in the average healthy subject is such that glucose will not appear in the urine until the blood glucose level exceeds 180 mg/dL. As a result, reagent tapes (Tes-Tape or Dextrostix) designed to detect the presence of glucose in the urine are not sensitive enough to establish a diagnosis of early DM (Smith *et al.*, 2005).

The current diagnostic criteria used for the diagnosis of diabetes and intermediate hyperglycaemia have been in place globally for almost a decade and are widely accepted.

However, in 2003 the ADA modified its recommendations resulting in discrepancies between its recommendations and those of the WHO. Although attention has focused on the difference in fasting plasma glucose levels for defining IFG, there are a number of important differences between the ADA and WHO recommendations which may result in differences in an individual's classification of glucose tolerance (Table 2-5).

These include:

- fasting plasma glucose value used to define IFG
- inclusion of 2–h plasma glucose value in defining IFG
- requirement for fasting plasma glucose level in defining IGT

fasting plasma glucose as the recommended method for diagnosing asymptomatic diabetes by ADA whereas WHO recommends the oral glucose tolerance test (WHO., 2006).

Table 2-5 Comparison of 1999 WHO and 2003 ADA diagnostic criteria

	WHO 1999	ADA 2003
Diabetese Fasting glucose 2–h glucose*	≥7.0mmol/l or ≥11.1mmol/l	≥7.0mmol/l or ≥11.1mmol/l
IGT Fasting glucose 2–h glucose	<7.0mmol/l (if measured) And ≥7.8 and <11.1mmol/l	Not required ≥7.8 and <11.1mmol/l
IFG Fasting glucose 2–h glucose	6.1 to 6.9mmol/l and (if measured) (measurement recommended)	5.6 to 6.9mmol/l Measurement not recommended (but if measured should be <11.1 mmol/l)

For decades, the diagnosis of diabetes has been based on glucose criteria, either the FPG or the 75-g OGTT. In 1997, the first Expert Committee on the Diagnosis and Classification of Diabetes Mellitus revised the diagnostic criteria, using the observed association between FPG levels and presence of retinopathy as the key factor with which to identify threshold glucose level. The Committee examined data from three cross-sectional epidemiologic studies that assessed retinopathy with fundus photography or direct ophthalmoscopy and measured glycemia as FPG, 2-h PG, and A1C. These studies demonstrated glycemic levels below which there was little prevalent retinopathy and

above which the prevalence of retinopathy increased in an apparently linear fashion. The deciles of the three measures at which retinopathy began to increase were the same for each measure within each population. Moreover, the glycemic values above which retinopathy increased were similar among the populations. These analyses helped to inform a new diagnostic cut point of ≥126 mg/dl (7.0 mmol/l) for FPG and confirmed the long-standing diagnostic 2-h PG value of ≥200 mg/dl (11.1 mmol/l) (ADA, 2012).

Table 2-6 Criteria for the diagnosis of diabetes

A1C ≥6.5%. The test should be performed in a laboratory using a method that is NGSP certified and standardized to the DCCT assay.*
OR
FPG ≥126 mg/dl (7.0 mmol/l). Fasng is defined as no caloric intake for at least 8 h.*
OR
2-h plasma glucose≥200mg/dl (11.1mmol/l) during anOGTT. The test should be performed as described by theWorld HealthOrganizaon, using a glucose load containing the equivalent of 75 g anhydrous glucose dissolved in water.*
OR
In a patient with classic symptoms of hyperglycemia or hyperglycemic crisis, a random plasma glucose ≥200 mg/dl (11.1 mmol/l).

*In the absence of unequivocal hyperglycemia, criteria 1–3 should be confirmed by repeat testing.

The Committee (comprising members appointed by the American Diabetes Association [ADA], the European Association for the Study of Diabetes [EASD] and the International Diabetes Federation [IDF] recommended that diagnosis in type 2 diabetes should now usually be made solely on the basis of an HbA1c confirmed to be ≥6.5% (48mmol/mol), without the need to measure a plasma glucose concentration in the subject. A ‘subdiabetic “high risk” state’ would exist for subjects with an HbA1c of 6.0–6.4% (42–46mmol/mol) (Kilpatrick and Winocour, 2010). The WHO Consultation concluded that Glycated haemoglobin (HbA1c) can be used as a diagnostic test for diabetes, provided that stringent quality assurance tests are in place and assays are standardised to criteria aligned to the international reference values, and there are no conditions present which preclude its accurate measurement. An HbA1c of 6.5% is recommended as the cut point for diagnosing diabetes. A value less than 6.5% does not exclude diabetes diagnosed using glucose tests. The expert group concluded that there is

currently insufficient evidence to make any formal recommendation on the interpretation of HbA1c levels below 6.5% (WHO., 2011).

The National Diabetes Information Clearinghouse (NDIC., 2008) determine the tests that are used for diagnosis of diabetes as follows:

- A fasting plasma glucose (FPG) test measures blood glucose in a person who has not eaten anything for at least 8 hours. This test is used to detect diabetes and prediabetes.
- An oral glucose tolerance test (OGTT) measures blood glucose after a person fasts at least 8 hours and 2 hours after the person drinks a glucose-containing beverage. This test can be used to diagnose diabetes and prediabetes.
- A random plasma glucose test, also called a casual plasma glucose test, measures blood glucose without regard to when the person being tested last ate. This test, along with an assessment of symptoms, is used to diagnose diabetes but not prediabetes.
- Test results indicating that a person has diabetes should be confirmed with a sec-ond test on a different day.

2.2.4. Classification of Diabetes

The current classification attempts to encompass both etiology and clinical stages of the disease and is useful clinically. It includes four main types or clinical classes. The two main types are:

- Type 1 (results from ß-cell destruction, usually leading to absolute insulin deficiency) which accounts for 5–10% of all cases.
- Type 2 (results from a progressive insulin secretory defect on the background of insulin resistance) which accounts for 90-95% of all individuals with diabetes.
- The third group consists of other less common types of diabetes that are caused or associated with certain specific conditions and/or syndromes, e.g., genetic defects in ß-cell function, genetic defects in insulin action, diseases of the exocrine pancreas (such as cystic fibrosis), and drug or chemical induced (such as in the treatment of AIDS or after organ transplantation).

- Gestational diabetes mellitus (GDM) diagnosed during pregnancy (ADA., 2008; WHO., 2003).

2.2.4.1. *Type 1 Diabetes*

Type 1 diabetes is caused by an absolute deficiency in insulin production. It is thought to arise from autoimmune destruction of the β-cells of the pancreas in genetically susceptible individuals, and constitutes ~ 10% of diabetes in the United States. Although this form of diabetes is more common in childhood, it can occur at any time in life (Fowler, 2010). The type 1 diabetes mellitus (previously referred to as insulin-dependent diabetes mellitus (IDDM) or juvenile onset diabetes) results from a progressive cellular mediated autoimmune destruction of the pancreatic β-cells that leads to complete insulin deficiency (Knip and Siljander, 2008). Type I diabetes mellitus is an autoimmune disease. The pancreas shows lymphocytic infiltration and destruction of insulin-secreting cells of the islets of Langerhans, causing insulin deficiency. Approximately 85% of patients have circulating islet cell antibodies, and the majority also have detectable anti-insulin antibodies before receiving insulin therapy (ADA., 2008).

It is Immune-mediated diabetes. This form of diabetes, which accounts for only 5–10% of those with diabetes, previously encompassed by the terms insulin dependent diabetes mellitus (IDDM), type 1 diabetes, or juvenile-onset diabetes, results from a cellular- mediated autoimmune destruction of the β-cells of the pancreas. Markers of the immune destruction of the β-cell include islet cell autoantibodies (ICAs), insulin autoantibodies (IAAs), autoantibodies to glutamic acid decarboxylase GAD (GAD65), and autoantibodies to the tyrosine phosphatases IA-2 and IA-2b. One and usually more of these autoantibodies are present in 85–90% of individuals when fasting hyperglycemia is initially detected (ADA, 2012; Taplin and Barker, 2008). Also, the disease has strong HLA associations, with linkage to the DQA and DQB genes, and it is influenced by the DRB genes. These HLA-DR/ DQ alleles can be either predisposing or protective. In this form of diabetes, the rate of b-cell destruction is quite variable, being rapid in some individuals (mainly infants and children) and slow in others (mainly adults). Some patients, particularly children and adolescents, may present with ketoacidosis as the first manifestation of the disease. Others have modest fasting hyperglycemia that can rapidly

change to severe hyperglycemia and / or ketoacidosis in the presence of infection or other stress (ADA, 2012; Todd *et al.*, 2007; Undlien *et al.*, 2001; WTCCC, 2007).

2.2.4.2. *Type 2 diabetes*

Type 2 DM is a metabolic, chronic and systemic disease characterized by hyperglycemia resulting from insulin resistance and impaired insulin secretion. It is characterized by the abnormal metabolism of carbohydrates, proteins and fats. Insulin resistance refers to decreased tissue sensitivity to insulin making insulin less effective at stimulating glucose uptake by the tissue (Andrus *et al.*, 2003).

The insulin insensitivity is usually evidenced by excess body weight or obesity, and exacerbated by over-eating and inactivity. It is commonly associated with raised blood pressure and a disturbance of blood lipid levels. The insulin deficiency is progressive over time, leading to a need for lifestyle change often combined with blood glucose lowering therapy (NICE., 2011). In type 2 diabetes impaired two-phasic pancreatic insulin secretion from betacells and decreased insulin action lead to elevated glucose levels. It has been estimated that approximately 50-60% of beta-cell insulin secretion capacity is lost by the time diabetes is clinically diagnosed (Butler *et al.*, 2003). Hyperglycemia has been proposed to lead to an accumulation of a large number of reactive oxygen species in the beta-cells, with subsequent damage to the cellular components (Stumvoll *et al.*, 2005). There is a strong correlation between the consumption of refined carbohydrates and the prevalence of type 2 diabetes (Gross and Li, 2004). The development of type 2 diabetes results from the interaction between an individual's genetic background and environment (Gerich, 1998).

The dyslipidemia involves high levels of triacylglycerides and circulating fatty acids originating from the diet or accelerated lipolysis in adipocytes. Direct exposure of muscle cells to these fatty acids impairs insulin-mediated glucose uptake and, therefore, may contribute to insulin resistance. Within the last decade, a hypothesis was proposed to explain the pathogenesis of T2DM that connects the disease to a state of subclinical chronic inflammation (Oguntibeju, 2013). Keller, (2006) explained the major role of overweight and obesity to insulin resistance and, subsequently, the development of type 2

DM. The pathogenic mechanisms of free fatty acids and excessive amount of adipocytes are associated with impaired insulin sensitivity.

Type 2 diabetes mellitus (T2DM) is currently considered as a global health problem where about six people die every minute from the disease worldwide. This rate will make T2DM one of the world's most prevalent causes of preventable mortality (Wild *et al.*, 2004). According to the Centers for Disease Control and Prevention, there was a 765% increase in type 2 diabetes cases from the year 1935 to 1996 (Gross and Li, 2004).

2.2.4.3. *Other Types of diabetes .*

A number of other types of diabetes exist. A person may exhibit characteristics of more than one type. For example, in latent autoimmune diabetes in adults (LADA), also called type 1.5 diabetes or double diabetes, people show signs of both type 1 and type 2 diabetes. Diagnosis usu-ally occurs after age 30.

Other types of diabetes include those caused by

- genetic defects of the beta cell, such as maturity-onset diabetes of the young (MODY) and neonatal diabe-tes mellitus
- genetic defects in insulin action, resulting in the body's inability to control blood glucose levels, as seen in leprechaunism and the Rabson-Mendenhall syndrome
- diseases of the pancreas or conditions that damage the pancreas, such as pancreatitis and cystic fibrosis
- excess amounts of certain hor-mones resulting from some medical conditions—such as cortisol in Cushing's syndrome—that work against the action of insulin
- medications that reduce insulin action, such as glucocorticoids, or chemicals that destroy beta cells
- infections, such as congenital rubella and cytomegalovirus
- rare autoimmune disorders, such as stiff-man syndrome, an autoimmune disease of the central nervous system
- genetic syndromes associated with diabetes, such as Down syndrome and Prader-Willi syndrome (NDIC., 2008).

Pre-diabetes indicates a condition that occurs when a person's blood glucose levels are higher than normal but not high enough for a diagnosis of Type 2 diabetes. Many people

destined to develop Type2 diabetes spend many years in a state of pre-diabetes. Some very uncommon cases of diabetes are caused by the body's tissue receptors not responding to insulin (Rother, 2007; WHO., 1999).

2.2.4.4. *Gestational diabetes mellitus (GDM)*

Gestational diabetes mellitus (GDM) is defined as any degree of glucose intolerance with onset or first recognition during pregnancy (Fraser and Heller, 2007; Metzger *et al.*, 2007). Gestation diabetes mellitus resembles Type 2 diabetes in several respects, involving combination of relatively inadequate insulin secretion and responsiveness. It occurs in about 2 to 5% of all pregnancies and may improve or disappear after delivery. Gestational diabetes is fully treatable but requires careful medical supervision throughout the pregnancy and if left untreated, can damage the health of the foetus or mother resulting in high birth weight, congenital cardiac or nervous system abnormalities and skeletal muscle malformation. About 20 to 50% of affected women develop Type 2 diabetes later in life. Increased foetal insulin may inhibit foetal surfactant production and cause Respiratory Distress Syndrome (Wild *et al.*, 2004). Deterioration of glucose tolerance occurs normally during pregnancy, particularly in the 3rd trimester. There is significant morbidity associated with GDM including intrauterine foetal death, congenital malformations, neonatal hypoglycemia, jaundice, prematurity and macrosomia (Cosson, 2010; Kim, 2010).

Pregnancy induces progressive changes in maternal carbohydrate metabolic process. As pregnancy advances insulin resistance and diabetogenic stress due to placental contra-insulin hormones necessitate compensatory increase in insulin secretion. When this compensatory mechanism fails due to pancreatic β cells inadequacy gestational diabetes develops. GDM affects 1-2% of all pregnancies. In majority of patients it is mild and can be adequately controlled with diet alone but a minority will require antidiabetogenic agents like glyburide or insulin (Sobrevia, 2013).

2.2.5. Diabetes Signs and symptoms

The classical symptoms of diabetes are hyperglycaemia, fatigue, weakness, dizziness, blurred vision, polyuria, polydipsia, polyphagia, and weight gain appear abruptly over

days or weeks in previously healthy non-obese children or young adults. In older patients the disease may manifest more gradually (Tierney *et al.*, 2005; Wild *et al.*, 2004).

The symptoms may develop quite rapidly in Type 1 diabetes particularly in children, however, in Type 2diabetes, symptoms usually develop much more slowly and may be subtle or completely absent. Type 1 diabetes may also cause a rapid yet significant weight loss and persistent mental fatigue. All of these symptoms except weight loss can manifest in Type 2 diabetes in patients whose diabetes is poorly controlled, although unexplained weight loss may be experienced at the onset of the disease (Wild *et al.*, 2004). Typical symptoms of excess thirst, fatigue, and weight changes may not be reported by older adults. The delay in reporting thirst in the presence of hyperglycemia can be due to subtle physiological changes, including decreased response to thirst, as part of the normal aging process. Urine diagnostic lab work may also be difficult to interpret because of changes in the glucose renal threshold, which are higher with advanced age (Terrio, 2009). Fatigue is a common complaint in this population and does not usually warrant concern. The older adult likely sees fatigue as a natural consequence of aging and therefore does not report this as a health concern.

2.2.6. Complications of diabetes mellitus

Diabetes mellitus is a syndrome characterized by hyperglycemia, polydipsia and polyuria and causes complications to the eyes, kidneys, and nerves. It is also associated with an increased incidence of cardiovascular disease (Pickup and Williams, 1991). T1DM is characterized by the slow progression towards the generation of some specific lesions of the blood vessels walls, affecting both small arterioles and capillaries (microangiopathy) and large arteries (macroangiopathy) (IDF, 2009).

Diabetes can affect many different organs in the body system and, after a while, can lead to very difficult complications. Complications from diabetes can be categorized as microvascular or macrovascular. Microvascular complications include nervous system damage (neuropathy), renal system damage (nephropatby) and eye damage (retinopathy) (Deshpande *et al.*, 2008). The “classical” diabetes microvascular complications are represented by diabetic retinopathy (DR) the main cause of blindness, diabetic nephropathy (DN) also known as renal disease, the main cause of renal substitution

therapy (dialysis or renal transplantation) in developed countries, and diabetic neuropathy (DPN) as reported (IDF, 2009). Macrovascular complications include cardiovascular disease, stroke, and peripheral vascular disease. Peripheral vascular disease may lead to bruises or injuries that do not heal, gangrene, and, ultimately, amputation (Deshpande *et al.*, 2008).

The chronic complications of diabetes can be subdivided into vascular and nonvascular complications (Powers, 2001). The vascular complications are further subdivided into microvascular (retinopathy, neuropathy, nephropathy) and macrovascular complications (coronary artery disease, peripheral vascular disease, cerebrovascular disease) (Meeuwisse-Pasterkamp *et al.*, 2008). The microvascular, neuropathic and macrovascular complications are a major health problem for patients with either IDDM or NIDDM (Herman and Crofford, 1998).

Multiple modifiable factors explain type 2 DM long-term complications including uncontrolled hyperglycemia, hypertension, dyslipidemia, smoking, eating habits, obesity and physical inactivity. Obesity, particularly abdominal adiposity, is associated with atherogenesis; also, it is linked to hyperglycemia, hypertension, dyslipidemia and hyperinsulinemia (Andrus *et al.*, 2003).

The major long-term complications of type 2 diabetes are retinopathy, nephropathy, neuropathy, and large-vessel atherosclerotic disease, including coronary artery disease, peripheral vascular disease, and cerebrovascular disease (Nathan, 1993).

Diabetic peripheral neuropathy is the most common complication in patients with diabetes, accounting for substantial morbidity and mortality and resulting in huge health care costs (Jin *et al.*, 2009).

2.2.7. Prevention and Treatment of diabetes mellitus

There is compelling clinical trial evidence that diabetes can be prevented or its onset can be delayed by lifestyle interventions, thus it is critical to delineate which are the best dietary strategies (Knowler *et al.*, 2002; Pan *et al.*, 1997; Tuomilehto *et al.*, 2001). There are national efforts to delay and prevent complications of diabetes such as retinopathy, nephropathy, neuropathy, and cardiovascular disease (Brownlee, 2005). The control of

blood sugar, blood pressure and lipid levels are very important in the prevention diabetic complications.

The most important strategy in the prevention of diabetic complications is the implementation of frequent blood glucose monitoring coupled with a well-planned regimen of insulin delivery. The degree of success noted in the DCCT trial in long-term glucose control translated into a significant reduction (50-70%) in the clinical incidence and progression of retinopathy, nephropathy, and peripheral neuropathy. Long-term normalization of blood glucose levels has been shown to decrease significantly the risk for the development and progression of the microvascular complications of the disease (The Diabetes and Complications Trial Research Group) (DCCTRG, 1993). The data that longterm tight glucose control prevents the development and progression of macrovascular disease (coronary artery disease, cerebral vascular disease, and peripheral vascular disease) is not as strong (Stratton *et al.*, 2000). Prevention of diabetes-related complications, self-monitoring of blood glucose, diet, physical activity, alcohol use and issues regarding insurance and following this three-monthly visits to their physician. Just after the diabetes education program patients were randomized to either a control group or to the intervention group receiving five individual sessions in one year. Health care providers, educated in motivational interviewing, conducted the sessions. The goal of the intervention was to help patients to recognize and address problem areas in the self-management of diabetes. Motivational interviewing is a pa- tient-centered directive clinical style for enhancing intrinsic motivation to change by exploring and resolving ambivalence (Miller and Rollnick, 2009).

2.2.7.1. *Nutrition therapy*

Nutrition therapy implemented appropriately contributes to important and essential outcomes in the management of diabetes. However, just as there is no one medical therapy appropriate for all individuals with type 1 or type 2 diabetes, there is no one prescription for nutrition therapy appropriate for all people with diabetes. Primary goals of nutrition therapy for diabetes are to improve glycemic, lipid, and blood pressure control, thus contributing to reduced risk for potential long-term complications of

diabetes and heart disease, and to improve the quality of life for individuals with diabetes (Franz and Evert, 2012).

The importance of diet in the context of diabetes medical nutrition therapy has been the topic of several reviews, but few have focused on diabetes prevention (Bantle *et al.*, 2008; Mann *et al.*, 2004). Recently, Buyken et al. (2010) and Spence et al., (2010) reviewed the role of diet in the prevention of diabetes, with particular emphasis on carbohydrates, and Risérus *et al*. (2009) reviewed the role of dietary fats.

Nutrition management has shifted from a prescriptive one-size fits all approach to a person-centred approach. A person-centred approach puts the person at the center of their care and involves assessing the person's willingness and readiness to change, tailoring recommendations to their personal preferences and joint decision making (Franz, 2004).

Type 1

The main components of these lifestyle interventions included weight loss, reduction in fat intake and increased physical activity. The most dominant predictor for Type 2 diabetes prevention is weight loss; every kilogram lost is associated with a 16 per cent reduction in risk (Hamman *et al.*, 2006). However, there is little evidence supporting the best approach for weight reduction in people at risk of Type 2 diabetes. The four major randomised trials used largely similar dietary approaches which were characterised by modest energy reduction and reductions in total and saturated fat intake.

In people receiving intensive treatment in the Diabetes Control and Complications Trial, a lower carbohydrate (37%) intake and higher total (45%) and saturated (17%) fat intakes were associated with worse glycemic control at year 5 compared to a higher carbohydrate (56%) intake (A1C values of 7.5 versus 7.0%, respectively), independent of exercise and BMI (Delahanty *et al.*, 2009). The authors suggest that the carbohydrate content is less critical than the total and saturated fat content, to which it is usually inversely related.

Type 2

Most trials of lifestyle interventions to prevent Type 2 diabetes use a combination of diet and physical activity and do not distinguish the individual contributions of each component. One trial has reported that there were no differences in progression to Type 2

diabetes in high risk individuals randomly allocated either diet alone, physical activity alone or a combination of the two (Pan *et al.*, 1997). A recent review also states that there is no significant difference between approaches incorporating diet, physical activity or both (Gillies *et al.*, 2007), although there is evidence that in the absence of weight loss, increased physical activity can reduce the incidence of Type 2 diabetes by 44 per cent (Hamman *et al.*, 2006).

A cross-sectional study of American Indians with type 2 diabetes in the Strong Health Study assessed dietary intake in 1,284 participants. Lower intake of carbohydrate (<35–40% of energy) and higher intakes of total (>25–30% of energy) and saturated fats (>13% of energy) were associated with poorer glycemic control. A lower fiber intake and a higher protein intake were marginally associated with poor glycemic control (Xu *et al.*, 2007).

The key strategies for achieving strict control of blood glucose levels are first of all frequent self-monitoring of blood glucose, followed by appropriate diet therapy, which is extremely important. During pregnancy, as pregnant women patients need to consume adequate energy, protein, and minerals. In Japan, according to the diet therapy recommended by the JSOG Committee on Nutrient and Metabolism Problems (Sugawa, 1985). Under the nutritional guidelines recommended by the Ministry of Health, Labour and Welfare (Ministry of Health, 2010), an additional calorie intake of 50 kcal, 250 kcal, 450 kcal is recommended for pregnant women during the first, second, and third trimester, respectively. Thus using these recommendations, it is reasonable to regard the currently recommended calorie intake for pregnant women as 25–30 kcal/kg+50 kcal, 25–30 kcal/kg+250 kcal, and 25–30 kcal/kg+450 kcal for the first, second, and third trimester, respectively.

2.2.7.2. *Weight loss*

Weight loss is important in people with Type 2 diabetes who are overweight or obese and should be the primary management strategy. Weight loss can also be an indicator of poor glycaemic control; the relationship between blood glucose and weight is not always straightforward. Weight gain is positively associated with insulin resistance and therefore weight loss improves insulin sensitivity (Davies *et al.*, 2003). Studies investigating the

effect of weight loss on glycaemic control in Type 2 diabetes have utilised low fat diets (also known as healthy eating), low carbohydrate diets, very low calorie liquid diets (VLCLD), meal replacements, commercial diets and increased physical activity (Kelly and Dyson, 2011). Some studies have reported that weight loss can prevent or delay the onset of diabetes in obese subjects. In the Nurses' Health Study (NHS) observational cohort, women who lost more than 5 kg over a 10-year period reduced their risk of diabetes by 50% or more (Colditz *et al.*, 1995). In the Swedish Obese Subjects study, the massive weight loss produced by bariatric surgery was associated with improvement in blood glucose and insulin levels as well as with a reduction in the incidence of diabetes (Sjostrom *et al.*, 1999).

2.2.7.3. *Physical activity*

Physical activity reduces insulin resistance, facilitating peripheral glucose utilization with consequent improvement of glycemic control while also helping to control weight gain during pregnancy. The recommended exercise prescription is low-impact physical activity, ideally being practiced daily for at least 30 minutes, which can be divided into three sessions of ten minutes each, keeping levels not exceeding 50% of the aerobic capacity of the patient (Sobrevia, 2013).

Increasing physical activity improves metabolic control in people with diabetes. Low level aerobic exercise (eg: brisk walking for half an hour per day) and physical resistance training have the following benefits:

- Improved glucose tolerance as insulin sensitivity increases
- Increased energy expenditure resulting in weight loss
- Increased feeling of well being
- Increased work capacity
- Improved blood pressure and lipid profiles.

Aerobic training which 'makes you puff ' and brings the heart rate up to 60–70% of maximum (220 – age [years] beats per minute) for a minimum of 30 minutes 3 or 4 times per week, establishes and maintains fitness and aerobic capacity. Active Australia recommends >150 minutes per week of moderate intensity physical activity (eg: walking). When prescribing a physical activity program a careful history should be taken.

Special attention needs to be paid to exertion-induced symptoms such as chest or abdominal discomfort or syncope. People with type 2 diabetes frequently have silent macrovascular disease. Isometric exercises such as heavy weight lifting (high load, low repetition) may increase blood pressure, increasing the risk of vitreous haemorrhage and sudden cardiac events. However, resistance programs using moderate weights and high repetition can be part of an exercise program for those with diabetes and have been shown to improve glycaemic control (Harris *et al.*, 2012).

Diabetes Australia determine Goals for optimum diabetes management and encourage all people with diabetes to reach these goals (Table 2-7).

Table 2-7 Goals for optimum diabetes management

BGL	**Ideal 4.0–6.0 mmol/L (fasting)** **NHMRC 6.1–8.0 mmol/L (fasting)**
HbA1c	≤7%
Total cholesterol	<4.0 mmol/L*
HDL-C	>1.0 mmol/L*
LDL-C	<2.0 mmol/L*
Non-HDL-C	<2.5 mmol/L*
Triglycerides	<2.0 mmol/L*
Blood pressure	≤130/80 mm Hg
BMI	18-24.9 kg/m2 where appropriate
Urinary albumin excretion	<20 µg/min (timed overnight collection) <20 mg/L (spot collection) <3.5 mg/mmol: women <2.5 mg/mmol: men (albumin creatinine ratio)
Cigarette consumption	Zero
Alcohol intake	≤ 2 standard drinks (20 g) per day for men and women
Physical activity	At least 30 minutes walking (or equivalent) 5 or more days/week (Total ≥150 minutes/week)

* National Vascular Disease Prevention Alliance (Harris *et al.*, 2012)

2.2.7.4. *Medication*

In May 2009 the National Institute of Health and Clinical Excellence (NICE) issued a guideline on the use of newer agents for type 2 diabetes, the role of which in management is becoming clearer. Key issues in selecting the best option are:

- Metformin is recommended first line for most type 2 patients
- Sulphonylureas have a more immediate effect at reducing blood glucose levels in symptomatic patients and may also be first choice for insulin-deficient individuals

- Sulphonylureas, glitazones and insulin may all cause weight gain and obesity is often already a problem
- Sulphonylureas may put the patient at risk of hypoglycaemia with implications for driving
- There appears to be increased risk of distal fractures in women using glitazones
- Glitazones may cause fluid retention, exacerbating or precipitating heart failure
- Exenatide may assist with weight reduction
- Sitagliptin is licensed for triple therapy (with metformin and a sulphonylurea) but vildagliptin is currently only licensed for use in combination with one of these other agents
- Failure of the HbA1c to respond to exenatide or a gliptin after an appropriate interval requires withdrawal of the drug (Holt and Kumar, 2010). The major classes of hypo-glycaemic agents and their current role in therapy are illustrated in (Table 2-8).

Table 2-8 Major classes of hypo-glycaemic agents and their current role in therapy.

Drug class and mode of action	Examples	Advantages	Disadvantages	Place in management
Biguanides: Reduce hepatic Gluconeogenesiss. Increases insulin sensitivity Reduces carbohydrate absorption from the GI tract	Metformin	Weight neutral No risk of hypoglycaemia Possible cardiovascular benefits beyond hypoglycaemic Effects Inexpensive	Gastrointestinal side effects Limit its usefulness	First line in the majority of patients particularly those with insulin resistance
Sulphonylureas: Increase endogenous insulin production	Gliclazide Glimepiride Glibenclamide	Generally well tolerated Inexpensive Effective at reducing HbA1c and blood glucose in symptomatic patients	Risk of hypoglycaemia Weight gain	Usually second line in patients uncontrolled on metformin alone, but can be used alone in those unable to tolerate metformin or patients who are insulin-deficient
Glitazones: Increase tissue sensitivity to insulin	Pioglitazone (Rosiglitazone)	Usually well tolerated May be used in combination with other oral therapies and	Take up to 12 weeks for maximum effect Risk of distal fractures in Women May	Usually second line in patients wishing to avoid the more significant weight gain with sulphonylureas, or

		with insulin	cause fluid retention and so precipitate heart failure in susceptible individuals Small degree of weight gain	third line in those still inadequately controlled on two agents (but insulin should be considered in such cases)
Incretin mimetics: Increase the release of endogenous insulin following carbohydrate ingestion Reduce release of pancreatic glucagon Delay gastric emptying	Exenatide Liraglutide	Actually reduce weight Can be used with either metformin, a sulphonylurea, or both	Injectable Expensive Use with sulphonylurea substantially increases risk of hypoglycaemia Nausea and vomiting is common	Could be used second line in a patient wishing to avoid weight gain but can also be added in to a combination of metformin and sulphonylurea
DPP-4 Inhibitors: Delay the clearance of natural incretins	Sitagliptin Vildagliptin	Weight neutral Sitagliptin can be used as triple therapy with metformin and a sulphonylurea	Relatively expensive Not always effective enough at reducing HbA1c	Can be added in second or third line (only sitagliptin licensed for triple therapy)
Meteglinides: Stimulate the release of pre-formed endogenous insulin	Repaglinide Nateglinide	Specifically aimed at post-prandial hyperglycaemia Rapid action	Multiple doses required - before each meal	Repaglinide may be used as monotherapy Nateglinide only Licensed for use with metformin
Alpha-glucosidase inhibitors: Delay the digestion and absorption of ingested carbohydrate	Acarbose	Reduces post-prandial hyperglycaemia Weight neutral	Poorly tolerated due to gastrointestinal side effects including flatulence	Can be used alone or in combination with metformin and/or sulphonylurea

(Holt and Kumar, 2010)

2.3. Oxidative stress

Oxidative stress (OxS) is an unavoidable aspect of aerobic life. It is the result of an imbalance between the production of reactive oxygen species (ROS) and antioxidant defenses in living organisms (Nishida, 2011). Under normal conditions, a dynamic equilibrium exists between the production of reactive oxygen species (ROS) and the antioxidant capacity of the cell (Granot and Kohen (2004); Stohs, 1995). Oxidative stress is defined as an imbalance in prooxidants and antioxidants, which results in

macromolecular damage and disruption of redox signaling and control (Sies and Jones, 2007). Oxidative stress is a situation when steady-state ROS concentration is transiently or chronically enhanced, disturbing cellular metabolism and its regulation and damaging cellular constituents" (Lushchak, 2011).

The first step in addressing the role of oxidative stress (OxS) in diabetic complications is to define OxS. It is often defined as a shift in the pro-oxidant antioxidant balance in the pro-oxidant direction (Figure 2-4). This definition of Oxidative Stress is more descriptive than quantitative and chemical in nature. Philosophically, it implies a null point, a balance point at which there is no OxS-OxS occurs only when the balance is shifted toward the pro-oxidant direction (packer, 2000b).

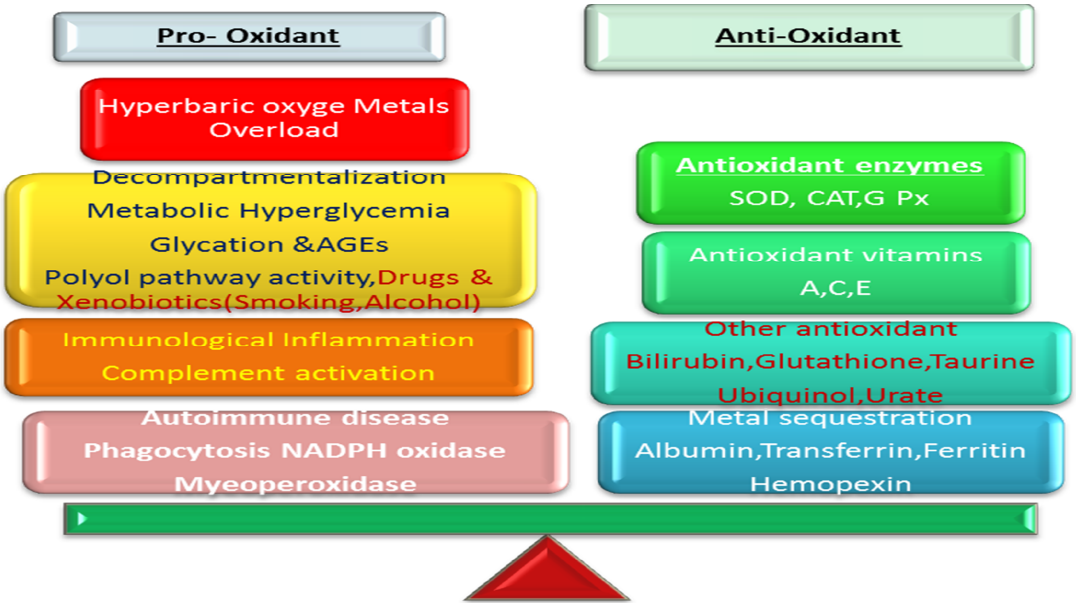


Figure 2-4 Some factors determining the status of OxS in biological systems

A new definition of oxidative stress was advanced by Dean P. Jones (*Antioxidants & Redox Signaling*) as a disruption of redox signaling and control that recognizes the occurrence of compartmentalized cellular redox circuits. Recognition of discrete thiol redox circuits led Jones to provide this new definition of oxidative stress. Measurements of GSH/GSSG, cysteine/ cystine, or thioredoxin reduced /thioredoxin oxidized provide a quantitative definition of oxidative stress. Redox status is thus dependent on the degree to which tissue-specific cell components are in the oxidized state (Packer and Sies, 2008).

Considerable research has focused on free radical (1-electron) mechanisms in macromolecular damage. Based on the xanthine oxidase kinetics, which show that univalent reduction of O_2 to superoxide anion radical is always a minor fraction of the bivalent reduction to H_2O_2 (Fridovich, 1970; packer, 2000b). Generally, oxidative stress can result from two mechanisms:

a. Diminished antioxidants, e.g. mutations affecting antioxidant defence enzymes (such as CuZnSOD, MnSOD, and glutathione peroxidase) or toxins that deplete such defenses. For example, many xenobiotics are metabolized by conjugation with GSH; high doses can deplete GSH and cause . Depletions of dietary antioxidants and other essential dietary constituents can also lead to oxidative stress.

b. Increase production of ROS/RNS/RCS,, e.g. by exposure to elevated O_2 or $\downarrow O_2$, the presence of toxins that are metabolised to produce ROS/RNS or are themselves reactive species, e.g. NO_2• or excessive activation of natural ROS/RNS-producing systems, e.g. inappropriate activation of phagocytic cells in chronic inflammatory diseases such as rheumatoid arthritis and ulcerative colitis).

2.3.1. Free radicals

Free radical is any species capable of independent existence that contains one or more unpaired electrons on the outer atomic or molecular orbital. Molecular oxygen possesses at external molecular orbital two unpaired electrons with parallel spins. According to the Pauli exclusion principle, which states that there are no two identical fermions occuping the same quantum state simultaneously, the electrons are located at different molecular shells (Lushchak and Semchyshyn, 2012).

The chemical definition for free radicals (oxidants) is ‘‘atoms or groups of atoms with one or more unpaired electrons.’’ This property makes them very unstable and highly reactive, trying to capture the needed electron from other compounds to gain stability. When the ‘‘attacked’’ molecule loses its electron, it becomes a free radical itself, beginning a chain reaction. Some free radicals, such as the toxic oxyradical species hydroxyl radical ($^{\cdot}OH$) or the less reactive superoxide radical ($O_2^{\cdot-}$), arise normally during mitochondrial oxidative metabolism (Surh, 2007).

Free radicals are defined as molecules having an unpaired electron in the outer orbit.12 They are generally unstable and very reactive. Examples of oxygen free radicals are superoxide, hydroxyl, peroxyl (RO_2•), alkoxyl (RO•), and hydroperoxyl (HO_2•) radicals. Nitric oxide and nitrogen dioxide (•NO_2) are two nitrogen free radicals. Oxygen and nitrogen free radicals can be converted to other non-radical reactive species, such as hydrogen peroxide, hypochlorous acid (HOCl), hypobromous acid (HOBr), and peroxynitrite ($ONOO^-$). ROS, reactive nitrogen species (RNS), and reactive chlorine species are produced in animals and humans under physiologic and pathologic conditions (Evans and Halliwll, 2001).

Free radicals also can be defined as the atoms, molecules, or ions with unpaired electrons in an open shell configuration. Sometimes, these free radicals may bear some charge, either positive, negative, or zero. They also play a significant role in combustion, atmospheric chemistry, polymerization, plasma chemistry, and many other chemical processes. Free radicals may generate different kinds of chemical and biological reactions in the body (Bano *et al.*, 2012).

2.3.1.1. *Generation of free radical*

Free radicals are mainly generated by various metabolic pathways such as lipid and peroxidation, gluconeogenesis, and glucuronidation. Generation of free radicals is first converted to hydrogen peroxide which is further reduced to water. This detoxification occurs during oxidative stress, where in the oxygen generates inside the body. The superoxide released by the oxidative phosphorylation pathway is the result of multiple enzymes; superoxide dismutase catalyzes the first step, and then various peroxides help in removing the hydrogen peroxide (Bano *et al.*, 2012).

Free radicals can be formed biological systems in many different ways such as (Halliwell, 2006).

- Homolytic cleavage of covalent bond of normal molecule, with each fragment retaining one of paired electrons.

$$X \square Y \rightarrow X^{\square} + Y^{\square}$$

- Losing of single electron from normal molecule.

$$X{:}Y \rightarrow X^{+} + Y^{-}$$

- Addition or Gaining of single electron to normal molecule.

$$X + e^- \rightarrow X^-$$

Oxygen is required for the generation of all ROS, RNS, and reactive chlorine species (Rassaf *et al.*, 2002). The major reactions for the production of oxygen and nitrogen free radicals in the body are illustrated in (Figure2-5).

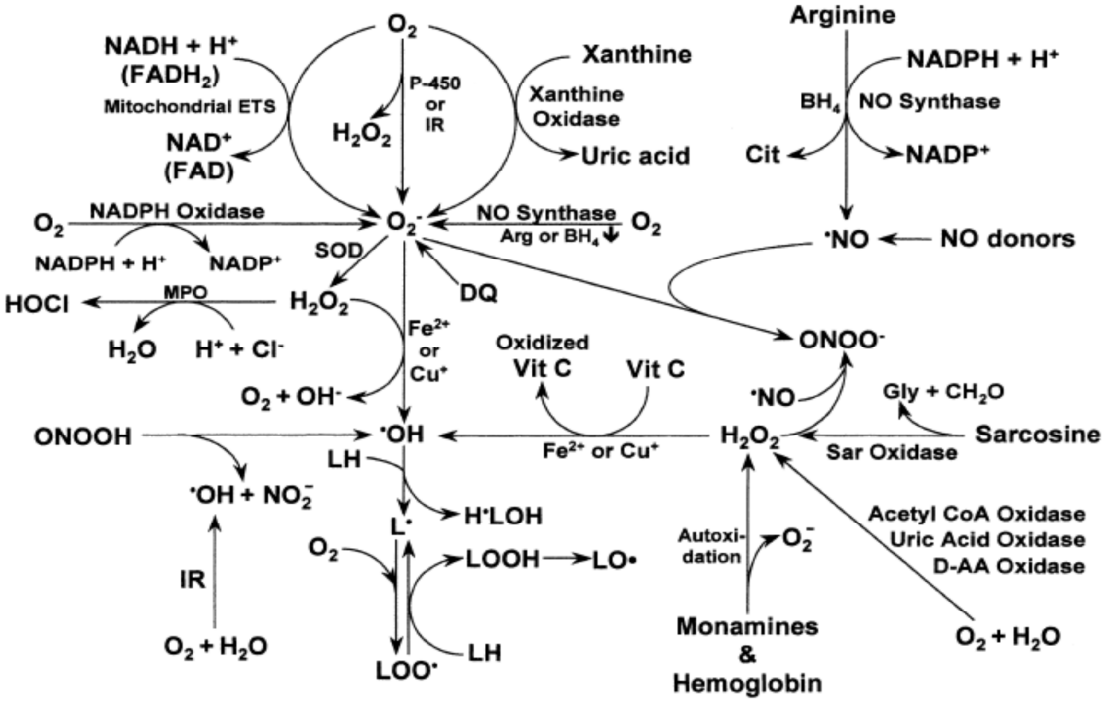


Figure 2-5 Production of oxygen and nitrogen free radicals and other reactive species in mammalian cells (Fang *et al.*, 2002).

2.3.1.2. *Classification of free radicals*

Radicals (often, but unnecessary named as free radicals) are chemical species

possessing an unpaired electron in the outer shell of the molecule so they are highly chemical reactive. Free radicals classification can be made by different criteria, a classification that includes the vast majority of radicals would be: (Bolojan, 2012):

1. Reactive oxigen species
2. Nitrogen reactive species
3. Aromatic compounds
4. Quinonic and semiquinonic compounds
5. Nucleic acids
6. Thiyl radicals

Reactive oxygen species (ROS) is a collective term used for a group of oxidants, which are either free radicals or molecular species capable of generating free radicals. Intracellular generation of ROS mainly comprises superoxide ($O2^{\cdot-}$) radicals and nitric oxide ($NO^{\cdot}$) radicals. Under normal physiologic conditions, nearly 2% of the oxygen consumed by the body is converted into $O_2^{\cdot-}$ through mitochondrial respiration, phagocytosis, etc (Winterbourn, 2008). ROS includes superoxide ($O2^{\bullet-}$), hydroxyl, peroxyl, alkoxy, and nitric oxide (NO) free radicals (Stohs, 1995). Superoxide is the first reduction product of molecular oxygen, and it is an important source of hydroperoxides and deleterious free radicals (Fridovich, 1986). The simultaneous transfer of four electrons to molecular oxygen is an essential step in many biochemical reactions involved in the aerobic metabolism of eukaryotic cells. This reaction leads directly to the formation of H_2O without generating reactive intermediates (Babior, 2000; Miller and Britigan, 1997). ROS percentage increases during infections, exercise, exposure to pollutants, UV light, ionizing radiation, etc. NO·, is an endothelial relaxing factor and neurotransmitter, produced through nitric oxide synthase enzymes. NO· and O2·− radicals, are converted to powerful oxidizing radicals like hydroxyl radical (·OH), alkoxy radicals (RO·), peroxyl radicals (ROO·), singlet oxygen ($1O_2$) by complex transformation reactions. Some of the radical species are converted to molecular oxidants like hydrogen pe-roxide (H_2O_2), peroxynitrite (ONOO·), hypochlorous acid (HOCl). Sometimes these molecular species act as source of ROS (Kunwar Amit, 2011).

