TESIS DOCTORAL

Role of beer in the modulation of degenerative processes. *In vivo* and *in vitro* integrative studies of the DNA protective activity, survival increase, antiproliferative potential and modulation of genetic methylation status

Papel de la cerveza en la modulación de procesos degenerativos. Estudios integrados *in vivo* e *in vitro* de la actividad protectora del ADN, aumento de supervivencia, potencial antiproliferativo e intervención en el estatus de metilación genética

María Tania Merinas Amo

2017

TITULO: *Role of beer in the modulation of degenerative processes. In vivo and in vitro integrative studies of the DNA protective activity, survival increase, antiproliferative potential and modulation of genetic methylation status*

AUTOR: *María Tania Merinas Amo*

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Trabajo realizado en el Departamento de Genética de la Universidad de Córdoba para optar al grado de Doctor en Biociencias y Ciencias Agroalimentarias por la Licenciada en Biología:

María Tania Merinas Amo

Dirigido por:

Dra. Ángeles Alonso Moraga

Dr. Fernando Calahorro Núñez

TÍTULO DE LA TESIS:
Role of beer in the modulation of degenerative processes. *In vivo* and *in vitro* integrative studies of the DNA protective activity, survival increase, antiproliferative potential and modulation of genetic methylation status.
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DOCTORANDA: María Tania Merinas Amo.

INFORME RAZONADO DEL/DE LOS DIRECTOR/ES DE LA TESIS

La Tesis Doctoral de Dña. María Tania Merinas Amo se ha llevado a cabo en el Departamento de Genética de la Universidad de Córdoba. Su desarrollo se ha ajustado a los plazos inicialmente previstos y ha permitido a la doctoranda adquirir una sólida formación en diversas disciplinas de la Genética aplicándolas de un modo transversal. Durante la realización de la Tesis, Dña. María Tania Merinas Amo ha confirmado una excelente aptitud y vocación por la investigación científica y ha demostrado poseer una excelente capacidad de trabajo, organización e integración así como de redacción de trabajos científicos. La doctoranda ha conseguido otorgar un valor añadido a la cerveza como alimento con potencial nutracéutico, asignándole papeles importantes en la lucha contra procesos degenerativos como: la seguridad, la protección genómica, la influencia en la longevidad, la quimioprevención, el daño a nivel genómico en células tumorales, la inactivación de macroautofagia de células inmortalizadas, y estudios de los cambios en el estado de metilación genómico en secuencias repetitivas. El papel de la cerveza y algunos de sus componentes bioactivos en la modulación de procesos degenerativos correspondiente a su primer capítulo de Tesis "*In vivo* and *in vitro* studies of the role of lyophilised blond Lager beer and three bioactive components in the modulation of degenerative processes" ha sido publicado en dos artículos independientes:

- "*In vivo* and *in vitro* studies of the role of lyophilised blond Lager beer and some bioactive components in the modulation of degenerative processes", en Journal of Functional Foods. DOI: 10.1016/j.jff.2016.09.014. Tania Merinas-Amo, Inmaculada Tasset-Cuevas, Antonio M. Díaz-Carretero, Ángeles Alonso-Moraga, Fernando Calahorro.
- "Role of Choline in the Modulation of Degenerative Processes: *In Vivo* and *In Vitro* Studies", en Journal of Medicinal Food. DOI: 10.1089/jmf.2016.0075. Tania Merinas-Amo, Inmaculada Tasset-Cuevas, Antonio M. Díaz-Carretero, Ángeles Alonso-Moraga, Fernando Calahorro.

Así mismo, está preparando cinco artículos más que se corresponden con los subsiguientes capítulos de la tesis.

Por todo ello, autorizamos la presentación de la Tesis Doctoral.

Córdoba, 26 de Enero de 2017
Firma de los directores

Fdo.: Ángeles Alonso Moraga

Fdo.: Fernando Calahorro Núñez

Dra. Ángeles Alonso Moraga y Dr. Fernando Calahorro Núñez

INFORMAN:

Que el trabajo titulado "Role of beer in the modulation of degenerative processes. *In vivo* and *in vitro* integrative studies of the DNA protective activity, survival increase, antiproliferative potential and modulation of genetic methylation status" ("Papel de la cerveza en la modulación de procesos degenerativos. Estudios integrados *in vivo* e *in vitro* de la actividad protectora del ADN, aumento de supervivencia, potencial antiproliferativo e intervención en el estatus de metilación genética") realizado por Dña. María Tania Merinas Amo bajo la dirección de la Dra. Ángeles Alonso Moraga y el Dr. Fernando Calahorro Núñez, puede ser presentado para su exposición y defensa como Tesis Doctoral en el Departamento de Genética de la Universidad de Córdoba.

Firmado en Córdoba, a 26 de Enero de 2017

Fdo. Ángeles Alonso Moraga | Fdo. Fernando Calahorro Núñez

Nota: esta Tesis Doctoral se presenta en inglés debido a su carácter internacional. La doctoranda Dña. María Tania Merinas Amo realizó una estancia durante tres meses en la universidad "Czech University of Life Sciences Prague" (Republica Checa), cumpliendo con los requisitos exigidos para dicha mención internacional. Para facilitar la lectura, las referencias de Introducción, Capítulos y Discusión General aparecen al final de cada uno de ellos.

Firstly, I would like to express my sincere gratitude to my principal supervisor Prof. Ángeles Alonso for the continuous support to my PhD study and related research, for her patience, motivation, and immense knowledge. Her guidance helped me during all the time of research and writing of this thesis. I would like to extend my sincere gratitude to my second supervisor Dr. Fernando Calahorro for the good advice, assistance and friendship. They have been invaluable on both academic and a personal level, for which I am extremely grateful. I could not have imagined having better advisors for my PhD study.

I owe my deepest gratitude to the exceptional faculty in the Crop Sciences and Agroforestry Department. I would like to give special thanks to Prof. Ladislav Kokoska and all his lab team. They each have made my time at Prague (Czech Republic) more positive and enjoyable.

I cannot express enough thanks to my family for their continued support and encouragement. Specially, I am grateful to my parents and sister for the countless times that you helped me throughout my life; the completion of this project and many others could not have been accomplished without their support.

Last of all, I would like to thanks everyone else who helped contribute to this project and to have a personal growth. Sorry for no named each one but I do not want to take the risk of forgetting any of my special students, lab mates and/or friends which whom I have the pleasure of have lived unforgettable moments.

Tania Merinas Amo

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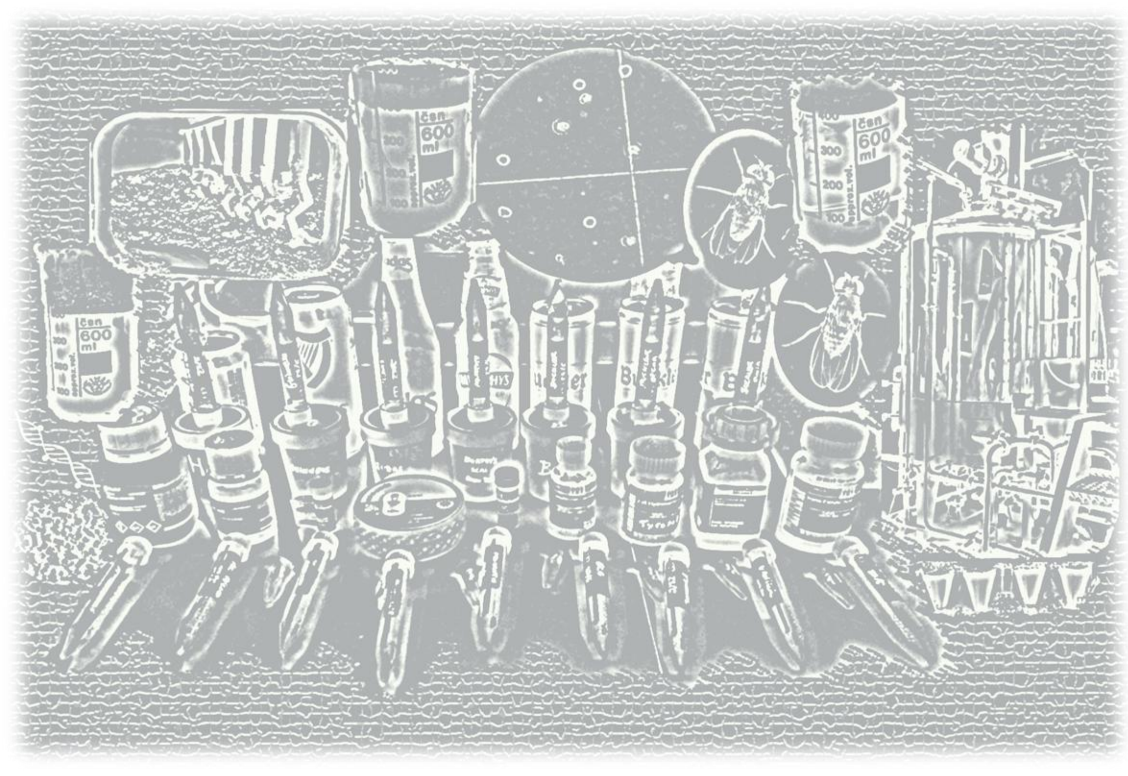

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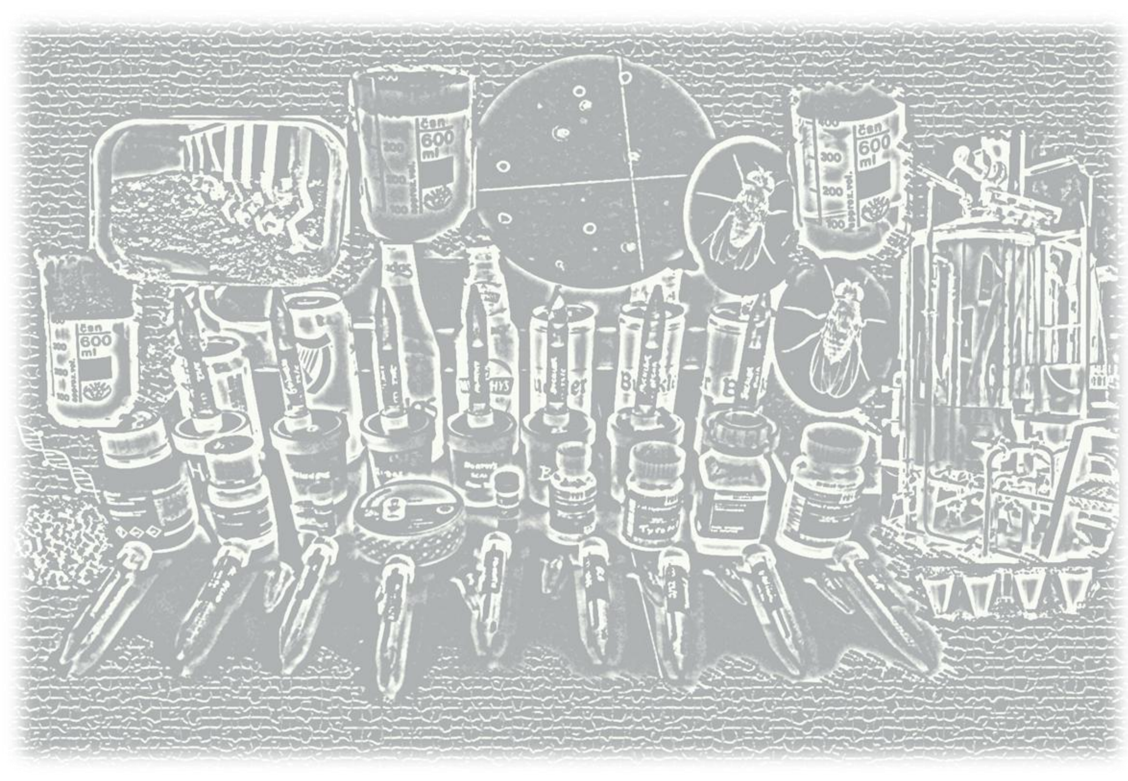
ABBREVIATIONS



AFB	Alcohol-free beer (containing ≤ 0.5% alcohol by volume)
Alu	SINE (Short Interspersed Nuclear Elements)
BAC	Blood Alcohol Concentrations
CpG	Cytosine-Phospate-Guanine dinucleotide
Ct	Cycle threshold
DNMT	DNA Methyltransferase
ECG	(-)-Epicatechin gallate
Etop	Etoposide
Ferulic	trans-Ferulic Acid
flr^3	*flare* genetic marker
HL-60	Human Leukaemia-60 cell line
Humulone	iso-alpha humulone
IC_{50}	Inhibitory Concentration 50
ID_{50}	Inhibitory Doses 50
IP	Inhibition Percentages
iTaq	DNA polymerase
IX	Isoxanthohumol
LAB	Low-alcohol beer (containing no more than 1.2% alcohol by volume)
LBAB	Lyophilised Blond Ale Beer
LBAFLB	Blond Alcohol-free beer
LBLB	Lyophilised Blond Lager Beer
LINE-1	Long Interspersed Elements
LPBAFLB	Pale-blond Alcohol-free beer
LSAB	Lyophilised Stout Ale Beer
LSAFLB	Stout Alcohol-free beer
LSLB	Lyophilised Stout Lager Beer
MA	Macroautophagy
mwh	*multiple wing hairs* genetic marker
NIH3T3	mouse fibroblast cells
qMSP	quantitative Methylation-Specific PCR
ROS	Reactive Oxygen Species
Sat-α	Satellite-alpha DNA
SMART	Somatic Mutation And Recombination Test
TM	Tail Moment
Tyrosol	4-(2-Dihydroxyethyl)phenol
Vanillic	4-Hydroxy-3-methoxybenzoic acid (Vanillic acid)

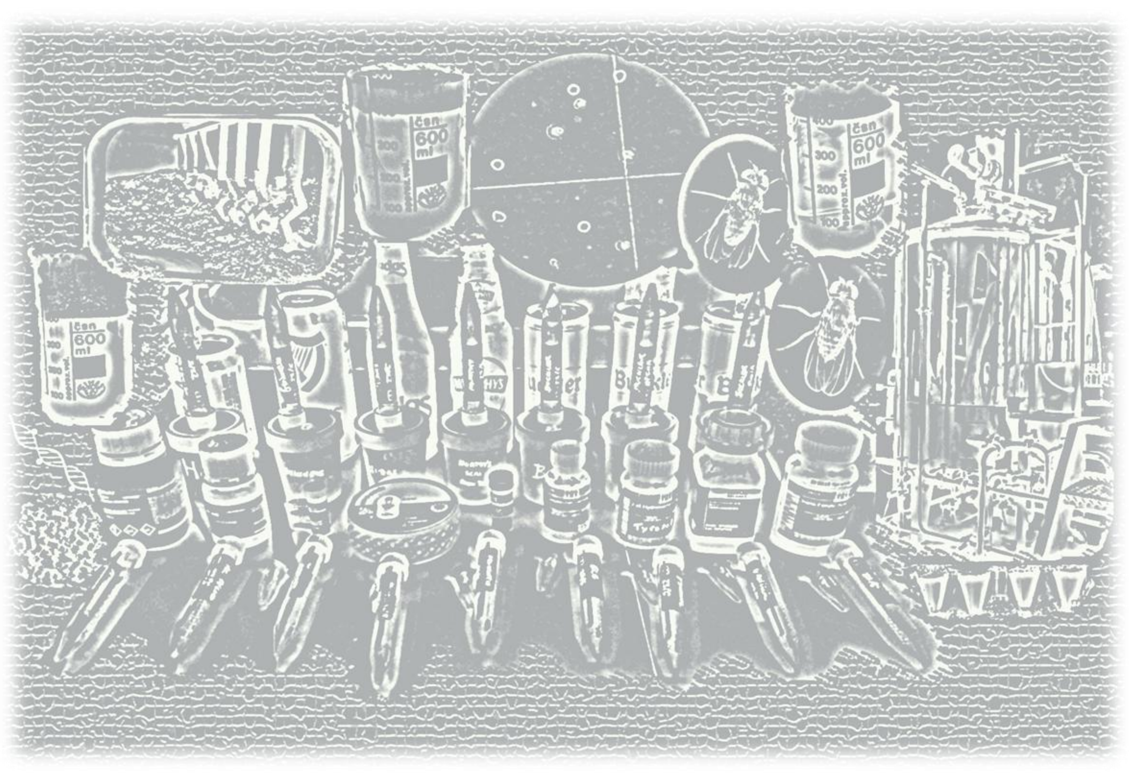
CHEMICAL COMPOUNDS

Agarose (PubChem CID: 11966311); Choline (PubChem CID: 6209); Ethanol (PubChem CID: 702); Ethidum bromide (PubChem CID: 14710); Folic acid (PubChem CID: 6037); Hydrogen peroxide (H_2O_2) (PubChem CID: 784); Iso-alpha humulone (humulone) (PubChem CID: 442911); Isopropanol (PubChem CID: 3776); Isoxanthohumol (PubChem CID: 513197); L-glutamine (PubChem CID: 5961); NaCl (PubChem CID: 5234); Propidium iodide (PubChem CID: 104981); SDS (PubChem CID: 3423265); Trans-Ferulic Acid (PubChem CID: 445858); Trypan blue (PubChem CID: 101417452); Xanthohumol (PubChem CID: 639665); 4-(2-Dihydroxyethyl)phenol (tyrosol) (PubChem CID: 10393); 4-Hydroxy-3-methoxybenzoic acid (PubChem CID: 8468); (-)-Epicatechin gallate (PubChem CID: 107905).

KEYWORDS

Antigenotoxicity; Beer; Brewing; Choline; Cytotoxicity; Czech beer; DNA damage; *Drosophila melanogaster*; Ferulic; Folic acid; HL-60 cell line; Human; Humulone; Isoxanthohumol; Longevity; Macroautophagy; Methylation status; Toxicity; Tumour cells; Tyrosol; Vanillic; Xanthohumol.

SUMMARY



Diet, among others lifestyle factors, is one of the most important environmental agents that exert a significant effect on health and disease risk. Here, we have evaluated the current third-most popular drink over the world: beer, and some of its bioactive compounds.

Despite beers are considered as a beverage with important properties on health, deep studies about the genotoxicological activities of this drink ant its compounds are missing. In these work, a new data corpus has been added to science by providing more information about the promising properties that beer shows as a nutraceutic substance in animal and human model systems. Multilevel assays were used to analyse the safety and protective effects against an oxidative toxin in the *in vivo Drosophila* model organism, as well as the chemopreventive potential and the induction of DNA damage ability in the *in vitro* tumour cells (human leukaemia HL-60) and in immortal mouse fibroblast NIH3T3 cells of beers and its components. Besides, a pilot clinical study in humans was carried out to study the role of a blond beer on the modifications of methylation status patterns of repetitive sequences. Here, we present a set of biological screening assays where several points related to degenerative processes can be checked. Briefly, the used methodology is below mentioned:

i) *In vivo* assays using the *Drosophila* model allowed evaluating:

- Toxicity and antitoxicity: the survival percentage of individuals with respect to their concurrent positive or negative controls is evaluated when *Drosophila* is treated either with different concentrations of a single substance or it is combined with an oxidative toxin (H_2O_2).
- Genotoxicity and antigenotoxicity: somatic mutation and recombination wing spot test evaluation of *Drosophila* treated at larval stage with different concentrations of a single substance or a combined substance with H_2O_2. This assay allows us to determine the mutagenic or antimutagenic activity of the studied sample at chromosomic level.
- Longevity: effects on the life extension and quality of life of *Drosophila* populations feed over time with different concentrations of substances.

ii) *In vitro* assays using the HL-60 promyelocytic cells and immortal NIH3T3 fibroblasts models allow us to evaluate:

- Cytotoxicity: capacity of substances to inhibit the tumour cells growth treated with different concentrations.
- DNA damage: ability to induce proapoptotic DNA fragmentation and/or DNA damage at single cell levels by inducing cell comets.
- Methylation status: capacity to modify epigenome by studying the methylation status pattern of DNA repetitive sequences (Alu, LINE and satellite repetitive elements) by treating tumour cells with beer and its components.
- Macroautophagy activity: the status of cellular homeostasis by studying the number of autophagosomes and autophagolysosomes formed in treated NIH3T3 fibroblast.

iii) Clinical trial in human volunteers to evaluate the modifications in the methylation status pattern of genome repetitive sequences due to moderate drinking beer.

Some specific summary points can be highlighted drawn from the present work:

1.- Raw materials and brewing conditions revealed key points for the final activities of beers. They showed a general safety and protective effects induced in the *in vivo* tests and chemopreventive and DNA damage activity in the *in vitro* assays for the different treatments.

2.- Lyophilised beers showed a safe character and protective ability against an oxidant agent in *D. melanogaster*, as well as chemopreventive potential against HL-60 cells.

3.- Studies in *D. melanogaster* at genetic level did not show genotoxic activity by any studied beer, except for LPABAFLB and LSAFLB. Furthermore, beers protected DNA in combined treatments with H_2O_2.

4.- Chronic studies on *Drosophila* populations treated with different lyophilised beers over time showed an improvement of longevity and healthspan properties induced by all the studied beers, except for LSAB.

5.- Genome wide screening on HL-60 treated cells with different lyophilised beers showed a hypermethylation status at molecular level of repetitive sequences, except for AFBs. In a pilot clinical study, moderate intake of blond lager beer induced a hypermethylation status in repetitive sequences of humans.

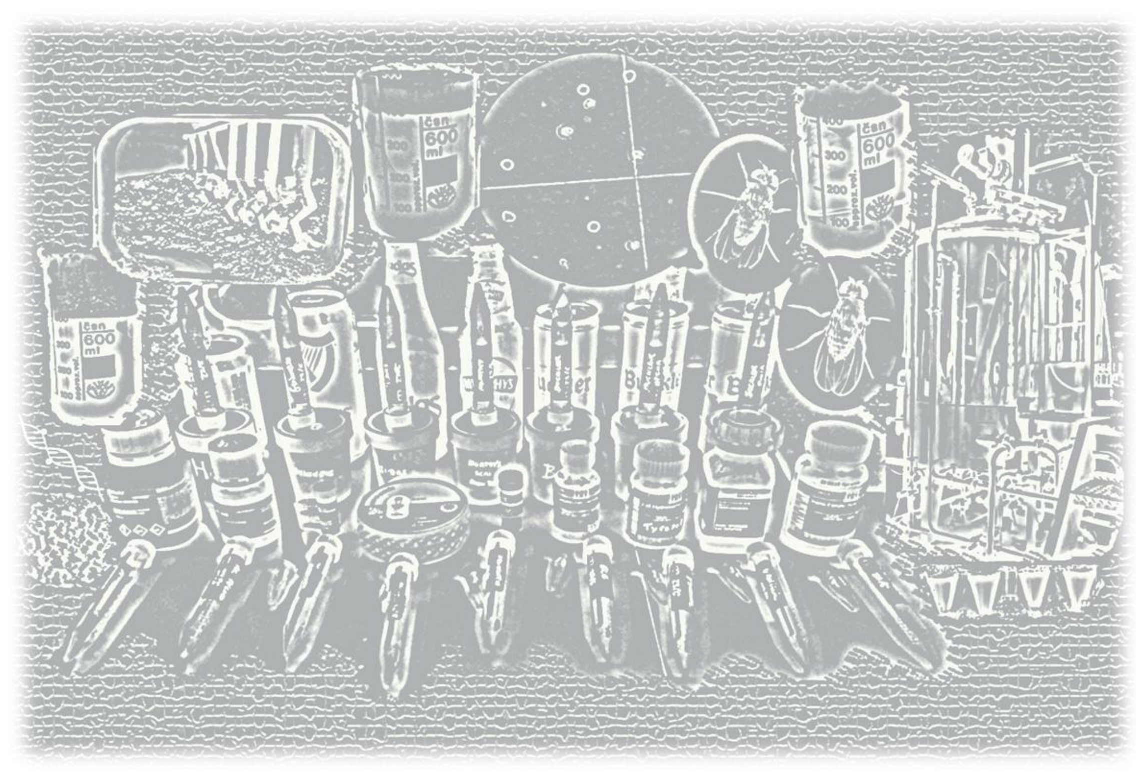
6.- Individual bioactive compounds of beers cannot be completely linked to the biological activities attributed to the full beverage. Despite of the final properties exhibited by full beers are not caused by any additive nor synergic effects of the studied compounds, in general, xanthohumol and humulone appear to be the most influencer molecules among all the single compounds studied. Both phenols are contained in hops; therefore, the addition of hops in the brewing process could be considered as one of the key points for the improvement of the beneficial properties of beers.

7.- Cytotoxic and macroautophagy studies with LBLB, choline, folic acid and xanthohumol in the NIH3T3 immortal cells showed the same inhibition growth pattern as in the HL-60 cells and an inhibition of MA in NIH3T3 cells except for the highest concentration assayed of folic acid.

Based on the analysis of the results obtained for the *in vivo* assays in the *Drosophila* model organism, the *in vitro* ones in the HL-60 cell line and the clinical studies in human as well, a general conclusion on the potential nutraceutic of lyophilised beers can be drawn. Nevertheless, the complete biological activities attributed to beers cannot be exclusively linked to any particular class of constituents due to their high complexity

and variability in the polyphenolic pattern. Besides, the brewing conditions like raw materials, wort composition, yeast strains and fermentation parameters, among others factors, influence the final outcome of the obtained beverage.

RESUMEN



La dieta, entre otros factores del estilo de vida, es uno de los agentes medioambientales más importantes que ejercen efectos sobre la salud y sobre el riesgo de adquirir enfermedades. En el presente trabajo hemos evaluado la tercera bebida más popular actualmente en todo el mundo: la cerveza, así como algunos de sus componentes bioactivos.

A pesar de que la cerveza es considerada como una bebida con importantes propiedades sobre la salud, no se conocen muchos estudios sobre su actividad genotoxicológica y la de sus compuestos. En el presente estudio se han añadido nuevos datos a la ciencia, aportando información sobre las prometedoras propiedades como sustancia nutracéutica que la cerveza muestra en modelos animales y humanos. Se han usado ensayos a diferentes niveles para analizar la seguridad y el efecto protector frente a una toxina oxidativa en el organismo modelo *in vivo Drosophila*, así como el potencial quimiopreventivo y la capacidad de inducir daño en el ADN en células tumorales *in vitro* (células de leucemia humana HL-60) y en células inmortales de fibroblastos de ratón NIH3T3, tanto de la cerveza como de sus componentes. Además, se llevó a cabo un ensayo clínico piloto en humanos para estudiar el papel de una cerveza rubia en la modificación del estado de metilación de secuencias repetitivas. Presentamos una variedad de ensayos biológicos de rastreo mediante los cuales se pueden detectar diferentes puntos relacionados con procesos degenerativos. La metodología usada se resume a continuación:

i) Los ensayos *in vivo* usado el modelo *Drosophila* han permitido evaluar:

- Toxicidad y antitoxicidad: se evalúa el porcentaje de individuos supervivientes con respecto a su control positivo o negativo cuando *Drosophila* es tratada con diferentes concentraciones tanto de las sustancias simples como combinadas con una toxina oxidativa (H_2O_2).
- Genotoxicidad y antigenotoxicidad: evaluación mediante el análisis de mutaciones somáticas y recombinaciones en células en expansión clonal de alas de *Drosophila* tratadas en su estado larvario con diferentes concentraciones de la sustancia simple o combinada con H_2O_2. Este ensayo nos permite determinar la actividad mutagénica o antimutagénica de los compuestos estudiados a nivel cromosómico.
- Longevidad: efectos sobre la extensión y calidad de vida de vida en poblaciones de *Drosophila* alimentadas durante toda su vida con las sustancias a diferentes concentraciones.

ii) Los ensayos *in vitro* usando los modelos celulares promielocíticos HL-60 y fibroblastos inmortales NIH3T3 permiten evaluar:

- Citotoxicidad: capacidad de las sustancias para inhibir el crecimiento celular a diferentes concentraciones.
- Daño en el ADN: capacidad para inducir fragmentación proapoptótica del ADN y/o daño en el ADN a nivel de células individuales induciéndoles cometas.
- Estado de metilación: capacidad de la cerveza y sus compuestos de modificar el epigenoma de células tumorales mediante el estudio de los patrones de estado de

metilación de secuencias repetitivas del ADN (secuencias repetitivas Alu, LINE y satélite).
- Actividad macroautofágica: análisis del estado de homeostasis celular mediante el estudio del número de autofagosomas y autofagolisosomas formados en fibroblastos NIH3T3 tratados.

iii) Estudios clínicos en humanos voluntarios para evaluar las modificaciones en el patrón de estado de metilación de secuencias repetitivas del genoma debido a ingesta modera de cerveza.

Algunas de las conclusiones específicas que se derivan del presente trabajo son:

1.- Las materias primas y las condiciones del procesado de la cerveza se han revelado como puntos críticos para las propiedades finales de las cervezas. Éstas muestran, de forma general, efectos seguros y protectores en los distintos estudios *in vivo*, además de actividad quimiopreventiva y daño en el ADN en los ensayos *in vitro* para los diferentes tratamientos.

2.- Las cervezas liofilizadas mostraron tener un carácter seguro y capacidad protectora frente a un agente oxidante en *D. melanogaster*, además de potencial quimiopreventivo frente a células HL-60.

3.- Estudios a nivel genético en *D. melanogaster* mostraron que las cervezas estudiadas no poseen actividad genotóxica, excepto las LPABAFLB y LSAFLB. Además, tratamientos combinados de las cervezas con H_2O_2 indicaron ciertos niveles de protección al ADN.

4.- Estudios crónicos en poblaciones de *Drosophila* tratadas durante toda su vida con diferentes cervezas liofilizadas mostraron capacidad de estas bebidas para aumentar la longevidad y calidad de vida, excepto la LSAB.

5.- Un rastreo a lo largo del genoma de células HL-60 tratadas con diferentes cervezas liofilizadas mostraron un estado de hipermetilación de las secuencias repetitivas a nivel molecular, excepto para AFBs. En un estudio clínico piloto, el consumo moderado de cerveza rubia lager indujo un estado de hipermetilación en secuencias repetitivas de humanos.

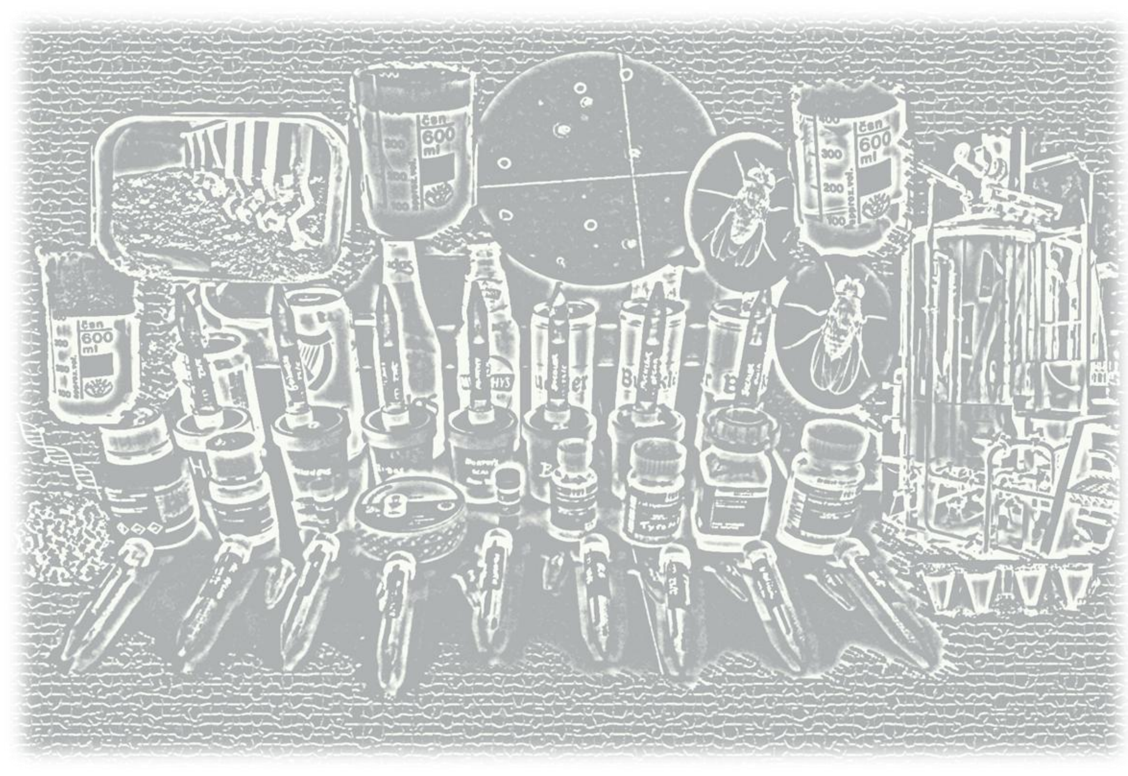
6.- A nivel individual, los compuestos bioactivos de las cervezas no se pueden asociar con las actividades biológicas atribuidas a la bebida en su conjunto. Aunque las propiedades finales mostradas por las cervezas no fueron causadas ni por un efecto aditivo ni sinérgico de los compuestos estudiados, en general, de entre todos los compuestos individuales estudiados, el xanthohumol y la humulona parecen ser las moléculas con mayor influencia. Ambos fenoles se encuentran en el lúpulo, por lo que la incorporación de éste en el proceso de fabricación de la cerveza puede ser

considerado como un punto clave en la mejora de las propiedades beneficiosas de las cervezas.

7.- Estudios de citotoxicidad y macroautofagia con LBLB, colina ácido fólico y xanthohumol en células inmortales NIH3T3 han mostrado el mismo patrón de inhibición de crecimiento celular que las mostradas en las células HL-60 así como inhibición de la actividad macroautofágica en células NIH3T3, excepto para la concentración más alta ensayada de ácido fólico.

Basándonos en los resultados obtenidos tanto en los ensayos *in vivo* del organismo modelo *Drosophila*, en los ensayos *in vitro* en la línea celular HL-60, como en los estudios clínicos en humanos, podemos destacar el potencial nutracéutico de las cervezas liofilizadas como conclusión general. Sin embargo, debido a la alta complejidad y variabilidad de tipos fenólicos, las propiedades biológicas atribuidas a las cervezas no están ligadas a un tipo particular de compuesto. Además, las condiciones de fabricación así como son la materia prima, composición del mosto, cepas de levadura y tipo de fermentación, entre otros factores, influyen en el resultado final de la bebida obtenida.

INTRODUCTION



1. DIET AND CANCER

Cancer is a disease characterised by self-sufficiency in growth signal, resistance to growth inhibition, limitless replicative potential, evasion of apoptosis, sustained angiogenesis, and tissue invasion and metastasis (Hanahan and Weinberg 2011). Cancer is caused by a variety of factors and offers a variety of targets for intervention and inhibition at every stage such as antioxidant activity, xenobiotics metabolism, anti-mutagenic, anti-inflammatory and anti-hormonal mechanisms, anti-proliferative activities, induction of terminal cell differentiation and apoptosis (Reddy et al. 2003).

Only a minority of cancers are caused by germ-line mutations, whereas the vast majority (90%) are linked to somatic mutations and environmental factors (Anand et al. 2008; Martin-Moreno et al. 2008). Furthermore, because of the adoption of behaviours and lifestyle factors, such as tobacco, obesity, alcohol, sedentary lifestyle, high-fat diet, radiation, and infections, it is expected an increasing of cancer worldwide (Anand et al. 2008; Messina and Hilakivi-Clarke 2009; Shu et al. 2009; Torre et al. 2015).

Diet is one of the most important environmental factors that exert effect on health and disease risk. The important role of diet in health is known centuries ago when Hippocrates proclaimed "Let food be thy medicine and medicine be thy food". A wide range of investigations support the potential role of nutrition at both preventive and therapeutic levels (Bingham and Riboli 2004; Boffetta et al. 2010; Esteller 2008; Fraga et al. 2007; Jiménez-Chillarón et al. 2012; Lundstrom 2012; Milner 2008; Milner 2006).

The development of strategies to use foods and dietary supplements with genetic-intervention potential, physical improvement and cognitive performance to reduce the risk of chronic diseases are one of the current main priorities (Kim et al. 2008; Milner 2006; Ordovas et al. 2007; Panagiotakos et al. 2007).

The bioactive food components (estimated more than 25,000 in human consumption) may arise from plants, animal sources, mushrooms or from the metabolism of food components by gastrointestinal bacteria. Many of these compounds have been identified as possible actors of the cancer process (Milner 2008; Milner 2006). The variety of dietary constituents may modify, either positively or negatively, cancer risk and tumour behaviour (World Cancer Research Fund 2007). Table 1 shows the relationship between the most relevant dietary factors with risk of major cancers. Antioxidants may be found in diet at significant amounts and confer protection against oxidative stress-related diseases (Aruoma 1998). Polyphenols are the most abundant dietary antioxidants in diets, distributed mostly in coffee, fruits, wine, vegetables, olive oil and beer. The regulate multiple cancer-inflammation pathways and epigenetic cofactors, are cost effective, exhibit low toxicity, and are readily available (Clifford 1999; Deorukhkar et al. 2007; Herrmann and Nagel 1989; Lee et al. 2011).

Table 1

Relationship between dietary factors with risk of major cancers (from Divisi et al. 2006); Greenwald et al. (2001); La Vecchia (2004); Miller (1990); Willett (2000)).

<table>
<tr><th>[1]</th><th>CR</th><th>Breast</th><th>Prost.</th><th>Lung</th><th>Stom.</th><th>Esop.</th><th>Oral</th><th>Panc.</th><th>Blad.</th><th>Kid.</th><th>End.</th><th>Liver</th></tr>
<tr><td>Macronutrients/energy balance</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>Carbohydrates & energy exposition</td><td>↑</td><td>↑</td><td>↑</td><td></td><td>↑</td><td></td><td></td><td>↑</td><td>↑</td><td>↑</td><td>↑</td><td></td></tr>
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<tr><td>Vitamin and Mineral Supplements</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>β-carotene</td><td></td><td>↓</td><td></td><td>|</td><td>↓</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
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<tr><td>Western</td><td>↑</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>Others</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>Coffee</td><td></td><td>↓</td><td></td><td></td><td></td><td></td><td></td><td>↑</td><td></td><td></td><td></td><td></td></tr>
<tr><td>Fish oil</td><td>↓</td><td>↓</td><td>↓</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
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<tr><td>Hot beverages</td><td></td><td></td><td></td><td></td><td></td><td>↑</td><td>↑</td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>Micronutrient deficit</td><td></td><td></td><td></td><td></td><td></td><td>↑</td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>Olive oil</td><td></td><td>↓</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>Salt, preserved food</td><td></td><td></td><td></td><td></td><td>↑</td><td>↑</td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
</table>

[1] CR: colorectal; Prost.: prostate cancer; Stom. : stomach cancer; Esop.: esophageal cancer; Panc.: pancreatic cancer; Blad.: bladder cancer; Kid.: kidney cancer End.: endometrial cancer.

↑: increasing of cancer risk; ↓: decreasing of cancer risk; |: contradictory/no significant results.

Many questions about the relation between food and cancer risk reduction remain unknown, including which specific dietary factors are most closely linked to cancer prevention, by what mechanisms diet components exert their effects, how food factors interact to affect cancer risk and what preventive steps can be taken to minimise adverse effects of factors that seem to increase disease risk. These questions have not yet been answered because of the complexity of cancer. In general, most of the recommendations includes high intake of fibre and a variety of fruits and vegetables, consumption of alcohol and salt only in moderation, reduced fat intake, and increased physical activity (Food ; Go et al. 2003; Greenwald et al. 2001; Ogimoto et al. 2000; Who and Consultation 2003).

2. NUTRACEUTICALS

The nutraceutical revolution began in the early 1980s, caused by the clinical benefits that calcium, fibre and fish oil showed in different clinical studies, and due to the awareness achieve by physicians and consumers about these substances (DeFelice 1995). The term nutraceutical is a hybrid or contraction of nutrition and pharmaceutical. Reportedly, it was coined in 1989 by DeFelice and the Foundation for Innovation in Medicine (Kalra 2003). Its definition was "any substance that may be considered a food or part of a food and provides medical or health benefits, including the prevention and treatment of disease. Such products may range from isolated nutrients, dietary, supplements and diets to genetically engineered 'designer' foods, herbal products, and processed foods such as cereals, soups, and beverages" (Keservani et al. 2010).

There is a wide range of purported nutraceutical substances, being the isoflavones, tocotrienols, allyl sulfur compounds, fibre, and carotenoids some of the more recognisable. Moreover, different classifications of nutraceuticals exit depending on one's interest and/or backgrounds: their potential as a food source to humans, their concentrations in foods, mechanism of action, chemical nature, among others (Wildman et al. 2016).

Some examples of dietary supplements as nutraceuticals are accepted by scientific community. Ascorbic acid, copper, selenium, manganese and zinc due to their antioxidant functions; calcium is related to bone health, colon cancer, and hypertension and cardiovascular disease; potassium has also been purported as hypertension reducer and thus improver cardiovascular health; folic acid is used to prevent spina bifida; β-carotene is used to prevent certain types of lung cancer; niacin is used to prevent recurrent heart attacks; pyridoxine is used to treat and prevent depression; vitamin A is used to treat measles; magnesium is used to treat hypertension; garlic is used to reduce atherosclerosis; fish oil is used for hypertension; cranberry juice is used to prevent urinary tract infections; antioxidants are used to reduce heart damage; and countless others (DeFelice 1995; Wildman et al. 2016).

3. ASSAYS

Being a potential nutraceutical one that holds a promise of a particular health or medical benefit, a nutraceutical substance should not be toxic and should also be able to prevent toxicity, avoid genetic oxidative damage, modulate the epigenome marks and induce cell death in a programmed manner in tumour cells, and maintain the epigenome marks in normal cells (Villatoro-Pulido et al. 2012). Recently, nutraceuticals are considered as active modulators of the carcinogenic process. Efforts are made for screening real preventive substances.

In the multistep process of carcinogenicity the toxic and genotoxic events are in the first steps. The early screening using prokaryotic systems gave us many false positive results as it seemed that every substance was genotoxic, eukaryotic systems depicted a more realistic panorama about this relationship. The *in vivo* somatic mutation and recombination assays like the wing spot test of *Drosophila*, are generally based on the clonal expansion of mutation induced in the imaginal discs of *Drosophila* larvae that are treated with the problem substance (Graf et al. 1984). Two genetic markers (*mwh* and flr^3) on the chromosome 2 affecting the number and shape of tricoma per cell are used. The transheterozygous larvae resulting from the cross of two strains *mwh* and flr^3 can be treated with many classes of compounds that can induce mutations and somatic recombinations between *mwh* and flr^3 and between flr^3 and the centromere. The induction/suppression of mutations can be observed in the emerging fly wings. The *Drosophila* animal model is a reliable system to test toxicity, genotoxicity and many degenerative processes (Graf et al. 1984) as more than 75% of human disease genes have a homologous gene in the *Drosophila* genome (Bier 2005; Lloyd and Taylor 2010).

Once the lack of toxicity and genotoxicity of a substance is established, it is desirable to test the ability for acting against the direct toxicity or the effects of reactive oxygen species. Oxygen, being an unavoidable molecule for living as it is associated to aerobic conditions, also represents a potential poison when it is produced in excess. ROS can damage DNA and are one of the more important genetic alterations that conduct finally to a cancer process. The *in vivo Drosophila* system of antitoxicity and antigenotoxicity using well-known strains with homogeneous genetic background is applied to test the ability to detoxify reactive oxygen species by some bioactive compounds (Anter et al. 2011; Graf et al. 1998).

Chemopreventive agents can counteract to the carcinogenic process either avoiding the toxicity against the DNA or acting as selective killer of transformed cells. In this stressed situation, the only defence is to have an exogenous source of antioxidants included in the diet. With this respect it is remarkable that all the countries keep traditions that include the ancestral medical use of vegetable foods and medicinal herbs. But, what can we do once the tumour start growing? The elimination of transformed cells is required. A food that avoids mutations is very convenient, but this food should

also have another activity that could have a selective cytotoxic activity against transformed cells.

The *in vitro* cytotoxicity assays, as the HL-60 model, uses exponential growing cancer cell lines treated with increasing concentrations of the problem substance. The cell viability is quantified after treatment and the tumour grow represented against the dose. The way by which a compound exerts the cytotoxic effect can be measured by evaluating the ability to induce DNA damage both as DNA internucleosomal fragmentation and as DNA strand breaks induction (Mateo-Fernández et al. 2016; Merinas-Amo et al. 2016).

Genome wide screening of genotoxic damage is an inclusive approach to understand the mechanisms underlying the cancer process. When scoring genetic or epigenetic mutations at single genes the portion of DNA scored is narrower than when recombinogenic events are scored. Although, when repetitive sequences are studied an excellent target for scoring wide DNA genomic portions raises.

The ability of food compounds to influence the epigenome in cancer cells has been studied and has also been related to the individual's risk for developing cancer. Since epigenetic changes can be reversed in the human lifespan, the epigenetic focus is a good tool for the dietary prevention/treatment of cancers.

Studies should be carried out to identify modifiable diet factor with epigenetic value in order to develop epigenetically based prevention strategies. The capacity of some foods and compounds to disrupt DNA methylation as an epigenetic process in HL-60 promyelocytic cancer cells can be evaluated. The cytotoxic activity and the methylation status of the LINE-1 retrotransposon promoter and other repetitive sequences become an excellent tool for screening the effects of foods on cancer cell lines like HL-60 cells. In theory, prevention or reversal of hypermethylation-induced inactivation of key tumour suppression genes or receptor genes by DNMT inhibitors could be an effective approach for cancer prevention (Roman-Gomez et al. 2008) as repetitive sequences account for 45% of the human genome.

Since interspersed repetitive elements (LINE 1 retrotransposons and Alu sequences) as well as tandem repeat centromeric and juxtacentromeric repeats (satellite sequences) contain numerous CpG dinucleotides, the methylation status of these sequences is relevant to understand global DNA methylation. It is generally thought that repetitive elements are heavily methylated in normal somatic tissues, but are methylated to a lesser extent in malignant tissues, driving the global genomic hypomethylation commonly found in human cancers. The hypomethylation affecting repeat sequences and transposable elements is believed to result in chromosomal instability and increased mutation events (Weisenberger et al. 2005).

We can ask for a little bit more and expect for a longer living if we use a nutraceutic. The survival trials are important when testing the safety of a substance as they represent the final outcome (quantitative trait and using the entire body and life of the insect) of a treatment. Because the important question for human is: does a compound increase the life and healthspan? At present, *Drosophila* is the animal model of choice for survival curves (Tasset-Cuevas et al. 2013). After *in vivo* and *in vitro* studies, clinical assays are needed to full evaluation of a substance as a potential nutraceutic (Sackett and Cook 1994).

4. HISTORY OF BEER

After humans abandoned their hunter-gatherer migratory existence and established agricultural communities, farming made grain and other essential foodstuffs were available in quantities which exceeded immediate needs. Brewing provided a ready use for grain, and a valuable line of defence against the pathogens in water (Kiple and Ornelas 2001; Wolf et al. 2008).

Although extensive archaeological evidence of beer production and consumption dates from 4000 to 3500 B.C. (Kiple 2000) the earliest date proposed for beer production is 8000 B.C. Researchers have suggested that the first beer was a batch of porridge left to sit too long (Wolf et al. 2008). The Sumerians are generally considered to be the first beer makers, but the Egyptians soon formalized the process, making bread from sprouted, dried grains, then breaking the bread into water and allowing it to ferment (Hornsey 2003). The following text, written by a Sumerian poet and found on clay tablet, from 1800 B.C. is the *Hymn to Ninkasi* (Damerow 2012). It includes one of the most ancient recipes for brewing beer:

Hymn to Ninkasi

Borne of the flowing water,
Tenderly cared for by the Ninhursag,
Borne of the flowing water,
Tenderly cared for by the Ninhursag,

Having founded your town by the sacred lake,
She finished its great walls for you,
Ninkasi, having founded your town by the sacred lake,
She finished it's walls for you,

Your father is Enki, Lord Nidimmud,
Your mother is Ninti, the queen of the sacred lake.
Ninkasi, your father is Enki, Lord Nidimmud,
Your mother is Ninti, the queen of the sacred lake.

You are the one who handles the dough [and] with a big shovel,

Mixing in a pit, the bappir with sweet aromatics,
Ninkasi, you are the one who handles the dough [and] with a big shovel,
Mixing in a pit, the bappir with [date] - honey,

You are the one who bakes the bappir in the big oven,
Puts in order the piles of hulled grains,
Ninkasi, you are the one who bakes the bappir in the big oven,
Puts in order the piles of hulled grains,

You are the one who waters the malt set on the ground,
The noble dogs keep away even the potentates,
Ninkasi, you are the one who waters the malt set on the ground,
The noble dogs keep away even the potentates,

You are the one who soaks the malt in a jar,
The waves rise, the waves fall.
Ninkasi, you are the one who soaks the malt in a jar,
The waves rise, the waves fall.

You are the one who spreads the cooked mash on large reed mats,
Coolness overcomes,
Ninkasi, you are the one who spreads the cooked mash on large reed mats,
Coolness overcomes,

You are the one who holds with both hands the great sweet wort,
Brewing [it] with honey [and] wine
(You the sweet wort to the vessel)
Ninkasi, (...)(You the sweet wort to the vessel)

The filtering vat, which makes a pleasant sound,
You place appropriately on a large collector vat.
Ninkasi, the filtering vat, which makes a pleasant sound,
You place appropriately on a large collector vat.

When you pour out the filtered beer of the collector vat,
It is [like] the onrush of Tigris and Euphrates.
Ninkasi, you are the one who pours out the filtered beer of the collector vat,
It is [like] the onrush of Tigris and Euphrates.

Early brewers were primarily women, mostly because it was deemed a woman's job. Mesopotamian men, of some 3,800 years ago, were obviously complete as clowns and had yet to realise the pleasure of brewing beer (Damerow 2012).

Using the above text, one could recreate the ancient recipe embedded within the poem. Thick loaves of bread called bappir were baked from several grains. Mixed with honey, the loaves were then twice baked until a granola like consistency was achieved, believing that the Sumerians stored this brew for later use. These loaves were added to a mash with a large addition of malt to ensure a proper conversion of starches. The mixture was then cooled naturally, not by modern techniques. The sweet liquid was strained away from the grains and transferred to the fermenter. Yeast was added and yielded a 3.5 percent alcohol by volume. After the fermentation, the beer was served in proper Sumerian style-sipped from bulky clay jugs using lengthy drinking straws (Wolf et al. 2008).

Beer early reached popularity. As much as 40% of Sumerian grain may have gone to make beer (Wolf et al. 2008). At all levels of society, Egyptians drank beer. Most people drank it daily, and it was a common offering to the gods (Hornsey 2003). In part, that may have been because few other beverages existed: no tea, coffee, wine, liquor, juice, nor water that was without bacterial contamination (Wolf et al. 2008). Beer provided a valuable addition to the diet consisting of an early nutritional supplement, composed of readily available starches and sugars, much like bread (Kiple 2000),various minerals, depending on the grain used and the soil, and valuable B vitamins (Hornsey 2003).

Travel and empire carried beer from the Middle East to Europe and Africa, but brewing also began spontaneously in many different locales. The Incas, for example, made corn-based alcohols, as well as those based on manioc and peanuts (Kiple 2000).

Different contributions like the use of hops to give a special flavour in the S. XII, the discovery of the pasteurization process some centuries later that allow the use of yeast, the Lager style find by chance in the S. XIX, the first stout beer "Guinness" made in Ireland at the end of the S. XVIII, the bottling machine in the S. XIX and the high technology development in the last century have contributed to the growth, improvement and variety of brewing as we currently know (Damerow 2012; Hornsey 2003; Wolf et al. 2008).

Nowadays, beer is the third most popular drink overall, after water and tea (Nelson, 2004) with high levels of annual per capita consumption in Europe (Figure 1). Being the total European consumption around 51,036 thousand kl (Kirin 2015).

Figure 1
Maps of beer consumption (litres per year per capita) in Europe.

Source: (Kirin 2015); [(1)] (Kirin 2014).

4.1 Beer composition

Brewers worldwide produce beer at an advanced technological level while keeping in mind the importance of tradition. Beer comprises hundreds of different compounds: some of them are derived from raw materials and pass unchanged through the brewing process, whereas others are produced during the process (Table 2).

Table 2
Composition of beer

Substance	Concentration	Number of compounds	Source or agent
Water	90-94%	1	-
Ethanol	3-5% v/v	1	Yeast, malt
Carbohydrates	1-6% w/v	~ 100	Malt
Carbon dioxide	3.5-4.5 g/l	1	Yeast, malt
Inorganic salts	500-4,000 mg/l	~ 25	Water, malt
Total nitrogen content	300-1,000 mg/l	~ 100	Yeast, malt
Organic acids	50-250 mg/l	~ 200	Yeast, malt
Higher alcohols	100-300 mg/l	80	Yeast, malt
Aldehydes	30-40 mg/l	~ 50	Yeast, hops
Esters	25-40 mg/l	~ 150	Yeast, malt, hops
Sulphur compounds	1-10 mg/l	~ 40	Yeast, malt, hops
Hop derivates	20-60 mg/l	> 100	Hops
Vitamin B compounds	5.0-10 mg/l	13	Yeast, malt

Source: Buiatti (2009).

Raw materials sources of all chemicals in beer are: water, malts (and its adjuncts), hops and yeast (Buiatti 2009). Their most important characteristics are described below:

- Water

Water is quantitatively the main ingredient of beers: it forms more than 90% and often even more than 94% of the final product (Wunderlich and Back 2009). The water must fulfil all legal requirements both chemically and microbiologically as well as satisfy the brewer's standards for clarity, taste, smell and lack of colour. The pH-value is especially important because different production steps only take place optimally at defined pH-values. Substantial amounts of ions are released from malt during mashing (Wunderlich and Back 2009).

The mineral content of brewing water has a very large effect on the properties of beer and gives an important contribution to the flavour of the finished product. The principal ions present in most brewing liquors are calcium, magnesium, sodium, potassium, sulphate, chloride, bicarbonate, carbonate and nitrate. Other elements are present but only in small amounts and are known as trace elements. It should be mentioned that brewing materials are also a source of some of these ions; most of the magnesium, potassium and phosphate in wort come from this source (Preedy 2011; Warnakulasuriya et al. 2002). Alkaline earth metals (in first order Ca^{2-} and Mg^{2-}) are important for the aspect of hardness in brewing liquor. Others like K^{-} often play a minor role. Generally, Ca^{2-} and Mg^{2-} are responsible for decreasing the pH-value. It is increased by hydrogen carbonate ions. Reactions with primary, secondary, and tertiary phosphates originating from malt inhibit this effect, but partly can also stimulate it. The relationship of pH-

value increasing and decreasing ions finds its expression in the residual alkalinity of brewing water (Buiatti 2009; Wunderlich and Back 2009).

- Malt: barley and other cereals

Malt is the germinated and dried barley and it is the grist component of more than 90% of beer produced in the world. Generally, barley (*Hordeum vulgare*) is quantitatively the second most important ingredient for beer. (Wunderlich and Back 2009). This raw material is the primary source of protein, lipid, carbohydrate, polyphenols present in beers (Preedy 2011).

Other crops like wheat, rye, triticale, spelt, and emmer are also suitable for brewing. They are added to barley malt. Recently, first trials in assessing alternative cereals and pseudo-cereals like sorghum for their suitability in malting are conducted. They resulted in brews with novel sensory and health aspects (Wunderlich and Back 2009). Alternative starch suppliers (malt substitutes) are interesting for their availability, profitability, and their special colour and aroma contribution. Often raw materials, like unmalted barley, wheat, rice, or corn are used. Sometimes starch, saccharine, glucose, and corresponding syrups are also used (Bamforth 2009; Briggs et al. 2004). Their application is regulated in every country. All these types of malts are the sources of several constituents present in beer such as, nitrogenous compounds, lipids, carbohydrates and vitamins.

- Hop

Hop (*Humulus lupulus L.*) gives beer its typical bitterness and hop aroma. Moreover it provides some protection against bacterial spoilage and it is fundamental for good foam formation. Further, hop contains pharmacologically active substances, for example it is said to be soporific, or sleep inducing. Of interest to the brewer are lupulin glands, with the exception of the tannins. Lupulin glands are located between spindle and bracts (Buiatti 2009; Wunderlich and Back 2009).

Three groups of substances are interesting from the brewing technological point of view: hop resins, flavouring agents, and polyphenols. Hop resins constitute about 10–20% of the hop dry weight. They represent the sum of all bittering substances. Other important components are the α- and β-acids, whose bittering potential differs markedly. α-acids are transformed into iso-α-acids during boiling. These iso-α-acids and their derivatives have significant bittering potential. β-acids have a low solubility in wort and beer. Thus, they contribute only a little to bitterness. Hop resins enhance physiological digestibility, foam stability, and bacteriostatic nature of wort and beer over and above the bittering potential (Buiatti 2009; Wunderlich and Back 2009).

Hop possesses approximately 0.4–2.0% flavouring agents per dry weight. These are essential oils that are responsible for the hop aroma and bouquet. More than 300 volatile substances have been identified up to now. Polyphenols (4–14% hop dry weight) also impact on beer quality. Additionally, low molecular polyphenols show antioxidative

properties among their benefits. Amounts of polyphenols and composition depend on hop variety, cultivation area, and climatic conditions (Preedy 2011).

- Yeast

Yeast (unicellular *fungi*) is the most important microorganism responsible of fermented beverages production and has a important impact on the quality of beer (Wunderlich and Back 2009). Many large and small breweries use several strains of *Sacharomyces cerevisiae* and/or *S. carlsbergensis* (*S. uvarum*) to produce different beers. properties of the beer produced strongly depend on yeast used. It plays a fundamental role in the synthesis and formation of several compounds found in beers. It produces not only ethanol and carbon dioxide but even other compounds (higher alcohols, organic acids, esters, aldehydes, ketones, sulphur compounds) which play a key role on the sensorial profile of beer (Buiatti 2009).

The following are the main criteria for a good brewing yeast: fermentation behaviour (bottom or top fermentation), flocculation (powdery or flocculent yeast), fermentation performance (fermentation rate, degree of fermentation), production, and degradation of side products (aroma development, diacetyl removal), as well as intensity of propagation (Preedy 2011).

4.2 Processing beer (brewing)

Brewing technologies worldwide are based on the same recipe, although brewers in some countries have more flexibility for selecting of starch supply. Nevertheless, barley is commonly used as the source of starch but it has to be malted to dissolve starch in the grains prior to brewing. Malting steps are steeping, germination, and kilning. Enzymes digest grain content during these processes and prepare starch for further processes. Heating during kilning produces colouring and flavouring substances. Further enzymes convert the starch of milled malt to fermentable sugars during mashing. This procedure results in wort that is boiled. Hops are added in this stage of boiling. Yeast converts sugars to alcohol during fermentation of cooled wort. After maturation and storage, beer is filtered and stabilized to inhibit quality deficiencies. These may be turbidity, decrease of flavour stability, or decrease of foam stability. Each production step influences decisively the resulting beer. So, an enormous variety of beers is possible that are all tasty, thirst quenching and healthy (Preedy 2011; Wunderlich and Back 2009).

Despite of the wide variety of styles of beer, their generally fall into two classifications: Lager or Ale beer. The two different categories of beer differ to each other in the type of yeast and the temperature used during their own brewing process. Lager beers use yeast strains that ferments best at cooler temperatures (below 5 °C), appropriate for bottom-fermented beers (*S. carlsbergensis*) they settle to the bottom of the fermentor after production of about 5% ethanol. Conversely, yeasts, typical for the production of top-fermented beers or Ale beers (*S. cerevisiae*), operate at room temperature and resist higher concentrations of ethanol, up to 12% (Granato et al. 2011).

Moreover, beers can exhibit colours ranging from pale to black due to the colour of malt(s) used and the caramelisation of sugars and Maillard-type reactions (De Keukeleire 2000; Kodama et al. 2006). During the malting process, drying continues until water content reaches 3.5–4% in pale malts and 1.5–2% in dark malts; and curing temperatures are high (95 °C) in a typical dark malt aroma whereas a curing temperatures range between 76 °C and 80 °C result for pale colour (Preedy 2011). Beers brewed with dark malts are often considered to have a longer shelf life than pale beers (Cantrell and Griggs 1996). Dark malts differ from pale malts in their content of coloured Maillard or caramelisation products formed during the roasting of malt by reactions involving proteins and carbohydrates (Woffenden et al. 2001). The overall effects of Maillard and caramelisation products on the oxidative stability of beer are unknown although positive correlations between antioxidant activity and malt colour have been observed due to the phenols compositions and the presence of Maillard components (Coghe et al. 2003; Woffenden et al. 2001).

There are many special malts and kilned raw grain that are similarly produced. All of them have a special impact on beer character (Wunderlich and Back 2009):

- Pale or dark wheat malt is used for wheat beer at grist ratios 50%. Flavour precursors get into wort and result in typical wheat beer aroma by top fermentation.
- Caramel malt awards beer a mouth filling, malty character, and dark colour. They are used in grist ratios between 2% and 10%.
- Roasted malt is added in grist ratios between 0.5% and 5%. It gives dark colour and roasting flavour.
- Roasted barley, roasted rye, and others originate from unmalted, but roasted crops. Resulting beers have a roasting, raw grain-like character. They are mostly used for stout and ale beers.

Recently, a worldwide interest in alcohol-free (AFB) and low-alcohol beers (LAB) is increasing. AFB and LAB are known to have a weaker aroma than standard beers (Brányik et al. 2012) with the difference to reduce their alcohol contents. Legally fixed maximum alcohol contents differ in countries, but AFB and LAB are considered to contents below ≤ 0.5% and no more that 1.2% alcohol per volume (Brányik et al. 2012). Athletes appreciate beer's typical properties, reduced calories, and the isotonic properties of most alcohol-free beers. Two procedures especially have been established for manufacturing non-alcoholic beer: physical methods based on removed the alcohol contend (dealcoholisation by vacuum rectification and evaporation, dialysis, reverse osmosis, membrane extraction, CO_2 extraction, pervaporation, adsorption or freeze), and biological processes based on limited alcohol formation (use of special yeast) (Anglerot 1994; Brányik et al. 2012; Etuk and Murray 1991; Von Hodenberg 1991). The most used yeast for alcohol-free beer production is *Saccharomycodes ludwigii*. It has the capacity to control the fermentation process due to its disability to ferment maltose and maltotriose, the prevailing fermentable sugars of all malt worts. Moreover, the fermentation of *S. ludwigii* is characterised by slow attenuation even at 20 °C which implies that the process does not require continuous monitoring (Brányik et al. 2012).

Literature is scarce to properly compare the impact on the product quality, as well as the economic aspects of processes producing LABs or AFBs. Nevertheless, it is clear that the limited fermentation process can be performed with common brewery equipment with shorter production time and fewer raw materials needed. Being the production costs for such LAB/AFBs the same or lower than for the regular beer, the only advantage of alcohol separation processes is their flexibility (start-up within hours, high productivity) and possibility to produce zero alcohol beers, which is hardly achievable by fermentative processes (Brányik et al. 2012).

Recently, special technologies were developed, for example, for enrichment of folic acid or xanthohumol in beer. Innovations like this, in addition to publicizing the health effects of beer ingredients, may strengthen consumer interest in a tasty and healthy beer (Preedy 2011; Wunderlich and Back 2009).

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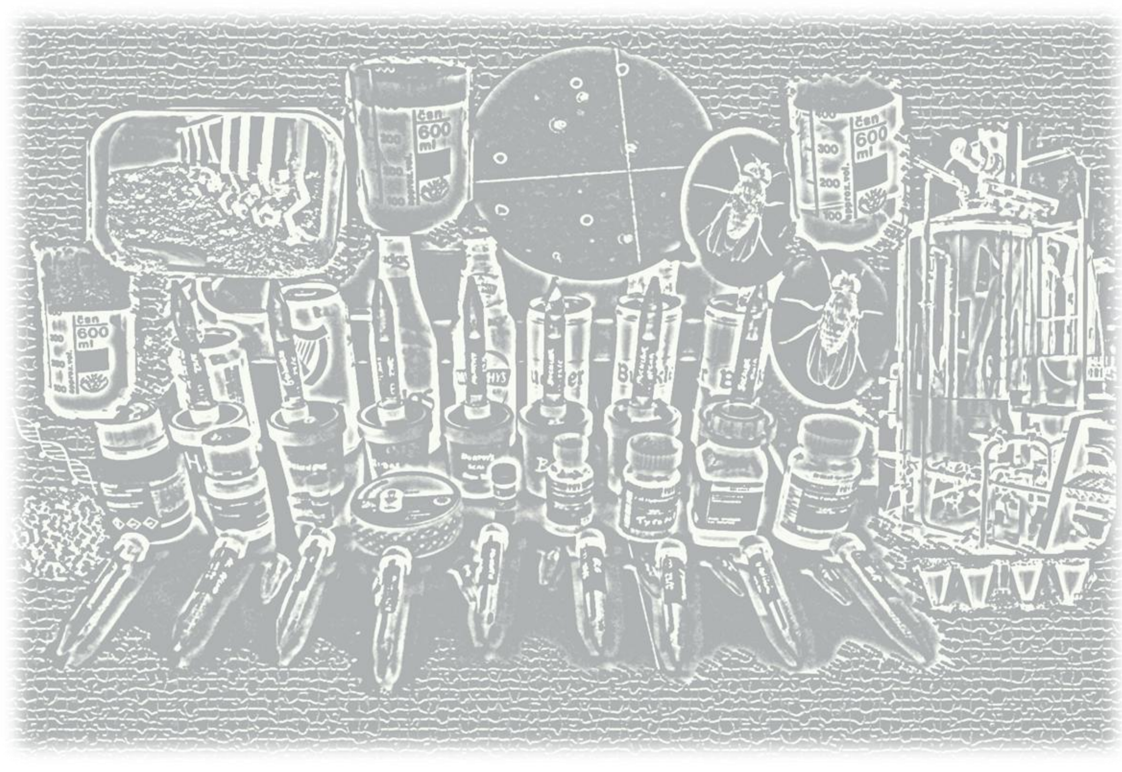
HYPOTHESIS



Beer is one of the most consumed alcoholic beverages around the world, being rich in nutrients such as carbohydrates, amino acids, minerals, vitamins and other compounds such as polyphenols (Arranz et al. 2012). Beers and its compounds are related to healthy properties as anti-bacterial, anti-inflammatory, anti-oxidative, anti-angiogenic, anti-melanogenic, anti-osteoporotic and anti-carcinogenic effects (Chen et al. 2014; Gerhäuser 2005; Reddy et al. 2003). It is well known that beer has been associated with a lower risk of suffering a cardiovascular disease. It is shown that beer consumption reduced mortality from coronary heart diseases in moderate alcohol consumers with respect to non-alcohol consumers (Rimm et al. 1991), and also the ability of beer against kidney stones formation (Curhan et al. 1996). Furthermore, there are accumulated evidences that support the health benefits of moderate consumption of alcohol as part of a healthy lifestyle, associated with lower rates of cardiovascular diseases, linked to polyphenol content of the alcoholic beverages (Bassus et al. 2004; Hertog et al. 1993). In contrast, other studies show evidence that alcohol increases the risk of mouth, oesophageal and liver cancers (Glade 1999). Some studies performing the Ames test suggest the possibility that the antimutagenic activity attributed to beer is a result of a cumulative effect of the individual beer constituents (Arimoto-Kobayashi et al. 1999).

The main natural antioxidants present in beer are polyphenols and melanoidins, particularly phenolic acids from barley (≈ 70%) and hop (≈ 30%) (Callemien and Collin 2009; Callemien et al. 2005; De Keukeleire et al. 1999; Goupy et al. 1999; Woffenden et al. 2001). The structural classes of polyphenols in beer include simple phenols, benzoic acid derivatives and cinnamic acid, coumarins, catechins, di- and tri-oligomeric proanthocyanidins, prenylated chalcones and α- and iso-α acids derived from hops (Arranz et al. 2012; Gerhäuser and Becker 2009). The composition and levels of phenolic compounds in beer could be different depending on the raw materials utilized, the brewing process and type of beer, and also on the changes during storage. Moreover, some alcohol-free beer usually has lower levels of phenolic constituents due to losses during the dealcoholization process. Due to the high complexity and variability in the polyphenolic pattern, biological activities attributed to beer consumption cannot be linked to one particular class of constituents (Gerhäuser and Becker 2009). Polyphenols that occur in beer in relatively high concentrations are: the phenol tyrosol, the benzoic acid derivative *p* -hydroxybenzoic acid, the cinnamic acid *p* -coumaric and ferulic acid, (-)-catechin and (-)-epicatechin, the proanthocyanidin dimers procyanidin B3 and prodelphinidin B3 and the flavanone isoxanthohumol (Gerhäuser and Becker 2009).

Epidemiological studies indicate the anti-proliferative activity of beer components with antioxidant properties in prostate cancer cells, showing a strong correlation between antioxidant potency and polyphenols content (Hevia et al. 2008). Many studies suggest that dietary flavonoids may have beneficial effects on human health and disease prevention, primarily attributed to their antioxidant properties (Stevens and Page 2004).
In the present thesis, we select 23 different types of beers according to its colour and fermented characteristics, as well as 3 processed-beer at different brewing steps were

studied. Furthermore, different raw materials and some bioactive compounds of beers were also selected. Details of all selected compounds are indicated below.

Types of beer and compounds studied

Details of the different types of beer studied are given in Table 3. Moreover, processed-beer in different brewing steps are analysed (Table 4). In order to be able to test from low to high amount of beer and, at the same time eliminate the alcohol factor, all samples were lyophilised in the present PhD dissertation.

Table 3

Details of studied beers.

Beer trade mark	Named in the text	Beer style / origin
"Heineken"	LBLB	Blond Lager
"Guinness"	LSAB	Stout Ale
"Judas"	LBAB	Blond Ale
"Murphy's Irish Red"	LSAB	Stout Lager
"Buckler 0,0"	LBAFLB	Blond Alcohol-free Lager
"Buckler 0,0 White"	LPBAFLB	Pale-blond Alcohol-free Lager
"Buckler 0,0 Black"	LSAFLB	Stout Alcohol-free Lager
"Pilsner Urquell"[1]	Pilsner Urquell	Blond Lager
"Staropramen Lezak"[1]	Staropramen Lezak	Blond Lager
"Staropramen Cerny Lezak"[1]	Staropramen Cerny	Stout Lager
"Staropramen Nealko"[1]	Staropramen Nealko	Alcohol-free Lager
"Staropramen Nefiltrovany"[1]	Staropramen Nefiltrovany	Blond Lager
"Lobkowicz Lezak Premium"[1]	Lobkowicz Lezak	Blond Lager
"Lobkowicz Cerny Premium"[1]	Lobkowicz Cerny	Stout Lager
"Lobkowicz Nealkoholicky Premium"[1]	Lobkowicz Nealko	Alcohol-free Lager
"Lobkowicz Psenicny Premium"[1]	Lobkowicz Psenicny	Wheat Lager
"Budweiser Budvar B:Original Svetly lezak"[1]	Budweiser Lezak	Blond Lager
"Budweiser Budvar B:Free Nealkoholicke pivo"[1]	Budweiser Nealko	Alcohol-free Lager
"Budweiser Budvar B:Cherry Tmavy lezak s visní"[1]	Budweiser Cherry	Cherry flavoured Lager
"Herold Svetly Breznicky Lezak"[1]	Herold Lezak	Blond Lager
"Herold Tmave specialni Pivo"[1]	Herold Cerny	Stout Lager
"Herold Psenicny Kvasnicovy Lezak"[1]	Herold Psenicny	Wheat Lager
"Herold Polotmave Specialni Pivo"[1]	Herold Polotmave	Pomegranate flavoured Lager

[1] Typical Czech beer.

Table 4
Details of the different processed-beers studied.

Processed-beer	Origin
Wort[(1)]	CULS[(4)] Prague brewery
Hopped wort[(2)]	CULS Prague brewery
Young beer[(3)]	CULS Prague brewery

[(1)] Sugar solution obtained after filtration of barley malt boiling.
[(2)] Liquid obtained after addition and boiling of hops to the wort, cooling and removal of spent hops.
[(3)] Liquid obtained after yeast addiction and fermentation during a week.
[(4)] Czech University of Life Science.

As raw materials, be studied the properties of malt, hops and yeast (Table 5).

Table 5
Details of raw materials studied.

Raw materials	Ingredient origin
Rest of mash[(1)]	CULS[(5)] Prague brewery
Blond malt	CULS Prague brewery
Dark malt	CULS Prague brewery
Sladek hop[(2)]	CULS Prague brewery
Saazer hop[(3)]	CULS Prague brewery
Saccharomyces uvarum[(4)]	CULS Prague brewery

[(1)] Rest of malt after boiling process.
[(2)] Hybrid aroma varieties of hop.
[(3)] Fine aroma hop.
[(4)] Lager yeasts sink to the bottom of the beer and ferment more slowly, preferring colder temperatures around 7-15 ºC (Goldammer 2000).
[(5)] Czech University of Life Science.

A total of 7 phenolic compounds in beers were studied due to their antioxidant characteristics and abundance in beers (Table 6). Further details of the different phenols are indicated below.

- Vanillic, 4-Hydroxy-3-methoxybenzoic acid, is an intermediate in the production of vanillin from ferulic acid (Civolani et al. 2000; Lesage-Meessen et al. 1996). Beers contain up to 3.6 mg/l of this phenolic acid constituent (Gerhäuser 2005).

- Ferulic, trans-Ferulic acid, is the main phenolic acid in barley, malted barley and beer, followed by *p*-coumaric, vanillic, caffeic, *p*-OH-benzoic and sinapic acids (Chunsriimyatav et al. 2010; MaIt and XgUMaIl 1992). Beers contain up to 6.5 mg/l of this phenolic acid constituent (Gerhäuser 2005). Ferulic acid, like many natural phenols, is an antioxidant *in vitro* in the sense that it is reactive toward free radicals such

as reactive oxygen species (ROS). ROS and free radicals are implicated in DNA damage, cancer, and accelerated cell aging. Animal studies and *in vitro* studies suggest that ferulic acid may have direct antitumor activity against breast cancer (Saulnier and Thibault 1999) and liver cancer (Gelinas and McKinnon 2006). Ferulic acid may have proapoptotic effects in cancer cells, thereby leading to their destruction (Gelinas and McKinnon 2006).

- Xanthohumol (XN, 2′,4′,6′,4-tetrahydroxy-3′-prenylchalcone), a prenylated chalcone isolated from the hop plant (*Humulus lupulus L.*), it is a beer polyphenol belonging to the group of chalcones (Saura-Calixto et al. 2011), which is used as an ingredient in the brewing process (Miranda et al., 2000). Xanthohumol was shown to scavenge reactive oxygen species and to inhibit superoxide anion radical formation (Ferguson 2001; Gerhauser et al. 2002). Furthermore, xanthohumol has been identified as a possible anticarcinogenic agent (Piendl and Biendl 2000), it can inhibit carcinogenesis at the initiation, promotion and progression steps of cancer, and protects DNA against genotoxicity and oxidative DNA damage (Plazar et al. 2008).

- Catechins (flavan-3-ols) are an important group of dietary antioxidants. Catechin derivatives in beer include monomers like (-)-catechin and (-)-epicatechin, but also their esters with gallic acid, that is catechin gallate and epicatechin gallate (ECG). Beer catechins are derived both from barley/malt (catechin and gallocatechin) as well as from hops (catechin and epicatechin) (Gerhäuser and Becker 2009).

- Isoxanthohumol (IX) which is a flavanone formed from the xanthohumol in hops during wort boiling (Piendl and Biendl 2000). Conventional beers contain up to approximately 3 mg of isoxanthohumol per litre, depending on hopping (quantity added and hop product used) and the brewing process employed (Biendl and Pinzl 2009). It has limited estrogenic activity. At the concentration found in beer, it is unlikely to have an estrogenic effect in breast tissue (Bolca et al. 2010).

- Humulone is a bitter-tasting chemical compound found in the resin of mature hops (*Humulus lupulus*) (De Keukeleire et al. 2003). Overall, it represents one of the most abundant classes of polyphenols in beer, with concentrations of up to 100 mg/l in very bitter English Ales ((De Keukeleire 2000). In addition to anti-inflammatory and antioxidative effects, the hop bitter acids also inhibit many gram positive bacteria (Bohra et al. 2008). According to Sánchez et al. (2010), hops have been frequently used as tranquillizer plant. Furthermore, many beer compounds can normalize the rhythm of the sleep in addition to stimulate it. This sedative capacity is due to its bitter acids, particularly the alpha acid component (Chen et al. 2014).

- Tyrosol, 4-(2-hydroxyethyl) phenol, is a natural phenolic antioxidant abundant in wine, virgin olive oil, vermouth and beer, and has gained increasing attention as a dietary antioxidant (Dewapriya et al. 2013). This aromatic alcohol which is produced by

fermentation of the amino acid tyrosine, was detected in exceptionally high concentrations up to 40 mg/l in beers (Tressl et al. 1976). As an antioxidant, tyrosol can protect cells against injury due to oxidation (Giovannini et al. 1999). Furthermore, studies suggest that tyrosol induces myocardial protection against ischemia related stress by inducing survival and longevity proteins that may be considered as anti-aging therapy for the heart (Samuel et al. 2008).

Table 6

Studied phenolic compounds in beers (modified from (Estruch et al. 2011; Gerhäuser and Becker 2009).

Structural classes	Phenolic compound	(mg/ml)
Benzoic acid derivatives	Vanillic acid	Up to 3.4
Cinnamic acids	Ferulic acid	Up to 6.5
Chalcones	Xanthohumol	0.002 - 1.2[a]
Flavonols (Catechins)	Epicatechin gallate	5 - 20
Flavanones	Isoxanthohumol	0.04 - 3.44[a]
Iso-alpha-acids	iso-n-Humulone 20-50%	0.6 - 100[b]
Simple phenols	4-(2-Hydroxyethyl)phenol (tyrosol)	Up to 40

[a] Highest levels are found in stout and porter beers (Stevens et al. 1999).

[b] In very bitter English ales (De Keukeleire 2000).

Moreover two vitamins B, choline and folic acid, were studied due mainly to its potential methyl donors role. Hence a dose controversial exits on choline and folate intake effects.

- Choline is a natural amine classified in the vitamin B complex and is a precursor for many other biologically important molecules (Institute of Medicine 1998). Choline is important for brain development as it is a precursor for the neurotransmitter acetylcholine, which is essential for the activity of cholinergic neurons (Blusztajn and Wurtman 1983; Zeisel and Blusztajn 1994). Choline is important for reducing the risk of neural tube defects (Zeisel and Niculescu 2006), for memory development (Zeisel 2006) and it has also been demonstrated to be an essential element for survival and normal growth of cultured cells (Chi-liang et al. 1999; Eagle 1955; Tercé et al. 1994; Yen et al. 2001). Furthermore, similar to folic acid, genomic stability role has been attributed to choline (Lu et al. 2012), because both are methyl donors and a high choline diet has been associated with a decreased risk of breast cancer (Xu et al. 2008).

- Folic acid, a member of the folate group (Jacques et al. 1999), is an essential B vitamin for DNA synthesis, methylation and repair (Ames 2001; Mason and Levesque 1996). Joshi et al. (2001) demonstrated the effective free radical scavenger activity of folates. The scavenging and repair of thiyl radicals by folic acid makes it a potential vitamin to be called as an antioxidant. Folate has been hypothesized as an anti-cancer

agent (Giovannucci 2002; Larsson et al. 2007a, b; Lin et al. 2008; Wei et al. 2005; Zhang et al. 2003).

Once the high consumption of beer since many years ago and their healthy properties are known, we have contrasted the hypothesis of considering beer as a potential nutraceutic determining which bioactive components are crucial in order to propose it as a promising biomedical beverage. For that purpose, the use of safety assays followed by studies on the protective effects that the tested compounds show in the *Drosophila* animal model were carried out. Moreover, chemopreventive studies in the *in vitro* model of human leukaemia cell line (HL-60) were implemented to evaluate de cytotoxic potential and DNA damage that our tested compounds induce in the tumour cells. Finally, a pilot *in vivo* human clinical trial was performed to examine the role of a blond Lager beer on the modulation of methylation levels of repetitive sequences in human leucocytes.

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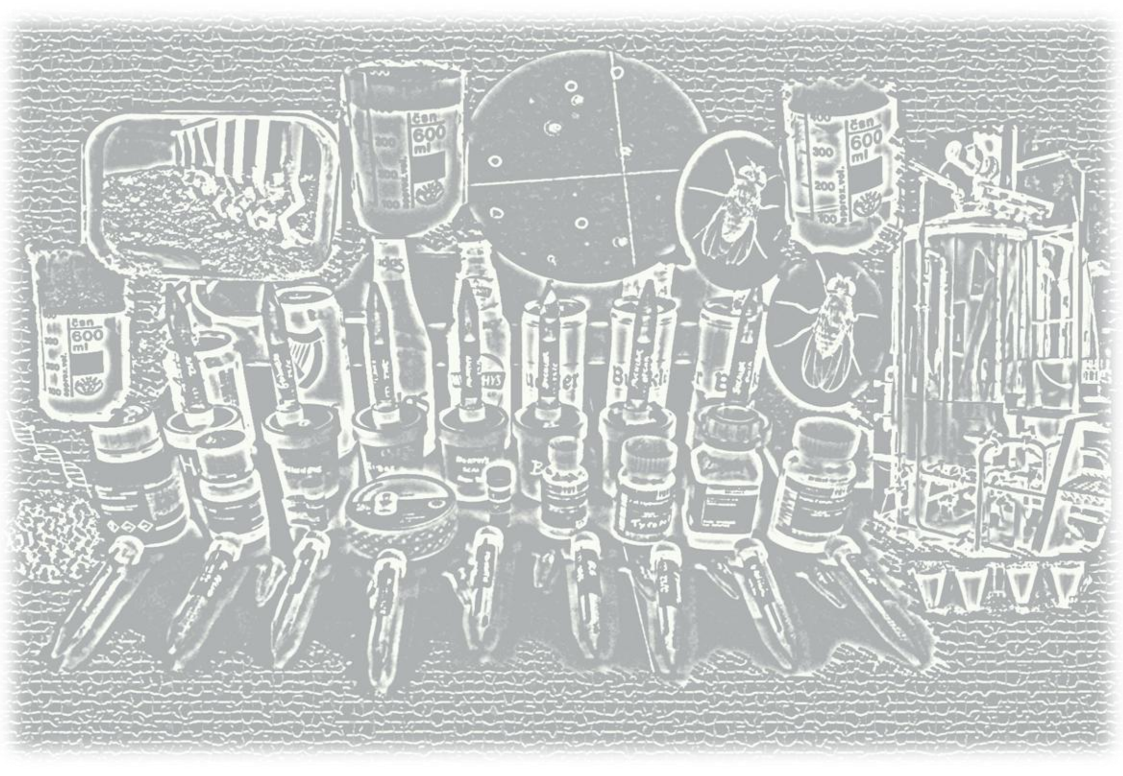
OBJETIVES



The main objective of the present thesis is to evaluate the nutraceutic potential of beers and its most important bioactive compounds.

For that propose, a genotoxicological screening by using *in vivo* and *in vitro* safety, protection, chemoprotective potential and DNA damage induction tests, as well as a pilot clinical study in human, were carried out. For this, the following specific objectives were established:
i) To determine the toxicity of the tested compounds studying the survival of *Drosophila melanogaster* model organism.
ii) To determinate the antitoxicity potential of beers and its compounds in *D. melanogaster* when it is fed with the tested substances in combined treatments with an oxidative toxin.
iii) To determinate the rate of mutation induction/reduction (genotoxicity) by the somatic mutation and recombination test in the *in vivo* model organism treated with the studied substances.
iv) To determinate the DNA protective effect in the somatic cells of *D. melanogaster* when substances are combined with hydrogen peroxide as genotoxine (antigenotoxicity) by the SMART test.
v) To determinate the ability of improve or not the longevity and healthspan in *D. melanogaster* fed over time with the studied substances.
vi) To determine the chemopreventive potential of the substances in tumour cells.
vii) To determine the ability of the substances to induce DNA damage in tumour cells by proapoptotic DNA fragmentation and/or single or double strand breaks way.
viii) To determine the ability of the substances to modify the methylation status of genome wide repetitive sequences in the HL-60 tumour cells.
ix) To evaluate the effects of beer daily intake on methylation status of repetitive sequences of healthy human DNA.

Different types of beers and their most important components, used for the brewing process and for taking part of the final product, were selected for the thesis objectives:

Various lager and ale as well as coloured, flavoured, filtrated and alcohol-free lyophilised type of beers from Heineken, Guinness, Judas, Murphy's, Buckler, Pilsner Urquell, Staropramen, Lobkowicz, Budweiser and Herold marks were selected for the study. Furthermore, three processed-beers from the three important brewing steps were studied to analyse the changes during the beer production.
As raw materials blond and dark malts, Sladek and Saazer hops and *Saccharomyces uvarum* yeast were selected.

Previously to be used, all complex mixtures were lyophilised in order to be able to test from low to high concentrations of beer and, at the same time to eliminate the alcohol factor.

In addition, a wide variety of single molecules belonging to different chemical categories were selected according to their abundance and role in the beers. Tyrosol, Vanillic acid, ferulic acid, epicatechin gallate, xanthohumol, isoxanthohumol and iso-α-humulone were selected as simple phenols. Besides, choline and folic acid were chosen as vitamins B and methyl donor compounds.

CHAPTER 1

In vivo and *in vitro* studies of the role of lyophilised blond Lager beer and three bioactive components in the modulation of degenerative processes

Published as two independent manuscripts:
- "*In vivo* and *in vitro* studies of the role of lyophilised blond Lager beer and some bioactive components in the modulation of degenerative processes", in Journal of Functional Foods. DOI: 10.1016/j.jff.2016.09.014.
- "Role of Choline in the Modulation of Degenerative Processes: *In Vivo* and *In Vitro* Studies", in Journal of Medicinal Food. DOI: 10.1089/jmf.2016.0075.

ABSTRACT

The aim of the present study was to examine the nutraceutic potential of lyophilised blond Lager beer, as well as three of its bioactive components: xanthohumol, choline and folic acid. Several toxicity, antitoxicity, genotoxicity, antigenotoxicity and longevity endpoints were checked in the SMART *in vivo Drosophila* system. Cytotoxicity in HL-60 promyelocytic and NIH3T3 mouse fibroblasts cells, proapoptotic DNA fragmentation, comet assay, macroautophagy activity and methylation status were tested in *in vitro* assays. Lyophilised blond Lager beer could be proposed as a substance with an important nutraceutical value, because of its hopeful results as a lifespan promoter, DNA protection against free radicals in the animal model and its chemopreventive activity in HL-60 cells and enhancer of macroautophagy at moderate doses in NIH3T3 immortal cells. Only xanthohumol mimicries the biological activities found in lyophilised beer.

1. INTRODUCTION

Cancer is originated by internal and external factors and offers a variety of targets for intervention and inhibition at every stage such as antioxidant activity, xenobiotics metabolism, anti-mutagenic, anti-inflammatory and anti-hormonal mechanisms, anti-proliferative activities, induction of terminal cell differentiation and apoptosis (Reddy et al. 2003). Many cases of cancer are related with lifestyle and environmental conditions, and it is estimated that one third of cancer deaths is due to dietary habits (Martin-Moreno et al. 2008). Drugs, chemicals, temperature and light are among the external environmental factors that can determine which genes are turned on and off, thereby influencing the way an organism develops and functions (Clayton and McKeigue 2001).

The reactive oxygen species possess a potent cytotoxic, mutagenic and carcinogenic effect and they are able to interact and alter genetic material (Benavente-Garcia et al. 1997; Kaur et al. 2006). Moreover, oxidative stress is involved in the pathology of many diseases, such as atherosclerosis, diabetes, neurodegenerative diseases, aging and cancer (Saura-Calixto et al. 2008).

Antioxidants may be found at significant amount in diet and confer protection against oxidative stress-related diseases (Aruoma 1998). Polyphenols are the most abundant dietary antioxidants, distributed mostly in coffee, fruits, wine, vegetables, olive oil and beer (Hertog et al. 1993). Specifically, the consumption of beer can contribute to the total intake of polyphenols and to the antioxidant capacity of the diet. Beer provides 7.2% of the daily dietary ingestion of polyphenols and accounts for around 3% of the total antioxidant capacity of the diet in some countries like Spain (Saura-Calixto et al. 2008), being the third most popular drink overall, after water and tea (Nelson 2004). Some representative bioactive compounds of beer are: xanthohumol, the principal flavonoid of hop plant (Plazar et al. 2007); choline, nitrogenous constituent of beer and essential for human growth; and folic acid or B9 vitamin, B vitamin constituent of beer (Buiatti 2009).

Beer is obtained from natural ingredients, which are related with health effects (Saura-Calixto et al. 2008). Beer constituents have been shown to possess various anti-bacterial, anti-inflammatory, anti-oxidative, anti-angiogenic, anti-melanogenic, anti-osteoporotic and anti-carcinogenic effects. Epidemiological studies on the association between beer drinking and skin disease are limited while direct evidence of beer compounds in clinical application is lacking. Potential uses of these substances in dermatology may include treatment of atopic eczema, contact dermatitis, pigmentary disorders, skin infections, skin ageing, skin cancers and photoprotections, which require an optimization of the biostability and topical delivery of these compounds (Chen et al. 2013). Beer has been associated with a lower risk of suffering a cardiovascular disease. It is shown that beer consumption reduced mortality from coronary heart diseases in moderate alcohol consumers with respect to non-alcohol consumers (Rimm et al. 1991), or the ability of beer against kidney stones formation (Curhan et al. 1996). Furthermore, there are accumulated evidences that support the health benefits of moderate

consumption of alcohol as part of a healthy lifestyle, associated with lower rates of cardiovascular diseases, linked to polyphenol content of the alcoholic beverages (Bassus et al. 2004; Hertog et al. 1993). In contrast, other studies show evidence that alcohol increases the risk of mouth, oesophageal and liver cancers (Fund 1997). Some studies performing the Ames test suggest the possibility that the anti-mutagenic activity attributed to beer is a result of a cumulative effect of the individual beer constituents (Arimoto-Kobayashi et al. 1999). According to Sánchez et al. (2010), hops have been frequently used as tranquillizer plant. Furthermore, many beer compounds can normalize the rhythm of the sleep in addition to stimulate it. This sedative capacity is due to its bitter acids, particularly the alpha acid component (Chen et al. 2013).

The main natural antioxidants present in beer are polyphenols and melanoidins, particularly phenolic acids from barley (≈ 70%) and hop (≈ 30%) (Callemien and Collin 2010; Callemien et al. 2005; Goupy et al. 1999; Woffenden et al. 2001). Epidemiological studies indicate the anti-proliferative activity of beer components with antioxidant properties in prostate cancer cells, showing a strong correlation between antioxidant potency and polyphenols content (Hevia et al. 2008). Many studies suggest that dietary flavonoids may have beneficial effects on human health and disease prevention, primarily attributed to their antioxidant properties (Stevens and Page 2004).

The present work focused on the biological activities of beer and three of its minor molecules belonging to the phenolic and B vitamin classes. A chalcone present in hops as the principal flavonoid: xanthohumol (Stevens et al. 1999); a natural amine classified in the vitamin B complex and a precursor for many molecules present in malt: choline (Institute of Medicine 1998; Zeisel and Da Costa 2009); and a folate contained in malt (Edelmann et al. 2013; Owens et al. 2007). All molecules are important distinctive compounds of beer with different structure-activity related characteristics. Moreover, free radical scavenging properties and antioxidant activity of all of them are reported as will be detailed below.

Xanthohumol (XN, 2′,4′,6′,4-tetrahydroxy-3′-prenylchalcone), a prenylated chalcone isolated from the hop plant (*Humulus lupulus L.*), is a beer polyphenol belonging to the group of chalcones (Saura-Calixto et al. 2008), which is used as an ingredient in the brewing process (Miranda et al. 2000). According to Ferguson (2001), the protection against oxidative damage of polyphenols is due to their ability to scavenge free radicals because of their interference with the oxidation of lipids, DNA and other molecules by rapid donation of a hydrogen atom to radicals ($RO^{\bullet} + PPH \rightarrow ROH + PP^{\bullet}$). The phenoxy radical intermediates are relatively stable, and also act as terminators of the propagation route by reacting with other free radicals ($RO^{\bullet} + PP^{\bullet} \rightarrow ROPP$). Xanthohumol was shown to scavenge reactive oxygen species and to inhibit superoxide anion radical formation (Gerhauser et al. 2002). Xanthohumol has been suggested as an effective constituent against atherosclerosis, increasing HDL-cholesterol levels (Hirata et al. 2012). Furthermore, xanthohumol can inhibit carcinogenesis at the initiation, promotion and progression steps of cancer, and protects DNA against genotoxicity and

oxidative DNA damage (Plazar et al. 2008). In addition, it has been shown an anti-proliferative activity in human cancer cell lines, including breast, colon, prostate, ovarian and blood cancer by inhibiting proliferation and inducing apoptosis (Benelli et al. 2012; Colgate et al. 2007; Delmulle et al. 2006; Gerhauser et al. 2002; Lust et al. 2005) (Pan et al. 2005). Finally, xanthohumol is associated with detoxification of reactive metabolites through induction of quinone reductase activity (Dietz et al. 2005; Miranda et al. 2000), also showing antigenotoxic capacity (Strathmann et al. 2010). All of these activities, contribute to the prevention of oxidative damage by scavenging reactive oxygen species (ROS) and nitrogen oxide (NO) production (Gerhauser et al. 2002; Miranda et al. 2000; Zhao and St Clair 2003), and might suggest xanthohumol as a cancer preventive agent in a long-term carcinogenesis model.

Choline is classified as an essential nutrient for humans and a minimum dietary intake is recommended (Institute of Medicine 1998). It is also an essential element for survival and normal growth of cultured cells (Eagle 1955; Terce et al. 1994; Yen et al. 2001; Yen et al. 1999). Other studies have suggested that choline is important for brain development (Blusztajn and Wurtman 1983), as choline is a precursor of the neurotransmitter acetylcholine, which is essential for the activity of cholinergic neurons (Nagler et al. 1968; Zeisel and Blusztajn 1994). Choline deficient diet leads to reduced secretion of liver triglyceride, resulting in accumulation of liver triglycerides (Yao and Vance 1988). In neuronal cell, the inhibition of phosphatidyl choline synthesis via NMDA (*N*-methyl-D-aspartate) receptor is a key early event in the process of cellular exocytosis that leads to necrotic cell death (Gasull et al. 2000), leading a cytoplasmic membrane damage caused by increased hydrolysis of membrane phopholipids (Farooqui et al. 1997). As well as folic acid, genomic stability and cell death properties has been attributed to choline (Lu et al. 2012). Furthermore, similar to folic acid, genomic stability has been attributed to choline (Lu et al. 2012), because both are methyl donors and a high choline diet has been associated with a decreased risk of breast cancer (Xu et al. 2008). On the other hand, studies have shown that a choline deficiency may have adverse effects like increased DNA damage and apoptosis in lymphocytes (da Costa et al. 2006), increased plasma homocysteine levels, which have been associated with cardiovascular disease (Homocysteine Studies Collaboration 2002), cognitive decline (Seshadri et al. 2002), bone fractures (van Meurs et al. 2004), development of fatty liver and liver muscle damage (Kohlmeier et al. 2005) and a reduced secretion of liver triglyceride, resulting in accumulation of liver triglycerides (Yao and Vance 1988), among others pathologies. Despite the beneficial effects of a daily intake of choline, a greater intake than the recommended dosage has been associated with harmful effects like nausea and diarrhoea (Deuster and Cooper 2006), body odour, sweating and salivation (Growdon et al. 1977; Lawrence et al. 1980; Zeisel et al. 1983), hypotension (Loffelholz 1981), hepatotoxicity (Nadkarni et al. 1992) and dermatitis (Fischer 1984). In rare cases, a large amount of choline consumption has been associated with depression (Davis et al. 1979) and with exacerbated symptoms of Parkinsonian patients (Gelenberg et al. 1979).

Folates belong to the B vitamin family. Folic acid and its constituents, pyrazine and pterin, can easily be reduced by hydrated electron and acetone ketyl radical (Moorthy and Hayon 1976) to the corresponding hydroderivatives in the pyrazine ring of the molecule. Furthermore, free radical intermediates have been suggested (Hemmerich 1968; Vonderschmitt and Scrimgeour 1967) in the chemical oxidation of reduced pterins by air, H_2O_2 or Fe^{3+}, as well as in the enzymatic oxidation of reduced pterin by phenylalanine hydroxylase. They are present in many natural foods such as green leafy vegetables, peas, beans, certain fruits and beer (Saxby et al. 1982), being the rank of folic acid in beer from 0.04 to 0.6 mg/l (Buiatti 2009). Folic acid, a member of the folate group (Jacques et al. 1999), is an essential B vitamin for DNA synthesis, methylation and repair (Ames 2001). Joshi et al. (2001) demonstrated the effective free radical scavenger activity of folates. The scavenging and repair of thiyl radicals by folic acid makes it a potential vitamin to be called as an antioxidant. Folate has been hypothesised as an anti-cancer agent (Giovannucci 2002; Larsson et al. 2007a, b; Lin et al. 2008; Wei et al. 2005; Zhang et al. 2003). Others studies revealed the role of folate in hyperhomocysteinemia and neuronal tube defects (Ferguson et al. 1998; Welch and Loscalzo 1998). Low-folic acid diets show an alteration in gene expression and increased DNA damage and it has been associated with the carcinogenesis process leading to a high incidence of colon cancer (Willett et al. 1990). Contrary, diets with high folate intake show a reduction of approximately 40% in the risk of colorectal neoplasms (Bailey et al. 2003). Recent *in vitro* studies show a beneficial effect on genomic stability and cell death (Lu et al. 2012). Nevertheless the meta-analysis in humans published by Wien et al. (2012) using 10 randomised controlled trials showed a borderline significant increase in frequency of overall cancer in the folic acid group compared to controls although formers epidemiological studies have shown that folate supplements can significantly reduce the risk of pancreatic cancer (Stolzenberg-Solomon et al. 1999) and breast cancer (Zhang et al. 1999). Hence a dose controversial exits on folate intakes effects.

A nutraceutical substance should be able to prevent genetic oxidative damage, increase the lifespan, and induce cell death in a programmed manner in tumour cells. Here, we have tested the nutraceutic potential of a lyophilised blond Lager beer (LBLB), as well as three of its bioactive components: xanthohumol, choline and folic acid. Several points related to degenerative processes have been checked: (i) *in vivo* assays using the *Drosophila* model, as 70% of human disease genes are conserved in this organism (Richardson et al. 2015), to evaluate the toxicity, antitoxicity, genotoxicity, antigenotoxicity and longevity induction and (ii) *in vitro* assays to evaluate the cytotoxic and proapoptotic DNA fragmentation activity, comet assay, macroautophagy activity and methylation status on the HL-60 promyelocytic and immortal NIH3T3 cell models.

2. MATERIAL AND METHODS

2.1 Sample preparation

A blond Lager beer (Heineken®) was selected for this study because, according to *"The Drinks Business"*, it is one of the world's most popular beers (Tepper 2012). Prior to carrying out the assays, beer was lyophilised (SCAI, University of Córdoba) and dissolved in distilled water in order to obtain the different concentrations tested. The lyophilised sample was stored, until use, at room temperature in a dark and dried atmosphere. Furthermore, xanthohumol, choline and folic acid were tested as single bioactive compounds.

2.2 Xanthohumol and folic acid content characterisation in LBLB sample

Xanthohumol and folic acid were tested as single bioactive compounds. The chromatographic behaviour of both compounds was analysed by the SCAI (University of Córdoba) using an HPLC/MS system to confirm their presence in the studied drink. Mass spectrometry (MS) analyses were performed on a triple-quadruple LC-MS equipment. The high performance liquid chromatography (HPLC) was interfaced to the MS via electrospray ionisation (ESI) in negative mode and by ions detection in the MRM (multiple reaction monitoring) mode in order to achieve the highest sensitivity as possible. The chromatographic separation of both compounds were performed in reverse phase (column C-18, 100 x 2.1 mm), with a gradient of water/formic acid and methanol.

Total flow: 0.25 ml/min; column temperature: 40 °C; injection volume: 5 µl.

2.3 Treatments and samples concentrations

The concentrations of LBLB used in the different assays were established taking into account the average daily food intake of *D. melanogaster* (1 mg/day) and the average body weight of *D. melanogaster* individuals (1 mg) (Ja et al. 2007). The concentration range for all tested substances was calculated in order to make it comparable to the beer daily intake for humans (192 ml of total beer/day for 70 kg human body weight (Kirin 2012).

2.4 *In vivo* assays

2.4.1 *Drosophila melanogaster*

The value of using *Drosophila* to investigate fundamental biological processes is increasing. This organism is revealing as an appropriate system as it is a complex multicellular organism in which many aspects of gene expression are parallel to those of humans. *Drosophila* substitute mammals in experiences with the distinct goal of uncovering insights directly relevant to human beings because it is a model for many human diseases, including cancer and ageing (Gonzalez 2013; Lints and Soliman 1988; Rudrapatna et al. 2012).

Two *Drosophila* strains were used, each with a hair marker in the third chromosome:

- *mwh/mwh*, carrying the recessive mutation *mwh* (*multiple wing hairs*) that produces multiple tricomas per cell instead of one (Yan et al. 2008).

• *flr*^{3}*/In (3LR) TM3, rip*^{p}*sep bx*^{34e}*e*^{s}*Bd*^{S}, where the *flr*^{3} (*flare*) marker is a homozygous recessive lethal mutation that produces deformed tricomas but is viable in homozygous somatic cells once larvae start the development (Ren et al. 2007).

Strains were maintained at 25 °C and 80% humidity in glass tubes with homemade meal (0.5 g NaCl, 12 g agar-agar, 100 g yeast, 25 g sucrose, 5 ml propionic acid, 3.5 ml of a 0.2% sulphate streptomycin solution and 1 litre of water) making changes three times per week.

Virgin females were obtained from these tubes, and were mated in order to perform the crosses for the different assays of toxicity, antitoxicity, genotoxicity, antigenotoxicity and longevity.

2.4.2 Toxicity and Antitoxicity Assays

Based on 2.3 Treatments and samples concentrations section, for the toxicity assays [(n of individuals born in each treatment / n of individuals born in the negative control) x 100] where n is number of flies born, the following ranks of concentrations were assayed: 3.125–50 mg/ml of LBLB and 0.00125–0.02 mM; 0.447–7.16 mM; and 0.00141–0.0226 mM of xanthohumol, choline and folic acid respectively, being the concentration of single molecules the correspondent to the concentration of LBLB assayed. The antitoxicity tests consisted of combined treatments of the same concentrations as in toxicity assays by adding the genotoxicant hydrogen peroxide at 0.12 M (Tasset-Cuevas et al. 2013) and comparing the percentage of emerging adults with the positive toxicant control.

Three independent experiments were carried out for each assay. Chi-square test in a Microsoft Office Excel 2007 was used to determine if the tested compounds significantly inhibited the survival of flies, with respect to the control. In the toxicity assay, statistical Chi-square values ($p < 0.05$) for the different concentrations tested were compared with respect to the negative control one; whereas the statistical of antitoxicity assay was analysed comparing the Chi-square values of the different concentrations with respect to the positive control value.

2.4.3 Genotoxicity and Antigenotoxicity Assays (SMART)

The genotoxicity assays were carried out following the method described by Graf et al. (1984). Briefly, trans-heterozygous larvae for *mwh* and *flr*^{3} genes were obtained by crossing 200 optimally virgin females (4 days old) of *flr*^{3} strain with 100 males of *mwh* strain. Four days after fertilization, females were allowed to lay eggs in fresh yeast medium (25 g of yeast and 4 ml of sterile distilled water) for 8 h in order to obtain synchronised larvae. After 72 h, larvae were collected, washed with distilled water, and placed, in groups of 100 individuals, in the treatment tubes where they were fed chronically with the different compounds. The treatment tubes contained 0.85 g of *Drosophila* Instant Medium (Formula 4–24, Carolina Biological Supply, Burlington, NC) and 4 ml of solutions with different concentrations of the substance to be tested

(3.125 and 50 mg/ml of LBLB; 0.00125 and 0.02 mM of xanthohumol; 0.447 and 7.16 mM of choline; and 0.00141 and 0.0226 mM of folic acid). The antigenotoxicity tests were performed following the method described by Graf et al. (1998), which consisted of combined treatments of genotoxin (0.12 M H_2O_2) and the same concentration used in genotoxicity assays of LBLB, xanthohumol, choline and folic acid. After emergence, adult flies were stored in 70% ethanol until the wings were mounted to the scrutiny of the mutations.

2.4.3.1 Mutations scoring, data evaluation and statistical analysis

Wings of emerging trans-heterozygous individuals (*mwh* flr^+/mwh^+ flr^3) for each control and concentration were mounted on slides using Faure's solution (30 g Gum Arabic, 20 ml glycerol, 50 g chloral hydrate and 50 ml water) and scored under a photonic microscope at 400x magnification. Similar numbers of male and female wings were mounted and wing hair mutations were scored from a total of 24,400 monotricoma wild type cells per wing (Moraga and Graf 1989). In the balancer-heterozygous genotypes (*mwh/*TM3, Bd^S), *mwh* spot phenotypes are produced predominantly by somatic point mutation and chromosome aberrations, since mitotic recombination between the balancer chromosome and its structurally normal homologue is a lethal event.

Wing hair spots were grouped into three different categories: a small single spot corresponding to one or two cells exhibiting the *mwh* phenotype that occur in the late stages of the mitotic division; a large single spot with three or more cells showing *mwh* or flr^3 phenotypes and occur in the early stages of larval development; a twin spot corresponding to two juxtapositioning clones, one showing the *mwh* phenotype and other the flr^3 phenotype. Small and large spots are originated by somatic point mutation, chromosome aberration, and somatic recombination, while twin spots are produced exclusively by somatic recombination between the flr^3 locus and the centromere.

The total number of spots was also determined and a multiple-decision procedure was applied to determine whether a result is positive, negative or inconclusive (Frei and Wurgler 1988). The frequencies of each type of mutant clone per wing were compared to the concurrent control and analysed applying the binomial Kastenbaum and Bowman Test (Frei and Wurgler 1995), without Bonferroni correction and at 5% level of significance. All inconclusive results were analysed with the nonparametric Mann Whitney *U*-test ($\alpha = \beta = 0.05$). The inhibition percentages (IP) for the combined treatments are calculated from total spots per wing with the following formula (Abraham 1994):

IP = [(single genotoxin - combined treatment) / single genotoxin] x 100

2.4.4 Lifespan Assays

Flies who undergo the longevity experiments exhibited the same genotype as in genotoxicity assays in order to compare both, genotoxicity and longevity results. The F_1

progeny from *mwh* and *flr*^{3} parental strains produced by a 24 h egg-laying in yeast medium was used to the trial.

All experiments were carried out at 25 °C according to the procedure described by Tasset-Cuevas et al. (2013). Briefly, synchronised 72 ± 12 hours old trans-heterozygous larvae were washed, collected and transferred in groups of 100 individuals into test vials containing 0.85 g of *Drosophila* Instant Medium and 4 ml of the different concentrations of the compounds to be assayed. Adult animals emerged from pupae were collected and sets of 10 individuals of the same gender were selected and placed into sterile vials containing 0.21 g of *Drosophila* Instant Medium and 1 ml of different concentrations of solution of the compounds to be tested (3.125–50 mg/ml of LBLB; 0.00125–0.02 mM of xanthohumol; 0.447–7.16 mM of choline; 0.00141–0.0226 mM of folic acid). Four replicates were followed during the complete life extension for each control and established concentrations. Alive animals were counted and the respective nourishment renewed twice a week.

The statistical treatment of survival data for each control and concentration was assessed with the SPSS Statistics 17.0 software (SPSS, Inc., Chicago), applying the Kaplan-Meier method. The significance of the curves was determined using the Log-Rank method (Mantel-Cox).

2.5 *In vitro* cytotoxicity assays

2.5.1 Cell culture conditions

The promyelocytic human leukaemia cell line HL-60 was grown in RPMI-1640 medium supplemented with heat-inactivated foetal bovine serum, L-glutamine at 200 mM and antibiotic-antimycotic solution with 10,000 units of penicillin, 10 mg of streptomycin and 25 µg amphotericin B per ml. Cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 (Shel Lab, Cornelius, OR, USA) (Gallagher et al. 1979). The cultures were plated at 2.5 x 10^4 cells/ml density in 10 ml culture bottles and passed every 2 days.

The immortal and non-tumour NIH3T3 cell line, derived from mouse fibroblast cells, were purchased from American Type Culture Collection (Manassas, VA) and cultured in a 37 °C incubator with 95% humidity and 5% CO_2 in complete Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Carlsbad, CA, USA) supplemented with 10% (v/v) heat-inactivated newborn calf serum (NCS) (Gibco, Carlsbad, CA, USA) and 1% penicillin/streptomycin/Fungizone mix. The cell culture was passed every 3 - 4 days by trypsination (0.05%). Only passages 10 - 25 were used.

The use of these two types of cells will allow us to study the effect of our compounds on a promyelocytic leukaemia and on an immortalised fibroblast cell model line. Although they belong to different organisms (human and mouse), results complemented each other and cytotoxicity assays will be comparable.

2.5.2 Cytotoxicity assay

HL-60 cells were placed in 96 well culture plates (2 x 10^4 cells/ml) and treated for 72 h either with LBLB, xanthohumol, choline or folic acid at different concentrations (7.8–250 mg/ml; 0.0012–0.02 mM; 0.447–7.16 mM; and 0.04–1.58 mM, respectively). Cell viability was determined by the trypan blue dye exclusion test and cells were counted with an inverted microscope at 100x magnification (AE30/31, Motic). Curves were plotted as survival percentage with respect to the control growing at 72 h. At least three independent repetitions of the assays were carried out to calculate means for statistical analysis.

NIH3T3 cells were plated in 96 well flat-bottom plates (BD Biosystems) at 1 x 10^4 cells per well and treated for 24 h either with LBLB, xanthohumol, choline or folic acid at different concentrations (7.8–250 mg/ml; 0.0012–0.02 mM; 0.22–7.16 mM; and 0.099–1.58 mM, respectively). Cell viability was measured using the CellTiter-Blue Cell Viability Assay (Promega, Madison, WI, USA). After treatments, 20 µl of CellTiter-Blue Reagent was added to each well and the cells were cultured for 2 h as changes in the fluorescence at 540 nm excitation and 590 nm emission wavelengths was measured using a Tecan, Infinite M200Pro microplate reader. Fluorescence intensity values were normalised to values of untreated wells. Curves were plotted as survival percentage of three independent experiments with respect to the control growing for 24 h.

2.5.3 Determination of DNA fragmentation

DNA internucleosomal fragmentation is observed by DNA laddering and it is associated to the activation of the apoptotic pathway in cancer cells as a hallmark of apoptosis affecting the genomic integrity (Wyllie 1980). HL-60 cells (1 x 10^6 cells/ml) were treated with different concentrations of LBLB, xanthohumol, choline or folic acid (15.625–250 mg/ml; 0.00125–0.02 mM; 0.447–7.16 mM; and 0.099–1.58 mM, respectively) for 5 h. Treated cells were collected and centrifuged at 603.72 x *g* (3,000 rpm) for 5 min, and DNA was extracted as follow: the cell pellet was resuspended in 900 µl of cell lysis buffer, then added 100 µl of SDS 10% and 25 µl of proteinase K solution (20 mg/ml) and incubated shaking for 5 h at 55 °C. After this, 432 µl of 5 M NaCl was added and the samples were centrifuged at 11336.52 x *g* (13,000 rpm) for 15 min. Supernatant was recovered into a fresh tube and 750 µl of cold isopropanol was added to precipitate DNA. Then, samples were centrifuged at 11336.52 x *g* (13,000 rpm) for 10 min, washed with 1 ml of 70% ethanol and DNA dried and resuspended in 20 µl of deionised water. Finally, 0.6 µl of 0.4 mg/ml RNase was added and incubated at 37 °C (6.0372 x *g*) (300 rpm) over night.

Total extracted DNA was quantified in a spectrophotometer (Nanodrop® ND-1000) and 1200 ng of DNA were loaded into a 2% agarose gel electrophoresis at 85 mA during 25 minutes, stained with ethidium bromide and visualised under UV light.

2.5.4 Comet assay

The DNA strand breaks induction was determined using the alkaline comet assay (pH < 13), following the method described previously (Husseini et al. 2000; Kumaravel and Jha 2006; Prosperini et al. 2013) with some modifications. Cells were treated with LBLB (7.8, 31.2 and 62.5 mg/ml), xanthohumol (0.0025, 0.005 and 0.02 mM), choline (0.895, 1.791 and 7.16 mM), and folic acid (0.099, 0.198 and 0.397 mM) for 5 h. After treatments, cells were rinsed in PBS, mixed with 0.75% low melting point agarose at 37 °C and transferred to frosted-end slides. Steps of lysis, alkaline electrophoresis, neutralization and dried were carried out according to Mateo-Fernández et al. (2016) protocol. Briefly, the slides were bathed into lysis solution (2.5 M NaCl, 100 mM Na-EDTA, 10 mM Tris, 250 mM NaOH, 10% DMSO, and 1% Triton X-100; pH 13) for 1 h at 4 °C and immersed into alkaline electrophoresis buffer (300 mM NaOH and 1 mM Na-EDTA; pH 13) for 20 min at 4 °C. Then an electrophoresis (20 V, 400 mM for 15 min) was performed and slides were submerged into neutralization buffer (0.4 M Tris-HCl buffer; pH 7.5). Finally, slides were dried overnight at room temperature in dark. DNA of 50 - 100 single cells was visualised by treating slides with 7 µl of a 10 µg/ml stock solution of propidium iodide (Kumaravel and Jha 2006). Comet images were analysed at 400x magnification using a Leica DM 2500 fluorescence microscope with green filter and an attached camera (JAI CV-M4CL). OpenCometTM freeware was used to score individual parameters for each cell.

Statistical analysis of data was performed using SPSS Statistic 17.0 software. The main parameters of the comet imaging are: total DNA, percentage of DNA in the comet and tail, tail length and tail moment (TM). The latter is the single most relevant index of DNA damage, which is calculated as the percent DNA in the tail multiplied by the distance between the means of the head and tail distributions (Husseini et al. 2000; Kumaravel and Jha 2006). Moreover, TM is considered appropriate for regulatory or inter-laboratory comparison studies (Kumaravel et al. 2009). The TM data were analysed applying a one-way ANOVA and post hoc Tukey's test to determinate the effect of choline on HL-60 cell DNA integrity. The level of $p \leq 0.05$ was considered statistically significant.

2.5.5 Macroautophagy activity evaluation

Macroautophagy (MA) is considered an inducible type of autophagy, whose essential role is the maintenance of cellular homeostasis and take part in the forefront of the response to stress positions against cellular aging. MA activity in NIH3T3 cells was evaluated as follows: for mCherry-GFP-LC3 conversion, NIH3T3 fibroblasts were transduced with a lentivirus carrying the tandem construct and cells were analysed at least one week after transduction to assure stable expression. 1×10^4 cells per well were plated in glass-bottom 96-well plates and fluorescence after the indicated treatment (7.8 mg/ml of LBLB; 0.0025 mM of xanthohumol; 0.22 mM of choline; and 0.19 mM of folic acid) was read in both channels using a high-content microscope (Operetta, Perkin Elmer). Moreover, the cytotoxic anticancer stressor Etoposide (Etop; 25 µM) was added to the NIH3T3 cells in order to analyse the growing behaviour of that cells treated with

the tested compounds. Images of 9 different fields per well were captured, which rendered an average of 2,000–3,000 cells counted. Nuclei, cell perimeter and puncta were identified using the manufacturer's software Columbus 2.4.2. Puncta positive for both fluorophores correspond to autophagosomes whereas those only positive for the red fluorophore correspond to autophagolysosomes. Autophagic flux was determined as the conversion of autophagosomes to autophagolysosomes (red-only puncta).

The statistical significance of macroautophagy activity for each compound was determined using the Student's t-test with the SPSS Statistics 17.0 software (SPSS, Inc., Chicago).

2.5.6 Methylation status

Genomic DNA was isolated in the same way as described in DNA fragmentation section. Bisulphite-modified DNA from the LBLB (15.625 and 250 mg/ml), xanthohumol (0.00125 and 0.020 mM), choline (0.447 and 7.16 mM), and folic acid (0.099 and 1.587 mM) treatments, using the EZ DNA Methylation-Gold™ Kit, was used as template for fluorescence-based real-time quantitative Methylation-Specific PCR (qMSP). The final reaction mixture with a total volume of 10 µl consisted of: 2 µl of deionised water, 5 µM of each forward and reverse primer, 2 µl of iTaq™ Universal SYBR® Green Supermix (Bio-Rad, containing antibody-mediated hot-start iTaq DNA polymerase, dNTPs, $MgCl_2$, SYBR® Green I dye, enhancers, stabilizers and a blend of passive reference dyes including ROX and fluorescein) and 25 ng of bisulphite converted genomic DNA.

qMSP conditions were as follows: one step at 95 °C for 3 minutes, 45 cycles at 95 °C for 10 seconds, 60 °C for 15 seconds, 72 °C for 15 seconds, another step at 95 °C for 30 seconds following by a 65 °C step during 30 seconds and finally a boost step from 65 °C to 95 °C for 95 seconds increasing 0.5 °C each 0.05 seconds. qMSP was carried out in 48 well plates in MiniOpticon Real-Time PCR System (MJ Mini Personal Thermal Cycler, Bio-Rad) and were analysed by Bio-Rad CFX Manager 3.1 Software.

In order to analyse a wide range of human genomic DNA repetitive elements were selected. While Alu and LINE sequences are interspersed throughout the genome, satellite DNA is confined to the centromere areas (Deininger et al. 2003; Ehrlich 2002; Ehrlich et al. 2003; Lee et al. 1997; Weiner 2002). All sequences had been obtained from Isogen Life Science. Alu M1, LINE-1 and Sat-α sequences were used (see Table 1 for detailed information (Weisenberger et al. 2005)).

The relative yielded results were normalised with the housekeeping sequence Alu C4 using the Nikolaidis et al. (2012) and Liloglou et al. (2014) comparative Ct method. Each sample was analysed in triplicate. One-way ANOVA and post hoc Tukey's tests were used to evaluate the differences between the tested compound, repetitive elements and concentrations.

Table 1

Primers information.

Reaction ID	GenBank number	Amplicon		Forward primer sequence 5' to 3' (N)	Reverse primer sequence 5' to 3' (N)	GC-content (%)	
		Start	End			Forward	Reverse
Alu C4	Consensus Sequence	1	98	GGTTAGGTA TAGTGGTTT ATATTTGTA ATTTTAGTA (36)	ATTAACTAA ACTAATCTT AAACTCCTA ACCTCA (33)	25	27.3
Alu M1	Y07755	5059	5164	ATTATGTTA GTTAGGATG GTTTCGATT TT (29)	CAATCGACC GAACGCGA (17)	27.6	58.8
LINE-1	X52235	251	331	GGACGTATT TGGAAAATC GGG (21)	AATCTCGCG ATACGCCGT T (19)	47.6	52.6
Sat-α	M38468	139	260	TGATGGAGT ATTTTTAAA ATATACGTT TTGTAGT (34)	AATTCTAAA AATATTCCT CTTCAATTA CGTAAA (33)	23.5	21.2

Source from Weisenberger et al. (2005).

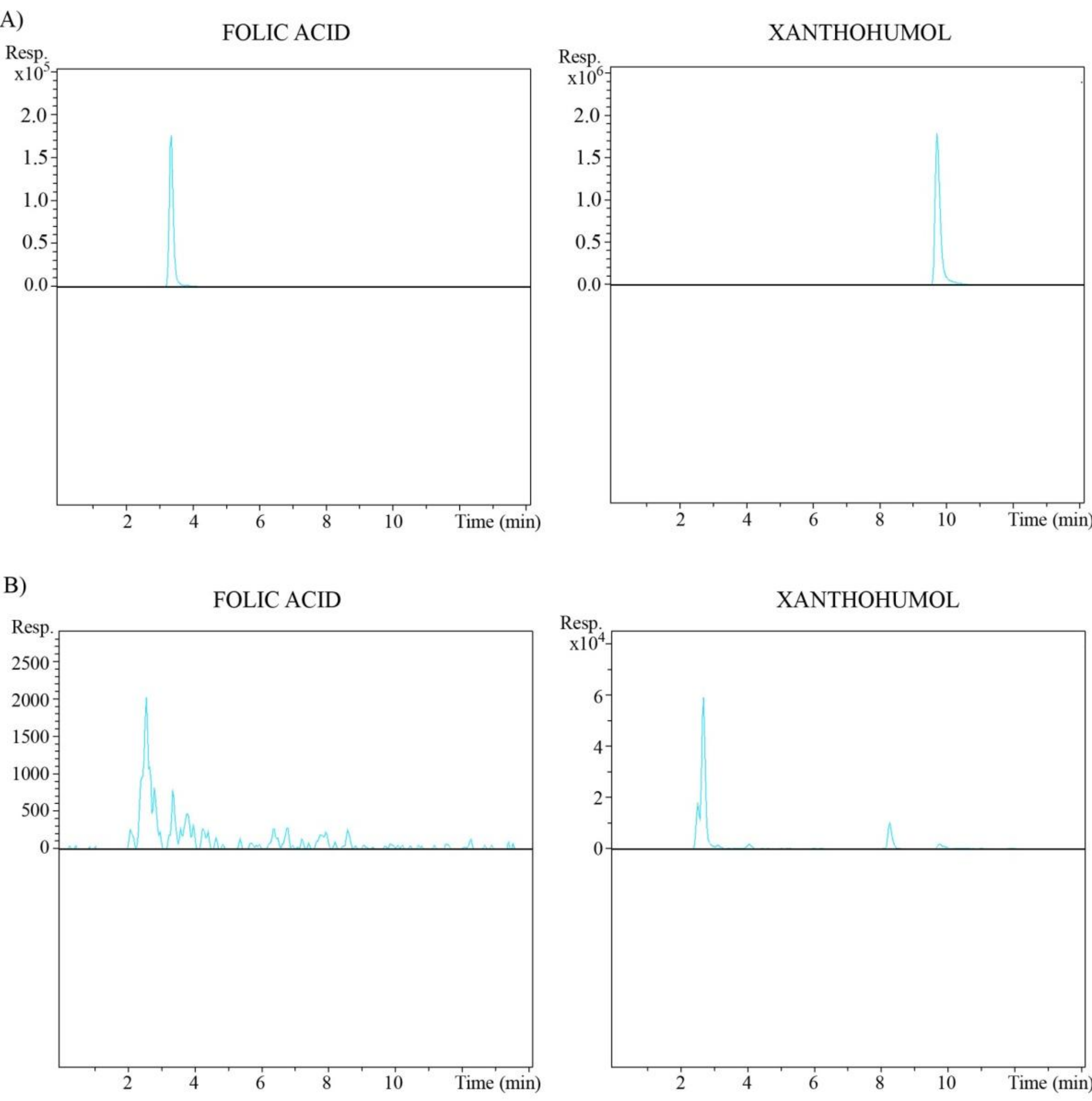
3. RESULTS AND DISCUSSION

3.1 Chemical characterisation

Results of the chromatographic behaviour for the individual bioactive compounds xanthohumol and folic acid are shown in Figure 1A. Moreover, their content in the full drink is represented in Figure 1B showing a value of 0.01 ppm and 0.02 ppm for xanthohumol and folic acid, respectively. These results agree with Gerhauser (2005) who detected a concentration of xanthohumol ranged between 0.002–1.2 mg/l and with Póo-Prieto et al. (2011) who detected a concentration rank between 0.016–0.026 mg/l of folic acid in 4–5.5% alcohol beers.

Figure 1

Sample compounds characterisation.



Chromatographic behaviour of folic acid and xanthohumol (A) analysed by an HPLC/MS system and their presence in the lyophilised blond Lager beer (LBLB) (B).

3.2 Toxicity and Antitoxicity

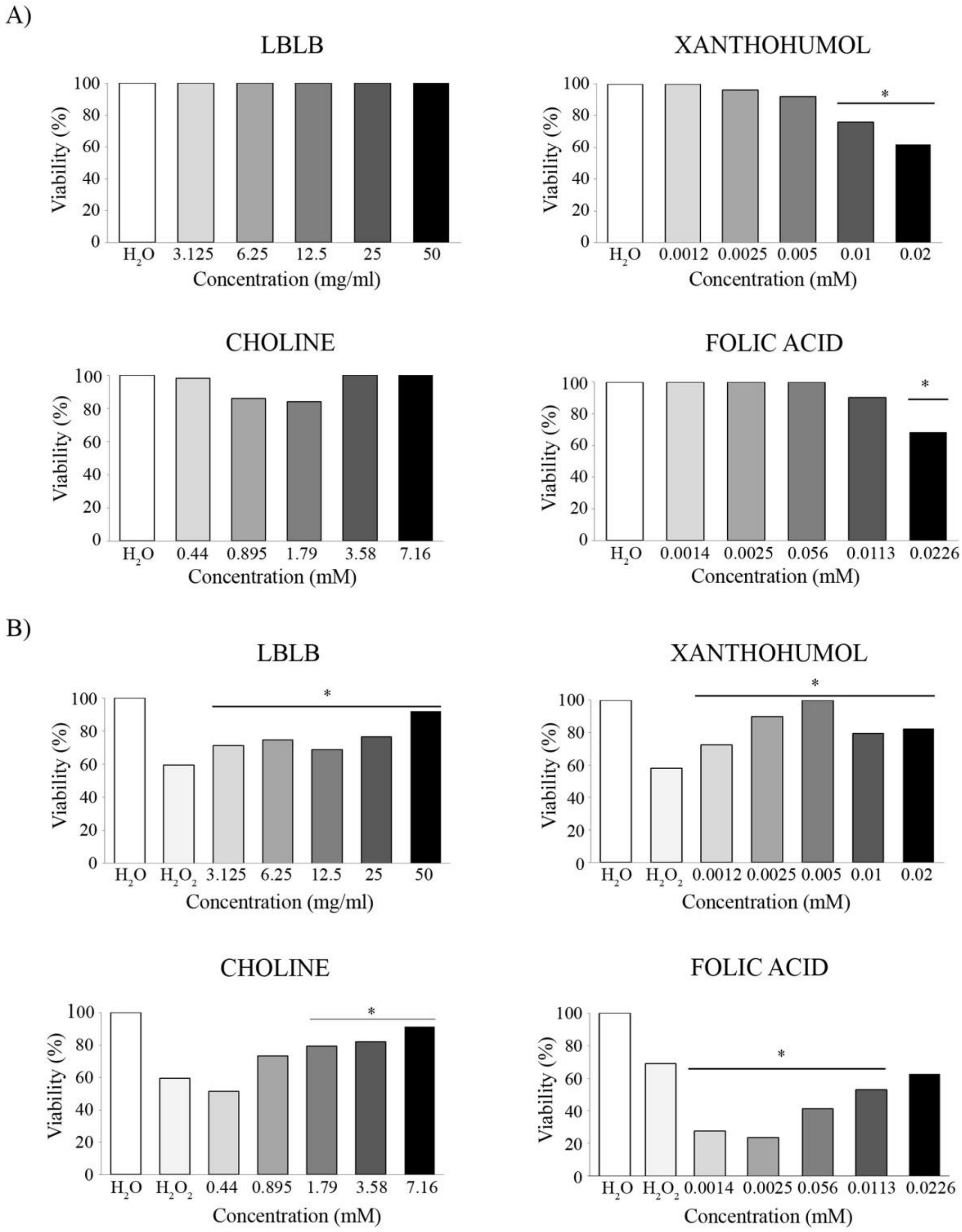
Toxicity of LBLB and the selected compounds has been assessed in the *Drosophila in vivo* model. Figure 2A shows the relative percentage of emerging adults after treating larvae with different concentrations of these substances. LBLB does not exhibit toxicity at the assayed concentrations with 100% survivals. To our knowledge, no previous studies of beer in *Drosophila* has been carried out. Choline shows a non-significant survival rate compared with their control. Recent work points out the critical role of choline in brain development (Meck and Williams 1997). Besides, getting adequate choline in the diet is important throughout life for optimal health in pregnancy and lactation (Zeisel 2006), neural tube defects (Shaw et al. 2004), memory development

(Glenn et al. 2008), heart disease (Olthof et al. 2005), inflammation (da Costa et al. 2006; Detopoulou et al. 2008) and cancers (Ackerstaff et al. 2003; Xu et al. 2008). Xanthohumol and folic acid are non-toxic substances for *Drosophila* larvae although the two highest and the highest assayed doses are significantly toxic, respectively. *In vivo* studies in different animal models have shown a safe activity of xanthohumol with respect to fertility, bone marrow, liver, exocrine pancreas, kidneys, muscles, thyroid, ovaries and suprarenal cortex (Avula et al. 2004; Hussong et al. 2005; Vanhoecke et al. 2005) and a protective activity (Dorn et al. 2010a; Dorn et al. 2012).

The antitoxicity assays revealed a differential behaviour of the substances. Figure 2B shows that hydrogen peroxide is toxic at 0.12 M with an average survival rate of 62% with respect to the water control; this result is in agreement with previous experiences of toxicity in *Drosophila melanogaster* when treated with H_2O_2 (Romero-Jimenez et al. 2005). LBLB, xanthohumol and choline are the most protective substances with positive dose-response effects. Xanthohumol would contribute to the prevention of oxidative damage by scavenging reactive oxygen species (ROS) produced by de toxicant H_2O_2 (Gerhauser et al. 2002; Miranda et al. 2000; Zhao and St Clair 2003) and prevents carbon tetrachloride-induced acute liver injury in rats (Pinto et al. 2012) and a protective activity has been reported in induced hepatic fibrosis is mice (Dorn et al. 2010a; Dorn et al. 2012). The antitoxic activity of choline against H_2O_2 found in our *in vivo* experiments would be in agreement with the well-known biological effects in human showing that choline deficient diets are related to liver and kidney degenerative diseases (Johnston 1939) and a dietary supplementation with choline induces a reduction of tissue injury and mortality in rat (Rivera et al. 1998) and juvenile carp (Wu et al. 2014), although an excess of choline intake could be toxic in humans (Li and Vance 2008). Our antitoxicity results showed that choline is able to protect against genomic damage induced by the H_2O_2 toxin. The combined treatments of folic acid and H_2O_2 showed a toxic synergic effect exerted by the two substances, except at the highest concentration tested. Although folic acid is generally regarded as not toxic for humans it may cause neurological injury when given to patients with undiagnosed pernicious anaemia (Butterworth and Tamura 1989). These findings would agree with our results for toxicity and antitoxicity assays of folic acid in the *Drosophila* animal model.

Figure 2

Toxicity (A) and antitoxicity (B) levels of lyophilised blond Lager beer (LBLB), xanthohumol, choline and folic acid in *Drosophila melanogaster.*



(A) Percentage of viability of *Drosophila* treated with different concentrations of the tested compounds. (B) Viability percentage of *Drosophila* tested with different concentrations of the tested compounds combined with 0.12 M H_2O_2. * indicates significant differences with respect to their concurrent control (Chi-square value higher than 3.84).

3.3 Genotoxicity and Antigenotoxicity

The Table 2 shows the results of genotoxicity assays in the SMART test for LBLB and the three single compounds under study. Negative control showed a frequency of mutations per wing of 0.157 which fall into the historical range for the wing spot test (Anter et al. 2011; Rojas-Molina et al. 2005). The final concentration of H_2O_2 used (0.12 M) has been demonstrated to exert a potent genotoxic effect capable to induce somatic mutations and mitotic recombination in *D. melanogaster* (Romero-Jimenez et al. 2005). The average frequency of total mutations per wing obtained in the treatment with H_2O_2 was 0.388. Single small, single large, twin and total clones were analysed in the wings of animals chronically treated for each concentration and compound.

The results showed that the LBLB, xanthohumol, choline and folic acid seem to have not genotoxic effect at the concentrations tested. The values of total mutation rates at the highest and lower concentrations of LBLB were similar or lower than the control mutation frequency (0.147 and 0.155 spots/wing, respectively) with no significant differences with the negative control (0.157 spots/wing). No dose effect is observed. Xanthohumol showed the lowest rate of mutations per wing among all the compounds under study at the highest concentration (0.051 spots/wing), encountering a dose-dependent effect more pronounced than in the case of folic acid (0.146 to 0.193 spots/wing). Choline seems to have not genotoxic effect at the tested concentrations with mutations rates not significantly different from that of water control (0.200 and 0.171 spots/wing for the highest and lowest concentration respectively. Although the lack of genotoxicity is expected due to the high phenol content of LBLB, this is the first time that has been assayed in the *in vivo Drosophila* system.

Vegetables contain many polyphenols and oligoelements with anti-mutagenic activity (Fernandez-Bedmar et al. 2011). The highest concentration tested for LBLB in the combined treatments was able to eliminate part of the genotoxic effect of H_2O_2, showing that the total mutation frequency is similar to the negative control (0.121 spots/wing for LBLB 50 mg/ml and 0.157 spots/wing for negative control), inhibiting around 68.8% of genotoxicity induced by H_2O_2. However, the lowest concentration of LBLB tested had a slight capacity to counteract the genotoxic effect of H_2O_2, obtaining a high total mutation frequency very similar to the value of the positive control (0.333 spots/wing in LBLB 3.125 mg/ml), showing only a 14.2% of inhibition (Table 2). Our results complement the *in vitro* assays of Rivero et al. (2005) who detected antigenotoxic activity in a dose dependent manner using calf thymus DNA damaged by ascorbic acid and copper. Other *in vitro* assays (HPRT mutagenicity test and the comet assay) performed in Chinese hamster lung fibroblasts give also anti-genotoxic activities of LBLB against genotoxicity of heterocyclic aromatic amines (Edenharder et al. 2002). It is remarkable that the *in vivo* genotoxicity/antigenotoxicity *Drosophila* wing spot test carried out in the present study does need neither metabolically competent mammalian cells nor metabolic bioactivation conditions of the *Salmonella* reversion assay. The high polyphenol content of beer would confers the antioxidant ability of LBLB, and that

would be the responsible for the positive effect of LBLB on the protection of DNA oxidative damage.

Table 2

Genotoxicity and antigenotoxicity of lyophilised blond Lager beer (LBLB), xanthohumol, choline and folic acid in the *Drosophila* wing spot test.

Compound	Nº of wings	Clones per wing (nº spots) [(1)] Small single clones (1-2 cells) m = 2	Large simple clones (> 2 cells) m = 5	Twin clones m = 5	Total clones m = 2	U-test [(2)]	IP (%) [(3)]
H_2O	38	0.157 (6)	0	0	0.157 (6)		
H_2O_2	36	0.305 (11)	0.083 (3)	0	0.388 (14) +		
SIMPLE TREATMENT							
LBLB (mg/ml)							
[3.125]	45	0.133 (6)	0	0.022 (1)	0.155 (7) i	Δ	
[50]	34	0.500 (2)	0.029 (1)	0.059 (2)	0.147 (5) i	Δ	
Xanthohumol (mM)							
[0.00125]	28	0.214 (6)	0.036 (1)	0	0.250 (7) i	Δ	
[0.02]	39	0.051 (2)	0	0	0.051 (2) -		
Choline (mM)							
[0.447]	41	0.097 (4)	0.073 (3)	0	0.171 (7)	Δ	
[7.16]	25	0.200 (5)	0	0	0.200 (5)	Δ	
Folic acid (mM)							
[0.00141]	31	0.193 (6)	0	0	0.193 (6) i	Δ	
[0.0226]	48	0.125 (6)	0.021 (1)	0	0.146 (7) i	Δ	
COMBINED TREATMENT WITH H_2O_2 (0.12 M)							
LBLB (mg/ml)							
[3.125]	36	0.306 (11)	0.028 (1)	0	0.333 (12) β		14.2
[50]	33	0.091 (3)	0.030 (1)	0	0.121 (4) β		68.8
Xanthohumol (mM)							
[0.00125]	40	0.175 (7)	0	0	0.175 (7) β		54.9
[0.02]	39	0.102 (4)	0.051 (2)	0	0.153 (6) β		60.6
Choline (mM)							
[0.447]	44	0.182 (8)	0.045 (2)	0	0.227 (10) β		41.5
[7.16]	38	0.079 (3)	0.052 (2)	0	0.131 (5) β		66.2
Folic acid (mM)							
[0.00141]	38	0.210 (8)	0	0.053 (2)	0.263 (10) β		32.2
[0.0226]	40	0.125 (5)	0	0	0.125 (5) β		67.8

[(1)] Statistical diagnosis according to Frei and Wurgler (1988). + (positive), - (negative) and i (inconclusive) versus negative control; β (significantly different) versus positive control. m: multiplication factor. Kastenbaum-Bowman Test without Bonferroni correction (Frei and Wurgler 1995), probability levels $\alpha = \beta = 0.05$. [(2)] Inconclusive results were resolved by Mann-Whitney U-test. Delta marker (Δ) means no differences between the treatment and the negative control (H_2O). [(3)] The inhibition percentage (IP) for the combined treatments were calculated from the total spots per wing according to Abraham (1994).

The total mutation rate in the combined treatments with xanthohumol indicated an anti-genotoxic effect, showing significant differences compared with positive control in both concentrations tested. Both 0.02 and 0.00125 mM concentrations, reduced the total mutation rates to a similar values as the negative control (0.153 and 0.175 spots/wing, respectively) (Table 2). This indicated that xanthohumol is able to partially inhibit the genotoxic activity of H_2O_2 (inhibition percentages of 54 and 58%, respectively). The hydroxil radicals generated by H_2O_2 could be directly reduced by xanthohumol by the donation of a hydrogen atom to radicals. Choline was able to inhibit the genotoxic activity of H_2O_2 in a dose dependent manner (41.5 and 66.2% for the lowest and highest concentrations respectively). Folic acid was also able to inhibit the induced mutations of the model genotoxin in combined treatments, by reducing its effect when decreasing the concentration, obtaining values of 67.8% (0.0226 mM) and 32.2% (0.00141 mM) of inhibition percentage (Table 2). Folic acid could protect bioconstituents like DNA from the free radical damage originated by hydroxyl radical at least by competitions (Joshi et al. 2001). The three single compound studied in the SMART assay individually explain the antigenotoxic activity of LBLB although the sum up of the three antigenotoxic values would overpass the complex mixture. The effect of isolated nutrients or other chemicals derived from food on chronic disease may not have the same beneficial effect as the whole nutrient does (Jacobs and Tapsell 2013). Xanthohumol is not genotoxic in the *in vitro* assays and is antigenotoxic in the *in vitro* comet assay using HepG2 cells (Plazar et al. 2007) and in the *Salmonella* microsomal assay (Kac et al. 2008) against procarcinogens and oxidative molecules (tert-butyl hydroperoxide). Our results using the *in vivo Drosophila* wing spot test will serve to confirm the high antigenotoxic activity of xanthohumol and folic acid. Our results of genotoxic inhibitory ability of choline *in vivo* due to its antioxidant activity against peroxide caused by its hydroxy amine groups (Saito and Ishihara 1997) are in agreement with previous *in vitro* studies on the genomic impact of choline deficiencies (Lu et al. 2012; Zeisel 2007). Folic acid status can influence background levels of DNA damage as it has a role in human chromosome breakage (Ames 2001; Reidy et al. 1983) and in the amelioration of the germ cell toxicity induced by methotrexate in mice (Padmanabhan et al. 2009).

3.4 Longevity assays

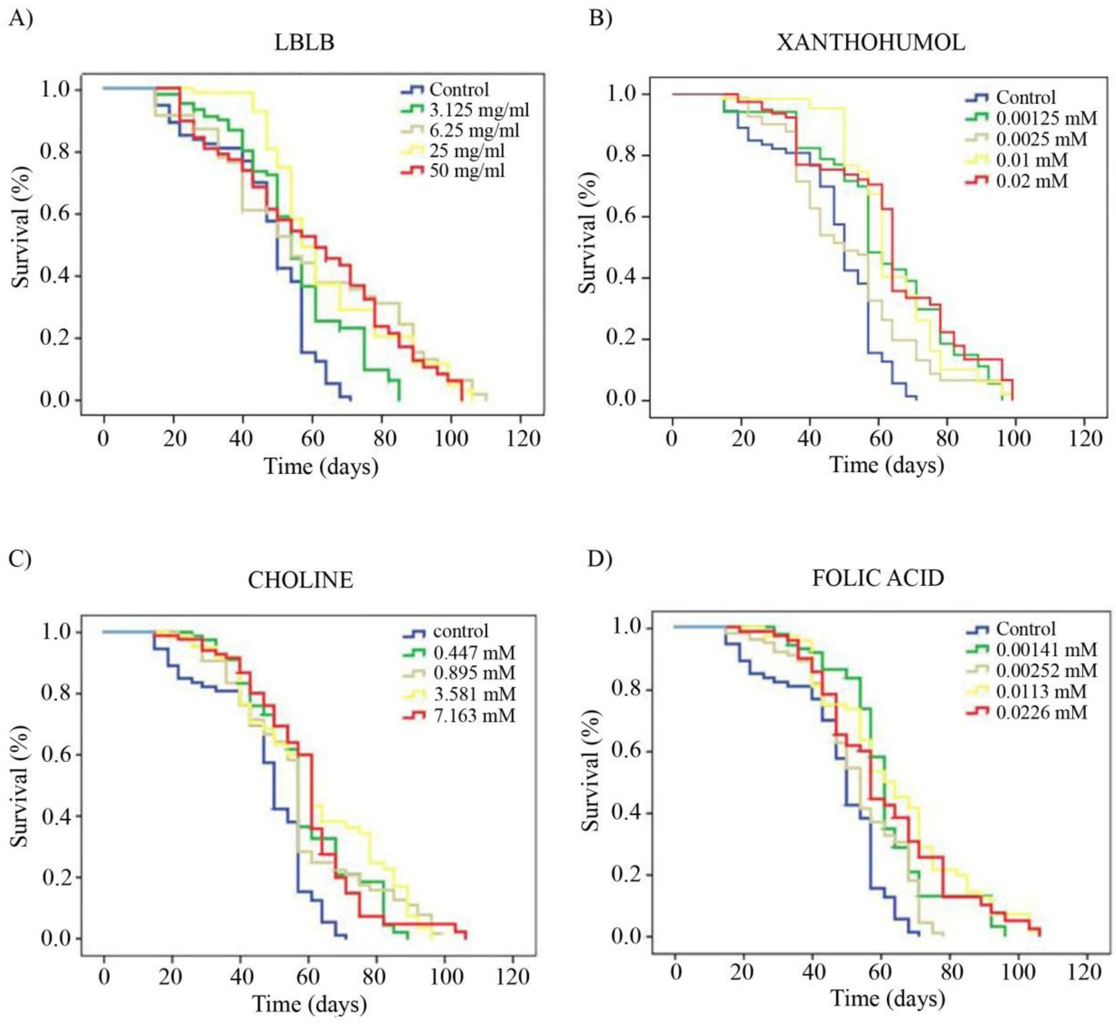
Longevity is a trait influenced by many factors, including gender, genetic variation, environmental influences, diet, lifestyle, access to health care and cultural influences. *Drosophila* revealed as an excellent system to investigate longevity-promoting properties of compounds and nutraceutical extracts as adults seem to show many of the cell senescence features seen in mammals (Fleming et al. 1992). It is well known that caloric restriction without malnutrition is a key strategy for increasing the lifespan (Guarente and Kenyon 2000). Nevertheless, there is available data on lifespan increase by adding nutraceutical substances to the *Drosophila* diet with no reduction of the total intake (Deshpande et al. 2014).

The entire lifespan curves obtained by the Kaplan-Meier method for each substance and concentration are shown in Figure 3. All the assayed compounds induced a lifespan

expansion in *Drosophila melanogaster* compared to the control (47 days average). The ranks of the average lifespan were: 55.3–64.9; 51.9–64.2; 57.2–62.1; and 53.7–63.8 days for the different concentrations of LBLB, xanthohumol, choline and folic acid, respectively. The rate of increased lifespan for all assayed products was +12.6 days with respect to the concurrent control, being the seconds higher concentrations which showed the longest-lived increases (17.6, 16.9, 14.9 and 16.5 days for LBLB, xanthohumol, choline and folic acid, respectively).