2.3.2. Biological effects of reactive oxygen species

Reactive oxygen species have plural effects in biological systems (Figure 2-4). These effects may be placed at least in four groups: (*i*) signaling, (*ii*) defense against infections, (*iii*) modification of molecules, and (*iv*) damage to cellular constituents. This division is rather relative and artificial, because in real cell they cannot be separated, i.e. they operate in concert. All these ways are based on ROS capability to interact with certain cellular components. The final effect of the interaction relies on the type of ROS and molecule it interacts with (Lushchak and Semchyshyn, 2012). Figure 2-6 shows reactive oxygen species produced in tissues.

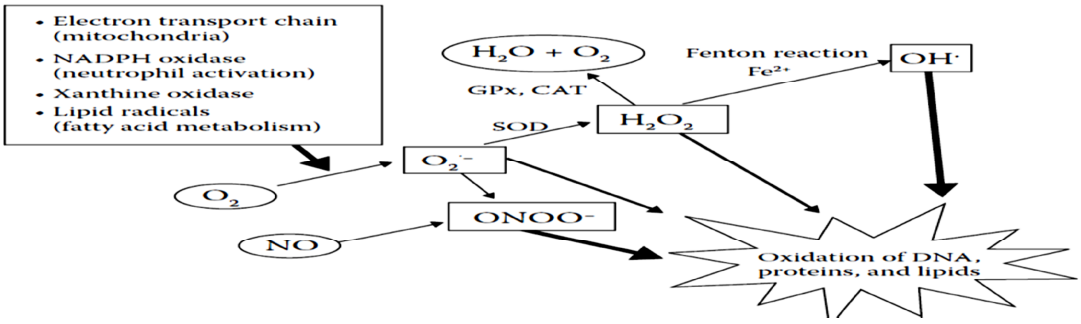


Figure 2-6 Reactive oxygen species produced in tissues (Packer and Sies, 2008)

2.3.2.1. *Protein oxidation*

Proteins are highly susceptible to oxidative damage. Modification of proteins is mainly initiated by hydroxyl radicals, leading to the oxidation of amino acid side chains (where sulfur-containing amino acids are found) and protein fragmentation (Stadtman and Levine, 2003; Berlett and Stadtman, 1997).

In cells, one-electron abstraction of molecules can yield sulfur-, oxygen-, carbon-, and nitrogen-centered free radicals. For example, removal of one electron (and a proton, H) from a –SH group of a protein by a radical species ($R^{\cdot}$) yields a sulfur centered free radical . If R· had just one unpaired electron, the reaction would convert it to a nonradical species. Another example is the one-electron removal from bis-allylic C–H bonds of polyunsaturated fatty acids (PUFAs) that yields a carbon centered free radical . This reaction can initiate lipid peroxidation damage of biological membranes (Marcelo, 2004)

$$R\cdot + Protein \rightarrow one - electron\ yransfer\ to\ R\cdot$$
$$\rightarrow Protein - S\cdot + R^- + H^+$$
$$R^{\cdot} + (PUFA) - (bis - allylic\ C - H\ bond \rightarrow (PUFA) - C^{\cdot} + RH$$

2.3.2.2. *Lipid Peroxidation*

Lipids when reacted with free radicals can undergo the highly damaging chain reaction of lipid peroxidation (LP) leading to both direct and indirect effects. During LP a large number of toxic byproducts are also formed that can have effects at a site away from the area of generation, behaving as ‘second messengers’. The damage caused by LP is highly detrimental to the functioning of the cell (Devasagayam *et al.*, 2003). It has widely been accepted that a chain reaction of free radicals causing lipid oxidation mediated by

reactive oxygen species is conveniently illustrated in the following three phases (Angelo, 1996):

Initiation:

$$RH + initiator \rightarrow R\cdot$$

$$ROOH + initiator \rightarrow ROO\cdot$$

Propagation:

$$R\cdot + O2 \rightarrow ROO\cdot$$

$$ROO\cdot + RH \rightarrow ROOH + R\cdot$$

Termination:

$$R\cdot + R\cdot \rightarrow R\text{-}R$$

$$ROO\cdot + R\cdot \rightarrow ROOR$$

In addition, the reaction chain is also terminated by some antioxidants or free radical scavengers. Transition metal ions, especially iron and copper, are powerful catalysts to initialize the process of the reaction. Additionally, lipoxygenase also acts as an initiator to induce the oxidation to generate the peroxides in food material containing lipid. Lipid peroxidation is a self-propagation reaction that leads to generation of lipid radicals and lipid peroxides. Cell membranes are phospholipid bilayers and proteins, which are the direct targets of lipid oxidation. As lipid oxidation of cell membranes increases, the polarity of lipid-phase and formation of protein oligomers increase but molecular mobility of lipids, number of SH groups, and resistance to thermodenaturation decrease (Halliwell *et al.*, 1992; Halliwell and Chirico, 1993).

2.3.2.3. *Oxidation of DNA*

Although DNA is a stable, well-protected molecule, ROS can interact with it and cause several types of damage such as modification of DNA bases, single and double strand DNA breaks, loss of purines (apu-rinic sites), damage to the deoxyribose sugar, DNA-protein cross-linkage and damage to the DNA repair system (Beckman and Ames, 1997). Activated oxygen and agents that generate oxygen free radicals, such as ionizing radiations, induce numerous lesions in DNA that causes deletion, mutations and other lethal genetic effects. Characterization of this damage to DNA has indicated that both sugar and base moieties are susceptible to oxidation, causing base degradation, single

strand breakage and cross links to proteins (Beckman and Ames, 1997). Reactive oxygen species can produce DNA oxidation by diverse procedures, such as through hydroxyl radicals, which are consequential from the lessening of H_2O_2 or through reactive nitrogen intermediates for instance peroxynitrate (Low *et al.*, 1997). Since oxidative stress is increased in the diabetic retina, the levels of oxidatively modified DNA and nitrosylated proteins are elevated, and antioxidant defense enzymes are impaired (Kowluru, 2005). Some of the negative effects that ROS can exert is DNA and RNA damage which can lead to an increased number of mutations, membrane lipid peroxidation, and protein tyrosine nitration , and after the cell sustains too many of these insults, it may become mutated or direct itself to apoptosis, giving way to the development of the previously mentioned oxidative stress-related diseases, like CVD, cancer diabetes and aging (Corpas *et al.*, 2008).

2.3.3. Oxidative stress and diabetes

The relationship of oxidative stress and diabetes mellitus can be described as a reciprocal effects relationship, where oxidative stress may be occurs as a result of diabetes mellitus, and in some cases, oxidative stress is a cause or one of the causes of diabetes mellitus and it plays a pivotal role in diabetes complications, and vice versa.

2.3.3.1. *The effects of diabetes in oxidative stress*

In DM patients, it has been suggested that low-density lipoprotein (LDL) oxidation contributes to LDL accumulation and, therefore, to an accelerated development of arteriosclerosis (Panasenko *et al.*, 1991). Moreover, the overproduction of ROS due to persistent hyperglycemia produces oxidative protein damage, which would be related to the pathogenesis of diabetic's complications (Martin-Gallan *et al.*, 2003). Damage to DNA may induce cell death or mutation that could give rise, several cellular divisions later, to cancer (Lindahl, 1993; Ames *et al.*, 1993). In fact hyperglycaemia can increase oxidative stress through several pathways and induce intracellular Reactive Oxygen Species (ROS) produced by the proton electrochemical gradient generated by mitochondrial electron transport chain and resulting in increased production of superoxide (Touyz, 2004).The other mechanism involves the transition metal catalyzed auto-oxidation of free glucose yielding superoxide anion and hydrogen peroxide

(Stephens *et al.*, 2009). There is also evidence that hyperglycaemia may comprise natural antioxidant defense. Under normal circumstances free radicals are rapidly eliminated by antioxidants such as reduced glutathione, vitamin C and vitamin E. Reduced glutathione content, as well as reduced vitamin E, have been reported among diabetics patients (Nishikawa *et al.*, 2000).

Despite the arguments that can be raised about the validity of some individual biomarkers, the sum of evidence from biomarkers reporting oxidative damage shows that such damage is increased in diabetes, affecting DNA, lipids, and proteins (glycoxidation), supporting the concept of increased oxidative stress in diabetes (Coininacini. L. *et al.*, 1997).

Evidence indicates that hyperglycemia-induced excessive mitochondrial $O_2^{\bullet -}$ production plays an important role in generating other radicals in diabetes mellitus (Nishikawa *et al.*, 2000). Other evidence indicates ROS are generated at each step of these reactions. Moreover, with excessive glucose in diabetes, it has been shown that glucose is diverted to other pathways such as sorbitol and hexosamine pathways where glucose is metabolized and ROS are generated (Figueroa-Romero *et al.*, 2008; Giacco and Brownlee, 2010).

On the other hand, the impaired antioxidant defense system, such as reduced levels of endogenous antioxidants, reduced/enhanced antioxidant enzyme activities and increased levels of oxidative stress markers such as MDA, is very common in diabetes mellitus (Erejuwa *et al.*, 2010; Rahimi, 2005).

Increased oxidative stress as measured by indices of lipid peroxidation and protein oxidation has been shown to be increased in both insulin dependent diabetes (IDDM), and non-insulin dependent (NIDDM) (Cederberg *et al.*, 2001; Laaksonen and Sen, 2000).

Accumulating evidence points to a number of mechanisms for the increase in the level of ROS in diabetes could be due to their increased production of free radicals, such as Superoxide (Dandona *et al.*, 1996), and/ or decreased destruction by nonenzymic and enzymic catalase (CAT), glutathione peroxidase (GSH-Px), and superoxide dismutase (SOD)] antioxidants. The level of these antioxidant enzymes critically influences the susceptibility of various tissues to oxidative stress and is associated with the development

of complications in diabetes. Also this is particularly relevant and dangerous for the beta islet, which is among those tissues that have the lowest levels of intrinsic antioxidant defenses (WEST, 2000; ROBERTSON, 2004; LENZEN *et al.*, 1996).

Cellular reactive oxygen species (ROS; superoxide and H_2O_2), especially when chronically raised to high levels and associated with hyperglycemia, are widely recognized to play an important pathophysiological role in diabetes and the chronic complications of diabetes as well as in the development of the disease (Packer and Sies, 2008; Lee *et al.*, 2008).

Insulin can stimulate oxidative stress by various mechanisms: the hormone induces production of H_2O_2 when activating its receptors. Insulin also stimulates the sympathetic nervous system, which leads to activation of neurotransmitters and their enzymatic systems, several of which induce oxidative stress (Kashiwagi *et al.*, 1999; McCarty, 2002).

Hyperglycemia activates many signaling mechanisms in cells. Four major pathways that can lead to cell injury downstream of hyperglycemia are illustrated. 1) Excess glucose shunts to the polyol pathway that depletes cytosolic NADPH and subsequently GSH. 2) Excess glucose also undergoes autooxidation to produce AGEs that impair protein function and also activate RAGEs that use ROS as second messengers. 3) PKC activation both further increases hyperglycemia and also exacerbates tissue hypoxia. 4) Overload and slowing of the electron transfer chain leads to escape of reactive intermediates to produce $O2^{-\bullet}$ as well as activation of NADH oxidase that also produces $O2^{-\bullet}$. A unifying mechanism of injury in each case is the production of ROS that impair protein and gene function. TCA, Trichloroacetic acid; PAI-1, plasminogen activator inhibitor-1(Figure 2-7) (Andrea *et al.*, 2004).

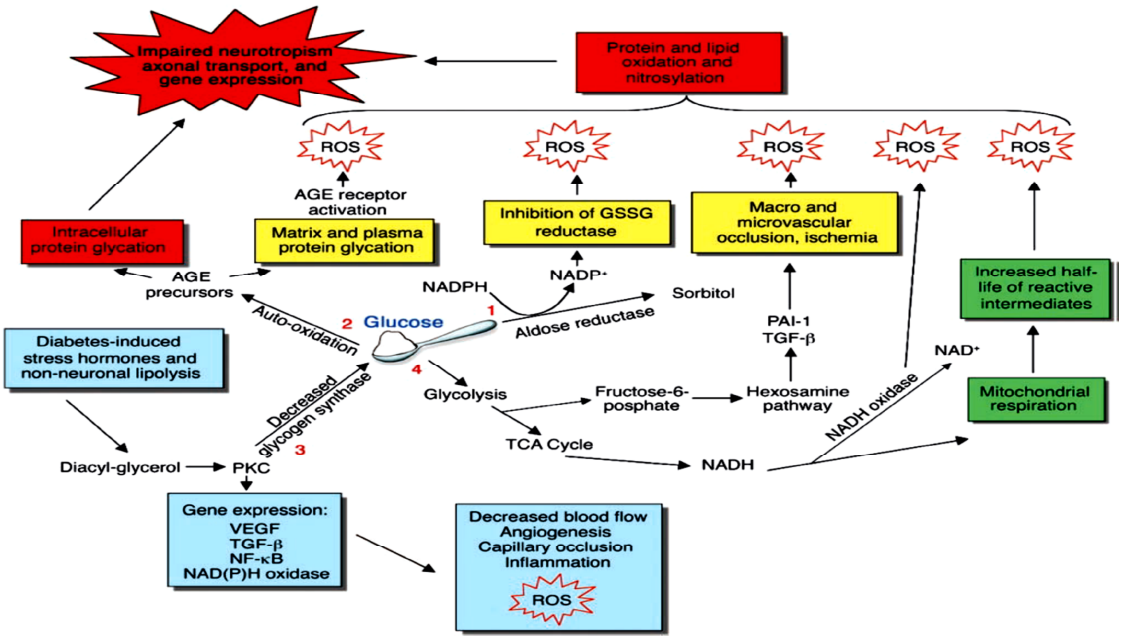


Figure 2-7 Hyperglycemia activates many signaling mechanisms in cells.

2.3.3.2. *The effects of oxidative stress on diabetes*

Increased oxidative stress can induce both type 1 and type 2 diabetes as well as its complications. Oxidative stress has been proposed as a major participant in the patophysiology of diabetic complications (Brownlee, 2005). Recently, some evidences suggest that oxidative stress may play an important role in the etiology of diabetes and diabetic complications (Shinn, 1998).

Oxidative stress shown to effect on diabetes by affect the two major mechanisms failing during diabetes: insulin resistance and insulin secretion. In insulin resistance, ROS and RNS affect the insulin signaling cascade (Rains and Jain, 2011). As with other ROS effects, low doses play a physiological role in insulin signaling. After insulin stimulation of its receptor in adipocytes, H_2O_2 is produced via NADPH oxidase, which by inhibits protein-tyrosine phosphatase 1B (PTP1B) catalytic activity, thus increasing tyrosine phosphorylation (Mahadev *et al.*, 2004), and shut down insulin signaling (Zick, 2004).

In insulin secretion, pancreatic beta-cells are especially sensitive to ROS and RNS, because their natural enzymatic antioxidant defenses are lower compared to other tissues such as liver. Moreover, they lack the ability to adapt their low enzyme activity levels in response to stress such as high glucose or high oxygen (Tiedge *et al.*, 1997). The excess

in mitochondrial superoxide production (by glucose oxidation pathway), activates uncoupling protein-2 (UCP-2), which lowers ATP/ADP relationship through proton leak in the beta-cell, and then reduces insulin secretion (Brownlee, 2003). ROS hyperproduction and excess mitochondrial metabolism resulting from hyperglycemia in the beta-cell may also alter mitochondrial shape, volume and behavior, uncoupling K-ATP channels from mitochondrial activity and thus altering glucose-induced insulin secretion (Drews *et al.*, 2010).

Recent study in Type 2 Diabetes subjects showed that ROS levels in liver positively correlated with increased expression of gluconeogenic enzymes resulting in increased liver glucose production (Takamura *et al.*, 2008). And cell based studies have reported that increased levels of ROS may disturb the translocation of GLUT4 to the cell membrane thus attenuating the insulin mediated glucose uptake in skeletal muscle and adipose tissue (Rudich *et al.*, 1998 ; Henriksen *et al.*, 2011). An implication of Oxidative stress has been first suggested when it was found that alloxan and STZ, used to induce diabetes in animals, destroyed pancreas by oxidative stress. In fact, by oxidative stress induces β-cell death ; this is favoured by an obvious low antioxidant potential of native β-cells (Ho and Bray, 1999; Lenzen *et al.*, 1999). *In vitro*, oxidative stress decreases the insulin gene promoter activity in HIT cells (Matsuoka *et al.*, 1997).

2.3.3.3. *Oxidative stress in diabetic complications*

Elevated levels of free radicals may cause cell membrane damage due to peroxidation of lipid membranes, protein glycation, and depletion of antioxidant defence enzyme systems all of which can lead to cell and tissue damage and thus contribute to the development and progression of diabetes related late stage complications (Maritim *et al.*, 2003). Diabetes-induced oxidative stress in target tissues for diabetic complications, including peripheral nerve, results from at least three mechanisms, including glucose autooxidation, formation of advanced glycation end products, and increased aldose reductase (AR) activity (Low, 1997). There is increasing evidence that the generation of reactive oxygen intermediates is also of major importance for the development of vascular complications in diabetes (packer, 2000b). Results derived from two decades of diabetes complications investigation point towards mitochondrial superoxide

overproduction as the main cause of metabolic abnormalities of diabetes (Giacco and Brownlee, 2010). As the cells of the retina contain high amounts of aldoketoreductase, they have high susceptibility to increase the polyol pathway in the presence of excess glucose, with concomitant decreases in NADPH (Brownlee, 2001). Free radicals generation-induced oxidative stress has produced the hyperglycemia-dependent endothelial dysfunction; it makes diabetes and its complication severe (Diederich *et al.*, 1994). Increased oxidative stress induces nuclear DNA strand breaks that, in turn, activate the enzyme poly ADN-ribose polymerase (PARP) leading to a cascade process that finally activates the four major pathways of diabetic complications:

(1) Increased aldose reductase activity and activation of the polyol pathway lead to increased sorbitol accumulation with osmotic effects, NADPH depletion and decreased bioavailability of nitric oxide (NO). (2) Activation of protein kinase C with subsequent activation of NF-kB pathway and superoxide-producing enzymes. (3) Advanced glycation end-products (AGEs) generation with alteration in the structure and function of both intracellular and plasma proteins. (4) Activation of the hexosamine pathway leads to a decrease in endothelial NO synthase (NOS_3) activity as well as an increase in the transcription of the transforming growth factor (TGF-β) and the plasminogen activator inhibitor-1 (PAI-1) as reported (Brownlee, 2005).

In addation, the reactions of free radical are essential for host defense mechanisms, as with neutrophils, macrophages, and other cells of the immune system, but if free radicals are overproduced, they cause tissue injury and cell death (Southorn, 1988). Diabetic neuropathy most likely comes from a mixture of micro vascular along with neuronal deficits, and oxidative stress participates in the breakdown of neuronal phenotype in experimental diabetic neuropathy (Sima *et al.*, 2004).

2.3.4. Biomarkers for oxidative stress

Oxidative stress can be monitored with several biomarkers (antioxidants and pro-oxidants) which can be assessed in plasma and/or erythrocytes (Passi *et al.*, 2001). Many different markers of oxidative stress have been proposed, including malonaldehyde and conjugated dienes in the blood, as well as alkanes and hydrogen peroxide in the breath (Kneepkens CM *et al.*, 1994; Kneepkens CM *et al.*, 1992).

Determination of markers of antioxidative defense as very sensitive parameters not only contributes to a better understanding of oxidative stress effect but on CVD development, diabetes and the treatment of these two diseases. Also, as the pathogenesis of both diabetes and cardiovascular disease involves oxidative stress, the use of antioxidants is an appealing therapy (Reilly *et al.*, 1996; Griendling and FitzGerald, 2003).

Many markers have been proposed, including lipid peroxides, malondialdehyde, and 4-hydroxynonenal as markers for oxidative damage to lipids; isoprostan as a product of the free radical oxidation of arachidonic acid; 8-oxoguanine (8-hydroxyguanine) and thymineglycol as indicators of oxidative damage to DNA; and various products of the oxidation of protein and amino acids including carbonyl protein, hydroxyleucine, hydrovaline, and nitrotyrosine. Lipid peroxide was assessed in clinical samples even in relatively early studies, and the analytical methods for this substance have improved (YOSHIKAWA and NAITO, 2002).

There is an increasing interest in using oxidative DNA lesions as in vivo biomarkers of oxidative stress. The developed analytical tools must have a very good sensitivity and specificity to detect levels of damage as low as one lesion per million nucleosides using a few mg of DNA (Halliwell, 2000; Collins *et al.*, 1996; Griffiths *et al.*, 2002; Collins *et al.*, 2004). The first three biomarkers have even been referred to as the “lipid triad”, and because they have been found in patients with CVD, it is also called the “atherogenic lipoprotein phenotype (Rizzo and Berneis, 2005; Grundy, 1998; Bestehorn *et al.*, 2010).

Proteins are targets of free-radical mediated oxidation, and the amino acids cysteine and methionine are highly susceptible to oxidation (Berlett and Stadtman, 1997). Interaction with ROS leads to protein carbonylation, which is a robust, stable, and general marker of oxidative stress (Dalle-Donne *et al.*, 2003b).

Antioxidant capacity of plasma can also be used as a marker of oxidative stress or REDOX status of live systems. A number of methods are used to measure plasma antioxidant capacity, some of the most popular are ferric reducing ability of plasma (FRAP), oxygen radical scavenging capacity (ORAC), total radical-trapping antioxidant

parameter (TRAP) and trolox equivalent antioxidant capacity (TEAC) among others (Grigelmo-Miguel *et al.*, 2009).

Evaluation of oxidative stress caused by reactive oxygen species (ROS) and reactive nitrogen species (RNS) is extremely important in clinical settings in the field of Anti-Aging Medicine. Attempts have been made to employ the evaluation of ROS-induced oxidative stress as a testing tool in Anti-Aging Medicine. However, at present, individual markers have not yet fully been qualified (Halliwell and Whiteman, 2004; Halliwell, 2009). Protein tyrosine nitration is a marker for oxidative stress, and it called nitrosative stress (Corpas *et al.*, 2008). Finally, Oxidative stress markers can be summaries according the nature of compounds in three groups (Table 2-9).

Table 2-9 Oxidative stress markers according the nature of compounds

1. Lipids and its derivatives	**2. DNA, PROTEIN, AND AMINO ACIDS**
- Measurement of Fat-Soluble Vitamins and Antioxidants(α-carotene, β-carotene, V.E., Tocophyrols, Lycopene, Sterols, Lutein + zeaxanthin, Retinol (vitamin A)) - Aldehydic Markers of Lipid Peroxidation in Biological Tissues (Crotonaldehyde (CRA), Malondialdehyde (MDA),..) - Measurement of Isofurans - Analysis of F2-Isoprostanes - Measurement of F4-Neuroprostanes - Lipid Peroxidation End Products (Methylglyoxal (MG), Cholesterol oxidation products, Oxidized Apolipoproteins in Oxidized HDL, Acrolein ,Oxidized low-density lipoprotein (oxLDL)) - Serum LPO (lipid peroxide) - HEL (hexanoyl lysine) - β-cryptoxanthin - An 4 -HNE (4-hydroxy-2-nonenal)monoclonal antibody - Polyunsaturated Fatty Acids (conjugated dienes ,arachidonic acid,..) and Corresponding Monohydroperoxy and Monohydroxy	- Synaptosomal Membrane Protein Oxidation - Free 3-Chlorotyrosine, 3-Bromotyrosine, Ortho-Tyrosine, and 3-Nitrotyrosine in Biological Fluids - Tyrosine Oxidation Products in Proteins and Tissues - Intestinal Epithelial Cells Using Manual Scoring and Ridit Analysis - Aldehydic DNA Lesions Using Aldehyde Reactive Probe - Neuroketal Protein Adducts - Isoketal Protein Adducts - 2'-Deoxyguanosine/8-Hydroxy-2_-Deoxyguanosine - 3-Nitrotyrosine in Human Plasma - 8-OHdG (8-hydroxy-deoxyguanosine) and its analogues - Thymidine glycol (TG) - Peroxynitrite-Induced DNA Modifications - Nitration Products in Human Plasma - Aliphatic Amino Acid Alcohols in Oxidized Proteins - Glutamate - S-Glutathionated Hemoglobin

3. REACTIVE OXYGEN SPECIES AND REACTIVE NITROGEN SPECIES
- Protein Carbonyl Levels—An Assessment of Protein Oxidation - Fluorogenic Analysis of H2O2 in Biological Materials - Detection of Reactive Oxygen Species by Flow Cytometry - Nitrite Determination by Colorimetric and Fluorometric Greiss Diazotization Assays: Simple, Reliable, High-Throughput Indices of Reactive Nitrogen Species in Cell-Culture Systems - Protein Carbonyl Determination Using Biotin Hydrazide - Real-Time, In-Vivo Measurement of Nitric Oxide Using Electron Paramagnetic Resonance Spectroscopic Analysis of Biliary Flow

The markers of Oxidative stress also may categorized according to the type of reactions to tow main groups which are Free Radicals and Antioxidants markers (Table 2-10).

Table 2-10 Markers of Oxidative Stress according reactions

A. FREE RADICAL DERIVED BIOMARKERS	B. ANTIOXIDANT BIOMARKERS
- Human Xanthine Oxidoreductase - Polyunsaturated Fatty Acids and Corresponding Monohydroperoxy and Monohydroxy Peroxidation Products - Products of Lipid Oxidation - Docosahexaenoic Acid Hydroperoxides in Retina - Lipid Hydroperoxide-Derived Protein Modification - Determining the Metabolic Pathways of Eicosanoids and for Evaluating the Rate-Controlling Enzymes - F2-Isoprostanes - Formation of Apolipoprotein AI-AII Heterodimers by Oxidation of High-Density Lipoprotein - Peroxynitrite-Induced DNA Modifications - Hydroxyl and 1-Hydroxyethyl Radicals - Aliphatic Amino Acid Alcohols in Oxidized Proteins - Glutamate - GSH and GSSG - Protein Carbonyl - N□ -(carboxymethyl)lysine (CML) as a Biomarker of Oxidative Stress in Long-Lived Tissue Proteins - S-Glutathionated Hemoglobin in Human Erythrocytes - Oxidized Cellular DNA and DNA Double-Strand Breaks - Antibodies Against Oxygen Free Radical-Modified DNA	- Multiple Redox-Active Metabolites - □ -Tocopherol Turnover in Plasma and in Lipoproteins - Uric Acid in Urine - Tocotrienols in Different Sample - □ -Carotene15,15'-Dioxygenase Activity - Ubiquinol/Ubiquinone Ratio - Catechol- and Pyrogallol-Type Flavonoids - Pyruvate Dehydrogenase Complex as a Marker of Mitochondrial Metabolism - Ceruloplasmin - Metallothionein and Oxidized Metallothionein - Fractionation of Herbal Medicine for Identifying Antioxidant Activity - Vitamins and some ions - Catalase activity - Glutathione peroxidase (GPx) - STAS (serum total antioxidant status) - Superoxide dismutase (SOD) - Retinol binding protein (RBP

2.3.5. Antioxidants

Antioxidants are compounds which, when present in low concentrations compared to oxidizable substrates, can quench free radicals and significantly delay or inhibit oxidation of the substrate and protect biological systems against potential harmful effects of free radicals (Arnao, 2000; Diplock *et al.*, 1998). Antioxidants also may be defined as substances those, when present at low concentrations compared to that of oxidizable substrates, significantly delay or inhibit oxidization of those substrates (Antolovich *et al.*, 2002). Antioxidants inhibit or delay the oxidation of other molecules by limiting either the initiation, or the propagation of oxidizing chain reactions (Velioglu *et al.*, 1998). Free radicals can be scavenged by several metalloenzymes (e.g., glutathione peroxidase, catalase, superoxide dismutase) as well as by the non-enzymatic antioxidant defence system (e.g., tocopherol, β-carotene, ubiquinol, vitamin C, glutathione, lipoic acid, uric acid, metallothionein, bilirubin) which quench their activity. Therefore, much attention of nutritionists is now focused on the possible role of the enhancement of the defenses against ROS (SIES, 1993).

According to Boskou and Elmadfa (1999) an effective antioxidant should:

1) Compete effectively with the substrate for the reactive intermediate.
2) Be able to be readily repaired by the biological system. If the antioxidant is destroyed irreparably at every encounter with the oxidizing species then the system will rapidly reach a state where it is no longer protected.
3) Be accessible to the reactive intermediate in the microenvironment, i.e, the antioxidant has to be located in the same microenvironment as the oxidizing radical species.
4) Be relatively unreactive to the substrate. The products should not be toxic to the system and must not take part in the reaction.
5) Be catalytic in its quenching mechanism. Ideally, the antioxidant should be generated once it has reacted with the radical species.

2.3.5.1. *Sources of Antioxidants*

a. Natural Antioxidant

Natural antioxidants are natural compounds, especially derived from dietary sources provide a large number of antioxidants. Some beverages such as tea are also rich sources

of antioxidants (Devasagayam *et al.*, 2003). The natural antioxidants are phenolic compounds (tocopherols, flavonoids, phenolic acids), nitrogen compounds (alkaloids, chlorophyll derivatives, amino acids, and amines), carotenoids or ascorbic acid. Synthetic antioxidants are phenolic structures with various degrees of alkyl substitution (Velioglu *et al.*, 1998). The nutritive antioxidants, including more than 500 carotenoids such as ß-carotene (sweet potatoes, carrots), lutein (kale, spinach), lycopene (tomatoes), the tocopherols (nuts, vegetable oils) of which a-tocopherol is the most active, and vitamin C (citrus fruits and juices), which exists in oxidised and reduced forms. The non-nutritive antioxidants include more than 2000 flavonoids such as quercetin, kaempferol, myricetin, apigenin and luteolin, which are commonly found in tea, onions and apples. Red wine is an important source of flavonoids in some populations (Fürst, 1997).

b. Synthetic antioxidants

Synthetic antioxidants such as butylated hydroxyanisole (BHA), and butylated hydroxytoluene (BHT), have been used as antioxidants since the beginning of this century. Synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), *tert*butylhydroquinone (TBHQ) and propylgallate (PG) are also used but are under strict regulation because of potential health hazards (Hettiarachchy and Qi, 1998). Synthetic antioxidants have been widely used to extend the shelf-life of various food materials. The current preference for synthetic antioxidants can be attributed to their proven effectiveness in a variety of food systems and their relative low cost when compared to natural antioxidants. The most commonly used synthetic antioxidants in the U.S. food industry include BHT, BHA and TBHQ (Boskou and Elmadfa, 1999).

2.3.5.2. *Types of Antioxidants*

Antioxidants can be classified as liposoluble or hydrosoluble antioxidants. Liposoluble antioxidants are located mainly on membranes or are associated with lipoproteins, while hydrosoluble antioxidants circulate more freely in the blood. Vitamin E, which is highly liposoluble, has a particular affinity for lipoproteins, whereas vitamin C, which is highly hydrosoluble, circulates freely with minimal protein binding (Cornelli, 2009).

Primary Antioxidants is formed of molecules of phenol, Tocopherols, gallic acid and its derivatives, flavonoids, and other compounds. Secondary (preventive) Antioxidants

prevent lipid oxidation in a different way, inhibiting the initiation by the fact that they oxidate themselves instead to oxidize lipid molecules or they turn intermediary or final products generated during the reactions into non-toxic forms (Song *et al.*, 2009).

Functionally, antioxidants can be grouped according to their preferential localization and Cornell, (2009), reported this for the formulation of antioxidant combinations. This classification identifies antioxidants as follows:

- Membrane antioxidants: these are represented by vitamin E, β-carotene, vitamin A, and are known also as lipophilic antioxidants. They have an affinity for membranes of cells and lipoproteins (low-density lipoprotein, very low density lipoprotein, high-density lipoprotein).
- Circulating antioxidants: these consist of vitamin C, amino acids, and polyphenols, which are also known as hydrophilic antioxidants. They are not heavily bound to proteins and may circulate freely in body fluids.
- Cytosol antioxidants: these are produced by cells. Members of this class are lipoic acid, squalene, coenzyme Q10. They are intermediates for the synthesis of endogenous molecules or macromolecules (cytochromes).
- System antioxidants: these are trace metals (such as Se and Zn) or amino acids (such as L-cysteine) (Cornelli, 2009).

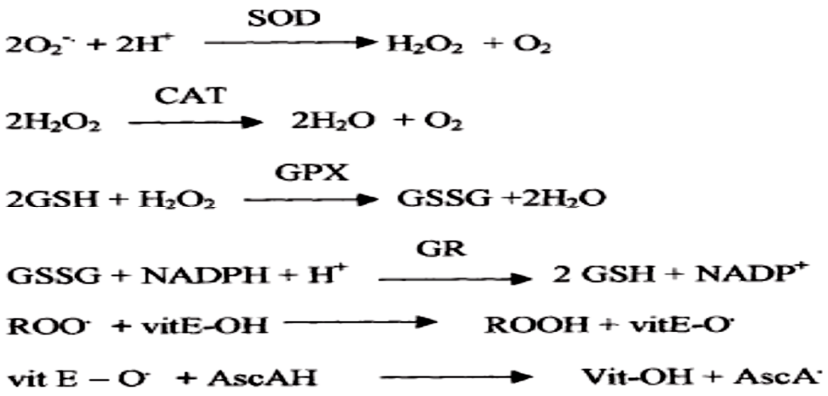
2.3.5.3. *Mechanisms of Antioxidant Defense*

Antioxidant defense mechanism involves both enzymatic and nonenzymatic strategies. Common antioxidants include the vitamins A, C and E, antioxidant minerals (copper, zinc, manganese, and selenium), and the cofactors (folic acid, vitamins B1, B2, B6, B12). They work in synergy with each other and against different types of free radicals. Vitamin E suppresses the propagation of lipid peroxidation; vitamin C with vitamin E inhibits hydroperoxide formation; metal complexing agents, such as penicillamine, bind transition metal involved in some reactions in lipid peroxidation and inhibit Fenton and Haber-weisstype reactions; vitamins A and E scavenge free radicals (Soltani, 2011).

Haber Weiss: $H_2O_2 + O_2^{\cdot-} \rightarrow O_2 + OH^- + {}^{\cdot}OH$

Fenton : $H_2O_2 + Fe^{2+} \rightarrow OH^- + \cdot OH + Fe^{3+}$

Enzymatic antioxidant system (Cu,Zn- and Mn-superoxide dismutase (SOD), catalase, glutathione (GSH) peroxidase (GPX), and GSH reductase (GR)) function by direct or sequential removal of ROS, thereby terminating their activities (Jakus, 2000).



The enzymatic antioxidant systems in mitochondria involve SOD, GPx, glutathione reductase (GR), catalase, glutaredoxin, thioredoxin, thioredoxin reductase (TrxR), and peroxiredoxin (PRx). Decreased activity of mitochondrial SOD and GPx were associated

with mitochondrial oxidative stress (Zang *et al.*, 2007). Catalase is also an important antioxidant enzyme that catalyzes the conversion of H2O2 to H2O. Catalase consists of four subunits, each of which contains a ferric (Fe+3) heme group bound to its active site (Bras *et al.*, 2005).

Several low molecular weight antioxidants are synthesized in vivo like GSH, lipoic acid, uric acid, taurine, bilrubin, □-keto acids, melatonin, coenzyme Q, histidinecontaining dipeptides, melanins, polyamines and plasmalogens. Among these antioxidants, GSH is one of the major cellular antioxidant (Amrit, 2008).

Among the various mechanisms for nutritional defense and disease prevention are: (i) ROS scavenging; (ii) reduction of peroxides and repair of peroxidized biological membranes; (iii) sequestration of iron to decrease ROS formation; (iv) utilization of dietary lipids (rapid energy production and ROS scavenging by short-chain fatty acids, ROS scavenging by cholesteryl esters); and (v) alternative biological pathways as occur in stomach cancer, multiple system organ failure and diabetes (Parke, 1999).

Antioxidant functions also include (1) quenching of reactive oxygen species, (2) regeneration of exogenous and endogenous antioxidants such as vitamins C and E and glutathione, (3) chelation of metal ions, and (4) prevention of membrane lipid

peroxidation and reparation of oxidized proteins. Because of its antioxidant properties, α -LA is known to be effective in both prevention and treatment of oxidative stress in a number of models or clinical conditions including diabetes (Lee *et al.*, 2008).

Four possible mechanisms have been suggested by which antioxidants function to reduce the rate of oxidation of fats and oils (Dauqan *et al.*, 2011). These are: 1) Hydrogen donation by the antioxidant. 2) Electron donation by the antioxidant. 3) Addition of the lipid to the antioxidant. 4) Formation of a complex between the lipid and antioxidant.

There are many biological and dietary constituents that show 'antioxidant' properties in vitro. For an antioxidant to have a physiological role, however, certain criteria must be met.

1. The antioxidant must be able to react with ROS found at the site(s) in the body where the putative antioxidant is found.
2. Upon interacting with a ROS, the putative antioxidant must not be transformed into a more reactive species than the original ROS.
3. The antioxidant must be found in sufficient quantity at the site of its presumed action in vivo for it to make an appreciable contribution to defense at that site: if its concentration is very low, there must be some way of continuously recycling or resupplying the putative antioxidant .

2.3.5.4. *Antioxidant and diabetes*

Endogenous cellular enzymatic and non-enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), or glutathione (GSH) are the most powerful antioxidants in the organism and have been shown to play an important role in diabetes (Vassort and Turan, 2010).

Several studies have addressed the possible participation of dietary antioxidants, such as vitamins, in ameliorating the diabetic state and retarding the development of diabetes complications (Cuerda *et al.*, 2011; Sheikh-Ali *et al.*, 2011). Many efforts have been made to prevent the development and progression of diabetes and cardiovascular disease by using materials with antioxidative properties. Among a variety of antioxidants, α-lipoic acid (α-LA) has recently received much research attention (Lee *et al.*, 2008). α -LA contains two sulfur molecules that can be oxidized or reduced. This feature allows α-LA

to function as a cofactor for several important enzymes as well as a potent antioxidant. α -LA functions as a cofactor of mitochondrial key enzymes such as pyruvate dehydrogenase and α -keto-glutarate dehydrogenase and thus has been shown to be required for the oxidative decarboxylation of pyruvate to acetyl-CoA, the critical step leading to the production of cellular energy (ATP) (Packer *et al.*, 1997; Reed, 1998). α-LA and its reduced form, DHLA, are powerful antioxidants (Packer, 1995). Routine supplementation with antioxidants, such as vitamins E and C and carotene, is not advised by the ADA because of lack of evidence of efficacy and concern related to long-term safety (ADA, 2012). Vitamins C, E, and A constitute the non-enzymatic defense against oxidative stress, by regenerating endogenous antioxidants. Vitamin C has a role in scavenging ROS and RNS by becoming oxidated itself. The oxidized products of vitamin C, ascorbic radical and dehydroascorbic radical are regenerated by glutathione, NADH or NADPH. In addition, vitamin C can reduce the oxidized forms of vitamin E and gluthatione (Garcia-Bailo *et al.*, 2011).Vitamin E is a fat-soluble vitamin which may interact with lipid hydroperoxides and scavenge them. It also participates, together with vitamin C, in gluthatione regeneration by interaction with lipoic acid (Evans *et al.*, 2002). The study of Srivatsan *et al.*, (2009) suggested that supplementation with dietary antioxidants especially antioxidant vitamins accompanied by change in lifestyle might help to reduce damage brought about by free radical toxicity in diabetes mellitus. Experimental study on diabetic rats suggested that nutritional vitamin E supplementation helps fatty acids metabolism and lower lipid peroxidation in rat tissues (Celik *et al.*, 2002).

2.4. Metabolic and Therapeutic effects of kefir as probiotic

Kefir is probiotic product made by fermentation of milk or sugary solution using mixture of bacteria and yeasts hold in matrix of polysaccharides and polypeptides calls kefir grains. It was produced and used in Middle Asian countries, Russia and Caucasia for many years. In these countries, kefir has been widely considered as a beverage and a medication for treatment of various illnesses (Kilic *et al.*, 1999). Historically, kefir has been recommended for the treatment of several clinical conditions such as gastrointestinal problems, hypertension, allergies, and ischemic heart disease. However, the variability

inherent in kefir production conditions in different assays makes it difficult to conduct comparisons between reported scientific results (Farnworth, 2005; Farnworth and Mainville, 2008; Rattray and O'Connell, 2011).

2.4.1. Productivity of bioactive components

Kefir and kefir grains contain and can provide several important bioactive compounds such as exopolysaccarides, organic acids, amino acids, fatty acids, vitamins, enzymes, bacteriocins, alcohols, which play significant and essential roles in nutrition and health improvement. Kefir grain fermentations from various substrates have been evaluated (Öner Z *et al.*, 2010; Magalhaes *et al.*, 2010a; Farnworth, 2005) and a wide variety of bioactive compounds have been observed, such as organic acids, CO_2, H_2O_2, ethanol, bioactive peptides, exopolysaccharides (kefiran), and bacteriocins. These compounds may act independently or together to produce the various health benefits attributed to kefir consumption (Öner Z *et al.*, 2010; Rattray and O'Connell, 2011; Garrote G. I. *et al.*, 2000). The analysis of the proteolytic activity of the strains isolated from kefir grains, revealed that proteinase-producing strains (Prt+) are often present. The active colonies (lactobacilli, lactococci, leuconostoc and yeasts) were shown to be 60% of the isolated population. From 276 *Lactobacillus* spp. strains, 49 were found to produce proteinases. These results suggested that *L. kefir* DR22x had cell-wall-bound proteinases with an extracellular location (Kabadjova-Hristova *et al.*, 2006). The best probiotic properties were observed in *L. acidophilus CYC 10051* and *L. kefiranofaciens CYC 10058. L. kefiranofaciens CYC 10058* produced an exopolysaccharide, which revealed that it was closely related to kefiran, a polysaccharide with antitumoral properties (Santos *et al.*, 2003). (Wang *et al.*, 2008), had characterized some of properties of polymer produced by *L. kefiranofaciens* ZW3 isolated from Tibet kefir. The strain produces a high amount of polymer having desirable physiochemical properties. The development of a "kefir-type" beverage is not the only industrial application that has been investigated. Kefir grains have also been studied regarding the production of single cell protein (SCP) in the bioconversion of cheese whey and their application in the food industry, to improve the sensory characteristics of certain products (Paraskevopoulou *et al.*, 2003). These beneficial effects that kefir and kefir grains have shown may be attributed to bioactive

ingredients within them. These include the exopolysaccharides, bacterial DNA, cell wall components, as well as peptides (Hong *et al.*, 2010; Thoreux and Schmucker, 2001; Vinderola *et al.*, 2006).

2.4.2. Antimicrobial activity

It is reported that Lactocoocus lactis DPC3147 a strain isolated from an Irish kefir grain produces a bacteriocin with a broad spectrum of inhibition (Ryan M.P. *et al.*, 1996). One study demonstrated that kefir possesses antibacterial effect against *Staphylococcus aureus (ATCC 29213), Bacillus cereus (ATCC 11778), Salmonella enteritidis (ATCC 13076), Listeria monocytogenes (ATCC 7644)* and *Escherichia coli (ATCC 8739)* . It has to be underlined that the inhibitoriest effect was seen against *Staphylococcus aureus*. On the other hand, it was seen that the antibacterial effect decreased or did not change during the storage at + 4 ^{o}C except of *Salmonella enteretidis*. The data presented in this study suggests that kefir may be a good antimicrobial agent in food technology for food safety (Ulusoy *et al.*, 2007). Forejt *et al.*, (2007), found that the counts of *Enterococcus faecalis* in the stool of individuals after kefir beverage consumption were significantly lower. It appears that kefir has a potential effect in changing the homeostasis of the oral ecosystem leading to the proliferation of a bacterial film (Cogulu *et al.*, 2010). In the study of Golowczyc *et al.*, (2011), two *L. kefir* strains that isolated from kefir showed to be different in their adhesion capacity after the process. In addition, although to a lesser extent compared to the fresh strain, it was demonstrated that rehydrated spray-dried *L. kefir 8321* retained the ability to protect against *Salmonella* invasion when both microorganisms are previously co-incubated. And in other studies reported that *L. kefir CIDCA 832, L. kefir CIDCA 8348, L. plantarum CIDCA 83114* were adhered to Caco-2 cells and protects epithelial cells against *Salmonella* invasion in vitro when they are previously co-incubated, and inhibits Salmonella and Escherichia coli in vitro, is able to adhere to Caco-2 cells (Golowczyc, 2008) and antagonizes *E. coli O157:H7* on Hep-2 cells (Hugo *et al.*, 2008). On the other hand, Chifiriuc et al., (2011) observed that all milk fermented with kefir grains had antimicrobial activity against *Bacillus subtilis*, *S. aureus*, *E. coli*, *E. faecalis* and *S.* Enteritidis, but did not inhibit *P. aeruginosa* and *C. albicans.*

2.4.3. Anti-diabetic activity

A major focus of current anti-diabetic research is the development of anti-hyperglycemic agents that are safe and free of negative side-effects.