Figure 3

Survival curves of *D. melanogaster* fed with different concentrations of lyophilised blond Lager beer (LBLB) (A), xanthohumol (B), choline (C) and folic acid (D) over time.



Longevity is a trait influenced by many factors, including gender, genetic variation, environmental influences such as diet, lifestyle, access to health care and cultural influences (Maklakov et al. 2008). Our results are in agreement with Tasset-Cuevas et al. (2013) and Villatoro-Pulido et al. (2012) who showed increases of lifespan by adding *Borago* seed oil and *Eruca* and sulforaphane. Although LBLB contains many nutrients including carbohydrate (Ferreira 2009) this increasing of caloric availability would

imply the concurrent intake of nutraceutical polyphenols (Gerhauser 2005). Studies with xanthohumol in murine model also agree with our results showing a significant increased lifespan by delaying neurological disorders caused by leukemic cells dissemination (Benelli et al. 2012). Nevertheless, in the case of using another model organism like *Caenorhabditis elegans*, *in vivo* studies showed that the inhibition of folate synthesis led to a dose-dependent increased in the animal lifespan (Virk et al. 2012).

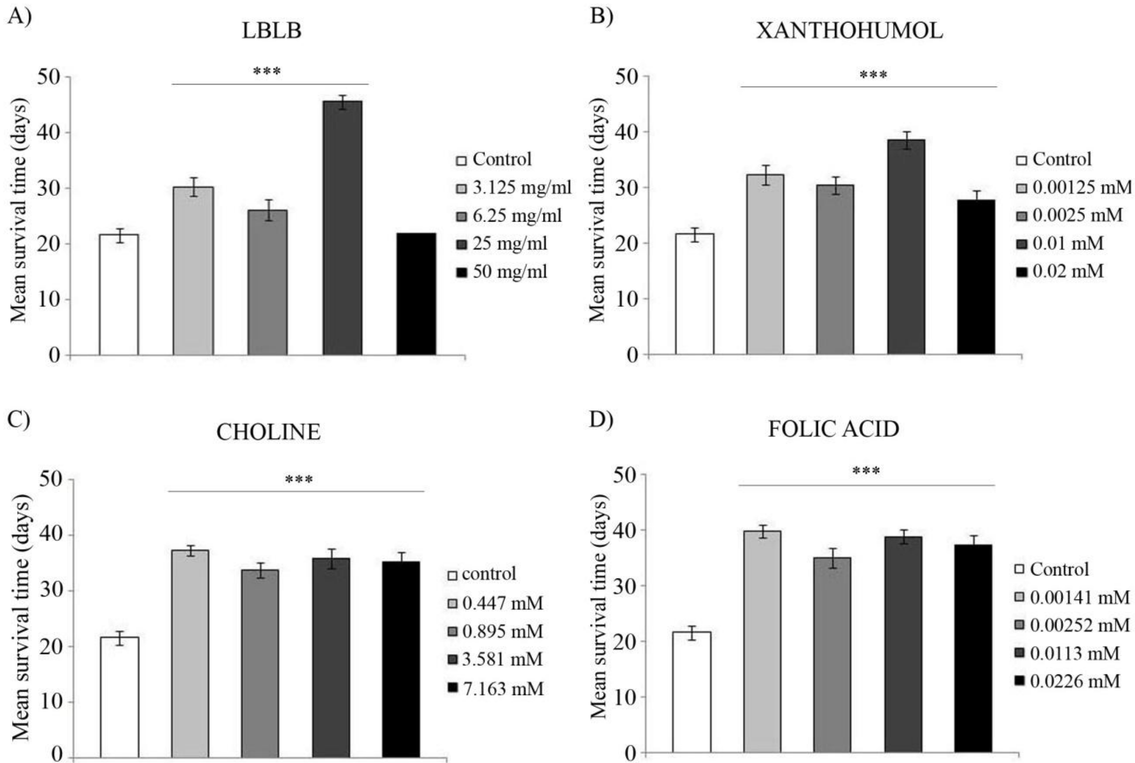
3.5 Healthspan assays

In order to know the quality of life of the *Drosophila* treated in the longevity assays, we studied the 25% of individual survival at the top of the lifespan curves obtained in the previous test for each substance and concentration tested. This part of the lifespan is considered as the healthspan of a curve, characterised by low and more or less constant age-specific mortality rate values (Soh et al. 2007). The results are shown in Figure 4.

All the assayed compounds induced a significant increase of healthspan in *Drosophila melanogaster* when compared to the control, with the exception of the higher concentration of LBLB (50 mg/ml) which is similar to the control (Table 3). Choline and folic acid showed similar mean survival time values. The increase ranges for these substances were between 14.1–19.1 and 15.2–20.2 days, for choline and folic acid respectively. On the other hand, LBLB and xanthohumol showed an irregular pattern in their mean survival time values with no dose-dependent effects, being the second-to-higher concentrations assayed of both products the concentrations that showed the maximum increases higher than 26 days for LBLB and 27 days for xanthohumol respect to the control. The rest of concentrations of LBLB and xanthohumol had increasing averages of 6.1–10.6 and 10.1–16.4 days, respectively.

Figure 4

Healthspan averages of *D. melanogaster* fed with different concentrations of lyophilised blond Lager beer (LBLB) (A), xanthohumol (B), choline (C) and folic acid (D) over time.



Mean of survival time in the highest 75% surviving population. *** indicates significant differences ($p < 0.001$), with respect to the concurrent control.

Table 3

Mean and significance of lifespan, healthspan and lifespan curves by gender.

Compound	Concentration		Mean Lifespan(1) (days)		Mean Healthspan(1) (days)		Lifespan curves by gender (days)				
							Females(1)		Males(1)		Gender sig.(2)
Control			47.242		19.769		51.202		49.914		0.386
LBLB	3.125	mg/ml	55.283	***	30.351	***	52.950	*	54.203	***	0.280
	6.25	mg/ml	59.111	***	25.833	**	82.950	***	44.774	ns	0.000
	25	mg/ml	64.895	***	45.658	***	85.398	***	56.875	**	0.000
	50	mg/ml	60.337	***	23.333	ns	82.632	***	47.480	ns	0.000
Xanthohumol	1.234	mM	61.569	***	36.168	***	80.500	***	51.514	*	0.000
	2.469	mM	51.921	*	29.842	***	51.356	ns	50.958	ns	0.954
	9.876	mM	64.159	***	46.686	***	60.523	***	68.409	***	0.051
	19.752	mM	63.490	***	32.619	***	63.070	***	63.416	***	0.817
Choline	0.447	mM	58.430	***	38.826	***	60.829	**	55.926	**	0.131
	0.895	mM	57.270	***	33.810	***	63.973	**	52.663	*	0.004
	3.581	mM	62.120	***	35.800	***	64.225	***	59.327	***	0.449
	7.163	mM	59.213	***	37.098	***	63.534	***	55.501	***	0.161
Folic acid	0.00141	mM	61.800	***	39.881	***	65.630	***	54.983	***	0.001
	0.00252	mM	53.680	***	34.920	***	53.618	*	53.696	**	0.299
	0.01130	mM	63.781	***	38.824	***	74.251	***	52.106	*	0.000
	0.02260	mM	60.880	***	37.333	***	67.434	***	49.324	ns	0.002

Mean lifespan for the different treatments assayed with the lyophilized blond Lager beer (LBLB) and its bioactive compounds, symbolised by asterisk (*) in Figure 5, were calculated by the Kaplan-Meier method and significance of the curves determined by the Log-Rank method (Mantel-Cox). (1)ns: non-significant ($p > 0.05$), *: significant ($p < 0.05$), **: significant ($p < 0.01$), ***: significant ($p < 0.001$). (2)Significant survival values between females and males caused by the different concentrations assayed, symbolised by arrows (→) in Figure 5.

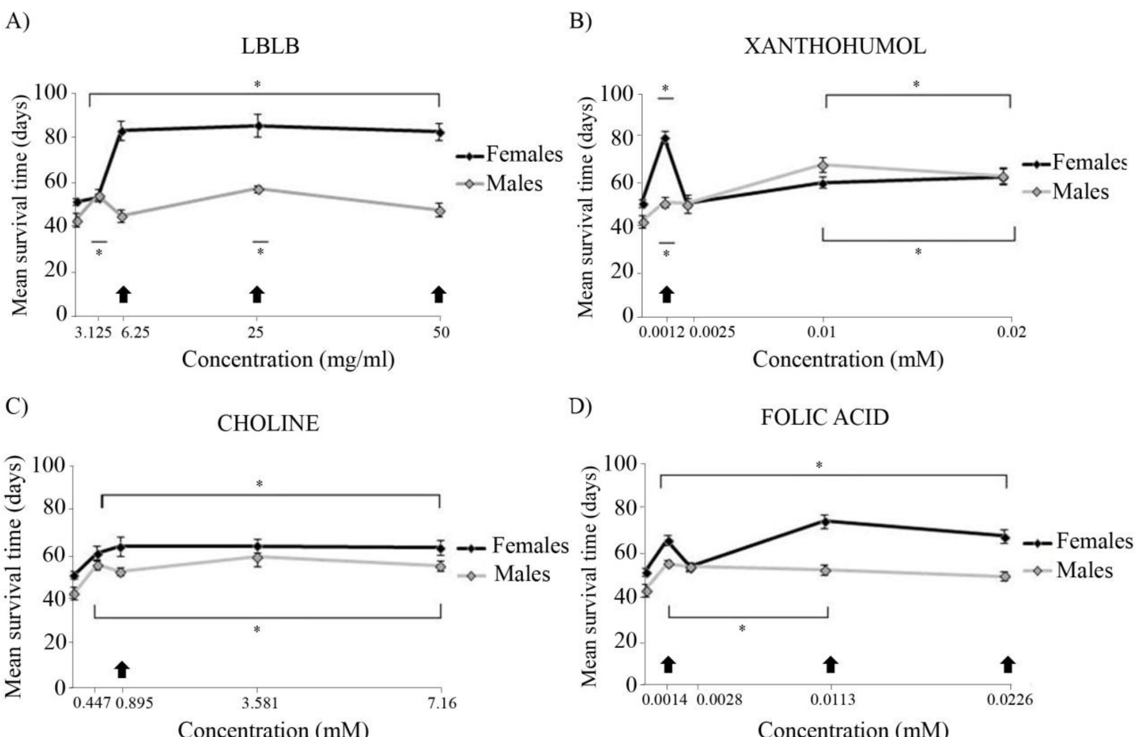
No previous results have been reported for the healthspan assay of beer and associated compounds. According to Breinholt et al. (1999); Hatch et al. (2000); Noroozi et al. (1998) only a small percentage of many dietary polyphenols is absorbed, although human studies suggest this may be adequate to exert a beneficial effect when there are mixed sources in the diet. We hypothesize that the antioxidant components of beer would counteract the oxidative radicals produced in the metabolism of the rest of the diet in the *Drosophila* longevity trials by increasing life and healthspan.

3.6 Gender studies

It is known that gender plays an important role on the human lifespan and has a differential behaviour depending on someone's lifestyle and specific fitness effects of nutrient intake on the lifespan are common (Maklakov et al. 2008). We analysed the effects that the different concentrations of LBLB and the simple compounds caused on the *Drosophila* survival time curves, taking into account the sex factor (Figure 5).

Figure 5

Survival gender comparison of *D. melanogaster* fed with different concentrations of lyophilised blond Lager beer (LBLB) (A), xanthohumol (B), choline (C) and folic acid (D) over time.



Significance between female and male curves with respect to their controls are symbolised by asterisk (*); significant survival values between females and males at the different treatments are symbolised by arrows (→).

All females and males treatment curves were significantly similar or longer than their corresponding water controls (Figure 5). In general, females were more favoured than males by the different treatments. From a total of 16 concentrations assayed, females leaded to males by 8 concentrations and were in balance with male's survival results by the others 8 ones left (Figure 5 and Table 3). The three highest concentrations assayed in females for LBLB produced a significantly increasing of survival greater than 30 days with respect to their negative control. Contrarily, males only had a significant increase of survival time in two of the four concentrations assayed with a survival time higher than 12 days with respect with their control. In the xanthohumol assay, both sexes showed significantly increases of survival in three of the four concentrations tested with a mean increasing of 16 and 18 days for female and male respectively. All the choline concentrations assayed showed significant increases of survival time with an average of 12 days for females and 13 days for males. Finally, in the folic acid test, females showed a significant increasing of survival time with a range of 14 days respect to their control while males did not exhibit a significant value for the highest concentration; the rank of survival time increased for males had a value of 10.7 days with respect to their control. In general, female increased 20.37, 4.49, 6.0 and 10.4 days

with respect to the male individuals at the treatments with LBLB, xanthohumol, choline and folic acid, respectively.
In the highest concentration of LBLB assayed, females far exceeded male's results in an average of 25 days. Xanthohumol and choline only showed a significant difference between females and males in one concentration. The lowest concentration of xanthohumol assayed showed an increasing of survival time for females of 20 days comparing to males results. Whereas choline second-lowest concentration increased the survival time in an average of only 3 days with respect to males. Folic acid showed significant differences between females and males in the lowest, medium and highest concentration assayed being the increasing of the survival time for females in relation to males over 8 days.

Genetic differences between males and females have a significant effect upon aging and lifespan. In general, where the heterogametic sex is male, as in humans and *Drosophila*, females tend to live longer than males (Fenech et al. 1994; Fox et al. 2003; Hazzard 1986; Smith and Warner 1989; Tower and Arbeitman 2009). Purine and folate metabolism genes have been argued as a potential target of sex-specific implication for lifespan in *Drosophila* (Bauer et al. 2006). It is important to point out that the findings from studies on *Drosophila*, mice, and *C. elegans* may not fully apply to humans. Gender related differences have already been identified e.g. estrogens, which are able to increase the expression of genes related to longevity (Fenech et al. 1994). We may yet find significant differences in the regulation and action of these pathways as we move from research on lower organisms like *C. elegans* to higher organisms like rodents and mammals (Pan and Chang 2012). Choline at lower concentrations, like other methyl donors such as purine and folate (Bauer et al. 2006), could be argued as a potential target of sex-specific implication for *Drosophila* lifespan. Besides, methyl-deficient diets cause fatty livers and promote liver carcinogenesis in rodents (Wainfan and Poirier 1992). On the other hand, a supplementation with choline improves hepatic steatosis (Buchman et al. 1995). Our results suggest that lower concentrations of choline could be an essential nutrient to prevent and protect the degenerative processes like ageing and DNA damage.

3.7 Cytotoxicity

LBLB and xanthohumol showed cytotoxic activity against HL-60 and NIH3T3 cells, folic acid enhanced HL-60 tumour growing and immortal NIH3T3 cells as well. Choline did not exert any effect in the *in vitro* cytotoxicity assays (Figure 6). The use of two types of model cell lines allowed us to obtain complementary conclusions on the mode of action of the compounds assayed. Methodologies for the different cells are used following the standard protocols described in the manufacturer's manuals, due to they belong to different suspension and adherence types of culture. LBLB showed a dose dependent response, with an increase of the cytotoxicity level according to increased concentration of LBLB, being the inhibitory concentration 50 (IC_{50}) of 125 mg/ml in the HL-60 cell line and the same pattern was observed when NIH3T3 cells are treated with lower concentrations of LBLB reaching the IC_{50} at 25 mg/ml. Xanthohumol

showed a slight (20%) inhibition at the lowest concentration (0.00125 mM) that is maintained when increasing the concentration, with a maximum inhibition of approximately 30% in HL-60 cells, meanwhile in NIH3T3 cells a 40% inhibition is achieved at 0.015 mM. Cytotoxicity curves of choline showed no inhibition. In relation to folic acid no inhibition was observed at any concentration tested, but contrarily a slight tendency to increase the cell growth is observed in both cell lines.

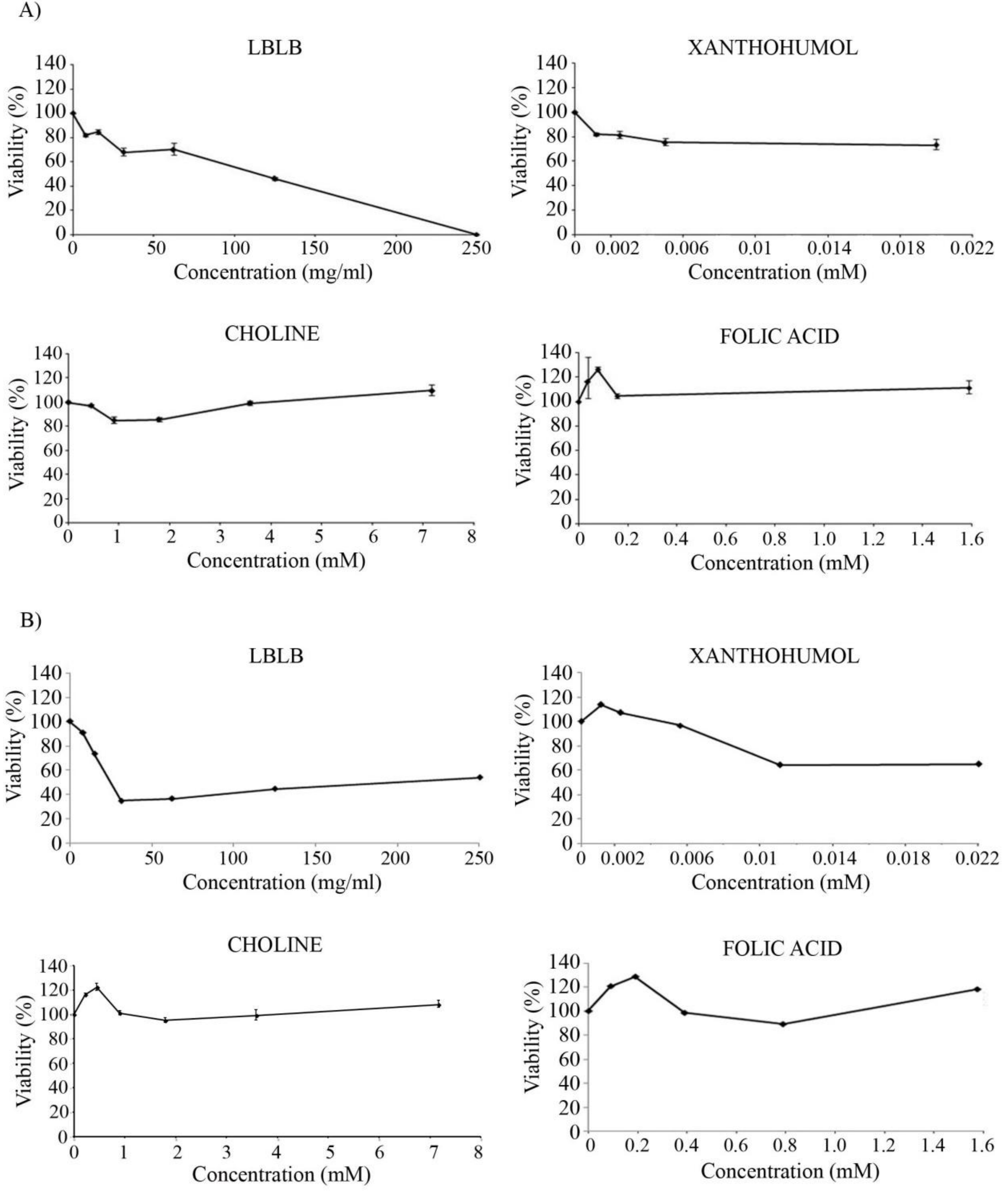
According to Gerhauser (2005), chemopreventive activities of beer and xanthohumol relevant to inhibition of carcinogenesis at the initiation, promotion and progression phases, were observed. This fact supports our results in the cytotoxic assays of beer and xanthohumol using promyelocytic HL-60 cells. Xanthohumol results of the present study are in agreement with the anti-proliferative activity in different human cancer cell lines (Colgate et al. 2007; Delmulle et al. 2006; Lust et al. 2005; Pan et al. 2005). This biological effect could be related to the anti-inflammatory properties by inhibition of cyclooxygenase activity and its anti-estrogenic activity (Subbaramaiah et al. 1997). A wide variety of studies correlated the chemoprotective activity of xanthohumol with its structure: xanthohumol treatment induced the mitochondrial-mediated pathways of programmed cell death in human colon carcinoma cells and caused a rapid breakdown of the mitochondrial membrane potential, and the release of cytochrome c, leading to apoptosis induction in BPH-1 cells (Strathmann et al. 2010). Some attempts have been made to relate the functional groups of several chalcones to the toxicity related activities: hydroxyl and prenyl xanthohumol substituents are associated with antioxidant properties, meanwhile the thiol reactivity of chalcones is likely to contribute to both cytotoxic and chemoprotective properties of these compounds (Go et al. 2005); alpha-methyl chalcones are believed to exhibit greater cytotoxic activity and inhibition of cell growth than unsubstituted analogues (Ducki 2009; Ducki et al. 1998). Studies about the relationship between cytotoxicity and structural-activities of these chalcones are not yet clear: meanwhile a substitution of the A ring drives chalcone activity and cytotoxicity, an OH-group at position 6' of the A-ring instead of a methoxy-group cannot be assigned to any specific activity, but reduced cytotoxicity of the resulting chalcone (Zenger et al. 2015). *In vivo* studies by Nozawa et al. (2004) demonstrated that the intake of beer significantly reduced the formation of colorectal tumours and preneoplastic lesions in Azoxymethane (AOM)-induced experimental carcinogenesis in male Fischer rats. However, considerable variations in inhibitory actions on tumours formation are observed among brands of beer, which may be due to the contents of components of beer (malt and hops extracts).

On the other hand, chemopreventive activities in LBLB and xanthohumol, relevant to inhibition of carcinogenesis at the initiation, promotion and progression phases, were observed in other studies (Gerhauser 2005). Xanthohumol has been characterised as a "broad-spectrum" cancer chemopreventive agent *in vitro* studies (Stevens and Page 2004). One of anticancer mechanism of xanthohumol is related with inhibition in the excessive production of prostaglandins, mediators of inflammation and with the abolishment of the stimulation of proliferation and angiogenesis (Bertl et al. 2004;

Gerhauser et al. 2002), as well as the inhibition of the activity of various cytochrome P450, which are involved in carcinogen activation (Gerhauser 2005; Gerhauser et al. 2002; Henderson et al. 2000; Miranda et al. 2000).

Figure 6

Effects of lyophilised blond Lager beer (LBLB), xanthohumol, choline and folic acid in *in vitro* assays.



A) Viability of HL-60 cells treated with LBLB, xanthohumol, choline and folic acid for 72 h. B) Viability of mouse fibroblast (NIH3T3) in the presence of different concentrations of LBLB and some of its components after 20 h. Each point represents the growing percentage respect to control mean +/- SE from three independent experiments.

Our results on genotoxicity of choline against HL-60 tumour cells are consistent with the conclusively demonstrated fact that choline metabolism is altered in a wide variety of cancers (Glunde et al. 2006), hence proposing novel anticancer therapies targeting choline metabolism. Experimental studies in mice established dose-effect relationship between choline deficiency and carcinogenic activity of diethanolamine (Leung et al. 2005), which is related with greater membrane fragility than cells grown in a control medium (da Costa et al. 2004).
The lack of cytotoxic activity of folic acid is in concordance with the last results which suggest that it could have promoter effect of cancer in humans. The meta-analysis published by Wien et al. (2012) using 10 randomised controlled trials showed a borderline significant increase in frequency of overall cancer in the folic acid group compared to controls. Contrarily, Joshi et al. (2001) proposed that folic acid could prevent cancer due to the free radical scavenging and antioxidant activity, being ubiquitous free radical scavengers believed to be partially responsible for their anticancer activity. On the basis of these epidemiological studies, some doubts about its overuse are arising.

3.8 DNA internucleosomal fragmentation

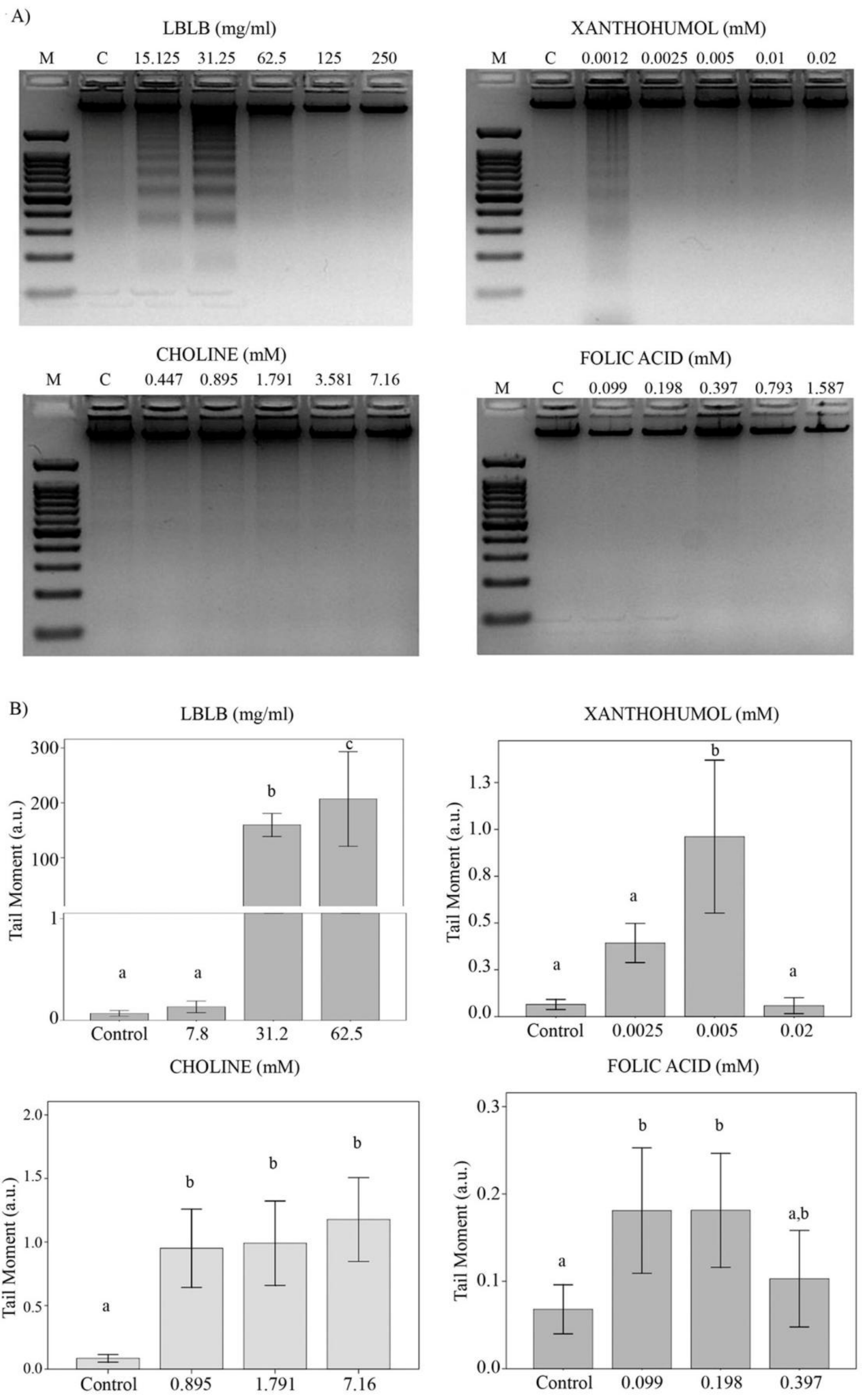
Figure 7A shows the electrophoresis of the genomic DNA of HL-60 cells treated with different concentrations of LBLB, xanthohumol, choline and folic acid. DNA internucleosomal fragmentation is represented by a DNA laddering and it is associated to the activation of the apoptotic way in cancer cells being a hallmark of the genomic integrity (Wyllie 1980). Fragmentation was observed in the two lowest concentrations of LBLB (15.125 and 31.25 mg/ml) and slightly in the intermediate concentration (62.5 mg/ml). On the other hand, the lowest concentration of xanthohumol (0.0012 mM) also induced the characteristic laddering as an apoptotic activity. Finally, choline and folic acid did not induce internucleosomal fragmentation.

According to Dorn et al. (2010b), xanthohumol treatment leads to apoptosis in activated human hepatic stellate cells (HSC) that is mediated by activation of caspase 3. Other studies reported, that xanthohumol treatment induced the mitochondrial-mediated pathways of programmed cell death in human colon carcinoma cells and caused a rapid breakdown of the mitochondrial membrane potential, and the release of cytochrome c, leading to apoptosis induction in BPH-1 cells (Strathmann et al. 2010).

Although we did not obtain significant results for the DNA damage assays (DNA internucleosomal fragmentation and comet assay), studies suggest that choline deficiency in humans is associated with significant DNA damage and with apoptosis in lymphocytes (da Costa et al. 2006). Finally, folic acid did not induce internucleosomal fragmentation. To our knowledge, this is the first time that LBLB and folic acid have been assayed in the *in vitro* DNA fragmentation. The results obtained in the internucleosomal fragmentation assays suggest that xanthohumol could account for most of the induction of proapoptotic DNA fragmentation of LBLB.

Figure 7

Effects of lyophilised blond Lager beer (LBLB), xanthohumol and folic acid in *in vitro* assays.



A) Internucleosomal DNA fragmentation in promyelocytic HL-60 cells treated during 5 h with different concentrations of LBLB, xanthohumol, choline and folic acid. M indicates DNA size marker. C indicates control treatment. B) DNA strand breaks induction in promyelocytic HL-60 cells treated during 5 h with different concentrations of LBLB, xanthohumol, choline and folic acid. DNA migration is reported as mean TM. The plot shows mean TM values and standard errors. Different letters mean different values after one-way ANOVA and post hoc Tukey's test.

3.9 Comet assay

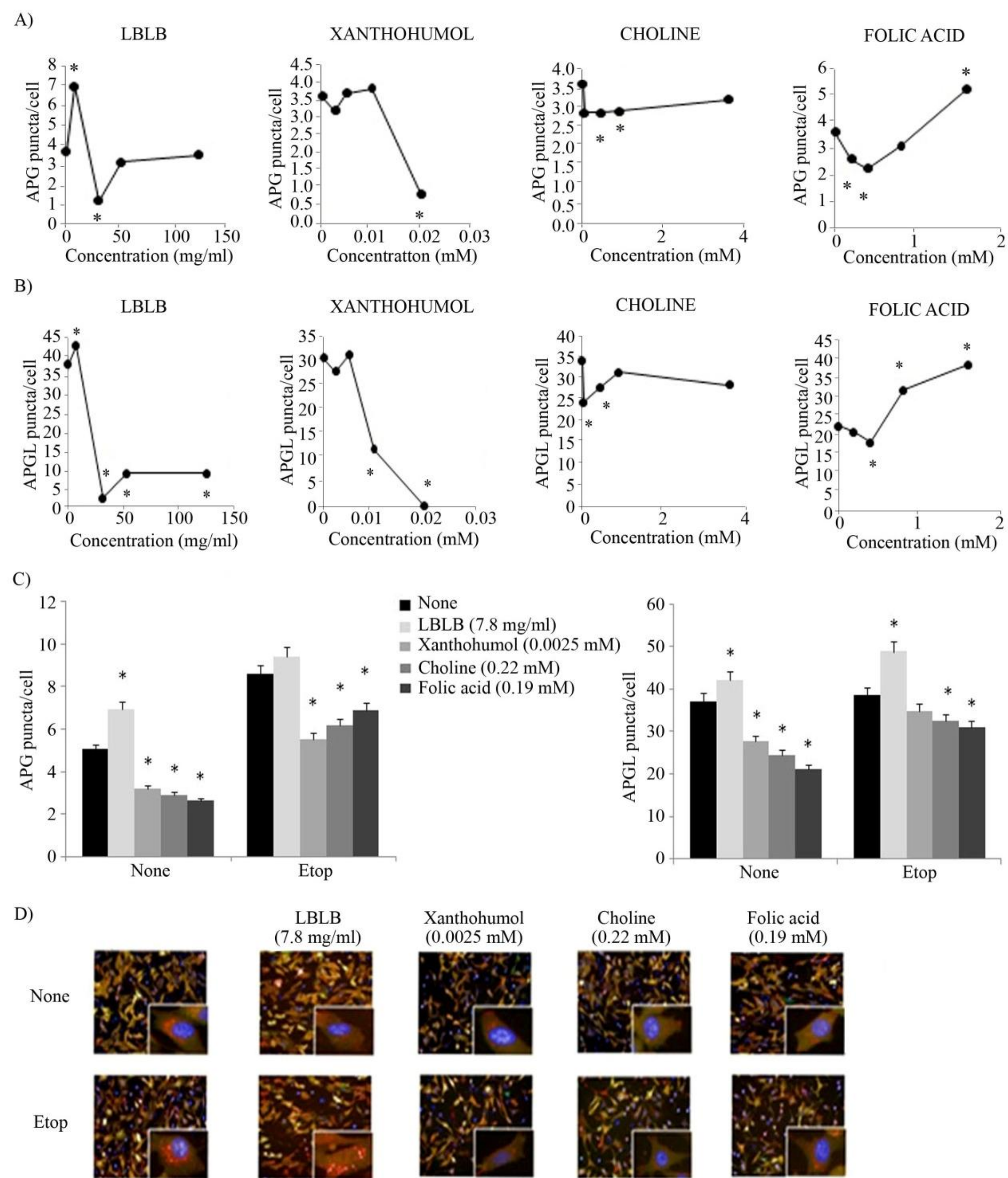
Results of the different tail moment (TM) for each treated cells are shown in Figure 7B. By using the comet assay we were able to readily identify apoptotic nuclei based on the characteristic morphological changes attributable to extensive DNA damage (Poe and O'Neill 1997). The comet assay tail moment measurements have been shown to correlate directly to the percent of viable cells as monitored using trypan blue exclusion for several human lymphoblast cell lines. Our results agree with the above correlations mentioned showing significant TM values correlated with the cytotoxicity ones that have been observed in the single cell gel electrophoresis. This fact could be caused by the double and/or the single strands breaks induced by the two highest concentrations assayed of LBLB, the 0.005 mM concentration of xanthohumol, the three assayed concentrations of choline and the two lowest tested folic acid concentrations. According to Fairbairn et al. (1996) the TM measurements are correlated with the cytotoxicity ones. Taking the DNA fragmentation into accounts, we could assume that comet assay shows single strands breaks in the xanthohumol and folic acid treatments because of the lack of DNA internuclear fragmentations showed in the genomic DNA electrophoresis of these compounds. On the other hand, double and single strands breaks are induced by LBLB as both DNA internucleosomal fragmentation and single cell gel electrophoresis comet assay show significant results. Studies with xanthohumol supported the significantly inhibited menadione-induced DNA single-trand breaks in Hepa1c1c7 cells pre-treated (Dietz et al. 2005). Moreover, studies affirm that choline has a significant impact on genomic stability and cell death, depending on its availability (Lu et al. 2012). Folic acid is essential for the synthesis and repair of DNA. Duthie and Hawdon (1998) studied the effects of folate depletion on DNA stability in normal human lymphocytes *in vitro*. They observed that lymphocytes cultured under folate-deficient conditions failed to grow normally compared with control cells. Moreover, cells deprived of folate were unable to efficiently repair oxidative DNA damage induced by hydrogen peroxide.

3.10 Macroautophagy activity

Results of LBLB, xanthohumol, choline and folic acid effect on MA are shown in Figure 8. MA is an important cellular pathway for maintain homeostasis, development, differentiation and survival of cells, where damaged organelles, proteins and intracellular pathogens are degraded. Defects in MA lead to altered metabolic homeostasis, protein quality control, among others. This effect vary depending on the site of autophagic blockage (Schneider and Cuervo 2014). It is known that MA decreases with age in almost all organisms studied and diet or specific nutrients affect MA activity (Hannigan and Gorski 2009).

Figure 8

Effect of lyophilised blond Lager beer (LBLB), xanthohumol, choline and folic acid on Macroautophagy activity.



Macroautophagy activity was measured in NIH3T3 cells stably expressing the tandem reporter mCherry-GFP-LC3-IL. Analysis was done using high content microscopy in 9 different fields per condition and well (approximately 3,500 total cells per condition). A) Quantification of the number of Autophagosomes (APG) and B) Autophagolysosomes (APGL) was performed. * significant ($p < 0.05$). C) Effect of Etoposide on Macroautophagy induced by the tested compounds. Cells were grown in absence or presence of the stressor (Etopo: 25 μM). Concentrations were selected on the basis of the results obtained in single treatments shown in Figure 8A-B. Quantification of the number of autophagyc vacuoles (AV: total red puncta), Autophagosomes (APG: total yellow puncta) and Autophagolysosomes (APGL: red but not green puncta) was performed. * significant ($p < 0.05$). D) Representative merged images of these cells and high magnification insets.

Our results show that LBLB at low concentration tested (7.8 mg/ml) induces an increase of MA activity, on the basis of these findings, moderate intake of beer could be helpful for restored the deficit on MA activity found in aging (Cuervo 2008; Cuervo et al. 2005). Limited dietary intake decreases the incidence of age-related disorders and increases lifespan in numerous animal experimental models from invertebrates to mammals (Masoro 2006). In the present research, the effects on *Drosophila* lifespan of low-moderates doses of LBLB are similar to a limited dietary intake as an increase of the lifespan is observed. These data fit to the MA significant activation at low-moderated LBLB doses in NIH3T3 cells. On the other hand, high-lipid content diets have an inhibitory effect on MA and chaperone mediated autophagy that explains how obesity and fatty liver disease disrupt this pathway (Rodriguez-Navarro and Cuervo 2012), so the daily intake of LBLB could protect against lipotoxicity.

Only one of components tested, folic acid acts as an activator of MA in immortal cells at high concentrations assayed. The intake of folic acid is controversial, during years was recommend for its effects on DNA synthesis and repair but recently (Wien et al. 2012) a relationship between folate intake and the frequency of overall cancer has been suggested.

Additionally we also analysed the effect of xanthohumol and choline on MA activity and we found that both compounds are inhibitors of MA at any concentration assayed with a more pronounced effect in the case of xanthohumol; we could define xanthohumol as responsible of the inhibitory effect of LBLB at higher concentrations; being these findings the confirmation of those of Delmulle et al. (2006) who could not detect any typical apoptotic morphological features like vacuoles. We have also observed that dietary choline inhibits macroautophagy activation. Once again, the importance of doses-effect is revealed when food or beverages are intended to be used as a nutraceutic. For that reason, moderate concentrations of folic acid and the other phenols, like those contained in beer, are acceptable. This is the first time that xanthohumol is proposed to be considered as a new family of autophagy inhibitors. Besides, it could be used in cancer therapy as a pharmacological inhibitor of MA. Moreover, the folic acid results allow to propose it as an activator of MA, so it could be used in aging as a pharmacological up regulation of MA. In this context more studies are needed to clarify the doses and time point of folic acid administration. Further studies are needed to understand this apparent paradox as choline exerts benefits on longevity and at the same time inhibits MA activity.

Furthermore, the DNA damaging topoisomerase inhibitor (etoposide) in control cells induce an increase on MA. This response is still maintained in the presence of all treatments analysed at lower concentration (Figure 8).

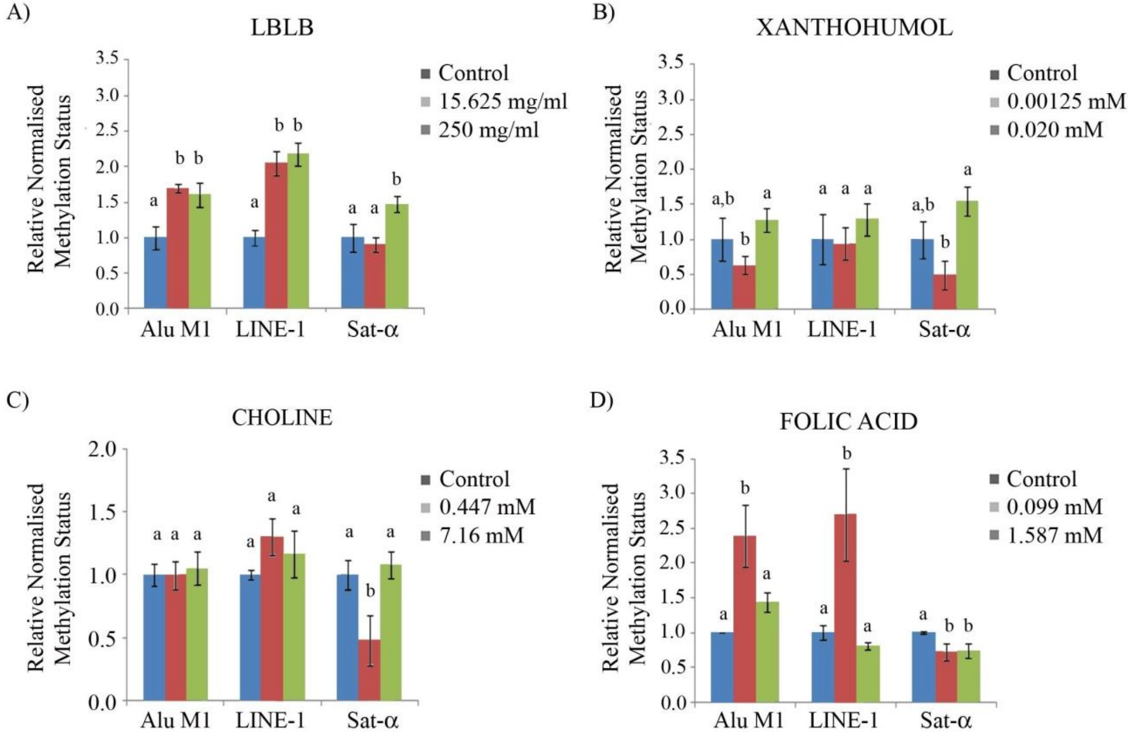
3.11 Methylation status

The relative normalised expressions of the three repetitive sequences (Alu M1, LINE-1 and Sat-α) studied in HL-60 cells treated with different concentrations of LBLB,

xanthohumol, choline and folic acid are shown in Figure 9. LBLB showed a significant hypermethylated status in all the tested concentrations except in the lowest concentration (15.625 mg/ml) for the Sat-α repetitive sequence, with respect to the concurrent control. Xanthohumol did not exhibit a highlight diversity in the modulation of the methylation status as both concentrations assayed showed methylation levels similar to the normalised control except the lowest concentration (0.00125 mM) in the Alu regions and the highest (0.020 mM) concentration in Sat-α regions that showed a significant demethylation and methylation status, respectively. Choline did not exhibit modulation of the methylation status as both concentrations assayed showed methylation levels similar to that of the normalised control. The only exception was for the lowest concentration of choline in the LINE-1 and Sat-α regions that showed a significant hypermethylation and demethylation status, respectively. Folic acid exhibited a methylation status in the lowest concentration tested (0.099 mM) in Alu and LINE-1 regions and a demethylation status for that concentration in the Sat-α sequences, with respect to the normalised control.

Figure 9

Relative normalised expression data of each repetitive elements studied in HL-60 cells treated with different concentrations of lyophilised blond Lager beer (LBLB) (A), xanthohumol (B), choline (C) and folic acid (D).



Relative normalised expression data of each repetitive element (Alu M1, LINE-1 and Sat-α). Different letters mean different values after one-way ANOVA and post hoc Tukey's test.

Overall, all the assayed substances act as preventive by blocking repetitive sequences of HL-60 tumoral cells as it changes the usual hypomethylation status of these elements in

cancer cells to a methylated status, contrary to the general hypermethylation that tumoral cells exhibit (Roman-Gomez et al. 2008). This fact agree with the assays about methylation levels in the Alu, LINE-1 and Satellite repetitive DNA sequences in normal and tumoral cells obtained by Chalitchagorn et al. (2004); Ehrlich et al. (2003); Roman-Gomez et al. (2008); Schmid (1991). The study on the epigenetic effects of choline treatments in tumoral cells did not shows any significant blocking effect of choline on repetitive sequences on HL-60 tumoral cells, except for the hypermethylation in LINE-1 sequences and the hypomethylation in Sat-α sequences caused by 0.447 mM choline. According to Zeisel (2007) and Locker et al. (1986), DNA methylation is influenced by the availability of choline: an excess of choline caused hypermethylation, which is associated with gene silencing or reduction of gene expression and common genetic polymorphisms; meanwhile, a choline deficiency is associated with DNA hypomethylation, which is related with health consequences in brain development and birth defects, fatty liver, muscle damage, elevation of plasma homocysteine and others problems in adults. Furthermore, it is known that LINE sequences are moderately dispersed-repetitive DNA, mainly transposable elements, that may be involved in regulation of gene expression. On the other hand, alpha-satellite DNA are highly repetitive sequences located in heterochromatic regions around the centromere/telomere, with structural or organisational roles, role in chromosome pairing, involvement in cross-over or recombination and junk. Taking into account the importance of choline requirements and the functions of the different repetitive sequences studied, an intake of choline lower than the recommendation ones (as 0.447 mM concentration) could have important effect on the demethylation pattern, especially in the satellite DNA repetitive sequences, as our results showed. Studies about the effect of folic acid on genomic DNA methylation suggest that folic acid plays a critical role in the prevention of chromosome breakage and hypomethylation of DNA (the hypomethylation can be reversed by folic acid) (Fenech 2001; Pufulete et al. 2005). Furthermore, the biochemical role of folate in DNA synthesis, repair, and methylation are well established (Joshi et al. 2001). No previous studies about the properties that beer and xanthohumol have on the modification on the DNA methylation status have been reported. Studies have been carried out using diet components as possible modulator agents of DNA methylation in cancer cells. Not only methyl donor agents are able to modify epigenetic patterns of DNA but also some phenolics. The modulation of CB_1 tumour suppressor gene expression by extra virgin olive oil phenolic compounds via epigenetic mechanism, both *in vitro* and *in vivo*, is proposed to provide a new therapeutic avenue for treatment and/or prevention of colon cancer (Di Francesco et al. 2015). Further studies on other repetitive sequences could be useful for better understand the methylating role of xanthohumol on promyelocytic cells found in the present research. Epigenetic therapy against harmful effects of diet components could be a potential tool in chemotherapy because epigenetic alterations are reversible in contrast to genetic defects (Miyamoto and Ushijima 2005). Diets components could act in a dual way by increasing or decreasing the methylation levels depending on the specific gene promoter or the genomic region.

4. CONCLUSIONS

In the present study, we evaluated the effects of LBLB and some of its components at three individual, cellular and DNA level. All data together indicated that: i) the intake of LBLB at moderate doses could be recommend because it is neither toxic nor genotoxic, exerts genomic protection against hydrogen peroxide, increases lifespan, improves healthspan, inhibits tumour cells growth, induces DNA proapoptotic fragmentation and activates MA (only at low concentration); ii) Xanthohumol protects against genotoxicant, improve the extension and quality of life of *D. melanogaster* and it is an inhibitor of MA activity; iii) Choline is not toxic either genotoxic, protects from genotoxicant damage, increases lifespan and healthspan in the model animal; iv) Folic acid is not toxic, improve extension and quality of life and is an activator of MA. Despite the relations between the structure and the activities that chalcone and folate exhibit, the effect of isolated components of LBLB on the degenerative checkpoints studied has been demonstrated not to have the same beneficial effect as the whole complex mixture beer suggesting that most of the beneficial properties are exerted when there are mixed sources in the diet.

All the *in vivo* and *in vitro* assays carried out in the present paper of the role of LBLB suggest an important role on the modulation of degenerative processes. The safety (non-toxicity, non genotoxicity), the protection against the oxidative genotoxin H_2O_2 (antitoxicity, antigenotoxicity), the induction of life and healthspan extension, the cytotoxicity against leukaemia HL-60 cells and the induction of the proapoptotic DNA fragmentation and DNA damage in individual cells of LBLB has been established. The bioactive compounds (xanthohumol, choline and folic acid) contained in beer showed most of the activities of beer as a complex mixture. Some interesting exceptions have to be pointed out: choline does not inhibit tumour growth and rarely modifies the methylation status of repetitive sequences of tumoral cells; folic acid is not antitoxic, but it exert a synergistic activity with H_2O_2 and has no cytotoxic activity but contrarily it seems to increase the tumour cells HL-60 growing. In conclusion, results suggest that daily moderate consumption of beer may be healthy due to the properties that the full beverage shows.

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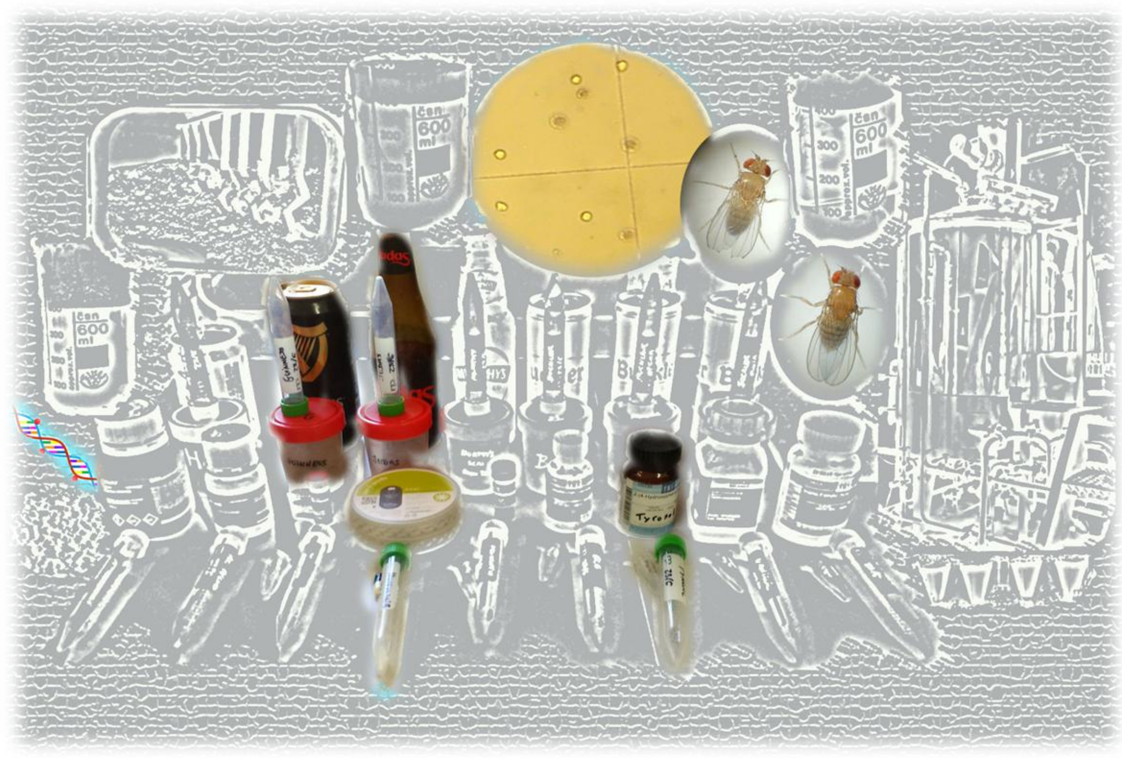
CHAPTER 2



Toxicological and epigenetic studies of two types of Ale beer, tyrosol and iso-alpha humulone

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ABSTRACT
Beer is the third-most popular drink overall, after water and tea. Although many benefits drawn from its consumption are claimed, the epidemiological records are contradictory with respect to cancer prevention. The objective of the present study was to evaluate the biological activities related to degenerative processes of two types of lyophilised Ale beers (blond and stout) as well as two of their bioactive compounds (tyrosol and iso-alpha humulone). *In vivo* toxicity, antitoxicity, genotoxicity, antigenotoxicity, lifespan and healthspan assays using *Drosophila melanogaster* and *in vitro* cytotoxicity, DNA damage and methylation status assays using HL-60 cell were carried out. The safety of the four substances has been confirmed as lyophilised blond Ale beer (LBAB), lyophilised stout Ale beer (LSAB), tyrosol and humulone are not toxic neither genotoxic. Moreover, they revealed the ability to protect the individuals against oxidative stress and inhibit the genotoxic activity of H_2O_2 with the exception of tyrosol. With respect to the degenerative process indicators lifespan and healthspan, tyrosol was the only compound that did not exert any influence on the life extension of *Drosophila*; LBAB induced a significant lifespan extension in *D. melanogaster*; LSAB and its distinctive compound humulone induced a reduction of longevity. The *in vitro* assays showed cytotoxic activity of LBAB, LSAB and tyrosol against HL-60 cells. Moreover, proapoptotic DNA fragmentation or DNA strand breakage was observed for both types of beers and humulone at different concentrations. Besides, lyophilised Ale beers and tyrosol exhibit an increasing genome wide methylation status while humulone exhibits a demethylation status in cancer cells repetitive sequences. The results obtained let us to propose lyophilised Ale beers as a safe potential nutraceutic beverages throughout a moderate consumption are able to prevent toxicity, to avoid genetic oxidative damage, to induce tumour cells death in a programmed manner, and to modulate the methylation status of tumour cells.

1. INTRODUCTION

Dietary constituents can influence carcinogenesis process at various stages through a variety of mechanisms. Although little is known about the mechanisms underlying cancer prevention, a number of food patterns, specific foods and some nutrients are believed to be particularly chemopreventive (Martin-Moreno et al. 2008; Reddy et al. 2003)

Nonetheless, both genes and environments play essential roles in human development. The expression of genes in an organism can be influenced by external and internal factors (Doll and Peto 1981). Drugs, chemicals, temperature, and light are among the external environmental factors that can determine which genes are turned on and off, thereby influencing the way an organism develops and functions (Clayton and McKeigue 2001).

Beer is the world´s most widely consumed alcoholic beverage, and is the third-most popular drink overall, after water and tea (Walker et al. 2012). Beer is a complex alcoholic beverage made from barley (malt), hop, water and brewer's yeast, rich in nutrients (carbohydrates, amino acids, minerals, and vitamins), as well as in non-nutrient components such as phenolic acids and flavonoids (Piendl 1989). Around 30% of polyphenols from beer comes from hops (Chen et al. 2014). Various brewing parameters like the variety of barley and the malting process, temperature and pH during mashing, sparging, boiling, the variety of hops added during wort-boiling, and yeast fermentation, influence the type and quality of beer.
Until the sixteenth century ale (top fermentation) was the main type of beer in Europe. To produce an ale type of beer, the fermentation is carried out using a "top-fermenting" yeast at temperatures from 20 °C to 25 °C, followed only by a short period of aging, or none at all (Buiatti 2009).

Some beer compounds are highly recommended for improving health. These compounds have been shown to possess various anti-bacterial, anti-inflammatory, anti-oxidative, anti-angiogenic, anti-melanogenic, anti-osteoporotic and anti-carcinogenic effects. Epidemiological studies on the association between beer drinking and skin disease are limited and direct evidence of beer compounds used in clinical application is lacking. Potential uses of these substances in dermatology may include treatment of atopic eczema, contact dermatitis, pigmentary disorders, skin infections, skin ageing, skin cancers and photoprotections, which require an optimization of the biostability and topical delivery of these compounds. Further studies are needed to determine the bioavailability of these compounds and their possible beneficial health effects when taken by moderate beer consumption (Chen et al. 2013).

An important aspect is the association of beer consumption with lower risk of suffering a cardiovascular disease. Approximately, 50% of blood cholesterol comes from diet (Muller et al. 2007). It is shown that beer consumption reduced mortality from coronary heart diseases in moderate alcohol consumers with respect to non-alcohol consumers

(Rimm et al. 1991) and the ability of beer against kidney stones formation (Curhan et al. 1996).

Phenolics and some B vitamins are argued to be the health promoter compounds of beer in reference to degenerative diseases (Gerhauser 2005). Some of them are derived from raw materials (water, malts, hops and yeast) and pass unchanged through the brewing process, others are produced during the process (Preedy 2011).

We focus our research in two phenolics presents in beers: tyrosol as the most abundant simple phenol present in all beer and iso-alpha humulone as a specific phenolic constituent of hops and found at high levels in stout beers.

Tyrosol, 4-(2-hydroxyethyl)phenol, is a natural phenolic antioxidant abundant in wine, virgin olive oil, vermouth and beer, and has gained increasing attention as a dietary antioxidant (Dewapriya et al. 2013). This aromatic alcohol which is produced by fermentation of the amino acid tyrosine, was detected in exceptionally high concentrations up to 40 mg/l in beers (Tressl et al. 1976). Several *in vitro* chemical studies have pointed out that tyrosol shows weaker anti-oxidative strength than its hydroxyl form, hydroxytyrosol (2-(30,40-dihydroxyphenyl)ethanol), due to the absence of the ortho-diphenolic group in its chemical structure (Mateos et al. 2003; Rietjens et al. 2007). Nevertheless, several cellular experimental models reported potent biological activities for tyrosol regardless of its weak anti-oxidative strength (Giovannini et al. 1999; Samuel et al. 2008; Sun et al. 2012). Cañuelo et al. (2012) found that tyrosol increases lifespan and stress resistance in the nematode *Caenorhabditis elegans*. This finding could support to the high longevity associated with the Mediterranean diet, more specifically, with olive oil and wine consumption. In biological systems, tyrosol is readily absorbed and retained for a prolonged period. An *in vivo* analysis of the protective activity of tyrosol against ischaemic brain injury has shown that rats treated with 30 mg/kg of tyrosol exhibited a significant protective effect against sensory motor dysfunction (Bu et al. 2007).

Humulone is a bitter-tasting chemical compound found in the resin of mature hops (*Humulus lupulus*) (De Keukeleire et al. 2003). Overall, they represent one of the most abundant classes of polyphenols in beer, with concentrations of up to 100 mg/l in very bitter English Ales (De Keukeleire 2000). The most important class of hop compounds are the hop acids, which are distinguished as alpha-acids (humulones) and beta-acids (lupulones) (Gerhauser 2005). Beta-acids are extremely sensitive to oxidation and do not survive the brewing process, whereas the transformations of the humulones during wort boiling have been studied in great detail. The most important chemical conversion during the brewing of beer is the isomerisation of alpha-acids to iso-alpha-acids; this process is involved in the generation of the bitter flavour of beer (Verzele and De Keukeleire 2013).
In addition to anti-inflammatory and antioxidative effects, the hop bitter acids also inhibit many gram positive bacteria (Bohra et al. 2008). Humulone can suppress the

TNF-a-dependent cyclooxygenase-2 induction with an IC_{50} as low as about 30 nmol/l in comparison to dexamethasone which does it at 1 nmol/l (Yamamoto et al. 2000). In mouse skin-stimulated with the tumour promoter '12-O-tetradecanoylphorbol-13-acetate (TPA)', topical application of humulone exhibited an anti-carcinogenic effect via suppressing TPA-induced activation of NF-jB (Lee et al. 2007). As a potent angiogenic inhibitor, humulone was shown to (a) significantly prevent *in vivo* angiogenesis in chick embryo chorioallantoic membrane in a dose dependent manner, (b) inhibit *in vitro* tube formation of vascular endothelial cells, (c) suppress the proliferation of endothelial cells and the production of vascular endothelial growth factor in endothelial and tumour cells (Shimamura et al. 2001). Furthermore, hops have been frequently used as tranquillizer plant. This sedative capacity is due to its bitter acids, particularly the alpha acid component. Many beer compounds can normalize the rhythm of the sleep in addition to stimulate it (Sánchez et al. 2010).

Many foods are claimed for medicinal purposes. A nutraceutical is any substance that is a food or a part of a food and provides medical or health benefits, including the prevention and treatment of disease. A nutraceutical substance should not be toxic and it should be able to prevent toxicity, avoid genetic oxidative damage, modulate the epigenome marks and induce cell death in a programmed manner in tumour cells, and maintain the epigenome marks in normal cells (Anter et al. 2011).

The main aim of our study is to evaluate the biological effects on degenerative processes of two types of lyophilised Ale beers (a blond and a stout one) and two of their most important bioactive components: tyrosol and humulone, in order to explore the possibility for propose them as a substances with therapeutic potential and consider them not only as an essential element for the daily diet. For that purpose, an integrative study of the biological activity at individual, cellular and molecular levels drawn from the *in vivo* and *in vitro* assays was carried out using two model systems. The *Drosophila* animal model, characterised by having more than 70% of human disease homologous genes (Richardson et al. 2015), is a reliable system to test toxicity, genotoxicity, longevity and many degenerative processes (Graf et al. 1984). Moreover, using *in vitro* model of human leukaemia cells (HL-60), we studied the effect of this compound on the growth inhibition of tumoral cells, DNA damage (internucleosomal fragmentation as double strand breaks DNA laddering associated with activation of the apoptotic pathway or single/double strands breaks in cells) and the modulation of methylation status.

2. MATERIALS AND METHODS

2.1. Samples preparation and simple compounds

A blond Ale beer (Judas®) and a stout Ale beer (Guinness®) were selected for this study. Furthermore, 4-(2-Dihydroxyethyl)phenol (tyrosol) (TCI) and iso-alpha humulone (humulone) (Aroxa™) were tested as single bioactive compounds.

Before to carry out the assays, beers were lyophilised (SCAI, University of Córdoba) and dissolved in distilled water in order to obtain the different tested concentrations. The lyophilised extracts were stored at room temperature in a dark and dried atmosphere until use.

The concentrations of beers were established taking into account the average daily food intake of *Drosophila* (1 mg/day) and the average body weight of *D. melanogaster* individuals (1 mg) (Ja et al. 2007). The concentration range for all tested substances was calculated in order to make it comparable to the beer daily intake for humans (192 ml of total beer/day for 70 kg human body weight) (Kirin 2012).

2.2. *In vivo* assays

Drosophila melanogaster

The value of using *Drosophila* to investigate fundamental biological processes is increasing. This organism is revealing as an appropriate system as it is a complex multicellular organism in which many aspects of gene expression are parallel to those of humans. *Drosophila* substitute mammals in experiences with the distinct goal of uncovering insights directly relevant to human beings because it is a model for many human diseases, including cancer and ageing (Gonzalez 2013; Lints and Soliman 1988; Rudrapatna et al. 2012).

Two *Drosophila* strains were used, each with a hair marker in the third chromosome:

• *mwh/mwh*, carrying the recessive mutation *mwh* (*multiple wing hairs*) that, in homozygosis, produces multiple tricomas per cell instead of one per cell (Yan et al. 2008).

• $flr^{3}/In\ (3LR)\ TM3,\ rip^{p}sep\ bx^{34e}e^{s}Bd^{S}$, where the flr^{3}(*flare*) marker is a homozygous recessive lethal mutation that produces deformed tricomas because is viable in homozygous somatic cells once larvae start the development (Ren et al. 2007).

Strains were maintained at 25 °C and 80% humidity in glass tubes with homemade meal (0.5 g, NaCl, 24 g agar-agar, 100 g yeast, 25 g sucrose, 5 ml propionic acid, 3.5 ml of a 0.2% sulphate streptomycin solution and 1l of water) making changes three times per week. Virgin females were obtained from these tubes, and were crossed in order to perform the crosses for the different assays.

2.2.1. Toxicity and Antitoxicity Assays

In the toxicity assays (number of individuals born in treatment/number of individuals born in the negative control) x 100, the following ranks of concentrations were assayed: 3.125 - 50 mg/ml for lyophilised blond Ale beer (LBAB) and lyophilised stout Ale beer (LSAB), 0.127–2.026 mM for tyrosol and 1.207–77.25 mM for humulone, being the concentration of single molecules the correspondent to the concentration of beers assayed. The antitoxicity tests consisted of combined treatments of the same concentrations as in toxicity assays by adding the genotoxicant hydrogen peroxide at 0.12 M (Sigma, H1009) (Tasset-Cuevas et al. 2013).

Chi-square test in a Microsoft Office Excel 2007 was used to determine if the tested compounds significantly inhibited the survival of flies. Negative control values were considered as those expected in Chi-square formula used in toxicity assay and positive control values in antitoxicity assay. The negative controls were prepared with medium and distilled water and positive controls with medium and 0.12 M H_2O_2 as an oxidative genotoxicant (Anter et al. 2010).

2.2.2. Genotoxicity and Antigenotoxicity Assays (SMART)

The genotoxicity assays were carried out following the method described by Graf et al. (1984). The treatment tubes contained 0.85 g of *Drosophila* Instant Medium (Formula 4-24, Carolina Biological Supply, Burlington, NC) and 4 ml of different concentrations of the substance to be tested (3.125 and 50 mg/ml of LBAB and LSAB; 0.127 and 2.026 of tyrosol; 2.414 and 77.25 of humulone). The antigenotoxicity trials were performed following the method described by Graf et al. (1998), which consisted of combined treatments of genotoxin (0.12 M H_2O_2) and the same concentration used in genotoxicity assays of LBAB, LSAB, tyrosol and humulone. After emergence, adult flies were stored in 70% ethanol until the wings are mounted to the scrutiny of the mutations.

Wings of emerging trans-heterozygous individuals (*mwh* flr^+/mwh^+ flr^3) for each control and concentration were mounted on slides using Faure's solution and scored under a photonic microscope at 400x magnification. Wing hair spots were grouped into three different categories: small single spots corresponding to one or two cells exhibiting the *mwh* phenotype that occur in the late stages of the mitotic division; large single spots with three or more cells showing *mwh* or flr^3 phenotypes that occur in the early stages of larval development; or twin spots corresponding to two juxtapositioning clones, one showing the *mwh* phenotype and other the flr^3phenotype. Small and large spots are originated by somatic point mutation, chromosome aberration, and somatic recombination, while twin spots are produced exclusively by somatic recombination between the flr^3 locus and the centromere.

A total of 361 and 358 wings were mounted and analysed for the genotoxicity and antigenotoxicity treatments, respectively. The total number of spots was also determined and a multiple-decision procedure was applied to determine whether a result is positive, inconclusive or negative (Frei and Wurgler 1988). The frequencies of each type of mutant clone per wing were compared to the concurrent control and analysed applying the binomial Kastenbaum and Bowman Test (Frei and Wurgler 1995), without Bonferroni correction and at 5% level of significance. All inconclusive and positive results were analysed with the nonparametric U-test of Mann, Whitney U-test ($\alpha = \beta = 0.05$). The inhibition percentages (IP) for the combined treatments are calculated from total spots per wing with the following formula (Abraham 1994):

IP= [(single genotoxin - combined treatment)/ single genotoxin] x100

2.2.3. Lifespan Assays

All experiments were carried out at 25 °C according to the procedure described in Tasset-Cuevas et al. (2013). Sets of 25 individuals of the same sex were selected and placed into sterile vials containing 0.21 g of *Drosophila* Instant Medium and 1 ml of different concentrations of solution of the compounds to be tested (3.125–50 mg of LBAB and LSAB; 0.127–2.026 mM of tyrosol; 2.414–77.25 mM humulone). Four replicates were followed during the complete life extension for each control and concentrations established. Alive animals were counted and the respective nourishment renewed twice a week.

In order to know the quality of life of treated *Drosophila* in the longevity trials, the upper 25% of lifespan survival curves was studied. This part of the lifespan is considered as the healthspan of a curve, characterised by low and more or less constant age-specific mortality rate values (Soh et al. 2007).

The statistical treatment of survival data for each control and concentration was assessed with the SPSS Statistics 17.0 software (SPSS, Inc., Chicago), applying the Kaplan-Meier method. The significance of the curves was determined using the Log-Rank method (Mantel-Cox).

2.3. *In vitro* cytotoxicity assays

2.3.1. Cell culture and incubation conditions

The promyelocytic human leukaemia cell line HL-60 was grown in RPMI-1640 medium (Sigma, R5886) supplemented with heat-inactivated foetal bovine serum (Linus, S01805), L-glutamine at 200 mM (Sigma, G7513) and antibiotic-antimycotic solution (Sigma, A5955). Cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 (Gallagher et al. 1979). The cultures were plated at 2.5 x 10^4 cells/ml density in 10 ml culture bottles and passed every 2 days.

2.3.2. Cytotoxicity assay

HL-60 cells were placed in 96 well culture plates (2 x 10^4 cells/ml) and treated for 72 h with LBAB, LSAB, tyrosol and humulone at different concentrations (3.125–250 mg/ml for beers; 0.127–2.026 mM for tyrosol; 1.207–38.62 mM for humulone). This wide range was selected in order to assess the cytotoxic doses ranging the inhibitory concentration 50 (IC_{50}) when possible. This allows the assessment of a wide range of concentrations in the *in vitro* cytotoxicity assays, with the aim to predict acute *in vivo* lethality.

Cell viability was determined by the trypan blue dye exclusion test in a Neubauer chamber at 100X magnification (AE30/31, Motic). Curves were plotted as survival percentage of three independent experiments with respect to the control growing for 72 h.

2.3.3. Determination of DNA fragmentation

HL-60 cells (1 x 10^6/ml) were treated with different concentrations of lyophilised beers, tyrosol or humulone (3.125–250 mg/ml; 0.127–2.026 mM; 0.603–154.5 mM, respectively) for 5 h. Treated cells were collected and centrifuged at 3000 rpm for 5 min, and DNA was extracted according to the procedure described in Merinas-Amo et al. (2016). Briefly, cell pellet was resuspended in lysis buffer and incubated in SDS 10% and proteinase K solution. Steps of precipitation with NaCl and isopropanol were followed by a 70% ethanol DNA washing step, and finally a RNAse incubation step over night.

DNA was quantified in a spectrophotometer (Nanodrop ND-1000) and 1200 ng of DNA was subjected to a 2% agarose gel electrophoresis at 85 mA for 25 min, stained with 7 µl ethidium bromide and visualized under UV light.

2.3.4. Comet assay

The DNA strand breaks induction was determined using the alkaline comet assay (pH < 13), following the method described previously (Husseini et al. 2000; Olive and Banath 2006; Prosperini et al. 2013) with some modifications. Cells were treated with LBAB and LSAB (7.56, 31.25 and 62.5 mg/ml), tyrosol (0.127, 0.253 and 0.506 mM) and humulone (1.207, 4.828 and 19.31 mM) for 5 h. After washing steps in PBS, a concentration of 6.25 x 10^5 cells/ml were mixed with 0.75% low melting point agarose (Sigma, A4018) and transferred to frosted-end slides. Steps of lysis, alkaline electrophoresis, neutralization and dried were carried out according to Mateo-Fernández et al. (2016) protocol. Finally, DNA of 50 - 100 single cells was visualized by treating slides with 7 µl of a 10 µg/ml stock solution of propidium iodide (Sigma, P4170). Comet images were analysed at 400x magnification using a Leica DM 2500 fluorescence microscope with green filter and an attached camera (JAI CV-M4CL). The OpenComet plugging from ImageJ (NIH) was used to score individual parameters for each cell.

Statistical analysis of data was performed using SPSS Statistic 17.0 software. The main parameters of the comet imaging are: total DNA, percentage of DNA in the comet and tail, tail length and tail moment (TM). The latest is the single most relevant index of DNA damage, which is calculated as the percent DNA in the tail multiplied by the distance between the means of the head and tail distributions (Husseini et al. 2000; Olive and Banath 2006). Moreover, TM is considered appropriate for regulatory or inter-laboratory comparison studies (Kumaravel et al. 2009). The Tail Moment data were analysed applying a one-way ANOVA and post hoc Tukey's test to determinate the effect of the compounds on HL-60 cell DNA integrity. $p \leq 0.05$ was considered statistically significant.

2.3.5. Methylation status

Genomic DNA was isolated in the same way as described in DNA fragmentation section. Bisulphite-modified DNA from the LBAB (15.625 and 250 mg/ml), LSAB (15.625 and 250 mg/ml), tyrosol (0.127 and 2.025 mM) and humulone (9.65 and 38.725

mM) treatments, using the EZ DNA Methylation-Gold Kit, was used as template for fluorescence-based real-time quantitative Methylation-Specific PCR (qMSP). A qMSP were carried out according to the protocol described by Merinas-Amo et al. (2016) in 48 well plates in MiniOpticon Real-Time PCR System (MJ Mini Personal Thermal Cycler, Bio-Rad) and was analysed by Bio-Rad CFX Manager 3.1 Software.

We selected repetitive elements in order to analyse a wide range of human genomic DNA. While Alu and LINE sequences are interspersed throughout the genome, satellite DNA is confined to the centromere areas (Deininger et al. 2003; Ehrlich 2002; Lee et al. 1997; Weiner 2002). The relative yielded results were normalised with the housekeeping sequence Alu C4 using the Nikolaidis et al. (2012) and Liloglou et al. (2014) comparative Ct method. Each sample was analysed in triplicate. One-way ANOVA and post hoc Tukey's tests were used to evaluate the differences between the tested compound, repetitive elements and concentrations. The selected Alu M1, LINE-1 and Sat-α sequences have been obtained from Isogen Life Science (see Table 1 for detailed information (Weisenberger et al. 2005)).

Table 1

Primers information.

Reaction ID	GenBank number	Amplicon		Forward primer sequence 5' to 3' (N)	Reverse primer sequence 5' to 3' (N)	GC-content (%)	
		Start	End			Forward	Reverse
Alu C4	Consensus Sequence	1	98	GGTTAGGTA TAGTGGTTT ATATTTGTA ATTTTAGTA (36)	ATTAACTAA ACTAATCTT AAACTCCTA ACCTCA (33)	25	27.3
Alu M1	Y07755	5059	5164	ATTATGTTA GTTAGGATG GTTTCGATT TT (29)	CAATCGACC GAACGCGA (17)	27.6	58.8
LINE-1	X52235	251	331	GGACGTATT TGGAAAATC GGG (21)	AATCTCGCG ATACGCCGT T (19)	47.6	52.6
Sat-α	M38468	139	260	TGATGGAGT ATTTTTAAA ATATACGTT TTGTAGT (34)	AATTCTAAA AATATTCCT CTTCAATTA CGTAAA (33)	23.5	21.2

Source from Weisenberger et al. (2005).

3. RESULTS AND DISCUSSION

3.1. *In vivo*

3.1.1. Toxicity and Antitoxicity

Toxicity of LBAB, LSAB, tyrosol and humulone has been assessed in the *Drosophila in vivo* model. Figure 1A shows the relative percentage of emerging adults after treating larvae with different concentrations of these substances. A non-significant survival rate compared with the control is shown, except for the highest concentration of tyrosol tested (2.026 mM) that exhibited toxic effect on the *Drosophila* viability with a 83% survival rate.

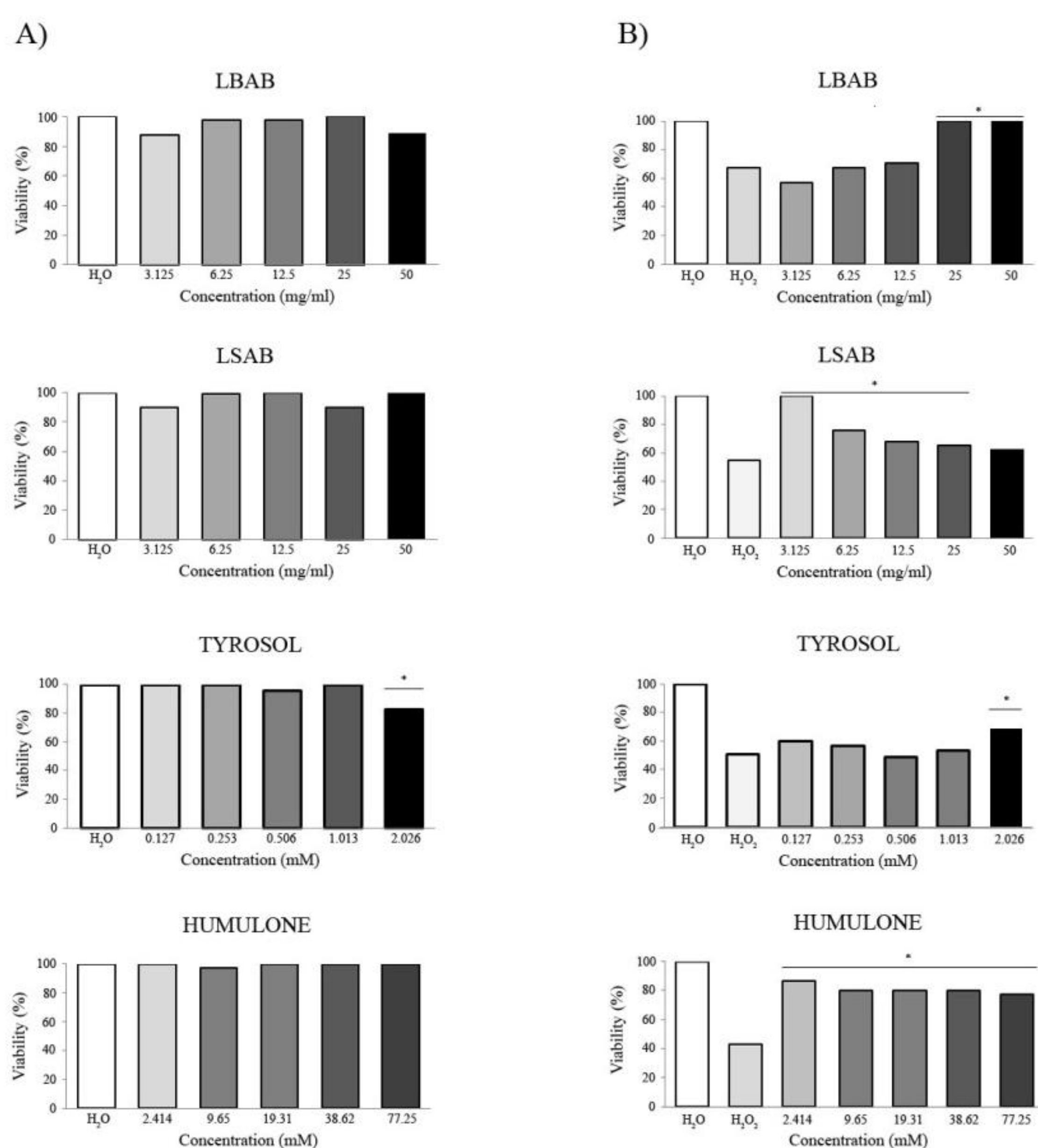
The antitoxicity assays revealed the ability of the LBAB, LSAB, tyrosol and humulone to protect the individuals against an oxidative stress at the tested concentrations. Figure 1B shows that the positive control H_2O_2 is toxic at 0.12 M with an average survival rate of 54% with respect to the water control; this results is in agreement with previous experiences of toxicity in *Drosophila melanogaster* treated with H_2O_2 (Romero-Jimenez et al. 2005). According to Gorjanovic et al. (2010), the possibility to use peroxide as uniform equivalent to evaluate the antioxidative capacity of beer as an equivalent based on decrease of H_2O_2 anodic limiting current can serve as criterion of antioxidant activity. LBAB showed a significant protective effect in the two highest assayed concentrations (25 and 50 mg/ml). Contrarily, LSAB showed a significant protective effect at all tested concentrations, except in highest one (50 mg/ml). The combined treatments of tyrosol and H_2O_2 showed significant protective effect only in the highest concentration assayed (2.026 mM). Finally, humulone was the most protective substance with significant values for all the assayed concentrations.

Unfortunately, not much information about Ale beers activities on toxicity and antitoxicity are found. To our knowledge, there are no previous studies about the effects that Ale beer cause on the survival and protection of *D. melanogaster*. By comparing our results with other studies on Lager beer (Merinas-Amo et al. 2016) be could confirm the non-toxic activity of both types of beers, although Lager beer shows a highest protective effect against H_2O_2 than the Ale beers.

Tyrosol has been linked to antimicrobial/toxic activity against several bacterial strains (Medina et al. 2006). Furthermore, the hop acids have pronounced bacteriostatic activity; they strongly inhibit the growth of Gram-positive bacteria. This remarkable bioactivity is of importance for killing micro-organisms during wort boiling, which ultimately leads to a sterile beer (De Keukeleire et al. 1999). These antimicrobial activities of phenols are not related to the safe properties that beers showed in our toxicity test in the *Drosophila* higher organism. This differential behaviour of tyrosol from procaryotic with respect to toxicity confirms the high potential of use *Drosophila* as a model organism.

Figure 1

Toxicity (A) and antitoxicity (B) levels of lyophilised blond Ale beer (LBAB), lyophilised stout Ale beer (LSAB), tyrosol and humulone in *Drosophila melanogaster.*



Data are expressed as percentage of surviving adults with respect to 300 untreated 72-hour-old larvae from three independent experiments treated with different concentrations of LBAB, LSAB, tyrosol and humulone (A) and combined treatment with 0.12 M H_2O_2 (B). *: significant differences with respect to the positive control. Chi-square value higher than 3.84.

Several studies affirmed that reactive oxygen species (ROS), and consequently oxidative stress, are closely associated with diverse diseases (Halliwell and Gutteridge 2007). The position and number of hydroxyl groups and double bonds, as well as the ortho-3,4-dihydroxy moiety in the chemical structure of flavonoids, are important for their antioxidant and free radical scavenging activities (Rice-Evans et al. 1996). It has been also reported that tyrosol has moderate but stable antioxidant activity (Yamanaka

et al. 1997). Moreover, studies in kidney cells showed that tyrosol at 25–2500 μM, does not exert any protective effect probably because of its lower scavenging ability (Loru et al. 2009), results which agree with ours. On the other hand, studies with coloured final products of the Maillard reaction which are non-enzymatically formed during the roasting of malt, indicate peroxyl radical scavenging potential (Liégeois et al. 2000). This fact could be the responsible for the stronger antitoxic potential of LSAB at lower concentrations with respect to the LBAB. Therefore, and due to their polyphenols content, beers could be a source of bioactive compounds with suitable free radical scavenging properties. Moreover, our results are in agreement with the antioxidant ability of blond and stout Ale beers found by Tafulo et al. (2010) and Granato et al. (2011).

For these reasons, it can be said that beers are not toxic and safe in the *Drosophila melanogaster* system. In fact, and due to its polyphenols, they are antitoxic and protect against the oxidative toxin H_2O_2.

3.1.2. Genotoxicity and Antigenotoxicity

Table 2 shows the results of genotoxicity and antigenotoxicity assays in the SMART test. Negative control showed a frequency of mutations per wing of 0.157, which fall into the historical range for the wing spot test (Anter et al. 2011). The concentration of H_2O_2 used (0.12 M) has been demonstrated to exert a potent genotoxic effect capable to induce somatic mutations and mitotic recombination in *D. melanogaster* (Romero-Jimenez et al. 2005) at a rate of 0.388 spots/wing.

The results showed that LBAB, LSAB, tyrosol and humulone seem to have no genotoxic effect at the tested concentrations. The total mutation rates at the lowest and highest concentration of LBAB, LSAB, tyrosol and humulone (0.238 and 0.273 spots/wing; 0.154 and 0.289 spots/wing; 0.395 and 0.175 spots/wing; 0.150 and 0.250 spots/wing, respectively) were not significantly higher than the negative control (0.157 spots/wing). Moreover, LBAB, LSAB and humulone were able to inhibit the genotoxic activity of H_2O_2 with an inhibition percentage of 48.45–59.02, 22.68–57.21 and 11.85–25.51 at the lowest and highest tested concentrations, respectively. On the other hand, tyrosol showed no significant differences with respect to the positive control exhibiting non antigenotoxic effects.

The antioxidant activity of the phenols was related to several biological activities, including antigenotoxicity (Anter et al. 2010). An evaluation of the antigenotoxic potential of beer components against carcinogens contained in the human diet, namely heterocyclic amines (HCAs) was carried out by Arimoto-Kobayashi et al. (2006). Their results suggested that beer components act in a protective manner against the genotoxic effects of heterocyclic amines in *in vivo* systems. Studies with tyrosol showed that it was non-genotoxic in the SMART test and it also showed antigenotoxic activity against H_2O_2 induced damage at 140 μM (Anter et al. 2010). This finding is only comparable to our lower concentration. There are not data available on the *in vivo* antigenotoxic

activity of humulone. Our results show for the first time the *in vivo* ability to protect DNA from oxidative damage of toxines. As we mentioned above, its antigenotoxic activity could be due to the scavenging activity shown by this molecule.

Table 2

Genotoxicity and antigenotoxicity of lyophilised blond Ale beer (LBAB), lyophilised stout Ale beer (LSAB), humulone and tyrosol in the *Drosophila* wing spot test.

		Clones per wing (nº spots) [1]					
Compound	Number of wings	Small single clones (1-2 cells) m = 2	Large simple clones (> 2 cells) m = 5	Twin clones m = 5	Total clones m = 2	Mann Whitney U-test [2]	Inhibition percentage (%) [3]
H_2O	38	0.157 (6)	0	0	0.157 (6)		
H_2O_2	36	0.305 (11)	0.083 (3)	0	0.388 (14) +		
SIMPLE TREATMENT							
LBAB (mg/ml)							
3.125	39	0.154 (6)	0	0	0.154 (6) i	Δ	
50	38	0.263 (10)	0.026 (1)	0	0.289 (11) i	Δ	
LSAB (mg/ml)							
3.125	42	0.190 (8)	0.047 (2)	0	0.238 (10) i	Δ	
50	44	0.159 (7)	0.091 (4)	0.022 (1)	0.273 (12) i	Δ	
Tyrosol (mM)							
0.127	40	0.263 (10)	0.105 (4)	0.026 (1)	0.395 (15) i	Δ	
2.026	40	0.175 (7)	0	0	0.175 (7) i	Δ	
Humulone (mM)							
2.414	40	0.150 (6)	0	0	0.150 (6) i	Δ	
77.25	40	0.125 (5)	0.125 (5)	0	0.250 (10) i	Δ	
COMBINED TREATMENT WITH H_2O_2 (0.12 M)							
LBAB (mg/ml)							
3.125	40	0.275 (11)	0	0.025 (1)	0.300 (12) γ		22.68
50	42	0.095 (4)	0.071 (3)	0	0.166 (7) γ		57.21
LSAB (mg/ml)							
3.125	40	0.175 (7)	0.025 (1)	0	0.200 (8) γ		48.45
50	44	0.159 (7)	0	0	0.159 (7) γ		59.02
Tyrosol (mM)							
0.127	40	0.325 (13)	0.075 (3)	0	0.400 (16) λ	Δ	-3.09
2.026	40	0.325 (13)	0.100 (4)	0	0.425 (17) λ	Δ	-9.53
Humulone (mM)							
2.414	38	0.342 (13)	0	0	0.342 (13) γ		11.85
77.25	38	0.289 (11)	0	0	0.289 (11) γ		25.51

[1] Statistical diagnosis according to Frei and Wurgler (1988). + (positive) and i (inconclusive) versus negative control; γ (significantly different) and λ (inconclusive) versus positive control. m: multiplication factor. Kastenbaum-Bowman Test without Bonferroni correction, probability levels $\alpha = \beta = 0.05$. [2] Inconclusive results were resolved by Mann Whitney U-test. Delta marker (Δ) means no differences between the treatment and the concurrent control. [3] The inhibition percentages for the combined treatments were calculated from total spots per wing according to Abraham (1994).

3.1.3. Lifespan

Longevity is a trait influenced by many factors, including gender, genetic variation, environmental influences, diet, lifestyle, access to health care and cultural influences. *Drosophila* revealed as an excellent system to investigate longevity-promoting properties of compounds and nutraceutical extracts as adults seem to show many of the cell senescence features seen in mammals (Fleming et al. 1992). It is well known that caloric restriction without malnutrition is a key strategy for increasing the lifespan (Guarente and Kenyon 2000). Nevertheless, there is available data on lifespan increase by adding nutraceutical substances to the *Drosophila* diet with no reduction of the total intake (Deshpande et al. 2014).

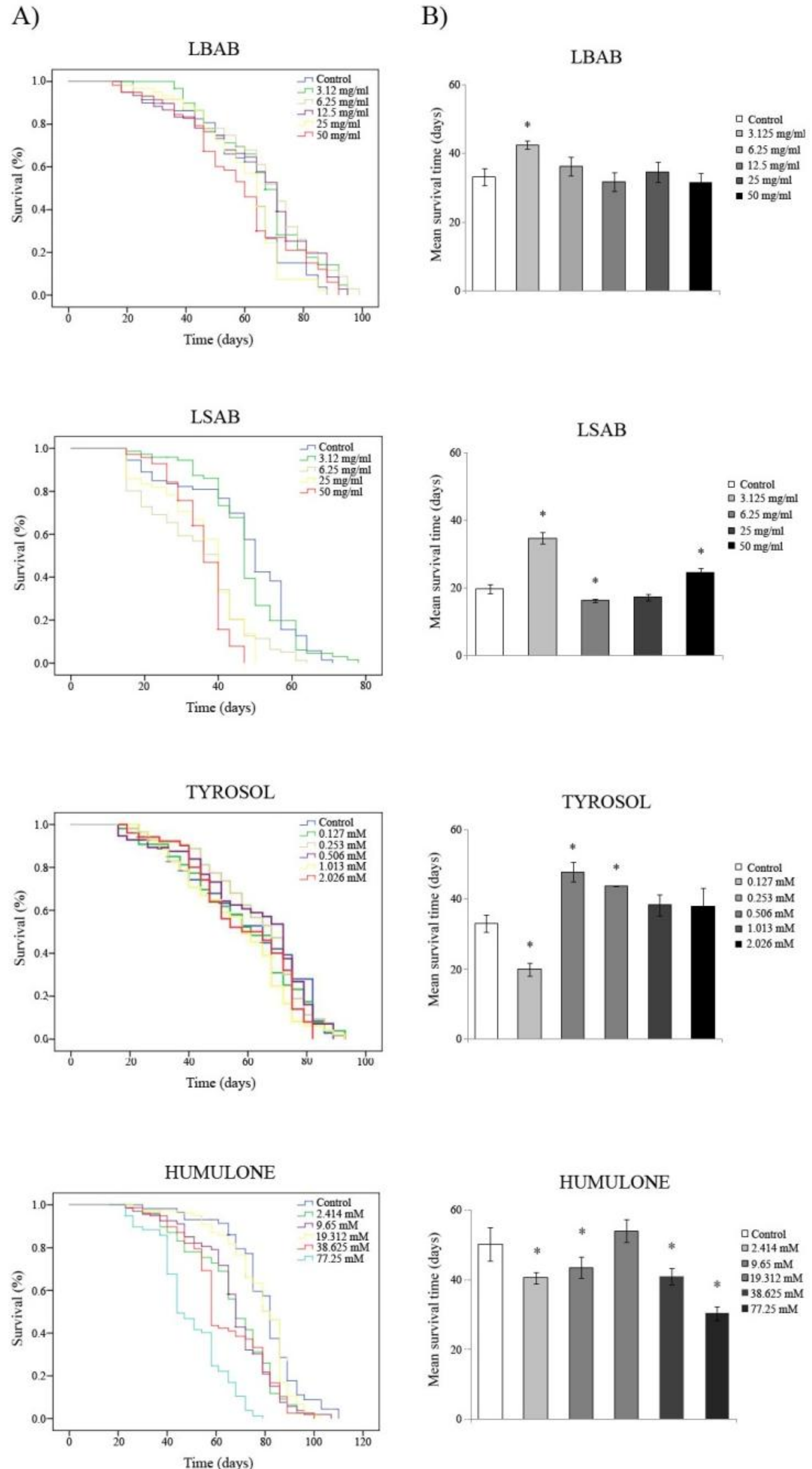
The entire lifespan curves obtained by the Kaplan-Meier method for each substance and concentration are shown in Figure 2A. LBAL showed a significant increase of lifespan of *Drosophila* at three lowest concentrations (3.125, 6.25 and 12.5 mg/ml) with an average of longevity increase of 6 days with respect to their concurrent control. Tyrosol did not significantly affect the longevity of *Drosophila* (longevity ranging between 56.2–63.046 days for the different concentrations) with respect to the control (60.333 days average) (Table 3).

On the other hand, LSAB significantly decreased the lifespan in over 13 days respect to their concurrent control, being the lowest concentration the only one that maintains the lifespan extension. Humulone was the substance that showed the strongest decrease of lifespan with a significant range between 12–30 days of lifespan reduction respect to its control assay (Table 3). A relationship between the characteristic phenol of beers and the activity of the full drink is also observed in ageing trials. LBAB either equals or improves the effect of tyrosol in the longevity treatments and LSAB the humulone ones.

Cañuelo et al. (2012) reported that the inclusion of 250 µM tyrosol increases lifespan and stress resistance in the nematode *Caenorhabditis elegans*. This finding agree with our result which confirmed an extension of *Drosophila* longevity at 0.253 and 0.506 mM treatments. Our results of humulone support the stout beer ones and let us hypothesize that humulone could be in part responsible of the results obtained in stout beer and, consequently, these type of ale beer should not be included into the group of nutraceutic substances. However, no previous research has been found for the Ale beer and humulone lifespan. For that reason, it is important to take with caution the present results and carry out additional research using other organisms and biological target.

Figure 2

Survival parameters of *Drosophila melanogaster* fed with different concentrations of lyophilised blond Ale beer (LBAL), lyophilised stout Ale beer (LSAB), tyrosol and humulone. A) Survival curves and B) Healthspan averages.



A) Survival curves of *Drosophila melanogaster*. B) Mean of survival time in the highest 75% surviving population. * indicates significant differences ($p < 0.001$) with respect to the control.

Table 3

Mean and significances of lifespan and healthspan curves.

Compound	Concentration	Mean Lifespan [1] (days)	Mean Healthspan [1] (days)
LBAB	Control	60.104	33.229
	3.125 mg/ml	66.176 *	42.500 **
	6.25 mg/ml	66.149 *	36.378 ns
	12.5 mg/ml	66.688 *	31.750 ns
	25 mg/ml	59.474 ns	34.634 ns
	50 mg/ml	58.600 ns	31.756 ns
LSAB	Control	47.242	19.769
	3.125 mg/ml	47.636 ns	34.728 ***
	6.25 mg/ml	34.323 ***	16.348 **
	25 mg/ml	35.918 ***	17.313 ns
	50 mg/ml	35.607 ***	24.671 **
Tyrosol	Control	60.333	33.154
	0.127 mM	58.664 ns	20.000 **
	0.253 mM	63.046 ns	47.923 ***
	0.506 mM	62.018 ns	43.857 ***
	1.013 mM	56.273 ns	38.364 ns
	2.026 mM	58.588 ns	38.083 ns
Humulone	Control	79.853	50.156
	2.414 mM	66.779 ***	40.510 **
	9.65 mM	67.702 ***	43.529 **
	19.312 mM	77.522 ns	54.035 ns
	38.625 mM	64.205 ***	41.017 **
	77.25 mM	49.824 ***	30.323 ***

Means were calculated by the Kaplan-Meier method and significance of the curves was determined by the Log-Rank method (Mantel-cox). [1] ns: non-significant ($p > 0.05$), *: significant ($p < 0.05$), **: highly significant ($p < 0.01$), ***: very highly significant ($p < 0.001$).

3.1.4. Healthspan

The mean survival time of healthspan for each substance and concentration are shown in Figure 2B and Table 3. LBAB did not show significant healthspan changes, except at the lowest concentration that increased significantly the quality of life in over 9 days with respect to the water control. LSAB induced a significant improvement of healthspan in *Drosophila melanogaster* compared to the control at the lowest and highest concentration assayed (3.125 and 50 mg/ml) with an increase of 15 and 5 days respectively, whereas the second-to-lower concentration (6.25 mg/ml) showed a slight significant decrease of healthspan in this model organism. Tyrosol showed a significant improvement of healthspan at 0.253 and 0.506 mM with an increase of 14 and 10 days respectively, whereas the lowest concentration (0.127 mM) showed a significant

decrease of healthspan in *Drosophila*. Humulone is the compound which exhibited the worst results because of their significant decrease of healthspan in *Drosophila* at all the assayed concentrations except at medium concentration (19.312 mM) which result is similar to the control one. The reductions ranged between 7–20 days.

This is the first time that a study of healthspan is carried out using a food supplementation with LBAB, LSAB, tyrosol and humulone. Not all the substances should necessarily increase the lifespan of *Drosophila*. Assays with borage oil were carried out to evaluate its healthy effects, showed positive effects. In contrast, the gamma-linolenic acid, which is one of the most important borage oil bioactive components, did not show theses effects (Tasset-Cuevas et al. 2013). This pattern is similar to that we observed in the LSAB and humulone assays: the food (complex mixture) exhibits positives effects whereas a single compound does not show the same healthy effect to *Drosophila*.

3.2. *In vitro*

3.2.1. Cytotoxicity

All substances assayed showed cytotoxic activity against HL-60 cells (Figure 3). LBAB, LSAB and tyrosol showed a dose dependent response, with an increase of the cytotoxicity level according to increased concentration of the compound, being the inhibitory concentration 50 (IC_{50}) lower than 62.5 mg/ml, 15.625 mg/ml and 0.127 mM, respectively. Cytotoxicity curve of humulone showed a strong dose-dependent increase of inhibition until 4.828 mM, at this point a tendency to increase the cell growth is observed until 19.3125 mM and finally the curve descended inducing cell death at 38.625 mM concentration (IC_{50} value under 2.414 mM).

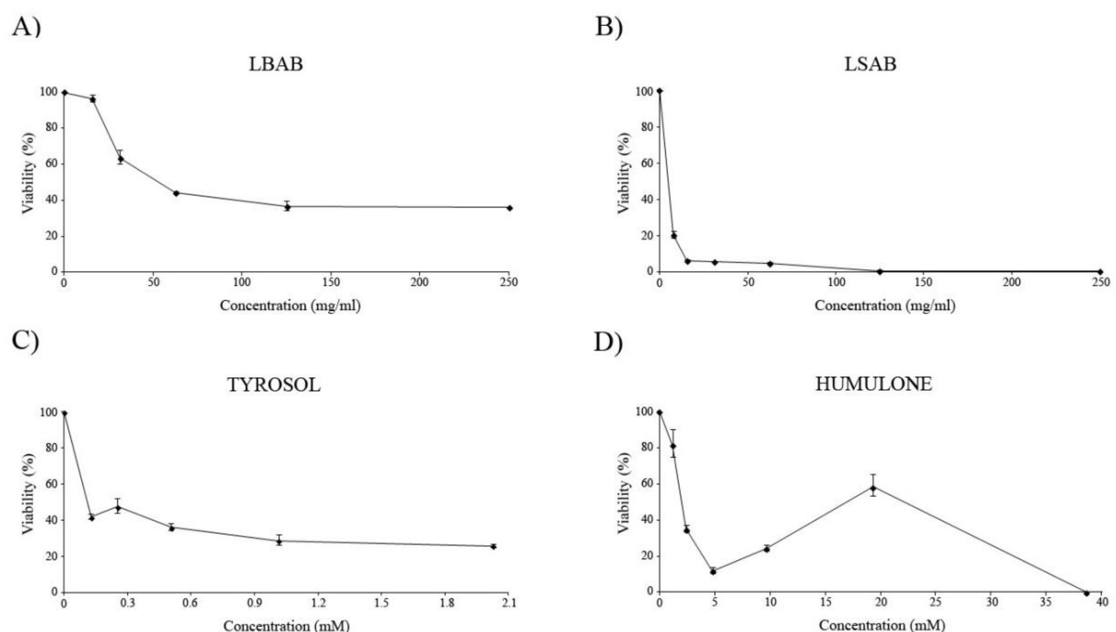
There are conflicting reports concerning the relationship between beer consumption and cancer risk. Riboli et al. (1991) have reported that beer consumption is not associated with colon cancer, but Kato et al. (1990) have shown that beer drinkers have an increased risk to colorectal cancer. An inverse association between moderate beer consumption and endometrial cancer was suggested by Swanson et al. (1993). However, a connection between beer consumption and lung cancer was suggested by Potter et al. (1992). *In vitro* studies indicate the anti-proliferative activity of Lager beer components with antioxidant properties in prostate cancer cells and HL-60 promyelocytic cancer cells, showing a strong correlation between antioxidant potency, polyphenols content and antiproliferative activity (Hevia et al. 2008; Merinas-Amo et al. 2016). The present cytotoxic results of both types of Ale beers, are support the epidemiological date on safety and the *in vitro* results of Lager beer.

In relation to the phenolic compounds, researches about tyrosol in HL-60 cells, salivary gland and Caco-2 cells indicate that tyrosol is not cytotoxic or with a low level of cytotoxicity (Anter et al. 2010; Babich and Visioli 2003). These results do not agree with ours of tyrosol, maybe because of the different and extreme concentrations tested by the researchers (lower than 140 μM or 10 mM, respectively).

An *in vitro* research on the biological activity of hop acids suggests that humulone exhibits significant activity against some human breast and lung cancer cells (Tyrrell et al. 2010). The active form of vitamin D (VD_3) inhibits proliferation and induces differentiation of myelomonocytic leukaemia cells, but its clinical use is limited by the adverse effect of hypercalcemia. According to Honma et al. (1998), humulone inhibits bone resorption. Since humulone is a less-toxic inhibitor of bone resorption, the combination of humulone and VD_3 may be useful in differentiation therapy of myelomonocytic leukaemia. According to Gerhauser (2005), chemopreventive activities of hop derived beer constituents, as bitter acids, reported to be relevant in inhibiting carcinogenesis at the initiation, promotion and progression phases. These fact supports our results of the cytotoxic assays of humulone.

Figure 3

Effect of lyophilised blond Ale beer (LBAL), lyophilised stout Ale beer (LSAB), tyrosol and humulone on cell viability.



Viability in promyelocytic human leukaemia cells (HL-60) treated with different concentrations of LBAL (A), LSAL (B), tyrosol (C) and humulone (D) for 72 h. Each point represents the growing percentage with respect to its control. Values are mean +/- SE from three independent experiments.

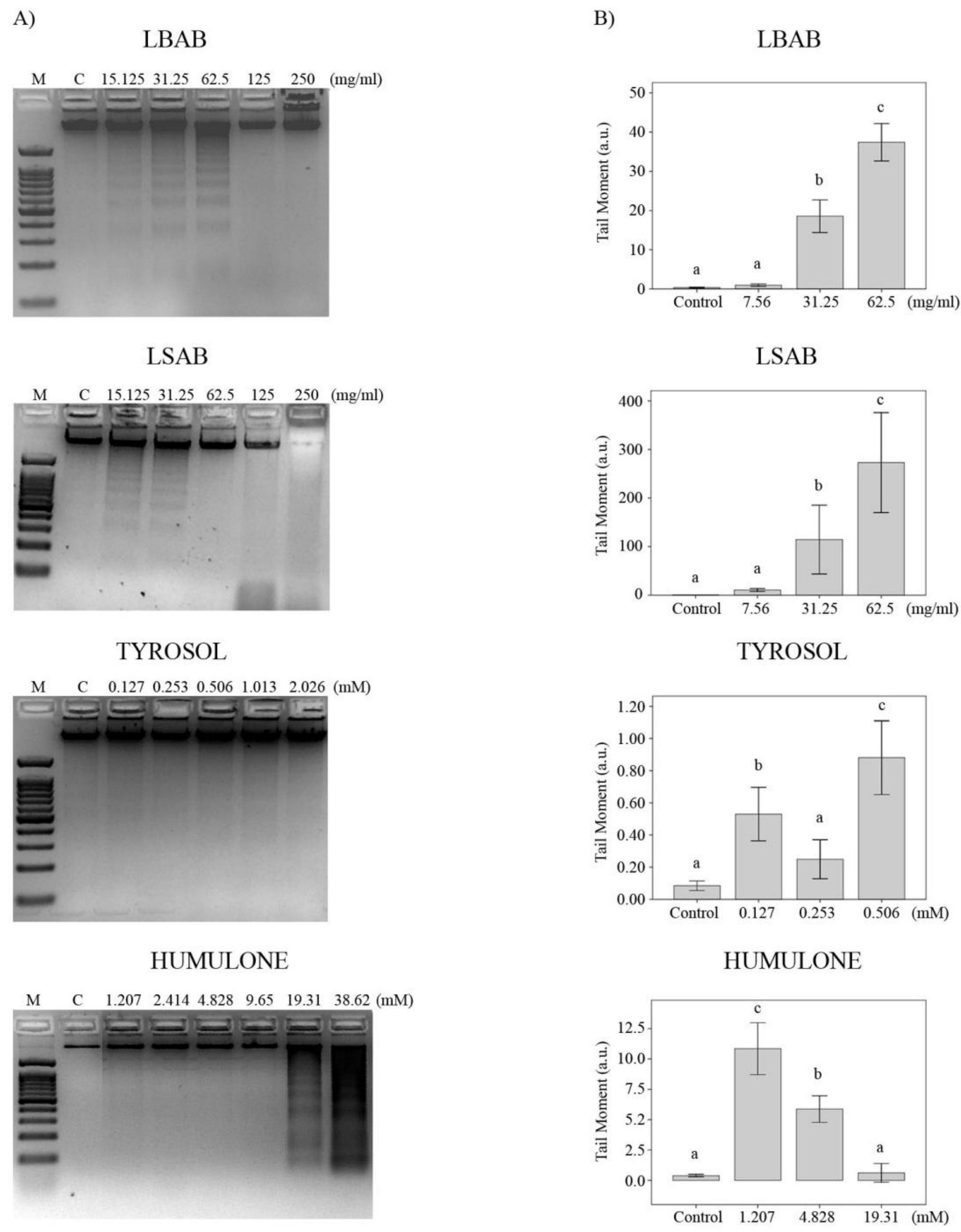
3.2.2. DNA fragmentation

Figure 4A shows the electrophoresis of the genomic integrity of HL-60 cells treated with different concentrations of LBAB, LSAB, tyrosol and humulone. DNA internucleosomal fragmentation is represented by a DNA laddering and it is associated to the activation of the apoptotic way in cancer cells. Fragmentation was observed in the third lowest concentrations of LBAB (15.625, 31.25 and 62.5 mg/ml) and in the two lowest concentration of LSAB (15.625 and 31.25 mg/ml). In addition, the highest concentrations of humulone (19.312 and 38.725 mM) were those that shown DNA

fragmentation. On the other hand, tyrosol did not induce internucleosomal fragmentation at any assayed concentrations.

Figure 4

DNA-induced damage in promyelocytic HL-60 cells treated with different concentrations of lyophilized blond Ale beer (LBAL), lyophilized stout Ale beer (LSAB), tyrosol and humulone for 5 h. A) Internucleosomal DNA fragmentation. B) DNA strand breaks induction.



A) M indicates DNA size marker, C indicates Control treatment. B) Alkaline comet assay (pH < 13) of HL-60 cells treated. DNA migration is reported as mean TM. Values are mean +/- SE. Different letters in treatments mean differences respect to the negative control after one-way ANOVA and post hoc Tukey's test.

To our knowledge, any study with LBAB and LSAB was carried out in order to analyse the DNA fragmentation patter that Ale beers cause in cancer cells. Studies with Lager beers showed a fragmentation effect at the concentrations of blond Lager beer (15.625 and 31.25 mg/ml) and slightly in the intermediate concentration (62.5 mg/ml) (Merinas-Amo et al. 2016). Our results were similar to the previous results with Lager beers. Studies about tyrosol reveals its capacity to prevent apoptotic human keratynoxytic cell death against UVB radiation confirming its beneficial properties and a possible application in the sun skin protection (Salucci et al. 2014) and a prevention of ischemia/reperfusion-induced apoptosis in H9c2 cells has also been described (Sun et al. 2012). Furthermore, Anter et al. (2010) studied the effect of tyrosol in the DNA of HL-60 cells. They showed that, at 8.75–140 µM concentrations this phenol did not induced proapoptotic DNA damage. Although we assayed higher concentrations, we agree with the results that tyrosol did not activate the apoptosis way in treated tumoral cells. In relation to humulone effects in HL-60 cells, a study supported that the potent antioxidative activity might cause apoptosis (Tobe et al. 1997). These results are in agreement to ours that showed a specific DNA fragmentation caused by humulone wich acts as an inductor of internucleosomal proapoptotic laddering.

3.2.3. Comet assay

Results of the tail moment (TM) for different concentrations of choline-treated HL-60 cells are shown in Figure 4B. A positive dose-dependent response is observed for LBAB and LSAB treatments with significant double and/or the single strands breaks at the two highest concentrations assayed (31.25 and 62.5 mg/ml). In the tyrosol treatments, the 0.127 and 0.506 mM concentrations showed significant TM with respect to the control. Furthermore, humulone exhibited a negative dose-dependent effects in the comet assay showing significant strands breaks at lowest concentrations (1.207 and 4.828 mM).

By using the comet assay we were able to readily identify, at unicellular level, apoptotic nuclei based on the typical morphological changes attributable to extensive DNA damage (Poe and O'Neill 1997). According to Fabiani et al. (2012), the frequencies distribution of DNA damage can be divided into five classes depending on the TM values as follows: class 0 (TM < 1; no damage), class 1 (TM 1–5; slightly damaged), class 2 (TM 5–10; medium damage), class 3 (TM 10–20; highly damaged) class 4 (TM >20; completely damaged). Based on this classification, all concurrent control values fell into class 0 (TM < 1); LBAB 31.25 and 62.25 mg/ml showed a TM value of 18 and 37, what means that respectively induce a high and complete clastogenicity damage in HL-60 cells; LSAB induced damage at all the tested concentration with a TM value of 10.6 for the lowest, 96 for the medium and 272 for the highest concentration tested; tyrosol induced TM values between 0.24 and 0.7 (class 0) no causing damage on cells; humulone, caused a highly damage at 1.207 mM (TM values of 10.84) and a medium damage at 4.828 mM (TM values of 5.8); and finally, the rest of concentrations no commented previously fell into class 0.

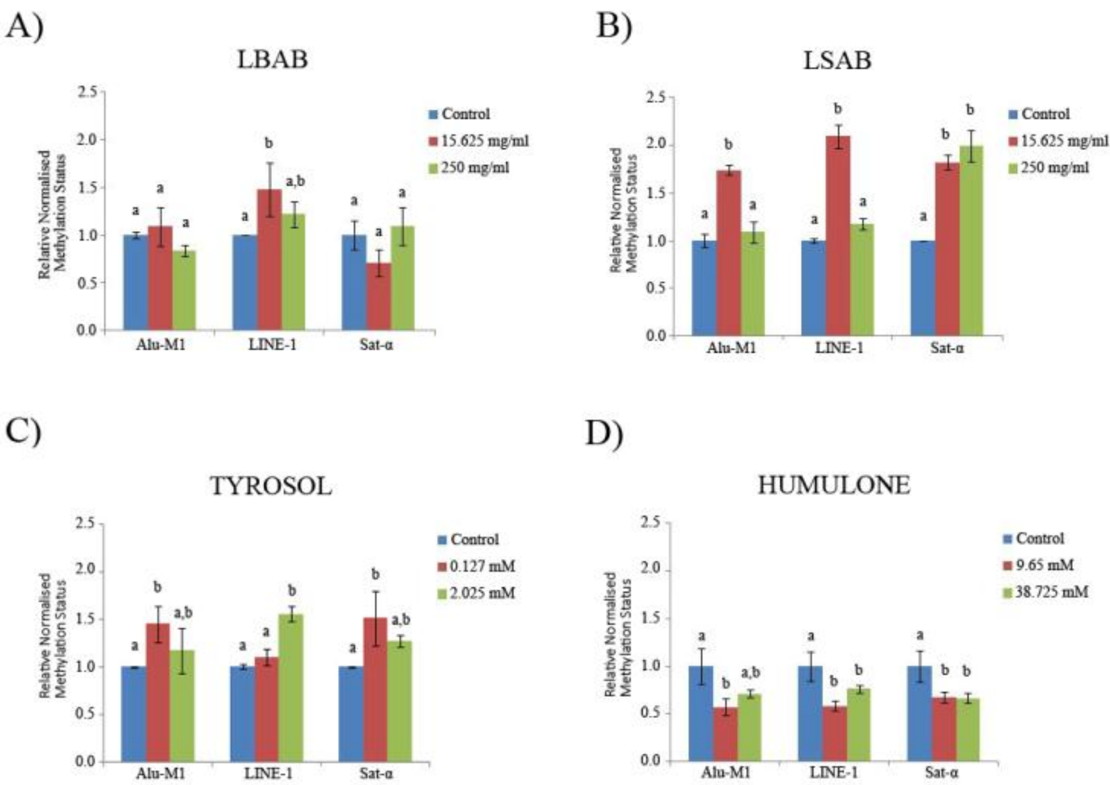
The comet assay TM measurements have been shown to correlate with the cytotoxicity ones (Fairbairn et al. 1996). Our results agree with the above correlations mentioned showing significant TM values correlated directly to the percent of viable cells as monitored using trypan blue exclusion for HL-60 cells. Moreover, the results of DNA fragmentation fit with those obtained in comet assay since TM values higher than 30 mean that apoptosis mechanisms has been induced (Fairbairn et al. 1995). Only the highest concentration of LBAB and the two highest ones of LSAB showed TM > 30 confirming the death of leukaemia cells by apoptosis. The rest of compounds and concentrations tested induce cell death by a necrosis mechanism.

3.2.4. Methylation status

The relative normalized expression of the three repetitive sequences (Alu M1, LINE-1 and Sat-α) studied in HL-60 cells treated with different concentrations of LBAB, LSAB, tyrosol humulone are shown in Figure 5. LBAB showed a significant hypermethylated status only in the lowest concentration tested (15.625 mg/ml) for the LINE-1 repetitive sequence, with respect to the concurrent control. LSAB showed a significant hypermethylation in 15.625 mg/ml treatments on all repetitive sequences and also at the highest concentration (250 mg/ml) for Sat-α sequence, with respect to their controls. Tyrosol showed a general tendency to methylate leukaemia cells with a significant hypermethylation at 0.127 mM for Alu-M1 and Sat- α sequences and at 2.050 mM for LINE-1 sequence, with respect to their controls. On the other hand, humulone exhibited a demethylation induction on the concentrations with respect to their controls.

There is not previous studies about methylation status with Ale beers, tyrosol and humulone. At present, the mechanisms controlling epigenetic methylation DNA modification are complex (Herman and Baylin 2003). Endoparasitic repetitive sequences refrain from jumping around thanks to the repression of DNA methylation (Esteller 2005). Due to the effects that methylation causes in the repetitive sequences of the genome, we could say that Ale beers and tyrosol prevents for DNA epigenetic damages as a methylaltion of repetitive sequences is understood as a genomic protective mechanism (Roman-Gomez et al. 2008; Weisenberger et al. 2005). These effects are not shown when HL-60 cells are treated with humulone. Our results show that LINE and SINE (Alu) sequences are prone to be methylated in HL-60 cells, due to its genomic context or characteristics. Centromeric areas (Sat) seemed to be more protected and inaccessible. Our results in leukaemia cells showed an increase of general methylation. Besides of that and bearing in mind the idea of tumoral cells demethylation, it would indicate that beers and its compounds act as preventive agents cutting off the repetitive sequences of tumoral cells (Roman-Gomez et al. 2008).

Figure 5
Relative normalized expression data of each repetitive element.



Relative normalised expression data of each repetitive element (Alu M1, LINE-1 and Sat-α). Different letters mean different values after one-way ANOVA and post hoc Tukey's test.

4. CONCLUSIONS

It is very important to study the different consequences of daily diet. In the present study, we evaluated some biological activities of two Ale beers (LBAB, LSAB) and its most abundant biophenols (tyrosol and humulone) at the three individual, cellular and DNA level.

All the *in vivo* and *in vitro* assays carried out suggest an important role on the modulation of degenerative processes by lyophilised blond and stout Ale beers. The safety (non-toxic and non-genotoxic properties), the protection against the oxidative genotoxine H_2O_2 (antitoxic and antigenotoxic activities), the extension of lifespan in *D. melanogaster* (only for LBAB), the induction of proapoptotic DNA fragmentation and DNA damage in individual tumour cells and the modulation of methylation status has been established. The phenolic alcohol contained in beer showed most of the activities of the blond beer as a complex mixture: it showed antitoxic effect at higher concentrations, the curve of cells growing was similar to the beer one and induced a hypermethylation status in tumour cells. The hop acids showed similar activities to the stout beer as a complex mixture: it showed antitoxic effect at lower concentrations, reduced the lifespan of the animal model and induced a 100% inhibition of tumour cells growing. On the other hand, some interesting exceptions have to be pointed out: tyrosol

showed a maintenance of lifespan in *Drosophila*, did not induced proapoptotic DNA fragmentation or DNA strand breaks and humulone induced single strand breaks at lower concentration whereas DNA damage induced by beers was at higher concentrations; different patterns in the methylation status has been observed for the compounds with respect to beers. For that reasons, the biological activities attributed to beer consumption cannot be linked to one particular class of constituents due to their high complexity and variability in the polyphenolic pattern, and also because the brewing is influenced by the raw materials, wort composition, yeast strains and fermentation conditions, among others factors. In conclusion, results suggest that daily moderate consumption of beer may be healthy due to the properties that the full beverage shows.

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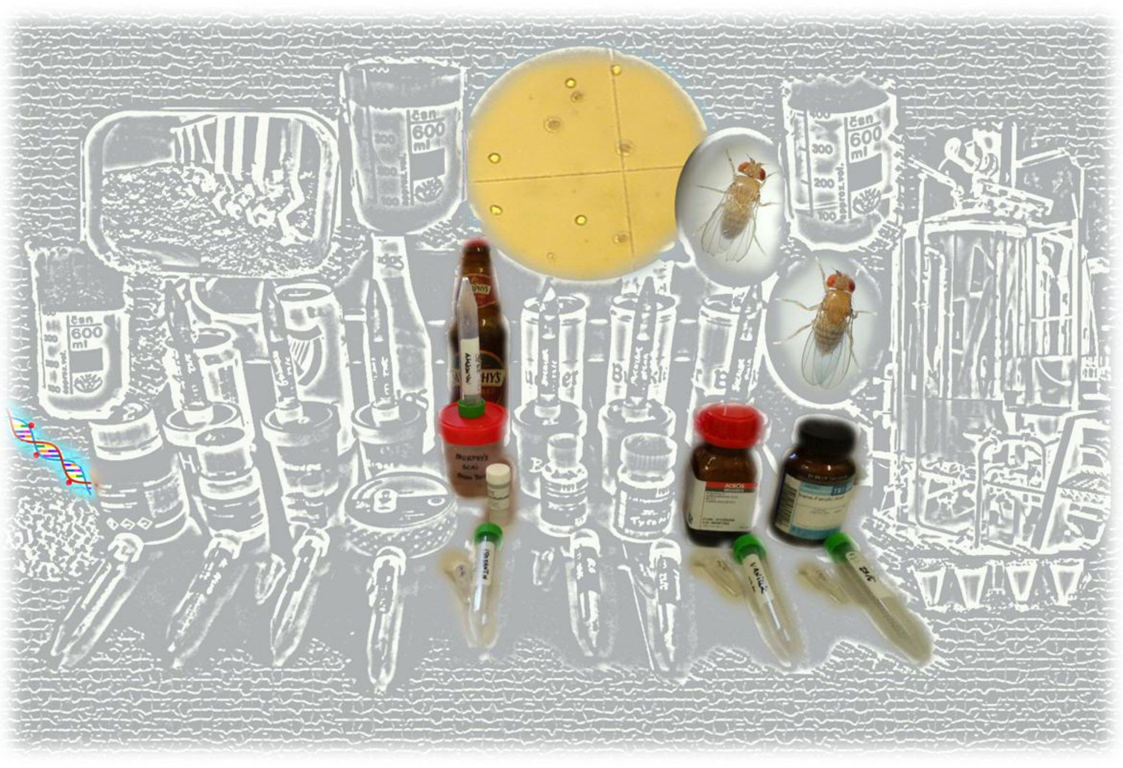
CHAPTER 3



Lyophilised stout Lager beer and three of its most distinctive phenols: a biological and toxicological study

ABSTRACT

In vivo and *in vitro* assays were carried out in order to analyse the nutraceutic potential of a lyophilised stout Lager beer, as well as three of their most characteristic bioactive compounds (isoxanthohumol, ferulic and vanillic acids). The *in vivo* results showed that, all substances assayed are non-toxic substances. Moreover, they revealed the ability to protect the individuals against an oxidative stress at some tested concentrations. Also, all substances have not genotoxic effects and were able to inhibit the genotoxic activity of H_2O_2. With respect to the degenerative process, all compounds induced a significant lifespan extension in *D. melanogaster* at some of the tested concentrations, except for the 0.062 mM of vanillic that significantly induce a reduction of longevity. The *in vitro* assays showed that LSLB and ferulic present a cytotoxic activity against HL-60 cells. Moreover, DNA damage was observed for beer, isoxanthohumol and vanillic at different concentrations, although only beer is able to induce death by the apoptotic way. Besides, lyophilised stout Lager beer, isoxanthohumol and ferulic exhibit an increasing genome wide methylation status while vanillic exhibits a demethylation status in cancer cells repetitive sequences. The results let us to propose lyophilised stout Lager beer as a nutraceutic beverage.

1. INTRODUCTION

Beer consumption has been associated with a wide range of beneficial health effects like reduction of the cardiovascular disease, decrease of kidney stones formation, chemopreventive activity, antioxidant capacity and physiological effects, among others (Bassus et al. 2004; Chen et al. 2013; Granato et al. 2011; Muller et al. 2007; Piazzon et al. 2010).

Beer is a very complex beverage that contains, apart from water, a multitude of nutritionally valuable substances, primarily minerals, B group vitamins, and polyphenols. Some of the compounds are derived from raw materials (water, malts, hops and yeast) and others are produced during the brewing process (Buiatti 2009). Depending on the fermentation conditions (mainly time and temperature) beers could be classified into two major types, Ale and Lager: Ale beers use a type of yeast that ferments best at warmer temperatures, whereas Lager beers use special yeast that ferments best at cooler temperatures. Moreover, beers can exhibit colours ranging from pale to black due to the colour of malt(s) used and the caramelisation of sugars and Maillard-type reactions (De Keukeleire 2000; Kodama et al. 2006). Dark malts differ from pale malts in their content of coloured Maillard or caramelisation products formed primarily during roasting of malt by reactions involving proteins and carbohydrates (Woffenden et al. 2001). Carbonyl and amino are the compounds involved in the Maillard reactions, which include reducing carbohydrates and the free amino groups of amino acids, peptides or proteins (Hodge 1953). Burnt, caramel and Madeira-like flavours are the commonly flavours originated from the Maillard reaction (Câmara et al. 2004). The Maillard reaction can cause either deterioration or enhancement of food quality (Friedman 1996; Henle 2005; Silván et al. 2006), by the formation of antinutritional and toxic Maillard reaction products (MRPs) with mutagenic (Yen et al. 1993), carcinogenic (Yen and Tsai 1993) and cytotoxic (Vagnarelli et al. 1991) effects; or beneficial MRPs compounds with antioxidant capacity (Woffenden et al. 2001).

The beer compounds have been shown to possess different biological activities as anti-bacterial, anti-inflammatory, anti-oxidative, anti-angiogenic, anti-melanogenic, anti-osteoporotic and anti-carcinogenic effects, among others (Arranz et al. 2012; Chen et al. 2013; Chiva-Blanch et al. 2013; Pal et al. 2003; Stocker and O'Halloran 2004; Vinson et al. 2003). Precisely, blond Lager beer has shown to be safe, protective against oxidative genetic damage and inductor to lifespan extension (Merinas-Amo et al. 2016).

The purpose of the present research is to evaluate the biological effects that a lyophilised stout Lager beer (LSLB) and three of its most important bioactive phenols (isoxanthohumol, ferulic and vanillic acid) have on degenerative processes in order to propose it as a substance with nutraceutic potential. The role of the different phenols selected on the activities of the complex mixture (stout Lager beer) is also evaluated.. For that purpose, a general overview of the biological activity at individual, cellular and molecular levels drawn from the *in vivo* and *in vitro* assays we carried out using the *Drosophila melanogaster* and human leukaemia cell (HL-60) models organisms.

2. MATERIALS AND METHODS

2.1. Samples preparation and simple compounds

A stout Lager beer (Murphy's Iris red®) was selected for this study. Murphy's Irish red is a type Lager beer produced by using "bottom-fermenting" process, roasted, with soft and fruity taste. Before to carry out the assays, beer was lyophilised (SCAI, University of Córdoba) and stored until use at room temperature in a dark and dried atmosphere. Lyophilised beer (LSLB) was dissolved in distilled water in order to obtain the different concentrations to be tested.

Furthermore, three single bioactive compounds were tested:

i) Isoxanthohumol (IX) which is a flavanone formed from the xanthohumol in hops during wort boiling (Piendl and Biendl 2000). Conventional beers contain up to approximately 3 mg of isoxanthohumol per litre, depending on hopping (quantity added and hop product used) and the brewing process employed (Biendl and Pinzl 2009).

ii) Trans-Ferulic acid (Ferulic) is the main phenolic acid in barley, malted barley and beer, followed by *p*-coumaric, vanillic, caffeic, *p*-OH-benzoic and sinapic acids (Chunsriimyatav et al. 2010; MaIt and XgUMaIl 1992). Beers contain up to 6.5 mg/l of this phenolic acid constituent (Gerhäuser 2005).

iii) 4-Hydroxy-3-methoxybenzoic acid (Vanillic) is an intermediate in the production of vanillin from ferulic acid (Civolani et al. 2000; Lesage-Meessen et al. 1996). Beers contain up to 3.6 mg/l of this phenolic acid constituent (Gerhäuser 2005).

The concentrations of beers were established taking into account the average daily food intake of *Drosophila* (1 mg/day) and the average body weight of *D. melanogaster* individuals (1 mg) (Ja et al. 2007). The concentration range for all tested substances was calculated in order to make it comparable to the beer daily intake for humans (192 ml of total beer/day for 70 kg human body weight) (Kirin 2012). Furthermore, the assayed concentrations for the single compounds are the equivalent concentrations contained in the full beer.

2.2. *In vivo* assays

The *Drosophila* animal model, characterised by having approximately 75% of the genes involved in human diseases related or similar sequences to *D. melanogaster* (Lloyd and Taylor 2010), is a reliable system to test toxicity, genotoxicity and many degenerative processes (Graf et al. 1984). There are many studies linking *Drosophila* use as a model for different human pathologies (Foriel et al. 2015; Maximino et al. 2015; Plantié et al. 2015; Venken et al. 2016), including Parkinson's and Alzheimer's, which are regarded as neurodegenerative disorders (Bilen and Bonini 2005), and cardiovascular diseases (Wolf et al. 2006), immunologic and intestinal infections (Kim and Lee 2014), and other human pathologies including cancer (Gonzalez 2013).

In the present work, two *Drosophila* strains were used, each with a hair marker in the third chromosome: (i) *mwh/mwh*, carrying the recessive mutation *mwh* (*multiple wing hairs*) that in homozygosis produces multiple tricomas per cell instead of one per cell

(Yan et al. 2008), and (ii) *flr*^{3}/*In (3LR) TM3, rip*^{p}*sep bx*^{34e}*e*^{s}*Bd*^{S}, where the *flr*^{3}(*flare*) marker is a homozygous recessive lethal mutation that produces deformed tricomas but is viable in homozygous somatic cells once larvae start the development (Ren et al. 2007).

2.2.1. Toxicity and Antitoxicity Assays

All compounds (LSLB, IX, ferulic and vanillic) were tested at five concentrations: 3.125–50 mg/ml; 0.0036–0.059; 0.0135–0.216 and 0.007–0.125 mM, respectively. The antitoxicity tests consisted of combined treatments of the same concentrations as in toxicity assays by adding the genotoxicant H_2O_2 at 0.12 M (Tasset-Cuevas et al. 2013).

Emerging adults of all groups were counted and toxicity was determined as the percentage of hatched individuals in each treatment compared with the negative control. Antitoxicity was assessed using the same procedure and experimental concentrations as in toxicity assays, but in combined treatments with 0.12M H_2O_2 and comparing the percentage of emerging adults with the positive toxicant control. Chi-square test in a Microsoft Office Excel 2007 was used to determine if the tested compounds significantly affected the survival of flies.

2.2.2. Genotoxicity and Antigenotoxicity Assays (SMART)

The genotoxicity assays were carried out following the method described by Graf et al. (1984). The treatment tubes contained 0.85 g of *Drosophila* Instant Medium (Formula 4-24, Carolina Biological Supply, Burlington, NC) and 4 ml of different concentrations of the substance to be tested (3.125 and 50 mg/ml of LSLB; 0036 and 0.059 mM of IX; 0.0135 and 0.216 mM of ferulic; 0.007 and 0.125 mM of vanillic). The antigenotoxicity tests were performed following the method described by Graf et al. (1998), which consisted of combined treatments of genotoxin (0.12 M H_2O_2) and same concentration used in genotoxicity assays of LSLB, IX, ferulic and vanillic. After emergence, adult flies were stored in 70% ethanol until the wings are removed and mounted on slides using Faure's solution. Mutant spots were scored under a photonic microscope at 400x magnification. The frequencies of each type of mutant clone per wing (single, large or twin spot) were compared with the concurrent control.

A total of 362 to 354 wings were mounted and analysed for the genotoxicity and antigenotoxicity treatments, respectively. The total number of spots was also determined and a multiple-decision procedure was applied to determine whether a result is positive, inconclusive or negative (Frei and Wurgler 1988). The frequencies of each type of mutant clone per wing were compared to the concurrent control and analysed applying the binomial Kastenbaum and Bowman Test (Frei and Wurgler 1995), without Bonferroni correction and at 5% level of significance. All inconclusive and positive results were analysed with the nonparametric U-test of Mann, Whitney U-test ($\alpha = \beta = 0.05$). The inhibition percentages (IP) for the combined treatments are calculated from total spots per wing with the following formula (Abraham 1994):

IP = [(single genotoxin - combined treatment) / single genotoxin] x100

2.2.3. Lifespan Assays

All experiments were carried out at 25 °C according to the procedure described in Tasset-Cuevas et al. (2013). Sets of 25 individuals of the same gender were selected and placed into sterile vials containing 0.21 g of *Drosophila* Instant Medium and 1 ml of different concentrations of solution of the compounds to be tested (3.125–50 mg of LSLB; 0.0036–0.059 mM of IX; 0.0135–0.216 mM of ferulic; 0.007–0.125 mM of vanillic). Two replicates were followed during the complete life extension for each control and concentrations established. Alive animals were counted and the respective nourishment renewed twice a week.

In order to know the quality of life of treated *Drosophila* in the longevity trials, the upper 25% of lifespan survival curves was studied. This part of the lifespan is considered as the healthspan of a curve, characterised by low and more or less constant age-specific mortality rate values (Soh et al. 2007).

The statistical treatment of survival data for each control and concentration was assessed with the SPSS Statistics 17.0 software (SPSS, Inc., Chicago), applying the Kaplan-Meier method. The significance of the curves was determined using the Log-Rank method (Mantel-Cox).

2.3. *In vitro* assays

Using the *in vitro* model of human leukaemia cells (HL-60) we studied the effect of the compounds on the growth inhibition of tumoral cells, DNA damage (internucleosomal fragmentation as double strand breaks DNA laddering associated with activation of the apoptotic pathway or single/double strands breaks in cells) and the modulation of methylation status.

2.3.1. Cell culture and incubation conditions

The promyelocytic human leukaemia cell line HL-60 were grown in RPMI-1640 medium (Sigma, R5886) supplemented with heat-inactivated foetal bovine serum (Linus, S01805), L-glutamine at 200 mM (Sigma, G7513) and antibiotic-antimycotic solution (Sigma, A5955). Cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 (Gallagher et al. 1979). The cultures were plated at 2.5 x 10^4 cells/ml density in 10 ml culture bottles and passed every 2 days.

2.3.2. Cytotoxicity assay

HL-60 cells were placed in 96 well culture plates (2 x 10^4 cells/ml) and cultured for 72 h supplemented with LSLB, IX, ferulic and vanillic at different concentrations (3.125–250 mg/ml; 0.0036–0.059 mM; 0.0135–0.216 mM; 0.007–0.125 mM, respectively). Moreover, a combination of the three bioactive compounds were assayed in order to understand the effects of the phenols together. This wide range was selected in order to assess the cytotoxic doses ranging the inhibitory concentration 50 (IC_{50})

when possible. This allows the assessment of a wide range of concentrations in the *in vitro* cytotoxicity assays, with the aim to predict acute *in vivo* lethality.

Cell viability was determined by the trypan blue dye exclusion test in a Neubauer chamber at 100x magnification (AE30/31, Motic). Curves were plotted as survival percentage of three independent experiments with respect to the control growing for 72 h.

2.3.3. Determination of DNA fragmentation

HL-60 cells (1 x 10^6/ml) were treated with different concentrations of lyophilised beer, IX, ferulic and vanillic (3.125–250 mg/ml; 0.0036–0.059 mM; 0.0135–0.216; 0.007–0.125 mM, respectively) for 5 h. Treated cells were collected and centrifuged at 3000 rpm for 5 min, and DNA was extracted according to the procedure described in Merinas-Amo et al. (2016). Briefly, cell pellet was resuspended in lysis buffer and incubated in SDS 10% and proteinase K solution. Steps of precipitation with NaCl and isopropanol were followed by a 70% ethanol DNA washing step, and finally a RNAse incubation step over night.

DNA was quantified in a spectrophotometer (Nanodrop ND-1000) and 1200 ng of DNA was subjected to a 2% agarose gel electrophoresis at 85 mA for 25 min, stained with ethidium bromide and visualized under UV light.

2.3.4. Comet assay

Cells were treated with LSLB (7.56, 31.25 and 62.5 mg/ml), IX (0.0036, 0.0073 and 0.0147 mM), ferulic (0.0135, 0.027 and 0.054 mM) and vanillic (0.007, 0.015 and 0.031 mM) for 5 h. After washing steps in PBS, a concentration of 6.25 x 10^5 cells/ml were mixed with 0.75% low melting point agarose (Sigma, A4018) and transferred to frosted-end slides. Steps of lysis, alkaline electrophoresis, neutralization and dried were carried out according to Mateo-Fernández et al. (2016) protocol. Finally, at least 50 single cells from each treatment were visualized by treating slides with 7 µl of a 10 µg/ml stock solution of propidium iodide (Sigma, P4170). Comet images were analysed at 400x magnification using a Leica DM 2500 fluorescence microscope with green filter and an attached camera (JAI CV-M4CL). The OpenComet plugging from ImageJ (NIH) was used to score individual parameters for each cell.

Statistical analysis of data was performed using SPSS Statistic 17.0 software. The Tail Moment (TM) data were evaluated by a one-way ANOVA. The post hoc Tukey's test was applied to asses on the effect of the compounds on HL-60 cell DNA integrity. Statistical significance was considered at $p \leq 0.05$.

2.3.5. Methylation status

Genomic DNA was isolated in the same way as described in DNA fragmentation section. Bisulphite-modified DNA from the LSLB (15.625 and 250 mg/ml), IX (0.0036 and 0.059 mM), ferulic (0.0135 and 0.216 mM) and vanillic (0.007 and 0.125 mM)

treatments, using the EZ DNA Methylation-Gold Kit, was used as template for fluorescence-based real-time quantitative Methylation-Specific PCR (qMSP). A qMSP were carried out according to the protocol described by Merinas-Amo et al. (2016) in 48 well plates in MiniOpticon Real-Time PCR System (MJ Mini Personal Thermal Cycler, Bio-Rad) and was analysed by Bio-Rad CFX Manager 3.1 Software.

Alu-M1, LINE-1 and Sat-α were selected as repetitive elements to analyse a wide range of human genomic DNA. While Alu and LINE sequences are interspersed throughout the genome, satellite DNA is confined to the centromere areas (Deininger et al. 2003; Ehrlich 2002; Lee et al. 1997; Weiner 2002) (see Table 1 for detailed information (Weisenberger et al. 2005)).

The relative yielded results were normalised with the housekeeping sequence Alu C4 using the Nikolaidis et al. (2012) and Liloglou et al. (2014) comparative Ct method. One-way ANOVA and post hoc Tukey's tests were used to evaluate the differences between the tested compound, repetitive elements and concentrations in three independent replicates.

Table 1

Primers information.

Reaction ID	GenBank number	Amplicon start	Amplicon end	Forward primer sequence 5' to 3' (N)	Reverse primer sequence 5' to 3' (N)	GC-content (%)	
						Forward	Reverse
ALU-C4	Consensus Sequence	1	98	GGTTAGGTATAGTGGTTTATATTTGTAATTTTAGTA (36)	ATTAACTAAACTAATCTTAAACTCCTAACCTCA (33)	25	27.3
ALU M1	Y07755	5059	5164	ATTATGTTAGTTAGGATGGTTTCGATTTT (29)	CAATCGACCGAACGCGA (17)	27.6	58.8
LINE-1	X52235	251	331	GGACGTATTTGGAAAATCGGG (21)	AATCTCGCGATACGCCGTT (19)	47.6	52.6
Sat-α	M38468	139	260	TGATGGAGTATTTTTAAAATATACGTTTTGTAGT (34)	AATTCTAAAAATATTCCTCTTCAATTACGTAAA (33)	23.5	21.2

Source from Weisenberger et al. (2005).

3. RESULTS

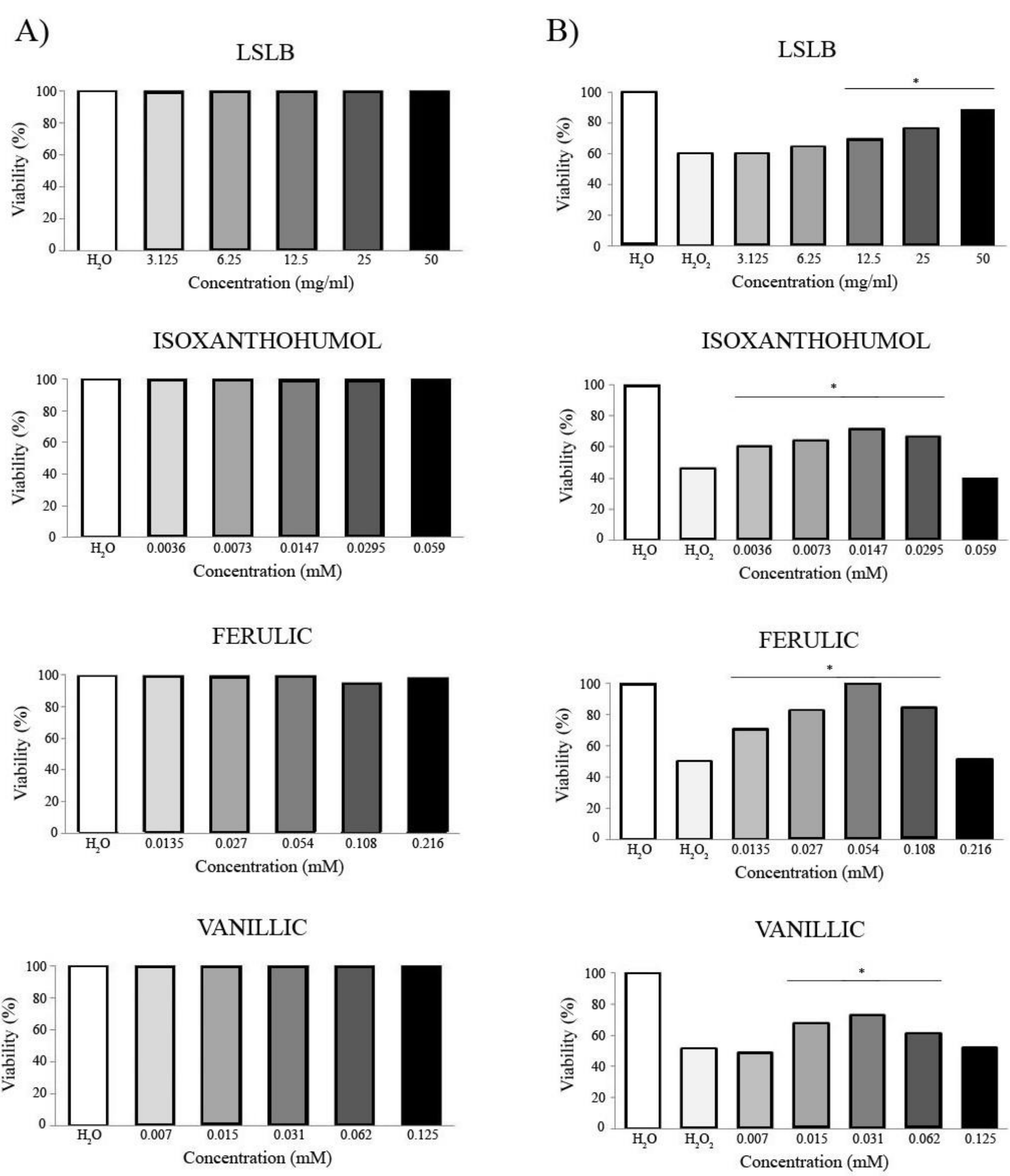
3.1. *In vivo*

3.1.1. Toxicity and Antitoxicity

Toxicity of LSLB, IX, ferulic and vanillic has been assessed in the *Drosophila in vivo* model. Figure 1A shows the relative percentage of emerging adults after treating larvae with different concentrations of these substances. A non-significant survival rate compared with the control is shown at any of the concentrations tested, exhibiting a non-toxic effect on the *Drosophila* viability.

Figure 1

Toxicity (A) and antitoxicity (B) levels of lyophilised stout Lager beer (LSLB), isoxanthohumol, ferulic and vanillic in *Drosophila melanogaster.*



Data are expressed as percentage of surviving adults with respect to 300 untreated 72-hour-old larvae from three independent experiments treated with different concentrations of LSLB, isoxanthohumol, ferulic and vanillic (A) and combined treatment with 0.12 M H2O2 (B). *: significant differences with respect to the positive control. Chi-square value higher than 3.84.

The antitoxicity assays revealed the ability of the LSLB, IX, ferulic and vanillic to protect the individuals against an oxidative stress at the tested concentrations. Figure 1B shows that the positive control H_2O_2 is toxic at 0.12 M with an average survival rate of 52% with respect to the water control. LSLB showed a significant protection against the genotoxicant at three highest concentrations tested (12.5, 25 and 50 mg/ml). IX and ferulic showed a significant protective effect in all the concentrations tested, except at the highest one (0.059 mM and 0.216 mM, respectively). The combined treatments of vanillic and H_2O_2 showed significant protective effect in the three middle concentrations (0.015, 0.031 and 0.062 mM).

3.1.2. Genotoxicity and Antigenotoxicity

Table 2 shows the results of genotoxicity and antigenotoxicity assays in the SMART test. Negative control showed a frequency of mutations per wing of 0.157, which fall into the historical range for the wing spot test (Anter et al. 2011). The concentration of H_2O_2 used (0.12 M) has been demonstrated to exert a potent genotoxic effect capable to induce somatic mutations and mitotic recombination in *D. melanogaster* (Romero-Jimenez et al. 2005) at a rate of 0.388 spots/wing.

The results showed that LSLB, IX, ferulic and vanillic seem to have no genotoxic effect at the tested concentrations. The total mutation rates at the lowest and highest concentration of LSLB, IX, ferulic and humulone (0.068 and 0.214 spots/wing; 0.315 and 0.268 spots/wing; 0.375 and 0.309 spots/wing; 0.243 and 0.375 spots/wing, respectively) were not significantly higher than the negative control (0.157 spots/wing). Moreover, LSLB, IX and ferulic were able to inhibit the genotoxic activity of H_2O_2 with an inhibition percentage of 48.45–47.42, 56.89–48.45 and 38.91–35.56 at the lowest and highest tested concentrations, respectively. Vanillic showed no significant differences with respect to the positive control exhibiting non antigenotoxic effects at highest concentration (0.125 mM) and a weak effect at the lowest concentration with an inhibition percentage of 3.35 at the lowest concentration tested (0.007 mM).

Table 2

Genotoxicity and antigenotoxicity of lyophilised stout Lager beer (LSLB), isoxanthohumol, ferulic and vanillic in the *Drosophila* wing spot test.