Functional foods and nutraceuticals have become prescribed widely to prevent the occurrence of diabetes mellitus, and to attenuate the complications of hyperglycemia in diabetic patients, that is because of their effectiveness, limited side effects, and relatively low cost.

Fermented food, their components, and their microorganisms have described to have hypoglycemic effects. Kefir can potentially be a useful food choice for patients with diabetes who are required to control their blood glucose levels (Kong, 2009). The results of Maeda *et al.*, (2004a) showed that kefiran had a hypoglycemic effect in KKAy mice. The daily administration of viable *Lactobacillus rhamnosus* GG cells decreased the blood glucose level in a genetic type 2 diabetes model, KK-Ay mice (Honda *et al.*, 2011). Anti-diabetic effects of lactic acid bacteria on KK-Ay mice have been reported for *Lactobacillus casei* (Matsuzaki *et al.*, 1997), and (dahi) containing L. acidophilus NCDC14 and *L. casei* NCDC19 on high fructose induced diabetic rats (Yadav *et al.*, 2007), and for dahi product on streptozotocin induced diabetes in rats (Yadav, 2008).

2.4.4. Anti-hyeperlipidimic Anti hypercholesterolemic and effects

In addition kefir, like several fermented dairy products, is a useful cholesterol-lowering product, and increasing the propionic acid in the fecal flora while not altering the cholesterol synthesis. With regard to the cholesterol metabolism, it has been observed that the fermented product prevented increased liver triacylglycerol and cholesterol levels but had no effect on plasma cholesterol levels(Oda and Hashiba, 1994; St-Onge *et al.*, 2000). One study observed that a 28-day kefir-supplemented diet had no effect on total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, or triacylglycerolconcentrations nor on cholesterol fractional synthesis rates (St-Onge *et al.*, 2000). Previous results, demonstrated that use of kefir as a probiotic in drinking water increases live weight, lowers total cholesterol and total lipid thus suggesting that its use in human diets may have beneficial effects (Cenesiz S. *et al.*, 2008). Results of recent investigation, on effects of *Lactobacillus plantarum MA2* isolated from Tibet kefir on

lipid metabolism and intestinal microflora of rats fed on high-cholesterol diet, showed that *L. plantarum* MA2 feeding significantly lowered serum total cholesterol, low density lipoprotein cholesterol, and triglycerides level, while there was no change in high-density lipoprotein cholesterol. In addition, liver total cholesterol and triglycerides was also decreased. However, fecal cholesterol and triglycerides was increased significantly ($P<0.05$) in comparison with the control (Wang *et al.*, 2009).

(Quiro´ s *et al.*, 2005), reported the ACE-inhibitory properties of kefir made from caprine milk. Sixteen peptides were identified and synthesized to evaluate their ACE-inhibitory activity. Two of these peptides, with the sequences PYVRYL and LVYPFTGPIPN, showed a potent ACE-inhibitory activity with IC50 values of 2.4 and 27.9 _*M*, respectively.

2.4.5. Antioxidant activity

Antioxidative activities such as the inhibition of ascorbate autoxidation, reducing activity, the scavenging effect of superoxide anion radicals and hydrogen peroxide of kefir samples were determined. Kefirs produced from whole soymilk had the highest inhibition rate of ascorbate autoxidation. Reducing activities of kefir samples, expressed as equivalent amounts of cysteine, were found statistically different and elevated by increased soymilk ratio. Results of the inhibition of superoxide radical generation of cow, cow/soy and soymilk kefir samples were found statistically different. However, the effect of fermentation on this activity neither with kefir grain nor culture was significant (KESENKAŞ *et al.*, 2011).

There is increasing in antioxidative activities of soymilk after fermentation with lactic acid bacteria and bifidobacteria (Wang *et al.*, 2006). and Probiotic Yeast and Bacteria (Rekha and Vijayalakshmi, 2008). Kefir could play an antioxidant role and its effectiveness depended on the dosage and time of application in Coruh trout (Can *et al.*, 2012). Milk-rice kefir displayed high significantly greater of antioxidant activity comparing with BHA as standard (Sirirat and Jelena, 2010). Milk-kefir and soymilk-kefir possess significant antimutagenic and antioxidant activity (Liu *et al.*, 2005b). There are variations among antioxidant properties of kefir samples produced from different cow/soy milk mixtures related to soymilk ratio in kefir milk

(KESENKAŞ *et al.*, 2011). Fermented soy whey extracts exhibited a potentially antioxidant activities (Monajjemi *et al.*, 2012).

2.4.6. Anti-inflammation and anti-allergenic effects

It had been found that, the expression of phospholate-nuclear factor-κB (p-NFκB), TNF-α, p-p38 and Bad protein products were reduced or retarded in the Kefir I- or II-treated allergy group. The GM080-treated allergy group exhibited significantly lower p-JNK, JNK1/2, phospholate-Ikappa B (p-IκB), Bax and Bad protein products than the Kefir I and Kefir II allergy groups. These results indicate that LAB can reduce inflammation and prevent apoptosis of cardiomyocytes in the heart of OVA-induced allergy mice (Wang *et al.*, 2012).

Oral administration of milk kefir and soymilk kefir for 28 days significantly increased the fecal populations of *bifidobacteria* and *lactobacilli*, while it significantly decreased those of Clostridium perfringens. Milk kefir and soymilk kefir also significantly decreased the serum OVA-specific IgE and IgG1 levels for both groups, but not those of the IgG2a analogues. Consumption of milk kefir and soymilk kefir suppressed the IgE and IgG1 responses and altered the intestinal microflora in our supplemented group, suggesting that milk kefir and soymilk kefir may be considered among the more promising food components in terms of preventing food allergy and enhancement of mucosal resistance to gastrointestinal pathogen infection (Liu. J. *et al.*, 2006a).

In addition, milk whey formula supplemented with *Lactobacillus rhamnosus* may alleviate some aspects of atopy and intestinal inflammation among infants with allergic symptoms (Majamaa and Isolauri, 1997). Although contradictory results have been obtained (Wheeler *et al.*, 1997), the majority of the reported results indicate that fermented dairy products, including kefir, possess antiallergy properties (Cross *et al.*, 2001; Umeda *et al.*, 2005).

Biokefir meets all the criteria of functional foods and can be used in the prevention and treatment of various acute and chronic inflammatory conditions and nutritional allergies (Figler M *et al.*, 2006).

2.4.7. Improve foods digestion and Lactose Intolerance

Several microorganisms in the kefir starter culture can improve lactose digestion in a manner similar to yogurt (De Vrese *et al.*, 1992). HERTZLER and CLANCY,(2003), evaluated lactose digestion from kefir in adults with lactose maldigestion. This study demonstrated that plain kefir improved lactose digestion just as well as plain yogurt. The improved lactose digestion from kefir was accompanied by a concomitant improvement in flatus symptoms that was comparable with the improvement observed from plain yogurt. Previous study indicated the addition of kefir into a normal diet could benefit protein digestion, due to the increased activity of the intestinal dipeptidase. Furthermore, kefir would benefit the basal glycemic index because the intestinal sugar Na+-dependent uptake is diminished (Urdaneta *et al.*, 2007). The diet containing kefir and fresh grains had a b-galactosidase activity of 4.4 U/l, which was identified as being responsible for the hydrolysis of lactose in the intestine, thus yielding galactose that can be absorbed. Kefir itself contains no galactose (Alm, 1982). It has also been shown that fermented milk products have a slower transit time than milk, which may further improve lactose digestion .(Vesa *et al.*, 1996; Labayen, 2001).

2.4.8. Stimulate The Immune System

A study, demonstrated that a kefir supplemented diet affects the intestinal mucosal and systemic immune responses to intraduodenal CT differently in young and old rats. Most importantly, these data suggested that orally administered kefir enhances the specific intestinal mucosal immune response against CT in young adult, but not in senescent rats (Thoreux and Schmucker, 2001).

Kefir and pasteurized kefir were able to modulate the mucosal immune system in a dose-dependent manner. Kefir was administered 10-times more diluted than pasteurized kefir, but it induced an immunomodulation of similar magnitude, indicating the importance of cell viabilty. The results suggest that a Th1 response was controlled by Th2 cytokines induced by kefir feeding. Pasteurized kefir would induce both Th2 and Th1 responses (Vinderola *et al.*, 2005). Fermented milks administered to mice resulted in significant effects on various immune responses such as increased IgA-producing cells in a dose-dependent way (YOSHIDA *et al.*, 2004; Perdigon *et al.*, 1999). The effect of kefir

exopolysaccharides on the immune system may be dependent on whether the host is healthy or has developed any tumours. Furukawa *et al.,* (1996) incubated kefir grain polysaccharides with Peyer's Patch (PP) cells from tumour-bearing mice and found that the supernatant of this mixture enhanced proliferation of splenocytes from normal mice and increased the mitogenic activities of lipopolysaccharides (LPS) and phytohaemagglutinin- P (PHA-P) in splenocytes. They concluded that the polysaccharide stimulated PP cells, causing them to secrete water-soluble factors that, in turn, enhanced the mitogenic response of thymocytes and splenocytes in normal mice.

2.4.9. Antitumor activity

Studies have shown that it can induce apoptosis of breast cancer cells, malignant T-lymphocytes, and colorectal cancer cells (EL-Hayek, *2011 ;* Maalouf *et al.,* 2011*)*. As a product of traditional yoghurt, the cell-free fraction of could inhibit proliferation of SGC7901 cells in vitro. Both cell cycle regulation and apoptosis were detected in SGC7901 cell lines treated with the cell-free fraction of Tibetan Kefir. Thus, Tibetan Kefir has the potential to be a functional food, and should be further evaluated clinically in vivo in patients with gastric *cance*r (Gao. Jie *et al.,* 2013). When we compared the serum proinflammatory cytokine levels of the two groups at the baseline and following the third and the sixth cycles, we again found no statistically significant difference ($p>0.05$). Kefir consumption at the mentioned doses made no statistically significant effect on serum proinflammatory cytokine levels and on the incidence of mucositis development in cancer patients (Topuz *et al.*, 2008).

3. MATERIALS AND METHODS

3.1. *In vitro* Experiments

3.1.1. Determination of microbiological composition of water kefir

3.1.1.1. *Origin and maintenance of the kefir grain starter*

Kefir grains abstained from laboratory of microbiology of the University of Evry (France), and were propagated at room temperature by daily transfer in mineral water-sugar solution (60 g/l), in glass bottle with semi-tightened plastic cover.

3.1.1.2. *Preparation of media cultures*

Lee medium (Lee et al. 1974) contained: 10 g tryptone/l, 10 g yeast extract/l, 5 g lactose/l, 5 g sucrose/l, 3 g CaCO3/l, 0±5 g K2HPO4 /l, 0±02 g BCP} and 18 g agar}l, pH 7. Acetic acid bacteria medium AAB (Carr and Passmore, 1979) contained: 30 g yeast extract/l, 22 mg BCG/l, 20 g ethanol/l and 20 g agar/l, pH 7. LIA agar Containing (per liter): Pancreatic digest of casein 20.0g, Lactose 10.0g, Agar 10g, Yeast extract 5.0g, NaCl 4.0g, Gelatin 2.5g, Ascorbic acid 2.5g, Sodium acetate 1.5g, pH 7.0 ± 0.1 at 25°C. Differential Broth for Lactic Streptococci containing (per liter): Agar10g, Sodium citrate 20.0g, Arginine 5.0g, digest of casein 5.0g, Yeast extract 5.0g, K2HPO4 1.0g, Bromcresol Purple 0.02g, Skim milk (11% solution) 35.0mL, pH 6.2 ± 0.2 at 25°C. LB Agar containing (per liter): Agar 20.0g, Glucose 20.0g, Beef extract 10.0g, Pancreatic digest of casein 10.0g, Yeast extract 5.0g, K_2HPO_4 2.0g, Tween 80 1.0g, Acetate buffer 80.0mL, Tomato juice, filtered 40.0mL, pH 6.8 ± 0.2 at 25°C. Acetate Agar (BAM M3) containing (per liter): Agar 20.0g, NaCl 5.0g, Sodium acetate 2.0g, KH2PO4. 1.0g, $(NH4)_2PO_4$. 1.0g, $MgSO_4$ 0.2g, Bromthymol blue 0.08g, pH 6.7 ± 0.2 at 25°C. YGC Medium (Yeast Extract Glucose Citrate Medium) (ATCC Medium 216) containing (per liter): Beef extract 10.0g, Glucose 10.0g, Peptone 10.0g, Ammonium citrate 5.0g, Yeast extract 5.0g, Sodium acetate, 2.0g, Tween 80 1.0g, $MgSO_4.7H_2O$ 0.2g, $MnSO_4 \cdot 4H_2O$ 0.05g, pH 6.5 ± 0.2 at 25°C. Acetate Agar (per liter): Meat extract 50.0g, Glucose 10.0g, Peptone 5.0g Yeast extract 5.0g, Sodium acetate buffer100.0mL, Tween™ 80 0.5mL, Sodium acetate 24.0g, Agar 5.0g, pH 5.4 ± 0.2 at 25°C. Hoyer's Medium (per liter): $(NH_4)_2SO_4$ 1.0g, KH_2PO_4 0.9g, $MgSO_4.7H_2O$ 0.25g, K_2HPO_4 0.1g, $FeCl_3.6H_2O$ 0.02g,

Ethanol solution 200.0mL. all medias contents were mixed and heated gently to poiling and then poured into flacons, and autoclaved at 120 °C for 15 min (Ronald, 2004).

3.1.1.3. *Microbial counts*

Serial dilutions of water kefir were in sterile saline solution (0.85 % NaCl, pH 7) Serial dilutions of the suspended samples were used for microbial enumerations by plated on MRS Agar (De Man *et al.*, 1960), M 17Agar, AAB, YGC medium (Carr and Passmore, 1979), and on potato dextrose agar, YGA (Merck, Darmstadt, Germany) with 0.14% added lactic acid (Mossel *et al.*, 1995) for enumeration *lactobacillus*, lactic acid *Lactococcus*, *Acetobacter*, *Leuconostoc* and the yeasts respectively (all the media were purchased from Merck). Plates were incubated anaerobically during three days at 37 °C for the *lactobacilli*, aerobically for two days at 37 °C and five days at 22 °C for the lactic acid *Lactococcus* and the yeasts respectively. Results were expressed as colony forming units per mL (cfu mL^{-1}) of kefir.

3.1.1.4. *Isolation of water kefir microorganisms*

Water kefir was suspended in sterile saline solution (0.85 % NaCl, pH 7) and microorganisms were isolated in several elective and selective media that have been developed for the isolation of *Lactobacillus* species rom mixed populations of lactic acid bacteria and other bacteria in kefir as follows: MRS for lactobacillus spp. Azide Agar (Azide Glucose Agar), M17, MBM for *Lactococcus lactis*, *Streptococcus sp*, *Leuconostoc* SP., YGC Medium (Yeast Extract Glucose Citrate Medium), Acetate Agar for *Leuconostoc* species, *Leuconostoc* species and *Pediococcus species*. (Yeast Extract Glucose Citrate Medium with Cysteine) *Leuconostoc mesenteroides*. Yeast Glucose Agar, Hoyer's Medium, Carr's Ethanol Medium (LMG Medium 228) for *Acetobacter aceti, Acetobacter pasteurianus*. Lee's Agar for *lactococci and Lactobacillus bulgaricus*. Differential Broth for Lactic *Streptococci* for *Lactobacillus lactis*, *Lactobacillus lactis subspecies cremoris*, Tomato Juice Milk Agar, LIA agar + azide for *Enterococcus faecalis*. Lactobacillus bulgaricus Agar (LB Agar) for *Lactobacillus bulgaricus*. Universal Medium (YM), Wort Agar, Malt Agar for Yeasts. *Lactobacilli* strains were grown anaerobically for three days at 37 °C, and *lactococci, Acetobacter, Enterococcus,* and *Leuconostoc,* strains were incubated aerobically for four days at 37 °C, and yeasts

strains were incubated for five days at 22 °C, for the lactic acid. All microorganisms were used immediately for further analysis.

3.1.1.5. *Identification of water kefir microorganisms*

3.1.1.5.1. Morphological analysis

Isolates were examined for colony morphology (Size, Shape, Elevation, consistency, Edge, Color or pigment, thickness, Surface texture, form, structure, opacity emulsifiable) and cell appearance shape (coccus or rod), grouping, arrangement, size, gram stain, endospore, capsule, sides, ends, irregular forms) carried out according to De la maza *et al.*, (1997) and Barrow and Feltham, (1993).

3.1.1.5.2. Physiological Analysis

The physiological analysis such as catalase activity, Oxygen Requirement, Gelatin liquefaction, Milk curdle, motility and production of CO2 from glucose in broth with a Durham tube, Growth at 10, 15 °C and 45 °C, growth at pH 4, 7.2 and 9.6, growth in presence of 6.5%- 8% NaCl, 0.1% MBM, 4%-10% Ethanol and resistance 30 min of heating at 60◦C. The genus and species names of bacterial strains were determined for each organism following the criteria of Bergey's Manual of Systematic Bacteriology (Holt *et al.*, 1994; Bergan and Norris, 1978).

3.1.1.5.3. Biochemical Analysis

Additional biochemical tests and API 50 CH and API 20 tests (bioMerieux) were also used for bacterial strains identification, *Lactococcus* and *Enterococcus* and *Lactobacillus* and *Leuconostoc*.

3.1.1.5.4. Yeast Identification

Yeasts were identified by morphological, physiological criteria and their biochemical characteristics, Colony (Surface, Margin, Colour, Elevation, Size) Cells shapes, size, method of multiplication. And Sugar assimilation(Maltose, Galactose, Sucrose, Lactose, Raffinose), Growth in(Ethanol, Methanol, 10%NaCl, 16%NaCl, 0.01% Cyclohexamide, Citrate) and Sugar fermentation (Raffinose, Maltose, Glucose, Galactose, Sucrose ,Lactose ,Starch) Growth at 30^{o}C, 37^{o}C, and 40^{o}C)and (Deak, 2008; Barnett *et al.*, 2000).

3.1.1.6. *Statistical analysis*

Experimental results recorded were the means ± standard deviation (SD) of triple determinations. The obtained data were subjected to analysis by One-way ANOVA and the differences between means were at the 5% probability level using Duncan's new multiple range tests using SAS software V. 9 eds.(SAS, 2001).

3.1.2. Optimize nonalcoholic water kefir producing

3.1.2.1. *Production of nonalcoholic kefir*

Before the grains were used as starter inoculum. Kefir grains were reactivated five times by inoculated into (60 g/l) mineral water-sugar solution, and incubated at 25°C for 24 h, the grains were separated by using a sieve. 5% (w/v) of active kefir grains were inoculation in sugar solution (6.5% w/v) in mineral water, 5g/l of fresh apple pieces was added, the mixture was placed in glass bottle with plastic cover (not closed tightly), and incubated at (10,15,20,25, and 37°C) for (12,18,24,48,72 and 84h), it was stirred and mixed in intervals of 5h. Apples pieces were deducted and kefir grain were sieved after fermentation period by with plastic siever, and washed with sterile water to be used in other process, and the water kefir drink was stored at 4 °C until being used to evaluate its alcohol contents, microbial and chemical properties.

3.1.2.2. *Measurement of Alcohol*

The spectrophotometric quantification was based on oxidation of ethanol to ethanoic acid by reacting it with an excess of potassium dichromate in acid presence of sulfuric acid and acetate buffer pH 4.3. In which dichromate ($Cr_2O_7^{--}$, Cr(VI)) which is yellowish in color is reduced by ethanol to a bluish green chromic (Cr^{+++}, Cr(III)) product according the following reaction:

$$3C_2H_5OH + 8H_2SO_4 + 2K_2Cr_2O_7 \rightarrow 3CH_3COOH + 11H_2O + 2Cr_2(SO_4)_3 + 2K_2SO_4$$

(Yellow color) → (Green color)

The absorbance of the produced products measured at 560-600 nm (580) nm.

The samples of water kefir that produced in different fermentation conditions were analyzed to determine their contents of alcohol by dichromate spectrophotometric method (Sumbhate *et al.*, 2012). In brief 5 ml of water kefir samples and ethanol standard

solution containing (1.6 mg/mL), added to 5 ml of potassium dichromate solution (40 mg/mL), 5 mL of acetate buffer (pH 4.3) and 25 mL of 1N sulfuric acid was added in 50 mL of volumetric flask. The mixture was shaken gently for 1 min and incubated for 120 min at room temperature resulted in formation of green colored reaction product. The absorbance was read at 578 nm, the concentration of alcohol in water kefir samples was calculated using equation.

$$Ethanol\ concentration\% = \left({}^{Cs}/_{Cwk}\right)\left({}^{Awk}/_{As}\right) \times 100$$

Where Cs = Concentration of standard, Cwk = Concentration of water kefir sample, As = Absorbance of standard, Awk = Absorbance of water kefir sample. This procedure was repeated in triplicates for each samples of water kefir.

3.1.3. Determination of physiochemical properties of water kefir

3.1.3.1. Titratable Acidity and pH determination

Titratable acidity of water kefir was determined using a potentiometer end point method, briefly 10 ml of kefir was transferred to a beaker containing 100 ml of distilled water, a magnetic stirrer used to expel the carbon dioxide when the contents were heated to 45 °C, cooled to 20 °C, and titrated with 0.1 N NaOH to pH 8.2 (Nieisen, 1998). The acidity was expressed as percent of lactic acid.

The pH of the water kefir was determined at 25 °C using a pH meter with a combined glass electrode and temperature probe (Radiometer, Copenhagen, Denmark) was used. The pH meter was calibrated using standard buffer solutions at pH 4.0 and 7.0 (Merck, Darmstadt, Germany). Each analysis carried out in triplicate.

3.1.3.2. Determination of nutritional composition of water kefir

Moisture content of water kefir was determined by drying at 100 °C to constant weight. Protein contents of water kefir were determined by kjeldahl method (Owusu-Apenten, 2002). The total fat in the kefir was measured using Rose-Gottlieb method according to IDF Standard 1D (IDF, 1996). And carbohydrates were determined by a phenol-sulfuric acid colorimetric assay (Dubois Michel *et al.*, 1951) with glucose as standard. Dry matter (DM) was determined according to IDF Standard No. 4 (IDF, 1986). Ash content was

determined by heating in a muffle furnace for about3–5 h at 600°C to constant weight (Harbers, 1998). Each chemical measurement was made in triplicate.

3.1.3.3. Determination of trace minerals

Trace minerals content of water kefir and kefir grains were measuring by Atomic Absorption Spectrometry method, CO_2 was removed from water kefir samples by transferring the sample to a large flask and shaking, and then by pouring back and forth between large containers. And abnormal suspended matter or sediment present were removed by filtration, the samples were homogenized, And 2.0000 g of the homogenized samples of water kefir and kefir grains were weighed into a porcelain crucibles and place in a cold muffle furnace with the chimney vent open, and allowed to heat up to 450-550°C. the vent then closed and maintained at this temperature overnight. Crucibles were removed from the furnace and allowed to cool, then 15 drops of HCl added from a polythene Pasteur pipette, all samples were moisten. HCl then was gently evaporated off using a fume cupboard at moderate heat, then the crucibles removed and cooled. After that the residue dissolved in 0.1 M HCl, and transferred quantitatively to a 10-ml volumetric flask. Elements standards solutions made up in 0.1 M HCl covering the expected ranges in the sample solutions and analyzed by AAS (spectrophotomètre d'absorptim atomique. AI 1200. Aurora. Canada) according to the instrument manufacturer's instructions.

The concentration of trace minerals in the samples calculated as follows, the sample solution is of 2 g in 10 ml, therefore the concentrations in µg ml–1 of the trace element should be multiplied by 5 to give the concentration in µg g–1 of the trace element in the sample (Faithfull, 2002). All samples were examined in triplicate.

3.1.4. Determination of Antioxidant Activity of water kefir

3.1.4.1. Materials

Kefir grains abstained from laboratory of microbiology of the university of Evry (France), Table sugar and apples fruit were purchased from the local market of Telemcen city (Algeria). 2,2,-diphenyl-1-pricrylhydrozyl (DPPH), butylated hydroxyanisole (BHA), ascorbic acid , Sodium phosphate buffer, potassium ferricyanide, trichloroacetic acid (TCA), ferric chloride, were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Hydrogen peroxide (H2O2 30%) was obtained from Mallinckrodt Baker (Phillipsburg, NJ, USA). HPLC grade methanol and ethanol were purchased from Fisher Scientific (Fair Lawn, NJ, USA). All reagents were of analytical grade.

3.1.4.2. Preparation of water kefir

Water kefir prepared by inoculation sugar solution (6.5% w/v) in mineral drinking water with 5% (w/v) of water kefir grains, 5g/l of fresh apple pieces was added, the mixture was placed in glass bottle with plastic cover (not closed completely), incubated at 21 °C for 24 h, and the glass bottles was stirred and mixed in intervals of 5h. in th end of incubation period, apples pieces were deducted and kefir grain were sieving by with plastic siever, and washed with sterile mineral water to be used in other process, and the water kefir drink was stored at 4 °C until being used to evaluate its antioxidant activity.

3.1.4.3. Preparation of water kefir sample

Samples of water kefir were then filtered with Whatman No. 1 filter paper, The resulting supernatants were then dried under reduced pressure by rotary evaporator At 45 °C, The dry matter then collected ,weighted, dissolved in distilled water, and stored at 4 °C until their analysis.

3.1.4.4. Determination of DPPH radical-scavenging activity

The antioxidant activity of the water kefir samples was measured in terms of hydrogen donating or radical scavenging ability, using the stable radical, DPPH (Brand-Williams *et al.*, 1995). A methanolic solution (50 µl) of the water kefir samples at various concentrations (0.125–5 mg/ml) was placed in a cuvette, and 2 ml of 6×10-5 M methanolic solution of DPPH was added, the mixture was incubated in the dark for 30 min at ambient temperature. After that the decrease in absorbance at 517 nm was determined by spectrophotometer for all samples. Methanol and samples was used to zero the spectrophotometer. And Ascorbic acid and BHA were used as positive reference standards. The absorbance of the DPPH radical without kefir samples used as the control. The inhibitory percentage of DPPH by the kefir samples was calculated according to the following equation:

$$\boldsymbol{Scavenging\ effect}(\%) = \frac{\boldsymbol{Ac - As}}{\boldsymbol{Ac}} \times \mathbf{100}\%$$

where Ac is the absorbance of the DPPH solution and As is the absorbance of the DPPH solution after the addition of samples. All tests were operated in triplicate.

3.1.4.5. Measurement of inhibition of ascorbate autoxidation

Inhibition of ascorbate autoxidation was measured by the method described by (Mishra and Kovachich, 1984). A 0.1 ml of water kefir samples (0.125–5 mg/ml) was added to 0.1 ml of ascorbate solution (5.0 mM) and 9.8 m phosphate buffer (0.2 M, pH 7.0). The mixture was incubated at 37°C for 10 min and then the absorbance was measured at 265 nm. A 0.1 ml of distilled water used as control. And BHA was used as positive reference standard. All tests were operated in triplicate and the ascorbate autoxidation inhibition rate of kefir samples were calculated as follows:

$$\boldsymbol{Inhibition\ effect}\ (\%): = \left[\frac{\boldsymbol{Absorbance}_{(sample)}}{\boldsymbol{Absorbance}_{(control)}} - \mathbf{1}\right] \times \mathbf{100}\%$$

3.1.4.6. Measurement of reducing power activity

The reducing power of water kefir samples was determined according to the method of Oyaizu, (1986).Various concentration of kefir samples (2.5 ml) (0.125–5 mg/ml) were mixed with an equal volume of sodium phosphate buffer (200 mM; pH 7) and 1% potassium ferricyanide solution. The mixture was incubated at 50°C for 20 min. After that, an equal volume of 1% trichloroacetic acid was added to the mixture, which was then centrifuged at 5000 rpm for 10 min. The upper layer (5 ml) was diluted with 5 ml of distilled water and 1 ml of a 0.1% ferric chloride solution, and then the absorbance of the solution was measured at 700 nm. Ascorbic acid was used as positive reference standard. Increased absorbance of the reaction mixture indicates greater reducing power of the kefir sample. All samples were assayed in triplicate.

3.1.4.7. Determination of Scavenging effect of water kefir on hydroxyl radicals.

Hydroxyl radical-scavenging activity (HRSA) of water kefir extract was measured according to the salicylic method with some modifications that described by Xiang et al., (2012). In brief the reaction mixtures in a final volume of 4 ml contained, 1 ml H_2O_2 (8.8 mM/l), 1 ml $FeSO_4$ (9 mM/l), 1 ml salicylic (9 mM/l), and 1 ml of water kefir sample solution in a concentration gradient. The H2O2 was added to the mixture and the reaction

was started up at last. The reaction mixture was incubated at 37 °C for 60 min and then centrifuged at 6,000 g for 15 min. The absorbance (A) of the reaction solutions at 510 nm was measured. The hydroxyl radical-scavenging ratio was calculated by the following formula:

$$\boldsymbol{Scavenging\ rate}\ (\%) = \{\boldsymbol{A0} - (\boldsymbol{Ax} - \boldsymbol{Ax0})\} \times \frac{\boldsymbol{100}}{\boldsymbol{A0}}$$

A control contained all the reaction reagents except the samples was prepared and measured as A0, Ax was the result of samples and Ax0 is the absorbance for background, i.e., the reaction mixture without H2O2. The procedures carried out in triplicates of tow experiment.

3.1.4.8. Determination of total antioxidant capacity

The total antioxidant capacity (TAOC) of fresh water kefir and its extract at different concentrations (0.125, 0.625, 1.25, 1.875, 2.5, 3.7. and 5.00 mg/ml) was evaluated by the method of ammonium molybdate that described by Miladi Sonia and Damak Mohamed (2008). An aliquot of 0.1 ml of sample solution (1 mg/ml) was combined with 1 ml of reagent solution (600 mM sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated in a boiling water bath at 95°C for 90 min. After the samples had cooled to room temperature (25 □), the absorbance of the aqueous solution of each was measured at 695 nm against a blank. A typical blank solution contained 1 ml of reagent solution and the appropriate volume of the same solvent used for the sample and it was incubated under the same conditions. A standard curve of α-tocopherol was made by measuring the absorbance of several concentration of α-tocopherol that processed by the same procedures for the samples. The antioxidant capacity was calculated by the standard curve of α-tocopherol, and it was expressed as the number of equivalents of α-tocopherol (µg/g of extract). The procedures carried out in triplicates of tow experiment.

3.1.4.9. Statistical analysis

Experimental results recorded were the means ± standard deviation (SD) of triple determinations. The obtained data were subjected to analysis by One-way ANOVA and the differences between means were at the 5% probability level using Duncan's new multiple range tests using SAS software V.9 eds. (SAS, 2001).

3.2. In vivo Experiments

3.2.1. Evaluation of Anti-hyperglycemic and anti-hyperlipidemic activities of Water kefir as probiotic on Streptozotocin-induced diabetic Wistar Rats

3.2.1.1. *Preparation of Water Kefir dilutions for rats treatment*

Water kefir grains that obtained from laboratory of YALACTA (France), were washed with distilled water, and inoculated (5% (w/v)) in (6.5% w/v) sugar solution with mineral drinking water. 5g/l of fresh apple pieces (purchased from the local market of Tlemcen city-Algeria) were added. The mixture was placed in glass bottle with plastic cover (not closed completely), and incubated at 21°C for 24 h. It was stirred and mixed in intervals of 5h. Apples pieces were deducted and kefir grains were sieving after fermentation period by filtration through a plastic sieve and washed to be used in other process. Water kefir drink was taken to consumption by experimental rats, and different dilutions were prepared with drinking water (10%, 20%, and 30% of water kefir) according to experiments design, water kefir dilutions were used in the same day.

3.2.1.2. *Animals and diets*

All aspects of the experiment were conducted according to the guidelines provided by the ethical committee of the experimental animal care at Tlemcen University. Adult Wistar rats, weighing 200 to 250g, were obtained from Pasteur institute (Algeria) and were housed at 20 °C individually in polypropylene cages and maintained on a 12:12-h light/dark cycle in the animal husbandry, Department of Biology, Faculty of life and Natural Sciences, ABBT University, ALGERIA. They were fed a commercial chow manufactured by the O.N.A.B (Food National office of Cattle, Remchi, Tlemcen). ONAB diet is composed of corn oil, cakes soya bean, a complex minerals and vitamins. The rats were allowed to free access to drinking water *ad libitum*. Before the study, rats were fed with AIN-93Standard diet (REEVES *et al.*, 1993) as presented in Table (1-3) for two weeks in order to adapt to diet.

Table 3.2-1 Composition of the Standard diet

Ingredients	**g/kg Diet**	Energy values
Casein (> 90 % protein)	200	
Corn starch	400	
Sucrose	232	
Lipid	70	
Cellulose	50	
Mineral mix (AIN-93G-MX]	35	
Vitamin mix (AIN-93-VX)	10	
L-Cystine	3	

Table 3.2-2 Mineral mix (AIN-93G-MX) that supplies the recommended concentrations of elements for the AIN-93G diet

Ingredient	**g/kg mix**
Essential mineral element	
Calcium carbonate, anhydrous, 40.04% Ca	357.00
Potassium phosphate, monobasic, 22.76% P; 28.73% K[1]	196.00
Potassium citrate, tri-potassium, monohydrate, 36.16% K	70.78
Sodium chloride, 39.34% Na, 60.66% Cl	74.00
Potassium sulfate, 44.87% K; 18.39% S	46.60
Magnesium oxide, 60.32% Mg	24.00
Ferrie citrate, 16.5% Fe	6.06
Zinc carbonate, 52.14% Zn	1.65
Manganous carbonate, 47.79% Mn	0.63
Cupric carbonate, 57.47% Cu	0.30
Potassium iodate, 59.3%	I 0.01
Sodium selenate, anhydrous, 41.79% Se	0.01025
Ammonium paramolybdate, 4 hydrate, 54.34% Mo	0.00795
Potentially beneficial mineral element	
Sodium meta-silicate, 9 hydrate, 9.88% Si	1.45
Chromium potassium sulfate, 12 hydrate, 10.42% Cr	0.275
Lithium chloride, 16.38% Li	0.0174
Boric acid, 17.5% B	0.0815
Sodium fluoride, 45.24% F	0.0635
Nickel carbonate, 45% Ni	0.0318
Ammonium vanadate, 43.55% V	0.0066
Powdered sucrose	221.026

[1]This amount of potassium phosphate supplies only 1561mg P/kg diet. The remainder (1440 mg) comes from casein, which contains an average of 0.72% P. The recommended amount of total phosphorus in the diet is 3000 mg/kg.

Table 3.2-3 Contribution of vitamins to AIN-93G and AIN-93M diets when the recommended vitamin mix AJN-93-VX is fed at 10 g/kg of the diet

Vitamin	***U/kg diet***
Nicotinic acid, mg	30
Pantothenate, mg	**15**
Pyridoxine, mg	6
Thiamin, mg	5
Riboflavin, mg	6
Folie acid, mg	2
Vitamin K, µg	750
D-Biotin, µg	200
Vitamin B-12, µg	25
Vitamin A, IU	4000
Vitamin 03, IU	1000
Vitamin E, IU	75

3.2.1.3. *Induction of Diabetes*

STZ was dissolved in cold 0.01 M citrate buffer, pH 4.5 and always prepared freshly for immediate use within 5 minutes. Rats were fasted for overnight to induce diabetes by intraperitoneal (ip) injection of streptozotocin (STZ) at the dose of 60 mg/kg body weight. The normal control group was given citrate buffer without STZ. Animals were allowed to drink 5% glucose solution overnight to overcome the drug-induced hypoglycemia. The development of diabetes was confirmed after 48 hours of STZ injection. The animals with fasting blood glucose level more than 200 mg/dl were considered as diabetic and included in this study. Body weight and glycemia were measured weakly. For glucose assay, blood samples were collected from the tail-tip of the rats.

3.2.1.4. *Experiment Design and Treatment*

After one week of diabetes induction, rats were divided into four groups. Each group consisted of six rats. All groups feed with standard diet a long of experiment period and received water kefir with drinking water as follows (Figure 3-1):

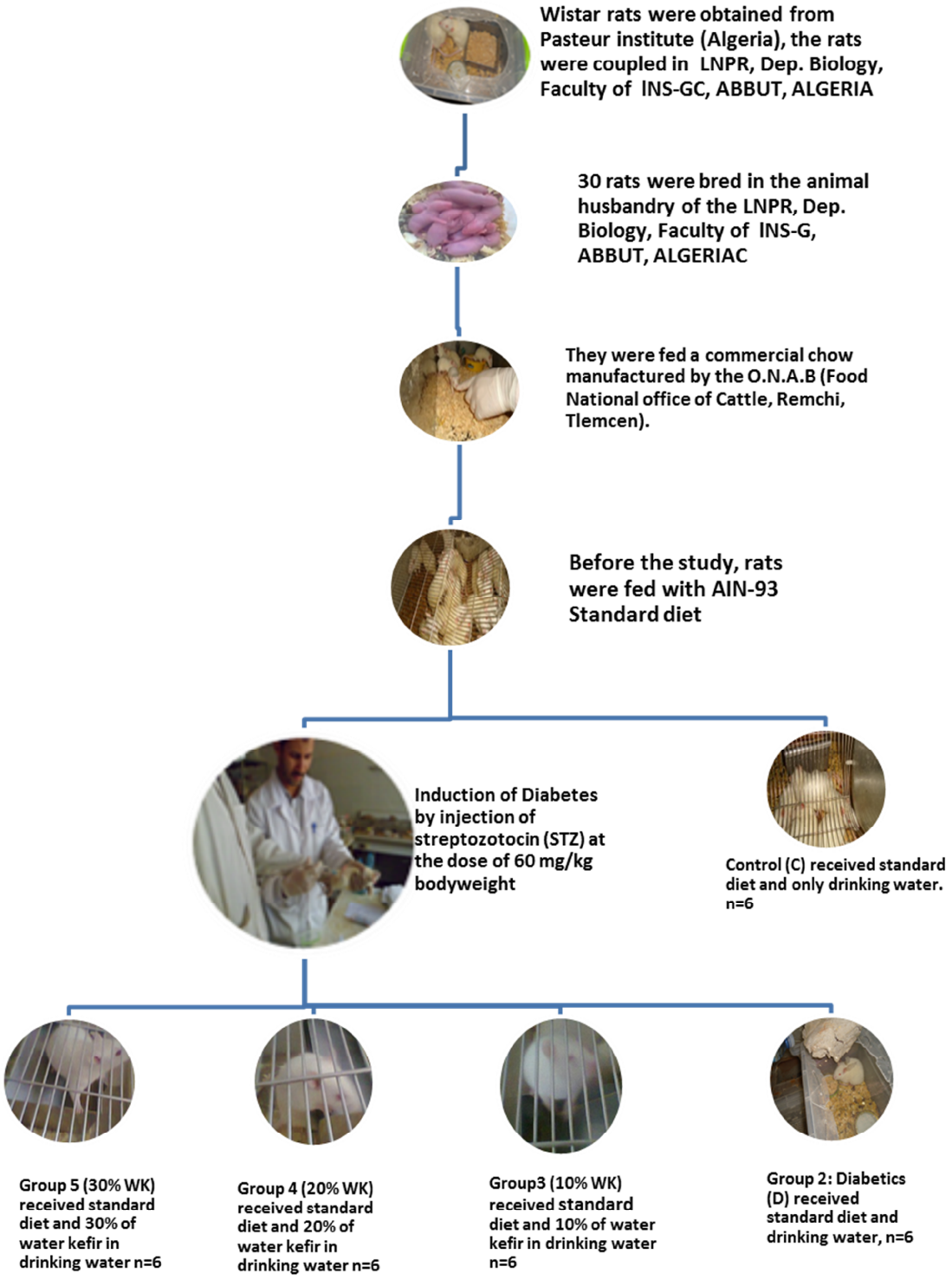


Figure 3-1 Animals and experimental design

Group1 serve as Normal Control (NC) received standard diet and only drinking water. Group 2: Diabetics (D) received standard diet and drinking water, Group 3- 5 (10% WK;

20% WK and 30% WK) received standard diet and 10%, 20%, and 30% of water kefir in drinking water respectively. The period of treatment was 35 days. Animals allowed to access to food and water or water kefir ad libitum.

At the end of the experiment and after overnight fasting, rats from each group were anaesthetized with intraperitoneal injection of sodium pentobarbital (60 mg/kg of body weight). The blood was drawn from the abdominal aorta, and serum was used for glucose and lipid profiles determinations.

3.2.1.5. *Analytical measurements*

Blood glucose levels were measured by the glucose-oxidase method using an Accu-chek blood glucose meter. Total cholesterol (TC), Triglyceride (TG), high-density lipoprotein (HDL)-cholesterol levels were measured in serum samples by using enzymatic method kits (Roche Diagnostics). The lipoprotein cholesterol sub-fractions in the serum, HDL, LDL, VLDL were estimated by precipitation with sodium phosphotungstate-magnesium chloride and sodium dodecyle sulphate reagents.

3.2.1.6. *Statistical analysis*

Statistical analysis of data was performed using SAS software version 9.1 (SAS, 2001). Significance of differences were carried out by the analysis of variance (ANOVA) general linear models-Univariate method followed by Post Hoc Multiple Tukey and LSD Tests at $p < 0.05$. Results are represented as mean with standard errors of mean (mean ± SEM).

3.2.2. Determination of the effects of water kefir in oxidative stress of streptozotosin induced diabetic rats

3.2.2.1. *Preparation of blood samples (Plasma and Lysate)*

Blood samples, were taken following an overnight fasting period for determining the parameters of oxidative stress were collected in tubes containing ethylenediamine-tetraacetic acid (EDTA), centrifuged for 15 min at 3000 rpm, and plasma was carefully separated.

Plasma is taken for the proportioning of the parameters of the oxidant /antioxydant statute (the rates vitamin C, Malondialdehyde, hydroperoxides and proteins carbonyl).

The erythrocyte pellets were washed three times with physiological water, and 0.5 ml of the cell suspension was diluted with 2 ml cold water to lyse the erythrocytes for 15 min in the ice. To 0.2 ml lysate 1.8 ml water and ethanol/chloroform (3:5/v:v) were then added to precipitate haemoglobin. The cellular remains are eliminated by centrifugation with 4000 rpm during 15 min.

The supernatant was used for determination of antioxidant enzyme of erythrocyte (Catalase).

3.2.2.2. *Malondialdehyde (lipid peroxidation) determination*

Lipid peroxidation in the plasma was evaluated by slightly modified spectrophotometric method based on the reaction between MDA and thiobarbituric acid (TBA) (Nourooz-Zadeh *et al.*, 1994; Ohkawam H *et al.*, 1979; Wasowicz *et al.*, 1993). Briefly, 100 µL of 0.8% thiobarbituric acid in water (w/v) and 500 µL of 20% trichloracetic acid (w/v) were added to 100 µL of the samples, and the mixture was incubated in a water bath at 100°C for 1 hour. The samples were then cooled and 25 µL of 5 mol/L HC1 was added (final pH 1.6-1.7), then samples were washed once with 4.0 mL of n-butanol . The n-butanol layer was separated by centrifugation (6000 rpm for 20 minutes). The amount of pink pigment reflecting the amount of thiobarbituric acid reactive substance was measured spectrophotometrically at a wavelength 532 nm with molar extinction coefficient $\varepsilon 532=1.56\times105\ (mol/L)^{-1} \bullet cm^{-1}$. The level of LP is expressed as µmol MDA /mL of plasma. The test carried out in triplicates for all samples.

3.2.2.3. *Determination of protein carbonyl level*

Determination of carbonyl level in proteins is used as an index of the extent of protein oxidative damage. Determination of protein carbonyl level was carried out based on the spectrophotometric detection of the reaction between 2,4-dinitrophenyl hydrazine (DNPH) with Protein Carbonyl to form protein hydrazone as previously described (Levine *et al.*, 1990) with minor modification. Briefly, one ml of haemolysate was placed in one glass tube and 3 ml of 8 mM 2, 4- dinitrophenylhydrazine (DNPH) in 3 M HCL was added. The tubes were incubated for 50 min at 37 °C in the dark and vortexed every 10 min. Then, 3 ml of 25% (w/v) trichloroacetic acid was added and the tube was left on ice for 8 min and then centrifuged at 3000 rpm for 10 min in a tabletop centrifuge

to collect the protein precipitates. This pellet was washed 3 times using 3ml 15% trichloroacetic acid. Next, the pellet was washed 3 times with 4 ml of ethanol-ethyl acetate (1:1), (v/v). The final precipitate was dissolved in 2 ml 5M sodium hydroxide solution and was incubated for 15 min at 37°C with mixing. Any insoluble materials were removed by repeated centrifugation. Carbonyl level was calculated from the peak absorbance of the spectra at 355-390 nm, using an absorption coefficient of (21,5 (mmol/l). cm -1) or 22000 M-1 cm-1. Results were expressed as mmol/ml of plasma. The test carried out in triplicates for all samples.

3.2.2.4. *Measurement of hydroperoxides*

Total plasma hydroperoxide concentrations were measured using the FOX2 assay essentially as described by (Nourooz-Zadeh *et al.*, 1994) with minor modifications. The FOX2 reagent was prepared by dissolving 38 mg of XO and 440 mg of BHT in 450 mL HPLC-grade methanol with stirring. Ammonium ferrous sulphate (49 mg) was quickly dissolved in 50 mL of 250 mmol/L sulphuric acid and was added to the methanol solution. The final FOX2 reagent comprised of 100 µmol/L XO, 4 mmol/L BHT, 25 mmol/L sulphuric acid and 250 µmol/L ammonium ferrous sulphate in methanol (90 %, v/v). The extinction coefficient of the FOX2 reagent at 560 nm used was 3.86 × 104 M-1cm-1. For each plasma sample, 900 µL aliquots (quadruplicates) of plasma were incubated (30 minutes, 20-25°C) in 10 µL methanol to generate a test sample. Another incubation (30 minutes, 20-25°C) with the FOX2 reagent was performed. After centrifugation at 2400 rpm for 10 min the absorbance of the supernatants was determined at 560 nm in an spectrophotometer. The hydroperoxide concentration of each sample was calculated from the difference of the absorbance of the blank and test samples.

3.2.2.5. *Estimation of Catalase*

The activity of catalase was assayed by the method of Sinha, (1972). Dichromate in acetic acid was reduced to chromic acetate, when heated in presence of hydrogen peroxide with the formation of per chromic acid as an unstable intermediate. The chromic acetate formed was measured at 590 nm. Catalase was allowed to split H_2O_2 for different periods of time. The reaction was stopped at different time intervals by the addition of dichromate acetic acid mixture and the remaining H_2O_2 was determined by measuring chromic acetate spectrophotometrically after heating the reaction mixture. In brief, to 0.9

ml of phosphate buffer (0.01 M, pH 7), 0.1 ml of hemolysate samples and 0.4 ml of H_2O_2 (0.2 M) were added. The reaction was stopped after 15, 30, 45 and 60 seconds by adding 2 ml of dichromate acetic acid reagent (Potassium dichromate 5% in water and glacial acetic acid was mixed in the ratio 1:3. From this 1 ml was diluted again with 4 ml of acetic acid). The tubes were kept in a boiling water bath for 10 minutes, and then cooled. The color developed was read at 530 nm. Standards in the concentration range of 20-100 µmoles was processed for the test.

The activity of catalase was expressed as U/ml for plasma (U- µmoles of H_2O_2 Utilised / second). The test carried out in triplicates for all samples.

3.2.2.6. *Measurement of vitamin C.*

The concentration of vitamin C in the serum was estimated by the phenyl-hydrazine spectrophotometry method (Lowry *et al.*, 1945). For the measurement procedure, 0.3 ml of plasma was added to 1.2 ml of 5% trichloroacetic acid and then centrifuged at 3000 rpm for 10 min. Clear supernatant of 0.96 ml was treated with 0.4 ml of dinitrophenylhydrazine-thiourea-copper sulphate (DTC) reagent (3g of 2,4 dinitrophenyl hydrazine, 0.4 g thiourea and 0.05 g of copper sulphate were dissolved in 100 ml of 9 N Sulphuric acid) and heated at 60°C for 1 hr in a water bath. Immediately after incubation, the sample was chilled in ice-cold water and 1.6 ml of 65% sulphuric acid solution was added gradually. The procedure was repeated with 0.3 ml of working standard solution of ascorbic acid as well as with 0.3 ml of reagent blank. Absorbance of sample and standard were read against reagent blank at 520 nm with a spectrophotometer. The level of vitamin C was expressed as mg/dl of plasma.

4. RESULTS

4.1. In vitro results

4.1.1. Microbial composition of water kefir

4.1.1.1. *Microbial counts of water kefir*

Count of microbial population on different culture media from water kefir in cfu mL-1 is shown in Table 4.1. Lactic acid bacteria in Nutrient Agar gave the major viable count (2.19×109 cfu mL-1), and *lactococcus* in M17 was the second with asymptotic number (1.38×109 cfu mL-1), while lactobacillus in MRS came 3rd (5.28×108 cfu mL-1), and *Acetobacter* in AAB (2.50×107 cfu mL-1), and *Leuconostoc* in YGC (2.34×107 cfu mL-1), while the yeasts were the fewer population in water kefir (1.29×107 and 7.85×106 cfu mL-1) in YGA and PDA respectively.

Table 4-1 Viable cell counts (cfu mL-1) of water kefir on different media.

Media	Microbial groups	Viable count (cfu mL^{-1})	
		Mean	S.D
Nutrient Agar	*Lactic acid bacteria*	2.19×10^{9}	±0.13
MRS	*lactobacillus*	5.28×10^{8}	±1.06
M17	*lactococcus*	1.38×10^{9}	±1.44
AAB	*Acetobacter*	2.50×10^{7}	±0.35
YGC	*Leuconostoc*	2.34×10^{7}	±2.52
YGA	*yeasts*	1.29×10^{7}	±3.82
PDA	*yeasts*	7.85×10^{6}	±5.19

Data are the main values ± standard deviation

4.1.1.2. *Microbial composition of water kefir*

In the first stage of identification process of water kefir microorganisms, 156 colony were selected to present all colonies categories from the various mediums (Nutrient Agar, MRS ,M17,AAB,YGC,YGA, and PDA) which used in enumeration step. According colonies morphology characteristics, these colonies classified to13 similar colonies categories. All colonies were streaked in the corresponding selecting medias culture for purification. The types of microorganisms which purified and identified in water kefir are summarized in table 4.2.

Table 4-2 The types of microorganisms of water kefir

Microorganisms category	Number of strains	Microorganisms Types
Cocci strains (g+)	2	*Enterococcus* *Pediococcus*
Coccobacilli strains (1 g - and 2 g+)	3	*Leuconostoc* *Acetobacter* *Lactococcus*
Bacilli strains (g+)	6	*L. kefiranofaciens* *L.kefirgranum* *L. bulgaricus* *L. parakefir* *L. casi* *L.kefr*
Yeast strains	2	*Candida* *Saccharomyces*

The colonies then were stained with gram stain and taken to microscopic tests, three types of bacteria and two types of yeast were distinguished according to their cellular properties and gram staining, the mixture of all types of bacteria and yeasts showed in figure 4.1

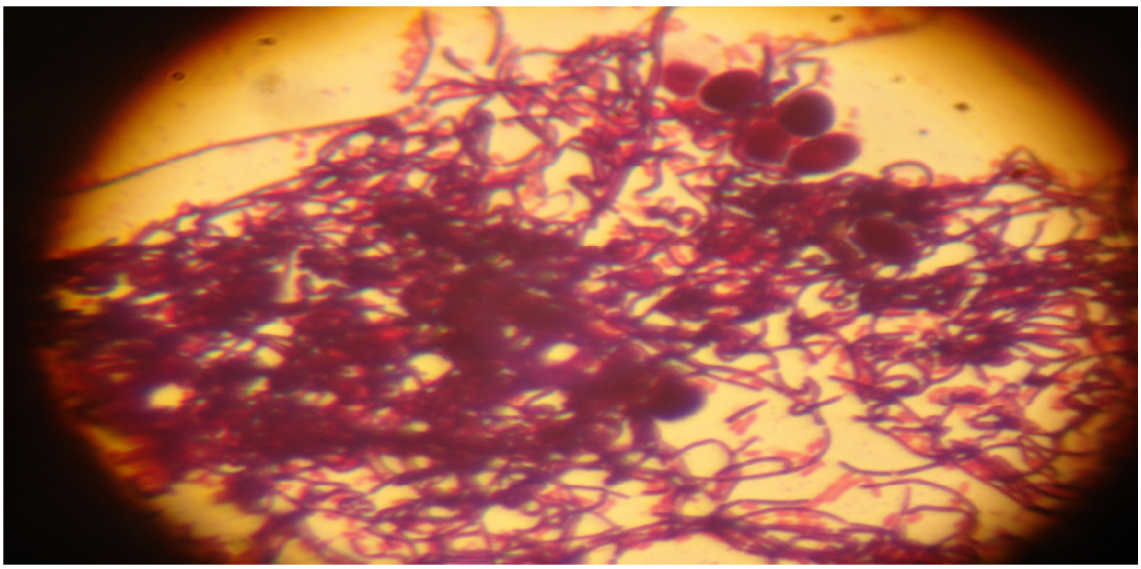


Figure 4.1 Microorganisms Mixture of Water Kefir

The three categories of microorganisms that found in water kefir are cocci (spherical), bacilli (rod), and coccobacilli (ovoid), both of cocci and bacilli groups were only gram

positive while coccobacilli group were contained the both of positive and negative gram staining bacteria.

4.1.1.2.1. Cocci (spherical) bacteria

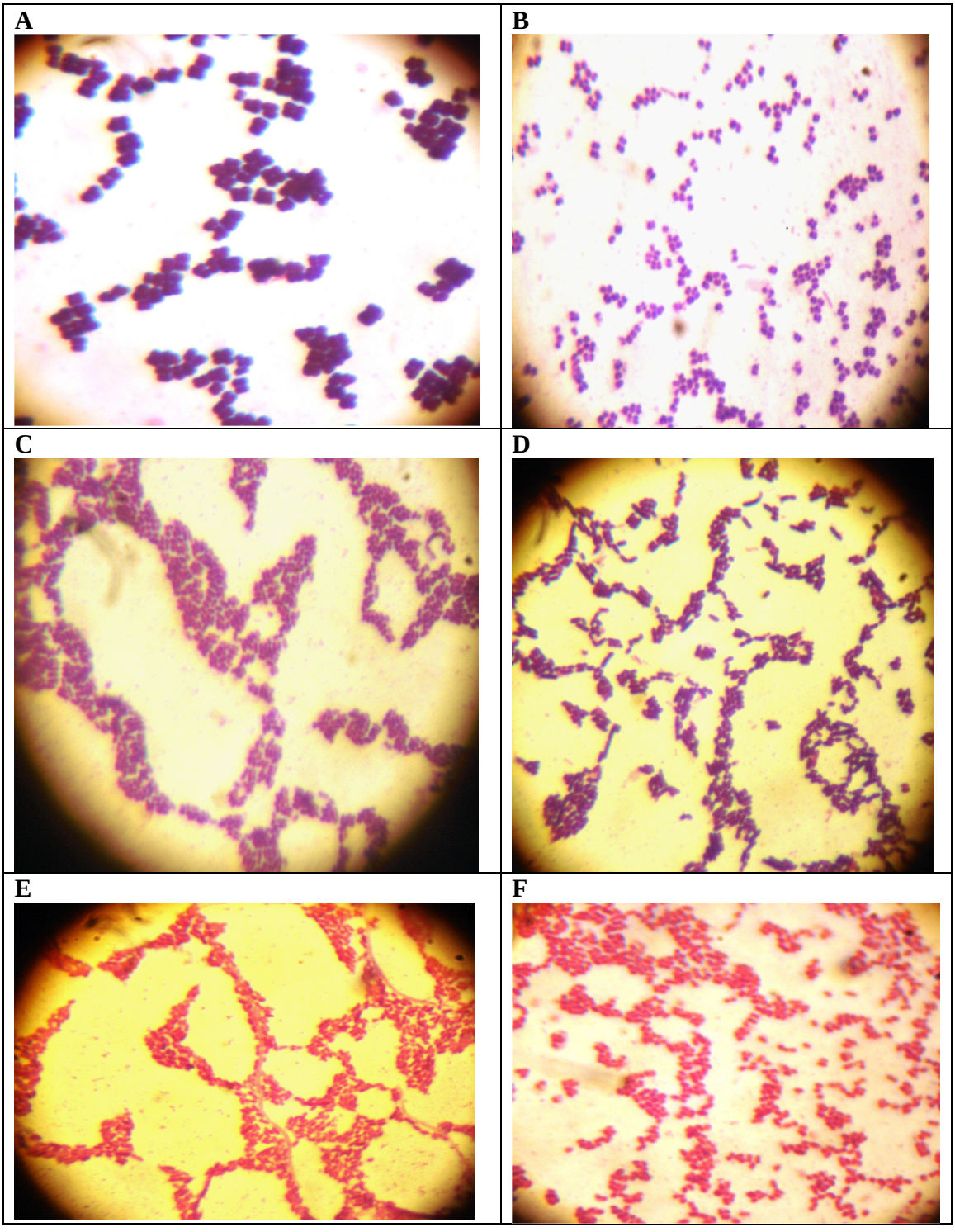
The first type of bacteria (5 isolates) according the results were, grown in M17,MRS, Acetate, Agar, with Small colonies (2 to 3 mm), spherical, raised, edges, white iridescent, grayish thin in color, moist smooth, while its cells were cocci, relatively large, occurred singly, pairs, and tetrad, non-capsulated, gram-positive, catalase negative, not grown at 4°C but it can grew weakly at 10 °C and grew well at 45 °C, it can't resisted for 30 min at 60◦C, and weren't grew in 6. 5% - 8% NaCl and 0.1% MBM, it were grew in pH 4, and pH 7.2, but can't grew in, pH9.6, and not grew in the presence of 4%-10% ethanol. It were non motile , nonspore forming, facultative in oxygen requirement, arginine positive, can't produce gas from glucose, non-gelatin liquefied, made milk curdled, and non-reducing AAB to Yellow. Based on this results and by comparing it with the Bergey's manual of systematic bacteriology and other previous studies this bacteria can be described as *Pediococus spp*. (Figure 4.2.A,B).

The results revealed other type of bacteria (3 isolates) which were, had colony morphology from agar plates as follows, medium sized, spherical, convex, entire , pale trans, smooth, bright orange on nutrient agar, and white in Lee's and Azide Agar, its cells were Cocci ovoid, in size (0.5 - 1.5 µm), pair, short chains, non-capsulated, non-spore forming and non-motile, it were gram-positive, catalase negative, not grown at 4°C and 45 °C, but it can grew at 10 °C, can't resisted for 30 min at 60◦C, it weren't grew in 6. 5% - 8% NaCl and 0.1% MBM, it were grew in pH 7.2, but can't grew in pH 4, pH9.6, and 4%-10% ethanol. It were facultative in oxygen requirement, arginine negative, gas produced from glucose, gelatin liquefied, made milk curdled, and non-reducing AAB to Yellow. by referring to the Bergey's Manual of Systematic Bacteriology and other previous studies we can describe this type of bacteria as *Lactococcus lactis* (Figure 4.2.C,D).

The third bacterial strain (3Isolates) that determined the results were, isolated in leuconostoc Acetate, Yeast Extract Glucose Citrate Medium with Cysteine, and Azide Agar, its size were 4-7.5 mm, its colonies were Circular, Convex, entire, marginated, flat,

Pale Yellow in color, dark pink ,shiny, semitrans, and slimy Smooth. The cells were observed to be coccobacilli shaped, medium sized, Single, pairs and short chains , and aggregated sometimes (Figure 4.2.G,H), non-capsulated, bacterial showed gram strain - positive, catalase negative, their growth was occurred at 4°C relatively weak, well grown at 10 °C, whereas no growth were seen at 45 °C, it can't resisted for 30 min at 60◦C, one of the strains (4.2.G) showed growth the presence of 6. 5% NaCl , and both of strains weren't grow in 8% NaCl and no growth in 0.1% MBM, it were grew in pH 7.2, but can't grew in pH 4 and pH9.6, and not grew in the presence of 4%-10% ethanol. Bacteria were motile, non-spore forming, facultative in oxygen requirement, arginine positive, produced gas from glucose, non-gelatin liquefied, made milk curdled, and can reduce AAB to Yellow. Based on this results and by comparing it with the Bergey's manual of systematic bacteriology and other previous studies this bacteria can be described to be *Leuconostoc spp.(mesenteroids, cremoris) and Leuconostoc mesenteroides.*

Other strain of bacteria (5 Isolates) were isolated in MBM, and LIA, and Tomato Juice Milk Agar medium, the colonies were small, Spherical, raised, irregular, white pale, yellowish , smooth. the cells chowed to take cocci (spherical) medium size, occurred in pairs and short chains, non-capsulated, they are aerobic, facultatively anaerobic, non-sporing, non-motile, catalase negative and produce gas from glucose, one of these strains showed growth at 4, 10 and 45 °C the other were only grew in 10 °C, both of strains were resistance for 30 min at 60 °C, and also the both can grow in the presence of 6.5% NaCl, 8% NaCl and 0.1% MBM, and at pH 7.2, 9.6, whereas no growth seen at pH 4, and in presence of 4%-10% Ethanol, it were arginine positive, non-gelatin liquefied, milk curdled, and non-reducing AAB to Yellow. These results conduce to *Enterococcus Sp* (Figure 4.2.I,J).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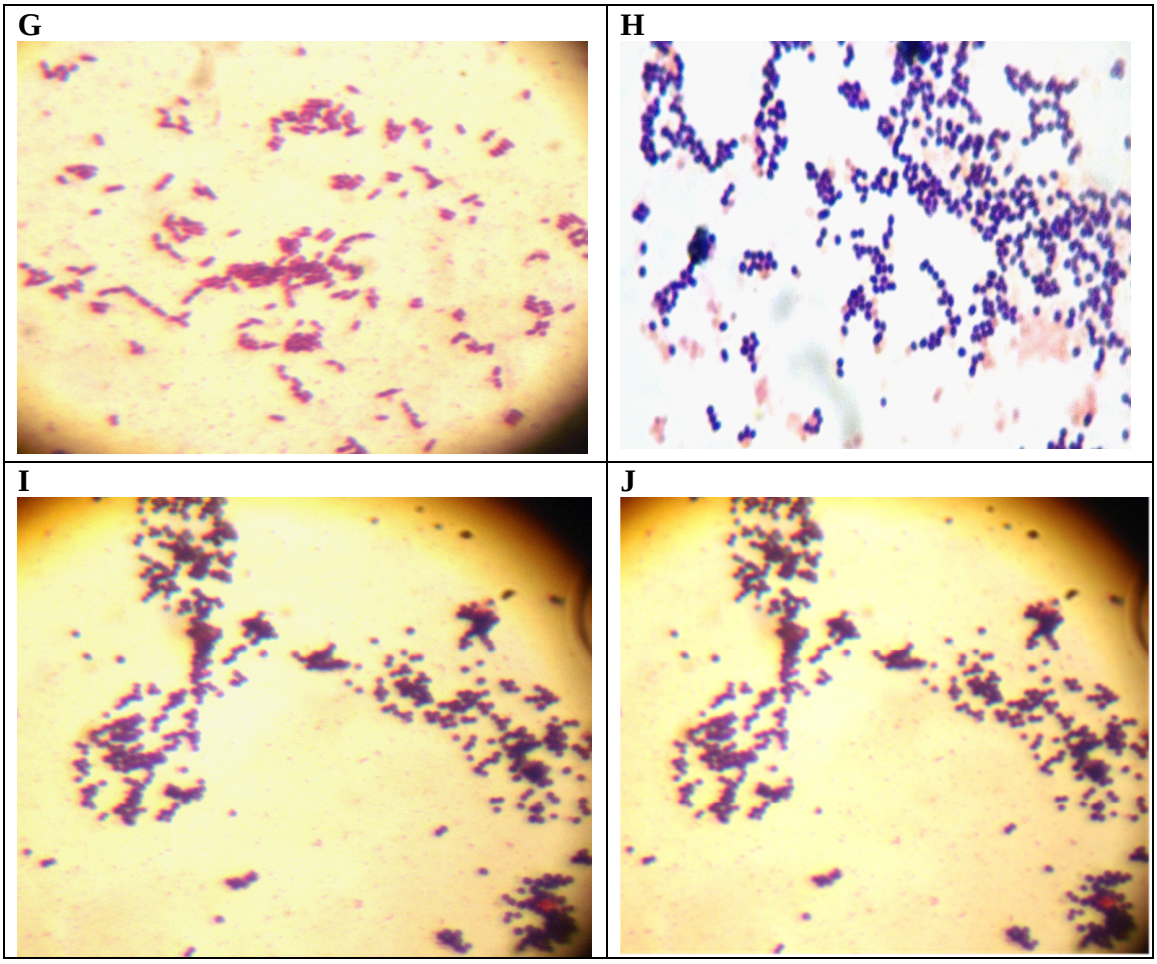


Figure 4.2 . The morphological characteristics of cocci bacteria in water kefir *A.Pediococus spp*. In M17, **B.** *Pediococus* spp. In Acetate Agar, **C.** *Lactococcus lactis*, in Acetate Agar, **D.** *Lactococcus lactis* in **M17** **E.** *Acetobacter spp*. In Hoyer's Medium, **F.** *Acetobacter spp.* In Carr's Ethanol Medium, **G.** *Leuconostoc spp.(mesenteroids, cremoris)*, **H**. L. *mesenteroides* in Yeast Extract Glucose Citrate Medium with Cysteine, **I.** *Enterococcus* in LIA agar**, J.** *Enterococcus Sp* in Tomato Juice Milk Agar**.**

4.1.1.2.2. Coccobacillus bacteria (Acetobacter spp)

The results of physiological and morphological analysis determined a type of bacteria (8 Isolates) that characterized by the following, its colony were tiny plated, adhered ,convex, entire, pale, blue conducted, and smooth colonies, it were tended to be shiny reflex, in addition, it is convert the AAB medium color to pale yellowish after 48 h of growth. While its cells were gram negative, coccobacillus, ovoid, singly, in paired arrangements, or in chains rarely, it size between 0.6 - 1.8 µm, Non capsulated, catalase was positive and nigative, it were grew and not in ethanol with 4% -10% concentrations, in modified Carr media after 24 and 48 hours incubation periods, and it was capable to

produce acetic acid increasingly during its growth in Carr media with different concentration of ethanol. Formed clear zones around the colonies. It were non motile, grew aerobically, non-spore forming, Arginine negative, it was able to Gelatin liquefaction, and Milk curdle. It were not grew in 4°C and 40 °C, and grew weakly in 10°C and it were Optimally grew at 30°C, it were not resisted for 30 min at 60°C, it can't grew in the presence of 6.5%-8% NaCl, and 0.1% MBM. it can't grew in pH 4 and pH9.6, but it were grew well in pH 6- 7.2. by referring to the Bergey's Manual of Systematic Bacteriology and other previous studies we can describe this type of bacteria as *Acetobacter* , and from the different in cell and colony *characters*, in addition to the growth in ethanol presence and catalase reaction, tow strains of *Acetobacter* can be distinguished as *A. acetia* and *A. pasteurianusa.* As in in figure 4.2. E. which reveal *Acetobacter acetia.* In Hoyer's Medium, and figure 4.2.F. *Acetobacter pasteurianusa* In Carr's Ethanol Medium. The results of morphological, physiological, and biochemical analysis of isolated cocci and coccobacillus bacteria, are Annexed in (Annex 1).

4.1.1.2.3. Lactobacilli bacteria

Four isolates were determined to present one of kefir bacilli bacteria, these isolates have colony morphology from agar plates, size (0.5-2.5mm mm), spherical shape, elevation convex irregular, entire edge, white-yellow, gray, sparkling in color and opacity, and smooth slimy surface texture. cells morphology were, rod shape, thin and long, pair, tall chains arrangement, non-capsulated cells, with round ends, parallel sides (Figure 4.3.A,B). It were Gram positive, Catalase negative, no growth showed at 4°C and some of it were grew at 45 °C, and weak growth occurred at 10 °C, it can't resisted for 30 min at 60°C, absence of growth in 6.5% , 8% NaCl, and 0.1% MBM, no and weak growth t low pH 4 and high pH 9.6, but it were grew in pH 7.2, while there were no grow at 4%-10% ethanol. The isolates were non motile, Facultative oxygen requirements, non-spore forming, Arginine negative, with das production from glucose, non-gelatin liquefied, milk curdle, and not appeared to convert AAB to yellow color. According this findings and by the guidelines of bacteriology references, this bacteria gain high probability to be *Lactobacillus bulgaricus.*

From the results of physiological and morphological analysis a type of bacilli bacteria group (13 Isolates) can be determined by its characters which were, the colonies were Spherical , Convex, bulging, entire, condensed, White, transparent to translucent, opaque , Smooth to rough, ropy, and dry, their size small to medium (0.5 to 3.0 mm) in diameter , the cells were Rod , Single, pair, short chain occasionally, truncate, parallel, capsulated, (Figure 4.3.D), it were gram-positive, catalase negative, no motile, and non-spore forming long rods (0.8 to 1.2 µm in width and 3.0 to 20.0 pm in length) , it weren't able to growth at 4°C ,10 ◦ C , 45 ◦C and can't resisted for 30 min at 60◦C, and can't grew in 6.5- 8% NaCl and 0.1% MBM, not grew in pH 4, pH9.6, but it were grew in pH 7.2the minimum growth were in the (pH 5 . 5), and weren't grew 4%-10% Ethanol, no motility were observed, facultative in oxygen requirement, non-spore forming, arginine negative, gas produced from glucose, gelatin liquefied, made milk curdled, and non-reducing AAB to Yellow. by referring to the Bergey's Manual of Systematic Bacteriology and other previous studies we can describe this type of bacteria as *Lactobacillus kefiranofaciens subsp. kefiranofaciens subsp. nov.* (Figure 4.3.C,D).

Another type of bacteria (9 Isolates) were grown in MRS with colonies in 0.5-2.0 mm size, and Circular irregular shapes, flat elevation, entire edge, opaque in appearance, and white color, with Smooth creamy surface texture. Its cells were Rod shaped, short, Single and paired arrangement, Non-capsulated, round ends, and bulging sides (Figure 4.3. E,F). It were non motile, grew aerobically and facultative, spore forming, Arginine positive, it were able to gelatin liquefying, and Milk curdle, it didn't convert AAB color. Bacteria strain were gram-positive, catalase negative, not grown at 4°C ,10 °C and 45 °C, it can't resisted for 30 min at 60◦C, and weren't grew in 6. 5% - 8% NaCl and 0.1% MBM, it were grew weakly in acid media in pH 4, and good in natural media pH 7.2, but can't grew in basic media pH9.6, and not grew in the presence of 4%-10% ethanol. This results when exposed to the scientific literatures of microbiology leading to describe this bacteria as *Lactobacillus. Parakefir.*

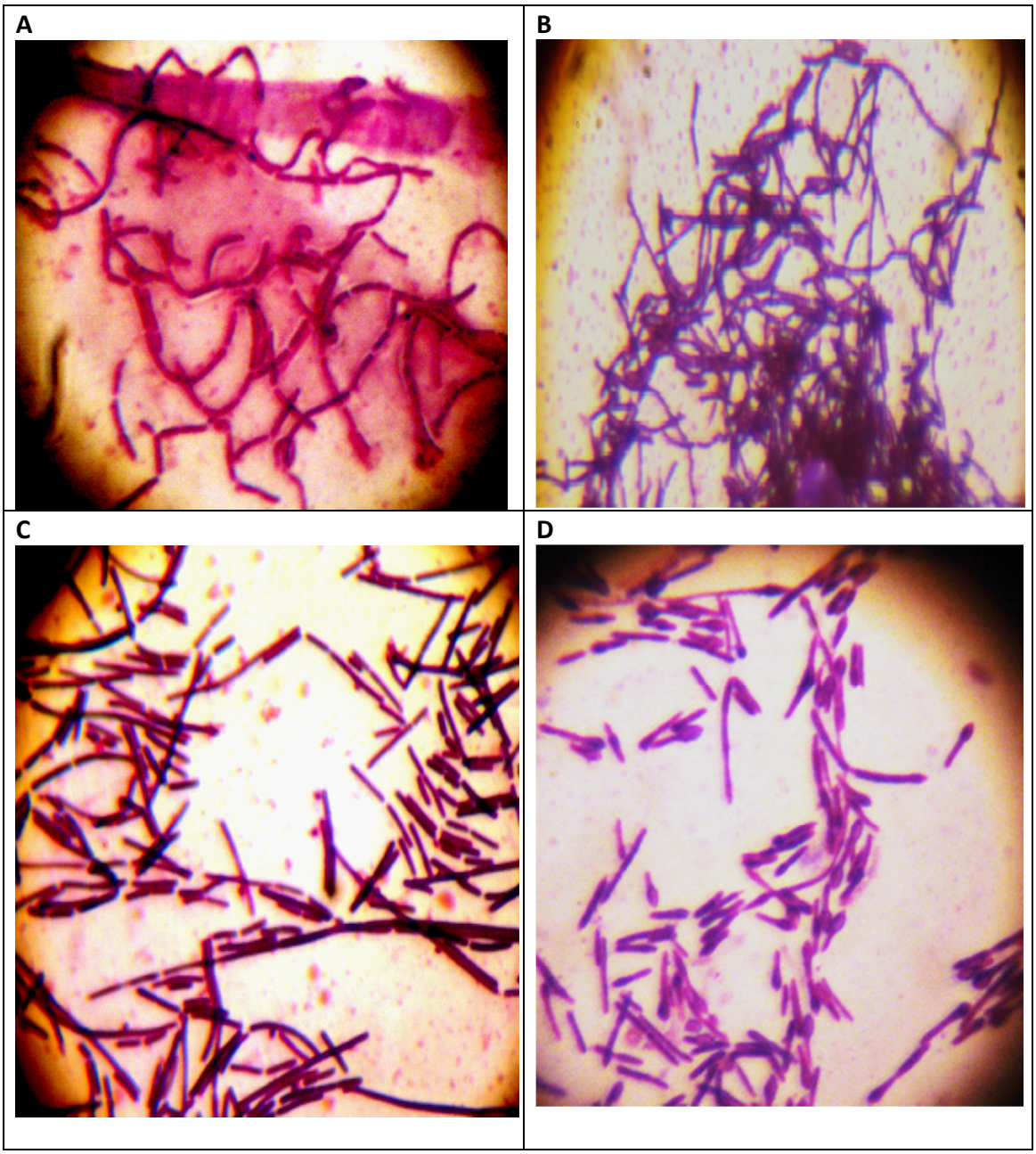
The second bacilli strain (8 Isolates) were characterized by Spherical colonies 0.5-3mm in diameter, convex, bulging, with entire edge and condensed, white color , opaque, the surface texture were smooth dry. The cells shape were rod, single, pair, short chain

arrangement, non-capsulated, with truncate ends, and parallel bulging sides (Figure 4.3.G,H). It were Gram positive, Catalase negative, no growth showed at 4°C and 45 °C, and weak growth occurred at 10 °C, it can't resisted for 30 min at 60°C, absence of growth in 6.5% , 8% NaCl, and 0.1% MBM, no growth t low pH 4 and high pH 9.6, but it were grew in pH 7.2, while there were no grow at 4%-10% ethanol. The isolates were non motile, Facultative oxygen requirements, non-spore forming, Arginine negative, with das production from glucose, gelatin liquefied, milk curdle, and not educing AAB to yellow color. This properties gave these isolates preponderance to be *Lactobacillus kefiranofaciens subsp. kefirgranum subsp. nov*. in compare with the references.

The other lactic acid cultures were (3 Isolates) referred to genus Lactobacillus. Its morphological, cultural and biochemical characteristics were, very small colonies, spherical shaped and adherence, entire edge, Pale, yellow colored, slimy Surface texture. Its cells were rods, very short Single, pair, short chain arrangement, non-capsulated, pointed ends, concave sides. It is gram positive, catalase negative, this species belongs to the group of the thermophilic *lactobacilli*, where a growth was occurred at 4°C relatively weak, well grown at 10 °C, whereas no growth were seen at 45 °C, it can't resisted for 30 min at 60◦C, and weren't showed growth the presence of 6. 5% , 8% NaCl and no growth in 0.1% MBM, it were grew in pH 4, 7.2, but can't grew in pH9.6, and not grew in the presence of 4% -10% ethanol. These bacteria culture were non motile, aerobic and facultative oxygen requirements, non-spore forming, Arginine positive, with gas production from glucose, non-gelatin liquefied, milk curdle, and not appeared to convert AAB color. As a result of the investigations the strain could be determined as *Lactobacillus paracasei* (Figure 4.3.I,J).

As a result of the taxonomic studies 6 isolates was determined as Lactobacillus kefir. Its morphological, cultural and biochemical characteristics were, very small colonies, spherical in shape, Plain in elevation, entire edge, pale, with rough surface texture. Its cells were straight rod, medium in size, single, pair arrangement, non-capsulated, rounded ends, straight and regular sides (Figure 4.3.K,L). These bacteria culture were non motile, aerobic and facultative oxygen requirements, non-spore forming, Arginine positive, with gas production from glucose, non-gelatin liquefied, milk curdle, and not appeared to convert AAB color. It is gram positive, catalase negative, their growth was not occurred

at 4°C and 10 °C, whereas weak growth were seen at 45 °C, it can't resisted for 30 min at 60◦C, and weren't showed growth the presence of 6. 5% , 8% NaCl and no growth in 0.1% MBM, this species belongs to the group of the acidophilic lactobacilli, where it were grew in pH 4, 7.2, but can't grew in pH9.6, and not grew in the presence of 4% - 10% ethanol. The results of morphological, physiological, and biochemical analysis of isolated Lactobacilli bacteria, are Annexed in (Annex 2).



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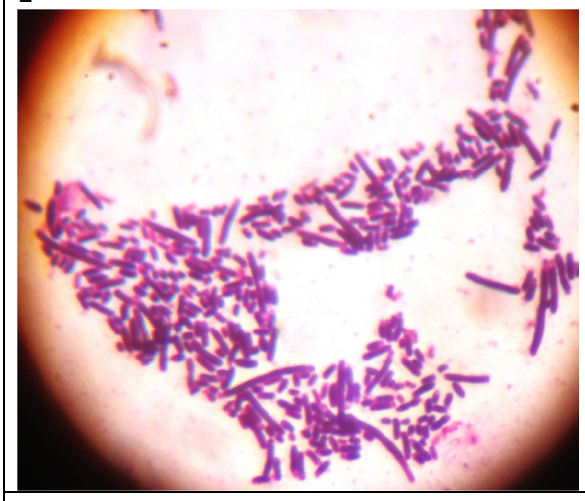

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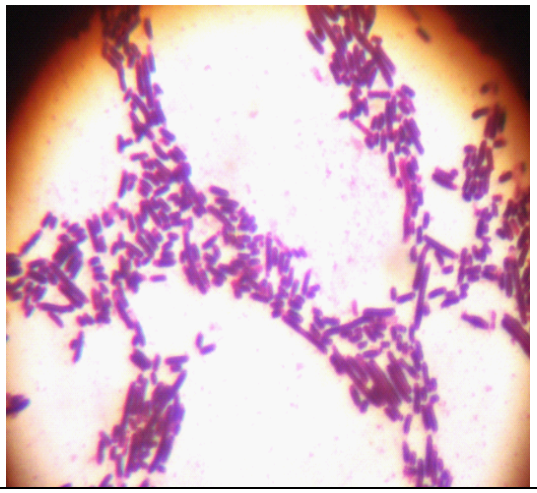

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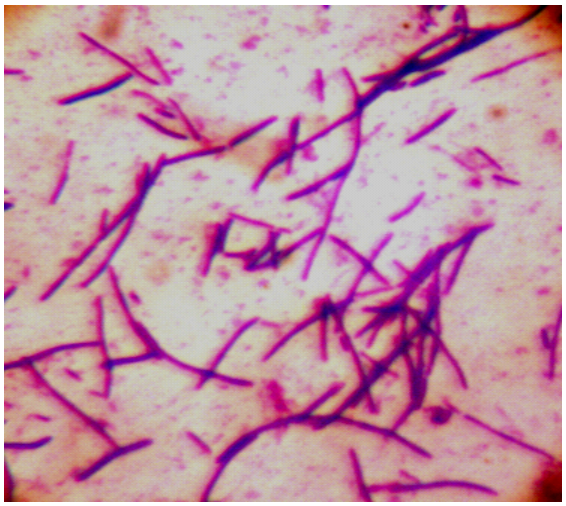

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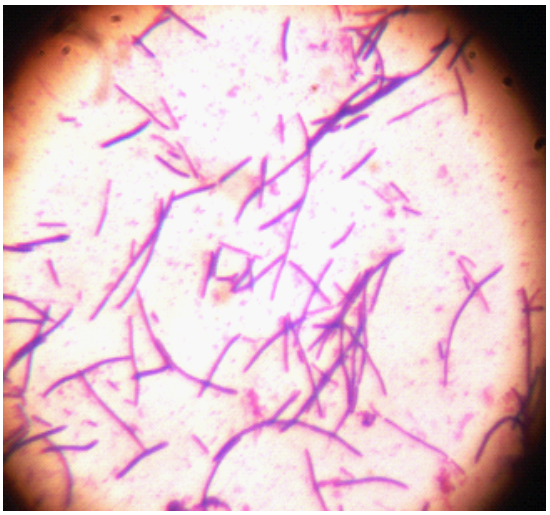

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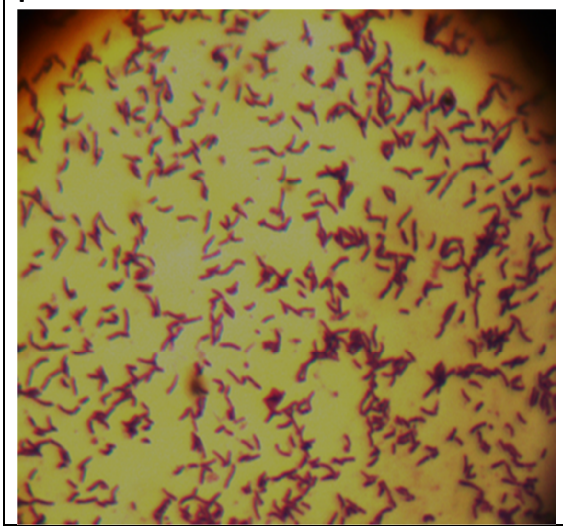

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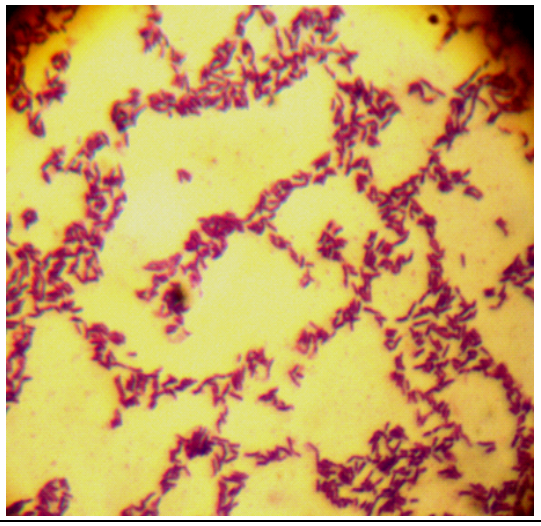

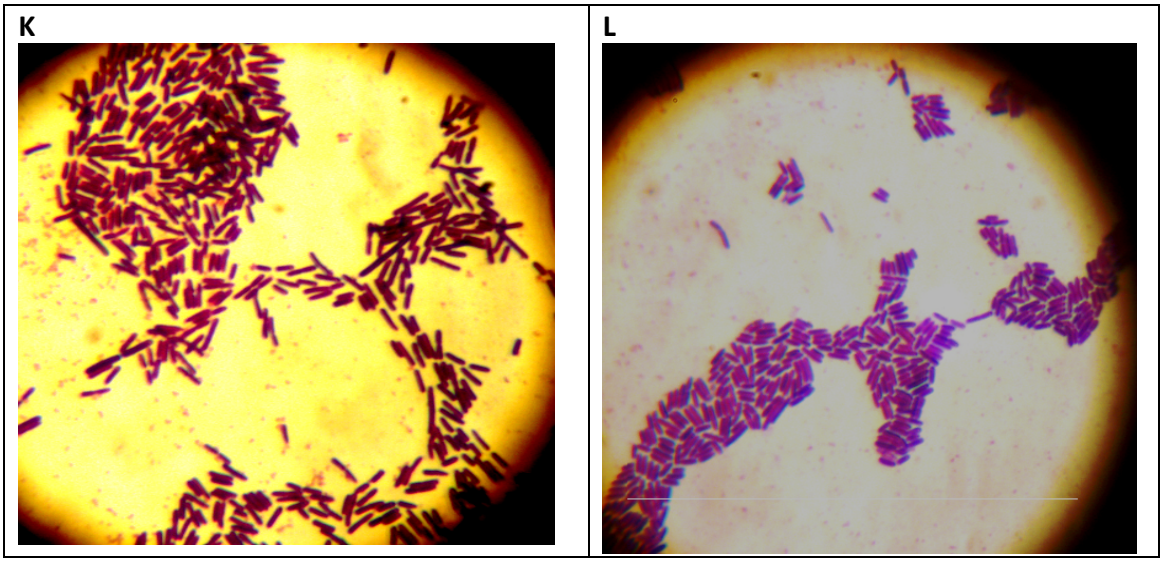

Figure 4.3 The morphological characteristics of cocci bacteria in water kefir

A. *Lactobacillus bulgaricus* in Lee's Agar, **B.** *Lactobacillus bulgaricus* in LB Agar, **C.** *Lactobacillus kefiranofaciens subsp. kefiranofaciens subsp. nov*.in MRS, **D**. *Lactobacillus kefiranofaciens subsp. kefiranofaciens subsp. nov*. with capsules, **E,F.** *Lactobacillus. Parakefir* in MRS, **G,H.** *Lactobacillus kefiranofaciens subsp. kefirgranum subsp. nov*. in MRS **I**,**J.** *Lactobacillus paracasei* in MR **K,L.** *Lactobacillus kefir* in MRS.

4.1.1.2.4. Yeast strains

The morphological properties of the colonies and cells of yeasts that isolated from water kefir are demonstrated in Figure 4.4.

Yeasts strains were isolated by using Universal Medium (YM), Wort Agar, Malt Agar for identification. The isolates tested for morphological and physiological properties, the results separated the yeasts in kefir according their characteristics to tow types, the first type (8 Isolates) were, have white colonies, with rough and granular surface texture, apiculate , adhere margin, convex elevation, medium to large in size (figure 4.4.A). Their cells were, oval cells with single lateral budding, simple Pseudohyphae, spheroidal. Cylindrical, ellipsoid, long-winded, single, paired, and aggregated in clumps, the mode of asexual reproduction was fission, lateral unipolar budding, and it is film forming yeast (Figure 4.4.C,E). Yeast were able to assimilate Maltose, Galactose, Sucrose, but can't assimilate both of Lactose and Raffinose. It were grew in the presence of Ethanol, 10% NaCl, 0.01% Cyclohexamide, and Citrate, while there no growth were shown in methanol and 16% of NaCl. It were able to ferment each of Raffinose, Maltose, Glucose,

Galactose , and Sucrose but not Lactose and Starch. It can grow in Growth at 30°C, while there were no growth at 37°c and 40°c were occurred. There were applying a strong inoculum into the center of YM agar plate and judged after 3–4 wk. the strain could be determined as *Candida.*

The other yeast were white colonies, with luster, smooth surface texture, entire margin, convex elevation, small to medium in size (figure 4.4.B). it cells were, large oval cells, round/oval ascospores, ellipsoid , it aggregated single, paired, and in clumps, the mode of asexual reproduction was miulilateral bipolar, and multipolar budding (Figure 4.4.D,F). It can produce non diffusible red, orange, and pink carotenoid pigments. It can assimilate Maltose, Galactose, Sucrose, and Raffinose, but can't assimilate Lactose. It were grew in the presence of Ethanol, 10% NaCl, while there no growth were shown in methanol and 16% of NaCl, and also in not grew in each of 0.01% Cyclohexamide, and Citrate in the contrast with the previous yeast. It were able to ferment each of Raffinose, Maltose, Glucose, Galactose , Sucrose, and Starch in the contrast of the previous yeast, but not Lactose. It can grow in Growth at 30°C, 37°c and 40°C in contradistinction with the other yeast which were not grew at the high temperature 37°c and 40°C . this yeast can be described to be *Saccharomyces cerevisiae.* The results of morphological, physiological, and biochemical analysis of isolated yeasts from water kefir, are Annexed in (Annex 3).