Compound	Number of wings	Clones per wing (nº spots) [1] Small single clones (1-2 cells) m = 2	Large simple clones (more than 2 cells) m = 5	Twin clones m = 5	Total clones m = 2	Mann Whitney U-test [2]	Inhibition percentage (%) [3]
H_2O	38	0.157 (6)	0	0	0.157 (6)		
H_2O_2	36	0.305 (11)	0.083 (3)	0	0.388 (14) +		
SIMPLE TREATMENT							
LSLB (mg/ml)							
3.125	44	0.068 (3)	0	0	0.068 (3) -		
50	42	0.143 (6)	0.071 (3)	0	0.214 (9) i	Δ	
Isoxanthohumol (mM)							
0.036	38	0.228 (8)	0.079 (3)	0.026 (1)	0.315 (12) i	Δ	
0.059	41	0.195 (8)	0.073 (3)	0	0.268 (11) i	Δ	
Ferulic (mM)							
0.0135	40	0.325 (13)	0.050 (2)	0	0.375 (15) i	Δ	
0.216	42	0.190 (8)	0.119 (5)	0	0.309 (13) i	Δ	
Vanillic (mM)							
0.007	37	0.081 (3)	0.162 (6)	0	0.243 (9) i	Δ	
0.125	40	0.300 (12)	0.075 (3)	0	0.375 (15) i	Δ	
COMBINED TREATMENT WITH H_2O_2 (0.12 M)							
LSLB (mg/ml)							
3.125	40	0.100 (4)	0.100 (4)	0	0.200 (8) β		48.45
50	44	0.181 (8)	0.023 (1)	0	0.204 (9) β		47.42
Isoxanthohumol (mM)							
0.036	40	0.15 (6)	0.025 (1)	0	0.175 (7) β		56.89
0.059	40	0.175 (7)	0.025 (1)	0	0.200 (8) β		48.45
Ferulic (mM)							
0.0135	38	0.210 (8)	0.026 (1)	0	0.237 (9) β		38.91
0.216	40	0.200 (8)	0.050 (2)	0	0.250 (10) β		35.56
Vanillic (mM)							
0.007	40	0.325 (13)	0.025 (1)	0.025 (1)	0.375 (15) β		3.35
0.125	36	0.361 (13)	0.111 (4)	0	0.472 (17) λ	Δ	-21.65

[1] Statistical diagnosis according to Frei and Wurgler (1988). + (positive) - (negative) and i (inconclusive) versus negative control; β (significantly different) and λ (inconclusive) versus positive control. m: multiplication factor. Kastenbaum-Bowman Test without Bonferroni correction, probability levels α = β = 0.05. [2] Inconclusive results were resolved by Mann Whitney U-test. Delta marker (Δ) means no differences between the treatment and the concurrent control. [3] The inhibition percentages for the combined treatments were calculated from total spots per wing according to Abraham (1994).

3.1.3. Lifespan

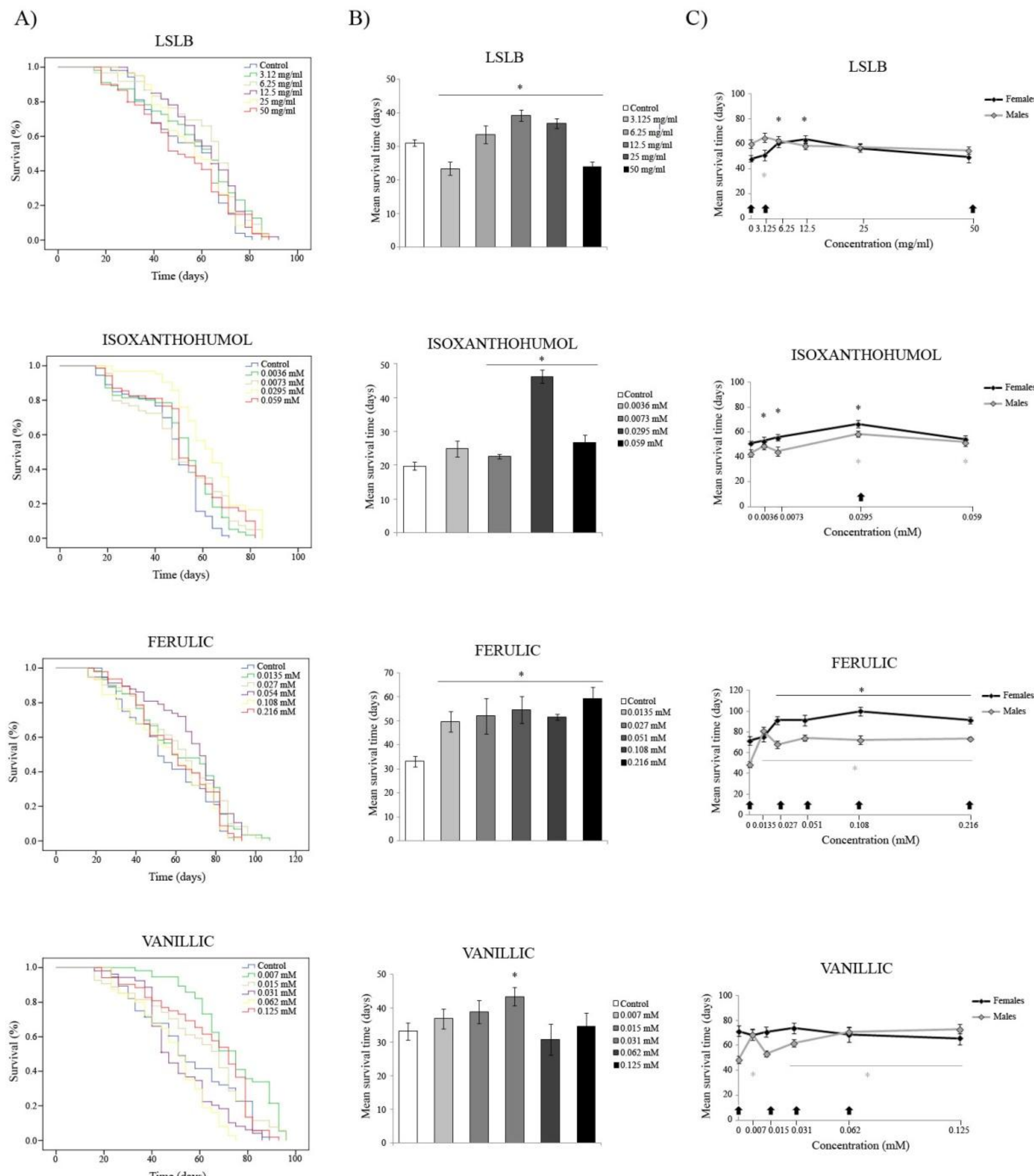
The entire lifespan curves obtained by the Kaplan-Meier method for each substance and concentration are shown in Figure 2A. Furthermore, the mean survival time of lifespan for each substance and concentration are shown in Table 3. LSLB showed a significant increase of life extension in *D. melanogaster* feed with concentrations of 3.125, 6.25 and 12.5 mg/ml, with respect to their concurrent control. IX induced an increasing of longevity in *Drosophila* in all the concentrations tested, with an average range of 49.81–3.14 days for the different assayed concentrations with respect to the control (47.24 days average). Ferulic only showed significant effects on lifespan at 0.027 and 0.054 mM with an extension of life in 6 and 11 days, respectively, with respect to the control. Finally, vanillic only was able to improve the longevity at the lowest concentration (0.007 mM); even more, the phenol induced a reduction of 6 days in the *Drosophila* life extension treated with the 0.062 mM concentration, compared with the control treatment.

3.1.4. Healthspan

The mean survival time of healthspan for each substance and concentration are shown in Figure 2B and Table 3. LSLB induced a significant improvement of healthspan in *D. melanogaster* compared to the control in the three middle concentrations assayed with an increase of 3, 9 and 6 days respectively, whereas the lowest and highest concentrations (3.125 and 50 mg/ml) showed a significant decrease of healthspan in this model organism. IX showed a significant improvement of healthspan at 0.0073, 0.0295 and 0.059 mM with an increase of 3, 27 and 7 days with respect to the control, respectively. Ferulic is the compound which exhibited the best results because of their significantly improvement of healthspan in *Drosophila* in all the assayed concentrations, with a ranges of 16–26 days in the quality of life. Vanillic only exhibited an improvement of healthspan at 0.031 mM, with respect to the control.

Figure 2

Survival parameters of *Drosophila melanogaster* fed with different concentrations of lyophilised stout Lager beer (LSLB), isoxanthohumol, ferulic and vanillic. A) Survival curves, B) Healthspan averages and C) Gender comparison.



A) Survival curves of *D. melanogaster*. B) Mean of survival time in highest 75% surviving population. * indicates significant differences ($p < 0.001$) with respect to the control. C) Significance between female and male curves with respect to their controls are symbolised by asterisk (*); significant survival values between females and males at the different treatments are symbolised by arrows (→).

Table 3

Mean and significances of lifespan, healthspan and lifespan curves by gender.

Compound	Concentration	Mean Lifespan [1] (days)		Mean Healthspan [1] (days)		Lifespan curves by gender (days)				
						Females [1]		Males [1]		Gender sig. [2]
LSLB	Control	54.375		30.983		48.19		60.32		0.026
	3.125 mg/ml	57.617	*	23.382	*	50.972	ns	65.126	*	0.001
	6.25 mg/ml	61.547	***	33.556	*	60.696	***	62.661	ns	0.536
	12.5 mg/ml	61.181	**	39.240	***	63.601	***	58.429	ns	0.387
	25 mg/ml	57.067	ns	36.800	***	56.433	ns	57.700	ns	0.499
	50 mg/ml	52.024	ns	24.067	***	49.500	ns	54.636	ns	0.041
Isoxanthohumol	Control	47.242		19.769		51.202		42.914		0.386
	0.0036 mM	51.064	*	24.882	ns	53.093	*	48.967	ns	0.392
	0.0073 mM	49.816	*	22.571	*	55.679	*	44.486	ns	0.150
	0.0295 mM	62.686	***	49.207	***	66.428	***	58.541	**	0.016
	0.059 mM	53.141	***	26.692	***	54.405	ns	52.000	*	0.967
Ferulic	Control	60.243		33.154		71.370		48.708		0.000
	0.0135 mM	78.094	***	49.700	**	75.308	ns	80.778	***	0.790
	0.027 mM	79.240	***	52.024	***	91.217	***	68.003	***	0.000
	0.054 mM	82.289	***	54.620	**	91.211	***	74.192	***	0.000
	0.108 mM	86.350	***	51.637	***	99.851	***	72.318	***	0.000
	0.216 mM	83.239	***	59.286	***	91.360	***	73.571	***	0.000
Vanillic	Control	60.243		33.154		71.370		48.708		0.000
	0.007 mM	68.454	ns	36.882	ns	68.389	ns	68.646	***	0.202
	0.015 mM	62.360	ns	38.918	ns	70.714	ns	53.326	ns	0.000
	0.031 mM	68.251	ns	43.500	***	73.824	ns	62.000	***	0.003
	0.062 mM	69.881	*	30.629	ns	68.593	ns	70.932	***	0.042
	0.125 mM	69.363	ns	34.633	ns	65.714	ns	73.005	***	0.765

Means were calculated by the Kaplan-Meier method and significance of the curves were determined by the Log-Rank method (Mantel-cox). [1] ns: non-significant ($p > 0.05$), *: significant ($p < 0.05$), **: highly significant ($p < 0.01$), ***: very highly significant ($p < 0.001$). [2] Significant survival values between females and males caused by the different assayed concentrations symbolised by arrow in Figure 2C.

3.1.5. Gender comparison

Figure 2C showed the effects that the different concentrations of LSLB, IX, ferulic and vanillic caused on the survival time curves for female and male individuals.

In general, females individuals lifespan was the most benefited in the different treatments in relation to male individuals assays lifespan. From a total of 23 concentration assayed, females lived significantly more than males in 11 concentrations, lived the same time than male in 10 ones and lived less time in only 2 concentrations (symbolized by arrows in Figure 2C, ↑).

Analysing the lifespan behaviour of gender with respect to their concurrent controls (symbolized by asterisks in Figure 2C, *), LSLB showed significant results in the 6.25 and 12.5 mg/ml concentrations for females and in the 3.125 mg concentration for males,

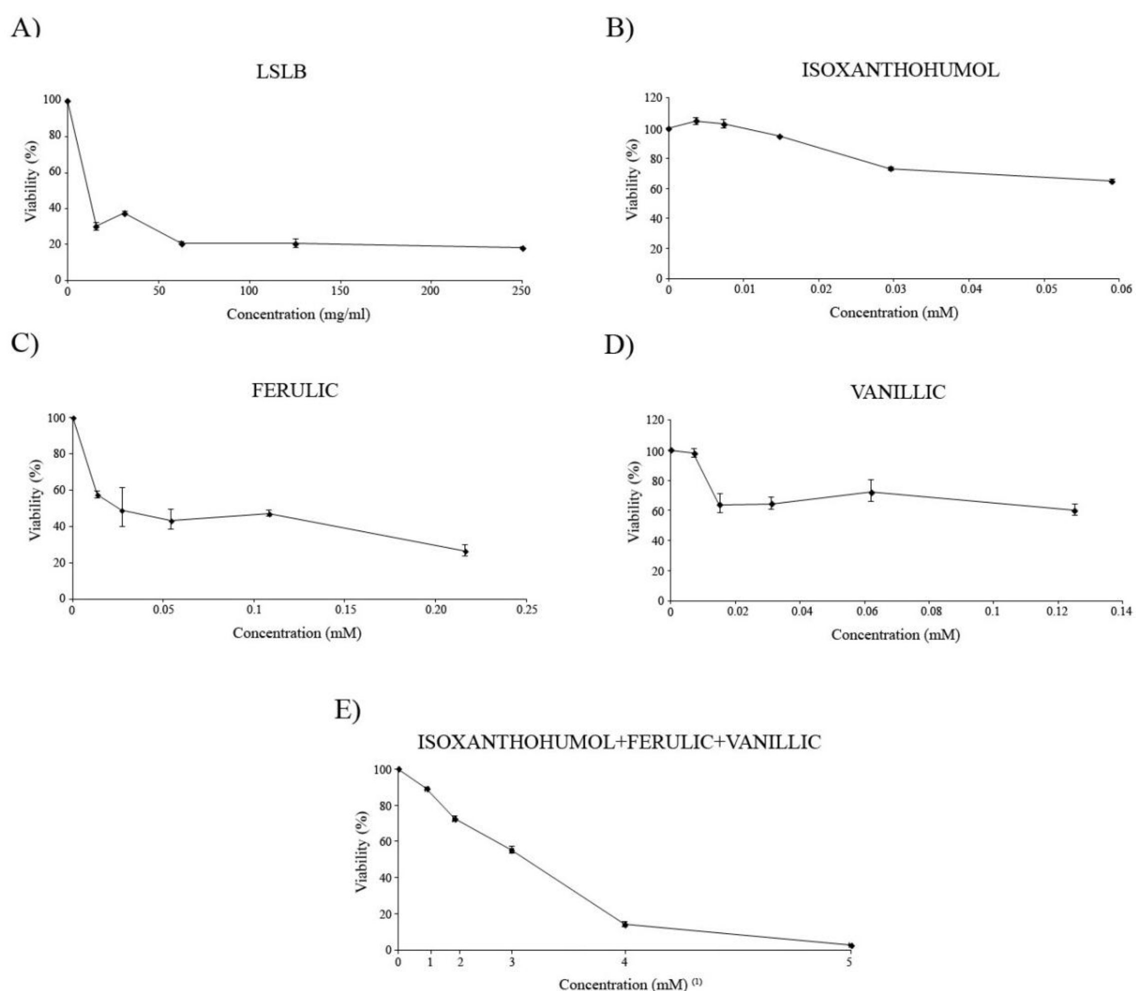
with an increase of *Drosophila* lifespan of 12, 13 and 5 days, respectively, with respect to their concurrent control. IX induced a significant increase of survival time at all concentration tested in females, except in the highest one (0.059 mM), with an average of survival increasing between 3–16 days, with respect to the control. Furthermore, IX showed a significantly increase of *Drosophila* lifespan of males at two highest concentrations (0.0295 and 0.059 mM), with an increasing of mean survival time in 10 and 16 days respectively, with respect to the control. Ferulic showed a significant extension of lifespan in all the concentrations assayed, except in the lowest one (0.0135 mM) for females. Ferulic induced an increasing of *Drosophila* lifespan ranged between 20–28 days for females and 20–32 days, with respect to their concurrent controls. Treatments with vanillic showed a significant increase of *Drosophila* lifespan at all tested concentrations of males, except at the second-lowest one (0.015 mM), with an increasing of life ranged between 5–25 days with respect to the control.

3.2. *In vitro*

3.2.1. Cytotoxicity

All substances assayed showed cytotoxic activity against HL-60 cells (Figure 3). LSLB and ferulic showed an increase of the cytotoxicity level according to increased concentration tested of the compound, being the inhibitory concentration 50 (IC_{50}) lower than 15.125 mg/ml and 0.027 mM, respectively. Cytotoxicity curve of IX and vanillic showed a tendency to decrease cells growing, although these compounds were no able to reach the IC_{50} value at any of the tested concentrations. Finally, the cytotoxicity of the three phenols combined exerted a synergistic effect with an inhibition of cells growing reaching the IC_{50} value at the medium concentration tested (0.0156, 0.054 and 0.031 mM of IX, ferulic and vanillic, respectively).

Figure 3
Effect of lyophilised stout Lager beer (LSLB), isoxanthohumol, ferulic and vanillic on cell viability.



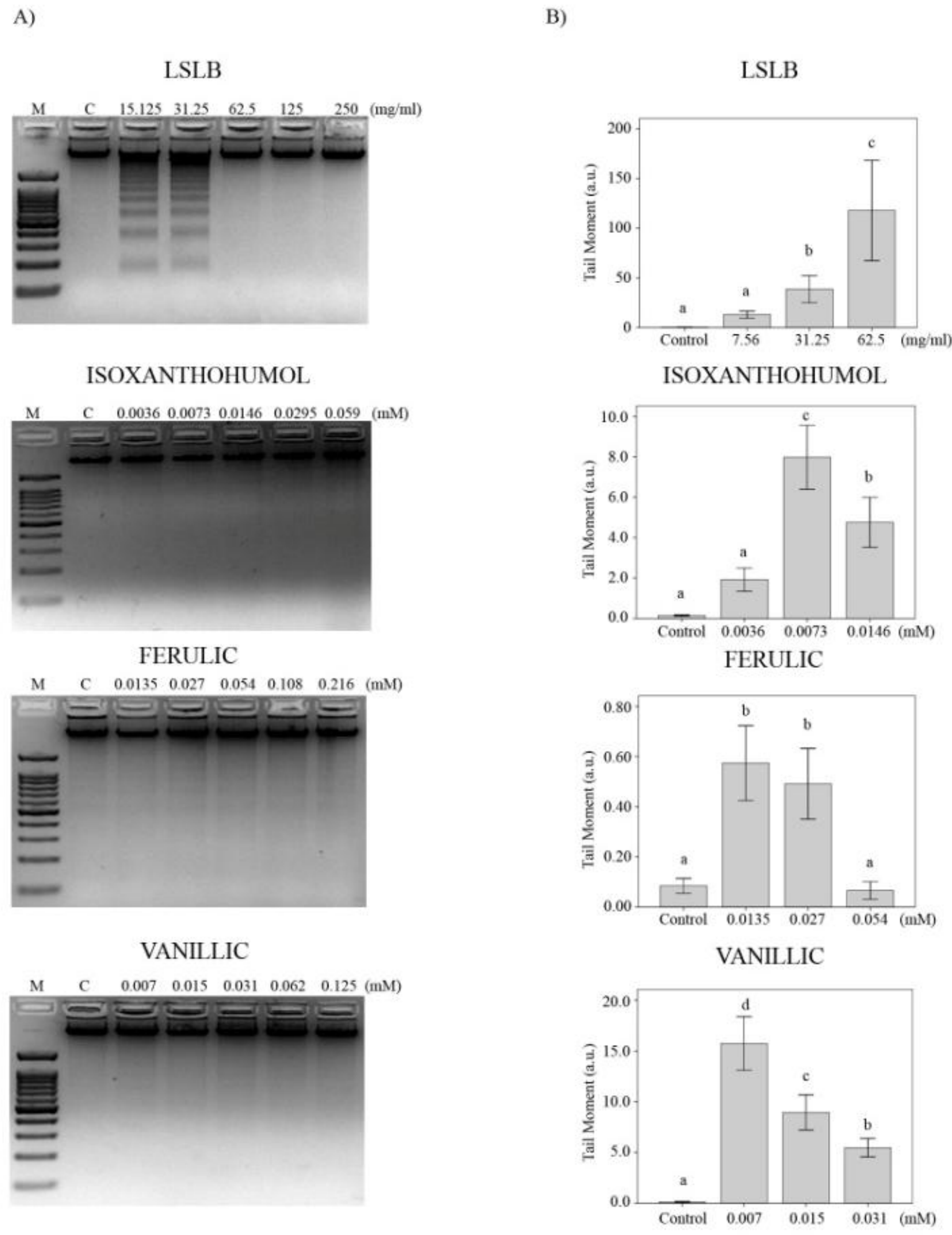
Viability in promyelocytic human leukaemia cells (HL-60) treated with different concentrations of LSLB (A), isoxanthohumol (B), ferulic (C), vanillic (D) and mixture treatment of isoxanthohumol+ferulic+vanillic (E) for 72 h. Each point represents the growing percentage with respect to its control. Values are mean +/- SE from three independent experiments. [(1)] value 1, 2, 3, 4 and 5 correspond to the lowest, three intermediates and highest concentrations respectively assayed for each single compound once their mixture is assayed.

3.2.2. DNA fragmentation

Figure 4A shows the electrophoresis of the genomic integrity in HL-60 cells treated with different concentrations of LSLB, IX, ferulic and vanillic. DNA internucleosomal fragmentation is represented by a DNA laddering and it is associated to the activation of the apoptotic way in cancer cells. Fragmentation was observed in the two lowest concentration of LSLB (15.625 and 31.25 mg/ml). IX, ferulic and vanillic did not induce internucleosomal fragmentation at any assayed concentrations.

Figure 4

DNA-induced damage in promyelocytic HL-60 cells treated with different concentrations of lyophilised stout Lager beer (LSLB), isoxanthohumol, ferulic and vanillic for 5 h. A) Internucleosomal DNA fragmentation. B) DNA strand breaks induction.



M indicates DNA size marker, C indicates Control treatment. B) Alkaline comet assay (pH < 13) of HL-60 cells treated. DNA migration is reported as mean TM. Values are mean +/- SE. Different letters in treatments mean differences respect to the negative control after one-way ANOVA and post hoc Tukey's test.

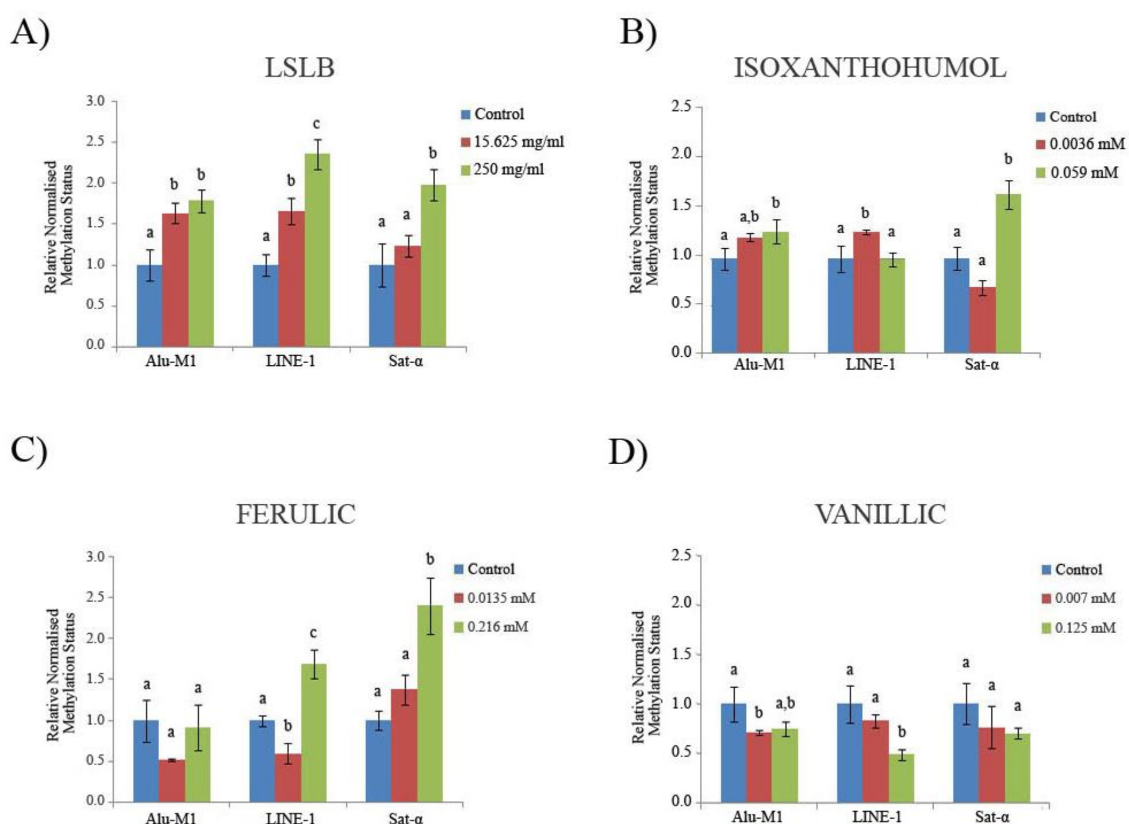
3.2.3. Comet assay

Results of the tail moment (TM) of HL-60 cells treated with different concentrations of LSLB, IX, ferulic and vanillic are shown in Figure 4B. A positive dose-dependent are observed in LSLB treatments with a significant double and/or the single strands breaks in the two highest concentrations assayed (31.25 and 62.5 mg/ml). In the IX treatments, the 0.0073 and 0.0146 mM concentrations showed significant TM with respect to the control. Furthermore, ferulic and vanillic exhibited a negative dose-dependent effects in the comet assay showing significant strands breaks at highest concentrations for ferulic and in all the tested concentrations for vanillic (0.0135 and 0.027 mM, and 0.007, 0.015 and 0.031 mM, respectively).

3.2.4. Methylation status

The relative normalized expression of the three primers (Alu-M1, LINE-1 and Sat-α) studied in HL-60 cells treated with different concentrations of LSLB, IX, ferulic and vanillic are shown in Figure 5. LSLB showed a significant hypermethylated status in both assayed concentrations (15.625 and 250 mg/ml), with respect to the control. IX showed a significant hypermethylation in 0.059 mM for Alu M1 and Sat- α sequences, and at 0.0036 mM for LINE-1 repetitive sequence, with respect to their concurrent controls. Ferulic showed a significant hypermethylation status at highest concentration (0.216 mM) in the LINE-1 and Sat-α repetitive sequences. Furthermore, at lower concentration tested (0.0135 mM), ferulic showed a significant hypomethylation activity in the LINE-1 sequences, with respect to the concurrent control. On the other hand, vanillic showed a general tendency to demethylate leukaemia cells with a significant hypomethylation at 0.007 and 0.125 mM for Alu M1 and LINE-1 sequences, respectively, with respect to their controls.

Figure 5
Relative normalised expression data of each repetitive element.



Relative normalised expression data of each repetitive element (Alu-M1, LINE-1 and Sat-α). Different letters mean different values after one-way ANOVA and post hoc Tukey's test.

4. DISCUSSION

Beer is one of the oldest beverages in the world and provides a valuable addition to the diet due to its components that confer healthy properties to the drink. Among all the ingredients of the diets, we focus our study on a stout Lager beer and three of its distinctive compounds in order to understand its effect on health.

Our toxicity results showed a significant non-toxic effect of LSLB, IX, ferulic and vanillic on the *Drosophila* viability. Moreover, all compounds showed a significant protection against the genotoxicant at different concentrations. This results are in agreement with previous experiences of toxicity in *Drosophila melanogaster* treated with H_2O_2 (Romero-Jimenez et al. 2005) and with blond beer (Merinas-Amo et al. 2016). According to Gorjanovic et al. (2010), the possibility to use peroxide as uniform equivalent to evaluate the antioxidative capacity of beer as an equivalent based on decrease of H_2O_2 anodic limiting current can serve as criterion of antioxidant activity.
Studies with coloured final products of the Maillard reaction which are formed non-enzymatically during the roasting of malt, indicate peroxyl radical scavenging potential (Liégeois, Lermusieau, & Collin, 2000). The formation of the Maillard intermediate, is clearly correlated with the alcohol content and colour. Vanderhaegen et al. (2004),

confirmed that levels of the Maillard intermediate are higher in beers with higher alcohol content and darker colour. The malts used for dark beers are produced by heating germinated barley at higher temperatures, which results in the formation of more Maillard intermediates (Coghe et al. 2004). This fact could be the responsible of the stronger antitoxic potential of LSLB at highest concentrations. Moreover, our results are in agreement with the antioxidant ability of blond and stout Ale beers found by Tafulo, Queirós, Delerue-Matos, and Sales (2010) and Granato, Branco, Faria, and Cruz (2011).

Taking into account the phenols studied, researches confirm the antioxidant effect showed by isoxanthohumol, ferulic acid and vanillic as they act as scavenger of free radicals (Cuvelier et al. 1992; Nardini et al. 2006; Sánchez-Moreno et al. 1998; Srinivasan et al. 2007; Żołnierczyk et al. 2015). Therefore, and due to their polyphenols content, beers could be a source of bioactive compounds with suitable free radical scavenging properties.

Results showed a non genotoxic effect of LSLB, IX, ferulic and vanillic at the tested concentrations. Moreover, LSLB, IX and ferulic were able to inhibit the genotoxic activity of H_2O_2. Vanillic exhibited non antigenotoxic effects at highest concentration (0.125 mM) and a weak effect at the lowest concentration tested (0.007 mM). The antioxidant activity of the phenols was related to several biological activities, including antigenotoxicity (Anter et al. 2010). An evaluation of the antigenotoxic potential of beer components against carcinogens contained in the human diet, namely heterocyclic amines (HCAs) was determined by Arimoto-Kobayashi et al. (2006). Their results suggested that beer components act in a protective capacity against the genotoxic effects of heterocyclic amines *in vivo*. As we mentioned above, its antigenotoxic activity it could be due to the scavenging activity shown (Romero-Jimenez et al. 2005). Furthermore, typical flavours has been associated with the type of malt used and the malting conditions (Coghe et al. 2004), which could be the main sources of the antigenotoxic effects of beer, specially for IX which is the most abundant phenol in stout beers (Gerhäuser 2005). Our results agree with similar genotoxicity studies on Lager beer (Merinas-Amo et al. 2016).

Longevity is a trait influenced by many factors, including gender, genetic variation, environmental influences, diet, lifestyle, access to health care and cultural influences. *Drosophila* revealed as an excellent system to investigate longevity-promoting properties of compounds and nutraceutical extracts as adults seem to show many of the cell senescence features seen in mammals (Fleming et al. 1992). It is well known that caloric restriction without malnutrition is a key strategy for increasing the lifespan (Guarente and Kenyon 2000). Nevertheless, there is available data on lifespan increase by adding nutraceutical substances to the *Drosophila* diet with no reduction of the total intake (Deshpande et al. 2014).

Studies with blond Lager beer affirm the healthy properties of beer improving the longevity of *Drosophila* up to 17 days with respect to the control (Merinas-Amo et al. 2016). All the substances did not necessarily increase the lifespan of *Drosophila*. Assays with borage oil were carried out in order to known its healthy effects, showed positives effects. In contrast, the gamma-linolenic acid, which is one of the most important borage oil bioactive components, did not show theses effects (Tasset-Cuevas et al., 2013). This pattern is similar to that we observed in the LSAB and humulone assays (chapter 2 of the present work): the food (complex mixture) exhibits positives effects whereas a single compound does not show the same effect. On the other hand, our results on gender comparisons agree with Fenech et al. (1994), who affirm that, where the heterogametic sex is male as in human and *Drosophila*, female individuals live more than males ones. However, no previous research has been found to could compare our positive results of longevity, healthspan and gender comparisons on *Drosophila* fed with LSLB, IX, ferulic and vanillic acids. For that reason, it is important to deal with these results with caution and make additional assays with other organisms.

There are conflicting reports concerning the relationship between beer consumption and cancer risk. Riboli et al. (1991) have reported that beer consumption is not associated with colon cancer, but Kato et al. (1990) have shown that beer drinkers have an increased risk to colorectal cancer. An inverse association between moderate beer consumption and endometrial cancer was suggested by Swanson et al. (1993). However, a connection between beer consumption and lung cancer was suggested by Potter et al. (1992). Others epidemiological studies indicate the anti-proliferative activity of beer components with antioxidant properties in prostate cancer cells, showing a strong correlation between antioxidant potency and polyphenols content (Hevia et al. 2008).
Moreover, phenolic acids are said to posses physiological and pharmacological functions. IX has been demonstrated to have potential cancer chemopreventive properties (Gerhäuser 2005). Cuvelier et al. (1992) and Sánchez-Moreno et al. (1998) described the anticancer effect of ferulic acid *in vitro*. Vanillic acid is evaluated as suppressor of snake venom (Dhananjaya et al. 2006) and carcinogenesis (Vetrano et al. 2005). Our cytotoxic results of LSLB, IX, ferulic and vanillic acids, are based on the chemopreventive ones. Moreover, it should be pointed the higher cytotoxic effect that three combined phenols showed on the cells growing than the effect of each phenols by a single way. This fact support the idea that full drink is resulted for the combination of the different compounds.

To our knowledge, any study with LSLB was carried out in order to analyse the DNA fragmentation patter that this beer cause in the cancer cells. Studies with a blond Lager beer showed a fragmentation effect in the two lowest concentrations of the beer (15.625 and 31.25 mg/ml) and slightly in the intermediate concentration (62.5 mg/ml) (Merinas-Amo et al. 2016), being our results in agreement with the previous one.
All our studied phenols showed a non-activation of the apoptosis way when tumoral cells where treated with them. The results agree with different researches with the phenols in a wide range of assays. Yang et al. (2007) demonstrated that IX induced

apoptosis when mature 3T3-L1 adipocytes where treated at concentrations of 75–100 μM. Our results agree with that results due to the highest concentration used in our assays was 0.059 mM. Ferulic acid is related with antiapopototic effects through reducing oxidative stress (Picone et al. 2009), regulating MAPK pathways (Jin et al. 2007) and inhibiting abnormal expression of apoptotic proteins (Balakrishnan et al. 2010). Vanillic acid is associated with a suppression of Neuro-2A cells apoptosis (Huang et al. 2008).

By using the comet assay we were able to readily identify apoptotic nuclei based on the characteristic morphological changes attributable to extensive DNA damage (Poe and O'Neill 1997). According to Fabiani et al. (2012), DNA damage can be divided into five classed depending on the TM values as follows: class 0 (TM < 1; no damage), class 1 (TM 1–5; slightly damaged), class 2 (TM 5–10; medium damage), class 3 (TM 10–20; highly damaged) class 4 (TM > 20; completely damaged). Based on this classification, all concurrent control values fell into class 0 (TM < 1); LBAB 31.25 and 62.25 mg/ml induced a completely damage in HL-60 cells (TM > 20); IX induced a slight damage at lowest concentration and a medium damage at two highest tested concentrations (TM 5–10 and TM 10–20, respectively); ferulic did not cause damage at any concentration felling into class 0 (TM < 1); vanillic acid induced highly damage at the lowest and medium damage at two highest concentrations with TM values between 6–15.

Moreover, the results of DNA fragmentation fit with those obtained in comet assay since TM values higher than 30 mean that apoptosis mechanisms has been induced (Fairbairn et al. 1995). Only the highest concentration of LSLB showed TM > 30 confirming the death of leukaemia cells by apoptosis. The rest of compounds and concentrations tested induce cell death by a necrosis mechanism.

Most of the DNA methylation in humans occurs in the cytosine of CpG dinucleotides (CpG islands). In a normal cell, they are unmethylated, and the gene is expressed if the required transcription factors are present (Esteller 2005). Due to the effects that methylation causes in the repetitive sequences of the genome, we could said that LSLB, IX at lowest concentration for LINE sequences and highest concentrations for Alu and Satellite sequences, and highest concentrations of ferulic for LINE and centromeric areas (Sat-α) prevents for DNA epigenetic damages as a methylation of repetitive sequences is understood as a genomic protective mechanism (Roman-Gomez et al. 2008; Weisenberger et al. 2005). These effects are not shown in vanillic acid assays. Besides of that and bearing in mind the idea of tumoral cells demethylation, it would indicate that beers and its compounds act as preventive agents cutting off the repetitive sequences of tumoral cells (Roman-Gomez et al. 2008).
Unfortunately, there are not previous studies about methylation status with stout beers, IX, ferulic and vanillic acids. Merinas-Amo et al. (2016) demonstrated that a lyophilised blond Lager beer induce hypermethylation in the DNA of tumoral cells in a wide range of repetitive sequences.

5. CONCLUSIONS

The obtained results let us to propose lyophilised stout Lager beer as a potential nutraceutic beverage due to their are able to prevent toxicity and to avoid genetic oxidative damage in the *D. melanogaster* eukaryotic model; and also has the ability to induce tumour cells death in a programmed manner and to modulate the methylation status of HL-60 leukaemia cells.

6. REFERENCES

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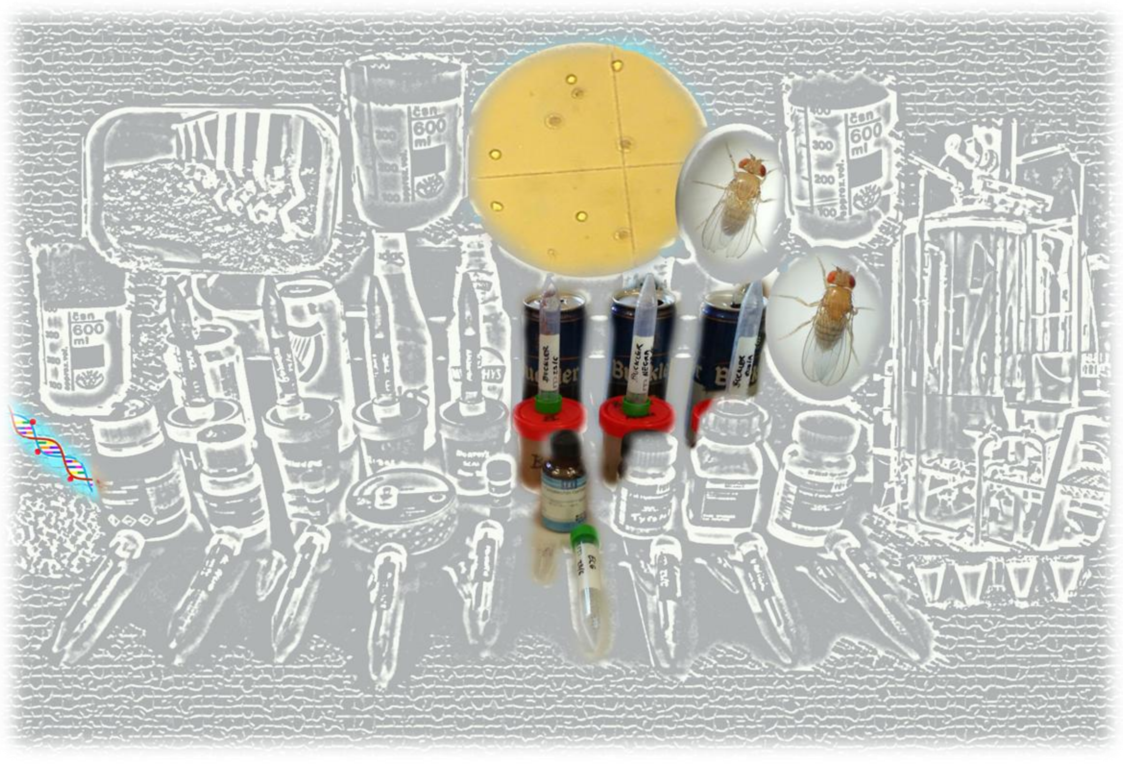
CHAPTER 4



Safety and protecting activities of alcohol-free beers

ABSTRACT
Nowadays, a general interest for improving health in order to get better conditions of life is increasing. Diet is one of the most important lifestyle that can modify health conditions. In the present study, we analyse the biological activities of three types of alcohol-free Lager beer (a blond, a pale-blond and a stout beer) as well as epicatechin gallate (ECG) as one of their most abundant phenol with the aim to propose them as a nutraceutic. For that purpose, we carried out safety assays followed by studies on the possible protective effects of the tested substances in the *Drosophila* animal model. Moreover, chemopreventive studies in the *in vitro* model of human leukaemia cell line (HL-60) were carried out to evaluate the cytotoxic potential and DNA damage that our tested compounds induced in the tumour cells. Results suggested the safety properties of all compounds, although pale-blond and stout beer showed a genotoxic activity only at the lowest concentrations assayed. Moreover, alcohol-free beers and phenol were able to protect against H_2O_2 oxidative damage as well as to induce an increase of longevity with an improvement of quality of life in the *in vivo* animal model assayed. Promising results were obtained as alcohol-free beers and ECG in the *in vitro* assays with human leukaemia cells as they were able to inhibit tumour cells growth, to induce DNA damage and to modify the methylation status of such a cancer cell line. To sum up, alcohol-free beers could be considered not only cause their interest as reduced-calories beer and isotonic properties, but they could also be recognised as nutraceutic substances.

1. INTRODUCTION

Beer is one of the oldest known beverages, which is not only considered as a beverage but it also provides a valuable added value to the diet. Beer consumption has been associated with a wide range of beneficial health effects like reduction of the cardiovascular disease, decrease of kidney stones formation, chemopreventive activity, antioxidant capacity and physiological effects, among others (Bassus et al. 2004; Chen et al. 2013; Muller et al. 2007; Piazzon et al. 2010). Moreover, it can be considered as a nutritional supplement with many bioactive molecules, composed of readily available starches and sugars, various minerals depending on the grain used and the soil, and valuable B vitamins (Hornsey 2003). About 20–30% of beer polyphenols originate from hops, whereas 70–80% is malt derived (Gerhäuser 2005).

Numerous proanthocyanidins, phenolic compounds formed from single monomer units like the (-)-epicatechin, are present in beer. Up to 30% of the proanthocyanidins present in beer are derived from hops (e.g. epicatechin gallate); the rest are derived from the malt (Gerhäuser and Becker 2009; Stevens et al. 2002). ECG [(-)-epicatechin gallate] is a 3-gallate ester form of (-)-epicatechin that, like other phenols, occurs in beer in relatively high concentrations (5–20 mg/l) (Gerhäuser and Becker 2009). Many studies support the health benefits of ECG due to its antioxidant properties and contribution to the reduced risk of cancer, obesity, and cardiovascular disease as well as to its anti-hypertensive effect, antibacterial and antiviral activity, ultraviolet protection to skin, and neuroprotective power, among others (Zaveri 2006). Furthermore, Bartolomé et al. (2000) and McMurrough and Baert (1994) recorded the presence of (-)-epicatechin in alcohol-free beers.

Despite of the wide variety of styles of beer, they generally fall into two classes: Lager and Ale beer. The two different categories of beer differ to each other in the type of yeast and the temperature used during the brewing process. Lager beers use special yeast that ferments best at cooler temperatures, whereas Ale beers use another type of yeast that ferments best at warmer temperatures (Granato et al. 2011). Moreover, beers can exhibit colours ranging from pale to black due to the colour of malt(s) used and the caramelisation of sugars and Maillard-type reactions (De Keukeleire 2000; Kodama et al. 2006). Beers brewed with dark malts are often considered to have a longer shelf life than pale beers (Cantrell and Griggs 1996). Dark malts differ from pale malts in their content of coloured Maillard or caramelisation products formed during the roasting of malt by reactions involving proteins and carbohydrates (Woffenden et al. 2001). The overall effects of Maillard and caramelisation products on the oxidative stability of beer are unknown although positive correlations between antioxidant activity and malt colour have been observed due to the phenols compositions and the presence of Maillard components (Coghe et al. 2003; Woffenden et al. 2001).

Recently, a worldwide interest in alcohol-free (AFB) and low-alcohol beers (LAB) is increasing. AFB and LAB are known to have a weaker aroma than standard beers (Brányik et al. 2012). Legally fixed maximum alcohol contents differ among countries,

but AFB and LAB are considered to content below 0.5% and no more that 1.2% alcohol per volume respectively (Brányik et al. 2012). Athletes appreciate beer's typical properties, reduced calories, and the isotonic properties of most alcohol-free beers.

Two procedures have been established for manufacturing non-alcoholic beer: (a) physical methods based on removing the alcohol content (dealcoholisation by vacuum rectification and evaporation, dialysis, reverse osmosis, membrane extraction, CO_2 extraction, pervaporation, adsorption or freeze), and (b) biological processes based on limited alcohol formation by using special yeast (Anglerot 1994; Brányik et al. 2012; Etuk and Murray 1991; Matson 1988; Von Hodenberg 1991). The most used yeast for alcohol-free beer production is *Saccharomycodes ludwigii*. It has the capacity to control the fermentation process due to its disability to ferment maltose and maltotriose, the prevailing fermentable sugars of all malt worts. Moreover, the fermentation of *S. ludwigii* is characterised by slow attenuation even at 20 °C which implies that the process does not require continuous monitoring (Brányik et al. 2012).

The available literature on comparisons of processes and their impact on the product quality and the economic aspects of brewing of LABs or AFBs is scarce. Nevertheless, it is clear that the arrested/limited fermentation process can be performed in a common brewery equipment and shorter production time and fewer raw materials are needed. Therefore, the production costs for such LAB/AFBs are the same or lower, than for the regular beer. Conversely, processes of the alcohol removal do require an extra equipment, relevant utilities and space, which mean additional investments and operating costs above the production costs of the regular beer to be dealcoholised. The advantage of alcohol separation processes is their flexibility (start-up within hours, high productivity) and possibility to produce zero alcohol beers, which is hardly achievable by fermentative processes, given by their nature (Brányik et al. 2012).

The composition and levels of phenolic compounds in beer vary strongly in dependence of the raw materials utilized, the brewing process and type of beer, and also change during storage. Alcohol-free beer usually has lower levels of phenolic constituents due to the differences in the duration of fermentation and the yeast strains employed in their brewing and to losses during the dealcoholization process (Bartolomé et al. 2000; Gerhäuser and Becker 2009).

The aim of the present study is to evaluate the safety and protective and chemopreventive activities of three types of alcohol-free Lager beers (a blond, a pale-blond and a stout beer-variety) and the common and most abundant phenol in all of them (ECG). For that purpose, the use of safety assays followed by studies on the protective effects that the tested compounds shown in the *Drosophila* animal model were carried out. Moreover, chemopreventive studies in the *in vitro* model of human leukaemia cell line (HL-60) were carried out to evaluate de cytotoxic potential and DNA damage that our tested compounds induced in the tumour cells.

2. MATERIALS AND METHODS

2.1. Samples preparation and simple compounds

A blond alcohol-free Lager beer (Bucker 0,0®) (LBAFLB), a pale-blond alcohol-free Lager beer (Pale Buckler 0,0®)(LPBAFLB) and a stout alcohol-free Lager beer (Stout Buckler 0,0®) (LSAFLB) were selected for this study. All beer are characterised by an alcohol volume ≤ 0.04% and the use of special yeast (*S. ludwigii*) during their brewing. It should be noticed that LPBAFLB is characterised by the addition of food additives like the E-414 stabiliser and the E-330 acidulant. Furthermore, epicatechin gallate (ECG) (TCI) was tested as single bioactive compound.

Beers samples were lyophilised before to carry out the assays (SCAI, University of Córdoba) and stored until use at room temperature in a dark and dried atmosphere. Lyophilised beers were dissolved in distilled water in order to obtain the different concentrations to be tested.

The concentrations tested of beers were established taking into account the average daily food intake of *Drosophila* (1 mg/day) and the average body weight of *D. melanogaster* individuals (1 mg) (Ja et al. 2007). Moreover, the concentration range was calculated in order to make it comparable to the beer daily intake for humans (192 ml of total beer/day for 70 kg human body weight) (Kirin 2012). Furthermore, the assayed concentrations for ECG are the equivalent to those contained in the full beer.

2.2. *In vivo* and *in vitro* model organisms used

2.2.1. *Drosophila melanogaster*

The fruit fly *D. melanogaster* is a well-known insect with long scientific history in biological sciences that has highly contributed to understanding the nuances of developmental biology, evolutionary concepts and, recently, to toxicology studies (Bhargav et al. 2008; Coulom and Birman 2004; Dean 1985; Hosamani 2013; Siddique et al. 2013). The unique characteristics that *Drosophila* possesses, such as a rapid and short life cycle (10–12 days at 25 °C), reliability, easy maintenance, propagation and manipulation, and cost efficient, make this dipteran an ideal model organism. Furthermore, *Drosophila* shows a consistent genetic similarity to humans, considering that approximately 75% of the genes involved in human diseases have related or similar sequences to *D. melanogaster* (Bier 2005; Lloyd and Taylor 2010).

Two *Drosophila* strains were used, each with a hair marker in the third chromosome:

- *mwh/mwh*, carrying the recessive mutation *mwh* (*multiple wing hairs*) that in homozygosis produces multiple tricomas per cell instead of one per cell (Yan et al. 2008).
- *flr*^{3}*/In (3LR) TM3, rip*^{p}*sep bx*^{34e}*e*^{s}*Bd*^{S}, where the *flr*^{3}(*flare*) marker is a homozygous recessive lethal mutation that produces deformed tricomas but is viable in homozygous somatic cells once larvae start the development (Ren et al. 2007).

2.2.2. HL-60 promyelocytic human leukaemia cells

The HL-60 cell line originated from a female patient with acute myeloid leukaemia (Collins 1987). The culture of peripheral blood leukocytes from this patient in conditioned medium resulted in the development of a growth-factor-independent immortal cell line with distinct myeloid characteristics (Gallagher et al. 1979). The promyelocytic human leukaemia cell line HL-60 proliferates in suspension culture with a doubling time of about 36 to 48 hours.

Cells were grown in RPMI-1640 medium (Sigma, R5886) supplemented with heat-inactivated foetal bovine serum (Linus, S01805), L-glutamine at 200 mM (Sigma, G7513) and antibiotic-antimycotic solution (Sigma, A5955). Cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 (Gallagher et al. 1979). The cultures were plated at 2.5 x 10^4 cells/ml density in 10 ml culture bottles and replaced three times per week.

2.3. Safety *in vivo* assays

3.3.1 Toxicity

The percentage of the number of individuals born in each treatment with respect to the number of individuals born in the negative control were analysed at the following ranks of concentrations for the different beers: 3.125–50 mg/ml. For ECG the concentrations used were those equivalents to the quantity of each compound in the full beer (0.0048–0.079 mM). The negative controls were prepared with medium and distilled water.

Chi-square test in a Microsoft Office Excel 2007 was used to determine if the tested compounds significantly affected the survival of flies. Negative control values were considered as those expected in Chi-square formula used in toxicity assay.

2.3.2 Genotoxicity

The genotoxicity assays were carried out following the method described by Graf et al. (1984). The treatment tubes contained 0.85 g of *Drosophila* Instant Medium (Formula 4-24, Carolina Biological Supply, Burlington, NC) and 4 ml of different concentrations of the substance to be tested (3.125 and 50 mg/ml of beers; 0.0048 and 0.079 mM of ECG). After emergence, adult flies were stored in 70% ethanol until the wings are mounted to the scrutiny of the mutations.

Wings of emerging trans-heterozygous individuals ($mwh\ flr^+/mwh^+\ flr^3$) for each control and concentration were mounted on slides using Faure's solution and scored under a photonic microscope at 400x magnification. Wing hair spots were grouped into three different categories: small single spots corresponding to one or two cells exhibiting the *mwh* phenotype that occur in the late stages of the mitotic division; large single spots with three or more cells showing *mwh* or flr^3 phenotypes that occur in the early stages of larval development; and twin spots corresponding to two juxtapositioning clones, one showing the *mwh* phenotype and other the flr^3phenotype. Small and large spots are originated by somatic point mutation, chromosome aberration,

and somatic recombination, while twin spots are produced exclusively by somatic recombination between the *flr*^{3} locus and the centromere.

A total of 207 were mounted and analysed for the genotoxicity treatments. The total number of spots was also determined and a multiple-decision procedure was applied to determine whether a result is positive, inconclusive or negative (Frei and Wurgler 1988). The frequencies of each type of mutant clone per wing were compared to the concurrent control and analysed applying the binomial Kastenbaum and Bowman Test (Frei and Wurgler 1995), without Bonferroni correction and at 5% level of significance. All inconclusive and positive results were analysed with the nonparametric U-test of Mann, Whitney U-test ($\alpha = \beta = 0.05$).

In the balancer-heterozygous genotypes (*mwh/TM3, Bd*^{S}), *mwh* spot phenotypes are produced predominantly by somatic point mutation and chromosome aberrations, since mitotic recombination between the balancer chromosome and its structurally normal homologue is a lethal event. To quantify the recombinagenic potency of the positive treatments, the frequency of *mwh* clones in the marker trans-hetorozygous wings (*mwh* single spots plus twin spots) was compared with the frequency of *mwh* spots in the balancer trans-heterozygous wings. The difference in *mwh* clone frequency is a direct measure of the proportion of recombination (Frei et al. 1992).
A total of 40 wings for each positive concentration (3.125 gm/ml) of LPBAFLB and LSAFLB with balancer marker (*mwh/Bd*^{S}) at their single treatments were also mounted. In order to quantify the percentage of genotoxic activity due to somatic recombinogenic events (R) of the substance the following formula was used (Valadares et al. 2008):

R = [1 − (*mwh* spots on the balancer wings / *mwh* spots on the marker wings)] x 100

2.4. Protection *in vivo* tests
2.4.1 Antitoxicity
The antitoxicity tests consisted in the study of the viability percentage of *Drosophila* treated with the same concentrations as in toxicity assays combined with the genotoxicant H_2O_2 at 0.12 M (Tasset-Cuevas et al. 2013). The positive controls were prepared with medium and 0.12 M H_2O_2 as an oxidative genotoxicant.

Chi-square test in a Microsoft Office Excel 2007 was used to determine if the tested compounds significantly affected the survival of flies. Positive control values were considered as those expected in Chi-square formula used in antitoxicity assay.

2.4.2 Antigenotoxicity
The antigenotoxicity trials were performed following the method described by Graf et al. (1998), which consisted of combined treatments of genotoxin (0.12 M H_2O_2) and the same concentration used in genotoxicity assays (3.125 and 50 mg/ml of beers; 0.0048 and 0.079 mM of ECG). After emergence, adult flies were stored in 70% ethanol until the wings are mounted to the scrutiny of the mutations and the same protocol of

genotoxicity is followed for mounted and scored the wings. A total of 196 wings were mounted and analysed. The inhibition percentages (IP) for the combined treatments are calculated from total spots per wing with the following formula (Abraham 1994):

IP= [(single genotoxin - combined treatment) / single genotoxin] x100

2.4.3 Longevity

All experiments were carried out at 25 °C according to the procedure described in Tasset-Cuevas et al. (2013). Sets of 25 individuals of the same sex were selected and placed into sterile vials containing 0.21 g of *Drosophila* Instant Medium and 1 ml of different concentrations of solution of the compounds to be tested (3.125–50 mg/ml of beers and 0.0048–0.079 mM of ECG). Four replica were followed during the complete life extension for each control and concentration established. Alive animals were counted and the respective media renewed twice a week.

In order to know the quality of life of treated *Drosophila* in the longevity trials, the upper 25% of lifespan survival curves was studied. This part of the lifespan is considered as the healthspan of a curve, characterised by low and more or less constant age-specific mortality rate values (Soh et al. 2007).

The statistical treatment of survival data for each control and concentration was assessed with the SPSS Statistics 17.0 software (SPSS, Inc., Chicago), applying the Kaplan-Meier method. The significance of the curves was determined using the Log-Rank method (Mantel-Cox).

2.5. Chemopreventive *in vitro* tests

2.5.1. Cytotoxicity assay

HL-60 cells were placed in 96 well culture plates (2 x 10^4 cells/ml) and treated for 72 h with the different beers (7.81–250 mg/ml for LBAFLB; 0.488–250 mg/ml for LPBAFLB and LSAFLB) and the equivalent concentrations for the ECG (0.0048–0.079 mM). This wide range was selected in order to assess the cytotoxic doses ranging including the inhibitory concentration 50 (IC_{50}) when possible. This allows the assessment of a wide range of concentrations in the *in vitro* cytotoxicity assays, with the aim to predict acute *in vivo* lethality.

Cell viability was determined by the trypan blue dye exclusion test in a Neubauer chamber at 100X magnification (AE30/31, Motic). Curves were plotted as the average survival percentage of three independent experiments with respect to the control growing for 72 h.

2.5.2. Tumour cells DNA damage evaluation

2.5.2.1. DNA fragmentation

HL-60 cells (1 x 10^6/ml) were treated with different concentrations of blond and stout selected lyophilised beers (15.625–250 mg/ml) and its equivalent concentration of ECG (0.0048–0.79 mM) for 5 h.

Treated cells were collected and centrifuged at 3000 rpm for 5 min, and DNA was extracted according to the procedure described in Merinas-Amo et al. (2016). Briefly, cell pellet was resuspended in lysis buffer and incubated in SDS 10% and proteinase K solution. Steps of precipitation with NaCl and isopropanol were followed by a 70% ethanol DNA washing step, and finally a RNAse incubation step over night.
DNA was quantified in a spectrophotometer (Nanodrop ND-1000) and 1200 ng of DNA was subjected to a 2% agarose gel electrophoresis at 85 mA for 25 min, stained with bromide ethidium and visualized under UV light.

2.5.2.2. Comet assay

Cells were treated with different concentrations of lyophilised beers (15.625, 31.25 and 62.5 mg/ml) and its equivalent concentrations of ECG (0.0048, 0.0097 and 0.0195 mM) for 5 h.

After washing steps in PBS, a concentration of 6.25 x 10^5 cells/ml was mixed with 0.75% low melting point agarose (Sigma, A4018) and transferred to frosted-end slides. Steps of lysis, alkaline electrophoresis, neutralization and dried were carried out according to Mateo-Fernández et al. (2016) protocol. Finally, DNA of 50–100 single cells was visualized by treating slides with 7 µl of a 10 µg/ml stock solution of propidium iodide (Sigma, P4170). Comet images were analysed at 400x magnification using a Leica DM 2500 fluorescence microscope with green filter and an attached camera (JAI CV-M4CL). The OpenComet plugging from ImageJ (NIH) was used to score individual parameters for each cell.

Statistical analysis of data was performed using SPSS Statistic 17.0 software. The Tail Moment data were evaluated by a one-way ANOVA. The post hoc Tukey's test was applied to asses on the effect of the compounds on HL-60 cell DNA integrity. Statistical significance was considered at $p \leq 0.05$ was.

2.5.2.3 Methylation status

Genomic DNA was isolated in the same way as described in DNA fragmentation section. Bisulphite-modified DNA from beers (15.625 and 250 mg/ml) and ECG (0.0048 and 0.079 mM) treatments, using the EZ DNA Methylation-Gold Kit, was used as template for fluorescence-based real-time quantitative Methylation-Specific PCR (qMSP). A qMSP were carried out according to the protocol described by Merinas-Amo et al. (2016) in 48 well plates in MiniOpticon Real-Time PCR System (MJ Mini Personal Thermal Cycler, Bio-Rad) and was analysed by Bio-Rad CFX Manager 3.1 Software.

Repetitive elements were selected in order to analyse a wide range of human genomic DNA. While Alu and LINE sequences are interspersed throughout the genome, satellites are confined to the centromere areas (Deininger et al. 2003; Ehrlich 2002; Lee et al. 1997; Weiner 2002). All sequences had been obtained from Isogen Life Science. Alu M1, LINE-1 and Sat-α sequences were used (see Table 1 for detailed information (Weisenberger et al. 2005)).

The relative yielded results were normalised with the housekeeping sequence Alu C4 using the Nikolaidis et al. (2012) and Liloglou et al. (2014) comparative Ct method. Each sample was analysed in triplicate. One-way ANOVA and post hoc Tukey's tests were used to evaluate the differences among the tested compound, repetitive elements and concentrations.

Table 1

Primers information.

Reaction ID	GenBank number	Amplicon start	Amplicon end	Forward primer sequence 5' to 3' (N)	Reverse primer sequence 5' to 3' (N)	GC-content (%)	
						Forward	Reverse
ALU-C4	Consensus Sequence	1	98	GGTTAGGTATAGTGGTTTATATTTGTAATTTTAGTA (36)	ATTAACTAAACTAATCTTAAACTCCTAACCTCA (33)	25	27.3
ALU M1	Y07755	5059	5164	ATTATGTTAGTTAGGATGGTTTCGATTTT (29)	CAATCGACCGAACGCGA (17)	27.6	58.8
LINE-1	X52235	251	331	GGACGTATTTGGAAAATCGGG (21)	AATCTCGCGATACGCCGTT (19)	47.6	52.6
Sat-α	M38468	139	260	TGATGGAGTATTTTTAAAATATACGTTTTGTAGT (34)	AATTCTAAAAATATTCCTCTTCAATTACGTAAA (33)	23.5	21.2

Source from Weisenberger et al. (2005).

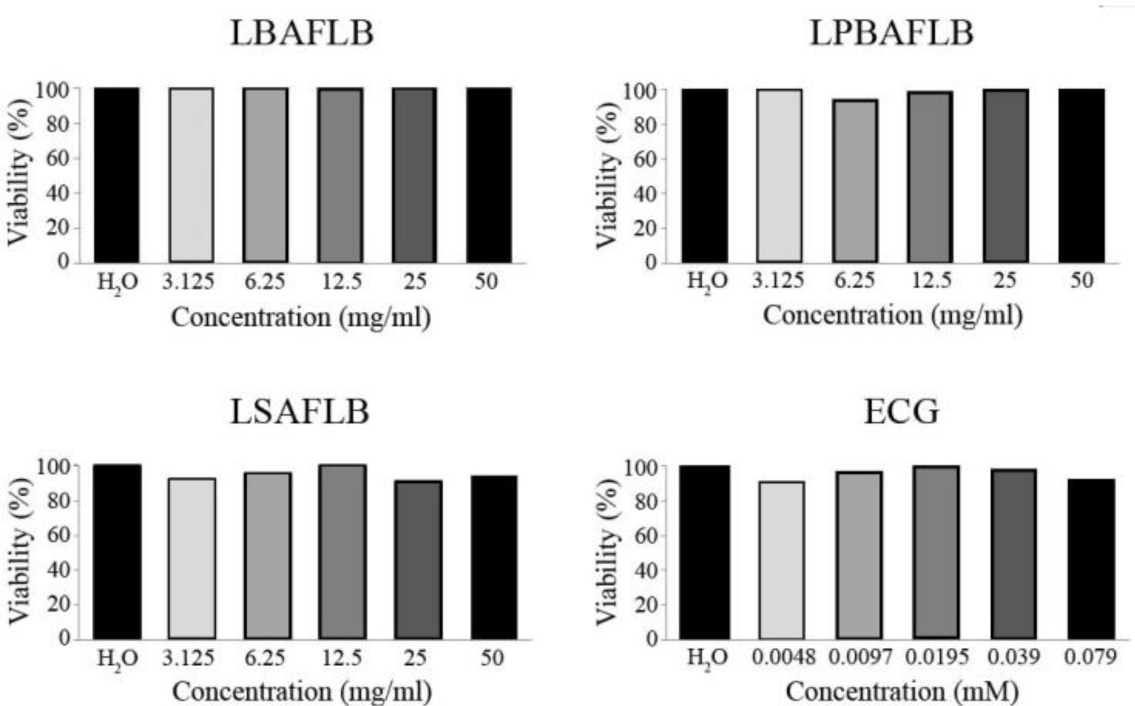
3. RESULTS AND DISCUSSION

3.1 Safety: Toxicity and Genotoxicity

Toxicity of LBAFLB, LPBAFLB, LSAFLB and ECG has been assessed in the *Drosophila in vivo* model. Figure 1 shows the relative percentage of emerging adults after treating larvae with different concentrations of these substances. A non-significant survival rate compared with the control is shown at any of the concentrations tested, exhibiting a non-toxic effect on the *Drosophila* viability.

Figure 1

Toxicity levels of lyophilised blond alcohol-free Lager beer (LBAFLB), lyophilised pale-blond alcohol-free Lager beer (LPBAFLB), lyophilised stout alcohol-free Lager beer (LSAFLB) and epicatechin gallate (ECG) in *Drosophila melanogaster*.



Data are expressed as percentage of surviving adults with respect to 300 untreated 72-hour-old larvae from three independent experiments treated with different concentrations of LBAFLB, LPBAFLB, LSAFLB and ECG.

Table 2 shows the results of genotoxicity assays in the SMART test. Negative control showed a frequency of mutations per wing of 0.157, which fall into the historical range for the wing spot test (Anter et al. 2011). The results showed that all the compounds assayed seem to have no genotoxic effect at the tested concentrations, except for the lowest concentrations (3.125 mg/ml) of the LPBAFLB and LSAFLB that showed a total mutation rate (0.575 and 0.452 spots/wings, respectively) significantly higher than the negative control (0.157 spots/wing). In the balancer-heterozygous genotype (*mwh*/TM3) *mwh* spots are produced predominantly by somatic point mutation and chromosome aberrations. By scoring *mwh*/TM3 balancers-heterozygous wings it is possible to quantify the recombinogenic potency of the positive control. The frequency of *mwh* clones on the marker transheterozygous wings (*mwh* single spots plus twin spots) was compared with the frequency of *mwh* spots on the balancer transheterozygous wings. The difference in *mwh* clone frequency is a direct measure of the proportion of recombination. Recombinogenicity values for genotoxic beers were 78.39% for LPBAFLB and 83.24% for LSAFLB, which are slightly higher than their respective recombinogenicity value induced by the control treatment (74.5%). Therefore, our compounds induced recombinogenic activity rather than point mutation activity.

Table 2
Genotoxicity of lyophilised blond alcohol-free Lager beer (LBAFL), lyophilised pale-blond alcohol-free Lager beer (LPBAFLB), lyophilised stout alcohol-free Lager beer (LSAFLB) and epicatechin gallate (ECG) in the *Drosophila* wing spot test.

Compound	No of wings	Clones per wing (nº spots) [1] Small single clones (1-2 cells) m = 2	Large simple clones (> 2 cells) m = 5	Twin clones m = 5	Total clones m = 2	F [2]	U-test [3]	R (%) [4]
SIMPLE TREATMENT (*mwh/flr³*)								
H_2O	38	0.157 (6)	0	0	0.157 (6)	0.650		76.92
LBAFLB (mg/ml)								
3.125	47	0.255 (12)	0.0212 (1)	0	0.276 (13) i	1.134	Δ	
50	45	0.288 (13)	0	0	0.288 (13) i	1.184	Δ	
LPBAFLB (mg/ml)								
3.125	40	0.450 (18)	0.15 (5)	0	0.575 (23) +	2.360		78.39
50	40	0.225 (9)	0.075 (3)	0	0.300 (12) i	1.230	Δ	
LSAFLB (mg/ml)								
3.125	42	0.357 (15)	0.095 (4)	0	0.452 (19) +	1.850		83.24
50	32	0.281 (9)	0.094 (3)	0	0.375 (12) i	1.665	Δ	
ECG (mM)								
0.0048	38	0.210 (8)	0.052 (2)	0	0.263 (10) i	1.079	Δ	
0.079	40	0.175 (7)	0.025 (1)	0	0.200 (8) i	0.820	Δ	
SIMPLE TREATMENT (*mwh*/TM3)								
H_2O [5]	80	0.040 (3)	0	0	0.040 (3)	0.150		
LPBAFLB (mg/ml)								
3.125	40	0.125 (5)	0	0	0.125 (5)	0.510		
LSAFLB (mg/ml)								
3.125	40	0.075 (3)	0	0	0.075 (3)	0.310		

(1) Statistical diagnosis according to Frei and Wurgler (1988). + (positive) - (negative) and i (inconclusive) versus negative control. m: multiplication factor. Kastenbaum-Bowman Test without Bonferroni correction, probability levels $\alpha = \beta = 0.05$.
(2) Frequency of clone formation per 10^5 cells: clones/wings/24,400 cells.
(3) Inconclusive results were resolved by Mann Whitney U-test. Delta marker (Δ) means no differences between the treatment and the concurrent control.
(4) Recombination percentage is calculated according to Valadares et al. (2008).
(5) Balancers-heterozygous wings.

Despite beer is known to have healthy properties as anti-bacterial, anti-inflammatory, anti-oxidative and it is associated with a lower risk of cardiovascular disease, not too much studies in the *Drosophila* model animal has been carried out with this beverage. A previous-similar study by Merinas-Amo et al. (2016) with an alcoholic lyophilised blond Lager beer showed that it does not exhibit toxicity at the assayed concentrations with a 100% survival with respect to the control; moreover, that beer showed non-

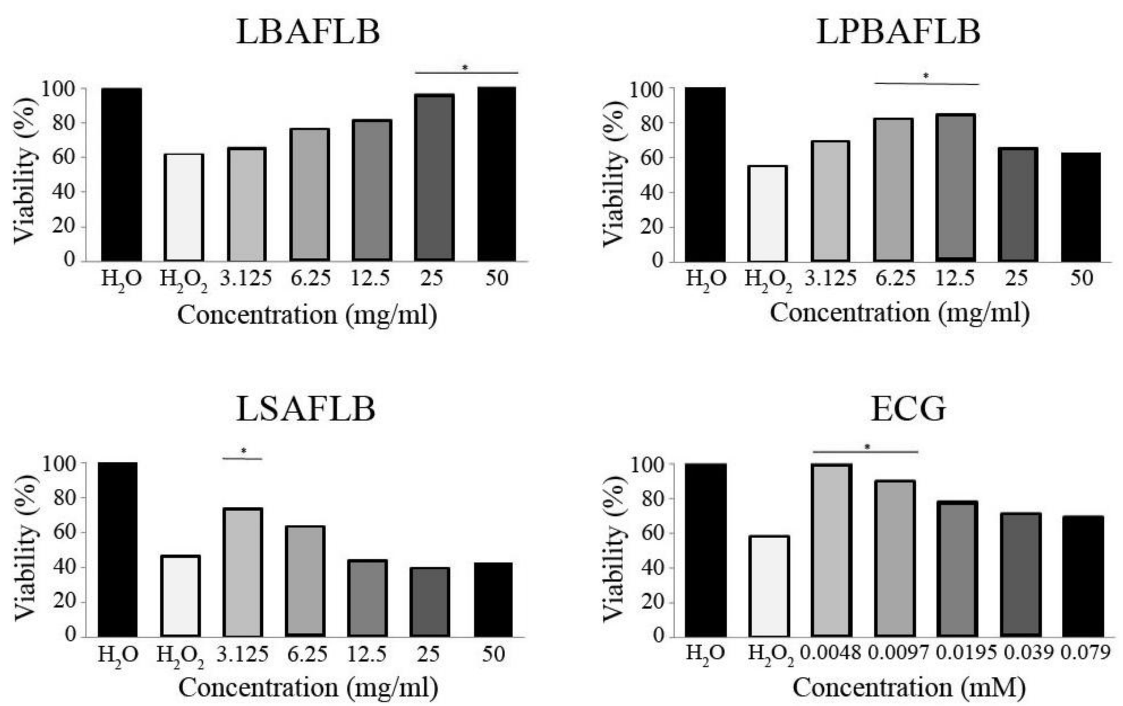
genotoxic effects in the *Drosophila* SMART test with significant similar or lower values of mutation rates at the different concentrations tested and compared with the negative control (Merinas-Amo et al. 2016).

3.2 Protection: Antitoxicity, Antigenotoxicity and Longevity

The antitoxicity assays revealed the ability of the LBAFLB, LPBAFLB, LSAFLB and ECG to protect the individuals against an oxidative stress at some of the tested concentrations. Figure 2 shows that the positive control H_2O_2 is toxic at 0.12 M with an average survival rate of 54% with respect to the water control. LBAFLB showed a significant protection against the genotoxicant at the two highest concentrations tested (25 and 50 mg/ml). LPBAFLB also showed a significant protective effect at 6.25 and 12.5 mg/ml concentrations. LSAFLB only showed a protective effect at the lowest concentration (3.125 mg/ml). The combined treatments of ECG and H_2O_2 showed significant protective effect in the two lowest concentrations (0.0048 and 0.079 mM).

Figure 2

Antitoxicity levels of lyophilised blond alcohol-free Lager beer (LBAFLB), lyophilised pale-blond alcohol-free Lager beer (LPBAFLB), lyophilised stout alcohol-free Lager beer (LSAFLB) and epicatechin gallate (ECG) in *Drosophila melanogaster*.



Data are expressed as percentage of surviving adults with respect to 300 untreated 72-hour-old larvae from three independent experiments treated with different concentrations of LBAFLB, LPBAFLB, LSAFLB and ECG combined with 0.12 M H_2O_2. *: Chi-square > 3.84 ($p < 0.05$).