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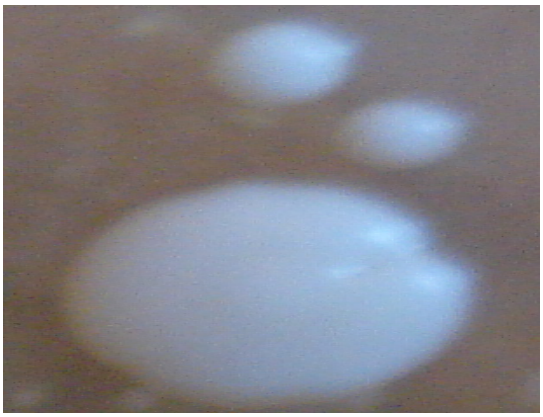

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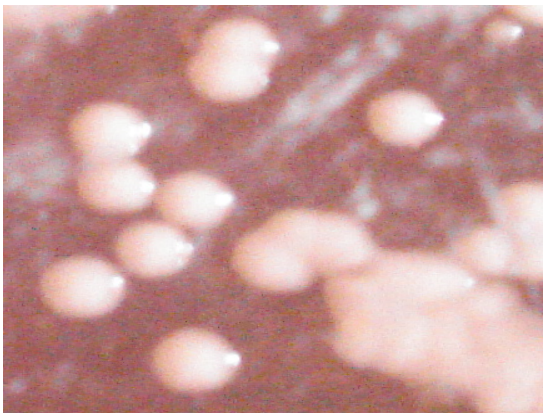

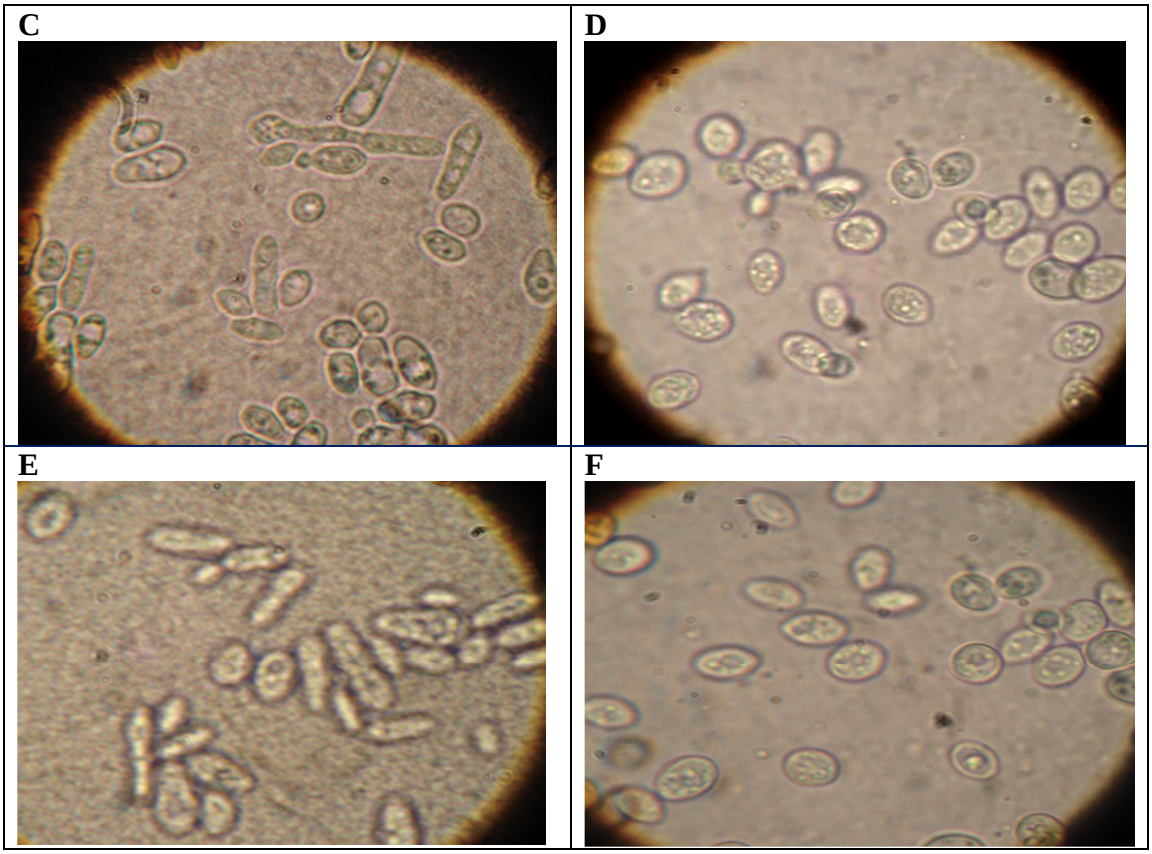

Figure 4.4 The morphological characteristics of yeasts in water kefir
A.Candida colonies in Universal Medium (YM), B. *Saccharomyces* colonies in Malt Agar, C. *Candida* cells in Malt Agar, D. *Saccharomyces cerevisiae* in Malt Agar, E. *Candida* cells grown in Wort Agar, F. *Saccharomyces cerevisiae* cells grown in Wort Agar.

4.1.2. Optimum production of non-alcohol water kefir

In this study, to optimization of non-alcohol water kefir production the fermentation process was carried out at 6.5% sucrose for various fermentation time (12,18,24,48,72, and 84h) at constant temperatures of 10, 15, 20, 25, and 37 °C. Figure 3.5. shows ethanol concentration (mg/ml) that produced in water kefir at different temperatures and in several incubation periods (h). It can be found from these results that the ethanol values increases slightly over the first 12 h of fermentation at all of temperature degrees, the increasing on ethanol concentration were high at the higher temperatures, followed by diminishing at 18 h with all temperature degrees. The lowest concentration of ethanol occurred with fermentation at 20°C and 18h which arrives to 1.06 mg/ml. whilst the mean final value concentration (14.63) obtained at 25°C and 84h.

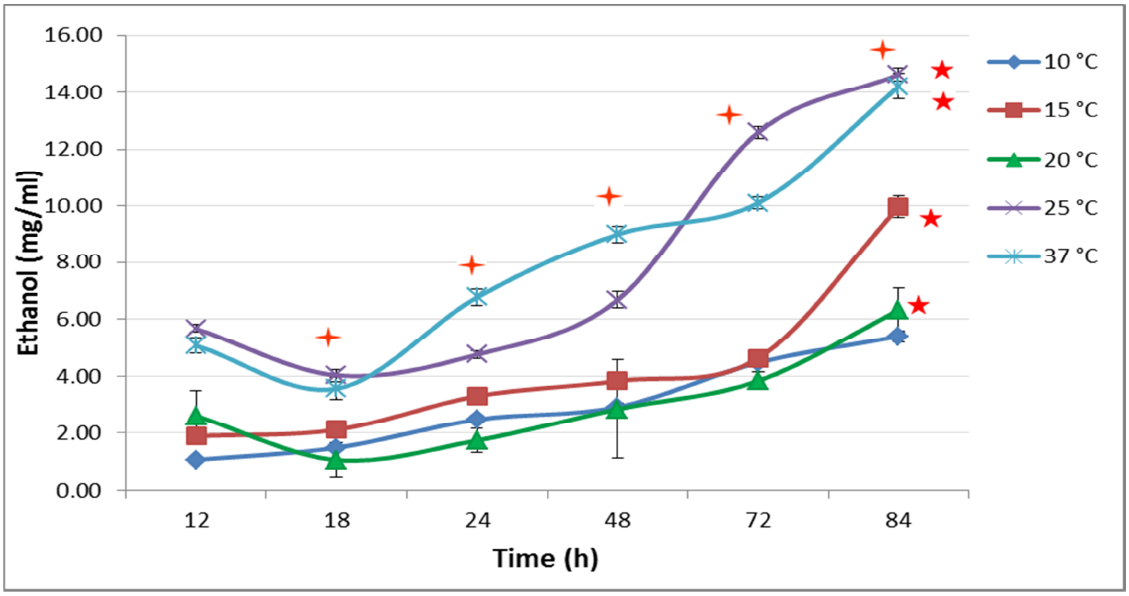

Figure 4.5 Ethanol changes of water kefir at different fermentation temperatures and times

Data are the mean ± SD

★= significant difference in compare with 10 □ at ($p \leq 0.05$)

✦ = significant difference in compare with 12 h at ($p \leq 0.05$)

The results of statistical analysis showed there are significant differences between ethanol production at different temperature degrees at level ($p \leq 0.05$) and the multiple Post Hoc analysis between groups display that there are significant differences between ethanol concentration at most of temperature degrees of fermentation accept that among 10 °C, 20 ° C and between 25 °C, 37 °C at 18h, where the differences are not significant at($p \leq 0.05$). It also shows there are significant differences of alcohol production through the time of fermentation at($p \leq 0.05$), whether in total or partially between groups by the multiple Post Hoc tests. The results of statistical analysis are illustrated in (Annex 4).

4.1.3. Physicochemical properties of water kefir

4.1.3.1. pH and Titratable acidity of water kefir

Titratable acidity development of water kefir during fermentation time expressed as lactic acid, and the changes of pH value of water kefir in different fermentation times exposed in Figure 3.6. Acidity of water kefir begun in (0.0225 g/100ml) at the beginning

of fermentation and increased as fermentation time was increasing to be (0.157 g/100ml) at the end of fermentation period. There is significant difference in kefir acidity between fermentation times at level ($P \leq 0.05$), the multiple comparisons displayed that the mean difference of acidity between all times of fermentation were significant at the (0.05) level, with the exception of that between each of 0h, 12h, and 12h,72, and 18 and both of 24h,72h, and also 24h,72h, where no significant of differences were found. The initial pH was 6.70 while the final is 3.84. The pH of water kefir drink was decreased significantly ($P \leq 0.05$) with the fermentation time increasing, between groups and within groups. This decreasing of pH was in parallel with the increasing of titratable acidity. The correlation between acidity and pH of water kefir is significant at the 0.01 level (2-tailed).

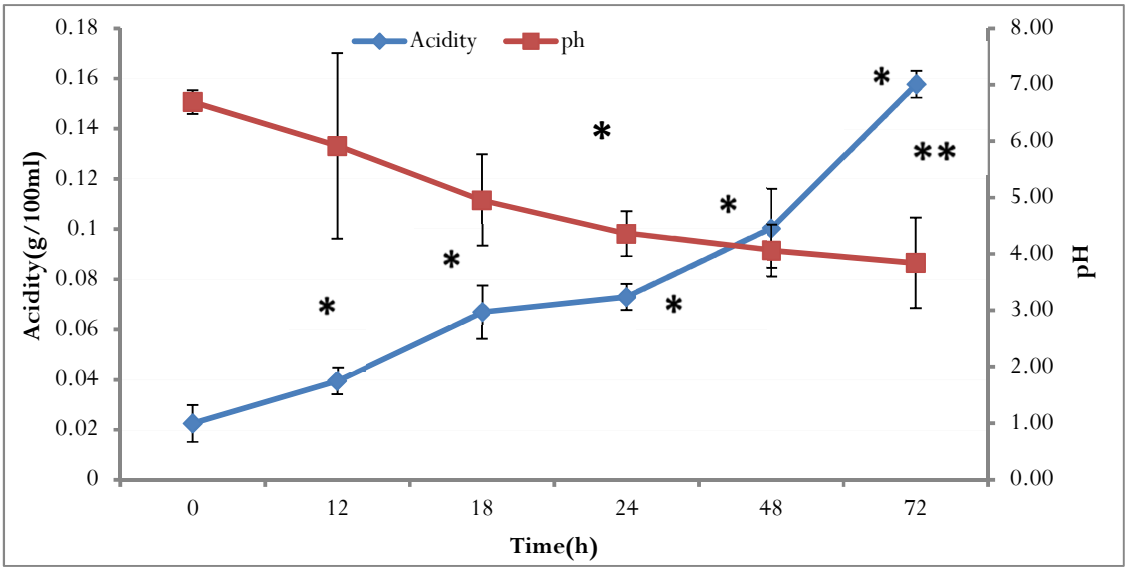

Figure 4.6 Titratable acidity development in water kefir during the time

Data are the mean ± SD for acidy and mean ± (SD ×10) for pH
* = The mean differences is significant at the ($p \leq 0.05$) level
** = Correlation is significant at the 0.01 level (2-tailed)

4.1.3.2. Chemical composition of water kefir and kefir grains

The chemical characteristics of water kefir and kefir grains are shown in Table 3.4. Moisture and dray matter in kefir gains and water kefir were 82.944%, 17.057% and 93.56% , 6.44% respectively, titratable acidity and pH were 0.025 (g/100ml), 6.21and 0.076(g/100ml), 3.98 of grains and kefir beverage respectively, Ash values were similar in kefir grains (0.65%) and water kefir (0.58%). Protein contents of water kefir was (0.31%) and it was (1.75%) for kefir grains, while carbohydrates which is mainly table

sugar in water kefir was (4.63%) and carbohydrates which contains of polysaccharides in kefir grains was (12.82%). The correlation between acidity and pH of water kefir is significant at the 0.01 level (2-tailed).

Table 4-3 Chemical composition of water kefir and kefir grains

Composition	Kefir grains	Water kefir
Moisture %	82.944±3.87	93.56±4.32
Dray Matter%	17.057±1.05	6.44±2.21
lipids %	1.82±0.06	0.97±0.8
DNF %	15.237±0.22	5.47±0.54
Protein %	1.75±0.03	0.31±0.034
Carbohydrates %	12.82±2.201	4.63±2.01
Ash %	0.65±0.004	0.58±0.006
Ethanol %	0.106	Nd
Titratable acidity(g/100ml)	0.025±0.002	0.076±0.0013
PH	6.21±0.44	3.98±0.73

Data are mean values ± standard deviation. Nd : not determined

4.1.3.3. Mineral content of water kefir and kefir grains

Mineral content of water kefir and kefir grains are illustrated in Table 3.4, the major mineral in kefir and kefir grains are Na (2150, 2240 ppm) followed by Ca (745,937ppm), Fe (67, 74), Cu (26, 34 ppm), Zn (21.5, 43.9 ppm), and pb (7.5, 12.9ppm), while Ag was not found in both of water kefir and kefir grains.

Table 4-4 Mineral content of water kefir and kefir grains

Mineral	water kefir		kefir gains	
	Mean	SD	Mean	SD
Ca (ppm)	745	25.76	937	17.63
Na(ppm)	2150	56.83	2240	44.09
Zn(ppm)	21.5	3.4	43.9	7.5
Ag(ppm)	0		0	0
Cu(ppm)	26	2.61	34	2.76
Fe(ppm)	67	7.54	74	6.44
Pb(ppm)	7.5	3.65	12.9	0.769

Data are mean values ± Standard deviation

4.1.4. Antioxidant activity of water kefir

4.1.4.1. Antioxidant activities of fresh water kefir drink

Antioxidant activities of fresh water kefir drink were measured using DPPH scavenging, Inhibition of Ascorbate autoxidation and reducing power methods to determine the antioxidant effects of fresh water kefir drink. The results are exposed in Table 3.4. Where the DPPH scavenging was 11%, and Inhibition of Ascorbate autoxidation was 7.5%, while the reducing power was 0.756 as the absorbance at 700nm, and Hydroxyl radical-scavenging (HRSA) was 9.167842%, while the total antioxidant capacity of the fresh water kefir was 262.58 (Eq. of α-tocopherol (µg/g of sample).

Table 4-5 Antioxidative activities of fresh water kefir drink

	Antioxidant activity of water kefir (Means)	±SD
DPPH scavenging %	11	0.797
Inhibition of Ascorbate autoxidation %	7.5	0.203
Reducing power (A.700 nm)	0.756	0.15
Hydroxyl radical-scavenging (HRSA%)	9.167842	1.54
Total Antioxidant Capacity (Eq. of α-tocopherol (µg/g of sample).	262.58	14,73

Results are mean values of triplicate determination ±S.D.

4.1.4.2. DPPH radical-scavenging activity water kefir

Figure 4.7. Illustrates the results of scavenging of DPPH by water kefir that produced by incubation of kefir grains with sugar solution at 21 °C for 24 h. in comparison with those of common synthetic antioxidants, ascorbic acid and BHA. As revealed in Figure 1, the DPPH radical scavenging effect of water kefir increased as the concentration of the water kefir increased. Water kefir samples at a concentration of (0.125–5 mg/ml) exhibited (9.88 – 63.17%) scavenging activity of DPPH radical. There are significant difference between all the concentration of water kefir at the 0.05 level. The results of statistical analysis of DPPH radical-scavenging activity water kefir, BHA, and ascorbic acid are posted in (Annex. 6)

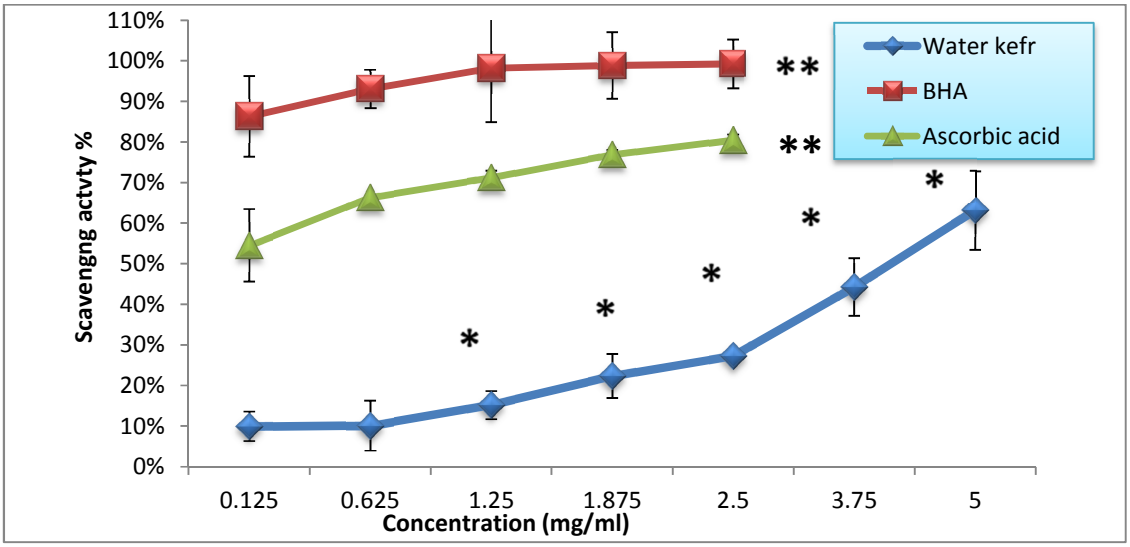

Figure 4.7 DPPH Scavenging effect of Water Kefir
Results are mean values of triplicate determination ±S.D

*. The mean difference is significant at the 0.05 level.

**. The mean difference is significant comparing with water kefir at the 0.05 level.

4.1.4.3. Inhibition of Ascorbate autoxidation by water kefir

In this study, the inhibition of ascorbate autoxidation was measured after incubation of ascorbate in phosphate buffer with and without of water kefir samples.

As in Figure. 4.8. Water kefir samples inhibit ascorbate autoxidation with the rate of 6.08% at the lowest concentration (0.153 mg/ml) and the top inhibition was 25.57% that appears with the highest concentration (5.00 mg.ml), These rates were dependent to the dose. The inhibition of autoxidation of ascorbate by BHA was ranged between (87.43%) at the concentration (0.125 mg/ml), and (99.27%) at the concentration (5.00 mg/ml). The results of statistical analyses of ANOVA showed that the mean difference of the inhibition of autoxidation of ascorbate by water kefir concentrations are significant at the $p \leq 0.05$ level, it is also significant for BHA. The results of statistical analysis of Inhibition of ascorbate autoxidation by water kefir and BHA are posted in (Annex 7)

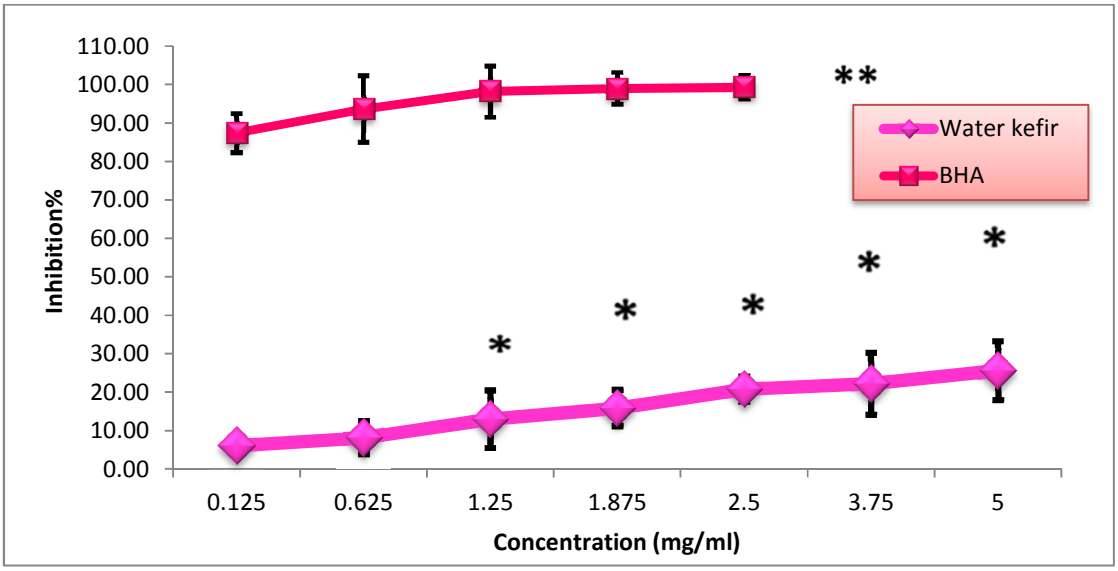

Figure 4.8 Inhibition of Ascorbic Acid autoxidation by Water Kefir
Results are mean values of triplicate determination ±S.D

*. The mean difference of concentrations are significant at the 0.05 level.

**. The mean difference is significant between water kefir and BHA at the 0.05 level.

4.1.4.4. Reducing power activity of water kefir

Figure 4.9 Expose the dose-response curves for reducing power of water kefir samples and the reference antioxidant ascorbic acid. Water kefir showed high ability to reduce Fe3+/ferricyanide complex to the ferrous ion form, the minimum absorbance at 700 nm that obtained by water kefir samples was 0.315 at the concentration (0.125 mg/ml) and the maximum was 1.969 at the concentration (5.00 mg/ml), and the minimum absorbance that occurred in the case of the standard antioxidant ascorbic acid was 1.786 at (0.125 mg/ml), while the maximum absorbance was 2.993 the highest concentration (5.00 mg .ml). Reducing power increased as the concentration of the tested samples increased. But the increasing in the absorbance of water kefir was little between the first and the second concentration the change was only 0.081, in comparing that of ascorbic acid where the different was 0.925, on the contrary the absorbance of water kefir had exposed to be increased after the second concentration up to the last concentration, while in ascorbic acid it seems to be stable after the second concentration where the noted increasing of absorbance were not exceed 0.300 from the second concentration until the last concentration. Statistical analysis results showed that the difference is significant at the

0.05 level between groups and the post hoc Tukey HSD and LSD tests showed that there are significant differences between all of concentration with in groups without exceptions for water kefir and ascorbic acid. The results of statistical analysis of Reducing power activity of water kefir and Ascorbic acid are posted in (Annex 8)

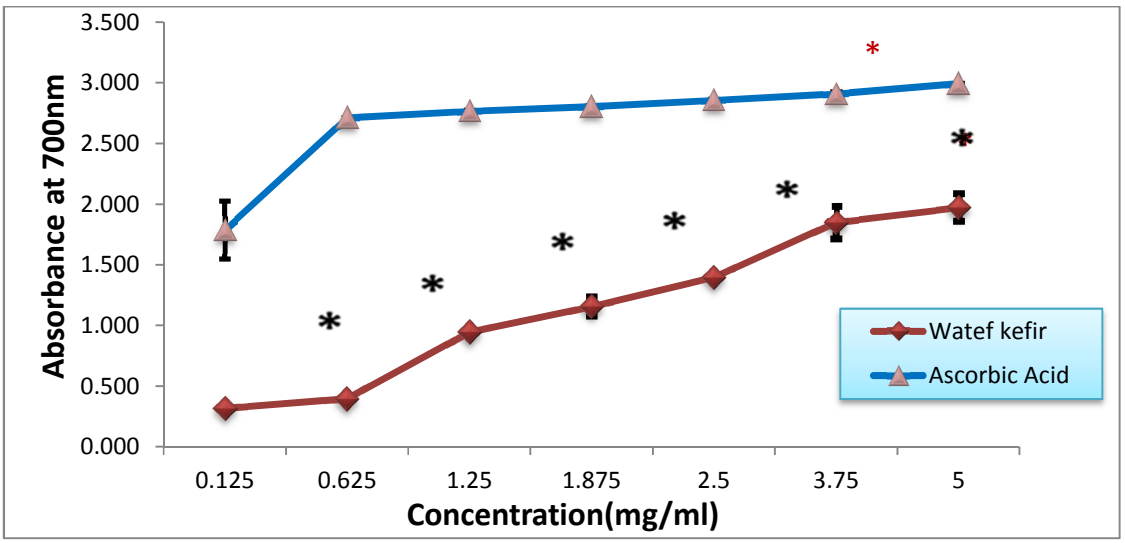

Figure 4.9 Reducing power activity of water kefir

Results are mean values of triplicate determination ±S.D

*. The mean difference is significant at the 0.05 level.

4.1.4.5. Scavenging effect of water kefir on hydroxyl radicals.

Hydroxyl radical-scavenging activity (HRSA) of water kefir extracts that determined using salicylic method is illustrate in Figure 4.10. As in the results the scavenging activity and the concentration of water kefir are in linear evenness positive relationship, where hydroxyl radical-scavenging activity were increased by the increasing of the concentration of water kefir. The minimum activity was 11.00% at 0.125 (mg/ml) of water kefir, and when concentration amplified five folds, the antioxidant activity was raised up by 15.445%, and the maximum hydroxyl radical-scavenging activity was 73.185% at the concentration 5.00 (mg/ml). The differences of hydroxyl radical-scavenging activity were appeared by statistical analysis of anova to be highly significant between the concentrations of water kefir which used in this study at level of ($p \leq 0.05$). The post hoc Tukey HSD and LSD tests showed that there are significant differences between all of concentration with in groups without exceptions by using hydroxyl

radical-scavenging activity as dependent variable, The correlation between hydroxyl radical-scavenging activity and water kefir concentration (Spearman's rho) was positive it was (0.954), and it was significant at the 0.01 level (2-tailed). The results of statistical analysis of scavenging effect of water kefir on hydroxyl radicals are posted in (Annex 9)

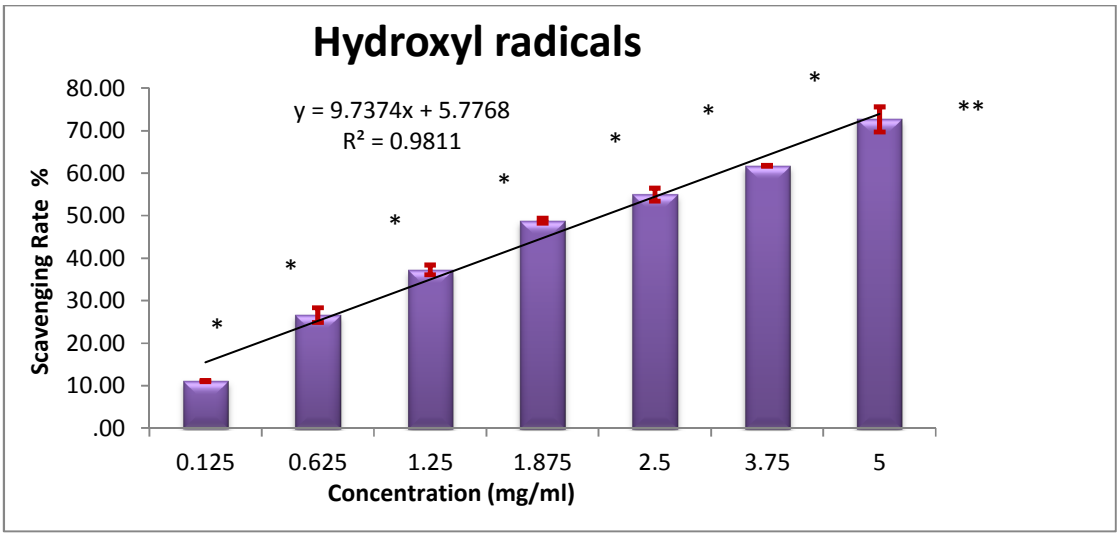

Figure 4.10 Scavenging effect of water kefir on hydroxyl radicals.

The results are the mains and standard deviation of triplicate experiment
* . The mean difference is significant at the 0.05 level.
**. Correlation is significant at the 0.01 level (2-tailed).

4.1.4.6. Total antioxidant capacity of water kefir (TAOC)

Figure 4.11. Demonstrate the total antioxidant capacity (TAOC) of the extract of water kefir at different concentrations (5, 25, 50, 75, 100 mg/ml) that was evaluated by the ammonium molybdate method, total antioxidant capacity expressed as (Eq. of α-tocopherol (µg/g of extract), the increasing of total antioxidant capacity are occurred in parallel with the increasing of the concentration of water kefir samples, the total antioxidant capacity was 232.85 (Eq. of α-tocopherol (µg/g of extract), in the lowest concentration of water kefir and it was amplified tow fold at the second concentration, the accumulating of total antioxidant capacity after the second and third concentration were steady at approximately 145 (Eq. of α-tocopherol (µg/g of extract)while it were elevated by 300 (Eq. of α-tocopherol (µg/g of extract) at the fourth, fifth, sixth, and the seventh concentration to be 1513.69 (Eq. of α-tocopherol (µg/g of extract) as the maximum total capacity antioxidant. The differences of Total antioxidant capacity of water kefir were

appeared by statistical analysis to be highly significant between the various concentrations of water kefir which used in this study at level of ($p \leq 0.05$). and the post hoc Tukey HSD and LSD tests showed that there are significant differences between the first concentration and all other concentrations, and between the second concentration and all the other concentrations with the exception of the third concentration, the third concentration was differ significantly from the both of the first and the last concentration, while its differences wasn't significant with both of the fourth, fifth, and sixth concentration, the fourth concentration was highly significant differ from the first two concentrations, while there are no significant difference were showed between it and all the other concentrations, the results also explained a significant differences between the highest concentration and all the other concentrations with the exception of the sixth at the 0.05 level, and the correlation between total capacity antioxidant and water kefir concentration (Spearman's rho) was positive equal (0.895), and it was significant at the 0.01 level (2-tailed). The results of statistical analysis of total antioxidant capacity are posted in (Annex 10).

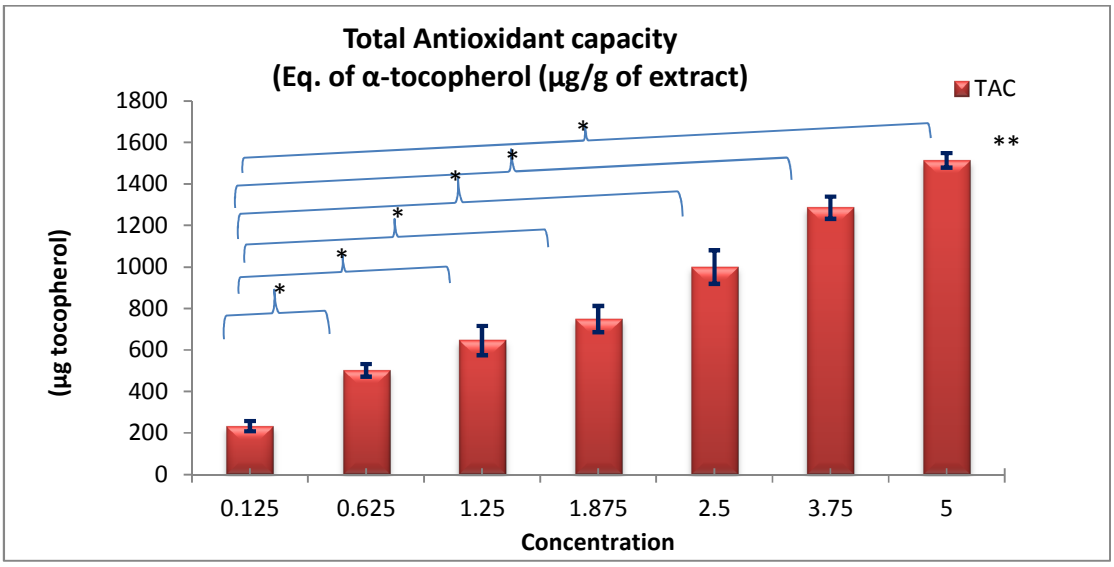

Figure 4.11 Total antioxidant capacity of water kefir (TAOC)
The results are the mains and standard deviation of triplicate experiment
* . The mean difference is significant at the 0.05 level.
**. Correlation is significant at the 0.01 level (2-tailed).

4.2. *In vivo* results

4.2.1. Anti-hyperglycemic activities of Water kefir as probiotic on Streptozotocin-induced diabetic Wistar Rats

4.2.1.1. The effect of water kefir on the body weight of rats

The effect of water kefir on the body weight of normal and STZ-induced diabetic Wistar rats, recorded for 35 days are shown in Figure 3.9. Body weight of normal control rats was increased during the period of experiment, on the contrary, with diabetic group which showed decreasing in body weight from the second week of experiment. The body weight of other groups treated with water kefir was ascended. The body weight of the rats treated with 10% of water kefir was ascending similarly with that of normal control, and 30%WK and 20%WK groups take the third and fourth order in body weight increasing. There were high significant differences between groups of treatment at ($p \leq 0.05$), the multiple comparisons of treatment groups showed that diabetic control group was differed significantly at ($p \leq 0.05$) from all of other groups. Although the changes of body weight of normal and diabetic rats during the time of experiment, there was no significant difference ($p \leq 0.05$) between body weight of rats respects with the time of study. But by host hoc tests, a significant difference ($p \leq 0.05$) was appear between body weight in the first day of study and that in sacrifice day.

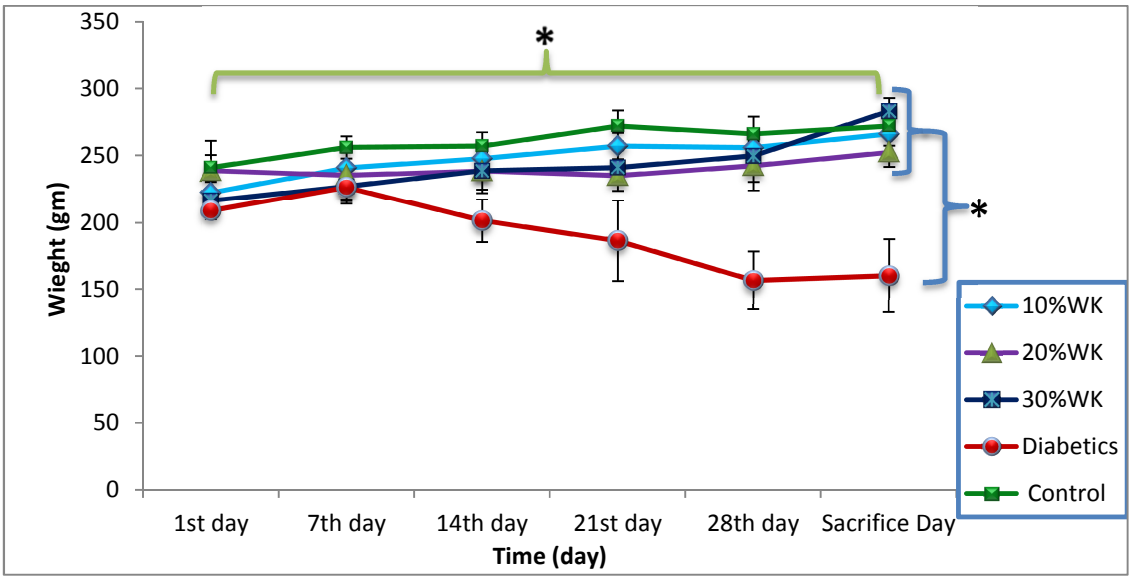

Figure 4.12 the change in body weight of normal and STZ-induced control and treated Wistar Rats.

Values are means ± SEM
* Statistically significant at $p \leq 0.05$.

4.2.1.2. The effects of water kefir in glucose concentration of rats

Anti-hyperglycemic effects of water kefir on normal control and STZ-induced diabetic rats which were measured every week over the entire experimental time are reported in Figure 3.10, Fasting blood glucose levels of diabetic control (DC) group were increased after the induction by streptozotocin and remained over 400 g/dl up to the end of study. Blood glucose levels of diabetic rats groups which gave water kefir instead of drinking water were found to be decreased, the initial glucose concentration was 401 g/dl, 411 g/dl, and 405 g/dl in 10% WK, 20% WK, and 30% WK group respectively. After one week of treatment, blood glucose concentration was reduced by 12 % in 10%WK group, 18.4% in 20% WK group, and 25% in 30% WK group, and at the end of experiment blood glucose concentration was diminished by 69.82 % in 10 %WK group, 71.36% in 20% WK group, and 71.79 % in 30% WK group from the initial concentrations.

Streptozotocin caused a significant increase in the glucose levels of experimental animals compared with normal control ($p \leq 0.05$). The reduction of blood glucose was high significantly ($p \leq 0.05$) in all water kefir received groups comparing with normal and diabetic control rats, but there were no significant differences between water kefir groups at ($p \leq 0.05$). Through the time of experiment, a great significant reducing ($p \leq 0.05$) were recorded in glucose levels of rats between the first day and each of the 14th, 21st, 28th, and sacrifice day; and between the 7th day and both of the 28th and sacrifice day.

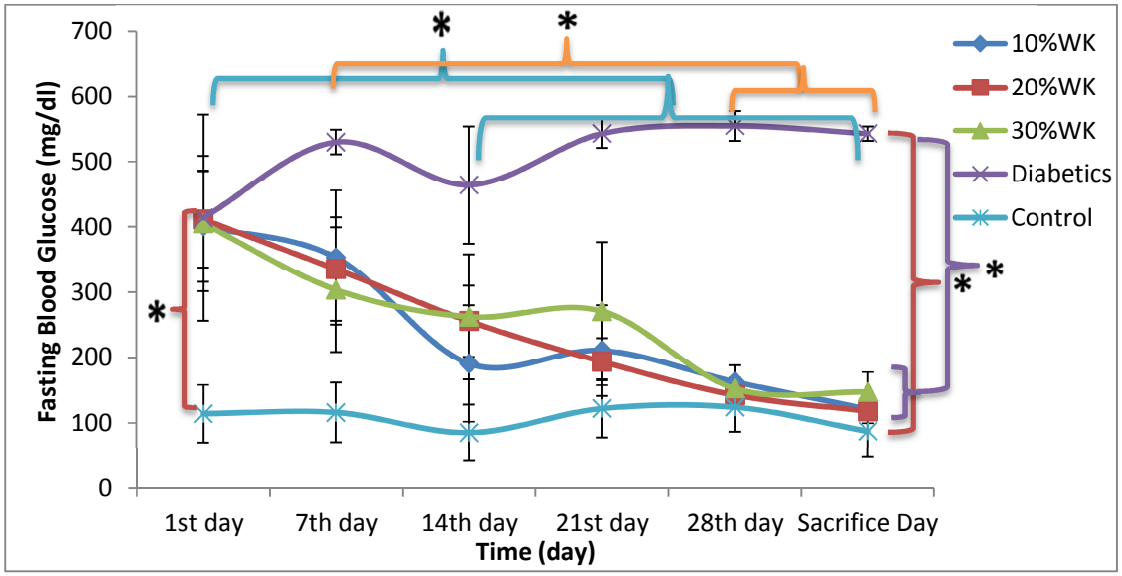

Figure 4.13 Glucose concentration of the normal rats, and STZ-induced diabetics control and treated rats

Values are means ± SEM

* Statistically significant at $p \leq 0.05$.

4.2.2. Anti-hyperlipidemic activities of Water kefir as probiotic on Streptozotocin-induced diabetic Wistar Rats

Figure 3.11 demonstrate the effect of water kefir in lipid profile in diabetics and normal wistar rats at the end of experiment. Total cholesterol (TC), Triglycerides (TG), low density lipoproteins, and very low density lipoproteins (VLDL) were found at high concentrations in diabetic rats, while the High density lipoproteins (HDL) in this group was the lowest concentration. In diabetic groups treated with 10, 20, and 30% of water kefir, all lipid profiles were outstandingly decreased in comparing with diabetics control group. Statistically, there were significant differences at ($p \leq 0.05$) in all the results of lipid markers between the rat groups of experiment. From Post Hoc Tests results, the decreasing of TC in the normal control and treated groups are statistically significant, the group which consumed 20% Water Kefir have a higher significance at ($p \leq 0.05$).The significant difference in TG presented only between diabetics and both of 20%WK and normal control groups. LDL were significantly reduced in all groups comparing with diabetics control with a preference of 30%WK and diabetics groups. On the other hand, HDL concentrations were significantly increased for all groups in opposing of diabetics

group at ($p \leq 0.05$), and only 20%WK group was differ significantly from diabetics in VLDL ($p \leq 0.05$).

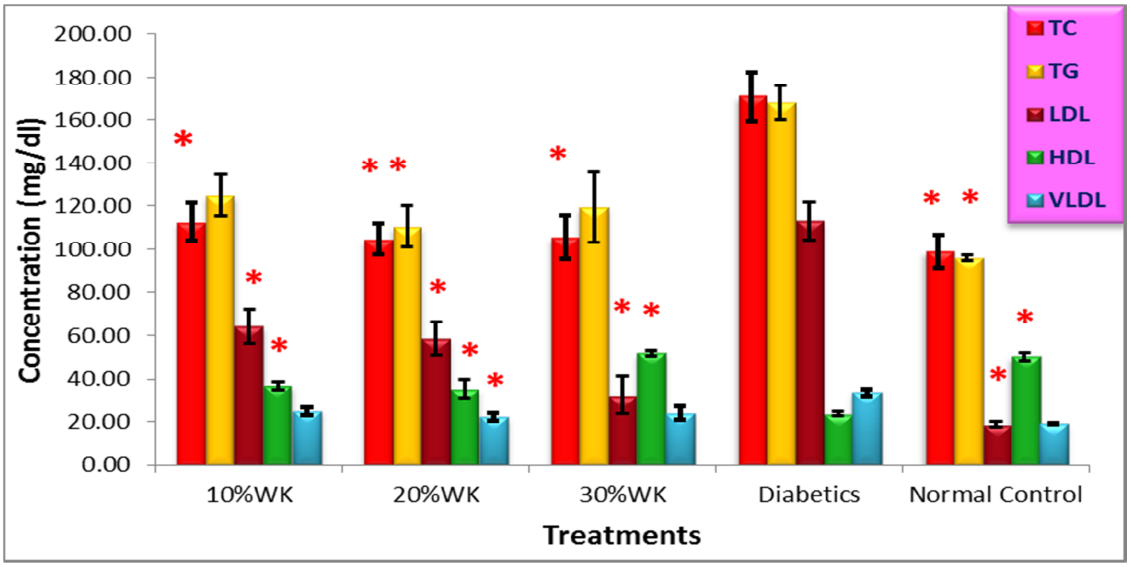

Figure 4.14 The concentration of lipids profile of normal rats, and STZ-induced diabetics control and treated Wistar Rats.
Water kefir **(WK),** Total cholesterol **(TC),** Triglycerides **(TG),** low density lipoproteins, and very low density lipoproteins **(VLDL),** High density lipoproteins **(HDL).**
Values are means ± SEM, * Statistically significant when compared to diabetics group at $p \leq 0.05$.

4.2.3. The effects of water kefir in oxidative stress of streptozotosin induced diabetic rats

4.2.3.1. Malondialdehyde (lipid peroxidation) contents in experimental rats

Lipid peroxidation results of streptozotocin-induced diabetic Wistar Rats and those which are treated with 10%, 20%, and 30% of water kefir, in addition to normal control rates are demonstrated in Figure 4.15. Lipid peroxidation is expressed as the concentration of Malondialdehyde (µmol/l). The concentrations of Malondialdehyde in the normal rats group were very low (0.30 µmol/l), while they raised in the Streptozotocin-induced diabetic Rats group that served as controls without of water kefir. In the streptozotocin-induced diabetic groups treated with water kefir, malondialdehyde levels were decreased compared to the diabetic group. It can be noted that administration of water kefir induced diminishing of 0.48 µmol/l malondialdehyde in 10% of Water Kefir (WK), 1.49 µmol/l,

in 20% WK and 1.02 µmol/l in 30% WK compared to control group. Statistical analysis indicated a significant difference at ($P \leq 0.05$) between diabetic group and control group, and between control rats and treated groups, also between diabetic group and each of these groups treated with water kefir at different concentrations. The results of Post Hoc Multiple Tukey and LSD Tests within groups showed that there were high significant between diabetic group and each of control, 20% WK, and 30% WK groups, while the difference between diabetic group and 10%WK group was low significant, and there were no significant different among the treated groups.

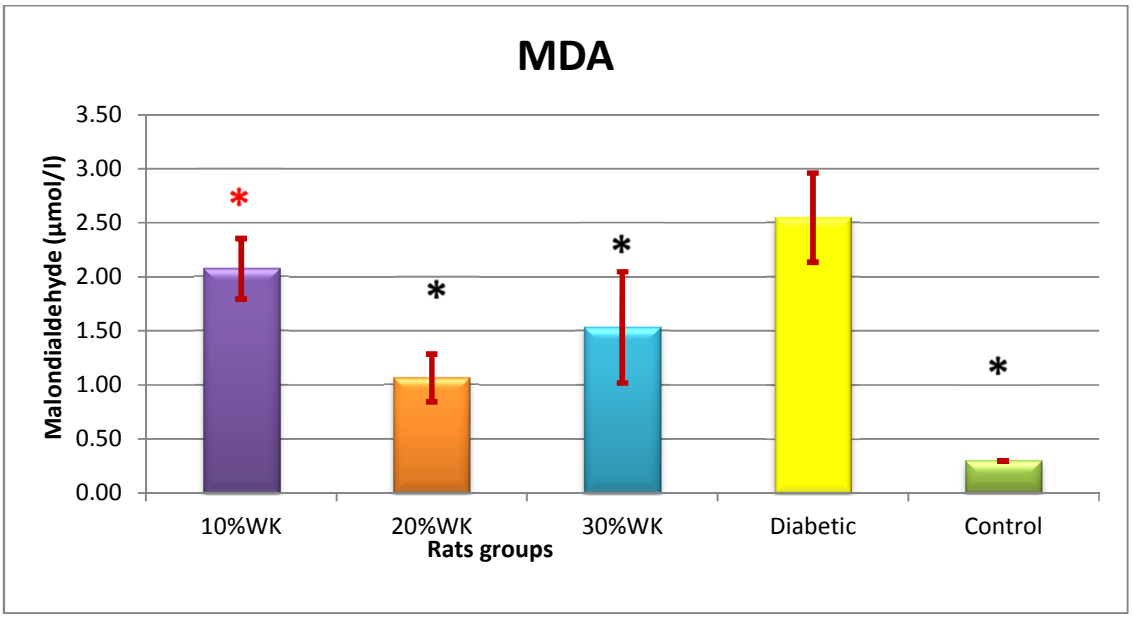

Figure 4.15 Lipids peroxidation concentration (Malondialdehyde, µmol/l) of control, STZ-induced diabetics, and treated Wistar Rats.
Results are represented as mean with standard errors of mean (mean ± SEM), n= 6
* Statistically significant when compared to diabetics group at p ≤0.05.

4.2.3.2. Protein carbonyl level in control and diabetic rats

Protein carbonyl level in haemolysate control and diabetic rats are illustrated in Figure 4.16. It is clear that protein carbonyls were accumulated in the streptozotosin-induced diabetic group, where of protein carbonyls were the highest reaching 4.756 mmol.l, on the other hand, protein carbonyl concentrations were low 1.650 mmol/l in the hemolysate of the control rats. The results showed significant diminishing of quantity of protein carbonyls in the rat groups drinking water kefir, where protein carbonyls in the group that

consumed 10% of water kefir was diminished by 1,419 mmol/l, it was reduced in the group having 20% of water kefir by 1.93 mmol/l, and finally the most important results were seen in the group of rats that drunk 30% of water kefir where protein carbonyls were dropped with considerable amounts (2.226 mmol/l) to be nearest to that in control rats. The analysis of variance (ANOVA) general linear models-Univariate results demonstrated high significant differences for diabetic group comparing with the control group. The water kefir treatment confirmed the own antioxidant activity, where treated groups had low values which significantly different from the diabetic group ($p \leq 0.05$).

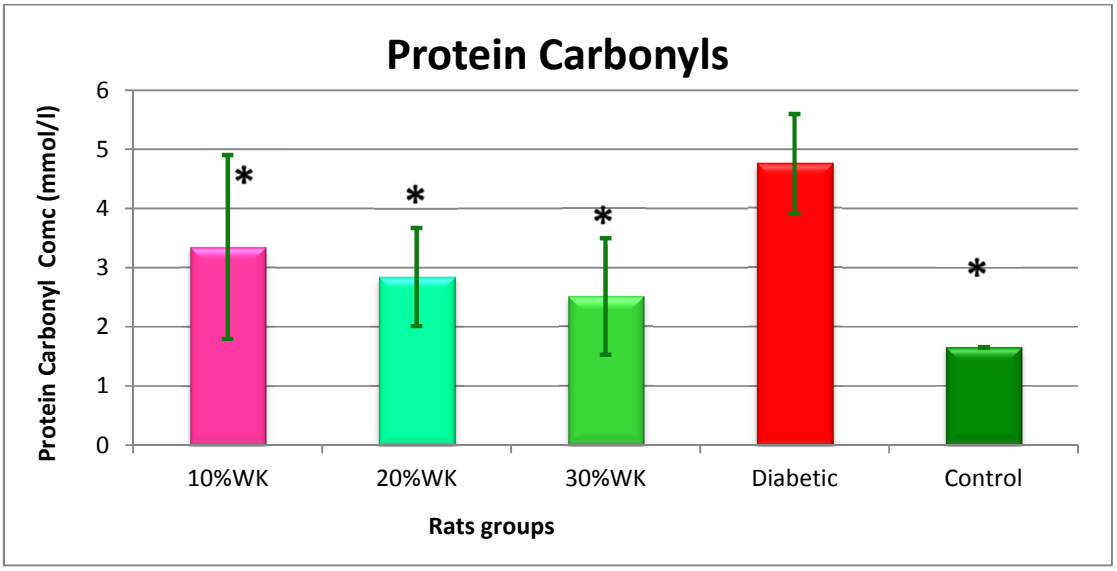

Figure 4.16 Protein carbonyl level in control and diabetic rats
Results are represented as mean with standard errors of mean (mean ± SEM), n= 6
* Statistically significant when compared to diabetics group at $p \leq 0.05$.

4.2.3.3. Hydroperoxide Amounts in control and diabetic rats

Figure 4.17 illustrate the results of Hydroperoxide Amounts in control, diabetic and water kefir treated rats. The results indicated a low quantity of Hydroperoxides ($ROOH^{\bullet}$) appeared in the control rats with concentration of (0.22 µmol/l) in plasma, and the largest concentration of hydroperoxides were found in diabetic rats (3.735 µmol/l). The administration of water kefir drink improved the oxidative stress stats in diabetic rats. As it is clear in the graph, 10% of water kefir scavenged 1.22 µmol/l from the plasma of the rats, and 20% of water kefir consumption hunted (1.72 µmpl/l) from the blood of these rats, while 30% of water kefir consumption inhibit the formation of (2.02 µmol/l) in

plasma of the last group. Treatment 30% of water kefir was the more effective treatment against the oxidation of biomolecules in rats blood, and then on oxidative stress. By analysis the results of hydroperoxides statistically, the data indicated high significant increasing in hydroperoxides among the diabetic rats comparing with the control rats at ($p \leq 0.05$). There are is significant differences between treated rat groups too at the same level, the significance was high in the group treated with 30% of water kefir. When comparing diabetics rats with both of 10% and 20% WK groups, the differences were moderated significantly. The results of Post Hoc Multiple Tukey and LSD Tests also showed that hydroperoxides concentrations in both of 20% and 30% WK were different significantly from 10% WK group ($p \leq 0.05$), but no differences were appeared between the groups 20% WK and 30% WK at the same level.

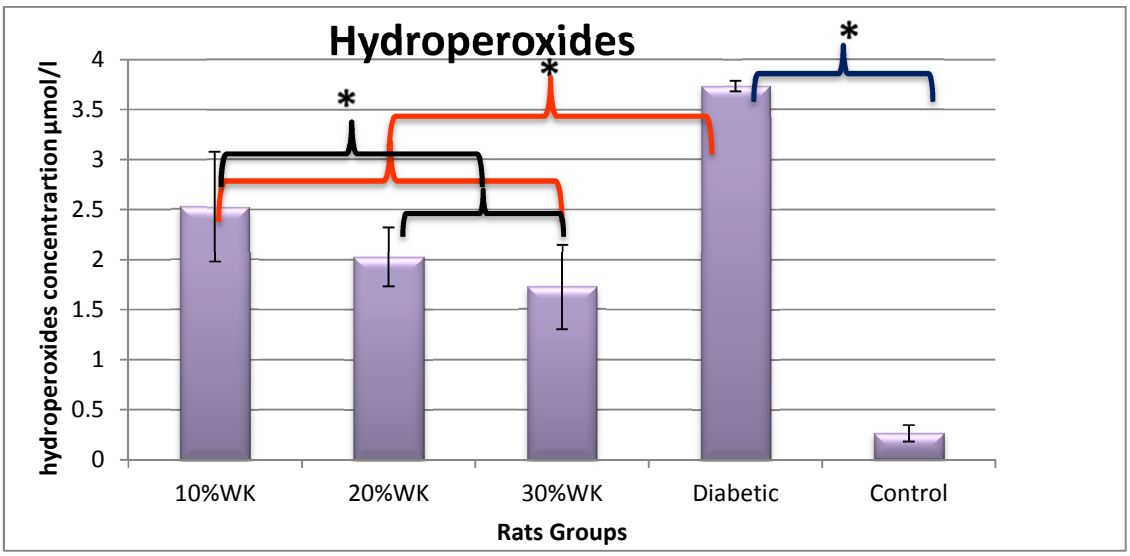

Figure 4.17 Hydroperoxides contents in control, diabetic and water kefir treated rats. Results are represented as mean with standard errors of mean (mean ± SEM) of triplicates, n= 6
* Statistically significant at $p \leq 0.05$.

4.2.3.4. Catalase activity in experimental rats

The results of the activity of catalase in control, diabetic and water kefir treated rats are given in Figure 4.18. Catalase activity was expressed as U/ml for lysate (U- µmoles of H_2O_2 Utilized / min). The antioxidant activity of blood enzymes, presented by catalase activity appeared to be elevated in the rats that have water kefir at concentration 10%WK

where it was increased by 8.7 U/ml comparing with diabetic group. Catalase activity was more effective in the group that administrated 20% of WK, its activity exceeded that of diabetic rats with 20.57 U/ml. The concentration 30% of water kefir exhibited excellent antioxidant activity, where catalase activity was twofold of its counterpart in diabetic values with increasing 37.56 U/ml. The improvement of antioxidant capacity of the rats that drunk water kefir was different significantly from the diabetic rats at ($p \leq 0.05$). There were also statistical significances in the variances of diabetic and control rats groups ($p \leq 0.05$). When comparing between the effects of the various concentrations of water kefir those employed in this experiment, no statistical differences had been shown among each treatments groups ($p \leq 0.05$).

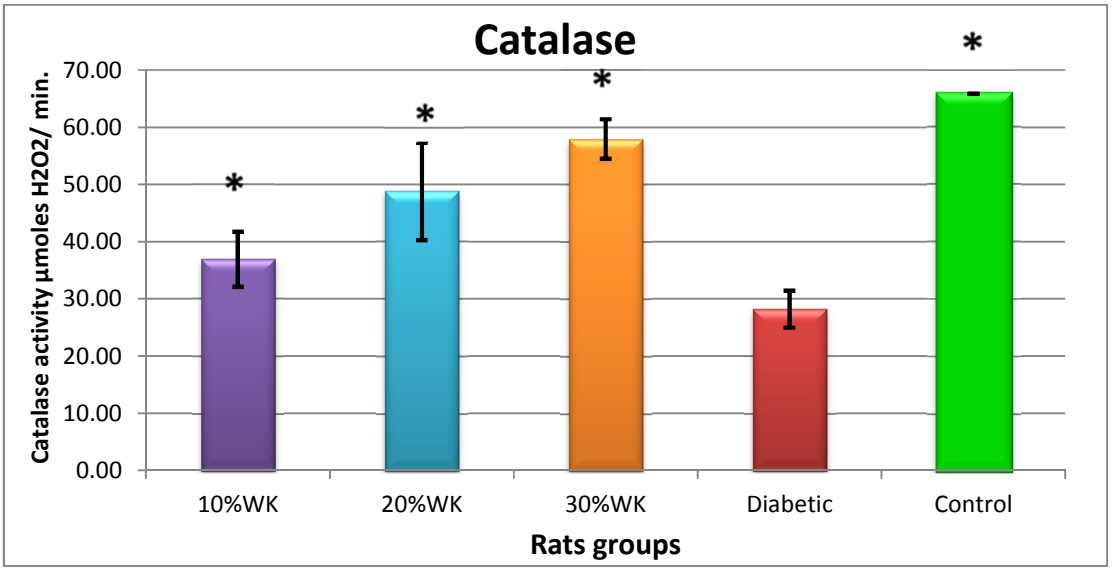

Figure 4.18 Catalase activity in control, diabetic and water kefir treated rats.
Results are represented as mean with standard errors of mean (mean ± SEM), n= 6

* Statistically significant when compared to diabetics group at $p \leq 0.05$.

4.2.3.5. Serum Vitamin C. in experimental rats

Figure 4.19 demonstrate the results of Vitamin C concentration in the blood serum of all of experimental rat groups. Vitamin C concentration in diabetic rats was low (10.61 µg/ml) in comparing with vitamin C levels in the control rats. The consumption of 10% of water kefir drink by diabetic rats resulted in raising of vitamin C concentration by 5.67 µg/ml in the serum of these rats. Drinking of 20% water kefir resulting elevation in vitamin C quantity by 8.47 µg/ml from that in diabetic rats, this value is approximate that

of control rats, while the largest quantity of vitamin C was gained by drinking of 30% of water kefir which was surpassed the value of vitamin C of diabetic rats by (15.97 µg/ml). It was surpassed vitamin C value in the control rats with (4.86 µg/ml). Statistically, the elevation of vitamin C in water kefir treated groups found to be significant from that of diabetic rats at level ($p \leq 0.05$). Normal rats were also differed from diabetic rats considerably ($p \leq 0.05$). In the same time, 30% WK group revealed high significant in variance from the other groups of rats ($p \leq 0.05$). Similarly, the alterations of control rats from the other groups were significant at level ($p \leq 0.05$). But 20% WK group was not varied importantly from 10% WK group at level ($p \leq 0.05$).

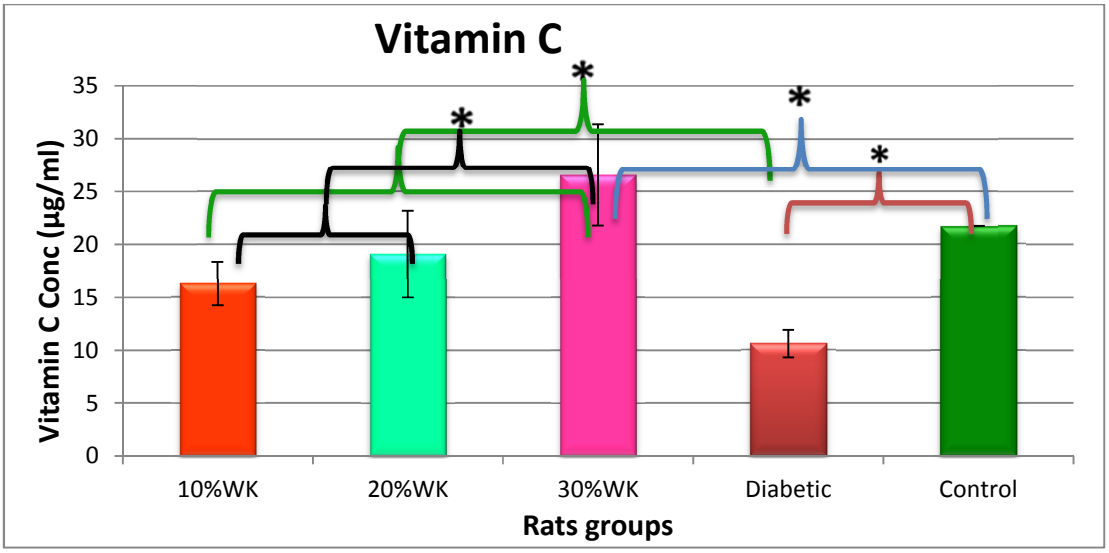

Figure 4.19 Serum Vitamin C concentrations of experimental rats

Results are represented as mean with standard errors of mean (mean ± SEM), n= 6

* Statistically significant group at $p \leq 0.05$.

5. DISCUSSION

Previous studies have investigated the composition of the microorganisms present in kefir grains and reported that Lactic acid bacteria was the most frequently bacteria found microorganism group, acidification of the substratum was mainly stimulated by the presence of lactic acid bacteria (Güzel-Seydim *et al.*, 2000). The amount of microorganisms in Portugese kefir grains were 10^9 cfu/ml lactobacilli,10^8 cfu/ml *lactococci*, and 10^6 - 10^9 cfu/ml yeast (Ferreira *et al.*, 2010). And in Irish kefir grains they were 10^6 cfu/ml lactobacilli, 10^9 cfu/ml *lactococci* and 10^6 cfu/ml yeast (Rea *et al.*, 1996), while in Argentinean kefir grains they were 10^8 cfu/g *lactobacilli* and 10^7 cfu/g yeast (Garrote *et al.*, 2001). Suriasih *et al.*, (2012), reported 9.51 (log cfu/ml) Lactic acid bacteria in MRS, 9.49 (log cfu/ml) Lactic acid bacteria in M17, and 6.44 (log cfu/ml) yeast in OGYE in kefir made from Bali cattle milk.

The results of this study for lactic acid bacterial counts in water kefir were in accordance with the study of Magalhães and Pereira, (2010) which reported that, the population of lactic acid bacteria in kefir were ranging from 10^9 – 10^{10} cfu/ml. But they were higher than that of Bergmann, *et al*. (2010), which were 4.5×10^4 CFU. g^{-1} in the kefir suspension of sugary kefir, and the results of Miguel *et al* (2011c) that reported that the counts of lactic acid bacteria that were 6.04 – 6.36 $\log_{10}$ cfu/g and 9.08- 9.18 $\log_{10}$ cfu/g. And they were higher in comparing with 5 x 10^6 (c.f.u./g) and 5 x 10^8 (c.f.u./g) for lactic acid bacteria in MRS that reported by Pidoux, (1989).

The count in this study of *lactococcus* in M17 was higher than that of Pidoux, (1989) which 3 x 10^7 (c.f.u./g), and that of Magalhães and Pereira, (2010) which was 8.41(log c.f.u./g) in sugary Brazilian kefir beverage. *Acetobacter* count in this study found in agreement with 8.31(log c.f.u./g) in the study of Magalhães and Pereira, (2010) and 1×10^7 in water kefir grains from different regions in brazil (Miguel *et al.*, 2011c). *Leuconostoc* amount in this investigation was lower than those of Magalhães and Pereira, (2010) that was 8.41 (log c.f.u./g). And yeast count of the present study were agreed with that of Miguel *et al* (2011c) 5.92 -8.30 $\log_{10}$ cfu/g in water kefir grains, and 7.31(log

c.f.u./g) of Magalhães and Pereira, (2010), while it was lower than that results of sugary kefir grains by Pidoux, (1989), where it was 1 x $l0^8$ (c.f.u./g) for yeasts in PDA. The differences in microbial count were caused by different incubation temperature during kefir preparation, these phenomenon were noted in this experiment, as well as at the study conducted by (Paramita, 2008). Other factors also may effect in microbial count in kefir, such as origins of kefir grains, handling and storage methods of kefir grains, in addition to the type and concentration of fermented media. In general, lactic acid bacteria were more numerous than yeast and acetic acid bacteria in milk kefir grains, although fermentation conditions can affect this pattern (Güzel-Seydim *et al.*, 2005).

Our results agreed with the results of the previous studies (Franzetti *et al.*, 1998; Gulitz *et al.*, 2011; Magalhaes *et al.*, 2010; Miguel, 2009; Miguel *et al.*, 2011c; Pidoux, 1989).

Identification and detection of different organisms from water kefir samples are very important in the food science and food industry for hygiene and monitoring purposes.

It is difficult to distinguish between *Leuconostoc lactis* and *Leuconostoc paramesenteroides* by their sugar fermentation patterns (Singh *et al.*, 2006). Identification results indicated that kefir grains from Taiwan contained a diverse spectrum of species and genera of microorganisms including *lactobacilli, lactococci* and *leuconostocs. Lb. kefiranofaciens* and *Lb. kefiri,* isolated from three Taiwanese kefir grains, were common LAB strains observed in kefir grains (Angulo *et al.*, 1993; Ninane *et al.*, 2007; Takizawa *et al.*, 1998), that even totally matched with the RFLP results demonstrated by Mainville et al., (2006). Simova *et al.*, (2002), isolated and identified LAB such as *L. brevis, L. delbrueckii* subsp. *Bulgaricus, L. lactis* and yeast in kefir, results indicated that *L. lactis* was a dominant microbe, 58–70% of the total microflora. A total of 11 lactic acid and non-lactic acid bacteria and yeast species were isolated and identified from kefir, among the isolated bacteria *L. brevis, L. Kefir, Leuconostoc mesenteroides, and Acetobacter aceti* were predominant over the rest of the bacteria, *Candida kefir* and *Saccharomyces cerevisiae* are the most commonly isolated species as compared to the rest or kefir isolated yeast (Motaghi *et al.*, 1997).

Angulo *et al.*, (1993) studied kefir samples of dairy products from eight domiciliary sources in the Galicia region, northwest of Spain, and found the following bacteria: *Lactobacillus brevis, Lactobacillus viridescens, Lactobacillus kefir, Lactobacillus fermentus, Lactobacillus casei* ssp. *rhamnosus, Lactobacillus casei* ssp. *tolerans, Lactobacillus casei* ssp. *pseudoplantarum, Lactobacillus acidophilus, Lactobacillus gasseri, Lactococcus lactis* ssp. *lactis, Leuconostoc* ssp., *Streptococcus salivarius* ssp. *thermophilus.* Among the yeasts, they identified *Torulaspora delbrueckii, and Saccharomyces cerevisae, Saccharomyces unisporus, Candida kefyr, Candida friedrichii, Kluyveromyces lactis,* and *Pichia fermentans*. The authors reported that the important differences in grain composition from the eight sources might be attributed to the different origins of the samples, among other factors. Such interfering factors seem to be mainly geographic and also due to the substratum used for grain proliferation.

The predominant species of microorganisms in kefir sample preparations–Grains in natural and lyophilized and fermented suspension (over 105 cfu/mL) were *Lactobacillus satsunensis, Lactobacillus casei, Lactobacillus plantarum* and *Lactobacillus nagelei* for bacteria, and *Zygosaccharomyes fermentati*, *Saccharomyces cerevisiae* and *Torulaspora delbrueckii* for yeasts (Bergmann *et al.*, 2010).

The predominant genus in water kefirs I and II was Lactobacillus, which accounted for 82.1% in water kefir I and 72.1% in water kefir II of the bacterial isolates. The most abundant species in water kefirs I and II were *Lactobacillus hordei* and *Lb. nagelii* followed by considerably lower numbers of *Lb. casei*. Other lactic acid bacteria (LAB) were identified as *Leuconostoc mesenteroides* and *Lc. citreum* in all three water kefirs. The most abundant species in water kefir III was *Lc. mesenteroides* (28%) and *Lc. Citreum* (24.3%). The *Acetobacter* species were more prevalent in consortium III. Cluster analyses of RAPD-PCR patterns revealed an interspecies diversity among the *Lactobacillus* and *Acetobacter* strains. Additionally, *Saccharomyces cerevisiae, Lachancea fermentati, Hanseniaospora valbyensis* and *Zygotorulaspora florentina* were isolated and identified by comparison of partial 26S rDNA sequences and FTIR spectroscopy (Gulitz *et al.*, 2011).

Our findings are agreed with that study specially in concerning with the presence of each of *Leuconostoc mesenteroides*, *Lactobacillus* and *Acetobacter, Lc. Citreum,* and *Saccharomyces cerevisiae.* But some of microorganisms that were mentioned in that study have not been isolated in the present study.

The main flora of water kefir had been described to be consisting predominantly of *Lb. brevis, Streptococcus lactis* and the *yeast S. cerevisiae* (Horisberger, 1969). Earlier investigations of the water kefir microbial structure, *Lactobacillus casei* was described as a very predominant species in water kefir (Franzetti et al., 1998; Pidoux, 1989). And also *Lactobacillus hilgardii* reported by Pidoux, (1989) to be the most predominant in the sugar kefir grains. This bacterial specie could not be detected in the water kefir and water kefir grains that analyzed in this study.

Acetic acid bacteria were identified in previous microbiological studies of water kefir but only in insignificant quantity (Franzetti *et al.*, 1998). In the contrary of our study where, acetic acid bacteria were found in higher amounts (2.50×10^{7} cfu mL^{-1}) in water kefir. Liu *et al*., (2004), isolated two strains of *L. lactis* from Tibetan kefir grains. *L. lactis* have been seen in good quantity in our study.

Several investigations have explored the microorganisms present in sugary kefir. The bacteria present include *Lactobacillus spp., Lactococcus spp., Leuconostoc spp., Enterobacter spp. and Gluconobacter spp*. and the yeasts present include *Saccharomyces spp., Zygosaccharomyces spp., Hahsenaspora spp., Hanseniaspora spp., Honsenizspora spp. and Candida spp.,* these species were identified across various different groups of sugary kefir grains (Waldherr *et al.*, 2010).

The microflora of Tibetan kefir grains was investigated by culture- independent methods. Denaturing gradient gel electrophoresis (DGGE) of partially amplified 16S rRNA for bacteria and 26S rRNA for yeasts, followed by sequencing of the most intense bands, The results showed that the dominant microorganisms were *Pseudomonas sp., Leuconostoc mesenteroides, Lactobacillus helveticus, Lactobacillus kefiranofaciens, Lactococcus lactis, Lactobacillus kefiri, Lactobacillus casei, Kazachstania unispora,*

Kluyveromyces marxianus, Saccharomyces cerevisiae, and *Kazachstania exigua.* The bacterial communities between three kinds of Tibetan kefir grains showed 78–84% similarity, and yeasts 80–92%. The microflora is held together in the matrix of fibrillar material composed largely of a water-insoluble polysaccharide (Jianzhong *et al.*, 2009). Water kefir in the present study were coincided with Tibetan kefir by presence of *Leuconostoc mesenteroides, Lactobacillus kefiranofaciens, Lactococcus lactis, Lactobacillus kefiri, Lactobacillus casei,* and *Saccharomyces cerevisiae.*

The microbial community of Tibetan kefir grains depends primarily on their source. It has been reported that Tibetan kefir grains contain *Lactobacilli, Lactococci* and yeast, and sometimes acetic acid bacteria, depending on the source of origin (Yang *et al.*, 2007; Zhou *et al.*, 2006).

Later, (Garrote et al., 2001), isolated *Lactococcus lactis ssp. lactis, Lactobacillus kefir, Lactobacillus plantarum, Acetobacter, Saccharomyces, Leuconostoc mesenteroides, Lactococcus lactis ssp. lactis biovar diacetylactis, Lactobacillus parakefir,* and *Kluyveromyces marxianus* in Argentine samples. However, qualitative differences were found in the four samples.

To some extent the differences in the composition of the water kefir consortia may be referred to different identification techniques used. The technique used for identification of lactic acid bacteria and acetic acid bacteria in the investigations done so far was API CH 50 (Franzetti *et al.*, 1998; Pidoux, 1989). Leroi and Courcoux (1996) also isolated less common strains from kefir kept in sugary water: *Lactobacillus hilgardü* and *Saccharomyces florentinus*. These data supports the hypothesis that different culture media and regions can present remarkable differences in the kefir microbiota.

Our results are very close to those of the above-mentioned authors with regard to isolated bacteria and yeasts.

Hsieh *et al.*, (2012), identified the microorganisms present in both the grains and filtrate and then evaluated their distribution. The structure of the grains was also observed by scanning electronic microscopy (SEM). The identification results indicated that there

were remarkable changes in microbial ecological profiles of the sugary kefir grains and their filtrates when brown sugar and milk were compared as fermentation media. Three lactic acid bacteria (LAB) species (*Leuconostoc mesenteroides, Lactobacillus mali* and *Lactobacillus hordei*) were found in the grains fermented using brown sugar. However, four species, named *Leu. mesenteroides, Lactococcus lactis, Bifidobacterium psychraerophilum* and *Enterococcus faecalis*, were identified in the grains fermented using either cow's or goat's milk.

In previous study, on the microorganisms of water kefir from eight Brazilian stats, the results revealed the presence of some bacteria, such as *Lactobacillus casei, lactobacillus kefir, L. rhamnosus, L.satsumensis, Gluconoacetobacter liquefaciens, Acetobacter lovaniensis, Acetobacter pasteurianus, Bacillus cereus*, and *Saccharomyces cerevisiae* (Miguel, 2009). The microorganisms of tibico (sugary kefir) from eight Brazilian stats were investigated, the bacteria genera included *lactobacillus, Acetobacter, Gluconobacter, and bacillus* and yeast strains included, *pichia, saccharomyces, kazachstania, candida, zygosaccharomyces, and yarrowia* (Miguel *et al.*, 2011c). And the microorganisms associated with sugary Brazilian kefir beverage were investigated using a combination of culture-dependent and -independent methods. A total of 289 bacteria and 129 yeasts were identified via phenotypic and genotypic methods. *Lb. paracasei* (23.8%) was the major bacterial isolate identified, followed by *Acetobacter lovaniensis* (16.31%), *Lactobacillus parabuchneri* (11.71%), *Lactobacillus kefir* (10.03%) and *Lactococcus lactis* (10.03%). *Saccharomyces cerevisiae* (54.26%) and *Kluyveromyces lactis* (20.15%) were the most common yeast species isolated (Magalhaes *et al*., 2010).

Our results are very similar to these findings with a few differences in some isolated bacteria and yeasts at level of species, where six of microorganisms that reported in these studies were found in water kefir in the present study which are *Lactobacillus paracasei, lactobacillus kefir, L.satsumensis, Acetobacter pasteurianus, Lactococcus lactis* and *Saccharomyces cerevisiae.*

Probiotics will have functional roles if the amount is minimally at 10^6 cfu, therefore the water kefir has met the requirements of research. However, the actual amount of

probiotic cells viable in a fermented dairy product, at the time of consumption and after transit through the digestive tract, is quite difficult to predict due the many variables and conditions associated with processing and digestion, but because kefir, especially traditionally produced, has a wide range of different bacterial strains and species, the adhesion to the intestinal mucosa might be improved over probiotic products with only one or two bacterial strains. Findings by Collado, *et. al*., (2007) have suggested that different probiotic combinations may enhance beneficial health effects due to synergistic adhesion effects; the combination of complimenting probiotic system and the protective nature incurred by an exopolysaccharide encapsulated cell could greatly increase the efficacy of a probiotic product, such as kefir.

The counts of yeasts in kefir grains decrease slightly in the range from 17 to 19 °C and thereafter increased progressively between (19-25) °C. In the range of temperature higher than 25 °C, there was a progressive decrease in the counts of yeasts, presented in kefir grains. This could indicate that at higher temperatures yeasts started to die (Zajsek and Gorsek, 2009).

The appropriate temperature for ethanol production is 14-15□ (Suihko *et al.*, 1993; Casey *et al.*, 1983). The optimum qO2 (specific oxygen uptake rate) for ethanol production was 14.3 or 66.7 mg . (g cell)$^{-1}$. h^{-l} (Taniguchi *et al.*, 1997).

The ethanol production rate in the early phase of the culture was relatively slow but rapidly increased after 12 h. Fermentation was completed after 24 h and the final concentration of ethanol was 14.2 g/l. For the control, the ethanol production rate increased rapidly after 6 h. The fermentation was completed after 18 h and the final concentration of ethanol was 13.7 g/l (Yu and Zhang, 2003). At lower culture temperatures, higher ethanol concentrations could be obtained after prolonged fermentation time (Nanba *et al.*, 1987).

Kefir drink contains lactic acid, mainly the L(+) form, of about 0.8-0.9%, as well as formic, succinic and propionic acids, CO2, ethyl alcohol, different aldehydes (propionic, acetic) and trace amounts of isoamyl alcohol and acetone (Libudzsis Z. and A., 1990).

Moreover, yeasts synthesize complex B vitamins and hydrolyze milk proteins, using oxygen to produce CO2 and ethanol (Lopitz-Otsoa A. *et al.*, 2006).

L. mesenteroides is another dominant microbe. It's a heterofermentative lactic acid bacteria producing aroma, can degrade lactose to lactic acid, acetic acid, ethanol and carbon dioxide, and degrade citric acid into diacetyl, endowing good flavor. In some countries, *L. mesenteroides*, as well as *L. lactis*, is often used to ferment dairy product, for example, buttermilk (Tamime, 2006).

The maximum specific ethanol production rates were observed between 30 and 45 ▫, and pH 4.0 -5.0 after 72 h incubation with different initial glucose concentrations (Lin *et al.*, 2012). Al-Talibi *et al.*, (1975), observed that the alcohol produced by *Saccharomyces cerevisiae* grown in Iraqi date juice increased with increasing sugar concentration in the juice from 10-25%. The growth pattern of sample treated with *Acetobacter aceti* was characterized by minimal cell concentration for *Saccharomyces cerevisiae* (SC) while *Saccharomyces pastorianus* was less sensitive to the presence of *Acetobacter aceti*. But the non - *Saccharomyces yeasts* (*Hansenula anomela* (HA) and *Candida tropicalis* (CA) indicated higher sensitivity to the presence of *Acetobacter aceti* and therefore was strongly inhibited (Ukwo *et al.*, 2010).

Our results reveal production of ethanol in the first 12 h of fermentation, and then the concentration of alcohol in water kefir was drooped at 18 h, in all of temperature degrees, and it was raised after fermentation period more than 24 h. These results are in coincidence with that of water kefir grain (tibi or tibico) which have been used to produce a refreshing beverage of low alcoholic and acetic content, when the fermentation duration is short, but when the process is prolonged this tibico beverage becomes an alcoholic beverage and later a tibico vinegar (Rubio *et al.*, 1993).

In the present study the minimum concentration of alcohol in water kefir was obtained at 20 °C after 18 h of fermentation, at this time the pH of water kefir was between 4.0 and 5.0 these results are corroborated by the other findings that showed, ethanol yield slightly decreased or was nearly constant with increasing the fermentation period using

media with initial pH 5.5 or 6.5. It was observed that the initial pH 4.0 or 5.0 negatively affected the production of ethanol by this yeast strain as compared to pH 5.5 - 6.5 (Hashem *et al.*, 2013). This study also showed that at the temperature 25 °C, the production of ethanol was low comparing with the highest temperature used, and that exactly what happened in our study where at the low temperature 10, 15, and 20 °C the production of alcohol was lower in comparing the high temperature of fermentation.

Based on our results decreasing of ethanol production in water kefir after 18 h are suggested to be due to the present of Acetobacter aceti in water kefir which consumed ethanol for their growth and the production of acetic acid, this assumption are corroborated by the increasing of acidity of water kefir in this time of fermentation which were duplicated at this time, in addition to that our results are supported by the study of Ukwo *et al.*, (2010), who observed that the inhibition of growth rate of yeast during fermentation was proved to be the result of acetic acid form by the oxidation of ethanol by *Acetobacter aceti* and not from the low pH as indicated by the use of hydrochloric acid.

From the results of present study, we can conclude that there are several factors and conditions to minimize the production of alcohol in kefir. These factors are, the short period of fermentation, the moderate temperature degree 18-20 °C, pH between 4.0 and 5.0, the presence of acetic acid bacteria in high quantities, semiaerobic conditions, and low concentration of sugar 6%.

Acetic acid is an intermediate in citrate metabolism, as well as one of the products of heterofermentative lactose metabolism and it can be produced by acetic acid bacteria by oxidation of ethanol (Østlie *et al.*, 2003; Hugenholtz, 1993).

These pH values in the present work were similar to those previously reported for milk kefir (García *et al.*, 2006; Motaghi *et al.*, 1997), and for novel whey-based kefir beverages (Magalhães *et al.*, 2011a), where, A sharp decrease in the pH was observed during the first 28 h, from an initial value of about 6.1 to 4.3 at 28 h, for all the substrates. Afterwards, the pH decreased slightly, reaching a final value of nearly 4.0. The presence

of acetic acid in the fermented beverages could be attributed to heterofermentative lactic acid and acetic acid cultures present in kefir grains microflora .

The low pH value (3.84) that occurred at the end of fermentation period as a result of the presence of *Lactobacillus* as the major bacterial species in the same time to the presence of *Acetobacter* species, which produce lactic acid and acetic acid respectively in kefir drink. During kefir preparation, lactic acid bacteria will convert lactose of the milk to obtain energy for their maintenance and growth, and released metabolites, primarily lactic acid, result in a decrease in the pH of the environment (kefir). Longer incubation time, means more time available for the lactic acid bacteria to metabolized the lactose of the milk, and so that more lactic acid produced, which contributed to much lower pH surrounding the lactic acid bacteria (Suriasih *et al.*, 2012).

The genus *Lactococcus* tends to grow faster than yeast in milk (Rea et al., 1996; Tamime, 2006). This genus hydrolyzes lactose, producing lactic acid and a suitable environment for yeast growth (Tamime, 2006).

Production of lactic acid has been linked with lactic acid bacteria metabolism and is of great importance due to its inhibitory effect on both spoilage and pathogenic microorganisms in kefir milk (Magalhães *et al.*, 2010).

The values of titratable acidity in the present study were consistent with the Results of (Cetinkaya and Elal mus, 2012) that were a mean of 0.8% L.A, ranging from 0.7% to 1.4% L.A.

In the present study, as expected, while the pH decreased, titratable acidity concentration increased progressively in water kefir during the time of fermentations, as a result of lactic acid and acetic acid formation by bacterial strains in water kefir grains. This agrees with the finding of Güzel-Seydim *et al*. (2000).

The results of chemical structure of water kefir and water kefir grains in the present investigation were similar to those recorded for grains and sugary Brazilian kefir beverage (Magalhães *et al.*, 2010), with a few differences particularly in the value of dry

matter of kefir grains which was 13.1, and in ash percent which was higher with three times in this study. The high acidity and law pH of water kefir were due to the production of lactic acid, acetic acid, CO_2 and alcohol by kefir microorganisms during fermentation process (Bakhshandeh *et al.*, 2011; Güzel-Seydim *et al.*, 2000). Protein value of kefir grains was six-fold that in water kefir and lipids content was twice as much as that in kefir beverage, that can be explained by the accumulation of microorganisms which are the source of these components in kefir grains and their distribution in water kefir. which might be correlated with the increase of microbial biomass during this process and consequently secretion of protein molecules, contributing to nutritional value of sugary kefir (Simova *et al.*, 2006). Average chemical composition of Bali cattle milk kefir was 5.68% protiens, 4.67% lipids, 0.89% Titratable acidity (Suriasih *et al.*, 2012).

Both the bacteria and yeasts are surrounded by a polysaccharide matrix, called kefiran, which is a water-soluble branched glucogalactan, and which has been reported to possess antibacterial, antimycotic and antitumor activity (Lee *et al.*, 2007).

L. bulgaricus HP1 strain, producing the polymer kefiran, was selected. The exopolysaccharide activity of the strain increased (1.7 times approx) in its associated growth with lactobacteria strains + a yeast strain, isolated from kefir grains. The reported results expand the understanding of “kefiran polymer” production by lactic acid bacteria isolated from kefir grains. They can form the basis for further studies on how to increase the exopolysaccharide activity of *L. bulgaricus* HP1, create conditions for intended synthesis of kefiran, as well as define the structure and physicochemical characteristics of the biopolymer (Frengova *et al.*, 2002).

Milk Kefir grains were reported to contain a range of (80- 90%) Water, (4.7-40%) Carbohydrates, (3.2-30%) Proteins, (0.3-1.69) Fats, (0.7-1.2%) Ash, and (10–20%) Dry matters (Abraham and De Antoni, 1999; Anine and Trevor, 2003; Bottazzi *et al.*, 1994; Garrote et al., 2001; Halle´ *et al.*, 1994; La Riviere and Kooiman, 1967; Libudzisz and Piatkiewicz, 1990; Liutkevičius and Šarkinas, 2004; Ottogalli *et al.*, 1973). Kefir grains have the chemical composition 89-90% water, 0.2% lipid, 3% proteins, 6% sugar (mainly polysaccharide) and 0.7% ash (Ozer and Ozer, 2000).

The chemical composition of kefir from different sources can be summaries as follows: Water 87.53- 90.63, Dray matter 5.3- 19.9, Proteins 1.36- 4.72%, Carbohydrates 1.1-18%, Fats 1.4- 3.59%, Ash 0.75-0.86%, Lactic acid 3.44- 7.56%, Acetic Acid 750-820 mg/kg, Ethanol 0.1- 0.94%, Titratable acid 0.25- 1.50%, and pH 3.41- 5.78 (Assadi *et al.*, 2000; Bakhshandeh et al., 2011; Cais-Sokolinska *et al.*, 2008; Chen *et al.*, 2009; Ergönül, 2007; Ertekin and Guzel-Seydim, 2010; Grønnevik *et al.*, 2011; Irigoyen *et al.*, 2005; Purnomo and Muslimin, 2012; Sady *et al.*, 2007).

The information of the chemical structure of water kefir is very poor, but also it seems that there are not data in chemical composition of water kefir grains were reported in the references literatures except that of Magalhães *et al.*,(2010), which only pointed on the moisture and dry matter.

To some extent, the chemical structure of water kefir was in the range of kefir composition that reported for other types of kefir with some differences which is referring to the difference in the types and structures of fermentation substrates which used in kefir manufacturing and the manner of production, in addition to the sources, types and structure of kefir grains.

Copper is widely distributed in biological tissues, where it occurs largely in the form of organic complexes, many of which are metalloproteins and function as enzymes. Copper enzymes are involved in a variety of metabolic reactions, such as the utilization of oxygen during cell respiration and energy utilization (Aras and Ataman, 2006). Calcium is the major component of bone and assists in teeth development, Iron is also required for the activity of certain enzymes involved in energy production and about 10% of the body pool of iron is used in this way (Aras and Ataman, 2006). The complexes of zinc and insulin in varying ratios are stored in pancreatic β-cells and released into the circulation via the portal vein (Scott and Fisher, 1938). Enzymes that do not contain a trace element as an integral part but are activated by metals such as Cu, Fe, and Ni respond to in vitro addition of several transition elements with a dose-dependent activation (Speck, 1949).