Table 3 shows the results of antigenotoxicity assays in the SMART test. The concentration of H_2O_2 used (0.12 M) has been demonstrated to exert a potent genotoxic

effect capable to induce somatic mutations and mitotic recombination in *D. melanogaster* (Romero-Jimenez et al. 2005) at a rate of 0.388 spots/wing.

Results showed that LBAFLB, LPBAFLB, LSAFLB and ECG were able to inhibit the genotoxic activity of H_2O_2 with an inhibition percentage of 27.32–42.78; 6.44–29.12; 25.25–35.56; and 16.24–9.79 at the lowest and highest tested concentrations respectively. There is an apparent contradictory result with the significant genotoxicity of the lower concentration of LPBAFLB and LSAFLB and the antigenotoxic activity observed against hydrogen peroxide. Nevertheless, we hypothesised that competitive oxidation mechanism can be held between hydrogen peroxide and the mutagenic compounds contained in the studied LPBAFLB and LSAFLB (Psomiadou and Tsimiduou 2002).

Table 3
Antigenotoxicity of lyophilised blond alcohol-free Lager beer (LBAFL), lyophilised pale-blond alcohol-free Lager beer (LPBAFLB), lyophilised stout alcohol-free Lager beer (LSAFLB) and epicatechin gallate (ECG) in the *Drosophila* wing spot test.

	Clones per wing (nº spots) (1)						
Compound	No of wings	Small single clones (1-2 cells) m = 2	Large simple clones (> 2 cells) m = 5	Twin clones m = 5	Total clones m = 2	F (2)	IP (%) (3)
H_2O	38	0.157 (6)	0	0	0.157 (6)	0.650	
H_2O_2	36	0.305 (11)	0.083 (3)	0	0.388 (14) +	2.049	
LBAFLB (mg/ml)							
3.125	39	0.231 (9)	0.0512 (2)	0	0.282 (11) β	1.156	27.32
50	45	0.155 (7)	0.066 (3)	0	0.222 (10) β	0.911	42.78
LPBAFLB (mg/ml)							
3.125	44	0.295 (13)	0.068 (3)	0	0.363 (16) β	1.490	6.44
50	40	0.025 (10)	0.025 (1)	0	0.275 (11) β	1.127	29.12
LSAFLB (mg/ml)							
3.125	34	0.235 (8)	0.059 (2)	0	0.290 (10) β	1.205	25.25
50	40	0.150 (6)	0.100 (4)	0	0.250 (10) β	1.025	35.56
ECG (mM)							
0.0048	40	0.275 (11)	0.050 (2)	0	0.325 (13) β	1.332	16.24
0.079	40	0.250 (10)	0.050 (2)	0.050 (2)	0.350 (14) β	1.434	9.79

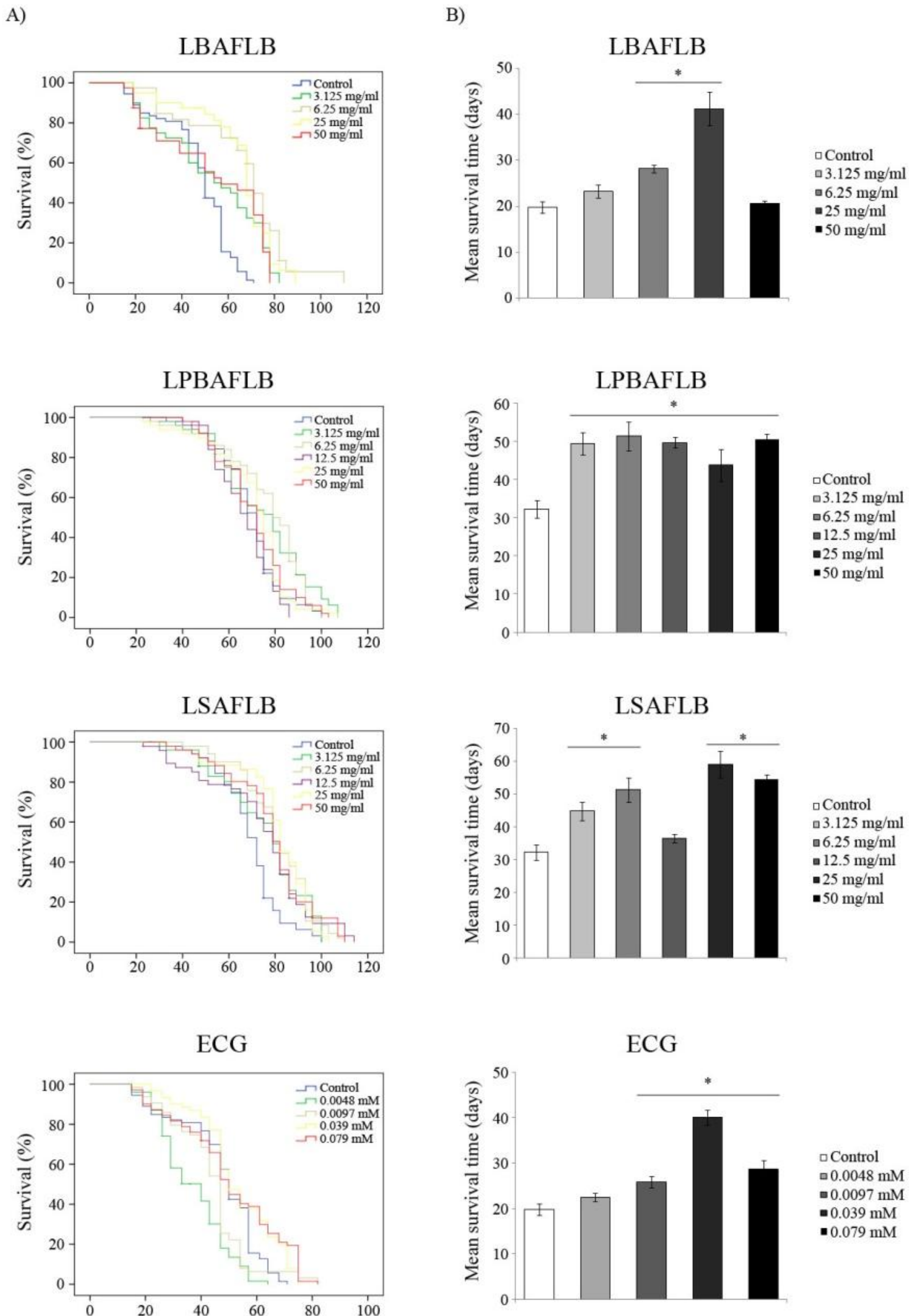
(1) Statistical diagnosis according to Frei and Wurgler (1988). β (significantly different) versus positive control. m: multiplication factor. Kastenbaum-Bowman Test without Bonferroni correction, probability levels α = β = 0.05.
(2) Frequency of clone formation per 10^5 cells: clones/wings/24,400 cells.
(3) The inhibition percentage for the combined treatments were calculated from total spots per wing according to Abraham (1994).

Longevity parameters assayed in the *D. melanogaster* model for the four compounds tested are shown in Figure 3 and Table 4. Survival curves showed either a similar or a significant increase of lifespan expansion for all compounds, with respect to their concurrent control, except for the two lowest concentrations of ECG (0.0048 and 0.0097 mM) that significantly reduced the lifespan of *D. melanogaster*. LBAFLB and LSAFLB showed a significant increase of life extension in *D. melanogaster* at all the tested concentrations with an average range of 52.90–66.38 days and 74.179–80.852 days, respectively, with respect to the control (47.242 and 69.324 days average, respectively). LPBAFLB showed a significant increase of life extension in *D. melanogaster* fed with concentrations of 3.125 and 6.25 mg/ml, with a life extension of 6 and 11 days respectively, with respect to the concurrent control. Finally, ECG was able to improve the longevity at two highest concentration (0.039 and 0.079 mM); although the phenol induced a reduction of 10–4 days in the *Drosophila* life extension treated with the respective 0.0048 and 0.0097 mM concentrations, when are compared with the control treatment.

Regarding the healthspan results, all substances are able to improve the quality of life of flies at some concentrations tested, with respect to their concurrent control. LBAFLB induced a significant improvement of healthspan in *D. melanogaster* compared to the control in the two central concentrations assayed (6.25 and 12.5 mg/ml) with an increase of 9 and 22 days respectively. LPBAFLB showed a significant improvement of healthspan at all the assayed concentration with an average range of 11–19 days with respect to the control. LSAFLB induced a significant improvement of quality of life in *Drosophila* at all assayed concentrations except at the middle concentration (12.5 mg/ml) with a range of 12–27 days, with respect to the control. ECG exhibited an improvement of healthspan at three highest concentrations (0.0097, 0.039 and 0.079 mM) with an increase of 6, 20 and 9 days with respect to the control, respectively.

Figure 3

Survival parameters of *Drosophila melanogaster* fed with different concentrations of lyophilised blond alcohol-free Lager beer (LBAFLB), lyophilised pale-blond alcohol-free Lager beer (LPBAFLB), lyophilised stout alcohol-free Lager beer (LSAFLB) and epicatechin gallate (ECG). A) Survival curves and B) Healthspan averages.



A) Survival curves of *D. melanogaster*. B) Mean of survival time in the highest 75% surviving population. * indicates significant differences ($p < 0.001$) with respect to the control.

Table 4
Mean and significances of lifespan and healthspan.

Compound	Concentration	Mean Lifespan [1] (days)		Mean Healthspan [1] (days)	
LBAFLB	Control	47.242		19.769	
	3.125 mg/ml	52.900	***	23.273	ns
	6.25 mg/ml	66.380	***	28.167	***
	25 mg/ml	64.994	***	41.231	***
	50 mg/ml	53.488	***	20.538	ns
LPBAFLB	Control	69.324		32.222	
	3.125 mg/ml	74.358	*	49.413	***
	6.25 mg/ml	76.310	**	51.308	***
	12.5 mg/ml	69.535	ns	49.667	***
	25 mg/ml	70.490	ns	43.700	*
	50 mg/ml	70.660	ns	50.417	***
LSAFLB	Control	69.324		32.222	
	3.125 mg/ml	75.782	**	44.786	***
	6.25 mg/ml	80.852	***	58.675	***
	12.5 mg/ml	74.179	**	36.500	ns
	25 mg/ml	80.686	***	59.077	***
	50 mg/ml	78.966	***	54.577	***
ECG	Control	47.242		19.769	
	0.0048 mM	37.187	***	22.500	ns
	0.0097 mM	43.899	*	25.800	***
	0.039 mM	53.070	*	39.962	***
	0.079 mM	50.647	*	28.619	***

Means were calculated by the Kaplan-Meier method and significance of the curves were determined by the Log-Rank method (Mantel-cox). [1] ns: non-significant ($p > 0.05$), *: significant ($p < 0.05$), **: highly significant ($p < 0.01$), ***: very highly significant ($p < 0.001$).

Most of the protective properties showed by beers have been related with the antioxidant activity of its phenols (Preedy 2011). Arimoto-Kobayashi et al. (2006) evaluated the antigenotoxic potential of beer components against carcinogens contained in the human diet suggesting their protective capacity. Moreover, the formation of the Maillard intermediate is correlated with the alcohol content and colour (Coghe et al. 2004; Vanderhaegen et al. 2004). Studies with coloured final products of the Maillard reaction which are formed non-enzymatically during the roasting of malt, indicate peroxyl radical scavenging potential (Liégeois et al. 2000). This fact, along with the presence of other phenols, could be the responsible for the stronger antitoxic potential of LSAFLB at lowest concentration. Moreover, our results are in agreement with the antioxidant ability of blond and stout beers found by Tafulo et al. (2010), Granato et al. (2011) and Merinas-Amo et al. (2016).

Researches confirm the antioxidant effect showed by ECG as it acts as a scavenger of free radicals and suppressor of oxidative stress (Rizvi et al. 2005; Siddique et al. 2014). Therefore, and due to their polyphenols content, beers could be a source of bioactive compounds with suitable free radical scavenging properties, like isoxanthohumol, ferulic acid, vanillic, folic acid, choline, among others (Cuvelier et al. 1992; Merinas-Amo et al. 2016; Sánchez-Moreno et al. 1998; Srinivasan et al. 2007; Żołnierczyk et al. 2015).

The alcoholic lyophilised blond Lager beer induced a lifespan expansion in *D. melanogaster* ranged between 55.3–64.9 days compared to the control (Merinas-Amo et al. 2016). Moreover, the healthspan was significantly increased with respect to the control, except for the highest concentration (50 mg/ml). We hypothesise that the antioxidant components of beer would neutralise the oxidative radicals produced during the metabolism of the rest of the diet in the *Drosophila* longevity trials by increasing life and healthspan.

Studies about the important effects of ECG in the inhibition of the development of insulin resistance and hyperglycaemia (Wu et al. 2004), coronary disease and atherogenesis (Miura et al. 2000) and its role on obesity as a therapeutic substance (Kao et al. 2000; Tian 2006; Zhang et al. 2006) confirm the positive properties that this phenol exhibits. Moreover, Gorinstein et al. (1997a); Gorinstein et al. (1997b) reported a beneficial effect of a special type of beer with an epicatechin content of 132- 2.5 mg/l on some blood parameters. In addition, age-associated diseases such as Parkinson disease were inhibited by ECG intake (Mandel et al. 2004; Nie et al. 2002). Our results agree with the health effects that beer and ECG exhibited. Furthermore, the role of ECG was studied on *Drosophila* in order to analyse its effect on neurons which are also present in humans (climbing ability, lipid peroxidation and apoptosis in the brain of flies) obtaining a promising effect when the phenol was supplemented at concentrations of 0.25, 0.50, and 1.0 µg/ml (Siddique et al. 2014). Our results agree with these similar safe assays. Moreover, we could relate these beneficial properties of the phenol with the safety and protective effects of the AFBs.

3.3 Chemoprotection: Cytotoxicity and DNA damage

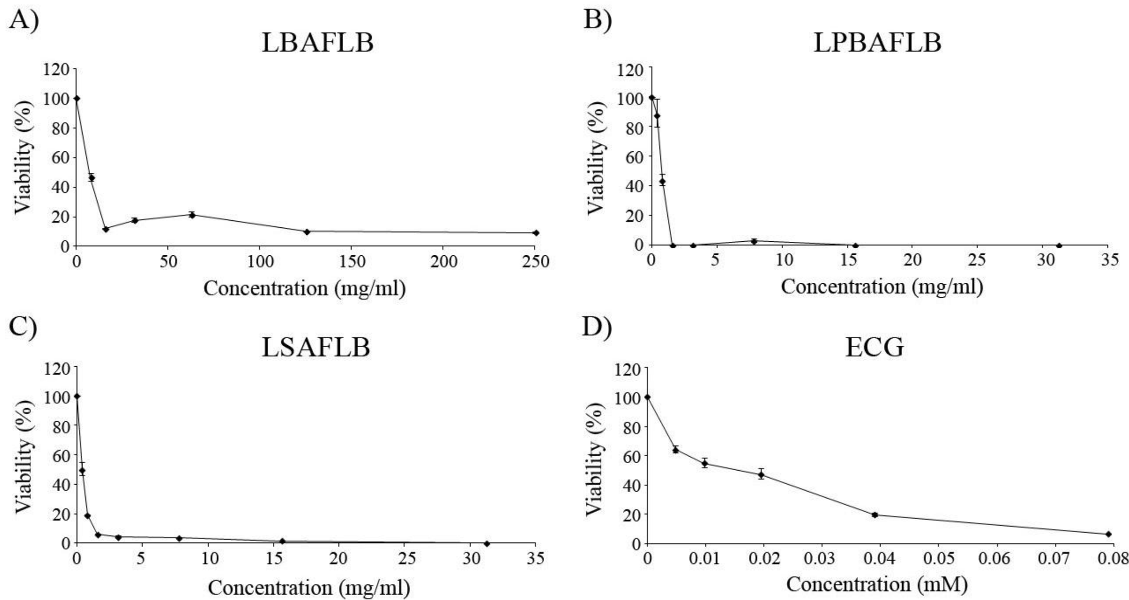
All substances assayed showed cytotoxic activity against HL-60 cells (Figure 4). LBAFLB showed an increase of the cytotoxicity level according to increased concentration tested of the compound, being the inhibitory concentration 50 (IC_{50}) 7.81 mg/ml. LPBAFLB and LSAFLB showed a stronger chemopreventive property inducing a total inhibitory tumour cells growing at 31.25 mg/ml, and reaching the IC_{50} value over a 0.488 mg/ml. Finally, the cytotoxicity of ECG exerted a progressive dose-dependent inhibition of cells growth reaching the IC_{50} value at the medium concentration tested (0.0195 mM).

There are conflicting reports concerning the relationship between beer consumption and cancer risk. Riboli et al. (1991) reported that beer consumption is not associated with

colon cancer; contrary, Kato et al. (1990) showed that beer drinkers have an increased risk to colorectal cancer and a connection between beer consumption and lung cancer was suggested by Potter et al. (1992). Nevertheless, an inverse association between moderate beer consumption and endometrial cancer was suggested by Swanson et al. (1993). *In vitro* studies indicate the anti-proliferative activity of Lager beer components with antioxidant properties in prostate cancer cells and HL-60 promyelocytic cancer cells, showing a strong correlation among antioxidant potency, polyphenols content and antiproliferative activity (Hevia et al. 2008; Merinas-Amo et al. 2016). The present cytotoxicity results of the different AFBs, support the epidemiological data on safety and the *in vitro* results of Lager beer. Several studies supported the beneficial effects of ECG on chemoprevention. In animals models studies , ECG was able to inhibit the growth tumours in colon (Baek et al. 2002; Hong et al. 2001; Weyant et al. 2001), breast (Bigelow and Cardelli 2006; Liao et al. 1995), prostate (Ahmad et al. 1997; Chung et al. 2001), head and neck (Lim et al. 2006), lung (Yang et al. 1998), and skin (Ahmad et al. 1997; Huang et al. 2005), among others multiple biological effects on human diseases. Our results agree with the chemopreventive potential of ECG against tumour cells growing. Moreover, we could hypothesise that ECG is the responsible in part for the chemopreventive activity of the alcohol-free beers.

Figure 4

Effect of lyophilised blond alcohol-free Lager beer (LBAFLB), lyophilised pale-blond alcohol-free Lager beer (LPBAFLB), lyophilised stout alcohol-free Lager beer (LSAFLB) and epicatechin gallate (ECG) on cell viability.



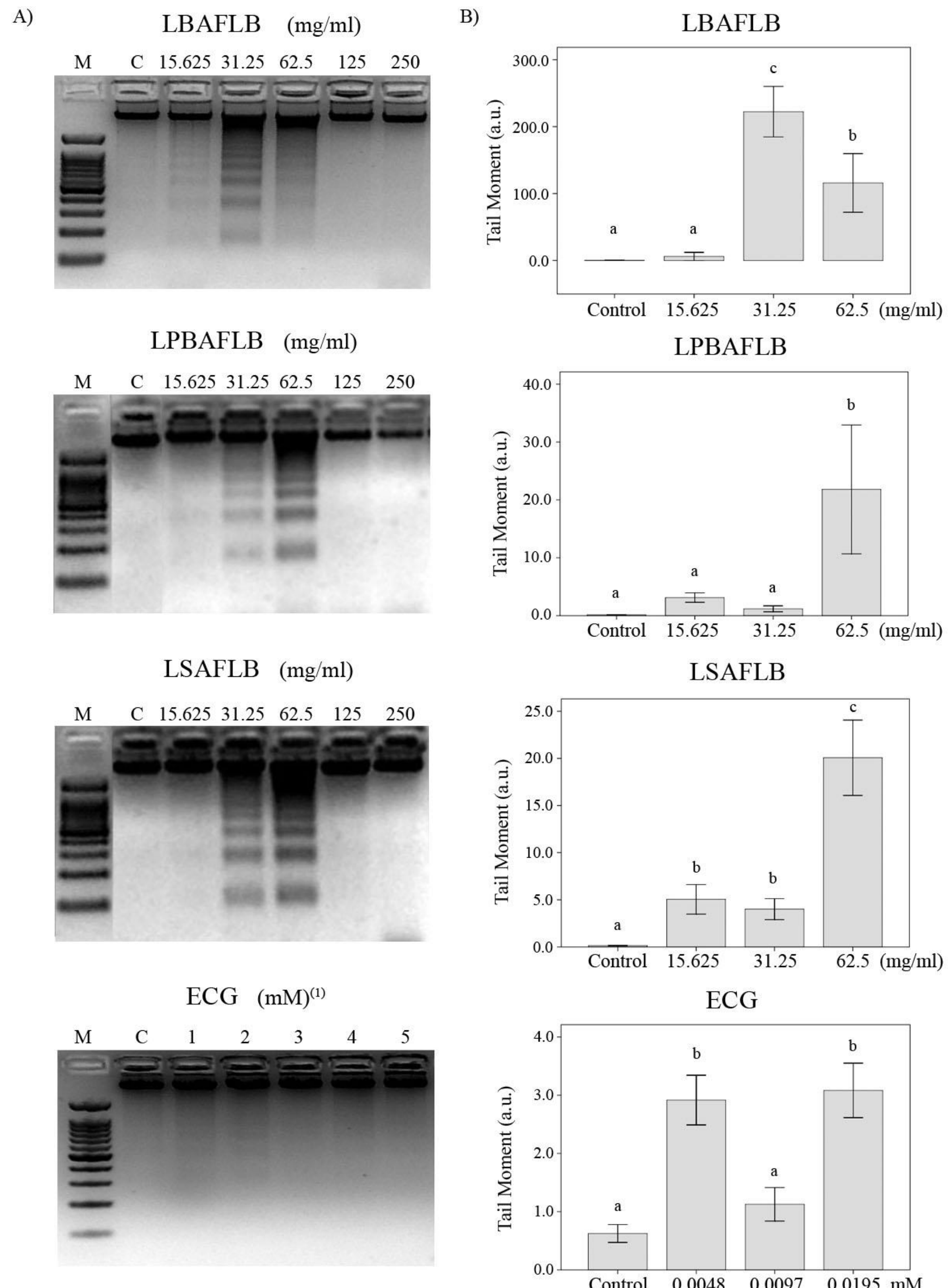
Viability in promyelocytic human leukaemia cells (HL-60) treated with different concentrations of LBAFLB (A), LPBAFLB (B), LSAFLB (C) and ECG (D) for 72 h. Each point represents the growing percentage with respect to its control. Values are mean +/- SE from three independent experiments.

DNA damage of tumour cells treated with different concentrations of our assayed compounds was evaluated by DNA internucleosomal fragmentation and DNA strand breaks induction (Figure 5), and as modification in the DNA methylation status of the HL-60 cells (Figure 6). DNA internucleosomal fragmentation is represented by a DNA laddering and it is associated to the activation of the apoptotic way in cancer cells. Fragmentation was observed in the third lowest concentrations of LBAFLB (15.625, 31.25 and 62.5 mg/ml) and in the two lowest concentration of LPBAFLB and LSAFLB (15.625 and 31.25 mg/ml). On the other hand, ECG did not induce internucleosomal fragmentation at any assayed concentration (Figure 5).That result do not allow us to establish any relation between ECG effects and the properties showed by the alcohol-free beers to induced DNA fragmentation. That fact could be due to the presence of other compounds that give the final properties to the AFBs. In a study with a blond alcohol beer, Merinas-Amo et al. (2016) related the property of beer to induce proapoptotic DNA fragmentation with the presence of xanthohumol.

By using the comet assay we were able to readily identify, at unicellular level, apoptotic nuclei based on the typical morphological changes attributable to extensive DNA damage (Poe and O'Neill 1997). According to Fabiani et al. (2012), the frequencies distribution of DNA damage can be divided into five classes depending on the TM values as follows: class 0 (TM < 1; no damage), class 1 (TM 1–5; slightly damaged), class 2 (TM 5–10; medium damage), class 3 (TM 10–20; highly damaged) class 4 (TM >20; completely damaged). Based on this classification, all concurrent control values fell into class 0 (TM < 1); LBAFLB 31.25 and 62.25 mg/ml showed a TM value near to 100, what means an induction of a high and complete clastogenicity damage in HL-60 cells; the same properties are showed by LPBAFLB that induced significant damage at the 62.5 mg/ml concentration with a TM value upper to 20; LSAFLB showed a slightly damage for the two lowest concentrations assayed (15.625 and 31.25 mg/ml) with a TM value lower than 5, and a high damage induction for the highest concentration (TM < 20); ECG induced TM values over 3 (class 1) at 0.0048 and at 0.0195 mM induced slight damage on cells (Figure 5). Although ECG induced a slight DNA damage (TM < 5), it is not the responsible of the high damage induced by alcohol-free beers (TM > 100 and TM > 20). That fact could be because of the presence of other compounds that give the final properties of AFBs.

Figure 5

DNA-induced damage in promyelocytic HL-60 cells treated with different concentrations of lyophilised blond alcohol-free Lager beer (LBAFLB), lyophilised pale-blond alcohol-free Lager beer (LPBAFLB), lyophilised stout alcohol-free Lager beer (LSAFLB) and epicatechin gallate (ECG) for 5 h. A) Internucleosomal DNA fragmentation. B) DNA strand breaks induction.

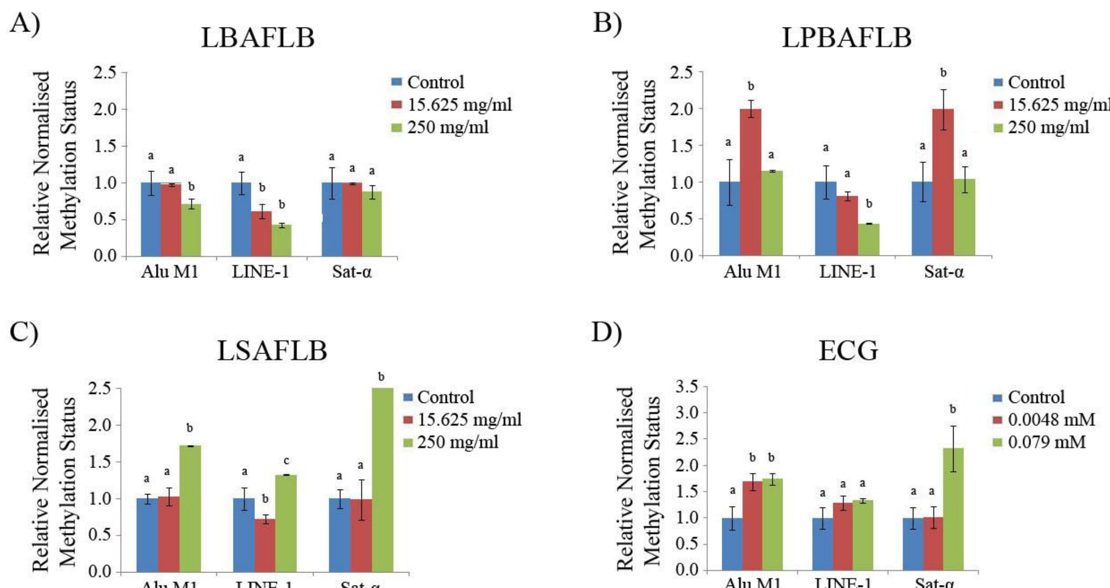


A) M indicates DNA size marker, C indicates Control treatment. [(1)] ECG: concentrations of 0.0048 mM (1), 0.0097 mM (2), 0.0195 mM (3), 0.039 mM (4) and 0.079 mM (5). B) Alkaline comet assay (pH < 13) of HL-60 cells treated. DNA migrations is reported as mean TM. Values are mean +/- SE. Different letters in treatment mean differences respect to the negative control after one-way ANOVA and post hoc Tukey's test.

Regarding the relative normalized expression of the three repetitive sequences (Alu M1, LINE-1 and Sat-α) studied in HL-60 cells treated with different concentrations of our compounds (Figure 6): LBAFLB showed a significant hypomethylated status in the highest concentration tested (250 mg/ml) for the Alu M1 and at both concentrations tested (15.625 and 250 mg/ml) for the LINE-1 repetitive sequence, with respect to the concurrent control; LPBAFLB showed a significant hypermethylation in 15.625 mg/ml treatments on Alu M1 and Sat-α repetitive sequences and at hypomethylation at the highest concentration (250 mg/ml) for LINE-1 sequence, with respect to their controls; contrarily, LSAFLB showed a hypermethylation in 250 mg/ml treatment for all the repetitive sequences, and a hypomethylation at the lowest concentration (15.625 mg/ml) for LINE-1 sequence; ECG showed a general tendency to methylate leukaemia cells with a significant hypermethylation at 0.0048 and 0.079 mM for Alu M1 and only at 0.079 mM for Sat- α sequences, with respect to their controls.

Figure 6

Relative normalised expression data of each repetitive element in treated HL-60 cells with different concentration of lyophilised blond alcohol-free Lager beer (LBAFLB) (A), lyophilised pale-blond alcohol-free Lager beer (LPBAFLB) (B), lyophilised stout alcohol-free Lager beer (LSAFLB) (C) and epicatechin gallate (ECG) (D).



Relative normalised expression data of each repetitive element (Alu M1, LINE-1 and Sat-α). Different letters mean different values after one-way ANOVA and post hoc Tukey's test.

To our knowledge, any study with AFBs was carried out in order to analyse the DNA fragmentation patter that this beer causes in the cancer cells. Studies with a blond Lager beer showed a fragmentation effect in the two lowest concentrations of the beer (15.625 and 31.25 mg/ml) and slightly in the intermediate concentration (62.5 mg/ml) (Merinas-Amo 2016), being our results in agreement with the previous one. Treatment with ECG in different tumour cells like human epidermis carcinoma (A431), human carcinoma karatinocyte (HaCaT), human prostate carcinoma (DU145), mouse symphoma

(L5178Y), human oral tumour cells (HSC-2) induced apoptosis by induction of DNA fragmentation, but not in normal NHEK cells and HGF-2 fibroblasts (Ahmad et al. 1997; Babich et al. 2005). Our results do not agree with the previous one, probably because we studied lower concentrations than the 40 µg/ml dose used by the previous authors.

The comet assay TM measurements have been shown to be correlate with the cytotoxicity ones (Fairbairn et al. 1996). Our results agree with the above correlations mentioned showing significant TM values correlated directly to the percent of viable cells as monitored using trypan blue exclusion for HL-60 cells. Moreover, the results of DNA fragmentation fit with those obtained in comet assay since TM values higher than 30 mean that apoptosis mechanisms has been induced (Fairbairn et al. 1995). Only the the two highest ones of LBAFLB showed TM > 30 confirming the death of leukaemia cells by apoptosis. The rest of compounds and concentrations tested induce cell death by a necrosis mechanism.

The mechanisms of controlling epigenetic DNA modifications are complex (Herman and Baylin 2003). Endoparasitic repetitive sequences refrain from jumping around thanks to the repression of DNA methylation (Esteller 2005). Most of the DNA methylation in humans occurs in the cytosine of CpG dinucleotides (CpG islands). In a normal cell, they are unmethylated, and the gene is expressed if the required transcription factors are present (Esteller 2005). Due to the effects that methylation causes in the repetitive sequences of the genome, we could said that only LPBAFLB at lowest concentrations for Alu M1 and Sat-α, LSAFLB at highest concentrations for all repetitive sequences studied and ECG at lowest concentration for Alu M1 and Sat-α and highest concentration for Alu M1 sequences prevents for DNA epigenetic damages as a methylation of repetitive sequences is understood as a genomic protective mechanism (Roman-Gomez et al. 2008; Weisenberger et al. 2005). Besides of that and bearing in mind the idea of tumoral cells demethylation, it would be suggested that beers and its compound act as preventive agents cutting off the repetitive sequences of tumour cells (Roman-Gomez et al., 2008).

Unfortunately, there are not previous studies about methylation status with AFBs or ECG. Merinas-Amo et al. (2016) demonstrated that a lyophilised blond Lager beer induce hypermethylation in the DNA of tumoral cells in a wide range of repetitive sequences. Fang et al. (2007) suggested that the effect of a single polyphenol is not significant in a normal dietary consumption, whereas the combination of polyphenols with dietary histone deacetylase inhibitors and the additive effect of different dietary chemicals may produce some effects. Furthermore, an excessive consumption of polyphenols in dietary supplements may affect DNA methylation status (Fang et al. 2007). Single or combined phenols contained in food should be taken into account in epigenetic-focussed therapies as not everything is worthy.

4. CONCLUSIONS

The different composition and brewing process of the drink could influence the final outcome of the full beer. Despite further researches must be done to check the properties of alcohol-free beers in other animals models and even in humans, results *in vivo* and *in vitro* with model organisms (animal and cell) are promising. Taking into account the previous ideas and results obtained in the present work, we could suggest that alcohol-free beers must be considered not only cause of their reduced calories, isotonic properties, but they could also be recognised as potential nutraceutic substances due to the safety, protective and chemopreventive results obtained herein.

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CHAPTER 5

Protective and chemopreventive effects of Czech beers and their constituents: *in vivo* and *in vitro* studies

ABSTRACT
Czech beer is unique because it is brewed using specific technology at specific latitude. Moreover, beers are characterised for being entirely produced in the area of the Czech Republic (selected raw materials, malting process and preparation of the beer). The aim of our study is to evaluate the protective and chemopreventive activities of a wide variety of Czech beers, as well as their raw specific materials (malts, hops and yeast) and processed-beer by products at three different steps during the brewing (wort, hopped wort and young beer). For that purpose, an *in vivo* assay of safety followed by a study about the protective effects against oxidative agent in the *Drosophila* animal model was carried out. Moreover, chemopreventive studies in the *in vitro* model of human leukaemia cells (HL-60) were performed to evaluate de cytotoxic and DNA damage potential that the tested compounds induce on the tumour cells. Results showed a safe effect for all the analysed substances and a general protective effect against hydrogen peroxide as an oxidative toxin, except for Staropramen Cerny, Staropramen Nefiltrovany, Lobkowicz Nealko, Budweiser Cherry, *S. uvarum*, highest concentration of Herold Cerny and lowest concentration of Sladek hop. Moreover, all compounds were able to inhibit the tumoral cells growth, except the rest of mash which is eliminated during the brewing process. The cytotoxic activity is due, in some cases, to the induction of proapoptotic DNA fragmentation damage in the HL-60 cells. The promising effects showed by Czech beers, due to its safety, protection against a toxin, chemopreventive potential and induction of DNA damage in tumour cells, allow us to propose Czech beer as a beverage with a nutraceutic potential.

1. INTRODUCTION

Beer is one of the oldest known beverages. Some 6,000 years ago, ancient text reveals the first beer production by Sumerians: "the sweetest grain, if baked, left out, moistened, forgotten, and then eaten, would produce an uplifting, cheerful feeling" (Damerow 2012). Different contributions like the use of hops, the use of yeast, the different fermentation methods and the high technology development in the last century have contributed to the growth, improvement and variety of brewing as we currently know (Damerow 2012; Hornsey 2003; Wolf et al. 2008).

Beer is not only considered as a beverage, but it provides a valuable added value to the diet. It acts as a nutritional supplement with many bioactive molecules, composed of readily available starches and sugars, various minerals and valuable B vitamins (Hornsey 2003). The brewing industry is a global business although the traditional European brewing regions with local varieties of beer are Germany, Belgium, the United Kingdom, Ireland, Poland, the Czech Republic, Scandinavia, the Netherlands and Austria (Marova et al. 2011).

The beer industry of Czech Republic (Bohemia) has a long history. The first Czech brewery was founded in 993 at Břevnov Monastery in Prague, although the first reference to brewing in the Czech lands was in 1088 when hops were used in the processing of beers. This first beer was a kind of cloudy wheat beer, using a top-fermenting yeast, which is known then as "bilé pivo" (white beer). Furthermore, a second type of beer was known as "staré pivo" (old beer) which was kept for longer, and was made with bottom-fermenting yeast.

As wars and conflicts swept through the region, many of the existing breweries were destroyed. The old brewery at Břevnov in Prague, was sacked along with the Břevnov Monastery in 1420, during the Hussite Wars, being rebuilt in 1720. However, for many centuries almost all of these breweries (at monasteries, in cities and in noble homes) were producing top-fermenting beers, many of them were made with wheat.

A new style of beer took popularity during this time: a crisp bottom-fermented beer. However, in Pilsen, the new brewery dried its malt using an English-style malt kiln, with indirect heat. The result was a beer which tasted crisp and clean, just like the so-called “Bavarian beer”. Instead of dark, the new brew from Pilsen shined with a beautiful golden colour. The change was swift and most of breweries changed their brewing process: the old brewery in Třeboň, South Bohemia, which had been making top-fermented beers since 1379, started using bottom fermentation in 1861. And the historic brewery at Břevnov Monastery, founded in 993, destroyed by the Hussites in 1420 and rebuilt by the Dientzenhofers in 1720, closed in 1889 and recently reopened again in 2013.

Consequently, there were so many milestones that were passed by the Czech brewery industry and at present it has paved way to a unique beer culture combining the Czech

language, the Czech culture, and Czech beer into one platform. Although the history of Czech beer got a little dark at times, it now looks to have a very happy ending (Wanninayake and Chovancová 2012; http://www.prague.eu/en/articles/history-of-czech-beer-10522).

Czech beer is unique because it is brewed using specific technology at particular latitude. Since October 2008 the EU has allowed the Czech Republic to use the "C*eské pivo/Czech Beer*" trademark (Commission 2008). Because of the specific character of Czech beer, due to the water, malt, hops and yeast used in the brewing, Czech brewers want recognition of the uniqueness of their beer. Czech beer is characterised for being entirely produced (selected raw materials, malting process and preparation of the beer) in the area of the Czech Republic. Beer production starts at the brewing house, where ground malt is mixed with water and mashed, which converts the unfermentable starch into fermentable sugars. The mashing process itself employs a one-mash to three-mash decoction method; infusion mashing is not used. At least 80% of the total malt grist is made up of malt produced from approved varieties, which guarantees the taste profile of "*Ceské pivo*". Depending on the conditions (time, temperature), pale or amber-coloured or even dark malts are obtained, the colour being due to caramelisation of sugars and to Maillard-type reactions. It is important to notice that the colour of beer is derived from the colour of the malt(s) used. Furthermore, it is obvious that coloured malts exhibited a distinct taste, which often is characteristic of particular dark beers.

After the mashing process has been completed, insoluble particles of malt have been separated through a process known as lautering. After filtration, the sugar solution, in brewers' jargon called *"wort"*, is transferred to the brewing kettle, where it is boiled with the addition of hops (*Humulus lupulus L.*). During this phase, which takes 60 to 120 minutes, an evaporation rate of at least 6% must be achieved. Hops can be added in up to three stages. The minimum quantity of Czech hops or products processed from them is 30% for pale lagers and at least 15% for other types of beer. Besides the formation of insoluble complexes with proteins and polypeptides, contributing to the colloidal stability of beer, hops sterilize the wort solution, which takes care of the bacteriological stability of beer. The most important asset of hops is the bitter taste conferred to, particularly, blond beers. Furthermore, hops are necessary for the stabilization of beer foam, while, on the other hand, the most precarious off-flavour in beer, called light-struck flavour, involves degradation of hop-derived components (Preedy 2011).

After wort boiling, the liquid known as "*hopped wort*" is cooled down to a pitching temperature of 6–10 °C and aerated. Depending on the temperature during fermentation and the nature of yeast collection at the end of the fermentation period, beers are distinguished as being produced by *"bottom fermentation"* or *"top fermentation"*. During the anaerobic phase yeast cells convert sugars to ethanol and carbon dioxide. Brewer's yeast used exclusively for bottom fermenting (*Saccharomyces cerevisiae subs. uvarum*) is then added. Fermentation takes place at a maximum temperature of 14 °C and this technological process is normally separated from secondary fermentation. A typical fermentation takes about one week thereby delivering a so-called *"green beer"*

or *"young beer"*, which is not drinkable, as a number of offending (bad taste and smell) compounds are formed during fermentation. The secondary fermentation process takes place at temperatures close to 0 °C. On completion of the process of maturation by secondary fermentation in tanks, the beer is filtered and casked, bottled, canned or tankered. It is also possible to make unfiltered beer. For prolonged conservation beers may be pasteurized. The entire beer production technology is continuously monitored (Commission 2008).

Worldwide interest in alcohol-free beers (AFB) is increasing. Athletes appreciate beer's typical properties, reduced calories, and the isotonic properties of most alcohol-free beers. Legally fixed maximum alcohol contents differ in countries. Arabic countries demand contents below 0.05% per volume. Germany allows contents below 0.5% per volume. Two procedures especially have been established for manufacturing non-alcoholic beer: physical and biological processes (Brányik et al. 2012). On the one hand, physical methods are based on gentle removal of alcohol from regular beer and require considerable investments into the special equipment for alcohol removal. The technologies applied for complete or partial ethanol removal from regular beers can be classified into two groups based on the principle of the separation process such as thermal and membrane processes. Besides the industrially applied methods of beer dealcoholisation (vacuum rectification and evaporation, dialysis, and reverse osmosis) there have been several other methods studied under laboratory conditions such as pervaporation (Mendes et al. 2008), adsorption on hydrophobic zeolites (Anglerot 1994), freeze concentration (Von Hodenberg 1991), membrane (Etuk and Murray 1991; Matson 1988) and supercritical CO_2 extraction (Brányik et al. 2012), After the removal process has been optimized, the sensorial quality of produced AFBs is usually good. Their further advantage is that they can remove ethanol from beers to vanishingly low levels.
On the other hand, the most widespread biological approaches are based on limited ethanol formation during the beer fermentation. They are usually performed in traditional brewery equipment and hence do not require additional investments, but their products are often characterized by worty off-flavours. Improvements taking advantage of special yeast increase the costs by the purchase, selection, or construction of the production organisms as well as by the need their propagation have to be separated. However, suitable tailor-made or selected microorganisms can contribute significantly to the product sensorial quality improvement. There are also AFB production processes (continuous fermentation with immobilized yeast) based on limited alcohol formation, which require special equipment and material (continuously operating bioreactor, carrier for cell immobilization). In this case, the higher investment costs have to be justified by the higher productivity of continuous processes. In general, the ethanol formation, which is intrinsic to the biological methods, makes impossible the production of AFBs with alcohol content close to zero.

The use of additives will be explained on the example of Czech alcohol-free beers (Brányik et al. 2012). Currently there are 30 AFB brands commercialized in the Czech

Republic (AFBs represented 2.92% of the Czech beer market in 2008). Among them, 26 brands are produced by the arrested/limited fermentation (at least one uses also a changed mashing process), two are fermented with special yeast and one is produced by vacuum rectification. According to information on the labels, 10 brands from the whole group of AFBs are produced only using traditional brewing raw materials, 9 contain one additive, 9 contain two additives, and 2 contain three or more additives. Recently, special technologies were developed, for example, for enrichment of folic acid or xanthohumol in beer. Innovations like this, in addition to publicizing the health effects of beer ingredients, may strengthen consumer interest in a tasty and healthy beer (Back, 2005a; Narziss, 2005).

Despite beers are considered years ago as beverages with important properties on health (Chen et al. 2014; Granato et al. 2011; Preedy 2011; Reddy et al. 2003; Saura-Calixto et al. 2008; Vinson et al. 2003), nowadays studies about the toxicological evaluation of beers are missing. Moreover, and due to the traditional history of beer in Czech Republic, in the present work we will try to add new data about the protective and chemopreventive properties of a wide variety of Czech beers, as well as its unique and differential raw materials (malts, hops and yeast) and processed-beer at three different steps during its brewing (wort, hopped wort and young beer). For that purpose, a previous assay of safety followed by a study on the protective effects that the tested compounds show in the *Drosophila* animal model were carried out. Besides, chemopreventive studies in the *in vitro* model of human leukaemia cells (HL-60) were carried out to evaluate the possible cytotoxic potential and DNA damage that our tested compounds could induce in the tumour cells.

- The fruit fly *D. melanogaster* shows a consistent genetic similarity to humans, considering that approximately 75% of the genes involved in human diseases have related or similar sequences to *D. melanogaster* (Bier 2005; Lloyd and Taylor 2010). It is a well-known insect with largest scientific history in biological sciences that has highly contributed to understanding the developmental biology, evolutionary concepts and recently toxicology (Bhargav et al. 2008; Coulom and Birman 2004; Dean 1985; Hosamani 2013; Siddique et al. 2013). The unique characteristics that *Drosophila* possesses, such as a rapid and short life cycle (10–12 days at 25 °C), reliability, cost efficient, easy maintain, and manipulation make this eukaryote an ideal model organism.
- The human model HL-60 cell line was originated from a female patient with acute myeloid leukaemia (Collins 1987). The culture of peripheral blood leukocytes from this patient in conditioned medium resulted in the development of a growth-factor-independent immortal cell line with distinct myeloid characteristics (Gallagher et al. 1979). The promyelocytic human leukaemia cell line HL-60 is world-wide used for many toxicity and cancer scientific purposes.

2. MATERIALS AND METHODS

2.1. Samples preparation and simple compounds

For our study, 16 samples of lager type beers commercially produced in Czech Republic were purchased at local market. Moreover, 6 raw material and 3 processed-beer samples were obtained from the research and teaching brewery of the Czech University of Life Sciences Prague. It consists of two-roll mill, brew house (steam heated kettle 12 hl, and lauter tun 14 hl), whirlpool 12 hl, wort cooler (10 hl/h), 6 fermentation tanks (24 hl each), and 14 lager tanks (22 hl). The standard brewing condition are as follows: 200 kg Pilsen malt, 2 mash process, 3 kg of Saazer hop variety, fermentation at 12°C for 7 days, yeast W96, maturation at 0°C for 3 months. The annually brewery production is 300 - 500 hl beer. Detailed characteristics of all 25 samples analysed in this study are summarised in Table 1. Beers samples were lyophilised using a Scanvac CoolSafe 110-4 freeze-drier (LaboGene, Denmark) before carry out the assays and stored until use at room temperature in a dark and dried atmosphere. Lyophilised beers were dissolved in distilled water in order to obtain the different concentrations to be tested.

The concentrations tested of beers were established taking into account the average daily food intake of *Drosophila* (1 mg/day) and the average body weight of *D. melanogaster* individuals (1 mg) (Ja et al. 2007). Moreover, the concentration range was calculated in order to make it comparable to the beer daily intake for humans (192 ml of total beer/day for 70 kg human body weight) (Kirin 2012).

Table 1

Samples of Czech beers and their ingredients type used in the present research.

Beer trade mark	Named in the manuscript	Beer type/ingredient origin
Pilsner Urquell	Pilsner Urquell	Blond
Staropramen Lezak	Staropramen Lezak	Blond
Staropramen Cerny Lezak	Staropramen Cerny	Stout
Staropramen Nealko	Staropramen Nealko	Alcohol-free
Staropramen Nefiltrovany	Staropramen Nefiltrovany	Blond
Lobkowicz Lezak Premium	Lobkowicz Lezak	Blond
Lobkowicz Cerny Premium	Lobkowicz Cerny	Stout
Lobkowicz Nealkoholicky Premium	Lobkowicz Nealko	Alcohol-free
Lobkowicz Psenicny Premium	Lobkowicz Psenicny	Wheat
Budweiser Budvar B:Original, Svetly lezak	Budweiser Lezak	Blond
Budweiser Budvar B:Free, Nealkoholicke pivo	Budweiser Nealko	Alcohol-free
Budweiser Budvar B:Cherry, Tmavy lezak s visni	Budweiser Cherry	Cherry flavoured
Herold Svétly Breznicky Lezak	Herold Lezak	Blond
Herold Tmave Speciálni Pivo	Herold Cerny	Stout
Herold Psenicny Kvasnicovy Lezak	Herold Psenicny	Wheat
Herold Polotmave Specialni Pivo	Herold Polotmave	Pomegranate flavoured
Rest of mash(1)	Rest of mash	CULS(8) Prague brewery
Blond malt	Blond malt	CULS Prague brewery
Dark malt	Dark malt	CULS Prague brewery
Sladek hop(2)	Sladek hop	CULS Prague brewery
Saazer hop(3)	Saazer hop	CULS Prague brewery
Saccharomyces uvarum(4)	*S. uvarum*	CULS Prague brewery
Wort(5)	Wort	CULS Prague brewery
Hopped wort(6)	Hopped wort	CULS Prague brewery
Young beer(7)	Young beer	CULS Prague brewery

(1) Rest of malt after boiling process.

(2) Hybrid aroma varieties of hop.

(3) Fine aroma hop.

(4) Lager yeasts sink to the bottom of the beer and ferment more slowly, preferring colder temperatures around 7–15 °C (Goldammer 2000).

(5) Sugar solution obtained after filtration of barley malt boiling.

(6) Liquid obtained after addition and boiling of hops to the wort, cooling and removal of spent hops.

(7) Liquid obtained after yeast addiction and fermentation during a week.

(8) Czech University of Life Science.

2.2. *In vivo* animal model and *in vitro* cellular culture used

2.2.1. *Drosophila melanogaster*

Two *Drosophila* strains were used, each with a hair marker in the third chromosome:

• *mwh/mwh*, carrying the recessive mutation *mwh* (*multiple wing hairs*) that in homozygosis produces multiple tricomas per cell instead of one per cell (Yan et al. 2008).

• flr^3*/In (3LR) TM3,* $rip^p sep\ bx^{34e} e^s Bd^S$, where the flr^3(*flare*) marker is a homozygous recessive lethal mutation that produces deformed tricomas but is viable in homozygous somatic cells once larvae start the development (Ren et al. 2007).

Strains were maintained at 25 °C and 80% humidity in glass tubes with homemade meal (0.5 g NaCl, 12 g agar-agar, 100 g yeast, 25 g sucrose, 5 ml propionic acid, 3.5 ml of a 0.2% sulphate streptomycin solution and 1 litre of water) making changes three times per week.

Virgin females were obtained from these tubes, and were mated in order to perform the crosses for the different assays of toxicity, antitoxicity, genotoxicity, antigenotoxicity and longevity.

2.2.2. HL-60 promyelocytic human leukaemia cells

Cells were grown in RPMI-1640 medium (Sigma, R5886) supplemented with heat-inactivated foetal bovine serum (Linus, S01805), L-glutamine at 200 mM (Sigma, G7513) and antibiotic-antimycotic solution (Sigma, A5955). Cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 (Gallagher et al. 1979) in a Shell Lab CO_2 incubator. The cultures were plated at 2.5 x 10^4 cells/ml density in 10 ml culture bottles and replaced three times per week.

2.3. Safety *in vivo* assay: Toxicity

The percentage of the number of individuals born in each treatment with respect to the number of individuals born in the negative control were analysed at the following ranks of concentrations for the different beers: 3.125–50 mg/ml. For malts, hops and yeast the concentrations used were those equivalent to the quantity of each compound in the full beer (0.0625–1 mg/ml for blond and dark malts; 0.00156–0.025 mg/ml for Sladek hop; 0.000625– 0.01 mg/ml for Saazer hop; 0.00023–0.00375 mg/ml for *S. uvarum*). The negative controls were prepared with medium and distilled water.

2.4. Protection *in vivo* test: Antitoxicity

The antitoxicity tests consisted in the study of the viability percentage of *Drosophila* treated with the same concentrations as in toxicity assays combined with the genotoxicant H_2O_2 at 0.12 M (Tasset-Cuevas et al. 2013). The positive controls were prepared with medium and 0.12 M H_2O_2 as an oxidative genotoxicant.

2.5. Chemopreventive *in vitro* tests

2.5.1. Cytotoxicity assay

HL-60 cells were placed in 96 well culture plates (2 x 10^4 cells/ml) and treated for 72 h with the different beers (1.25–250 mg/ml) and the equivalent concentrations for the raw materials (0.156–20 mg/ml for both malts; 0.00097–0.125 mg/ml for Sladek hop; 0.00039–0.05 mg/ml for Saazer hop; 0.00029–0.0375 mg/ml for *S. uvarum*).
This wide range was selected in order to assess the cytotoxic doses ranging the inhibitory concentration 50 (IC_{50}) when possible. This allows the assessment of a wide range of concentrations in the *in vitro* cytotoxicity assays, with the aim to predict acute *in vivo* lethality.

2.5.2. Tumour cells DNA damage evaluation

2.5.2.1. DNA fragmentation

HL-60 cells (1 x 10^6/ml) were treated with different concentrations of blond and stout selected lyophilised beers (1.25–250 mg/ml) and its equivalent concentration for the raw materials (0.156–20 mg/ml for both malts; 0.00097–0.125 mg/ml for Sladek hop; 0.00039–0.05 mg/ml for Saazer hop; 0.00029–0.0375 mg/ml for *S. uvarum*) for 5 h.
Due to the similar pattern observed for the different beers studied in the *in vivo* assays, we selected a blond and a stout beer to study the DNA damage evaluation. They were chosen according to today's most known and oldest Czech breweries: Pilsner Urquell (1842) and Smichov Staropramen (1869) (Kozák 2013).

Treated cells were collected and centrifuged at 3000 rpm for 5 min, and DNA was extracted according to the procedure described in Merinas-Amo et al. (2016b). Briefly, cell pellet was resuspended in lysis buffer and incubated in SDS 10% and proteinase K solution. Steps of precipitation with NaCl and isopropanol were followed by a 70% ethanol DNA washing step, and finally a RNAse incubation step over night.
DNA was quantified in a spectrophotometer (Nanodrop ND-1000) and 1200 ng of DNA was subjected to a 2% agarose gel electrophoresis at 85 mA for 25 min, stained with GelRed™ and visualized under UV light.

2.5.2.2. Comet assay

Cells were treated with different concentrations of lyophilised beers (7.56, 31.25 and 62.5 mg/ml) and its equivalent concentrations for the raw materials (0.625, 2.5 and 5 mg/ml for both malts; 0.0039, 0.0156 and 0.03125 mg/ml for Sladek hop; 0.00156, 0.00625 and 0.0125 mg/ml for Saazer hop; 0.00117, 0.0047 and 0.0094 mg/ml for *S. uvarum*) for 5 h. Like in the DNA fragmentation assay, we selected a blond (Pilsner Urquell) and a stout (Staropramen Cerny) Czech beer for this study.

After washing steps in PBS, a concentration of 6.25 x 10^5 cells/ml were mixed with 0.75% low melting point agarose (Sigma, A4018) and transferred to frosted-end slides. Steps of lysis, alkaline electrophoresis, neutralization and dried were carried out according to Mateo-Fernández et al. (2016) protocol. Finally, DNA of 50–100 single cells was visualized by treating slides with 7 µl of a 10 µg/ml stock solution of

propidium iodide (Sigma, P4170). Comet images were analysed at 400x magnification using a Leica DM 2500 fluorescence microscope with green filter and an attached camera (JAI CV-M4CL). The OpenComet plugging from ImageJ (NIH) was used to score individual parameters for each cell.

2.6. Statistics

2.6.1 Toxicity and Antitoxicity

Chi-square test in a Microsoft Office Excel 2007 was used to determine if the tested compounds significantly affected the survival of flies. Negative control values were considered as those expected in Chi-square formula used in toxicity assay. A total of 300 untreated 72-hour-old larvae from three independent experiments were treated with different concentrations of lyophilised Czech beers, raw materials and processed-beers.

2.6.2. Cytotoxicity

Cell viability was determined by the trypan blue dye (Sigma, T8154) exclusion test. Trypan blue was added to the cells at a 1:1 volume ratio and 20 µl of cells suspension was loaded into a Neubauer chamber. The cells were counted with an inverted microscope at 100X magnification (AE30/31, Motic). Curves were plotted as survival percentage of three independent experiments with respect to the control growing for 72 h.

2.6.3. Comet assay

Statistical analysis of data was performed using SPSS Statistic 17.0 software. The Tail Moment data were evaluated by a one-way ANOVA. The post hoc Tukey's test was applied to asses on the effect of the compounds on HL-60 cell DNA integrity. Statistical significance was considered at $p \leq 0.05$ was.

According to Fabiani et al. (2012), the frequencies distribution of DNA damage can be divided into five classes depending on the TM values as follows: class 0 (TM < 1; no damage), class 1 (TM 1–5; slightly damaged), class 2 (TM 5–10; medium damage), class 3 (TM 10–20; highly damaged) class 4 (TM >20; completely damaged).

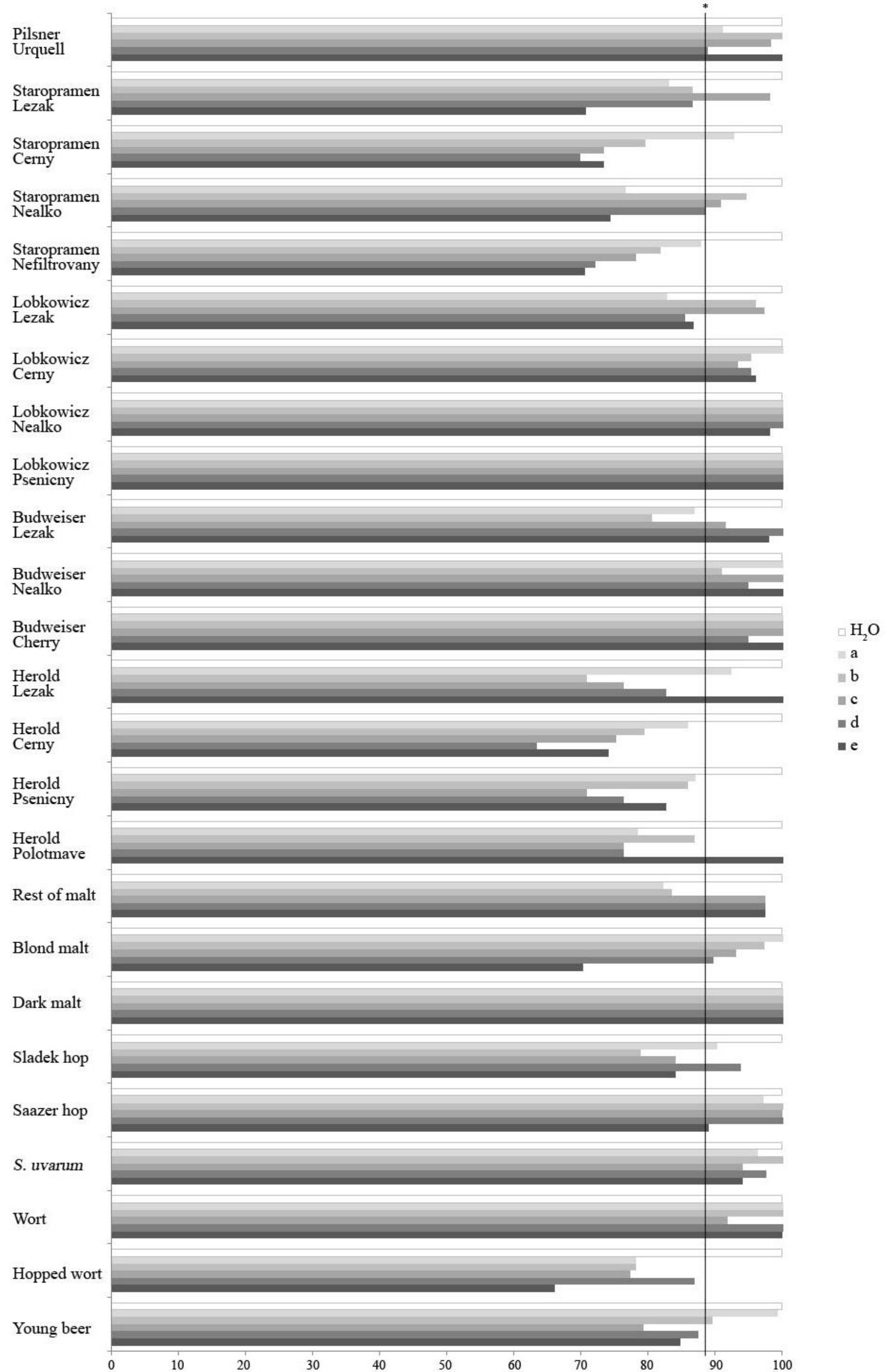
3. RESULTS AND DISCUSSION

3.1. Safety

Toxicity of Czech lyophilised beers, raw material and processed-beer has been assessed in the *Drosophila in vivo* model. Figure 1 shows the relative percentage of emerging adults after treating larvae with different concentrations of these substances. None of the substances reached the LD_{50} thus all of them can be considered non-toxic in the *Drosophila* animal model. Nevertheless, viability rates were different.

Figure 1

Toxicity levels of lyophilised Czech beers, raw materials and processed-beers in *Drosophila melanogaster*.



Data are expressed as percentage of surviving adults with respect to 300 untreated 72-hour-old larvae from three independent experiments treated with different concentrations of lyophilised Czech beers, raw materials and processed-beers. *: Chi-square value 3.84 ($p < 0.05$). Value a, b, c, d and e correspond to concentration of 3.125, 6.25, 12.5, 25 and 50 mg/ml for beers and it respective concentration for each single component.

The highest reduction of *Drosophila* viability caused by the tested beer samples and their constituents reached a 70% of survival, compared with the control. Taking into account that a toxic effect is considered when survival reaches a reduction under of 50% of survival, all our samples exhibited a non-toxic/safe effect on the model animal. On the other hand, if the survival of flies is analysed with a $p < 0.05$ (Chi-square value of 3.84) results showed different patterns: i) samples with a safety potential significantly similar to the control in all the concentrations (Pilsner Urquell; Lobkowicz Cerny; Lobkowicz Nealko; Lobkowicz Psenicny; Budweiser Nealko; Budweiser Cherry; Dark malt; Saazer hop; *S. uvarum* and Wort); ii) samples with safety potential at lowest/medium concentrations (Staropramen Cerny; Herold Lezak; Blond malt; Sladek hop and Young beer); iii) samples with safety properties at highest/medium concentrations (Rest of mash; Herold Lezak; Herold Polotmave; Budweiser Lezak; Sladek hop); iv) samples with safety properties at medium concentrations (Staropramen Lezak; Staropramen Nealko; Lobkowicz Lezak) and v) samples with no safety properties at any concentrations with respect to the control (Staropramen Nefiltrovany; Herold Cerny; Herold Psenicny; Hopped wort).

Many researches highlight the healthy value and potential uses of beers and its components, some of them are those derived from raw materials (water, malts, hops and yeast) and pass unchanged through the brewing process, and some others are produced during the process (Buiatti 2009). Chen et al. (2013) emphasised the potential uses of beer compounds in dermatology such as treatment of atopic eczema, contact dermatitis, pigmentary disorders, skin infections, skin ageing, skin cancers and photoprotections.
Sánchez et al. (2010), highlight the common use of hops as tranquillizer plant. Furthermore, many beer compounds can normalize the rhythm of the sleep in addition to stimulate it due to its bitter acids, particularly the alpha acid components (Chen et al. 2013). Furthermore, there are accumulated evidences that support the health benefits of moderate consumption of alcohol as part of a healthy lifestyle, associated with lower rates of cardiovascular diseases (Rimm et al. 1991), or the ability against kidney stones formation (Curhan et al. 1996). Vinson et al. (2003) showed that polyphenols present in both lager and dark beer inhibited atherosclerosis, decreased both total cholesterol and triglycerides in serum, acted as *in vivo* antioxidant by decreasing the oxidizability of low-density lipoprotein cholesterol and decreased atherosclerosis in cholesterol-fed hamsters. In contrast, other studies show evidence that alcohol increases the risk of mouth, oesophageal and liver cancers (Research and International 1997).

According to our best knowledge, this is the first study of toxicity/antitoxicity of Czech beers using *D. melanogaster*. Some studies performing the Ames test in other beers suggest the possibility that the antimutagenic activity attributed to beer is a result of a cumulative effect of the individual beer constituents (Arimoto-Kobayashi et al. 1999). Moreover, phenolics and some B vitamins are argued to be the health promoter compounds of beer in reference to degenerative diseases (Gerhäuser 2005). Our results agree with alleged safety properties of beer and its components adding more

information about the promising properties that specifically Czech beers show as nutraceutic substances in a model organism.

3.2. Antitoxicity

The antitoxicity assays revealed the ability of the Czech lyophilised beers, raw materials and processed-beers to protect the individuals against an oxidative stress at the tested concentrations. Figure 2 shows that the positive control H_2O_2 is toxic at 0.12 M with an average survival rate of 39.2% with respect to the water control.

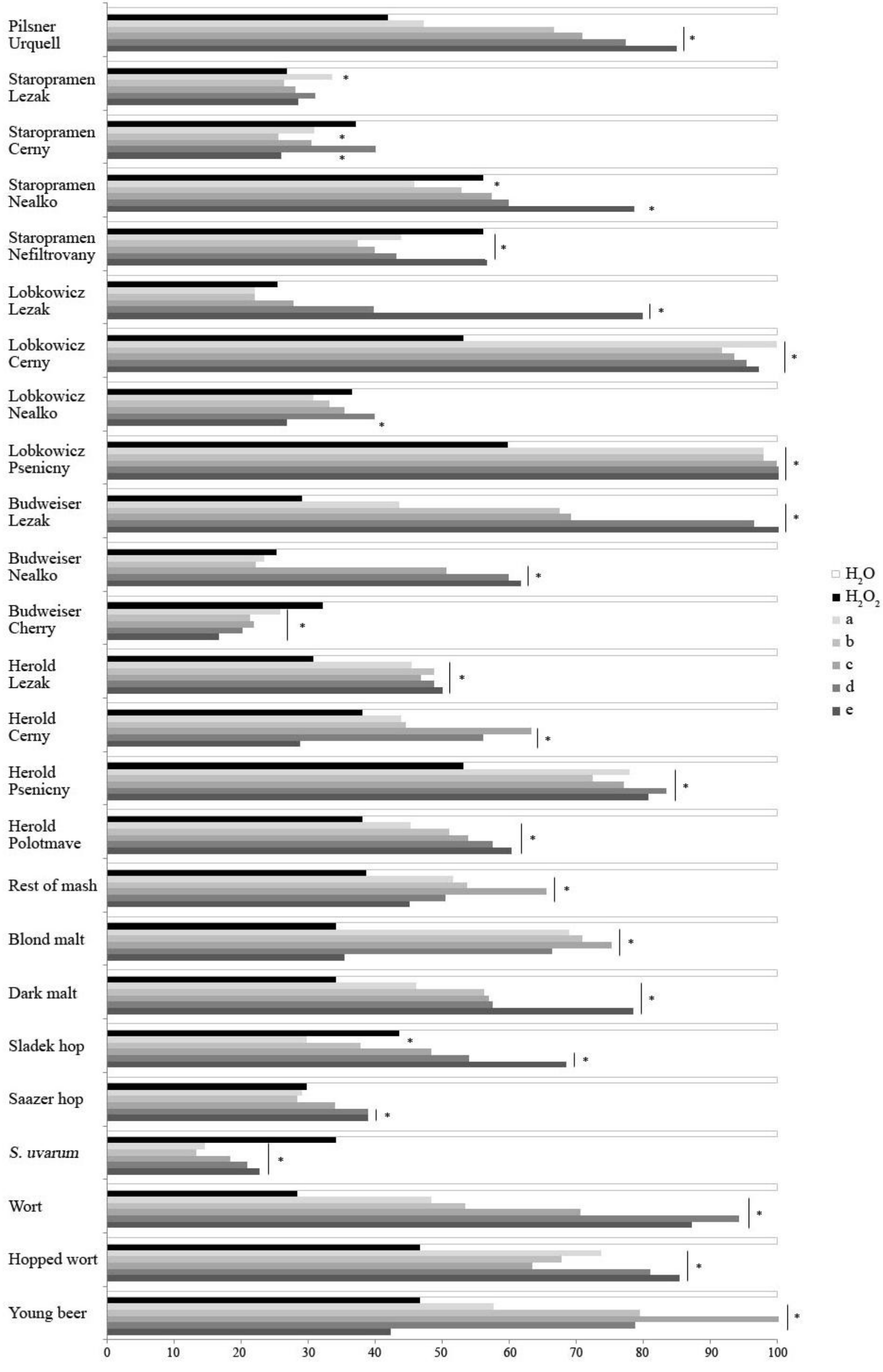
All compounds showed a protective effect with respect to their concurrent control except *S. uvarum*, Budweiser Cherry and Staropramen Nefiltrovany at all the tested concentrations. Moreover, not antitoxic activity against H_2O_2 was showed at lower concentrations of Sladek hop, Staropramen Nealko and Staropramen Cerny; and at higher concentrations of Herold Cerny, Lobkowicz Nealko and Staropramen Cerny. On the other side, a significant protective effect are shown at combined treatments with Pilsner Urquell, Lobkowicz Cerny, Lobkowicz Psenicny, Budweiser Lezak, Herold Lezak, Herold Psenicny, Herold Polotmave, rest of mash, blond malt, dark malt, wort, hopped wort and young beer; at lower concentration of Staropramen Lezak; at medium/higher concentration of Herold Cerny; and at higher concentration of Staropramen Nealko, Lobkowicz Lezak, Sladek hop, Saazer hop, Budweiser Nealko.

Because of the ability of beers to increase antioxidant capacity in humans, many studies are focused in the evaluation of their possible health benefits, since oxidative stress and consequently the presence of reactive oxygen species are closely associated with diverse types of diseases (Halliwell and Gutteridge 2015).

It is known that the antioxidant capacity of beer is mainly derived from polyphenols, Maillard compounds and vitamin C from malt and hops (Callemien and Collin 2010; Callemien et al. 2005; Goupy et al. 1999; Vinson et al. 1999; Woffenden et al. 2001) (Saura-Calixto et al. 2008; Vinson et al. 1999). Moreover, the Maillard reaction can cause both deterioration of food quality (Friedman 1996; Henle 2005; Silván et al. 2006), by the formation of anti-nutritional and toxic Maillard reaction products (MRPs) with mutagenic (Yen et al. 1993), carcinogenic (Yen and Tsai 1993) and cytotoxic (Vagnarelli et al. 1991) effects. Our results agree with this fact as both malts and hops showed protective effects against H_2O_2 with respect to their concurrent controls, except the lowest concentration of Sladek hop tested.

Figure 2

Antitoxicity levels of lyophilised Czech beers, raw materials and processed-beers in *Drosophila melanogaster*.



Data are expressed as percentage of surviving adults with respect to 300 untreated 72-hour-old larvae from three independent experiments treated with different concentrations of lyophilised Czech beers, raw materials and processed-beers combined with 0.12 M H_2O_2. *: Chi-square value higher than 3.84 ($p < 0.05$). Value a, b, c, d and e correspond to concentration of 3.125, 6.25, 12.5, 25 and 50 mg/ml for beers and it respective concentration for each single component.

The antioxidant power of beer depends on a number of parameters involved in brewing such as the variety of malt and malting process, temperature and pH during mashing, sparing, boiling, the variety of hops added during wort boiling and yeast fermentation (Saura-Calixto et al. 2008). According to Rivero et al. (2005), dark beer obviously contains more of antioxidant compounds than lager or alcohol-free beer, since it is precisely that they are responsible for its characteristic colour. Moreover, the antioxidant capacity of alcohol-free beer is considerably lower than that of lager (Saura-Calixto et al. 2008). On the other hand, no relationship has been found between the antioxidant capacity of different phenols and the degree of browning (Morales and Babbel 2002). Our results did not allow to establish any pattern about the characteristic of beers and their antioxidant potential: a stout, an unfiltered, an alcohol-free and a special cherry beer, together with *Saccharomyces* were the only four substances among a total of 26 samples studied which did not showed protective effect against the hydrogen peroxide oxidative model toxin. We hypothesised that antioxidant activities showed by beers are caused by the synergic activities of the different phenols that compose them.