The mean dietary intakes of children have been reported to be in the range 9–278 µg of lead per day and for adults 20–282 µg day^{-1}. A typically high dietary intake (e.g. 500 µg of lead per day) was found in one Indian investigation (Aras and Ataman, 2006). According to our results the lead contains in water kefir are adequate and in the save range.

In the dry matter of milk kefir grain the following macro- and microelements were found: macroelements (%): K (1,65); Ca (0,86); P (1,45); and Mg (0,30); microelements (mg/kg): Cu (7,32), Zn (92,7); Fe (20,3); Mn (13,0); Co (0,16); and Mo (0,33) (Liutkevičius and Šarkinas, 2004). Mineral contents of peanut milk kefir (µ/g) Zn 3.15, Fe 3.9, Mn 1.94, Cu 0.48, Na 206, K 712, Mg 225, and Ca 281, while 70% peanut milk kefir Zn 3.07, Fe 3.07, Mn 1.6, Cu 0.61, Na 281, K1063, Mg 187, Ca 630 and in whole-milk kefir Zn 5.86, Fe 1.16, Mn 0.21, Cu 0.78, Na 586, K 1529, Mg 97.7, Ca 1018 (Bensmira and Jiang, 2012).

It can note that water kefir is higher than each of peanut milk kefir and whole milk kefir and their mixture in its content of Na, Zn, Fe, and Cu, while whole milk kefir is higher than water kefir in Ca content, which come from raw milk. The difference of mineral content can be referred to the difference of water kefir types and sources and also to water and can sugar that used in water kefir production.

These results demonstrate that the concentration of major elements and micronutrients in water kefir and kefir grains were in Sufficient or normal ranges according to reference and standards values (Kalra, 1998). Water kefir appeared to contained the macro and micronutrient compounds in appropriate quantities with respect to the limitation of the raw materials that used in it preparation.

In a previous study, it was demonstrated that orally administered kefir not only inhibited tumor growth and induced an apoptotic form of tumor cell lysis, but it also reduced glutathione ferrous-ion chelating ability and superoxide dismutase activity. These findings have indicated that kefirs possess certain functionalities (Liu *et al.*, 2005b).

Natural antioxidants can also be used in nutraceutical applications as supplements (Nishino T and Ishikawa F. 1998). The ingestion of antioxidative supplements, or foods containing antioxidants, may reduce the oxidative damage on the human (Lin and Yen, 1999). Antioxidation activity is increased by fermentation process of foods and the additions of microorganisms. Lactobacillus strains could be useful as starter cultures to provide antioxidants in food and that fermented milk may serve as a delivery vehicle for antioxidative, probiotic lactobacilli from non-dairy sources (OSUNTOKI and KORIE, 2010).

The results of initial test of fresh water kefir drink that produced by fermentation of sugary solution by kefir grains for 24h exhibit that the fresh water kefir drink has ability to scavenge the radical, inhibited the autoxidation of ascorbate, reducing of iron ions, scavenging hydroxyl radicals, and it were also showed good total antioxidant capacity. Where water kefir in fresh state gained acceptable values in comparing with other antioxidants. These results indicate to the antioxidant potentials of water kefir. It is may be referred to the microorganisms and biomolecules that produced in water kefir.

The antioxidative properties of other probiotic strains have also been reported in healthy persons (Naruszewicz, 2002; Chamari, 2008). Studies using animal models of diabetes have also shown that Lactobacillus acidophilus and Lactobacillus casei attenuate oxidative stress and have antidiabetic effects (Harisa, 2009; Yadav, 2007). In an animal study, Yadav *et al.* (2007) reported that probiotic dahi, a fermented milk product containing *L. acidophilus* and *L. casei*, had an antioxidative effect on the liver and pancreas tissues of high fructose–induced diabetic rats and delayed the onset of glucose intolerance, hyperglycemia, and hyperinsulinemia. Recent trial showed that consuming 300 g/d of probiotic yogurt containing *L. acidophilus La5* and *B. lactis* Bb12 improved the antioxidant status and fasting blood glucose in patients with T2DM. These findings suggest that probiotic yogurt is a functional food that can exert antidiabetic and antioxidant properties (Ejtahed *et al.*, 2012).

DPPH radical is organic compound containing nitrogen and has proton free radical. DPPH solutions show a strong absorption at 517-250 nm appearing a deep violet colour.

The absorption vanishes and the resulting decolourization is stoichiometric with respect to degree of reduction (Blois, 1958; Yamaguchi *et al.*, 1998). Hence, this stable DPPH radical is a widely used as a substrate to evaluate the free radical scavenging ability or antioxidant properties of various samples (Ebrahimzadeh *et al.*, 2008; Brand-Williams *et al.*, 1995; Yen and Duh, 1994).

The Immediately increasing of the DPPH radical-scavenging activity of milk and soymilk kefir after kefir grains additions, indicating that some components of the antioxidants contained in the kefir grains were transferred to milk and soymilk, and after the incubation for 32 h, the DPPH radical-scavenging activities of milk-kefir and soymilk-kefir were significantly greater than those of milk and soymilk (2005b). Recent studies have reported the development of antioxidant activity in whey during fermentation and with lactic acid bacteria, and the antioxidant activity is strain-dependent (OSUNTOKI and KORIE, 2010; Sun J. *et al.*, 2010). Other study demonstrated that the DPPH-scavenging activity of soybean can be enhanced through fermentation with a certain microorganism (Sun *et al.*, 2009). And the results of Lin and Chang, (2000) indicate that the radical scavenging ability of the intact cells and intracellular extracts of B. longum and L. acidophilus contributes to the antioxidative effect. The scavenging effects % on DPPH radicals of the LAB fermentation product Seaweed oligosaccharide LAB fermentation product (SwOS-LAFP) seaweed and polysaccharide LAB fermentation product (SwPS-LAFP) were in the range of 36.2 ±2.2 to 79.6 ±1.0 (Wu *et al.*, 2010). The exopolysaccharides from the corn Stover-containing medium presented significantly strong hydroxyl and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity (Xiang *et al.*, 2012).

In this study, we found that Water kefir samples demonstrated excellent radical-scavenging activity when comparing with those of that of soy whey kefir, cow-milk and goat-milk kefir, rice milk kefir and milk whey kefir which showed respectively (>60,>90,40-90,6-30%) DPPH scavenging ratio, after considering the activity of milks and soy milk that was (80,55-60%) (Liu *et al.*, 2005a; Monajjemi *et al.*, 2012; Sirirat and Jelena, 2010). And that results which suggested that this activity may be attributable, in part, to the peptides deriving from milk proteins and soybean proteins (Liu *et al.*, 2005b).

Intracellular cell-free extract of all strains of lactic acid bacteria demonstrated antioxidative activity with inhibition rates of ascorbate autoxidation in the range of 7−12%. Antioxidative mechanisms including metal ion chelating ability, scavenge of reactive oxygen species, enzyme inhibition (Lin and Yen, 1999). It was found that the inhibition rate of cow/soy milk kefirs to inhibit ascorbate autoxidation ranged from 8.34–17.00% depending on soymilk ratio. that can explained by intensive effect of intracellular antioxidants of microorganisms (KESENKAŞ *et al.*, 2011). The development of antioxidant activity was strain-specific characteristic (Virtanen *et al.*, 2007). The ability of fermented soymilk to inhibit ascorbate autoxidation with inhibition rate in the range 11.92–16.38%, varied with the starter organism. and the simultaneous of lactic acid bacteria and *bifidobacteria*, exhibited a significantly higher inhibition rate of ascorbate autoxidation (Wang *et al.*, 2006).

Our results indicate a high ability of water kefir for inhibition ascorbate autoxidation, suggesting that antioxidant activity of water kefir attributed to its contains of lactic acid, acetic acid bacteria, and yeasts, it's also can referred to their simultaneously existing and their intracellular and extracellular metabolites.

Reducing power of compound have described by certain studies to be an important indicator for its own potential antioxidant activity (Liu *et al.*, 2002; Meir *et al.*, 1995). And may be associated with antioxidant activity (Yen *et al.*, 2000).

A number of studies have found that the reducing power of kefir and fermented milk and soymilk were significantly greater than milk and soymilk and suggested that certain metabolites, such as glutathione, that demonstrate superior reducing power might be produced during kefir fermentation and which could react with free radicals to stabilize and terminate radical chain reactions (Wang *et al.*, 2006; Yang *et al.*, 2000). Intracellular antioxidants sourced from kefir microflora, soymilk kefir samples (0:100) had approximately 3 times higher reducing activity (KESENKAŞ *et al.*, 2011)..

Our results conducts that water kefir possess great reducing compounds, which are outcome of the fermentation of sugar solution by kefir grains.

Although the conditions in the gastrointestinal tract are very complicated, the results from the study of Kaizu *et al.,* (1993) showed that the intracellular extract is antioxidative in vivo.

The results obtained from this study showed excellent antioxidant activity of water kefir and suggesting that this activity attributed to the presence of lactic acid, acetic acid bacteria, and yeasts in water kefir, it also can referred to their simultaneously existing, and their intracellular and extracellular metabolites and also to the products of its cell lysis.

So we conclude that water kefir could be an interesting source of natural antioxidants with good potential in health improving. Hence, more research are needed to conclusively prove antioxidant activity of water kefir.

The hydroxyl radical (´OH) constitutes the chemically most reactive species of `activated oxygen' formed by successive monovalent reduction of dioxygen (O2) in cell metabolism, and is primarily responsible for the cytotoxic effects of oxygen in plants, animals and micro-organisms, living in an oxygenic atmosphere (Halliwell and Gutteridge, 1989; Halliwell and Gutteridge, 1992). Hydrogen peroxide H_2O_2 is produced as by products during reduction of O2 to water. The reduction of O_2 to H_2O is a multi-step process. Initially oxygen reacts with one electron, superoxide is produced. If oxygen reacts with two electrons hydrogen peroxide is formed. As such superoxide may not be harmful to cell but it generates free radicals like OH, OR etc., which are extremely toxic to cells. Superoxide, OH, OR, H_2O_2 are collectively called as reactive oxygen species (ROS) (Rao, 2006).

The hydroxyl radical is an extremely reactive free radicals formed in biological systems and has been implicated as one highly damaging species in free radical pathology, capable of damaging almost every molecule found in living cells (Tedesco *et al.*, 2000). Detection of the hydroxyl radical was via a salicylate probe generated from a reaction between an iron(II)-EDTA complex and H_2O_2. The resultant hydroxyl radicals attack both the salicylate probe and the hydroxyl radical scavengers that are incubated in

the solution. Radical scavengers compete with salicylate for the hydroxyl radical produced (Mustafa *et al.*, 2008).

Our results revealed that, water kefir possesses the high hydroxyl radical scavenging ability at all of concentrations. The results of present study indicated that the scavenging activity and the concentration of water kefir are in linear evenness positive relationship, where hydroxyl radical-scavenging activity were increased by the increasing of the concentration of water kefir.

The result of this study suggest that, hydroxyl radical-scavenging activity of water kefir may attributed to the exopolysaccharides that produced by some bacterial strains in water kefir, this assumption previously confirmed (Xiang et al., 2012; Xu *et al.*, 2011) where, exopolysaccharides samples obtained from the two kinds of fermentation media exhibited a hydroxyl radical-scavenging activity in a dose-dependent manner. And Wu, et al., (Wu *et al.*, 2010) who found that, seaweed oligosaccharides fermented by two lactic acid bacteria, appeared to have hydroxyl radicals scavenging capability.

Likewise, hydroxyl radical-scavenging activity of water kefir can be referred to the yeasts of water kefir (Saccharomyces cerevisiae and Candida) where, yeast cell wall polysaccharides, β-D-glucans and α-D-mannans, have been previously demonstrated to reveal antioxidant properties (Križková *et al.*, 2001; Tsiapali *et al.*, 2001).

Microorganisms of water kefir such as, *Lb. delbrueckii ssp. Bulgaricus* can also scavenge hydroxyl radicals, as reported beforehand by Lin and Yen (1999) who demonstrated that *Lb*. *delbrueckii* ssp. *bulgaricus* Lb possesses the highest hydroxyl radical scavenging ability at 234 mM.

The ability of water kefir to scavenging hydroxyl radicals in this study appeared to be greater in comparing with the kefir that produced from Thai Jasmine rice milk (Sirirat and Jelena, 2010), where it was scavenging approximately 70% of hydroxyl radicals by with 25 mg/ml, while in only 5mg/ml of water kefir scavenging approximately 72.65% of hydroxyl radicals.

Total antioxidant capacity was estimated using phosphomolybdenum method based on the reduction of Mo (VI) to Mo (V) by the formation of a green phosphate/Mo (V) complex at acidic pH (0.6 to 1.0) with a maximum absorbance at 695 nm (Miladi and Damak, 2008).

In previous study, 25 bacterial strains demonstrated the radical scavenging activity with inhibition rate in the range of 3–53%. Among the strains *Leuconostoc and Lact. acidophilus ATCC 4356* showed higher inhibition rate in the range of 42–53*%. Lact. lactis strains, Lact. casei a*nd one kefir strain showed low inhibition rate in the range of 3–10%. The inhibition rate found among *Lactobacillus* strains varied from 12% to 42% and among *Lactococcus* strains varied from 3% to 22% (Virtanen *et al.*, 2007). Kaizu *et al.*, (1993) have screened 570 strains and 19 strains had antioxidative activity as determined by an assay using rat liver microsomes and thiobarbituric acid.

Bin and Baojun, (2013) reported, Brewer's yeast extract and 1800 kDa oat β-glucan were found to have the highest antioxidant activity in ORAC system, respectively. Therefore, it is suggested that further work could elucidate the possible antioxidant mechanism of β-glucan. And, he highest antioxidative activity strain screened by TBARS assay was *Lactobacillus sakei* PP6-S.

Certain results had revealed that fermented and unfermented soymilk and strawberry soymilk were free radical inhibitors or scavengers, acting possibly as primary antioxidants. They might have reacted with free radicals, particularly of the peroxy radicals, which are the major propagatous of the autoxidation chain of fat, thereby terminating the chain reaction (Gordon, 1990; Shahidim *et al.*, 1992). The intracellular antioxidantive peptides of the starter organism and their hydrogen-donating ability may also contribute to this increased reducing ability (Sung *et al.*, 2000).

It is reported that, the strain which demonstrated highest ORAC value was *Lactobacillus casei* JCM20024 (Yamamoto, 2009). There are extensively investigated antioxidant and the related antimutagenic/ antigenotoxic and anticancer properties of two cell wall polysaccharides isolated from the industrial yeast strains, *Saccharomyces*

cerevisiae and *Candida* utilis (Kogan *et al.*, 2008). After studying total antioxidative potential of unfermented and fermented product of soymilk and strawberry soymilk with probiotic cultures and yoghurt cultures individually and in combinations (Yadav *et al.*, 2012), concluded that highest antioxidation was shown by combination of probiotic cultures, followed by probiotic culture alone, yoghurt culture alone and unfermented product.

In the present study, Total antioxidant capacity of water kefir were to be highly significant between the various concentrations of water kefir which used, moreover the accumulating of total antioxidant capacity, were geminated by the increasing of water kefir concentrations.

However, from our results, Total antioxidant capacity of water kefir, suggested to be attributed to the bacterial strains, yeasts, and exopolysaccharides of water kefir. The fundamental mechanism underlying hyperglycemia in diabetes mellitus involves the over production of glucose (excessive hepatic glycogenolysis and gluconeogenesis) and or decreased utilization of glucose by the tissues (Latner, 1958).

Streptozotocin (STZ; N-nitro derivative of glucosamine) is a naturally occurring, broad spectrum antibiotic and cytotoxic chemical that is particularly toxic to the pancreatic, insulin producing beta cells in mammals (Hayashi *et al.*, 2006; Takeshita *et al.*, 2006). Streptozotocin [2-deoxy-2-(3-methyl-3-nitrosoureido)- D-glucopyranose] is a naturally produced antibiotic from *Streptomyces achromogenes* (Szkudelski, 2001). STZ-induced hyperglycemia is a widely used experimental model for screening the activity of hypoglycemic agents. In this model, hyperglycemia arises because of irreversible destruction of the b-islet cells of the pancreas by STZ, causing a reduction of insulin secretion. The generation of ROS and the subsequent increase of local oxidative stress, DNA methylation, and protein modification are suggested as the pathophysiological mechanisms of STZ-induced diabetes (Szkudelski, 2001; Beltramini *et al.*, 2006).

STZ-induced diabetes is characterized by severe loss in body weight and this reduction is due to loss or degeneration of structural proteins, as the structural proteins are known a

major contributor to body weight (Rajkumar *et al.*, 1991; Chen and Ianuzzo, 1982). Previous reports show that protein synthesis is decreased in all tissues due to decreased production of ATP and absolute or relative deficiency of insulin (Murray *et al.*, 2009).

This symptom was clear in our results which reveal significant reduction in body weight of diabetics rats comparing that of normal control. These results are in correspondence with the results of Judiono, *et al.*, (2012) who showed that the delta animal weight varied among the groups, except for the positive groups they gained with very small achievement bout 4.01 + 16.82 g. The result of the present study displayed improving in body weight by administration of water kefir, that may be referred to the presence of exopolysaccharides produced in kefir by its microorganisms, this effect of exopolysaccharides was reported in other studies (Kim *et al.*, 2001; Hwang, 2005).

In the contrast of our results, significant differences in body weight and food intake were not found among the experimental groups of KKAy mice and SD rats induced by a low-fiber diet when administrated exopolysaccharide (kefiran) from *lactobacillus kefiranofacins* (Maeda *et al.*, 2004a). While in our study, there were high significant differences between groups of treatment at ($p \leq 0.05$), the multiple comparisons of treatment groups showed that diabetic control group was differed significantly at ($p \leq 0.05$) from all of other groups. Although, there was no significant difference ($p \leq 0.05$) between body weight of rats respects with the time of study.

Our results showed that the rats group that drink 10% of water kefir gained high body weight in comparing with the tow other treated groups, although the increasing of body weight in control group still increasing higher than all groups in the study, this increasing in body weight which observed in parallel with the decreasing of water kefir concentration in drink water, can be referred to the carbohydrates in water kefir that diminish the appetite of rats, and give them satisfying feeling, an then weakening feed uptake by these rats which reflecting in their body weight.

Diseases in which prolonged elevated levels of VLDL, IDL, chylomicron remnants, or LDL occur in the blood (eg, diabetes mellitus, lipid nephrosis, hypothyroidism, and other

conditions of hyperlipidemia) are often accompanied by premature or more severe atherosclerosis. There is also an inverse relationship between HDL (HDL2) concentrations and coronary heart disease, and some consider that the most predictive relationship is the LDL: HDL cholesterol ratio (Peter and Kathleen, 2009). Hypercholesterolemia and hypertriglyceridemia have been reported to occur in streptozotocin diabetics rats (Sharma *et al.*, 1996; Pushparaj *et al.*, 2000).

Lactic acid bacteria fermented food display hypolipidemic effects by inhibiting cholesterol biosynthesis and decreasing lowdensity lipoproteins (Harbers, 1998; Kawase *et al.*, 2000).

Previous research suggests the reduction of inflammatory therapy on **β**-cells in pancreas contributed the synthesis of proinsulin to insulin by increased cell mass and insulin sensitivity (Donath *et al.*, 2009). Hariom , *et al.*, (2007) indicated that, the probiotic dahi-supplemented diet significantly delayed the onset of glucose intolerance, hyperglycemia, hyperinsulinemia, dyslipidemia, and oxidative stress in high fructose-induced diabetics rats, indicating a lower risk of diabetes and its complications. Kefir consumption modulate significantly blood glucose, antioxidants (SOD, Catalase, GPx), peroxidation lipids (MDA), immune response (cytokines IL1, IL6, IL10) and pancreatic**β**-cell function (Judiono *et al.*, 2012).

Lactobacillus, Bifidobacterium and *Streptococcus* strains, improve insulin resistance wild-type male C57BL6 mice (Ma *et al.*, 2008). Lim *et al.*, (2010) concluded that the extract from solid fermented materials stimulates blood glucose absorption into muscle cells, and the PI3kinase/Akt protein pathway is involved in signal transmission. Consumption of Nono (Nigerian fermented milk) produced by wild lactic acid bacteria may be helpful in diabetes management (laleye, igbakin and Akinyanju 2008). Recent findings indicate that specific strains of lactic acid bacterium can be expected to be beneficial for the management of type 2 diabetes (Honda *et al.*, 2012).

Our investigate disclosed that the regular administration of water kefir can expressively lower blood glucose concentration, because of water kefir

exopolysaccharides and water kefir contents of yeast and bacteria, that previously proven to have hypoglycemic activities (Honda *et al.*, 2011; Wu *et al.*, 2011). It also confirmed by the study of maeda, *et al.*, (2004a), where, the result of physiological effects of exopolysaccharide (kefiran) from *lactobacillus kefiranofacins* in their study had a significant effects on lowering blood glucose concentration in KKAy nice and improve defection in constipated SD rats.

The results of present study were in agreement with Yadav *et al.*, (2007) who found that, *Lactobacillus acidophilus* and *Lactobacillus casei* significantly delayed the onset of glucose intolerance, hyperglycemia, hyperinsulinemia and dyslipidemia, and that study which demonstrated that supplementation of the plain kefir with dose about 3,6 cc/200 g bw / day for 30 days in vivo study of Wistar rats STZ induced hyperglycemia, was significantly decreased blood glucose (Judiono *et al.*, 2012) . And the study that made on Lifeway® Low Fat Plain Kefir which showed a significantly lower total glucose AUC compared with the reference meal (glucose solution, $p< 0.0002$). The total blood glucose AUC of subjects consuming strawberry-flavored kefir ($p< 0.0023$) and orange-flavored kefir ($p< 0.0002$) was significantly lower compared with the reference meal (50 g of glucose solution) (Kong, 2009).

The results of our study suggest that polysaccharides that produced in water kefir by its bacteria contribute in the reducing of blood glucose concentration in the rats that drunk water kefir. This assumption is corroborating by the pervious findings which reported that, fermented milk, Kefram-Kefir augmented glucose uptake in L6 myotubes and the results which suggested the possibility of therapeutic use for type 2 diabetes mellitus. The active agent included in water-soluble fraction of Kefram-Kefir is stable at a high temperature and pH 4-10, small molecular in weight and anion charged. It is suggested two pathways of the glucose uptake augmentation by the watersoluble fraction of Kefram-Kefir from the treatment of wortmannin; (1) the active agent in Kefram-Kefir mainly augments glucose uptake via the insulin signaling pathway including PI 3-kinase and activates PI 3-kinase or other upstream molecules in the insulin signaling pathway, (2) the active agent in Kefram-Kefir also activates the PI 3-kinase independent pathway (Teruya *et al.*, 2002). And the study of Hwang *et al.*, (2005) which found that P. baumii

EPS administration led to the diabetogenic effect of STZ and significantly reduced the degree of diabetes. Previous result encouraged us to believe that oral administration of P. baumii EPS may have a potential benefit in preventing diabetes, since pancreatic damage induced by environmental chemicals and other factors is a cause of diabetes (Lo *et al.*, 2004). In addition to that study which reported that the structural feature of mushroom polysaccharides is also linked to the hypoglycemic activity. The (1-4)-linked and/or (1-6)-linked residues in a h-(1-6)-branched (1-3)-h-D-glucan were needed for the hypoglycemic effect in diabetic mice (Kiho *et al.*, 1994).

The supplementation by water kefir revealed a promising potentially to reduce hyperglycemia. The mechanism underlying is probably through its bioactive components such as exopolyssacharide, biomass, antioxidant properties.

Water kefir in our study, showed valuable effects such as antioxidation effects in vitro, enhancing of the antioxidant system in vivo, and also acting as an anti hyperlipidemic. Therefore, we considered that water to be effective for diabetes mellitus.

From the present study, it is suggested that the presence of various inorganic trace elements such as, zinc, copper, iron, potassium, and sodium could account for the hypoglycemic ability of water kefir. Where the role of inorganic elements like Zn, Cr, V, Fe, Cu, and Ni in the improvement of impaired glucose tolerance and their indirect role in management of diabetes mellitus are being increasingly recognized (Narendhirakannan *et al.*, 2005). And Levine *et al.*, (1983) has observed that there is tissue zinc deficiency in genetically obese, insulin-resistant diabetic mice. Also Faille *et al.*, (1983) suggested that abnormal zinc metabolism play a role in the pathogenesis diabetes and/or its complications.

Previous study reported that, in STZ-induced diabetes, the increase in blood glucose levels is usually accompanied by an increase in plasma cholesterol, triglycerides, LDL and VLDL and decreases in HDL (Mitra *et al.*, 1995). The results of Elsayed, (2010) indicated that, supplementation with *Bifidobacterium* and *Lactobacillus acidophilus* alone and in combination significantly decreased the mean value of serum glucose, total

cholesterol, triglycerides, LDL-C and VLDL-C and significantly increased HDL-C and insulin secretion as compared to control groups. And Lb. acidophilus had shown highest (79%) cholesterol removal activity followed by Lb. casei (67%) and Lc. lactis (29%). During the feeding trial, feeding milk fermented with Lb. acidophilus and Lb. casei exhibited decreased plasma total cholesterol levels (~47 and y49 mg/dl, respectively) after 8 days compared with those of control group (~63 mg/dl) (Jain *et al.*, 2009). Hosono and Tono-oka (1995) demonstrated that binding of dietary cholesterol by LAB cells in different fermented milk products varied among strains and species. They also suggested that differences in binding abilities might be due to chemical and structural properties of peptidoglycan layer of bacterial cell wall. In the present study, cholesterol removal ability also differed in different cultures such as Lb. acidophilus had shown highest cholesterol assimilation ability followed by Lb. casei and Lc. lactis. In addition dahi prepared with these bacterial species had shown a potent antidiabetic as well as anti-hypercholesterolemic effect in high fructose diet-fed rats (Yadav *et al.*, 2007; Yadav, 2008).

In this study, we have also observed an increasing in the concentration of TC, TG, LDL, and VLDL and in the same time decreasing of HDL concentration in streptozotocin induced diabetic rats. And Total cholesterol, triglycerides, LDL, and VLDL of the streptozotocin induced diabetes rats treated with water kefir (10%-30%) showed significant reduction, and improve the level of HDL cholesterol as compared to diabetics rats.

These results are in consonance with pervious study that showed supplementation kefir that produced by the fermentation of kefir grains in brown sugar containing distilled water, exerted some beneficial effects on lipoprotein profile, increasing HDL-c and reducing triacylglycerols of mice (Jascolka *et al.*, 2013). The enhancement in HDL-cholesterol that showed in our study is also in agreement with previous studies using milk-kefir and soyamilk-kefir in cholesterol-fed hamsters (Liu *et al.*, 2006b), and of milk fermented by culturing with various lactic acid bacteria and *saccharomyces cerevisiae* on serum and liver lipids of rats fed high cholesterol diets (Tamai *et al.*, 1996).

Similarly, several workers also reported that LAB and their fermented milk products have an ability to reduce media as well as blood cholesterol (Kimoto *et al.*, 2002; Taranto *et al.*, 2003). This might be due to the binding of dietary cholesterol with the bacterial cell wall in the food and/or intestinal tract before cholesterol can be absorbed into the body (Gilliland and Walker, 1990 ; Gilliland *et al.*, 1985).

But the results of study on human did not support the use of kefir in the management of hyperlipidemia. And showed that, neither kefir nor milk supplementation decreased total cholesterol, HDL-cholesterol, LDL-cholesterol, or triglyceride concentrations. Although that study suggested that the rise in propionate was not sufficient to inhibit acetate's actions as a precursor for cholesterol synthesis (St-Onge *et al.*, 2002). Thus, they concluded, the lack of effect of kefir on plasma cholesterol concentrations could have been due in part to a lack of sufficient increase in propionic acid concentrations, they also assumed that, the bacterial content of the kefir was not adequate to cause a sufficient rise in colonic bacterial content, and the type of bacteria present in kefir do not preferentially produce propionate over acetate and hence, would not inhibit cholesterol synthesis and lead to diminished circulating cholesterol levels.

L. casei and *Lactobacillus sp.* group (homologous to *Lb. acidophilus*) were another dominant homofermentative lactobacillus, producing lactic acid to make milk acid. They are probiotics, good at improving the intestinal environment. Our lab obtained a strain of *L. casei,* having effective activity of degrading cholesterol (Xiao and Dong, 2003). Significant variations in the ability to assimilate cholesterol in milk were observed among 6 kefir cultures. The amounts of cholesterol assimilated during 24 h of incubation and 48 h of storage ranged from 10.8 to 5.3 mg/dl of milk or from 84 to 41% of cholesterol in control milk (12.8 mg/dl) (Vujicic *et al.*, 1992).

Kluyveromyces marxianus YIT 8292 exhibited the most potent hypocholesterolemic activity among the yeasts that were tested. *K. marxianus* YIT 8292 significantly decreased not only plasma total cholesterol but also liver total cholesterol when administered as a dietary admixture at a concentration of 3%. In contrast, brewer’s yeast and baker’s yeast, which have been predominantly used for food, did not exhibit

hypocholesterolemic activity even when administered at a concentration of 10%. These results suggest that *K. marxianus* YIT 8292 may be utilized as a novel food material with the ability to contribute to the prevention of hypercholesterolemia (YOSHIDA *et al.*, 2004).

Our results likewise are in corroborate with the pervious study where, the intake of soured kefir was tested in the healthy rabbits to identify its plausible effects in serum cholesterol levels. Rabbits (n=10/group) were fed with kefir grains in natura mixed with reconstituted pelletized industrial rations during 30 days, following their growth and serum lipid assessments (total cholesterol, triglycerides, HDL, LDL, and VLDL) (Bissoli, 2005). The rabbits whom received kefir grains in natura had significantly lesser growth than the control group. Besides, the fraction of total cholesterol and HDL had significant increases, with a mean reduction of the Castelli II index (LDL/HDL ratio) for the kefir group. This datum suggest the increase of total cholesterol as due to the increase of serum HDL, as measured from the rabbit auricular veins. And the result of Cenesiz, *et al.*, (2008), that demonstrate that use of kefir as a probiotic in drinking water increases live weight, lowers total cholesterol and total lipid thus suggesting that its use in human diets may have beneficial effects. Similarly, total cholesterol, triglycerides, LDL-C, and VLDL-C were significantly decreasing by administration of kefiram from *lactobacillus kefiranofaciens* in Stroke-prone spontaneously hypertensive rats (SHRSP) (Maeda H. *et al.*, 2004b).

One mechanism by which colonic bacteria can alter cholesterol concentrations in plasma is via bile acid deconjugation. Deconjugated bile acids are excreted in the feces (Gilliland, 1990). whereas non-conjugated bile acids are recycled to the liver via the enterohepatic circulation. Their excretion in fecal material results in increased cholesterol use for *de novo* bile acid synthesis. Decreased cholesterol synthesis would result in decreased body cholesterol pools. This would in turn lead to lower total to LDL-cholesterol pools and diminish cardiovascular risk.

It is also suggested that reduced serum cholesterol concentration induced by kefir could be attributed to the fact that the deconjugation of bile acids by Lactobacillus spp.

increases a discharge of bile acids, which in turn increases the expenditure of cholesterol to produce bile acids, as well as precipitating cholesterol due to the low pH value of kefir (Brashears *et al.*, 1998; Tamai *et al.*, 1996) In addition, inhibition of 3HMG-Co A which is an intermediate of mevalonate during the synthesis of cholesterol from acetyl-Co A by fermented milk products has been suggested as the reason for the reduced level of cholesterol in serum (Sanders, 2000).

The data to support this mechanism, were obtained in the present study where, each of total cholesterol and other lipids concentrations of the rats that consumed water kefir, showed significant decreasing, comparing with diabetic group.

The increased total cholesterol and triglyceride levels observed in diabetic rats may be the result of impaired liver function caused by the damage done by streptozotocin, which acts either directly or indirectly by enhancing the plasma glucose level (Van Horn, 1996).

HDL-cholesterol was increased as a result of water kefir supplementation. This might reduce the risk of cardiovascular diseases and metabolic syndrome that are usually associated with diabetes mellitus.

In our study, diabetes was associated with increased total cholesterol, triglycerides, LDL, and lipid peroxidation MDA, which is a well-known marker of oxidative stress, and with significant decrease in the antioxidant enzymes catalase, and non-enzymes antioxidant vitamin . In addition, the diabetes caused marked increase in the level of protein carbonyl and significant increase in hydroperoxides diabetic rats.

The result also revealed decreased concentrations of TC, TG, LDL–Cholesterol, and VLDL–Cholesterol in diabetic and supplemented with water kefir compared with diabetic treated and not supplemented. Similar results have earlier been reported

Diabetes is one of the many pathological processes known to be related to an unbalanced production of reactive oxygen species, such as hydroxyl radicals (HO), superoxide anions (O_2) and hydrogen peroxide (H_2O_2). Reactive oxygen species generated during metabolism can enter into reactions that, when uncontrolled, can affect

certain processes leading to clinical manifestations (Kirkova *et al.*, 1995; Mates *et al.*, 2000). The increase in the blood carbonyl level is related to the oxidative damage of protein by elevated oxygen free radicals in the diabetic group. Increased blood malondialdehyde levels also suggest oxygen free radical-mediated damage of the membrane lipid (Qujeq Durdi *et al.*, 2005). Oxidative stress is the result of excess formation and/or insufficient removal of reactive oxygen species (ROS). Increased production of ROS oxidizes unsaturated fatty acids of cell membranes and produces lipid hydroperoxides by initiating a chain reaction (Halliwell and Chirico, 1993). Consequences of oxidative stress are adaptation or cell injury, i.e. damage to DNA, proteins and lipids, disruption in cellular homeostasis and accumulation of damaged molecules (Jakus, 2000).

Montilla *et al.*, (2005) reported that STZ triggered a drop in GSH concentration, an increase in lipid peroxidation levels, and reductions in key antioxidative enzymes in the brain, kidney and plasma.

During the oxidative stress, counter-acting antioxidant system plays role for gene expression of protective enzyme and the synthesis of other antioxidant molecules. To defense against the reactive oxygen species the ability of TAS decreases and maybe according to these levels decline. Use of probiotics may prevent the consumption of cell's antioxidant systems (Castex *et al.*, 2010). Reduced oxidative stress in the diabetic condition has been observed in experimental animals after the administration of certain polyphenols (Sanders *et al.*, 2001). Previous study's results indicate that when no particular oxidative stress occurred, *Pediococcus acidilactici* supplementation in the diet confers a higher overall antioxidant status (TAS) to the shrimp but seemed to have had little effect on the antioxidant defenses (Castex *et al.*, 2009). Starter cultures with free radical scavenger properties would be useful in the food manufacturing industry. They could benefit the consumer by providing another dietary source of antioxidants, or by providing probiotic bacteria with the potential of producing antioxidants during growth in the intestinal tract (Peran *et al.*, 2006; Rivero and Vidal, 2003).

Recent study concluded that Kombucha Tea Ferment (KT) which is a sour beverage prepared from the fermentation of black tea and sugar with a symbiotic culture of acetic acid bacteria and yeasts might protect liver and kidney from oxidative damage induced by exposure to cadmium and/ or γ-irradiation (Ibrahim, 2013).

Some species of *Lactobacillus* (*Lb*.) and *Streptococcus* (*Strep*.) have been reported to produce antioxidative activity (Phadtare, 2004; Carroll and Threadgill, 2007). Dahi (Indian yogurt) prepared with *Lactobacillus acidophilus, Lactobacillus casei* and *Lactococcus lactis* strains had shown a potent antioxidant activity in-vivo in diabetic rats fed for 56 days (Yadav, 2008). Interestingly, all three strains (*Lactobacillus acidophilus, Lactobacillus casei* and *Lactococcus lactis*) exhibited potent scavenging ability of cellular toxic substances i.e. lipid peroxidation end products (MDA) as well as H_2O_2 radicals (Jain *et al.,* 2009). Recent trial showed that consuming 300 g/d of probiotic yogurt containing *L. acidophilus* La5 and *B. lactis* Bb12 improved the antioxidant status and fasting blood glucose in patients with T2DM (Ejtahed *et al.*, 2012).

In the rats that oxidative stress induced by lead, the level of Malondialdehyde, vitamin E, and *β*-carotene were higher than control group, on supplementing kefir the levels become similar to control group, thus kefir may indeed prevent the oxidative damage induced by lead and strengthen antioxidant system (Ozcan and Cenesiz, 2009).

Oral vitamin C and vitamin E has the ability to lower the oxidative stress in eye (Peponis, 2002), and the vascular endothelia function get better in type1 and not type 2 diabetes (Beckman *et al.*, 2003).

Zinc deficiency is associated with insulin resistance, and zinc therapy is capable of modulating insulin action (Kinlaw *et al.*, 1983). Zinc may work as an antioxidant through superoxide dismutase. Zinc deficiency may also decrease the zinc/copper ratio and thereby increase the adverse effects of copper ions on oxidant stress in diabetes. Mn-SOD not only suppresses ONOO- production and tyrosine residue nitration, but also inhibits membrane lipid peroxidation and mtDNA damage (Stojanovi *et al.*, 2005).

In view of low activities of the SOD and GPX in diabetic patients it was concluded that supplementary trace elements such as Selenium, Copper, Zinc and Manganese, the essential components of the enzymes structures may be useful in preventing the development of diabetic complications (Rahbani-Nobar *et al.*, 1999).

The study of Judiono *et al.*, (2012) demonstrated to prove the hypotheses and build a new theory, that oral Clear kefir supplementation of 3,6 cc/200 g bw/ day given for 30 days, significantly affected blood glucose and antioxidants (SOD, GPX, Catalase) of the rats. MDA levels statistically decreased Çoruh Trout (Salmo coruhensis) that treated with different doses of kefir in water (Can *et al.*, 2012). Another study suggest that the mechanism of action of plain kefir in initially lowering blood glucose and pro inflamatory cytokine, also decrease subsequent effect of free radicals, and lipid peroxidation (Hadisaputro *et al.*, 2012).

Malondialdehyde (MDA) is one of many low molecular weight end-products of lipid hydoperoxide decomposition and is the most often measured as an index of lipid peroxidation (de Zwart and *et.al.*, 1999), malondialdehyde (MDA) is ‘ by far ’ the most popular indicator of oxidative damage to cells and tissue (Draper, 1990). The condensation of malondialdehyde with two molecules of 2-thiobarbituric acid (TBA) has been widely used to measure the extent of oxidative deterioration of lipids in biological and food systems (GRAY, 1978; Frankel and Neff, 1983; Melton, 1983).

In this study, we observed increased malondialdehyde amount in the erythrocytes of streptozotocin induced diabetic rats. Increased blood malondialdehyde levels also suggest oxygen free radical-mediated damage of the membrane lipid of diabetic rats, while there are significant decreasing in all of water kefir treated rats, in contrast with the pervious study which reported that blood anti-ox LDL antibodies were also similar in both groups, suggesting that kefir do not affect oxLDL formation in blood (Jascolka *et al.*, 2013).

But our results were agreed with the study of Can *et al.*, (2012), and Judiono *et al.*, (2012), and that study which focused on the effect of kefir on damage of carbon tetrachloride in rat, Güven et al. (Güven *et al.*, 2003), that found that GSH-Px enzyme

activity increased while MDA levels, which increased with carbon tetrachloride, was decreased by kefir.

Regarding to the results of MDA we found decreasing in MDA in all of water kefir treated groups, but it was more decreasing in 20% WK groups in comparing with the others, a previous studies reported that, MDA content has been shown to be unstable due to the presence of aldehyde oxidase in cells (Ward *et al.*, 1985). Also, the carbonyl group of MDA reacts with proteins, nucleic acids and amino groups of phospholipids (Chiu *et al.*, 1989). And the excess MDA produced as a result of tissue injury can combine with free amino groups of proteins (MDA reacts mainly with Lysine residues by Michael addition), producing MDA modified protein adducts (PALMIERI and SBLENDORIO, 2007). These various post-production fates of MDA may partially explain the observed decrease in MDA content in 20% WK groups.

Carbonylation of protein often leads to a loss of protein function, which is considered a widespread marker of severe oxidative stress, damage and disease-derived protein dysfunction (Dalle-Donne *et al.*, 2006). Protein carbonyls may be produced by the oxidation of some amino acid side chains (e.g., in Lys, Arg, Pro and Thr); by the formation of Michael adducts between Lys, His and Cys residues and α,β-unsaturated aldehydes, forming ALEs (Advanced Lipooxidation End Products); and by glycation/glycoxidation of Lys amino groups, forming advanced glycation end AGE products (Dalle-Donne *et al.*, 2003a; Aldini *et al.*, 2005). The generation of carbonyl molecules is the most general and widely used marker of severe protein oxidation both *in vitro* and *in vivo*, with different assays developed for the quantification of these species (Dalle-Donne *et al.*, 2003b). The chemical stability of protein carbonyls makes them suitable targets for laboratory measurements and is also useful for their storage: their stability during storage for 10 years at –80°C has been demonstrated (PALMIERI and SBLENDORIO, 2007). As a marker of oxidative damage to proteins, carbonyls have been shown to accumulate during aging, ischemia/reperfusion injury, chronic inflammation, cystic fibrosis and many of age-related diseases in some organisms (Levine, 2002).

In this study, it was observed an increasing in carbonyl content in the erythrocytes of streptozotocin induced diabetic rats. Furthermore, the increase in the blood carbonyl level is related to the oxidative damage of the protein by increased oxygen free radicals in the diabetic group.

The administration of water kefir showed significant diminishing in protein carbonyl in each of treated groups comparing with diabetic group, the protein carbonyl concentration of 30% WK group was similar with the normal control group, This effect on protein oxidation was also seen in previous in vitro studies that showed protective effects of kefir against oxidative damage of DNA, proteins and lipids (Teruya et al., 2002; Nagira *et al.*, 2002).

The majority of hydroperoxides generated in plasma were recovered in the LDL fraction. Furthermore, when isolated lipoproteins were subject to oxidation initiated by AAPH, very low- density lipoprotein and LDL showed the greatest propensity for hydroperoxide accumulation, whereas HDL seemed relatively resistant. Estimates for plasma and LDL peroxidation based upon techniques which measure total lipid hydroperoxides suggest that levels of hydroperoxides in plasma and LDL are far higher than that those estimates generated by ostensibly more selective techniques Higher levels of hydroperoxides in LDL than those reported by HPLC-chemiluminescence also seem in greater accordance with other available data concerning LDL oxidation. (Nourooz-Zadeh *et al.*, 1996).

The data of this work, correspondingly showed that the hydroperoxide concentration in the diabetic rats, was raised significantly comparing with control rats, this raising is due to the increasing in LDL cholesterol in the same group as a results of cellular damage by streptozotocen. While by the administration of water kefir drink hydroperoxide concentration were drooped in significant amount where it was diminishing in 30 % WK group to be half of that in the diabetic control, this improvement in hydroperoxides occurred in synchronism with the elevation of HDL and diminishing of glucose triglycerides, total cholesterol, and LDL concentrations in rats that have water kefir. This results are in coincidence with the results of recent study that showed that liver from KF

group mice showed a lower concentration of hydroperoxides compared to CT ones (Jascolka *et al.*, 2013).

Catalase is one of the most active antioxidant defense enzymes known for being highly cooperative with SOD and other H2O2 producers at high flux of hydrogen peroxide. Catalase (EC1.11.1.6; hydrogen peroxide: hydrogen peroxide oxidoreductase; CAT) is an iron porphyrin enzyme that catalyzes the breaking down of H_2O_2 to water and oxygen molecule (Patnaik *et al.*, 2013). Catalase is a cytosolic enzyme that converts H_2O_2 to water, and therefore its activity needs to be present when SOD is active. Myeloperoxidase is a peroxisomal enzyme that accelerates the conversion of H_2O_2 to highly reactive singlet oxygen as part of cellular antibacterial function (Weiss *et al.*, 1980).