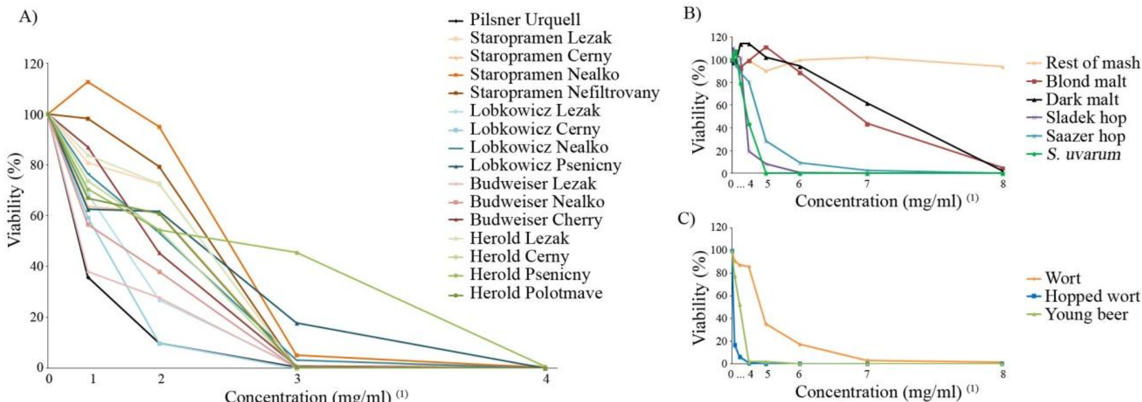
The antioxidants present in hops are also important for the brewing industry. A study confirms the different composition of both Saazer and Sladek hop resins (Krofta 2003). Saazer fine aroma hops have a similar composition of hop resins owing to genetic affinity. The content of α-bitter acids ranged between 3–4.5% w/w and is lower than the content of β-bitter acids (4.5–6.5% w/w). Sladek hybrid aroma varieties hop resins have a composition of 4–8% w/w of α-bitter acids and 3.5–8% w/w of β-bitter acids. Moreover, different relative percentage of antioxidant activity is observed in these hops which is correlated with the content of polyphenol substances (Krofta et al. 2008): the highest antioxidant activity within 70 to 80% rel. is exhibited by the variety Saazer, followed at a relatively considerable distance by Sladek variety with an antioxidant activity within 40 to 60% rel. Our results agree with this characterisation of hops, showing a stronger antitoxic potential for Saazer hop at lowest concentrations tested.

3.3. Cytotoxicity

All substances assayed showed cytotoxic activity against HL-60 cells except rest of mash which did not reach the inhibitory concentration 50 (IC_{50}) at any assayed concentration (Figure 3).

Figure 3

Effect of Czech lyophilised beers, raw materials and processed-beers on cell viability.



Viability in promyelocytic human leukaemia cells (HL-60) treated with different concentrations of Czech lyophilised beers (A), raw materials (B) and processed-beers (C) for 72 h. Each point represents the growing percentage with respect to its control. Values are mean from three independent experiments. [(1)] value 1, 2, 3, 4, 5, 6, 7 and 8 correspond to concentration of 1.95, 3.9, 7.81, 15.625, 31.25, 62.5, 125 and 250 mg/ml for beers and it respective concentration for each single component.

According to the IC_{50}, Czech lyophilised beer are classified as follow: i) Pilsner Urquell and Budweiser Lezak with a half maximal inhibitory concentration lower than 1.25 mg/ml; ii) Lobkowicz Cerny followed by Budweiser Nealko, then Lobkowicz Lezak and finally Budweiser Cherry, with a IC_{50} between 1.25 to 3.5 mg/ml; iii) Lobkowicz Nealko and Herold Cerny followed by Staropramen Cerny and Herold Polotmave, then Lobkowicz Nefiltrovany, followed by Herold Lezak, then Staropramen Nefiltrovany and finally Staropramen Nealko and Herold Psenicny with a IC_{50} ranged between 3.5 to 7.81 mg/ml. Despite this ranking, all beers were able to completely inhibit the tumour cells growth in a concentration that ranged between 7.81 to15.625 mg/ml. The most cytotoxic raw material is Sladek hop followed by *S. uvarum*, Saazer hop, blond malt and dark malt reaching an IC_{50} at concentrations lowest than the corresponded to 15.625 mg/ml in beer.

Scientific studies indicate the cancer-prevention activity of beer. However, considerable variations in inhibitory actions on tumours formation are observed among brands of beer, which may be due to the contents of the different components of beer (malt and hops extracts) and their antioxidant properties (Gerhäuser 2005; Hevia et al. 2008; Stevens and Page 2004).

In vivo studies by Nozawa et al. (2004) demonstrated that a moderate consumption of beer significantly reduced the formation of colorectal tumours and preneoplastic lesions in azoxymethane induced experimental carcinogenesis in male Fischer rats. Furthermore, studies with a type of lyophilised blond lager beer showed a chemopreventive dose dependent response, reaching an IC_{50} at 125 mg/ml in the HL-60 cell line and an IC_{50} at 25 mg/ml in the immortal NIH3T3 cells (Merinas-Amo et al. 2016b).

Our results agree with the cytotoxic effects of beers, showing that Czech beers exhibit a stronger chemopreventive potential compared to other brands of beers due to its reached a IC_{50} at lower concentrations than other beers.

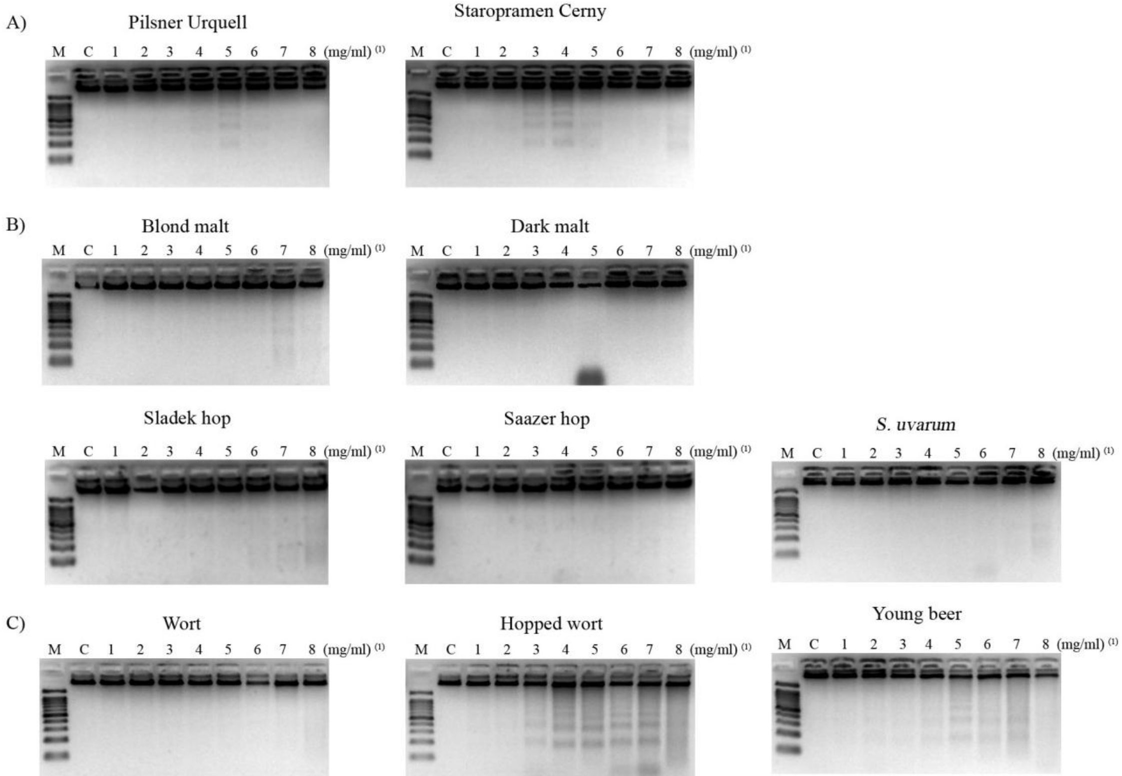
3.4. DNA fragmentation

According to Wyllie et al. (1980), DNA internucleosomal fragmentation is represented by a DNA laddering which is associated with the activation of the apoptotic way in cancer cells, being a hallmark of the genomic integrity. Figure 4 shows the electrophoresis of the genomic integrity in HL-60 cells treated with different concentrations of lyophilised blond and stout Czech beers, raw materials and processed-beers. DNA internucleosomal fragmentation is represented by a DNA laddering and it is associated to the activation of the apoptotic way in cancer cells.

Pilsner Urquell showed a slight patter of fragmentation when cells are treated at concentration of 15.625, 31.25 and 62.5 mg/ml. Moreover, Staropramen Cerny beer induced DNA damage in an apoptotic way at 7.81, 15.625 and 31.25mg/ml concentrations (Figure 4A). With respect to raw material, any of them showed DNA damage at the concentration tested (Figure 4B), except blond malt and *S. uvarum* that induced a very light patter of fragmentation at concentration 10 and 0.375 mg/ml (lane 7 and lane 8 in the agarose gel, respectively). Finally, processed-beer showed DNA fragmentation in a dose-dependent manner for hopped wort and young beer (Figure 4C).

A similar study with another brand of a lyophilised blond Lager beer showed that beer induced DNA fragmentation damage at 15.625, 31.25 and 62.5 mg/ml concentrations (Merinas-Amo et al. 2016b). The results obtained are similar to the Czech beers ones.
Moreover, our study with the raw materials and processed-beers allow us to suggest possible correlations between the modifications of beer and its compound properties during the brewing development. The most abundant phenols during the hopped wort process are xanthohumol and humulone, hop-phenols characterised by an induction of DNA damage as other researches indicated (Merinas-Amo et al. 2014; Merinas-Amo et al. 2016b). During the beer maturing, the characteristic of saccharomyces and other phenols are involved in the final outcome of beers, like ferulic acid, isoxanthohumol, vanillic acid, tyrosol, epicatechin gallate, among others reducing the proapoptotic DNA fragmentation activity as matured beers showed (Merinas-Amo et al. 2016a).

Figure 4
DNA-induced damage induced by internucleosomal DNA fragmentation in promyelocytic HL-60 cells treated with different concentrations of lyophilised blond and stout Czech beers (A), raw materials (B) and processed-beers (C) for 5 h.



M: DNA size marker; C: Control treatment; [(1)] value 1, 2, 3, 4, 5, 6, 7 and 8 correspond to concentration of 1.95, 3.9, 7.81, 15.625, 31.25, 62.5, 125 and 250 mg/ml for beers and it respective concentration for each single component.

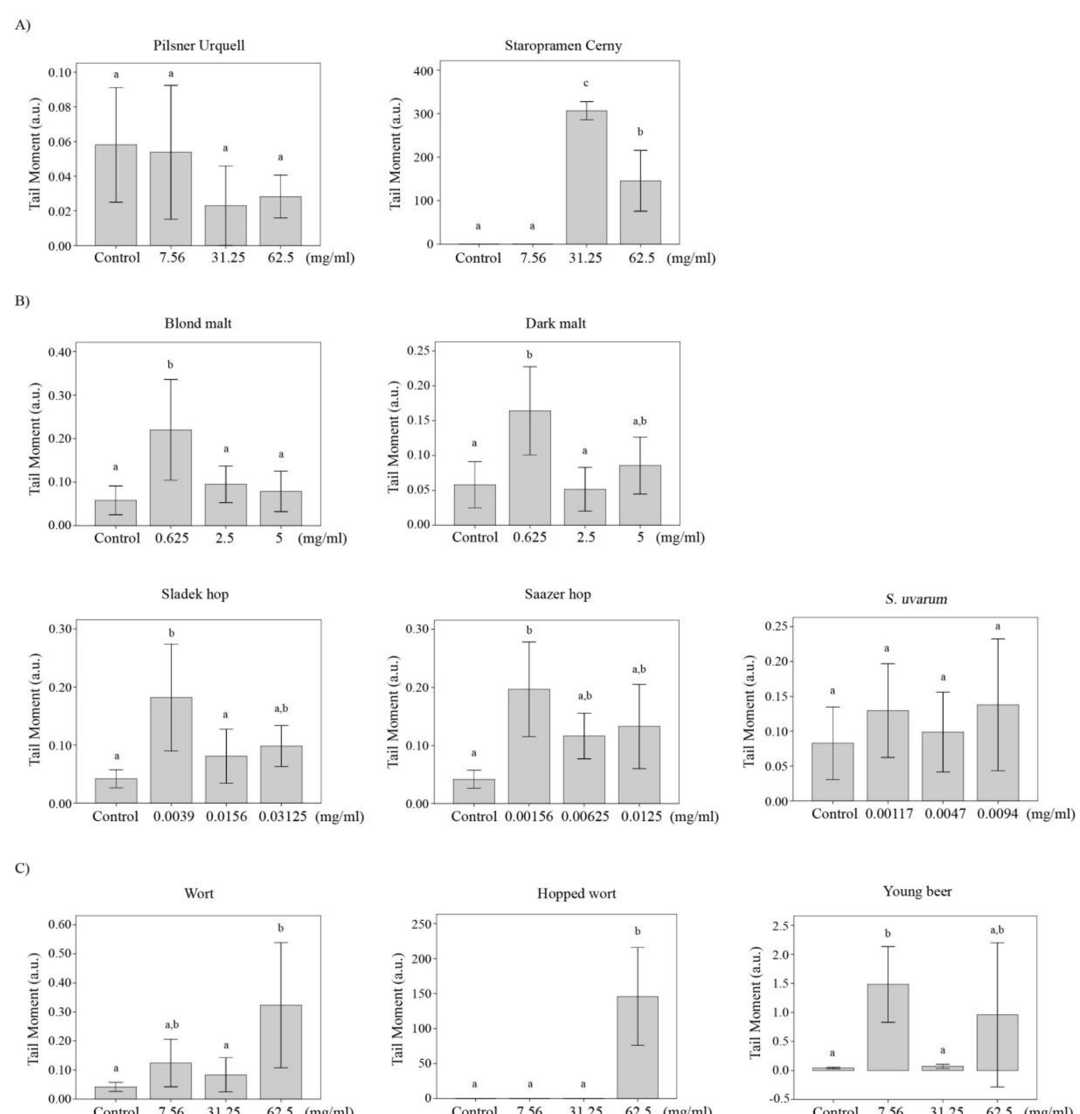
3.5. Comet assay

Results of the tail moment (TM) of HL-60 cells treated with different concentrations of blond and stout Czech lyophilised beers, raw materials and processed-beers are shown in Figure 5.

The comet assay allows to readily identify, at unicellular level, apoptotic nuclei based on the typical morphological changes attributable to extensive DNA damage (Poe and O'Neill 1997). Taking into account this classification of Fabiani et al. (2012), all concurrent controls values fell into class 0 (TM < 1) showing no DNA damage in untreated HL-60 cells. Pilsner Urquell, raw materials and wort did not induced DNA damage at single and/or double strands breaks showing TM values lower than 1. On the other hand, young beer showed a slight damage at lowest concentration assayed (7.56 mg/ml) with TM value included in class 1. Moreover, Staropramen Cerny and hopped wort completely induced DNA damage at medium and highest (31.25 and 62.5 mg/ml) and at highest concentration respectively, with a TM value higher than 100 compared with their concurrent controls.

Figure 5

DNA strand breaks induction in promyelocytic HL-60 cells treated with different concentrations of lyophilised blond and stout Czech beers (A), raw materials (B) and processed-beers (C) for 5 h.



Alkaline comet assay (pH < 13) of HL-60 cells treated. DNA migration is reported as mean TM. Values are mean +/- SE. Different letters in treatments mean differences respect to the negative control after one-way ANOVA and post hoc Tukey's test.

The results of DNA fragmentation fit with those obtained in comet assay since TM values higher than 30 mean that apoptosis mechanisms has been induced (Fairbairn et al. 1995). The concentrations 31.25 and 62.5 mg/ml of Staropramen Cerny and the highest concentration (62.5 mg/ml) of hopped wort tested showed a TM > 30 confirming the death of leukaemia cells by apoptosis as is observed in the lane 5 and 6 of fragmentation (Figure 4). The rest of compounds and concentrations tested showed a light or null DNA damage at double/single strands levels inducing cells death by a necrosis mechanism.

To our knowledge, no studies about the effects of beer and its components at individual cellular level have been carried out. A study with a different brand of blond lager beer by Merinas-Amo et al. (2016b) showed a significant damage with a TM higher than 100 when HL-60 cells were treated with different concentrations of beer. Despite we did not obtain the same results for the lager beer, similar clastogenic pattern is observed in the stout beer and hopped wort at highest concentrations tested. Moreover, the different composition of beers allows us to observe a strongest cytotoxic potential of full Czech beers compared with the studied blond lager beer, but not causing DNA damage at cells during the 5 h of treatment and inducing total cells death at 72 h. It could be mainly because of the raw materials used (water, malt, hop and yeast), as those produced in the Czech Republic area are considered as one of the highest-quality materials in the world.

4. CONCLUSIONS

The new data corps of our study contributes and supports the benefits showed by this beverage, due to its safety, protection against an oxidative toxin, chemopreventive potential and the induction of DNA damage in tumour cells.

Our pilot work results indicate that raw materials and brewing conditions are key points for the final properties of beers. Despite the promising properties showed for all Czech beers, Pilsner Urquell, Budweiser Lezak, Lobkowicz Cerny and Budweiser Nealko were the beverages with the best results obtained in the *in vivo* and *in vitro* assays. The possible cause could be found in the absence of Maillard reaction during the colouring of malt, which could cause both deterioration and enhancement of food quality, by the formation of antinutritional and toxic Maillard reaction products or beneficial compounds with antioxidant capacity.

Furthermore, the biological activities attributed to beer consumption cannot be linked to one particular class of constituents due to their high complexity and variability in the polyphenolic pattern, and also because the brewing is influenced by the raw materials, wort composition, yeast strains and fermentation conditions, among others factors.

We could purpose Czech beers as a drink with a nutraceutical potential although further research is needed to elucidate the contribution of beer to other diets, as well as its potential role in the prevention of chronic diseases, which is associated with the intake of antioxidants.

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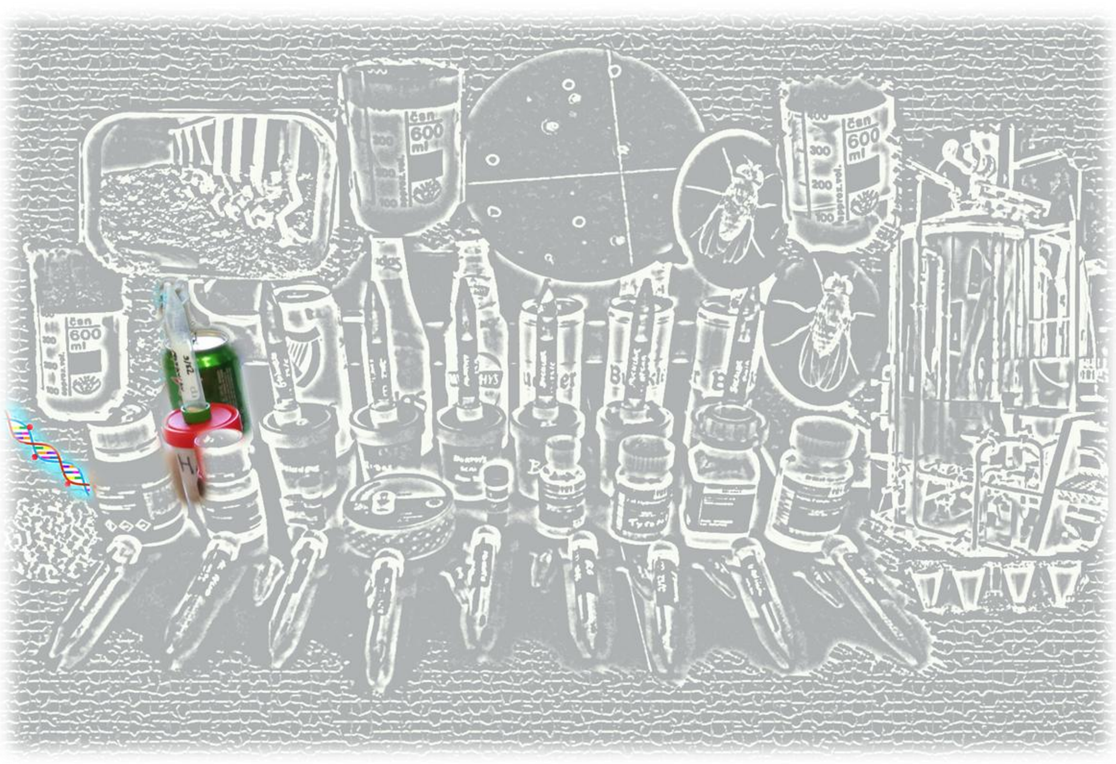
CHAPTER 6



A clinical pilot assay of beer consumption: modulation in the methylation status patterns of repetitive sequences

ABSTRACT
Epidemiological evidences show that moderated beer consumption is associated with the prevention of chronic diseases, including cancer. Epidemiological data suggest a relationship between the intake of antioxidants and cancer prevention, mainly due to the protection against free radicals. The purpose of the present study was to examine the role of a blond Lager beer on the modulation of methylation levels of endoparasitic repetitive sequences in a pilot *in vivo* human clinical trial. The assay was carried out in healthy volunteers aged between 18 and 28. The participants were given 330 ml of beer a day, during a period of 21 days. Peripheral blood samples were taken before and after the treatment period. DNA underwent bisulphite conversion and subsequent quantitative methylation specific PCR was carried out for three Alu, LINE-1 and Sat-α selected repetitive sequences. Results of the genome wide methylation status induced by beer indicated a general induction of methylation levels in first drinker healthy humans. Reversible epigenetic changes have been showed for usually beer-drinkers who decreased their usual daily beer-consumption. In conclusion, the present research enables us to propose beer as a potential nutraceutic beverage.

1. INTRODUCTION

Cancer is a disease characterised by self-sufficiency in growth signal, resistance to growth inhibition, limitless replicative potential, evasion of apoptosis, sustained angiogenesis, and tissue invasion and metastasis (Hanahan and Weinberg 2011). Cancer is caused by many factors and offers a variety of targets for intervention and inhibition at every stage such as antioxidant activity, xenobiotics metabolism, anti-mutagenic, anti-inflammatory and anti-hormonal mechanisms, anti-proliferative activities, induction of terminal cell differentiation and apoptosis (Reddy et al. 2003).

Many cancer cases are related with lifestyle and environmental conditions, and it is estimated that one third of cancer deaths is due to dietary habits (Martin-Moreno et al. 2008). Furthermore, because of the adoption of behaviours and lifestyle factors, such as tobacco, obesity, alcohol, sedentary lifestyle, high-fat diet, radiation, and infections, it is expected an increasing of cancer rate worldwide (Anand et al. 2008; Messina and Hilakivi-Clarke 2009; Shu et al. 2009; Torre et al. 2015). Precisely, the reactive oxygen species that possess potent cytotoxic, mutagenic and carcinogenic effects interact and alter genetic material (Benavente-Garcia et al. 1997; Kaur et al. 2006). Moreover, oxidative stress is involved in the pathology of many other degenerative diseases, such as atherosclerosis, diabetes, neurodegenerative diseases and aging (Saura-Calixto et al. 2008).

Diet is one of the most important environmental factors that exert effect on health and disease risk. A wide range of investigations support the potential role of nutrition at both preventive and therapeutic levels (Esteller 2008; Fraga et al. 2007; Jiménez-Chillarón et al. 2012; Lundstrom 2012; Milner 2008; Milner 2006). Recently, investigations are focused on epigenetic studies in order to develop new drugs with the ability to modulate (modify/revert) the methylation pattern in target genes as a tool to avoid cancer and other diseases, and arguably diet and environment-mediated epigenetic perturbations play a crucial role in cancer progression in humans (Dolinoy et al. 2007; Feinberg 2008; Herceg 2007; Link et al. 2010).

1.1 Diet and cancer

The important role of diet in health is known centuries ago when Hippocrates proclaimed "Let food be thy medicine and medicine be thy food". The development of strategies to use foods and dietary supplements with genetic potential, physical improvement and cognitive performance to reduce the risk of chronic diseases are one of the current main priorities (Kim et al. 2008; Milner 2006; Ordovas et al. 2007; Panagiotakos et al. 2007).

The bioactive food components (estimated more than 25,000 in human consumption) may arise from plants, animal sources, mushrooms or from the metabolism of food components by gastrointestinal bacteria. Many of these compounds have been identified as possible of the cancer process (Milner 2008; Milner 2006). The variety of dietary

constituents may modify, either positively or negatively, cancer risk and tumour behaviour (Fund and Research 2007).

Antioxidants may be found in diet at significant amounts and confer protection against oxidative stress-related diseases (Aruoma 1998). Polyphenols are the most abundant dietary antioxidants in diets, distributed mostly in coffee, fruits, wine, vegetables, olive oil and beer, that regulate multiple cancer-inflammation pathways and epigenetic cofactors, are cost effective, exhibit low toxicity, and are readily available (Clifford 1999; Deorukhkar et al. 2007; Herrmann and Nagel 1989; Lee et al. 2011).

1.2 Epigenetic and cancer

The term 'epigenetics' was first neologised by Conrad H. Waddington in 1942, and it is defined as reversible heritable changes in gene expression that occur without alteration in DNA sequence, but changes that are sufficiently powerful to regulate the dynamics of gene expression (Link et al. 2010). Epigenetic modifications do not take place in the primary DNA sequence, and are therefore potentially reversible, which makes them attractive targets for therapy (Lundstrom 2012). The most common modifications comprise DNA methylations (Fang et al. 2007), histone modifications (Su et al. 2012) and RNA interference (Esquela-Kerscher and Slack 2006).

From a clinical point of view, epigenetics offers a very promising and attractive opportunity for designing optimal chemopreventive and therapeutic strategies because of the epigenetic reversible regulation in early carcinogenesis. Epigenetically modified genes can eventually be restored throughout the lifespan and methylation silenced genes can be demethylated via dietary interventions (Link et al. 2010; Lundstrom 2012; Ong et al. 2012).

DNA methylation at the 5' position of the cytosine residues within CpG dinucleotides through addition of a methyl group is the best-known epigenetic mark (Issa and Kantarjian 2009). CpG dinucleotides often enriched in the promoter regions of genes, as well as regions of large repetitive sequences (e.g. centromeric repeats, LINE and Alu retrotransposon elements) (Bird 2002).

Short CpG-rich regions are also called as "CpG islands", and are present in more than 50% of human gene promoters (Wang and Leung 2004). DNA hypermethylation at the promoter region of many genes is proved to be responsible for silencing of more than 600 cancer-related genes and this number is still rising (Esteller 2007; Mulero-Navarro and Esteller 2008; Suzuki and Bird 2008). They include tumour suppressor genes, oncogenes as well as genes involved in the cell-cycle regulation, DNA repair, angiogenesis, metastasis and apoptosis (Herceg 2007). Oxidative stress (ROS, RNS) and inflammatory damage play an important role in epigenetic reprogramming of expression of cytokines, oncogenes and tumour suppressor genes, thereby setting up a ground for chronic inflammatory diseases and carcinogenesis (Aggarwal and Gehlot 2009; Cyr and Domann 2011; Grivennikov and Karin 2010). On the other hand, global

hypomethylation of the DNA is said to activate endoparasitic sequences and causes the global chromosome instability leading to various mutations and cancer progression (Esteller 2008).

1.3 Nutrition and epigenetic

Diet can influence the degree of methylation by modifying the availability of methyl donors and DNA methyltransferases (DNMTs) activity (Davis and Ross 2008; Hede 2006; Ross 2003). Some biologically active food constituents have been shown to affect the metabolic processes associated with energy metabolism through changes in DNA methylation status of genes directly or indirectly (Link et al. 2010). Nutrients like folic acid, B vitamins and SAM (S-adenosyl methionine) are key components of the methyl-metabolism pathway, and methyl-donating nutrient-rich diet can alter gene expression, especially during early development when the epigenome is first being established (Davis and Ross 2007, 2008; Link et al. 2010).

The use of foods to correct epigenetic defects is currently a new area for drug development in the field of cancer prevention and therapy. This epigenetic therapy could be useful due to the reversible characteristic of epigenetic marks (Miyamoto and Ushijima 2005).

1.4 Our work: Beer as an epigenetic nutraceutical for humans

The main purpose of this pilot study is to evaluate the nutrigenomic effects of beer on the methylation status of genome wide repetitive sequences of healthy humans in order to propose it as a promising biomedical compound.

Nowadays biomedical investigations are focused on epigenetic studies in order to carry out new drugs with the ability to modify the methylation pattern as a tool to avoid cancer and others diseases. The most exciting medical idea in epigenetics is that might be possible to take part in the junction between the genome and the environment, to modify the effects of deleterious gene expression. Different types of cancers are associated with methylation of different combination of tumour suppressor genes ((Feinberg 2008).

Beer is obtained from vegetal natural ingredients, which are related with health effects (Saura-Calixto et al. 2008), although there are conflicting studies about health and beer consumption. It is shown that beer consumption, reduced mortality from coronary heart diseases in moderate alcohol consumers with respect to non-alcohol consumers (Rimm et al. 1991), and that a greater intake of beer have been associated with a decreased kidney stones formation (Curhan et al. 1996). Furthermore, there are accumulated evidences that support the health benefits of moderate consumption of alcohol as part of a healthy lifestyle, associated with lower rates of cardiovascular diseases, linked to polyphenol content (Bassus et al. 2004). In contrast, other studies showed evidences that alcohol increases the risk of mouth, oesophageal and liver cancers (World Cancer Research Fund 1997).

Beer is a low-alcohol drink that contains natural and added antioxidants. Specifically, the consumption of beer contributes to the total intake of polyphenols and to the antioxidant capacity of the diet. Total consumption in Europe was 51,036 thousand kl in 2014 (Kirin 2015), providing about a 7.2% of the daily dietary ingestion of polyphenols and accounts for around 3% of the total antioxidant capacity of the diet (Saura-Calixto et al. 2008). The main natural antioxidants of beer components are polyphenols and melanoidins, particularly phenolic acids from barley (around 70%) and hop (about 30%) (Callemien and Collin 2010; Goupy et al. 1999). Epidemiological studies indicate the anti-proliferative activity of beer components with antioxidant properties in prostate cancer cells, showing a strong correlation between antioxidant potency and polyphenols content (Hevia et al. 2008). Moreover, the antioxidant activity of beer depends on the type, being Lager beer more antioxidant than the dealcoholised beers. Phenolic content and antioxidant activity of beer also depend on the quantity and quality of starting materials and also on the industrial brewing process itself (Piazzon et al. 2010). Low-alcohol beers contain much less polyphenols than the others, partly because these beers are brewed with lower original wort extract, whereas the rest of beers types are brewed with higher wort extract content (Krofta et al. 2008).

Moreover, beer is a very complex beverage rich in nutrients (carbohydrates, amino acids, minerals and vitamins), as well as in non-nutrient components such as phenolic acids and flavonoids (Piendl 1989). Methyl donor substances are included in the beer composition as the B vitamins folic acid and choline (Buiatti 2009). Both of them are associated to genome stability, methylation and DNA repair (Ames 2001; Bailey et al. 2003; Lu et al. 2012; Mason and Levesque 1996), whereas low intake of these compounds is related with adverse effects like increased DNA damage and carcinogenesis (da Costa et al. 2006; Willett et al. 1990). Nevertheless a meta-analysis in humans published by Wien et al. (2012) showed a borderline significant increase in frequency of overall cancer in the groups treated with folic acid compared to controls one, although formers epidemiological studies have shown that folate supplements can significantly reduce the risk of pancreatic cancer (Stolzenberg-Solomon et al. 1999) and breast cancer (Zhang et al. 1999). Precisely, in a previous study with human leukaemia HL-60 cells, treatments with folic acid exhibited a methylation status in the lowest concentration tested (0.099 mM) in Alu and LINE-1 regions and a demethylation status for that concentration in the Sat-α sequences, with respect to the normalised control (Merinas-Amo et al. 2016). Hence a dose controversial exits on folate intakes effects.

Although dietary methyl donors are included in some foods such as green leafy vegetables, peas, beans, certain fruits and beer for folic acid (Saxby et al. 1982); and eggs, liver, chicken, fish, legumes, cruciferous vegetables, milk and soya products for choline (Zeisel et al. 2003), beer is a good source for both folic acid and choline compounds (0.04-0.6 mg/l for folic acid (Buiatti 2009) and 5.7 mg/mg for choline (Howe et al. 2004)). Folate and choline being metabolically interrelated (Zeisel and Blusztajn 1994). Diminished folate availability increases demand for choline as a

methyl donor while decreased choline availability increases demand for folate methyl groups (Kim et al. 1994).

Human trials are necessary to validate the *in vivo* animal and *in vitro* experiences. Attending to gender, it is known that sex plays an important role and has a differential behaviour depending on someone's life style. The physiological differences between men and women such as body size, metabolism and hormones can affect the alcohol consumption and metabolism (Frezza et al. 1990; Sutker et al. 1983): (i) Differences in body water content between men and women: women have less water content than men (52% and 61% respectively). Given that, more alcohol can be diluted into the body of men and women could have more blood alcohol concentrations (BAC); (ii) Alcohol dehydrogenase (ADH): it is present in liver and stomach and it is responsible for metabolizing alcohol. Women ADH activity is lower which means that decomposition of alcohol is less effective than in men body; (iii) Hormonal differences between men and women can also affect the metabolism of alcohol.

The present work shows a preliminary set of data from a pilot study performed in a group of volunteers for a first step evaluation of the biological activity of beer on the human epigenome.

2. MATERIALS AND METHODS

2.1 Samples and sample preparation

The pilot clinical study was carried out in a total of 9 healthy volunteers (six women and three men), aged between 18 and 28. Every participant was given 330 ml of a blond Lager beer per day, during a period of 21 days. Volunteers shall avoid drinking any kind of alcoholic beverages and soft drinks. After informing the patients, they had to fulfil personal details (e.g. lifestyles, dietary habits, exposure to smoking, health) and signed in an informed consent form. At the beginning and at the end of the study a blood extraction was made by a qualified person. According to the Personal Data Protection Law 15/1999, of 13 December, our patient's data remains strictly anonymous.

2.2 Blood extraction

Blood was collected in a 2 ml BD vacutainer tube (BD Vacutainer K2E Plus blood collection tube, Becton Dickinson) and each tube was immediately inverted 8-10x to avoid blood clotting.

2.3 DNA isolation from peripheral blood

Genomic DNA was isolated using a Molecular Bio Laboratory® Genomic DNA Purification Kit which is based on a five-step process. The first step in the purification procedure cells and nuclei were lysed followed by a proteins salt-precipitation step leaving the high molecular weight genomic DNA in solution. In the third step, DNA is precipitated with isopropanol. Next, DNA is washed with ethanol 70% and finally it is hydrated with 65 µl of buffer. DNA isolated must be stored at 4 °C for later use.

2.4 Bisulphite conversion

The EZ DNA Methylation-Gold™ Kit integrates DNA denaturation and bisulphite conversion processes into one-step. The kit is based on a three-step reaction process between cytosine and sodium bisulphite resulting in cytosine being converted into uracil: an amount of 500 ng of our DNA sample, in a 20 µl total volume, is mixed with CT Conversion Reagent and thermo cycled at 98 °C for 10 min and 64 °C for 2.5 h (or 4 °C storage up to 20 h). A binding buffer step following by a wash step is carried out in an IC Column in order to stick the DNA to the binding solution of the column. Then a desulphonation buffer is used during 17 min to eliminate otherwise cumbersome precipitations. Finally a washing buffer step and an elution buffer are used to obtain our bisulphite converted DNA (it should be stored at or below -20 °C for later use).

2.5 Quantitative Methylation-Specific PCR (qMSP)

Bisulphite-modified DNA was used as template for fluorescence-based real-time quantitative Methylation-Specific PCR (qMSP). The final reaction mixture with a total volume of 10 µl consisted of: 2 µl of deionised water, 5 µM of each forward and reverse primer (Isogen Life Science BV), 2 µl of iTaq™ Universal SYBR® Green Supermix (Bio-Rad, it contains antibody-mediated hot-start iTaq DNA polymerase, dNTPs, $MgCl_2$, SYBR® Green I dye, enhancers, stabilizers and a blend of passive reference dyes including ROX and fluorescein) and 25 ng of bisulphite converted genomic DNA. qMSP conditions were as follows: one step at 95 °C for 3 min, 45 cycles at 95 °C for 10 sec, 60 °C for 15 sec, 72 °C for 15 sec, another step at 95 °C for 30 sec following by a 65 °C step during 30 sec and finally a boost step from 65 °C to 95 °C for 95 sec increasing 0.5 °C each 0.05 sec. qMSP was carried out in 48 well plates in MiniOpticon Real-Time PCR System (MJ Mini Personal Thermal Cycler, Bio-Rad) and was analysed by Bio-Rad CFX Manager 3.1 Software. The housekeeping primer Alu C4 was used as a reference to correct for total DNA input, also negative controls and multiple water blanks were used. Each sample was analysed in triplicate.

2.5.1 Primers

Repetitive sequences account for 45% of the human genome (Jordan et al. 2003; Lander et al. 2001). Since interspersed repetitive elements (LINE-1 retrotransposons and Alu sequences) as well as tandem repeat centromeric and juxtacentromeric repeats (satellite sequences) contain numerous CpG dinucleotides, the methylation status of these sequences is relevant to understand and evaluate global DNA methylation status. Alu sequences belong to the SINE family (Short Interspersed Nuclear Elements), and are the most abundant in the human genome, representing 10% of the whole genome (Weisenberger et al. 2005). One million of copies are found in introns regions (Mighell et al. 1997) and had a great influence on the human genome, in particular on its evolution (Häsler and Strub 2006). LINEs (Long Interspersed Nuclear Elements) are juxtacentromeric autonomous retrotransposons and comprise about 21% of the human genome; the most abundant active elements belong to LINE-1 family (Beck et al. 2010). Sat-α (Satellite alpha DNA) repeats are composed of tandem repeats of 170 bp DNA sequences and represent the main DNA component of every human centromere,

constituting about 5% of total human DNA (Waye and Willard 1986). The distribution of the variability of these repetitive sequences on human genome is mapped in Figure 1.

Figure 1
Structural variants (SVs) identified in two humans. SVs mapped onto chromosomal ideograms.

Structural variants mapped onto chromosomal (chr) ideograms. Left side: Log-scale size of an event (events > 1 Mb are drawn at same length, corresponding to the maximum length of a line); unmated insertions and simple insertions are depicted with 1-kb lines; line colours indicate repetitive sequences in +/- 3-kb window of the predicted breakpoint junction: red, SDs; blue, LINEs; yellow, LTRs; green, satellites; black, two or more repetitive elements with equal frequency; gray, no repeat association. Source from Korbel et al. (2007).

All Alu, LINE-1 and Sat-α sequences were obtained from Isogen Life Science (see Table 1 for detailed information).

Table 1

Primers information.

Reaction ID	GenBank number	Amplicon		Forward primer sequence 5' to 3' (N)	Reverse primer sequence 5' to 3' (N)	GC-content (%)	
		start	end			Forward	Reverse
Alu C4	Consensus Sequence	1	98	GGTTAGGTA TAGTGGTTTA TATTTGTAAT TTTAGTA (36)	ATTAACTAAA CTAATCTTAA ACTCCTAACC TCA (33)	25	27.3
Alu M1	Y07755	5059	5164	ATTATGTTAG TTAGGATGG TTTCGATTTT (29)	CAATCGACC GAACGCGA (17)	27.6	58.8
LINE-1	X52235	251	331	GGACGTATT TGGAAAATC GGG (21)	AATCTCGCGA TACGCCGTT (19)	47.6	52.6
Sat-α	M38468	139	260	TGATGGAGT ATTTTTAAAA TATACGTTTT GTAGT (34)	AATTCTAAAA ATATTCCTCT TCAATTACGT AAA (33)	23.5	21.2

Source from Weisenberger et al. (2005).

2.5.2 Statistics

The results of each C_T cycle were obtained from each qMSP. Data were normalised with the housekeeping Alu C4 using the Nikolaidis et al. (2012) and Liloglou et al. (2014) comparative C_T method:

- C_T of the target gene was normalised respect to referent gene (ΔC_T).
- ΔC_T of each experimental sample or reference ($\Delta C_{T,r}$) were compared with ΔC_T of the calibrator sample ($\Delta C_{T,cb}$): $\Delta\Delta C_T$.
- The relative value of each sample is defined by the formula:

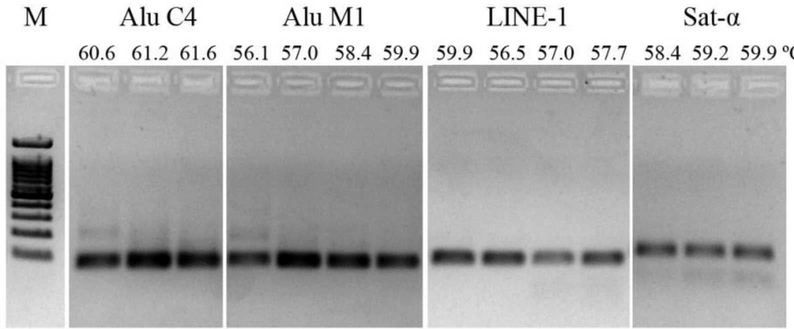
$$2^{-(\Delta C_{T,r} - \Delta C_{T,cb})} = 2^{-\Delta\Delta C_T}$$

3. RESULTS AND DISCUSSION

3.1 Primers studies

In order to optimise the analysis of the methylation status of Alu, LINE-1 and Sat-α repetitive sequences in the same PCR plate in human volunteers, the working temperature of the different primers was assessed (Figure 2). A temperature gradient was established showing that all repetitive sequences were compatibles at 60 °C.

Figure 2
Alu, LINE-1 and Sat-α gradient amplification temperatures.

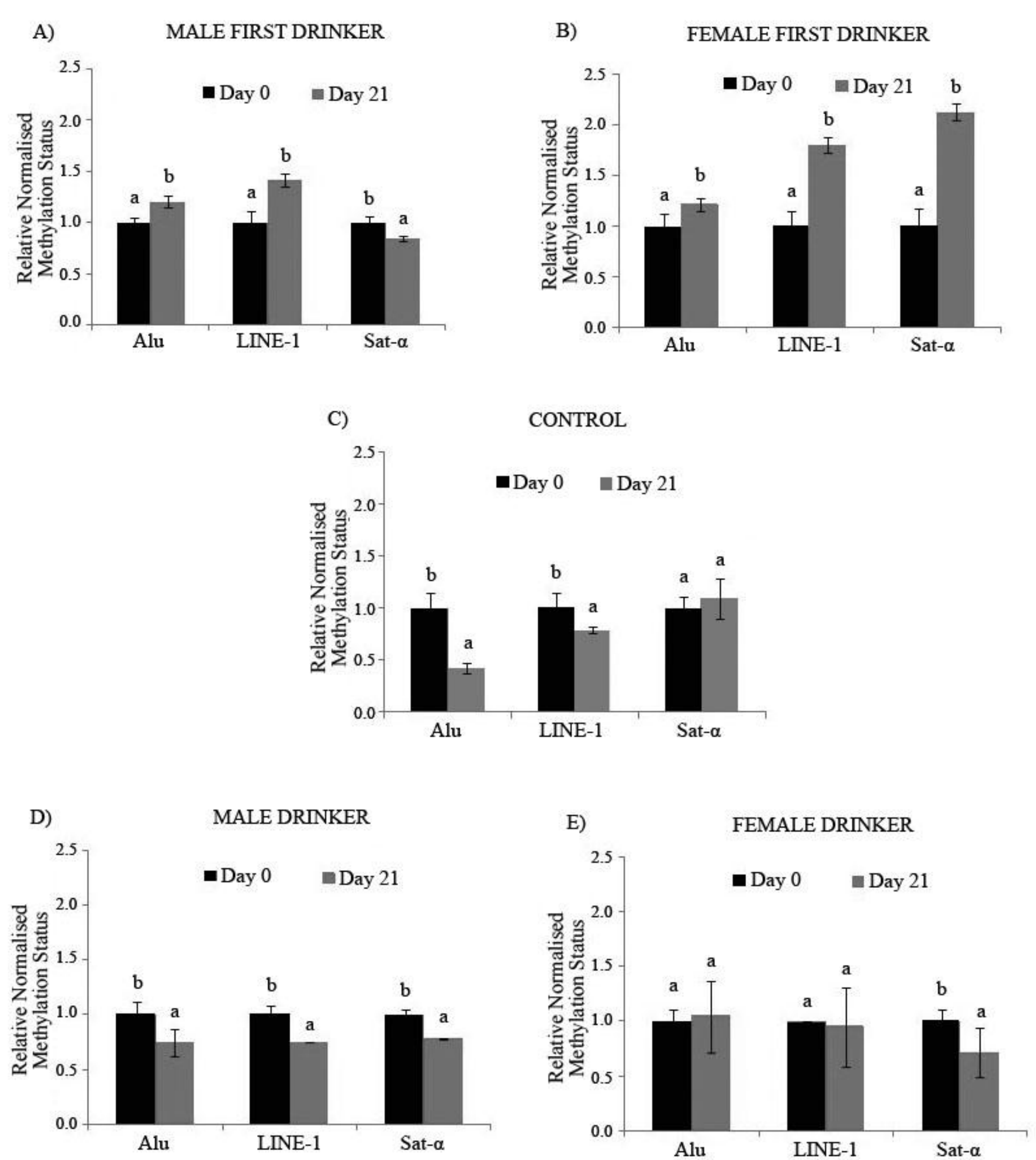


M indicates DNA size marker.

3.2 Epigenetic studies
Figure 3 shows the results for the DNA methylation patterns during the corresponded treatment. Five groups of healthy volunteer with different profiles have been established: male first drinker (A), female first drinker (B), control group (C), male drinker (D) and female drinker (E). The results obtained after 21 days of study showed:

- Male first drinker: this group consisted of non-drinker men that were given 330 ml of blond Lager beer during 21 days. As it is observed in Figure 3A, at the end of the assay both Alu and LINE-1 methylation levels increased, nevertheless, in Sat-α a slight demethylation is observed.
- Female first drinker: this group is composed of non-drinker women that were given 330 ml of blond Lager beer during 21 days. An increased methylation status is observed in all the repetitive sequences studied: in ascending order Alu, LINE-1 and Sat-α (Figure 3B).
- Control group: the volunteers of this group are kept before and during the study without alcohol and soft drinking. As it is observed in Figure 3C, both Alu and LINE-1 have a lower methylation level with reference to their control, whereas Sat-α has remained constant.
- Male drinker: this group of volunteers included those men who usually drink alcohol in their daily life. They were advised to restrict their drinking intake at only 330 ml of blond Lager beer a day. After 21 days, the results in Figure 3D showed an evident deal of demethylation in Alu, LINE-1 and Sat-α sequences. The expression level was down 30% with respect to the controls.
- Female drinker: this group of volunteers included woman who usually drink alcohol in their everyday life. They were advice to restrict their drinking intake at only 330 ml of blond Lager beer a day. After 21 days, the results in Figure 3E showed maintenance of the methylation level in Alu and LINE-1 and a demethylation in Sat-α sequence.

Figure 3
Methylation levels in the different assays-groups in healthy volunteer drinkers.



Relative normalised expression of human DNA methylation treated with blond Lager beer in Alu, LINE-1 and Sat-α repetitive sequences. (A) Male first drinker, (B) Female first drinker, (C) Control, (D) Male drinker and (E) Female drinker.

4. DISCUSSION

The ability of food compounds to influence the epigenome in cancer cells has been studied and has also been related to the individual's risk of developing cancer. Since epigenetic changes can be reversed throughout the human lifespan, the epigenetic focus is a good tool for the dietary prevention/treatment of cancers. Studies are carried out to identify modifiable diet factor with epigenetic value in order to develop epigenetically based prevention strategies (Arasaradnam et al. 2008; Berghe 2012; Bingham and Riboli 2004; Harvey 2008; Parasramka et al. 2012; vel Szic et al. 2010).

Tumoral cells are characterised by chromosome number variation, chromosome inactivation, oncogenes activation, gene silencing and inactivation of DNA repair systems (Jones and Baylin 2007; Roldán-Arjona and Ariza 2009). All these changes have their origin in the variability of genetic sequences and in the alteration of epigenetic marks. DNA methylation is an epigenetic modification of the genome and plays an important role in so-called "epigenetic" regulation of gene expression (Salozhin et al. 2005). The DNA methylation in plants and animals consists of the addition of a methyl group to the fifth carbon position of the cytosine pyrimidine ring via a methyltransferase enzyme in CpG dinucleotides (cytosine-phosphate-guanine) (Robertson 2005). It is generally a unidirectional process and will be inherited after cell division, associated with "closed" (inactive) chromatin state and, therefore, negative regulation of transcription (Antequera and Bird 1993). It is an epigenetic mark that shows the transcriptional gene silencing and plays an important role during development and in the genome defence against transposable elements (Suzuki and Bird 2008). There is great interest to correlate methylation of cytosine with external factors such as diet, life style and some specific clinical conditions in patients suffering cancer that are treated with hypomethylating agents (Yang et al. 2006). It is generally thought that repetitive elements are heavily methylated in normal somatic tissues, but are methylated to a lesser extent in malignant tissues, driving the global genomic hypomethylation commonly found in human cancers. Hypermethylation induced transcriptional silencing of tumour suppressor genes or receptor genes by DNMT inhibitors being a promising target for developing epigenetic drugs (Link et al. 2010; Roman-Gomez et al. 2008). The hypomethylation affecting repeat sequences and transposable elements is believed to result in chromosomal instability and increased mutation events (Weisenberger et al. 2005).

Even though the epigenetic changes in embryonic stem cells have much impact on the global epigenetic status of the organism, those epigenetic changes occurring in adult somatic cells day after day either by stochastic, genetic or environmental factor should be seriously taken into consideration (Aguilera et al., 2010). The maximal differences in DNA methylation profiles observed in aged monozygotic twins with a history of non-shared environments (Christensen et al. 2009; Fraga et al. 2005) highlight this interest. Given the fact that epimutations are related to a broad range of human diseases, the scope of epigenetic therapies is likely to expand in the coming years (Link et al. 2010).

CpG islands are thought to be normally unmethylated and are associated with at least 75% of human genes (Ioshikhes and Zhang 2000). CpG depletion is pronounced in LINE (long interspersed nuclear element) transposons and the LTRs (long terminal repeats) of endogenous retroviruses, which contain, respectively, only 18% and 19% of the CpG sites predicted by nucleotide composition. CpG retention in the SINE (short interspersed nuclear element) class is 41% of the expected value; this reflects the relatively recent accumulation of large numbers of primate-specific Alu SINE transposons, most of which have accumulated in the last 60 million years (Lander et al. 2002). The most severe CpG depletion (15% of expected) is seen in unannotated single-

copy sequences like oldest transposons, pseudogenes, and other sequences that have been evolving under the neutral rate for long periods of time and have been severely eroded by the accumulation of mutations (Rollins et al. 2006).

Our results provide evidences that beer could have a positive effect on quality of life and confer protection against degenerative processes. Clinical assays in healthy human volunteers showed methylation in repetitive sequence, ensuring a pro-healthy state. Moreover, the reversible characteristic of epigenetic has been shown in the usually drinker-volunteers who were restricted their daily beer intake during the trial. No previous studies about effect of beer in the DNA methylation status in human have been reported. In contrast, *in vivo* and *in vitro* studies has recently shown an increase of methylation levels induced by the two methyl donors founds in beer (folic acid and choline). Previous studies with the two vitamins and the blond Lager beer tested has been carried out in a tumoral cell line showing a similar increased patter of methylation of repetitive sequences in the HL-60 cells (Merinas-Amo et al. 2016; Merinas-Amo et al. 2017). These results suggested that beer and its bioactive compounds act as preventive blockers of repetitive sequences of tumoral cells, although different factors such as gender, diet or weight should also considered as important epigenome modifiers. Moreover, studies with dietary methyl deficiency of folate and choline in an animal model showed modifications in hepatic DNA methylation patterns and an induction of liver cancer (Poirier 1994). A study in mice revealed that only with early re-feeding of a methyl sufficient diet during methyl-deficiency-induced hepatocarcinoma in the animal can help mitigate aberrant DNA methylation defects, emphasizing that timing must to be considered in any intervention (Pogribny et al. 2006). On the other hand, some studies indicated that bioactive food compounds such as folate, polyphenols, selenium, vitamin B12, zinc, retinoids, fatty acids, isothiocyanates (ITCs) and allyl compounds affect gene expression through DNA methylation, histone modification processes and miRNA expression (Ho et al. 2011; Ong et al. 2012).

Increased inflammation is strongly associated with cancer (Aggarwal and Gehlot 2009) and, specifically, inflammatory bowel disease has been shown to be a driver of aberrant DNA methylation in the colon (Gunter and Leitzmann 2006; Issa and Kantarjian 2009). One potential mechanism for this effect is through the aberrant methylation of the p53 promoter via up-regulation of DNMT1 gene expression (Hodge et al. 2005; Hodge et al. 2001).

The influence of certain anti-inflammatory dietary elements (polyphenols, isothiocyanates, epicatechins) and micronutrients (folic acid, selenium) on heritable gene expression, activity of the epigenetic machinery, DNA methylation, chromatin remodeling or microRNAs has been demonstrated (Burdge and Lillycrop 2010; Delage and Dashwood 2008; Fang et al. 2007; Hauser and Jung 2008; Huang et al. 2011; Kontogiorgis et al. 2010; Link et al. 2010; Suzuki and Miyata 2006). Although recent evidences are emerging that specific combinations of polyphenols may be far more

effective in protecting against cancer than single compounds (de Kok et al. 2008; Harvey 2008). Our results using a complex mixture like beer support the last evidences.

The new genomic era is delivering a map cancer-associated epigenetic variation, specifically variation in DNA methylation and chromatin states (Brower 2011; Ernst and Kellis 2010; Ernst et al. 2011; Rakyan et al. 2011). The prevalence of reversible epigenetic changes in different cancers allows us to hope in epigenetic therapy for treatment.

5. CONCLUSIONS

Despite further clinical assays must be carried out in order to know the mechanism by which food influence the epigenetic modifications, our results using a moderate daily intake of beer evidence a promising increase of DNA methylation status in repetitive sequences distributed by a wide range of the human genome.

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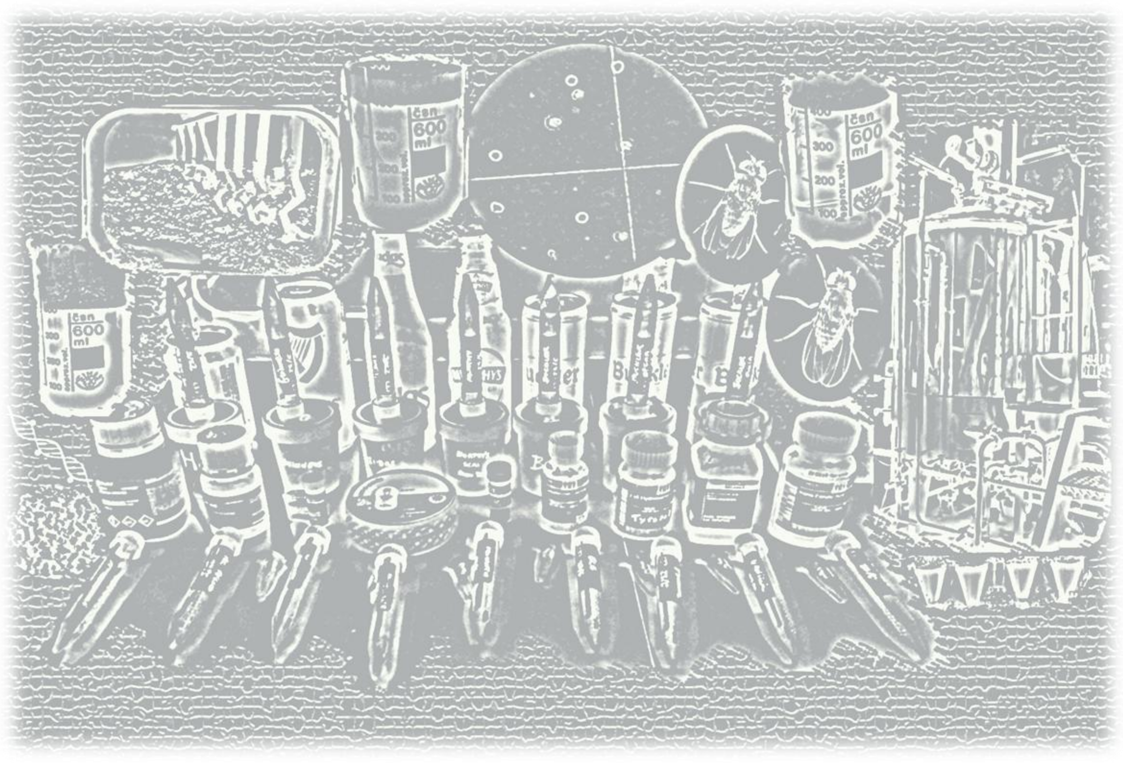
GENERAL DISCUSSION



Beer is one of the oldest beverages in the world and provides a valuable addition to the diet due to its components that confer healthy properties to the drink.

Many researches highlight the healthy value and potential uses of beers and its components, some of them are those derived from raw materials (water, malts, hops and yeast) and pass unchanged through the brewing process, and some others are produced during the process (Buiatti 2009).

Comparisons both *in vivo* and *in vitro* assays, among different lyophilised beers (Table 1 and Table 2), processed-beers and raw materials (Table 3), and beer compounds (Table 4 and Table 5) have been analysed in order to could clarify which components are the responsible of the health properties of beers. Finally, and due to the promising results obtained, a pilot clinical study with human was carried out to obtain new data that supported the properties of beers showed in the *in vivo* and *in vitro* tests.

1. LYOPHILISED BEERS

1.1 *In vivo* assays

Toxicity of beers has been assessed in the *Drosophila in vivo* model. The relative percentage of emerging adults after treating larvae with different concentrations of beers showed a non-significant survival rate compared with the control, exhibiting a general non-toxic effect on the *Drosophila* viability. Exception made for some concentrations of Staropramen Lezak, Staropramen Cerny, Staropramen Nealko, Lobkowicz Lezak, Budweiser Lezak, Herold Lezak and Herold Polotmave Czech beers, and for all tested concentrations of Herold Cerny and Herold Psenicny that exhibited toxic effect on the *Drosophila* viability, although with a survival rate upper to 60% which does not reach the toxic LD_{50}.

The antitoxicity assays revealed the ability of beers to protect the individuals against an oxidative stress with respect to the positive 0.12 M H_2O_2 control, except for Staropramen Cerny, lowest concentration of Staropramen Nealko, Satropramen Nefiltrovany, Losbkowicz Nealko, Budweiser Cherry and highest concentration of Herold Cerny Czech beers.

Results of genotoxicity and antigenotoxicity assays in the SMART test showed that the studied beers have no genotoxic effect at the tested concentrations with respect to the control, except for the lowest concentrations of LPBAFLB and LSAFLB which were able to induce somatic mutations and mitotic recombination in *D. melanogaster*. Moreover, beers were able to protect the individuals against an oxidative stress at tested concentrations with respect to the positive control in an average of 24% inhibition percentage. Most of the protective properties showed by beers were related with the antioxidant activity of its phenols (Granato et al. 2011; Preedy 2011; Tafulo et al. 2010). Arimoto-Kobayashi et al. (2006) evaluated the antigenotoxic potential of beer components against carcinogens contained in the human diet suggesting their protective capacity, suggesting that beer components act in a protective manner against the

carcinogens contained in the diet in *in vivo* system. Other *in vitro* assays (HPRT mutagenicity test and the comet assay) performed in Chinese hamster lung fibroblasts give also anti-genotoxic activities of beer against genotoxicity of heterocyclic aromatic amines (Edenharder et al. 2002).

Survival curves showed either a general similar or significant increase of lifespan expansion for all beers, with respect to their concurrent control, except for LSAB that significantly decreased the lifespan of *Drosophila* with respect to the concurrent control. Furthermore, an improvement of quality of life in *Drosophila* treated with the different assayed beers was shown with respect to the concurrent control, except, again, for the LSAB that shows different/spurious pattern in the healthspan depending on the concentrations tested.

According to our best knowledge, this is the first study using *D. melanogaster* to deeply learn the toxicological and ageing activities that a full drink like beer could show in this model animal.

Table 1
In vivo results of lyophilised beers.

	Toxicity	Antitoxicity	Geno toxicity	Anti-genotoxicity IP (%)(1)	Longevity	Healthspan
LBLB			0.155 0.147	**14.2** **68.8**		
LSAB			0.238 0.273	**48.48** **59.02**		
LBAB			0.154 0.289	**22.68** **57.21**		
LSLB			**0.068** 0.214	**48.75** **47.42**		
LBAFLB			0.276 0.288	**27.32** **42.78**		
LPBAFLB			**0.575** 0.300	**6.44** **29.12**		
LSAFLB			**0.452** 0.375	**16.24** **9.79**		

(continued)

(Table 1 continued)

	Toxicity	Antitoxicity		Toxicity	Antitoxicity
Pilsner Urquell			Lobkowicz Psenicny		
Staropramen Lezak			Budweiser Lezak		
Staropramen Cerny			Budweiser Nealko		
Staropramen Nealko			Budweiser Cherry		
Staropramen Nefiltrovany			Herold Lezak		
Lobkowicz Lezak			Herold Cerny		
Lobkowicz Cerny			Herold Psenicny		
Lobkowicz Nealko			Herold Polotmave		

(1) IP: inhibition percentage. Green colour means positive properties; Red colour means negative properties; Orange colour means positive and negative properties depending on the dose analysed. LBLB: lyophilised blond lager beer; LSAB: lyophilised stout ale beer; LBAB: lyophilised blond ale beer; LSLB: lyophilised stout lager beer; LBAFLB: lyophilised blond alcohol-free lager beer; LPBAFLB: lyophilised pale-blond alcohol-free lager beer; LSAFLB: lyophilised stout alcohol-free lager beer.

1.2 *In vitro* assays

The cytotoxic activity against HL-60 cells showed an inhibition of tumour cells growth for all assayed beers. Despite our convincing results in the human leukaemia cells, there are conflicting reports concerning the relationship between beer consumption and cancer risk. Riboli et al. (1991) have reported that beer consumption is not associated with colon cancer, but Kato et al. (1990) have shown that beer drinkers have an increased risk to colorectal cancer. An inverse association between moderate beer consumption and endometrial cancer was suggested by Swanson et al. (1993) and a connection between beer consumption and lung cancer was suggested by Potter et al. (1992). *In vivo* studies by Nozawa et al. (2004) demonstrated that a moderate consumption of beer significantly reduced the formation of colorectal tumours and

preneoplastic lesions in azoxymethane induced experimental carcinogenesis in male Fischer rats. All the results obtained in the present work with the HL-60 model would drive us in the direction of the chemoprotective activity of lyophilised beers.

DNA internucleosomal fragmentation is represented by a DNA laddering and it is associated to the activation of the apoptotic way in cancer cells being a hallmark of the genomic integrity (Wyllie et al. 1980). Most of beers induced DNA proapoptotic fragmentation patter at concentrations ranged between 15.625–62.5 mg/ml.

By using the comet assay we were able to readily identify apoptotic nuclei based on the characteristic morphological changes attributable to extensive DNA damage (Poe and O'Neill 1997). Base on the Fabiani et al. (2012) classification of nuclei, all control values fell into class 0 (TM < 1) and beers fell into class 4 (TM > 20) at highest concentrations tested. Moreover, the results of DNA fragmentation fit with those obtained in comet assay since TM values higher than 30 mean that apoptosis mechanisms has been induced (Fairbairn et al. 1995). The rest of compounds and concentrations tested induce cell death by a necrosis mechanism.

The relative normalised expressions of three repetitive sequences in HL-60 cells treated with different concentrations of beers showed an overall induction of hypermethylation by beers. Only alcohol-free beers did not showed that patter at the repetitive sequences studied, inducing a hypomethylation status in the DNA treated with LBAFLB and a controversial methylation status in cells treated with LPBAFLB and LSAFLB.

To our knowledge, no previous study with beer has been carried out in order to analyse the induction of DNA damage caused by this beverage in tumour cells.

Additional studies on cytotoxicity and macroautophagy (MA) process in NHI3T3 mouse fibroblast cells were carried out with LBLB and three compounds (choline, folic acid and xanthohumol) in order to know more about their health promoting properties. All compounds showed a similar behaviour pattern as they showed in the cytotoxic assay with HL-60 cells: only LBLB and xanthohumol were able to inhibit immortal cells growth whereas choline and folic acid induced a maintenance or increase of cells growing. With respect to MA, LBLB only induces an increase of MA activity at the lowest concentration tested whereas the rest of concentrations tested induce a high inhibition of MA; folic acid acts as an activator of MA in immortal cells at high concentrations assayed; choline and xanthohumol inhibit MA at any concentration assayed with a more pronounced effect in the case of xanthohumol. Finally, although at our best knowledge no previous studies on MA with beers has been described, we could hypothesise that daily intake of LBLB could protect against lipotoxicity and also we could define xanthohumol as responsible of the inhibitory effect of LBLB at higher concentrations

Table 2

In vitro results of lyophilised beers.

	Cytotoxicity	DNA fragmentation	Comet assay	Methylation status	Macroautophagy
Control					
LBLB					
LSAB					
LBAB					
LSLB					
LBAFLB					
LPBAFLB					
LSAFLB					

(continued)

(Table 2 continued)

	Cytotoxicity	DNA fragmentation	Comet assay		Cytotoxicity
Pilsner Urquell				Lobkowicz Psenicny	
Staropramen Lezak				Budweiser Lezak	
Staropramen Cerny				Budweiser Nealko	
Staropramen Nealko				Budweiser Cherry	
Staropramen Nefiltrovany				Herold Lezak	
Lobkowicz Lezak				Herold Cerny	
Lobkowicz Cerny				Herold Psenicny	
Lobkowicz Nealko				Herold Polotmave	

Green colour means positive properties; Red colour means negative properties; Orange colour means positive and negative properties depending on the dose analysed. LBLB: lyophilised blond lager beer; LSAB: lyophilised stout ale beer; LBAB: lyophilised blond ale beer; LSLB: lyophilised stout lager beer; LBAFLB: lyophilised blond alcohol-free lager beer; LPBAFLB: lyophilised pale-blond alcohol-free lager beer; LSAFLB: lyophilised stout alcohol-free lager beer.

2. PROCESSED-BEERS AND RAW MATERIALS

2.1 *In vivo* assays

Because of the ability of beers to increase antioxidant status in humans, lots of studies are focused in the evaluation of their possible health benefits, since oxidative stress and consequently the presence of reactive oxygen species are closely associated with diverse types of diseases (Halliwell and Gutteridge 2015). These researches support our toxicity results, which showed non-toxic properties by raw materials and processed-beers.

The final antioxidant power of beer depends on a number of parameters involved in brewing such as the variety of malt and malting process, temperature and pH during mashing, sparing, boiling, the variety of hops added during wort boiling and yeast

fermentation (Saura-Calixto et al. 2008). In our study, all raw materials and processed-beers showed antioxidant properties against H_2O_2 with respect to the positive control, except for *S. uvarum* and the lowest concentration of Sladek hop. Furthermore, the formation of the Maillard intermediate is correlated with the alcohol content and colour (Coghe et al. 2004; Vanderhaegen et al. 2004). Studies with coloured final products of the Maillard reaction which are formed non-enzymatically during the roasting of malt indicate peroxyl radical scavenging potential (Liégeois et al. 2000). According to Rivero et al. (2005), dark beer obviously contains more antioxidant compounds than lager or alcohol-free beer, due to they are the responsible of colour characteristic. On the other hand, no relationship has been found between the antioxidant capacity of different phenols and the degree of browning (Morales and Babbel 2002). Although our results showed a higher antioxidant capacity of dark malt that blond malt, that difference was not observed in full beers; probably due to the high variety of compounds present that could modified the isolated properties of beers components. Moreover, the antioxidant capacity of alcohol-free beer is considerably lower than the lager ones (Saura-Calixto et al. 2008), being our results in agreement with this point and showing lager beers a higher antitoxic activity than alcohol-free beers.

Different relative percentage of antioxidant activity is observed in hops, which is correlated with the content of polyphenol substances (Krofta et al. 2008): the highest antioxidant activity within 70 to 80% rel. is exhibited by the variety Saazer, followed at a relatively considerable distance by Sladek variety with an antioxidant activity within 40 to 60% rel. Our results agree with this characterisation of hops, showing a stronger antitoxic potential for Saazer hop at lowest concentrations tested. Sánchez et al. (2010), highlight the common use of hops as tranquillizer plant.

2.2 *In vitro* assays

Considerable variations in inhibitory actions on tumours formation are observed among brands of beer, which may be due to the contents of the different components of beer (malt and hops extracts) and their antioxidant properties (Gerhäuser 2005; Hevia et al. 2008; Stevens and Page 2004). Our results showed that all raw materials were able to inhibit the tumour cells growth, except rest of mash. The most cytotoxic raw material was Sladek hop followed by *S. uvarum*, Saazer hop and finally by blond and dart malts. Moreover, processed-beers also showed chemopreventive activity against HL-60 cells, being Hopped wort the most cytotoxic processed-beer, followed by young beer and by wort.

Analysing the different ways of induction of DNA damage, any raw materials tested showed proapoptotic DNA fragmentation, except blond malt and *S. uvarum* that induced a very light patter of fragmentation at concentration 10 and 0.375 mg/ml. Processed-beer showed DNA fragmentation in a dose-dependent manner for hopped wort and young beer. With respect to the comet assay, raw materials and wort did not induced DNA damage at single and/or double strands breaks; young beer showed a slight damage at lowest concentration assayed (7.56 mg/ml); and hopped wort

completely induced DNA damage at highest concentration (62.5 mg/ml), compared with the concurrent controls. To our knowledge, this is the first time that effects of raw material and processed-beers at individual cellular level have been carried out at individual cellular level.

Our study with the raw materials and processed-beers allow us to suggest possible correlations between the modifications of beer and the properties of its compounds during the brewing development. The most abundant phenols during the hopped wort process are xanthohumol and humulone, hop-phenols characterised by their ability to induce DNA damage. During the beer maturing, the characteristic of *Saccharomyces* and other phenols are involved in the final outcome of beers, like ferulic acid, isoxanthohumol, vanillic, tyrosol, epicatechin gallate, among others reducing the proapoptotic DNA fragmentation activity as matured beers showed.

In an overall view, *in vivo* toxicity and antitoxicity properties showed by lyophilised and processed-beers could be explained by the results obtained in the raw materials studied. Although some substances could be consider toxic if we compared the survival of flies with respect to the concurrent control with a significance of $p < 0.05$, none of the compounds reached the lethal doses 50 (LD_{50}), hence can be considered as a safe. On the other hand, most of lyophilised beers showed antitoxicity activities, except a Cerny, Nefiltrovany, Nealko and Cherry beer, being these results supported by the protective effects showed by the processed-beers and raw materials studied, except for *S. uvarum*.

The *in vitro* assays showed a completely chemopreventive activity for all lyophilised beers tested which is in agreement with the results obtained by the processed-beers and raw materials. Only rest of mash showed non cytotoxic activity against tumour cells, although mention should be made that this material is eliminated during the brewing process. With respect to the ability to induce DNA damage, all lyophilised beers are able to induce proapoptotic DNA fragmentation and also single strand breaks, expect for AFBs and Pilsner Urquell. Hopped wort, young beer, blond malt and Sladek hop are the only processed-beers and raw materials that shown ability to induce DNA damage. This lead us to hypothesise that during brewing process, beers increase their beneficial properties due to the compounds formed from the raw materials.

Table 3
In vivo and *in vitro* results of processed-beers and raw materials.

	In vivo		*In vitro*		
	Toxicity	Antitoxicity	Cytotoxicity	DNA fragmentation	Comet assay
Wort					
Hopped wort					
Young beer					
Rest of mash					
Blond malt					
Dark malt					
Sladek hop					
Saazer hop					
S. uvarum					

Green colour means positive properties; Red colour means negative properties; Orange colour means positive and negative properties depending on the dose analysed. *S. uvarum*: *Saccharomyces uvarum*.

3. BEER COMPOUNDS

3.1 *In vivo* assays

Most of the protective properties showed by beers were related with the antioxidant activity of its phenols (Preedy 2011). Chen et al. (2014) emphasised the potential uses of beer compounds in dermatology such as treatment of atopic eczema, contact dermatitis, pigmentary disorders, skin infections, skin ageing, skin cancers and photoprotections. Furthermore, many beer compounds can normalize the rhythm of the sleep in addition to stimulate it due to its bitter acids, particularly the alpha acid

components (Chen et al. 2014). Furthermore, there are accumulated evidences that support the health benefits of moderate consumption of alcohol as part of a healthy lifestyle, associated with lower rates of cardiovascular diseases (Rimm et al. 1991), or the ability against kidney stones formation (Curhan et al. 1996). Vinson et al. (2003) showed that polyphenols present in both lager and dark beer inhibited atherosclerosis, decreased both total cholesterol and triglycerides in serum, behaved as *in vivo* antioxidant by decreasing the oxidizability of low-density lipoprotein cholesterol and decreased atherosclerosis in cholesterol-fed hamsters. In contrast, other studies show evidence that alcohol increases the risk of mouth, oesophageal and liver cancers (World Cancer Research Fund 2007). Our toxicity results agree with the known health properties exhibited by beer compounds. Only the highest concentration assayed of folic acid, xanthohumol and tyrosol induced a decreased viability of *Drosophila* but always with a survival percentage of individuals upper to 60%.