Kirkman and Gaetani, (1984) demonstrated that mammalian catalase has bound NADPH, which becomes oxidized to NADP, and displaced by unbound NADPH in the course of both preventing and reversing the inactivation of catalase by its own substrate, H_2O_2 (Kirkman *et al.*, 1987). Thus, both means of disposal of H_2O_2 have been shown to be dependent on the availability of NADPH. A variety of studies provides evidence that one mechanism or the other is functioning to remove H_2O_2 in erythrocytes. In a previous study, reported here, they compared normal erythrocytes with those of a single subject with a genetic deficiency of catalase (Gaetani *et al.*, 1989). These results and recent results suggested that catalase disposes of half the H_2O_2 generated in erythrocytes (Gaetani *et al.*, 1994).

In addition to fatty acyl CoA oxidase, that generate hydrogen peroxide. H_2O_2 can generate toxic free radicals. Thus, these enzymes are confined to peroxisomes, where the H_2O_2 can be neutralized by the free radical defense enzyme, catalase. Catalase converts H_2O_2 to water and O_2 (Smith *et al.*, 2005). Hydrogen peroxide H_2O_2 is produced as by products during reduction of O_2 to water. The reduction of O_2 to H_2O is a multi-step process. Initially oxygen reacts with one electron, superoxide is produced. If oxygen reacts with two electrons hydrogen peroxide is formed. As such superoxide may not be harmful to cell but it generates free radicals like OH, OR etc., which are extremely toxic

to cells. Superoxide, OH, OR, H_2O_2 are collectively called as reactive oxygen species (ROS) (Rao, 2006). Peroxisomes are cytoplasmic organelles, similar in size to lysosomes, that are involved in oxidative reactions using molecular oxygen , these reactions produce the toxic chemical hydrogen peroxide (H_2O_2), which is subsequently used or degraded within the peroxisome by catalase and other enzymes (Smith *et al.*, 2005).

SOD and Catalse are the most important enzymes involved in amelioration the effects of oxygen metabolism (Pande and Flora, 2002). Catalase, an enzyme that occurs in almost all aerobically respiring organism, serves to protect cells from the toxic effects of hydrogen piroxides, by catalyzing the hydrogen peroxide to water and oxygen (Moosavi-Movahedi *et al.*, 1987).

Our results demonstrate a significant increasing were occurred in catalase activity in the rats that administrated water kefir with the various concentration, the increasing of catalase activities were in parallel with the concentration of water kefir in drinking water.

The present findings of increased catalase in are consistent with the studies of Jascolka et al., (Jascolka *et al.*, 2013), where catalase activity were reduced in liver of KF mice, and a different study related to effect of probiotics on oxidative stress and antioxidant status, Castex et al. (Castex *et al.*, 2009), determined that CAT activity in digestive gland was decreased by probiotic. In addition to a study on Çoruh Trout (Salmo coruhensis), where reported that, CAT activity, which is one of the antioxidant enzymes, decreased in treatment groups by kefir (Can *et al.*, 2012).

Likewise in this work, there was a sharp decreasing in catalase activity in diabetic rats, the reduction of antioxidant enzymatic activities were, in some cases, associated with decreased oxidative stress and free radical activities. These were based on the fact that the lower oxidative stress and the fewer antioxidant enzymes were produced (Rahmat *et al.*, 2006). The increased catalase activity observed in the lysate of diabetic rats reflects increased production of H_2O_2.

Vitamin C have been shown to possess several antioxidant properties and it is an important water-soluble antioxidant in biological fluids (Anitra and Balz, 1999). Vitamin

C may play an important role in physiological reactions such as mixed function oxidation involving incorporation of oxygen into a biochemical substrate. In addition, this vitamin is considered the most important antioxidant in extracellular fluids and its antioxidant function has been shown to efficiently scavenge superoxide, hydrogen peroxide, hydroxyl, peroxyl and singlet oxygen radicals. Deficiency in vitamin C causes damage of collagen synthesis in cellular basal membranes, structure of mucosal epithelium and increased capillary fragility (McDowell, 1989; Sies *et al.*, 1992; Burtis and Ashwood, 1994). Vitamin C may protect lipids and lipoproteins in cellular membranes against oxidative damage caused by toxic free radicals at early stage. The antioxidant function of vitamin C is related to its reversible oxidation and reduction characteristics. Thus, vitamin C may partially prevent certain types of hepatic cellular damage (McDowell, 1989; Sies *et al.*, 1992; Burtis and Ashwood, 1994; Parola *et al.*, 1992; Netke *et al.*, 1997).

In this study, the concentration of vitamin C in the plasma of the streptozotocin-induced diabetes rats decreased in significant manner in comparing with that of control rats, these results are analogous to results of a previous study that demonstrate that an enhanced oxidative stress is caused in these tissues by diabetes. Vitamin C concentrations of the brain, heart, lung, liver, kidney and plasma of the diabetic rats decreased significantly after 8 weeks compared with those of the control group (SUN *et al.*, 1999). And these study that reported a decrease of vitamin C in plasma of diabetic rats (Young *et al.*, 1995; Garg *et al.*, 1997). The decrease in vitamin C may be ascribed to its enhanced consumption by elevated oxidative stress caused by diabetes as evidenced by augmentation of tissue lipid hydroperoxides. An association between vitamin C and atherosclerosis has been suggested in studies that evaluated the relationship between vitamin C and cholesterol levels (Spittle, 1972; Dubic and Hunter, 1987). Relationship of ascorbic acid with hypocholesterolaemia in man and guinea pigs has been reported (Kurowska *et al.*, 2000). Ascorbic acid levels were also found to be lower in patients with diagnosis of cardiac infarction and diabetes mellitus (Chatterjea and Shinde, 2002).

A positive relation has been demonstrated between high plasma vitamin C level and reduction in complications of diabetes (Harding *et al.*, 2008). Vitamin C plays a central

role in the antioxidant protective system, protecting all lipids undergoing oxidation and diminishing the number of apoptotic cells (Sadi *et al.*, 2008).

In the present investigation, the increasing of vitamin C in the group of rats that administrate water kefir were significantly differ in comparing with the diabetic rats, this increasing in vitamin C concentration may be attributed to the production of vitamin C by the yeast strains that found in water kefir, which are able to synthesize L-Ascorbic Acid from glucose and arabinose, where it was shown that the d-AL-Ox of C*andida albicans* and *Saccharomyces cerevisiae* are able to convert not only l-arabinono-1,4-lactone but also l-galactono-1,4-lactone and l-gulono-1,4-lactone, in vitro, and synthesize vitamin C. (Hancock *et al.*, 2000; Sauer *et al.*, 2004), also, it has been shown that intact *S. cerevisiae* cells accumulate l-AA intracellularly upon incubation with l-galactose, which is most probably due to endogenous d-AL-Ox and d arabinose dehydrogenase activities (Hancock *et al.*, 2000). In additions yeasts are able to synthesize the l-AA 5-carbon analogue d-erythroascorbic acid. Although this substance structurally differs from l-AA, it possesses similar redox characteristics and therefore is thought to carry out l AA like functions in yeasts, e.g. as antioxidant (Huh *et al.*, 1998). These results are supported by the amelioration of lipid profiles in the same rats groups in our previous study, which also can be due to the presence of vitamin C in water kefir, these results are in accordance with the results of Owu *et al.*, (2006), that suggest that the administration of vitamin C in this model of established diabetes mellitus might be beneficial for the restoration of basal metabolic rate and improvement of lipid profile.

Vitamin C and kefir administration clearly reduced the severity of AOM induced liver lesions. Induction of MT expression was observed in the liver and kidneys, particularly in the centrilobular zones and renal cortex, mainly in the distal renal tubules, collecting tubules, Henle's loop, and medulla, respectively. In conclusion, vitamin C and kefir supplementation were found to be able to reduce the severity of hepatotoxic lesions (Sozmen *et al.*, 2005).

From the results of the present study, it revealed that the mechanism of water kefir initially played lowering blood glucose, in fact, it also decreased subsequently on free

radicals, lipid peroxidation, and enhancement of antioxidants in the blood of rats. These abilities are associated to bioactivity of water kefir.

The present study therefore suggest that, Antioxidant activity of water kefir are referred to the their microorganisms and to the polysaccharides that produced by some bacterial strains in kefir where, it were reported that, *Lactococcus lactis* ssp. *cremoris* which produced two EPSs with different sugar compositions and molecular mass: a neutral EPS of 106 Da and a smaller one of 104 Da with negatively charged phosphate groups (Marshall *et al.*, 1995). Another LAB, *Leuconostoc*, produces more biomass in the MRS medium than in the EPS medium, but the quantity of EPS produced in the EPS medium (22.5 g/L) is much higher than in the MRS (14 g/L) (Vijayendra *et al.*, 2008). This suggestion supported by the results of Tsai *et al.*, (2007) that demonstrate that polysaccharides in ASCS have antioxidant properties which may involve up-regulation of GST activity, maintenance of normal GSH/GSSG ratio, and scavenging of ROS.

These results also suggest that despite an increase in the activity of antioxidant enzymes there is an enhanced formation of lipid peroxidation product which could be due to a decrease in non-enzymic antioxidants. In conclusion, oxidative stress is associated with the development and progression of diabetes mellitus.

According to our pervious results of the microbiological and chemical composition of water kefir which revealed the water kefir contents of lactic acid bacteria and therefore lactic acid that produced by it, in addition to the yeasts and their prospective products as ascorbic acid, lactic acid and acetic acid. According to these results and the results of Devbhuti *et al.*, (2013) which found that enhancement MDA content due to etoposide was significantly suppressed when the liver homogenate was incubated with both the drug and antioxidants like ascorbic acid and lactic acid. It can be conclude that both ascorbic acid and lactic acid could significantly reduce the lipid peroxidation induction potential of etoposide due to their free radical scavenging capacity.

6. CONCLUSION

Based on belief in the beneficial effects of probiotics products to human health, and because of the probiotic and symbiotic properties of water kefir, the current study investigated different fermentation conditions to optimize nonalcoholic water kefir production, and the microbial and chemical properties of the produced water kefir determined, and antioxidant activity evaluated in vitro. In vivo study on the antidiabetic and antilipidimic effects of water kefir and its effects on oxidative stress of streptozotocin induced diabetic wistar rats also achieved, the major results of this study can be recapped as follows:

Three distinct microbial populations were identified in water kefir, lactic acid bacteria were the predominant, followed by Gram-negative bacteria from the *Acetobacter* genus and the yeasts. *Lactobacillus* was the most abundant bacterium followed by *lactococcus*, *Acetobacter*, and then *Leuconostoc*. While *Saccharomyces cerevisiae* was the predominant yeast strain. Microorganisms that found in water kefir was determined to include: The Cocci strains (g+) comprised *Pediococcus* and *Enterococcus*, Coccobacilli strains (g+) *is leuconostocs sp* and *Lactococcus*. While the Coccobacilli (g-) are *Acetobacter sp.* The Bacilli strains (g+) that defined in water kefir were: *l. kefiranofaciens, l.kefirgranum, l. bulgaricus, l. parakefir, l. casi, l.kefr*, and *L lactis.* And the yeast strains contains 2 strains: *Candida* and *Saccharomyces*. These data support the hypothesis that different culture media, method, source and regions can present significant differences in the kefir microbiological structure.

Because water kefir has met the requirements as probiotic, and because kefir, has a wide range of different bacterial strains and species, in addition to its yeast strains, kefir come to sight to be the opportune product which is possess the capacity to be symbiotic product with health benefits promising.

The research presented in this thesis, as the first work of its kind to determine the chemical and mineral composition of water kefir, indicated that water kefir contained the macro and micronutrient compounds in adequate quantities with respect to the

limitation of the raw materials that used in it preparation, and have potential as source of nutrients and organic acids, in addition to some important minerals. From the present study, it is concluded that the presence of various inorganic trace elements such as, zinc, copper, iron, potassium, and sodium in water kefir and water kefir grains, could account for the hypoglycemic, antioxidant, capabilities of water kefir.

In the present study the minimum concentration of alcohol in water kefir were obtained at 20 °C after 18 h of fermentation, at this time the pH of water kefir were between 4.0-5.0. Based in our results decreasing of ethanol production in water kefir after 18 h are suggested to be due to the present of *Acetobacter aceti* in water kefir which consumed ethanol for their growth and the production of acetic acid. From the results of present study, we can conclude several factors and conditions to minimize the production of alcohol in kefir which are, the short period of fermentation, the moderate temperature degree 18-20 °C, pH between 4.0 and 5.0, the presence of acetic acid bacteria in high quantities, semiaerobic conditions, and low concentration of sugar 6%.

Concerning antioxidant activity, the obtained results revealed high ability of water kefir for inhibit ascorbate autoxidation, DPPH scavenging, and reducing power. The result of this study also demonstrate hydroxyl radical-scavenging activity of water kefir, total antioxidant capacity of water kefir were to be highly significant between the various concentrations of water kefir which used, moreover the accumulating of total antioxidant capacity, were geminated by the increasing of water kefir concentrations. The results displayed excellent antioxidant activity of water kefir and suggesting that this activity attributed to the presence of lactic acid, acetic acid bacteria, and yeasts in water kefir, it also can referred to their simultaneously existing, and their intracellular and extracellular metabolites and also to the products of its cell lysis.

So we conclude that water kefir could be an interesting source of natural antioxidants with good potential in health improving.

Consumption of water kefir in (10%-30%) concentrations for 5 weeks has shown beneficial effect not only on blood glucose but also on body weight and lipid profiles of streptozotocin- induced diabetic rats. The mechanism underlying is probably through its

bioactive components such as exopolyssacharide, biomass, antioxidants such as lactic acid, acetic acid and ascorbic acid.

Total cholesterol, triglycerides, LDL, and VLDL of the streptozotocin induced diabetes rats treated with water kefir (10%-30%) showed significant reduction, and improve the level of HDL cholesterol as compared to diabetics rats. It is also suggested that reduced serum cholesterol concentration induced by kefir could be attributed to the fact that the deconjugation of bile acids by *Lactobacillus spp.* increases a discharge of bile acids, which in turn increases the expenditure of cholesterol to produce bile acids, as well as precipitating cholesterol due to the low pH value of kefir The data to support this mechanism, were obtained in the present study where, each of total cholesterol and other lipids concentrations of the rats that consumed water kefir, showed significant decreasing, comparing with diabetic group. HDL-cholesterol was increased as a result of water kefir supplementation. This might reduce the risk of cardiovascular diseases and metabolic syndrome that are usually associated with diabetes mellitus.

The administration of water kefir showed significant increasing in protein carbonyl, lipid peroxidation MDA, improvement in hydroperoxides occurred in synchronism with the elevation of HDL and diminishing of glucose triglycerides, total cholesterol, and LDL, significant increasing were occurred in catalase activity, the increasing of catalase activities were in parallel with the concentration of water kefir in drinking water. The increasing of vitamin C in the group of rats that administrate water kefir were significantly differ comparing with the diabetic rats, this increasing in vitamin C concentration may be attributed to the production of vitamin C by the yeast strains, which are able to synthesize L-Ascorbic Acid from glucose and arabinose. It can be conclude that both ascorbic acid and lactic acid could significantly reduce the lipid peroxidation induction potential of etoposide due to their free radical scavenging capacity.

And biomarkers of antioxidant status have stronger positive associations with dietary intake of antioxidants as water kefir.

In the present study, diabetes was associated with increased total cholesterol, triglycerides, LDL, and lipid peroxidation MDA, which is a well-known marker of oxidative stress, and with significant decrease in the antioxidant enzymes catalase, and non-enzymes antioxidant vitamin . In addition, the diabetes caused marked increase in the level of protein carbonyl and significant increase in hydroperoxides diabetic rats.

As overall conclusion, according to our results of the microbiological and chemical composition of water kefir which revealed water kefir contents of lactic acid bacteria and acetic acid bacteria, in addition to the yeasts and their prospective products such as ascorbic acid, lactic acid acetic acid, and exopolysaccharides. And from the results of *in vitro* and *in vivo* studies, which revealed that the mechanism of water kefir initially played lowering blood glucose, in fact, it also decreased subsequently on free radicals, lipid peroxidation, and enhancement of antioxidants in the blood of rats. These abilities are associated to bioactivity of water kefir. Water kefir, exhibited valuable effects such as antioxidation effects *in vitro*, enhancing of the antioxidant system *in vivo*, and also acting as an anti-hyperlipidemic and anti-diabetic. Therefore, water kefir can consider effective agent against diabetes mellitus, and other diseases.

Water kefir was found to be less cost hypoglycemic, antioxidant, and hypolipidemic effective and less time consuming. Therefore water kefir can potentially be a useful food choice for patients with diabetes who are required to control their blood glucose levels, and also for diminishing the risks of cardiovascular disease. These results also would suggest that the water kefir can be thought for weight control therapies and prophylactic actions against dyslipidemies. And because of water kefir antioxidant activity, it recommended to be consumed continually for prevention from several diseases such as cancer.

In additions, good hygienic practices should be applied throughout manufacture and at retail to ensure that contamination and pathogen growth do not occur in kefir.

Since the current study was the first comprehensive work on water kefir and its activities on the health, it fashioned a heads of filaments for new investigations, and opened board prospects for new scientific research. So, further studies are required to

determine anticancer, antimicrobial, and other effects of water kefir and water kefir grains. more research also are needed to conclusively prove antioxidant activity of water kefir. And several technics of microbial analysis are required to determine all of microorganisms in water kefir, additionally chemical analysis are wanted to measuring the composition of water kefir from amino acids, fatty acids, vitamins, organic acids, minerals, and other biomolecules.

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Annexes

Annex 1 Morphological and physiological properties of cocci strains in water kefir

Colony Number		1	2	3	4	5
Number of isolate		5	3	8	3	5
Media Culture		MBM, LIA	leucon Acetate, Azide Agar	AAB, Hyor ’s, YGA	Lee’s, Azide Agar	M17,MRS, Acetate, Agar
Colony Morphology (from agar plates)	Size(mm)	V	M 5.7 mm	tiny	M	Small 2 to 3 mm
	Shape	Spherical	Circular	Plated, Adhere	Spherical	Spherical
	Elevation	raised	Convex	Convex	Convex	raised
	Edge	irregular	entire marginated , flat	entire	entire	edges
	Color and Opacity	White pale yellowish	Pale Yellow pink ,shiny , semitrans	blue conducted. pale trans	Pale trans, bright orange	White iridescent , grayish thin
	Surface texture	Smooth	slimy Smooth	shiny reflex smooth	Smooth	moist smooth
Cell Morphology	Shape	Cocci spherical	coccobacilli	Coccobacil (ellipsoidal to rodshaped) ovoid	Cocci ovoid	Cocci
	Cell size (µm)	m	m	0.6 - 1.8 µm	0.5 - 1.5 µm	M-l
	Arrangement	pair + short chains	Single, pairs	singly, in pairs, or in chains rar	pair + short chains	singly, Pairs, Tetrad,

	Capsule staining	Non capsulated	Non capsulated	Non capsulated	Non capsulated	Non capsulated
Gram reaction (+/-)		+	+	-	+	+
Catalase reaction(+/-)		-	-	acetia + A. pasteurianusa–	-	-
Growth at	**4°C**	+-	w	-	-	-
	10 ˚C	+	+	w	+	w
	45 ˚C	+-	-	-	-	+
Resists	**30 min at 60˚C**	+	-	-	-	-
Growth in	**6.5% NaCl**	+	+ L. *mesenteroides* - cremoris	nd	-	-
	8% NaCl	+	-	-	-	-
	0.1% MBM	+	-	-	-	-
	pH 4	-	-	-	-	+
	pH 7.2	+	+	+	+	+
	pH9.6	+	-	-	-	-
	4%-10% Ethanol	-	-	A. acetia – pasteurianusa +	-	-
Motility		No	yes	no	no	N0
Oxygen Requirem		F	F	aerobic	F	aerobic
Spore forming		No	No	No	No	No
CO2 from glucose		+	+	+	+	-
Arginine		+	-	-	-+	+
Gelatin liquefaction		-	-	+	-	-

B

Milk curdle	+	+	+	+	+
Reducing AAB to Yellow	w	w	+	w	w
Bacterial type	**Enterococcus**	**leuconostoc**	**Acetobacter**	**Lactococcus**	**Pediococcus**

Annex 2 Morphological and physiological properties of lactobacilli strains in water kefir

Colony number		6	7	8	9	10	11
Number of isolate		13	8	4	9	3	6
Media culture		**MRS**	**MRS**	**MRS ,** Lee's,LB Agar	**MRS**	**MRS**	**MRS**
Colony Morphology (from agar plates)	**Size(mm)**	M-L m-l(0.3-3mm)	M-l(0.5-3mm)	M (0.5-2.5mm)	M (0.5-2.0 mm)	V small	V small
	Shape	Circle	Spherical	Spherical	Circular, irregular	spherical adherence	Spherical
	Elevation	convex	Convex, bulging	Convex irregular	flat	-	Plain
	Edge	irregular	entire, condensed	entire	entire	entire	entire
	Color and Opacity	Pale , transparent to , translucent	White, opaque	White-yellow, gray, sparkling	opaque, and white	Pale, yellow	Pale
	Surface texture	Smooth, rough cruddy	Smooth m dry	Smooth slimy	Smooth creamy	slimy	rough
Cell Morphology	**Shape**	Rod	Rod	rod	Rod	rods	Rod
	Cell size (µm)	M-tall	S-m	thin, long	short	v. Short	m

	Arrangement	Single, pair, short chain	Single, pair, short chain	Pair, tall chains	Single, pair	Single, pair, short chain	Single, pair
	Capsule	capsulated.	Non-capsulated	Non-capsulated	Non-capsulated	Non-capsulated	not
	End	truncate	truncate	Round	round	pointed	rounded
	Sides	parallel bulging	parallel	parallel	bulging	concave	Straight
Gram reaction (+/-)		**+**	**+**	**+**	**+**	**+**	**+**
Catalase reaction(+/-)		**_**	**-**	-	**-**	**-**	**-**
Growth at	**4°C**	**_**	**-**	**-**	**-**	**-**	**-**
	10 ˚C	**w**	**-**	**w**	**-**	**+**	**-**
	45 ˚C	-	-	+-	-	-	w
Resists	**30 min at 60˚C**	**-**	**-**	**-**	**-**	**-**	**-**
Growth in	**6.5% NaCl**	**_**	**-**	**-**	**-**	**-**	**-**
	8% NaCl	**_**	**-**	**-**	**-**	**-**	**-**
	0.1% MBM	**_**	**-**	**-**	**-**	**-**	**-**
	pH 4	**_**	**-**	**+,w**	**w**	**+**	**+**
	pH 7.2	**+**	**+**	**+**	**+**	**+**	**+**
	pH9.6	**-**	nd	**-**	nd	**-**	**-**
	4%-10% Ethanol	**-**	**-**	**-**	**-**	**-**	**-**
Motility		no	non	non	non	non	Non
Oxygen Requirem		F	F	F	F	F	F
Spore forming		NO	NO	NO	NO	NO	NO

Arginine		-	-	-	+	+	+
Gas from glucose		+	+	+	+	+	+
Gelatin liquefaction		+	+	-	+	+	+
Milk curdle		+	+	+	+	+	+
Reducing AAB to Yellow		-	-	-	-	-	-
Bacterial type		l. kefiranofaciens	*l.kefirgranum*	*l. bulgaricus*	l. parakefir	l. casi	l.kefr

Annex 3 Morphological, physiological, and biochemical analysis of isolated yeasts from water kefir.

Colony number	1	2
Number of isolate	8	11
Surface	rough, granular	smooth
Margin	Crispulate, Adhere	entire
Colour	white	white
Elevation	convex	convex
Size	m	s-m
Cells	oval cells with multilateral budding	large oval cells with multilateral budding; simple pseudohyphae spheroidal. cylindrical
colony + - - - -	*Candida*	*Saccharomyces*
Sugar assimilation		
Maltose	+	+
Galactose	+	+
Sucrose	+	+
Lactose	-	-
Raffinose	-	+
Growth in		
Ethanol	+	+
Methanol	-	-
10%NaCl	+	+
16%NaCl	-	-
0.01%Cyclohexamide	+	-
Citrate	+	-
Sugar fermentation	-	+
Raffinose	+	+

Maltose	+	+
Glucose	+	+
Galactose	+	+
Sucrose	+	+
Lactose	−	−
Starch	−	+
Growth at 30oC	+	+
Growth at 37oC	−	+
Growth at 40oC	−	+

Annex 4.1 The results of statistical analysis of alcohol production

Annex 4.1. ANOVA of Temperature degrees						
		Sum of Squares	df	Mean Square	F	Sig.
10 C	Between Groups	42.762	5	8.552	458.895	.000
	Within Groups	.224	12	.019		
	Total	42.986	17			
15 C	Between Groups	131.727	5	26.345	417.663	.000
	Within Groups	.757	12	.063		
	Total	132.484	17			
20 C	Between Groups	49.993	5	9.999	365.601	.000
	Within Groups	.328	12	.027		
	Total	50.322	17			
25 C	Between Groups	295.539	5	59.108	1313.953	.000
	Within Groups	.540	12	.045		
	Total	296.078	17			
37 C	Between Groups	220.784	5	44.157	413.438	.000
	Within Groups	1.282	12	.107		
	Total	222.066	17			

Annex 4.2. ANOVA of alcohol production during time

		Sum of Squares	df	Mean Square	F	Sig.
12	Between Groups	48.778	4	12.195	344.676	.000
	Within Groups	.354	10	.035		
	Total	49.132	14			
18	Between Groups	20.136	4	5.034	116.294	.000
	Within Groups	.433	10	.043		
	Total	20.569	14			
24	Between Groups	47.886	4	11.971	444.044	.000
	Within Groups	.270	10	.027		

	Total	48.155	14			
48	Between Groups	87.080	4	21.770	412.520	.000
	Within Groups	.528	10	.053		
	Total	87.608	14			
72	Between Groups	199.174	4	49.793	783.737	.000
	Within Groups	.635	10	.064		
	Total	199.809	14			
84	Between Groups	221.287	4	55.322	612.870	.000
	Within Groups	.903	10	.090		
	Total	222.190	14			

Annex 5 statistical results of water kefir acidity

Annex 5.1.Descriptive result of acidity and PH

Estimates

Dependent Variable	time	Mean	Std. Error	95% Confidence Interval	
				Lower Bound	Upper Bound
pH	0	6.697	.050	6.589	6.805
	12	5.917	.050	5.809	6.025
	18	4.953	.050	4.845	5.061
	24	4.360	.050	4.252	4.468
	48	4.060	.050	3.952	4.168
	72	3.843	.050	3.735	3.951
Acidity	0	.024	.005	.014	.034
	12	.042	.005	.032	.052
	18	.067	.005	.057	.077
	24	.077	.005	.067	.087
	48	.100	.005	.090	.110
	72	.158	.005	.048	.068

Annex 5.2. ANOVA of acidity

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.011	5	.002	33.191	.000
Within Groups	.001	12	.000		

Annex 5.2. ANOVA of acidity

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.011	5	.002	33.191	.000
Within Groups	.001	12	.000		
Total	.012	17			

Annex 5.3. ANOVA of pH

ph

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	19.042	5	3.808	516.593	.000
Within Groups	.088	12	.007		
Total	19.131	17			

Annex 5.4.Correlations between pH and acidity

		ph	acidity
ph	Pearson Correlation	1	-.795-**
	Sig. (2-tailed)		.000
	N	18	18
acidity	Pearson Correlation	-.795-**	1
	Sig. (2-tailed)	.000	
	N	18	18

**. Correlation is significant at the 0.01 level (2-tailed).

Annex 6 Descriptives of WK DPPH

Annex 6.1. Descriptives of WK DPPH

				95% Confidence Interval for Mean			
	Mean	Std. Deviation	Std. Error	Lower Bound	Upper Bound	Minimum	Maximum
.13	9.7500	.36290	.20952	8.8485	10.6515	9.34	10.03
.63	10.3667	.60699	.35044	8.8588	11.8745	9.89	11.05
1.25	15.1367	.33710	.19462	14.2993	15.9741	14.78	15.45
1.88	22.6167	.52444	.30278	21.3139	23.9194	22.27	23.22
2.50	27.0633	1.09769	.63375	24.3365	29.7902	25.87	28.03
3.75	44.3133	.70868	.40916	42.5529	46.0738	43.65	45.06
5.00	63.5333	.97705	.56410	61.1062	65.9605	62.79	64.64
Total	27.5400	18.85052	4.11352	18.9593	36.1207	9.34	64.64

Annex 6.2. ANOVA

WK DPPH

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	7099.744	6	1183.291	2332.855	.000
Within Groups	7.101	14	.507		
Total	7106.845	20			

Annex 7 WK inhibition% of ascorbate autoxidation results

Descriptives

Annex 7.1. WK inhibition% of ascorbate autoxidation

	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
				Lower Bound	Upper Bound		
.13	6.0800	.21517	.12423	5.5455	6.6145	5.87	6.30
.63	8.0833	.40673	.23483	7.0729	9.0937	7.76	8.54
1.25	12.9733	.74333	.42916	11.1268	14.8199	12.12	13.48
1.88	15.8167	.46522	.26860	14.6610	16.9723	15.45	16.34
2.50	20.7567	.32083	.18523	19.9597	21.5537	20.45	21.09
3.75	22.1967	.80525	.46491	20.1963	24.1970	21.28	22.79
5.00	25.5700	.76707	.44287	23.6645	27.4755	24.87	26.39
Total	15.9252	6.97558	1.52220	12.7500	19.1005	5.87	26.39

ANOVA

Annex 7.2. WK inhibition%

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	968.534	6	161.422	486.952	.000
Within Groups	4.641	14	.331		
Total	973.175	20			

Annex 8. WK Reducing power

Descriptives

Annex 8.1.WK Reducing power

	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
				Lower Bound	Upper Bound		
.13	.3153	.01528	.00882	.2774	.3533	.30	.33
.63	.3960	.00458	.00265	.3846	.4074	.39	.40
1.25	.9440	.00954	.00551	.9203	.9677	.93	.95
1.88	1.1574	.07759	.04480	.9647	1.3501	1.07	1.22
2.50	1.3947	.01079	.00623	1.3679	1.4215	1.39	1.41
3.75	1.8507	.13144	.07589	1.5242	2.1772	1.77	2.00
5.00	1.9690	.11225	.06481	1.6901	2.2479	1.87	2.09
Total	1.1467	.61888	.13505	.8650	1.4284	.30	2.09

Annex 8.2. ANOVA

WK Reducing power

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	7.587	6	1.265	243.460	.000
Within Groups	.073	14	.005		
Total	7.660	20			

Annex 9 Results of Hydroxyl radical activity of water kefir

Report

Annex 9.1. descriptive

VAR00001	Mean	Std. Deviation	Std. Error of Mean	N
.13	11.0785	.13469	.07777	3
.63	26.6269	1.72366	.99515	3
1.25	37.2323	1.16746	.67403	3
1.88	48.8152	.55715	.32167	3
2.50	54.9394	1.50589	.86943	3

3.75	61.7455	.09640	.05566	3
5.00	72.6457	2.98082	1.72098	3
Total	44.7262	20.18655	4.40507	21

Annex 9.2. ANOVA Table of hydroxyl radical

			Sum of Squares	df	Mean Square	F	Sig.
	Between Groups	(Combined)	8118.286	6	1353.048	598.512	.000
		Linearity	7424.015	1	7424.015	3283.966	.000
		Deviation from Linearity	694.271	5	138.854	61.421	.000
	Within Groups		31.650	14	2.261		
	Total		8149.936	20			

Annex 10 Total Antioxidant Capacity

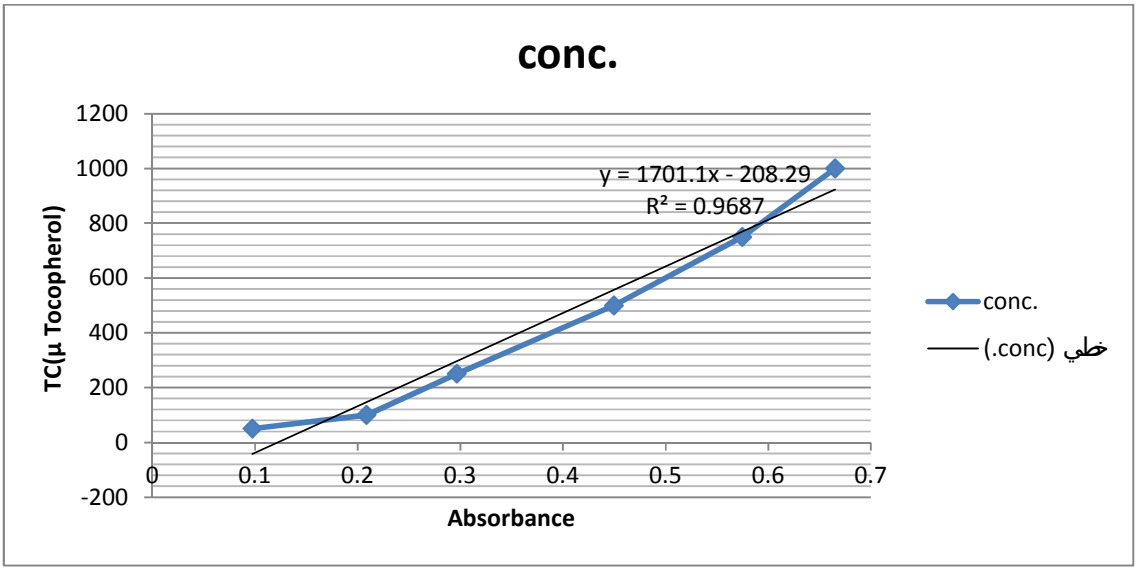
Annex 10.1.Report

VAR00001

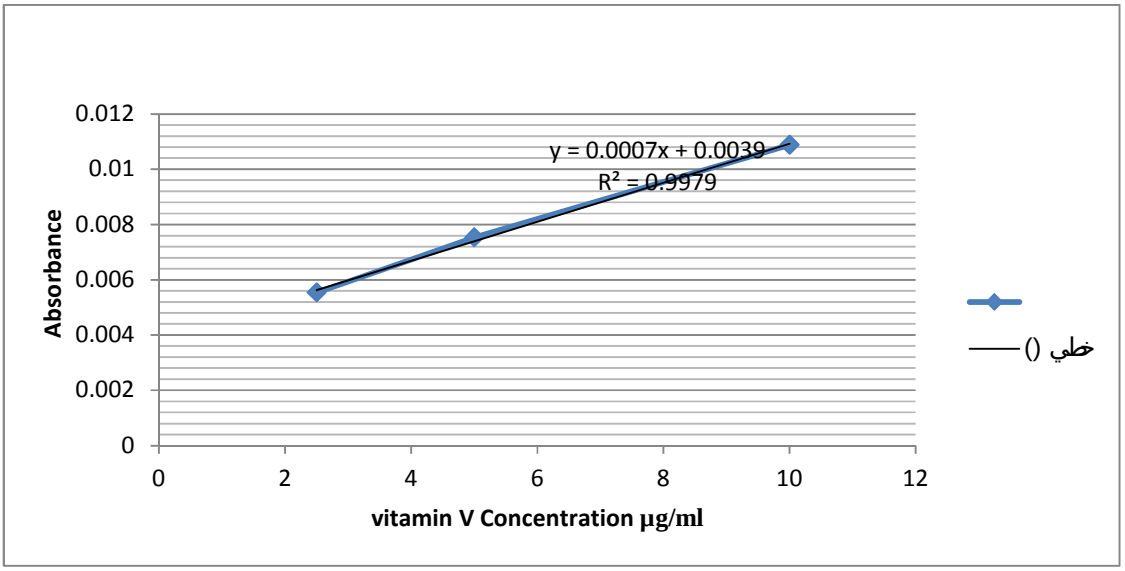
VAR00006	Mean	Std. Deviation	Std. Error of Mean	N
1.00	232.5784	61.26243	25.01028	6
2.00	501.6357	73.79455	30.12650	6
3.00	653.1956	159.05317	60.11645	7
4.00	757.2544	171.34984	76.62998	5
5.00	999.4910	198.22013	80.92303	6
Total	625.3624	291.33693	53.19060	30

Annex 10.2. ANOVA Table Of Total Antioxidant Capacity

			Sum of Squares	df	Mean Square	F	Sig.
VAR00001 * VAR00006	Between Groups	(Combined)	1949758.801	4	487439.700	23.816	.000
		Linearity	1907198.935	1	1907198.935	93.183	.000
		Deviation from Linearity	42559.866	3	14186.622	.693	.565
	Within Groups		511680.236	25	20467.209		
	Total		2461439.037	29			



Annex 11. Standard curve of tocopherol (total capacity antioxidant)



Annex 12. Standard curve of vitamin C

Annex 13.**ANOVA of Stress oxidative biomarkers**

A. MDA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	6.852	4	1.713	3.711	.038
Within Groups	5.078	11	.462		
Total	11.929	15			

B. Hydroperxide

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	13.411	4	3.353	2.329	.121
Within Groups	15.835	11	1.440		
Total	29.246	15			

C. Proteincarbonyl

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	11.142	4	2.786	.609	.664
Within Groups	50.293	11	4.572		
Total	61.436	15			

D. Catalase

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3270.064	4	817.516	1.366	.307
Within Groups	6581.843	11	598.349		
Total	9851.907	15			

E. Vitamin C

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	555.711	4	138.928	2.880	.074
Within Groups	530.585	11	48.235		
Total	1086.296	15			

الحمد لله الذي بنعمته تتم الصالحات

الملخص

كيفير الماء منتج تكافلي يصنع بتخمير محلول سكري بواسطة مزرعة من البكتيريا والخمائر مجتمعه في قالب من السكريات المتعددة تدعى (حبوب الكيفير). يعتقد انه يحتوي على الكثير من المواد الفعالة حيويا، و يستطيع المساهمة تحسين صحة الانسان. هدفنا في هذه الدراسة من خلال تعيين ظروف التخمر المثلى (الزمن، الاس الهيدروجيني، درجة الحرارة) الى إنتاج كيفير خالي من الكحول. علاوة على ذلك هدفنا الى تقييم التأثيرات المضادة للأكسدة والايضية لهذا المنتج. وتم فيها عزل وتعريف الاحياء المجهرية بناء على الاختبارات المورفولوجية و البيوكيميائية. ثم تم تقييم الفعالية المضادة للأكسدة علي مستوي المختبر بخمس طرق هي: الدي بي بي أتش، و تثبيط اكسدة الاسكوربات الذاتية، والقوة الاختزالية، و الهيدروكسيل الحر والسعة الكلية المضادة للأكسدة. وتم حث مرض السكري لدى فئران ويستار البالغة بالحقن داخل التجويف البطني (داخل الصفاق) بمادة الاستربتوزوتوسين، وأعطيت الكيفير لمدة خمسة اسابيع، وتم تقدير وزن الجسم ونسبة الجلوكوز ومستوى دهون الدم. كما تم تقدير دلائل الضغط التأكسدي وهي: المالونداياالديهيد، و الهيدروبيروكسيدات والبروتين كربونيل وفعالية الكاتالاز وفيتامين سي. تم التعرف على ثلاثة مجموعات رئيسية من الاحياء المجهرية في شراب الكيفير، شملت بكتيريا حمض اللاكتيك، وبكتيريا حمض الخليك، والخمائر. وأظهرت النتائج كميات هامة من المواد المغذية والمعادن في كيفير الماء، وأبدي كيفير الماء فعالية ممتازة ضد الأكسدة، كما بينت النتائج تحسنا معنويا في وزن الجسم ونسبة جلوكوز ودهون الدم والدلائل الحيوية للضغط التأكسدي لدي الفئران المعاملة مقارنة مع الفئران المريضة بالسكري والكنترول. تقود هذه النتائج الى إستنتاج أن كيفير الماء يمكن ان يكون منتج تكافلي واعد من الأحياء المجهرية النافعة، والمركبات الفعالة حيويا، ومضادات الاكسدة الطبيعية، ذو قدرة ممتازة على تحسين الصحة والمحافظة عليها. كما أن كيفير الماء لديه امكانية ممتازة كغذاء وظيفي مفيد لمرضى السكري، للسيطرة علي مستوى جلوكوز ودهون الدم وتحسين النظام الدفاعي في الجسم ضد الضغط التأكسدي.

الكلمات المفتاحية:

كيفير الماء الخالي من الكحول، مضاد ارتفاع السكر، مضاد زيادة الدهون، مضادات الاكسدة، المنتج التكافلي، فئران ويستار.

Abstract

Water Kefir is a symbiotic product made by sugar solution fermentation using a culture of bacteria and yeast held in a polysaccharide matrix called (kefir grains). It is believed to contain many bioactive substances, and it may contribute in human health improving. By optimize the fermentation conditions (Time, pH, Temperature), we tried to produce nonalcoholic kefir. Furthermore we aimed to investigate antioxidant and metabolic effects of this product. Microorganisms were isolated and identified on the basis of morphology and biochemical tests. We Also, determined the nutrients and minerals contents of this product. Antioxidant activity *in vitro* evaluated using five methods: DPPH, inhibition of ascorbate autoxidation, reducing power, hydroxyl radicals, and total antioxidant capacity. Diabetes was induced in adult *Wistar* rats by intraperitoneal injection of streptozotocin, and they were given kefir in drinking water for five weeks. Body weight, glucose and lipids levels were then measured. The parameters of oxidative stress, Malondialdehyde, catalase, protein carbonyl, hydroperoxides, vitamin C. were also examined. Three distinct microbial populations were identified in water kefir beverage including, lactic acid bacteria, Acetic acid bacteria, and yeasts.The results showed important amounts of nutrients and minerals in water kefir. Water kefir exhibited an excellent antioxidant activity. It also demonstrated significant improvement in body weight, glucose, lipids profiles, and oxidative stress biomarkers of treated rats comparing with diabetic and control ones. These results conduce to conclude that water kefir could be promisor symbiotic of beneficial microorganisms, bioactive compounds, and natural antioxidants with excellentcapability in health developingand maintenance. Water kefir can potentially be useful functional food for diabetics to control glucose lipids levels, and enhancing of defenses system against oxidative stress.

Key words: Nonalcoholic Water Kefir, anti-hyperglycemic, anti-hyperlipidimic, Antioxidant activity, Symbiotic, *Wistar* rats.

Résumé

Le kéfir eau est un produit symbiotique issu de la fermentation d'une solution sucrée à l'aide de bactéries et de levures maintenues dans une matrice de polysaccharides appelée (grains de kéfir). Il contient de nombreuses substances bioactives qui pourraient contribuer à l'amélioration de la santé humaine. En optimisant les conditions de fermentation (temps, pH, température) nous avons essayé de produire du kéfir non alcoolique. En outre, nous avons étudié les effets antioxydants et métaboliques de ce produit. Les micro-organismes ont été isolés et identifiés selon leur morphologie et par des tests biochimiques. Nous avons aussi déterminé les nutriments et les minéraux que renferme le produit. L'activité antioxydante in vitro a été évaluée par cinq méthodes : le DPPH , l'inhibition de l' auto-oxydation de l'ascorbate, le pouvoir réducteur des radicaux hydroxyles et la capacité antioxydante totale. Le diabète a été induit au préalable chez des rats Wistar adultes par une injection intra péritonéale de streptozotocine, et ils ont ensuite ingéré le kéfir eau pendant cinq semaines. Le poids corporel ainsi que le taux de glucose et de lipides ont été mesurés. Les paramètres du stress oxydatif, Le malondialdehyde, la catalase, les protéines carbonylées, les hydroperoxydes, la vitamine C ont été ensuite déterminés. Trois populations microbiennes distinctes ont été identifiées dans le breuvage du kéfir d'eau, à savoir : des bactéries lactiques, des bactéries acétiques et des levures. Les résultats ont montré d'importantes quantités de nutriments et de minéraux dans le kéfir eau. Ce dernier a montré une excellente capacité antioxydante .Il a également été démontré une amélioration significative du poids corporel, du taux glucose, des profils lipidiques et des bio marqueurs du stress oxydatif des rats traités en comparaison avec les rats diabétiques et les témoins. Les résultats obtenus nous mènent à déduire que le kéfir eau pourrait être un bon apport symbiotique de micro-organismes bénéfiques, de composés bioactifs et d'antioxydants naturels. Ainsi qu'un aliment fonctionnel utile pour les diabétiques pour le contrôle des taux de glucose et de lipides et pour renforcer le système de défense contre le stress oxydatif.

Mots-clés: kéfir d'eau non-alcoolique, anti-hyperglycémiant, anti-hyperlipidémiant, activité antioxydante, symbiotique, rats Wistar.