A wide range of researches evaluated the antigenotoxic potential of beer components against carcinogens contained in the human diet suggesting their protective capacity as scavenger free radicals and suppressor of oxidative stress (Arimoto-Kobayashi et al. 2006; Cuvelier et al. 1992; Granato et al. 2011; Rizvi et al. 2005; Sánchez-Moreno et al. 1998; Siddique et al. 2014; Srinivasan et al. 2007; Tafulo et al. 2010; Żołnierczyk et al. 2015). Our results agree with the antitoxic activity of beers components as they significantly protect against the toxic effect of H_2O_2 in combined treatments, except for folic acid.

According to a wide range of researches relating the antioxidant activity of phenols with several biological activities (Anter et al. 2010; Preedy 2011), our results agree with the no genotoxic and antigenotoxic activities of the studied phenols. Some early studies performing the Ames test suggested the possibility that the antimutagenic activity attributed to beer is a result of a cumulative effect of the individual beer constituents (Arimoto-Kobayashi et al. 1999). Moreover, phenolics and some B vitamins are argued to be the health promoter compounds of beer in reference to degenerative diseases (Gerhäuser 2005). Due to their polyphenols content, beers could be a source of bioactive compounds with suitable free radical scavenging properties, like isoxanthohumol, ferulic acid, vanillic, folic acid, choline, ECG, among others (Cuvelier et al. 1992; Rizvi et al. 2005; Sánchez-Moreno et al. 1998; Siddique et al. 2014; Srinivasan et al. 2007).

Survival curves showed either a general similar or significant increase of lifespan expansion for all compounds, with respect to their concurrent control, except for humulone that significantly decreased the lifespan of *Drosophila* and ECG that showed irregular pattern in longevity depending on the concentration analysed. Furthermore, a improvement of quality of life in *Drosophila* treated with the different assayed beers was shown with respect to the concurrent control, except for the humulone that induced a reduction of quality of life in all concentrations studied, and tyrosol that showed different patterns in healthspan depending on the concentrations tested. Taking into

account that humulone is the most abundant phenol of stout ale beer, this result support the longevity results above mentioned for LSAB.

Table 4
In vivo results of beer compounds.

	Toxicity	Antitoxicity	Geno toxicity	Anti-genotoxicity IP (%)[1]	Longevity	Healthspan
Folic acid			0.193 0.146	**32.2** **67.8**		
Choline			0.171 0.200	**41.5** **66.2**		
Xanthohumol			0.250 **0.051**	**54.9** **60.6**		
Tyrosol			0.395 0.175	-3.09 -9.53		
Humulone			0.150 0.250	**11.85** **25.51**		
Isoxanthohumol			0.315 0.268	**56.89** **48.45**		
Vanillic acid			0.243 0.375	**3.35** -21.65		
Ferulic acid			0.375 0.309	**38.91** **35.56**		
ECG			0.263 0.200	**16.24** **9.79**		

[1] IP: inhibition percentage. Green colour means positive properties; Red colour means negative properties; Orange colour means positive and negative properties depending on the dose analysed. ECG: epicatechin gallate.

3.2 *In vitro* assays

Tyrosol, humulone, ferulic and ECG phenols showed a potential cancer chemopreventive activity. Several studies supported the beneficial effects of tyrosol (Anter et al. 2010; Babich and Visioli 2003; Gerhäuser 2005), humulone (Gerhäuser 2005), ferulic (Cuvelier et al. 1992; Sánchez-Moreno et al. 1998) and ECG (Ahmad et al. 1997; Baek et al. 2002; Bigelow and Cardelli 2006; Chung et al. 2001; Hong et al. 2001; Huang et al. 2005; Liao et al. 1995; Lim et al. 2006; Weyant et al. 2001; Yang et

al. 1998) on chemoprevention. Furthermore, xanthohumol, isoxanthohumol and vanillic showed cytotoxic activity against HL-60 although they were not able to reach the IC_{50}. These results are in agreement with the anti-proliferative activity, chemopreventive potential and carcinogenesis effects showed by these phenols in different human cancer cell lines and described by several researches (Colgate et al. 2007; Delmulle et al. 2006; Dhananjaya et al. 2006; Gerhäuser 2005Lust et al. 2005; Pan et al. 2005; Subbaramaiah et al. 1997; Vetrano et al. 2005). On the other hand, cytotoxicity curves of choline and folic acid showed no inhibition and a slight tendency to increase the cells growth in some of the tested concentrations. Although controversial results have been found for these compounds, Glunde et al. (2006) demonstrated that choline metabolism is altered in a wide variety of cancers and Wien et al. (2012) showed a borderline significant increase in frequency of overall cancer in the folic acid group compared to controls.

Generally, our results suggest that beer compounds were not able to induced DNA damage in tumour cells. Only the lowest concentration of xanthohumol tested and highest concentrations of humulone were able to induce proapoptotic DNA fragmentation. Analysing the results of DNA damage at single/double strand breaks, slight or medium damage were induced by humulone, isoxanthohumol, vanillic and ECG phenols in the treated tumour cells. Finally, the DNA methylation status of HL-60 cells was switched to hypermethylation with tyrosol, isoxanthohumol and ECG treatments; different patterns of methylation modification were induced by folic acid and ferulic; and a general hypomethylation were caused by choline, xanthohumol, humulone and vanillic. An overall analysis of methylation patterns induced on the repetitive sequences by different treatments with lyophilised beers and beer compounds, showed a relative hypermethylation status of 15.5 and 17%, respectively.

Studies carried out with xanthohumol (Dietz et al. 2005; Dorn et al. 2010; Strathmann et al. 2010), choline (da Costa et al. 2006; Locker et al. 1986; Lu et al. 2012; Zeisel 2007), folic acid (Duthie and Hawdon 1998; Fenech 2001; Joshi et al. 2001; Pufulete et al. 2005), isoxanthohumol (Yang et al. 2007), ferulic (Balakrishnan et al. 2010; Jin et al. 2007; Picone et al. 2009), vanillic (Huang et al. 2008), ECG (Ahmad et al. 1997; Babich et al. 2005) support our results for the different effects obtained in the study of DNA damage in cancer cells. To our knowledge, for the rest of beer compounds tested there are no previous studies.

These results allows us to hypothesise that cytotoxic properties of lyophilised beers are not caused only by a single one beer compound. Although they do not show synergic activities, a threshold effect against tumoral cells growing is observed. Only in the case of the DNA fragmentation we could relate the effect of xanthohumol and humulone, both obtained from hop during brewing, as the main phenols that explain the activity of full beers. Finally, comet assay and methylation status does not seem to be associated with any particular beer compound. Probably, their joint activity is the responsible for the activity of full beers.

Table 5
In vitro results of beer compounds.

	Cytotoxicity	DNA fragmentation	Comet assay	Methylation status	Macroautophagy
Folic acid					
Choline					
Xanthohumol					
Tyrosol					
Humulone					
Isoxanthohumol					
Vanillic acid					
Ferulic acid					
ECG					

Green colour means positive properties; Red colour means negative properties; Orange colour means positive and negative properties depending on the dose analysed. ECG: epicatechin gallate.

4. PILOT STUDY IN HUMANS

Taking into account the health properties of beers, we carried out a clinical pilot study with humans. The aim of the study was to confirm the effects of beer in humans, specifically by analysing the modification in the DNA methylation status. Our results evidence a promising increase of DNA methylation status in repetitive sequences distributed by a wide range of the human genome using a moderate daily intake of beer. Further studies are need to confirm these results.

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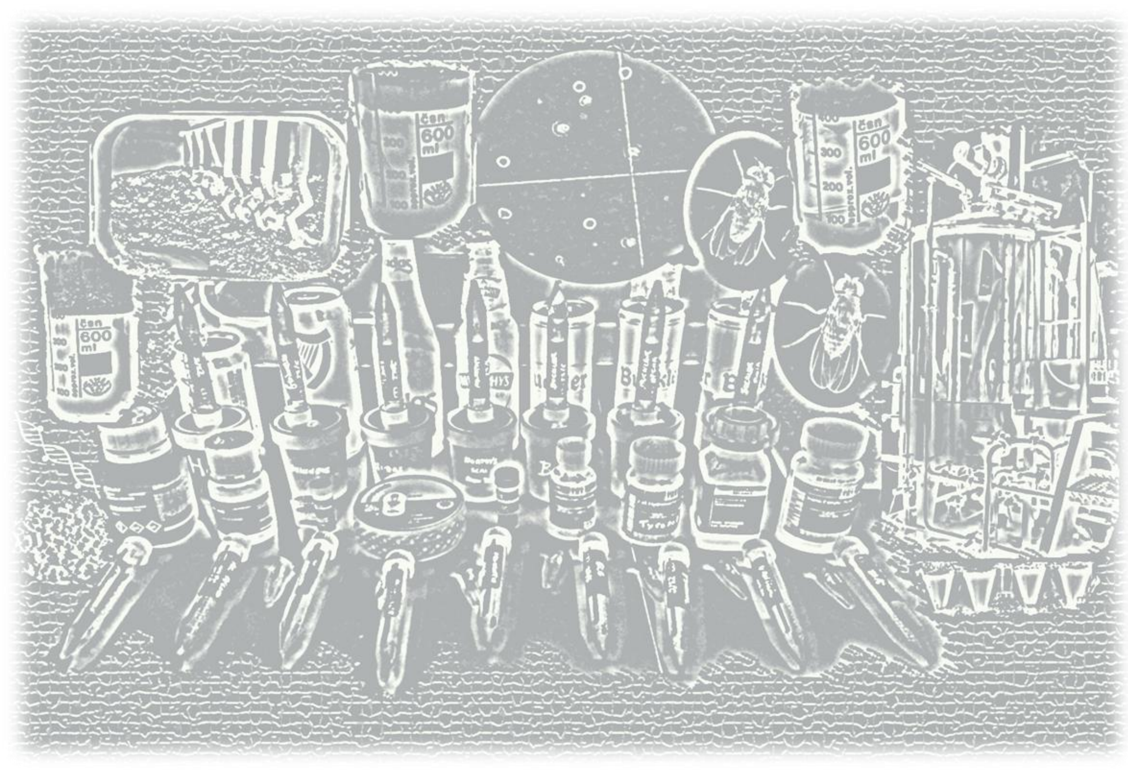
CONCLUSIONS



Based on the analysis of the results obtained for the *in vivo* assays in the *Drosophila* model organism, the *in vitro* ones in the HL-60 cell line and clinical studies in human as well, we can draw a general conclusion on the potential nutraceutic of lyophilised beers.

A new data corpus has been added to science by including more information about the promising properties that beers show as a nutraceutic substance in model systems. Data support, in general, the benefits showed by this beverage, due to its safety, protection against an oxidative toxin, chemopreventive potential and the induction of DNA damage in tumour cells.

Nevertheless, the complete biological activities attributed to beers cannot be exclusively linked to one particular class of constituents due to their high complexity and variability in the polyphenolic pattern. Besides, the brewing conditions like raw materials, wort composition, yeast strains and fermentation parameters, among others factors, influence the final outcome of the obtained beverage.

Some specific summary points can be highlighted drawn from the present work:

1.- Raw materials and brewing conditions are key points for the final activities of beers. Although some raw materials are slightly toxic (like malt, Sladek hop and consequently hopped wort, never reaching the LD_{50} at any concentration tested), the beer final product is non toxic. Additionally, the antioxidant capacity of all analysed substances used for obtaining mature beers (except for *Saccharomyces uvarum*) has been evident as well as their chemopreventive activity. Furthermore, the apoptotic way detected for the tumour cells treated with the different substances, is induced only when hops is added in the brewing process. Thus, during hopped wort process different modifications in the processing beer compositions have to bring about so the slight apoptotic damage become to a stronger one.

2.- Lyophilised beers show a safe character and protective ability against an oxidant agent in the *D. melanogaster*, as well as chemopreventive potential against HL-60 cells. None of the lyophilised beers reach a LD_{50} and all of them show protective activity against hydrogen peroxide except 4 out of the 23 studied beers. Moreover, all lyophilised beers are cytotoxic (9/9) showing an apoptotic way activation observed even at single cell level, although not all the samples exhibit a clear comet induction patter (6 out of 9 lyophilised beers).

3.- Studies in *D. melanogaster* at genetic level do not show genotoxic activity by any studied beer (except for the lowest concentration of LPABAFLB and LSAFLB); besides, a protective effects of genome against oxidative agent are observed when beers are combined with H_2O_2 exhibiting an IP ranged between 3.35–66.2%. For both genotoxic AFBs, a competitive oxidation activity is argued in the combined treatments.

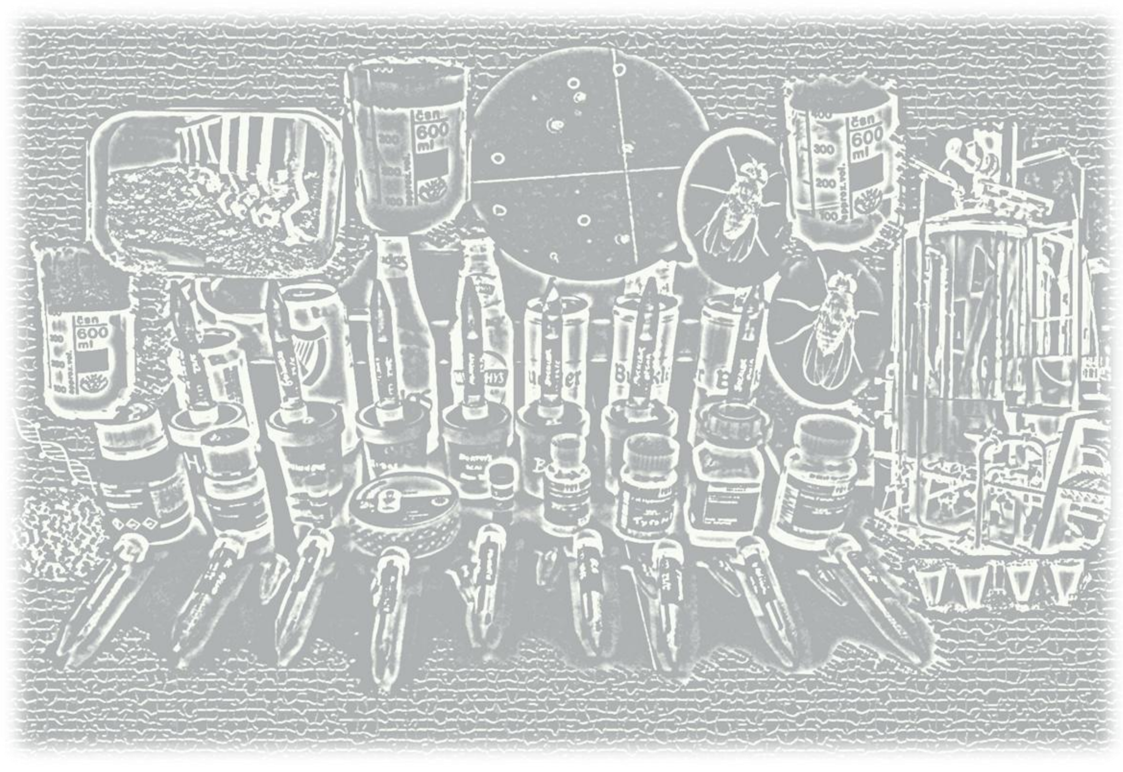
4.- Chronic studies on *Drosophila* populations treated with different lyophilised beers over time show an improvement of longevity and healthspan properties induced by all the studied beers (exception made of LSAB that significantly decreased the longevity parameters).

5.- Genome wide screening on HL-60 treated cells with different lyophilised beers show a hypermethylation status at molecular level of repetitive sequences, except for AFBs. Due to the promising results obtained in the blond lager beer, a pilot study with humans was carried out to assess the methylation status modifications, concluding that a moderate intake of blond lager beer induced a hypermethylation status in repetitive sequences of humans.

6.- Individual bioactive compounds of beers are not linked to the biological activities attributed to the full beverage. Neither a single compound nor the sum up of the different effects showed by the different assays can justify the results obtained in the full beers. Nevertheless, some phenols are key components of beers:
- Only folic acid shows no antitoxic activity against H_2O_2 in *Drosophila*. The rest of compounds exhibit a strong protective effect, although none of them or their combinations show the final antioxidant properties of beers.
- Humulone, one the most abundant phenols of stout ale beers, induces a strong reduction of longevity and healthspan in *D. melanogaster* supporting the results obtain for the LSAB.
- Xanthohumol (one of the most abundant phenols of blond beers) and humulone are the only compounds that are able to induce proapoptotic DNA fragmentation. Moreover, only humulone, isoxanthohumol, vanillic acid and ECG are the compound able to induce a slight DNA damage at single cell level.
- Despite of the final properties exhibited by full beers, they are not caused by any synergic effects of the studied compounds, in general, xanthohumol and humulone appear to be the most influencer molecules among all the single compounds studied. Both phenols are contained in hops; therefore, the addition of hops in the brewing process could be considered as one of the key points for the improvement of the beneficial properties of beers.

7.- On account of the promising results show by beer, xanthohumol, choline and folic acid, studies with the immortal cells (NIH3T3) on their effects on cytotoxicity and macroautophagy are carried out. Results show the same inhibition growth pattern showed in the HL-60 cells, as well as an inhibition of MA in NIH3T3 cells except for the highest concentration assayed of folic acid.

CONCLUSIONES



Basándonos en los resultados obtenidos tanto en los ensayos *in vivo* del organismo modelo *Drosophila*, en los ensayos *in vitro* en la línea celular HL-60, como en los estudios clínicos en humanos, podemos destacar el potencial nutracéutico de las cervezas liofilizadas como conclusión general.

Se ha añadido a la ciencia un nuevo cuerpo de datos aportando información sobre las prometedoras propiedades como sustancia nutracéutica que la cerveza muestra en sistemas modelo. En general los datos apoyan los beneficios mostrados por esta bebida debido a su seguridad, protección frente a una toxina oxidativa, potencial quimiopreventivo e inducción de daño celular en células tumorales.

Sin embargo, debido a la alta complejidad y variabilidad de tipos fenólicos, las propiedades biológicas atribuidas a las cervezas no están ligadas a un tipo particular de compuesto. Además, las condiciones de fabricación, así como la materia prima, composición del mosto, cepas de levadura y tipo de fermentación, entre otros factores, influyen en el resultado final de la bebida obtenida.

Algunas de las conclusiones específicas que se derivan del presente trabajo son:

1.- Las materias primas y las condiciones del procesado de la cerveza se han revelado como puntos críticos para las propiedades finales de las cervezas. Aunque alguna de las materias primas puede ser levemente tóxica (como la malta, lúpulo Sladek y consecuentemente el mosto con lúpulo, aunque sin llegar a alcanzar la dosis letal LD_{50}), el producto final no es tóxico. También ha quedado manifiesta la capacidad antioxidante de todas los compuestos utilizados para obtener la cerveza madura (excepto la de *Saccharomyces uvarum*), así como su actividad quimiopreventiva. Además, la vía apoptótica utilizada por las células tumorales tratadas con diferentes sustancias, sólo es inducida cuando el lúpulo es añadido al procesado de la cerveza. Por tanto, durante el procesado del mosto con lúpulo se deben dar diferentes modificaciones en la composición de la cerveza para que se pase de una leve daño apoptótico a un daño más pronunciado.

2.- Las cervezas liofilizadas muestran un carácter de seguridad y protección frente a agentes oxidantes en *D. melanogaster*, además de potencial quimiopreventivo frente a células HL-60. Ninguna de las cervezas liofilizadas alcanza la DL_{50} y todas muestran actividad protectora frente al peróxido de hidrogeno excepto 4 de las 23 cervezas estudiadas. Todas las cervezas son citotóxicas (9/9) mostrando una activación de la vía apoptótica, aunque a nivel unicelular no todas muestran un patrón claro de inducción de cometas (6 de 9 cervezas liofilizadas).

3.- Los estudios a nivel genético en *D. melanogaster*, no muestran actividad genotóxica de las cervezas estudiadas (excepto para la concentración más baja de LPABAFLB y LSAFLB); además se observa un efecto de protección del genoma frente a un agente oxidativo cuando las cervezas estudiadas se combinan con H_2O_2 mostrando un PI

(porcentaje de inhibición) entre 3.35–66.2%. En el caso de las AFBs se observa una oxidación competitiva en los tratamientos combinados.

4.- Estudios crónicos en poblaciones de *Drosophila* tratadas durante toda su vida con diferentes cervezas liofilizadas han mostrado la capacidad de éstas para aumentar la longevidad y calidad de vida (excepto para LSAB que disminuyó significativamente los parámetros de longevidad).

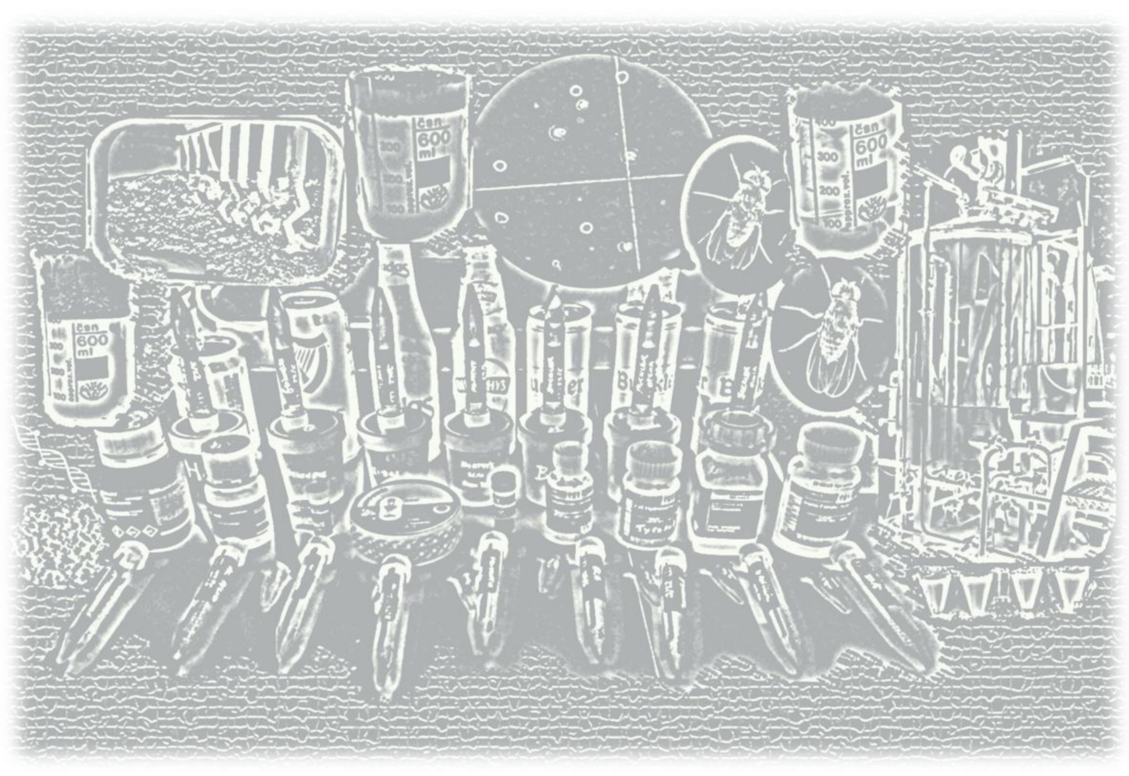
5.- Un rastreo a lo largo del genoma de células HL-60 tratadas con diferentes cervezas liofilizadas mostró un estado de hipermetilación de las secuencias repetitivas a nivel molecular, excepto para AFBs. Debido a los resultados tan prometedores obtenidos con la cerveza rubia lager, se realizó un estudio clínico piloto con humanos para evaluar las modificaciones en el estado de metilación, concluyéndose que un consumo moderado de cerveza rubia lager produce un estado de hipermetilación en secuencias repetitivas de humanos.

6.- Los compuestos bioactivos de las cervezas no se pueden asociar a nivel individual con las actividades biológicas atribuidas a la bebida en su conjunto. Ni los compuestos por sí mismos, ni la suma de sus efectos mostrados para los diferentes ensayos pueden justificar los resultados obtenidos en las cervezas. Sin embargo, algunos fenoles son componentes claves de las cervezas:
- El ácido fólico es la única molécula que no muestra actividad antitóxica frente al H_2O_2 en *Drosophila*. El resto de compuestos muestran un fuerte efecto protector, aunque ninguno de ellos ni sus combinaciones muestran las propiedades antioxidantes finales de la cerveza.
- La humulona, unos de los fenoles más abundantes de las cervezas negras tipo ale, induce una clara reducción de longevidad y calidad de vida en *D. melanogaster* apoyando los resultados obtenidos en LSAB.
- El xanthohumol (uno de los fenoles más abundantes de las cervezas rubias) y la humulona son los únicos compuestos capaces de inducir fragmentación apoptótica en el ADN. Además, sólo la humulona, el isoxanthohumol, el ácido vanílico y ECG son los compuestos capaces de inducir un leve daño en el ADN observable a nivel unicelular.
- Aunque las propiedades finales mostradas por las cervezas no son causadas por un efecto sinérgico de los compuestos estudiados, en general de entre todos los compuestos individuales estudiados, el xanthohumol y la humulona parecen ser las moléculas con mayor influencia. Ambos fenoles se encuentran en el lúpulo, por lo que la incorporación de éste al proceso de fabricación de la cerveza puede ser considerado como un punto clave en la mejora de las propiedades beneficiosas de las cervezas.

7.- Debido a la los prometedores resultados de cerveza, xanthohumol, colina y ácido fólico, se realizaron estudios sobre los efectos citotóxicos y macroautofágico en células inmortales (NIH3T3). Los resultados mostraron el mismo patrón de inhibición de crecimiento celular obtenido en las células HL-60, así como una inhibición de la

actividad macroautofágica en células NIH3T3, excepto para la concentración más alta ensayada de ácido fólico.

ANNEXES



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In vivo and *in vitro* studies of the role of lyophilised blond Lager beer and some bioactive components in the modulation of degenerative processes

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ABSTRACT

The aim of the present study was to examine the nutraceutic potential of lyophilised blond Lager beer, as well as two of its bioactive components: xanthohumol and folic acid. Several toxicity, antitoxicity, genotoxicity, antigenotoxicity and longevity endpoints were checked in the SMART *in vivo Drosophila* system. Cytotoxicity in HL-60 promyelocytic and NIH3T3 mouse fibroblasts cells, proapototic DNA fragmentation, comet assay, macroautophagy ac-tivity and methylation status were tested in *in vitro* assays. Lyophilised blond Lager beer could be proposed as a substance with an important nutraceutical value, because of its hopeful results as a lifespan promoter, DNA protection against free radicals in the animal model and its chemopreventive activity in HL-60 cells and enhacer of macroautophagy at mod-erate doses in NIH3T3 inmortal cells. Only xanthohumol mimics the biological activities found in lyophilised beer.

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Chemical compounds: Agarose (PubChem CID: 11966311); Etanol (PubChem CID: 702); Ethidum bromide (PubChem CID: 14710); Folic acid (PubChem CID: 6037); Hydrogen peroxide (H_2O_2) (PubChem CID: 784); Isopropanol (PubChem CID: 3776); L-glutamine (PubChem CID: 5961); NaCl (PubChem CID: 5234); Propidium iodide (PubChem CID: 104981); SDS (PubChem CID: 3423265); Trypan blue (PubChem CID: 101417452); Xanthohumol (PubChem CID: 639665).

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Abbreviations: Etop, Etoposide; *flr*[3], *flare* genetic marker; HL-60, human leukaemia-60 cell line; IC_{50}, inhibitory concentration 50; IP, inhi-bition percentages; LBLB, lyophilised blond Lager beer; LINE, long interspersed elements; MA, macroautophagy; *mwh*, *multiple wing hairs* genetic marker; NIH3T3, mouse embryonic fibroblast cells; qMSP, quantitative methylation-specific PCR; Sat-α, satellite-alpha DNA; SMART, somatic mutation and recombination test; TM, tail moment

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1. Introduction

Cancer is originated by internal and external factors and offers a variety of targets for intervention and inhibition at every stage such as antioxidant activity, xenobiotics metabolism, anti-mutagenic, anti-inflammatory and anti-hormonal mechanisms, anti-proliferative activities, induction of terminal cell differ-entiation and apoptosis (Reddy, Odhav, & Bhoola, 2003). Many cases of cancer are related with lifestyle and environmental conditions, and it is estimated that one third of cancer deaths is due to dietary habits (Martin-Moreno, Soerjomataram, & Magnusson, 2008). Drugs, chemicals, temperature and light are among the external environmental factors that can deter-mine which genes are turned on and off, thereby influencing the way an organism develops and functions (Clayton & McKeigue, 2001).

Antioxidants may be found at significant amount in diet and confer protection against oxidative stress-related dis-eases (Aruoma, 1998). Polyphenols are the most abundant dietary antioxidants, distributed mostly in coffee, fruits, wine, vegetables, olive oil and beer (Hertog, Feskens, Hollman, Katan, & Kromhout, 1993). Specifically, the consumption of beer can contribute to the total intake of polyphenols and to the anti-oxidant capacity of the diet. Beer provides 7.2% of the daily dietary ingestion of polyphenols and accounts for around 3% of the total antioxidant capacity of the diet in some coun-tries like Spain (Saura-Calixto, Serrano, & Pérez-Jiménez, 2008), being the third most popular drink overall, after water and tea (Nelson, 2004).

Beer constituents have been shown to possess various anti-bacterial, anti-inflammatory, anti-oxidative, anti-angiogenic, anti-melanogenic, anti-osteoporotic and anti-carcinogenic effects. Epidemiological studies on the association between beer drinking and skin disease are limited while direct evi-dence of beer compounds in clinical application is lacking. Potential uses of these substances in dermatology may include treatment of atopic eczema, contact dermatitis, pigmentary disorders, skin infections, skin ageing, skin cancers and photoprotections, which require an optimisation of the biostability and topical delivery of these compounds (Chen, Becker, Qian, & Ring, 2013). Beer has been associated with a lower risk of suffering a cardiovascular disease. It is shown that beer consumption reduced mortality from coronary heart diseases in moderate alcohol consumers with respect to non-alcohol consumers (Rimm et al., 1991), or the ability of beer against kidney stones formation (Curhan, Willett, Rimm, Spiegelman, & Stampfer, 1996). Furthermore, there are accu-mulated evidence that support the health benefits of moderate consumption of alcohol as part of a healthy lifestyle, associ-ated with lower rates of cardiovascular diseases, linked to polyphenol content of the alcoholic beverages (Bassus et al., 2004; Hertog et al., 1993). In contrast, other studies show evi-dence that alcohol increases the risk of mouth, oesophageal and liver cancers (Fund, 1997). Some studies performing the Ames test suggest the possibility that the antimutagenic ac-tivity attributed to beer is a result of a cumulative effect of the individual beer constituents (Arimoto-Kobayashi et al., 1999). According to Sánchez et al. (2010), hops have been frequently used as tranquillizer plant. Furthermore, many beer compounds

can normalise the rhythm of sleep in addition to stimulating it. This sedative capacity is due to its bitter acids, particularly the alpha acid component (Chen et al., 2013).

The main natural antioxidants present in beer are poly-phenols and melanoidins, particularly phenolic acids from barley (≈70%) and hop (≈30%) (Callemien & Collin, 2010; Callemien, Jerkovic, Rozenberg, & Collin, 2005; Goupy, Hugues, Boivin, & Amiot, 1999; Woffenden, Ames, & Chandra, 2001). Epi-demiological studies indicate the anti-proliferative activity of beer components with antioxidant properties in prostate cancer cells, showing a strong correlation between antioxidant potency and polyphenols content (Hevia, Mayo, Quiros, & Sainz, 2008). Many studies suggest that dietary flavonoids may have ben-eficial effects on human health and disease prevention, primarily attributed to their antioxidant properties (Stevens & Page, 2004).

The present work focused on the biological activities of beer and two of its minor molecules belonging to the phenolic and B vitamin classes: a chalcone present in hops as the princi-pal flovonoid: xanthohumol (Stevens, Taylor, & Deinzer, 1999); and folate contained in malt (Edelmann, Kariluoto, Nyström,
& Piironen, 2013; Owens, Clifford, & Bamforth, 2007). Both con-stituents were quantified in the selected lyophilised blond Lager beer (LBLB) and evaluated for their nutraceutic potential. Both molecules are important distictive compounds of beer with dif-ferent structure-activity related characteristics. Moreover, free radical scavenging properties and antioxidant activity of both of them are reported as will be detailed below.

Xanthohumol (XN, 2′,4′,6′,4-tetrahydroxy-3′-prenylchalcone), a prenylated chalcone isolated from the hop plant (*Humulus lupulus L.*), is a beer polyphenol belonging to the group of chalcones (Saura-Calixto et al., 2008), which is used as an in-gredient in the brewing process (Miranda et al., 2000). According to Ferguson (2001), the protection against oxidative damage of polyphenols is due to their ability to scavenge free radicals because of their interference with the oxidation of lipids, DNA and other molecules by rapid donation of a hydrogen atom to

radicals ($RO^{\bullet} + PPH \rightarrow ROH + PP^{\bullet}$). The phenoxy radical inter-mediates are relatively stable, and also act as terminators of the propogation route by reacting with other free radicals
($RO^{\bullet} + PP^{\bullet} \rightarrow ROPP$). Xanthohumol was shown to scavenge re-active oxygen species and to inhibit superoxide anion radical formation (Gerhauser et al., 2002). Xanthohumol has been sug-gested as an effective constituent against atherosclerosis, increasing HDL-cholesterol levels (Hirata et al., 2012). Further-more, xanthohumol can inhibit carcinogenesis at the initiation, promotion and progression steps of cancer, and protects DNA against genotoxicity and oxidative DNA damage (Plazar, Filipic,
& Groothuis, 2008). In addition, an anti-proliferative activity in human cancer cell lines, including breast, colon, prostate, ovarian and blood cancer by inhibiting proliferation and in-ducing apoptosis, has been shown (Benelli et al., 2012; Colgate, Miranda, Stevens, Bray, & Ho, 2007; Delmulle et al., 2006; Gerhauser et al., 2002; Lust et al., 2005; L. Pan, Becker, & Gerhauser, 2005). Finally, xanthohumol is associated with de-toxification of reactive metabolites through induction of quinone reductase activity (Dietz et al., 2005; Miranda et al., 2000), also showing antigenotoxic capacity (Strathmann et al., 2010). All of these activities, contribute to the prevention of oxidative damage by scavenging reactive oxygen species (ROS) and

nitrogen oxide (NO) production (Gerhauser et al., 2002; Miranda et al., 2000; Zhao & St Clair, 2003), and might suggest xanthohumol as a cancer preventive agent in a long-term car-cinogenesis model.

Folates belong to the B vitamin family. Folic acid and its con-stituents, pyrazine and pterin, can easily be reduced by hydrated electron and acetone ketyl radical (Moorthy & Hayon, 1976) to the corresponding hydroderivatives in the pyrazine ring of the molecule. Furthermore, free radical intermediates have been suggested (Hemmerich, 1968; Vonderschmitt & Scrimgeour, 1967) in the chemical oxidation of reduced pterins by air, H_2O_2 or Fe^{3+}, as well as in the enzymatic oxidation of reduced pterin by phenylalanine hydroxylase. They are present in many natural foods such as green leafy vegetables, peas, beans, certain fruits and beer (Saxby, Smith, Blake, & Coveney, 1982), being the rank of folic acid in beer from 0.04 to 0.6 mg/l (Buiatti, 2009). Folic acid, a member of the folate group (Jacques, Selhub, Bostom, Wilson, & Rosenberg, 1999), is an essential B vitamin for DNA synthesis, methylation and repair (Ames, 2001; Mason & Levesque, 1996). Joshi, Adhikari, Patro, Chattopadhyay, and Mukherjee (2001) demostrated the effective free radical savenger activity of folates. The scavenging and repair of thiyl radicals by folic acid makes folic acid a potential vitamin to be called as an antioxidant. Folate has been hypothesised as an anti-cancer agent (Giovannucci, 2002; Larsson, Giovannucci, & Wolk, 2007a, 2007b; Lin et al., 2008; Wei et al., 2005; Zhang et al., 2003). Other studies revealed the role of folate in hyperhomocysteinemia and neuroal tube defects (Ferguson et al., 1998; Welch & Loscalzo, 1998). Low-folic acid diets show an alteration in gene expression and increased DNA damage and it has been associated with the carcinogenesis process leading to a high incidence of colon cancer (Willett, Stampfer, Colditz, Rosner, & Speizer, 1990). Contrary, diets with high folate intake show a reduction of approximately 40% in the risk of colorectal neoplasms (Bailey, Rampersaud, & Kauwell, 2003). Recent *in vitro* studies show a beneficial effect on genomic sta-bility and cell death (Lu et al., 2012). Nevertheless the meta-analysis in humans published by Wien et al. (2012) using 10 randomised controlled trials showed a borderline significant increase in frequency of overall cancer in the folic acid group compared to controls although formers epidemiological studies have shown that folate supplements can significantly reduce the risk of pancreatic cancer (Stolzenberg-Solomon et al., 1999) and breast cancer (Zhang et al., 1999). Hence a controversial on dose effect exits on folate intakes.

A nutraceutical substance should be able to prevent genetic oxidative damage, increase the lifespan, and induce cell death in a programmed manner in tumour cells. Here, we have tested the nutraceutic potential of a lyophilised blond Lager beer (LBLB), as well as two of its bioactive components: xanthohumol and folic acid. Several points related to degen-erative processes have been checked: (i) *in vivo* assays using the *Drosophila* model, as 70% of human disease genes are conserved in this organism (Richardson, Willoughby, & Humbert, 2015), to evaluate the toxicity, antitoxicity, genotoxicity, antigenotoxicity and longevity induction and (ii) *in vitro* assays to evaluate the cytotoxic, proapoptotic DNA fragmentation activity, comet assay, macroautophagy activity and methyla-tion status on the HL-60 promyelocytic and immortal NIH3T3 cell models.

2. Material and methods

2.1. *Sample preparation*

A blond Lager beer (Heineken®) was selected for this study because, according to *"The Drinks Business"*, it is one of the world's most popular beers (Tepper, 2012). Prior to carrying out the assays, beer was lyophilised (SCAI, University of Córdoba) and dissolved in distilled water in order to obtain the differ-ent concentrations tested. The lyophilised sample was stored, until use, at room temperature in a dark and dried atmosphere.

2.2. *Xanthohumol and folic acid content characterisation in LBLB sample*

Xanthohumol and folic acid were tested as single bioactive com-pounds. The chromatographic behaviour of both compounds was analysed by the SCAI (University of Córdoba) using an HPLC/MS system to confirm their presence in the studied drink. Mass spectometry (MS) analyses were performed on a triple– quadrupole LC-MS equipment. The high performance liquid chromatography (HPLC) was interfaced to the MS via electrospray ionisation (ESI) in negative mode and by ions de-tection in the MRM (multiple reaction monitoring) mode in order to achieve the highest sensitivity as possible. The chromato-graphic separation of both compounds were performed in reverse phase (column C-18, 100 × 2.1 mm), with a gradient of water/formic acid and methanol.

Total flow: 0.25 ml/min; column temperature: 40 °C; injec-tion volume: 5 l.

2.3. *Treatments and samples concentrations*

The concentrations of LBLB used in the different assays were established taking into account the average daily food intake of *Drosophila melanogaster* (1 mg/day) and the average body weight of *D. melanogaster* individuals (1 mg) (Ja et al., 2007). The concentration range for all tested substances was calculated in order to make it comparable to the beer daily intake for humans (192 ml of total beer/day for 70 kg human body weight (Kirin, 2012).

2.4. In vivo *assays*

2.4.1. D. melanogaster

The value of using *Drosophila* to investigate fundamental bio-logical processes is increasing. This organism is revealing as an appropriate system as it is a complex multicellular organ-ism in which many aspects of gene expression are parallel to those of humans. *Drosophila* substitute mammals in experi-ences with the distinct goal of uncovering insights directly relevant to human beings because it is a model for many human diseases, including cancer and ageing (Gonzalez, 2013; Lints & Soliman, 1988; Rudrapatna, Cagan, & Das, 2012).

Two *Drosophila* strains were used, each with a hair marker in the third chromosome:

- *mwh/mwh*, carrying the recessive mutation *mwh* (*multiple wing hairs*) that produces multiple tricomas per cell instead of one (Yan et al., 2008).

- *flr*^{3}*/In (3LR) TM3, rip*^{p}*sep bx*^{34e}*e*^{s}*Bd*^{S}, where the *flr*^{3} (*flare*) marker is a homozygous recessive lethal mutation that produces deformed tricomas but is viable in homozygous somatic cells once larvae start the development (Ren, Charlton, & Adler, 2007).

Strains were maintained at 25 °C and 80% humidity in glass tubes with homemade meal (0.5 g NaCl, 12 g agar-agar, 100 g yeast, 25 g sucrose, 5 ml propionic acid, 3.5 ml of a 0.2% sul-phate streptomycin solution and 1 litre of water) making changes three times per week.

Virgin females were obtained from these tubes, and were mated in order to perform the crosses for the different assays of toxicity, antitoxicity, genotoxicity, antigenotoxicity and longevity.

2.4.2. *Toxicity, antitoxicity, genotoxicity and antigenotoxicity assays (SMART)*

Based on section 2.3, for the toxicity assays [(n of individuals born in each treatment/n of individuals born in the negative control) × 100] where n is number of flies born, the following ranks of concentrations were assayed: 3.125–50 mg/ml of LBLB and 0.00125–0.02 mM and 0.00141–0.0226 mM of xanthohumol and folic acid respectively, being the concentration of single mol-ecules the correspondent to the concentration of LBLB assayed.

The antitoxicity tests consisted of combined treatments of the same concentrations as in toxicity assays by adding the genotoxicant hydrogen peroxide at 0.12 M (Tasset-Cuevas et al., 2013) and comparing the percentage of emerging adults with the positive toxicant control. Chi-square test was used to de-termine if the tested compounds significantly inhibited the survival of flies. Negative control values were considered as those expected in Chi-square formula used in toxicity assay and posi-tive control values in antitoxicity assay (Martínez Becerra & Robles González, 1999). The negative controls were prepared with medium and distilled water and positive controls with medium and 0.12 M H_2O_2 as an oxidative genotoxicant (Anter et al., 2010).

The genotoxicity assays were carried out following the method described by Graf et al. (1984). Briefly, trans-heterozygous larvae for *mwh* and *flr*^{3} genes were obtained by crossing 200 optimally virgin females (4 days old) of *flr*^{3} strain with 100 males of *mwh* strain. Four days after fertilisation, females were allowed to lay eggs in fresh yeast medium (25 g of yeast and 4 ml of sterile distilled water) for 8 h in order to obtain synchronised larvae. After 72 h, larvae were collected, washed with distilled water, and placed, in groups of 100 in-dividuals, in the treatment tubes where they were fed chronically with the different compounds. The treatment tubes contained 0.85 g of *Drosophila* Instant Medium (Formula 4–24, Carolina Biological Supply, Burlington, NC) and 4 ml of solu-tions with different concentrations of the substance to be tested (3.125 and 50 mg/ml of LBLB; 0.00125 and 0.02 mM of xanthohumol; 0.00141 and 0.0226 mM of folic acid).

The antigenotoxicity tests were performed following the method described by Graf, Abraham, Guzman-Rincon, and Wurgler (1998), which consisted of combined treatments of genotoxin (0.12 M H_2O_2) and the same concentration used in genotoxicity assays of LBLB, xanthohumol and folic acid. After emergence, adult flies were stored in 70% ethanol until the wings were mounted to the scrutiny of the mutations.

2.4.2.1. Mutations scoring, data evaluation and statistical analysis. Wings of emerging trans-heterozygous individuals (*mwh flr*$^+$/*mwh*$^+$ *flr*^{3}) for each control and concentration were mounted on slides using Faure's solution and scored under a photonic microscope at 400× magnification. Similar numbers of male and female wings were mounted and wing hair mu-tations were scored from a total of 24,400 monotricoma wild type cells per wing (Moraga & Graf, 1989). In the balancer-heterozygous genotypes (*mwh*/TM3, *Bd*^{S}), *mwh* spot phenotypes are produced predominantly by somatic point mutation and chromosome aberrations, since mitotic recombination between the balancer chromosome and its structurally normal homo-logue is a lethal event.

Wing hair spots were grouped into three different catego-ries: *S*, a small single spot corresponding to one or two cells exhibiting the *mwh* phenotype that occur in the late stages of the mitotic division; *L*, a large single spot with three or more cells showing *mwh* or *flr*^{3} phenotypes and occur in the early stages of larval development; or *T*, a twin spot corresponding to two juxtapositioning clones, one showing the *mwh* pheno-type and other the *flr*^{3} phenotype. Small and large spots are originated by somatic point mutation, chromosome aberra-tion, and somatic recombination, while twin spots are produced exclusively by somatic recombination between the *flr*^{3} locus and the centromere.

The total number of spots was also determined and a multiple-decision procedure was applied to determine whether a result is positive, negative or inconclusive (Frei & Wurgler, 1988). The frequencies of each type of mutant clone per wing were compared to the concurrent control and analysed ap-plying the binomial Kastenbaum and Bowman Test (Frei & Wurgler, 1995), without Bonferroni correction and at 5% level of signinficance. All inconclusive results were analysed with the nonparametric Mann–Whitney *U*-test ($\alpha = \beta = 0.05$). The in-hibition percentages (IP) for the combined treatments are calculated from total spots per wing with the following formula (Abraham, 1994):

$$IP = [(\text{single genotoxin} - \text{combined treatment})\ \text{single} / \text{genotoxin}] \times 100$$

2.4.3. *Lifespan assays*

Flies who undergo the longevity experiments exhibited the same genotype as in genotoxicity assays in order to compare both genotoxicity and longevity results. The F_1 progeny from *mwh* and *flr*^{3} parental strains produced by a 24 h egg-laying in yeast medium was used to the trial.

All experiments were carried out at 25 °C according to the procedure described by Tasset-Cuevas et al. (2013). Briefly, syn-chronised 72 ± 12 hours old trans-heterozygous larvae were washed, collected and transferred in groups of 100 individu-als into test vials containing 0.85 g of *Drosophila* Instant Medium and 4 ml of the different concentrations of the compounds to be assayed. Adult animals emerged from pupae were col-lected and sets of 10 individuals of the same gender were selected and placed into sterile vials containing 0.21 g of *Drosophila* Instant Medium and 1 ml of different concentra-tions of solution of the compounds to be tested (3.125–50 mg/ml of LBLB; 0.00125–0.02 mM of xanthohumol; 0.00141–0.0226 mM of folic acid). Four replicates were followed during the

complete life extension for each control and established con-centrations. Alive animals were counted and the respective nourishment was renewed twice a week.

The statistical treatment of survival data for each control and concentration was assessed with the SPSS Statistics 17.0 software (SPSS, Ink., Chicago), applying the Kaplan–Meier method. The significance of the curves was determined using the Log-Rank method (Mantel-Cox).

2.5. **In vitro** *assays*

2.5.1. Cell culture conditions.

Cells were grown in RPMI-1640 medium supplemented with heat-inactivated foetal bovine serum, L-glutamine at 200 mM and antibiotic-antimycotic solution with 10,000 units of peni-cillin, 10 mg of streptomycin and 25 g amphotericin B per ml. Cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 (Shel Lab, Cornelius, OR, USA) (Gallagher et al., 1979). The cultures were plated at 2.5×10^4 cells/ml density in 10 ml culture bottles and passed every 2 days.

The immortal and non-tumour NIH3T3 cell line, derived from mouse fibroblast cells, was purchased from American Type Culture Collection (Manassas, VA) and cultured in a 37 °C incu-bator with 95% humidity and 5% CO_2 in complete Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Carlsbad, CA, USA) supplemented with 10% (v/v) heat-inactivated newborn calf serum (NCS) (Gibco, Carlsbad, CA, USA) and 1% penicillin/ streptomycin/Fungizone mix. The cell culture was passaged every 3–4 days by trypsination (0.05%). Only passages 10–25 were used.

2.5.2. Cytotoxicity assay.

HL-60 cells were placed in 96 well culture plates (2×10^4 cells/ ml) and treated for 72 h either with LBLB, xanthohumol or folic acid at different concentrations (7.8–250 mg/ml; 0.0012– 0.02 mM and 0.04–1.58 mM, respectively). Cell viability was determined by the trypan blue dye exclusion test and were counted with an inverted microscope at 100× magnification (AE30/31, Motic). Curves were plotted as survival percentage with respect to the control growing at 72 h. At least three in-dependent repetitions of the assays were carried out to calculate means for statistical analysis.

NIH3T3 cells were plated in 96 well flat-bottom plates (BD Biosystems) at 1×10^4 cells per well and treated for 24 h either with LBLB, xanthohumol or folic acid at the same concentra-tions above mentioned. Cell viability was measured using the CellTiter-Blue Cell Viability Assay (Promega, Madison, WI, USA). After treatments, 20 l of CellTiter-Blue Reagent was added to each well and the cells were cultured for 2 h as changes in the fluorescence at 540 nm excitation and 590 nm emission wave-lengths were measured using a Tecan, Infinite M200Pro microplate reader. Fluorescence intensity values were normalised to values of untreated wells.

2.5.3. Determination of DNA fragmentation.

HL-60 cells (1×10^6 cells/ml) were treated with different concentrations of LBLB, xanthohumol or folic acid (15.625– 250 mg/ml; 0.00125–0.02 mM and 0.099–1.58 mM, respectively) for 5 h. Treated cells were collected and centrifuged at 603.72 × *g* for 5 min, and DNA was extracted as follow: the cell pellet was resuspended in 900 l of cell lysis buffer, then added 100 l of SDS 10% and 25 l of proteinase K solution (20 mg/ml) and in-cubated by shaking for 5 hours at 55 °C. After this, 432 l of 5 M NaCl was added and the samples were centrifuged at 11,336.52 × *g* for 15 min. Supernatant was recovered into a fresh tube and 750 l of cold isopropanol was added to precipitate the DNA. Then, samples were centrifuged at 11336.52 × *g* for 10 min, washed with 1 ml of 70% ethanol and DNA was dried and resuspended in 20 l of deionised water. Finally, 0.6 l of 0.4 mg/ml RNase was added and incubated at 37 °C (6.0372 × *g*) over night.

Total extracted DNA was quantified in a spectrophotom-eter (Nanodrop® ND-1000) and 1200 ng of DNA were loaded into a 2% agarose gel electrophoresis at 85 mA during 25 minutes, stained with ethidium bromide and visualised under UV light.

2.5.4. Comet assay.

The DNA strand breaks induction was determined using the al-kaline comet assay (pH < 13), following the method described previously (Husseini, El-Fayoumi, O'Neill, Rapoport, & Pitt, 2000; Olive & Banath, 2006; Prosperini, Juan-Garcia, Font, & Ruiz, 2013) with some modifications. Cells were treated with LBLB (7.8, 31.2 and 62.5 mg/ml), xanthohumol (0.0025, 0.005 and 0.02 mM) and folic acid (0.099, 0.198 and 0.397 mM) for 5 h. After treatments, cells were rinsed in PBS, mixed with 0.75% low melting point agarose at 37 °C and transferred to frosted-end slides. Steps of lysis, alkaline electrophoresis, neutralisation and dried were carried out according to Mateo-Fernández, Merinas-Amo, Moreno-Millán, Alonso-Moraga, and Demyda-Peyrás (2016) pro-tocol. Finally, DNA of 50–100 single cells was visualised by treating slides with propidium iodide. Comet images were analysed at 400× magnification using a Leica DM 2500 fluorescence micro-scope with green filter and an attached camera (JAI CV-M4CL). OpenCometTM freeware was used to score individual param-eters for each cell.

Statistical analysis of data was performed using SPSS Sta-tistic 17.0 software. The Tail Moment (TM) data were analysed applying a one-way ANOVA and post hoc Tukey's test to de-termine the effect of choline on HL-60 cell DNA integrity. The level of $p \leq 0.05$ was considered statistically significant.

2.5.5. Macroautophagy activity evaluation.

Macroautophagy (MA) is considered an inducible type of au-tophagy, whose essential role is the maintenance of cellular homeostasis and take part in the forefront of the response to stress positions against cellular aging. MA activity in NIH3T3 cells was evaluated as follows: for mCherry-GFP-LC3 conver-sion, NIH3T3 fibroblasts were transduced with a lentivirus carrying the tandem construct and cells were analysed at least one week after transduction to assure stable expression. 1×10^4 cells per well were plated in glass-bottom 96-well plates and fluorescence after the indicated treatment was read in both channels using a high-content microscope (Operetta, Perkin Elmer). Moreover, the cytotoxic anticancer stressor Etoposide (Etopo; 25 M) was added to the NIH3T3 cells in order to analyse the growing behaviour of that cells treated with the tested com-pounds. Images of 9 different fields per well were captured, which rendered an average of 2000–3000 cells counted. Nuclei, cell perimeter and puncta were identified using the manufac-turer's software Columbus 2.4.2. Puncta positive for both

fluorophores correspond to autophagosomes whereas those only positive for the red fluorophore correspond to autolysosomes. Autophagic flux was determined as the con-version of autophagosomes to autolysosomes (red-only puncta).

The statistical significance of macroautophagy activity for each compound was determined using the Student's t-test with the SPSS Statistics 17.0 software (SPSS, Inc., Chicago).

2.5.6. Methylation status.

Genomic DNA was isolated in the same way as described in the DNA fragmentation section. Bisulphite-modified DNA from the LBLB (15.625 and 250 mg/ml), xanthohumol (0.00125 and 0.020 mM) and folic acid (0.099 and 1.587 mM) treatments, using the EZ DNA Methylation-Gold™ Kit, was used as template for fluorescence-based real-time quantitative Methylation-Specific PCR (qMSP). The final reaction mixture with a total volume of 10 l consisted of: 2 l of deionised water, 5 M of each forward and reverse primer, 2 l of iTaq™ Universal SYBR® Green Supermix (Bio-Rad, containing antibody-mediated hot-start iTaq DNA polymerase, dNTPs, $MgCl_2$, SYBR® Green I dye, enhanc-ers, stabilisers and a blend of passive reference dyes including ROX and fluorescein) and 25 ng of bisulphite converted genomic DNA.

QMSP conditions were as follows: one step at 95 °C for 3 minutes, 45 cycles at 95 °C for 10 seconds, 60 °C for 15 seconds, 72 °C for 15 seconds, another step at 95 °C for 30 seconds fol-lowed by a 65 °C step during 30 seconds and finally a boost step from 65 °C to 95 °C for 95 seconds increasing 0.5 °C each 0.05 seconds. QMSP was carried out in 48 well plates in MiniOpticon Real-Time PCR System (MJ Mini Personal Thermal Cycler, Bio-Rad) and was analysed by Bio-Rad CFX Manager 3.1 Software.

In order to analyse a wide range of human genomic DNA repetitive elements were selected. While Alu and LINE se-quences are interspersed throughout the genome, satellite DNA is confined to the centromere areas (Deininger, Moran, Batzer, & Kazazian, 2003; Ehrlich, 2002a, 2002b; Lee, Wevrick, Fisher, Ferguson-Smith, & Lin, 1997; Weiner, 2002). All sequences had been obtained from Isogen Life Science. Alu M1, LINE-1 and Sat-α sequences were used (see Supplementary Material 1 for detailed information (Weisenberger et al., 2005)).

The relative yielded results were normalised with the house-keeping sequence Alu C4 using the Nikolaidis et al. (2012) and Liloglou, Bediaga, Brown, Field, and Davies (2014) compara-tive CT method. Each sample was analysed in triplicate. One-way ANOVA and post hoc Tukey's tests were used to evaluate the differences between the tested compound, repetitive ele-ments and concentrations.

3. Results and discussion

3.1. Chemical characterisation

Results of the chromatographic behaviour for the individual bioactives (xanthohumol and folic acid) are shown in Fig. 1A. Moreover, their content in the full drink is represented in Fig. 1B showing a value of 0.01 ppm and 0.02 ppm for xanthohumol and folic acid, respectively. These results agree with Gerhauser (2005) who detected a concentration of xanthohumol ranged between 0.002 and 1.2 mg/l and with Póo-Prieto, Alonso-Aperte, and Varela-Moreiras (2011) who detected a concentration rank between 0.016 and 0.026 mg/l of folic acid in 4–5.5% alcohol beers.

3.2. Toxicity and antitoxicity

Toxicity of LBLB and the selected compounds has been as-sessed in the *Drosophila in vivo* model. Fig. 2A shows the relative percentage of emerging adults after treating larvae with dif-ferent concentrations of these substances. LBLB does not exhibit toxicity at the assayed concentrations with 100% survivals. Xanthohumol and folic acid are non-toxic substances for *Dro-sophila* larvae although the two highest and the highest assayed doses are significantly toxic, respectively. *In vivo* studies in dif-ferent animal models have shown a safe activity of xanthohumol withh respect to fertility, bone marrow, liver, exocrine pan-creas, kidneys, muscles, thyroid, ovaries and surrenal cortex (Avula et al., 2004; Hussong et al., 2005; Vanhoecke et al., 2005) and a protective activity (Dorn, Bataille, Gaebele, Heilmann, & Hellerbrand, 2010; Dorn, Heilmann, & Hellerbrand, 2012).

The antitoxicity assays revealed a differential behaviour of the substances. Fig. 2B shows that hydrogen peroxide is toxic at 0.12 M with an average survival rate of 62% with respect to the water control; this result is in agreement with previous ex-periences of toxicity in *D. melanogaster* when treated with H_2O_2 (Romero-Jimenez, Campos-Sanchez, Analla, Munoz-Serrano, & Alonso-Moraga, 2005). LBLB and xanthohumol are the most pro-tective substances with positive dose–response effects. Xanthohumol would contribute to the prevention of oxida-tive damage by scavenging reactive oxygen species (ROS) produced by the toxicant H_2O_2 (Gerhauser et al., 2002; Miranda et al., 2000; Zhao & St Clair, 2003) and preventing carbon tetrachloride-induced acute liver injury in rats (Pinto, Duque, Rodriguez-Galdon, Cestero, & Macias, 2012) and a protective activity has been reported in induced hepatic fibrosis is mice (Dorn et al., 2010, 2012). The combined treatments of folic acid and H_2O_2 showed a toxic synergic effect exerted by the two sub-stances, except at the highest concentration tested. Although folic acid is generally regarded as not toxic for humans it may cause neurological injury when given to patients with undi-agnosed pernicious anaemia (Butterworth & Tamura, 1989). These findings would agree with our results for toxicity and antitoxicity assays of folic acid in the *Drosophila* animal model.

3.3. Genotoxicity and antigenotoxicity

The Table 1 shows the results of genotoxicity assays in the SMART test for LBLB and the two single compounds under study. Negative control showed a frequency of mutations per wing of 0.157 which falls into the historical range for the wing spot test (Anter et al., 2011; Rojas-Molina, Campos-Sanchez, Analla, Munoz-Serrano, & Alonso-Moraga, 2005). The final con-centration of H_2O_2 used (0.12 M) has been demonstrated to exert a potent genotoxic effect capable to induce somatic mutations and mitotic recombination in *D. melanogaster* (Romero-Jimenez et al., 2005). The average frequency of total mutations per wing obtained in the treatment with H_2O_2 was 0.388. Single small, single large, twin and total clones were

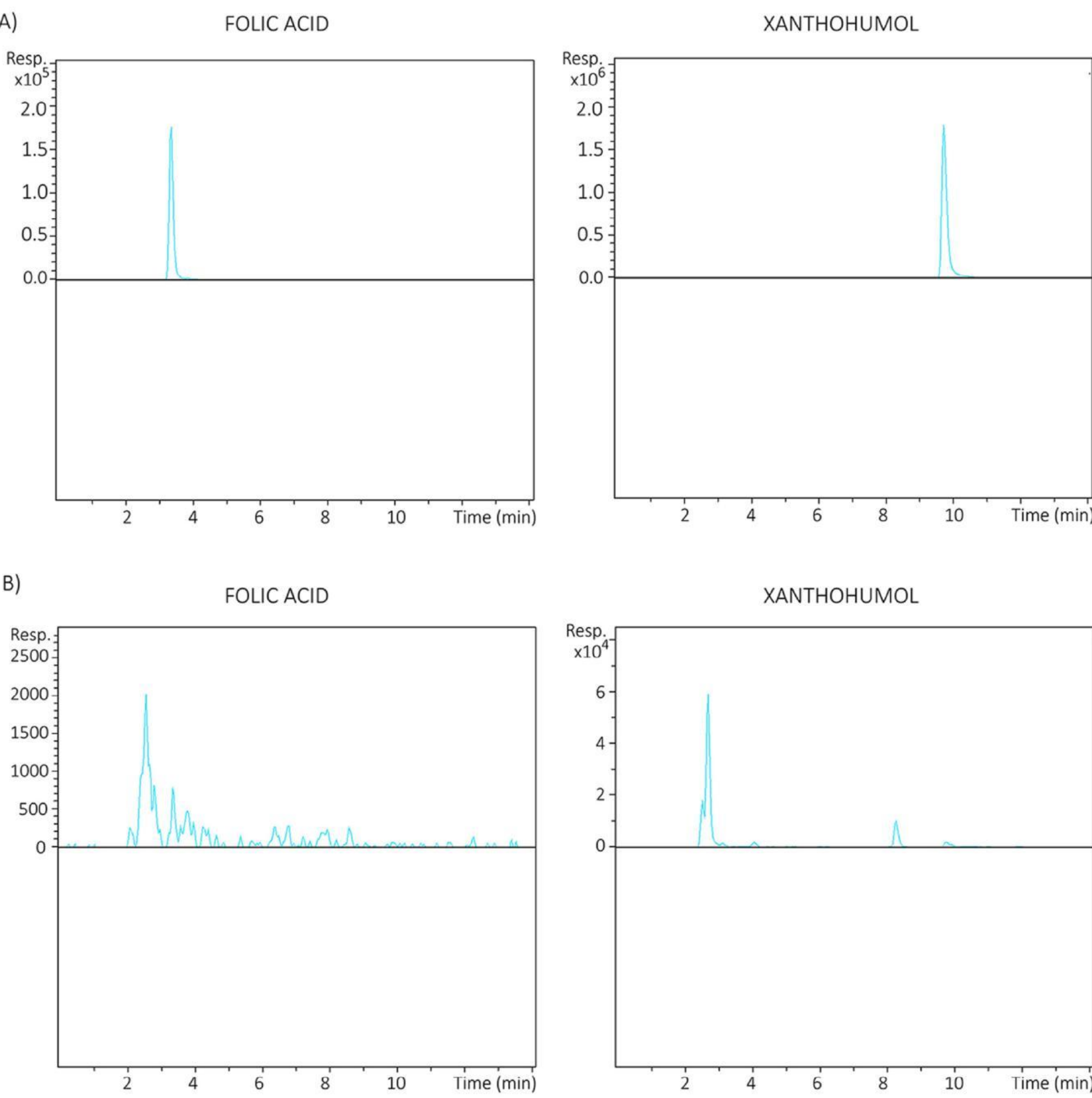


Fig. 1 – Sample compounds characterisation. Chromatographic behaviour of xanthohumol and folic acid (A) analysed by an HPLC/MS system and their presence in the lyophilised blond Lager beer (LBLB) (B).

analysed in the wings of animals chronically treated for each concentration and compound.

The results showed that the LBLB, xanthohumol and folic acid seem to have no genotoxic effect at the concentrations tested. The values of total mutation rates at the highest and lower concentrations of LBLB were similar or lower than the control mutation frequency (0.147 and 0.155 spots/wing, re-spectively) with no significant differences with the negative control (0.157 spots/wing). No dose effect is observed. Xanthohumol showed the lowest rate of mutations per wing among all the compounds under study at the highest concen-tration (0.051 spots/wing), encountering a dose-dependent effect more pronounced than in the case of folic acid. This is the first time that LBLB has been assayed in the *in vivo Drosophila* system.

Vegetables contain many polyphenols and oligoelements with antimutagenic activity (Fernandez-Bedmar et al., 2011). The highest concentration tested for LBLB in the combined treatments was able to eliminate part of the genotoxic effect of H_2O_2, showing that the total mutation frequency is similar to the negative control (0.121 spots/wing for LBLB 50 mg/ml and 0.157 spots/wing for negative control), inhibiting around 68.8% of genotoxicity induced by H_2O_2. However, the lowest concen-tration of LBLB tested had a slight capacity to counteract the genotoxic effect of H_2O_2, obtaining a high total mutation fre-quency very similar to the value of the positive control (0.333 spots/wing in LBLB 3.125 mg/ml), showing only a 14.2% of in-hibition (Table 1). Our results complement the *in vitro* assays of Rivero et al. (2005) who detected antigenotoxic activity in a dose dependent manner using calf thymus DNA damaged by ascorbic acid and copper. Other *in vitro* assays (HPRT mu-tagenicity test and the comet assay) performed in Chinese hamster lung fibroblasts also give antigenotoxic activities of LBLB against genotoxicity of heterocyclic aromatic amines (Edenharder, Sager, Glatt, Muckel, & Platt, 2002). It is remark-able that the *in vivo* genotoxicity/antigenotoxicity *Drosophila* wing spot test carried out in the present study does need neither metabolically competent mammalian cells nor metabolic bioactivation conditions of the *Salmonella* reversion assay. The

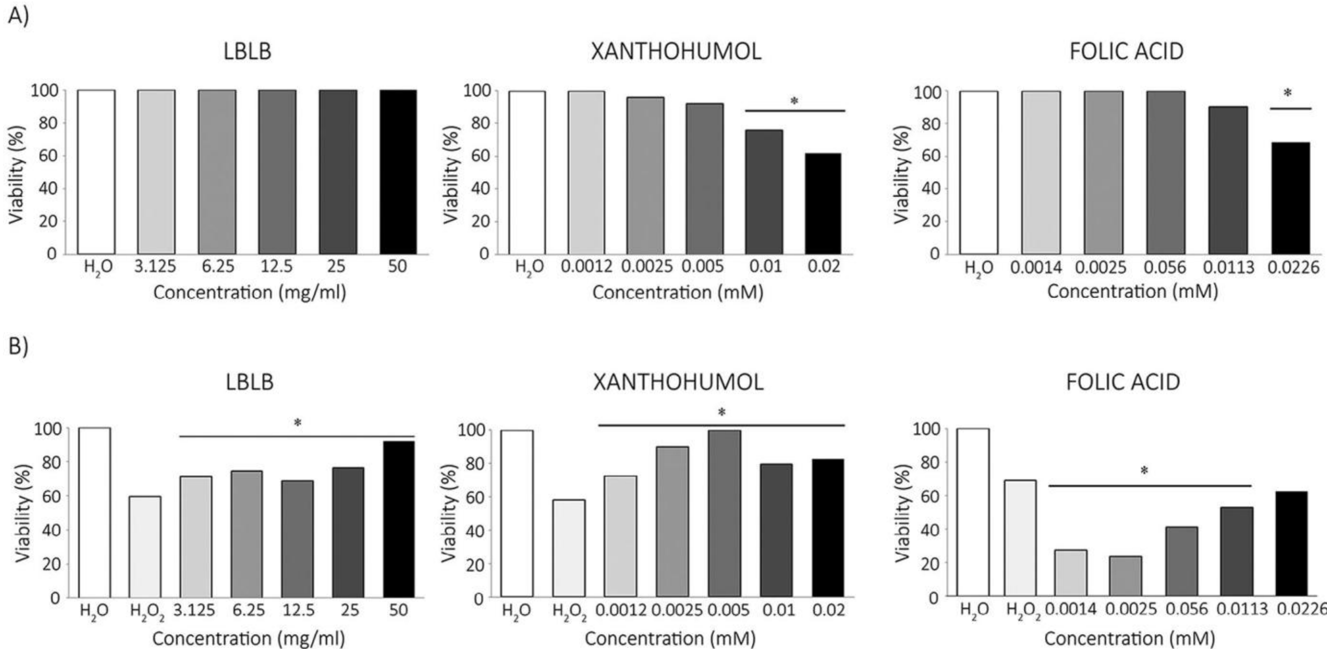


Fig. 2 – Toxicity and antitoxicity levels of lyophilised blond Lager beer (LBLB), xanthohumol and folic acid in *Drosophila melanogaster*. (A) Percentage of viability of *Drosophila* treated with different concentrations of the tested compounds. (B) Viability percentage of *Drosophila* tested with different concentrations of the tested compounds combined with the genotoxicant hydrogen peroxide at 0.12 M. *Indicates significant differences (one tail) with respect to their concurrent control (Chi-square value higer than 5.02) (Martínez Becerra & Robles González, 1999).

high polyphenol content of beer would confer the antioxi-dant ability of LBLB, and that would be responsible for the positive effect of LBLB on the protection of DNA oxidative damage.

The total mutation rate in the combined treatments with xanthohumol indicated an antigenotoxic effect, showing sig-nificant differences compared with positive control in both concentrations tested. Both 0.02 and 0.00125 mM concentra-tions reduced the total mutation rates to similar values as the negative control (0.153 and 0.175 spots/wing, respectively) (Table 1). This indicated that xanthohumol is able to partially inhibit the genotoxic activity of H_2O_2 (inhibition percentages of 54 and 58%, respectively). The hydroxyl radicals generated by H_2O_2 could be directly reduced by xanthohumol by the do-nation of a hydrogen atom to radicals. Folic acid was also able to inhibit the induced mutations of the model genotoxin in combined treatments, by reducing its effect when decreasing the concentration, obtaining values of 67.8% (0.0226 mM) and 32.2% (0.00141 mM) of inhibition percentage (Table 1). Folic acid could protect bioconstituents like DNA from the free radical damage originated by hydroxyl radical at least by competi-tions (Joshi et al., 2001). The effect of isolated nutrients or other chemicals derived from food on chronic disease may not have the same beneficial effect as the whole nutrient does (Jacobs & Tapsell, 2013). Xanthohumol is not genotoxic in the *in vitro* assays and is antigenotoxic in the *in vitro* comet assay using HepG2 cells (Plazar, Zegura, Lah, & Filipic, 2007) and in the *Salmonella* microsomal assay (Kac et al., 2008) against procarcinogens and oxidative molecules (tert-butyl hydroper-oxide). Our results using the *in vivo Drosophila* wing spot test will serve to confirm the high antigenotoxic activity of

xanthohumol and folic acid. Folic acid status can influence back-ground levels of DNA damage as it has a role in human chromosome breakage (Ames, 2001; Reidy, Zhou, & Chen, 1983) and in the amelioration of the germ cell toxicity induced by methotrexate in mice (Padmanabhan, Tripathi, Vikram, Ramarao, & Jena, 2009).

3.4. Longevity assays

Longevity is a trait influenced by many factors, including gender, genetic variation, environmental influences, diet, lifestyle, access to health care and cultural influences. *Drosophila* was re-vealed as an excellent system to investigate longevity-promoting properties of compounds and nutraceutical extracts as adult ones seem to show many of the cell senescence features seen in mammals (Fleming, Reveillaud, & Niedzwiecki, 1992). It is well known that caloric restriction without malnutrition is a key strategy for increasing the lifespan (Guarente & Kenyon, 2000). Nevertheless, there is available data on lifespan in-crease by adding nutraceutical substances to the *Drosophila* diet with no reduction of the total intake (Deshpande et al., 2014).

The entire lifespan curves obtained by the Kaplan–Meier method for each substance and concentration are shown in Fig. 3A. All the assayed compounds induced a lifespan expan-sion in *Drosophila melanogaster* compared to the control (47 days average). The ranks of the average lifespan were: 55.3–64.9; 51.9– 64.2 and 53.7–63.8 days for the different concentrations of LBLB, xanthohumol and folic acid, respectively. The rate of in-creased lifespan for all assayed products was +12.6 days with respect to the concurrent control, being the second highest con-centration which showed the longest-lived increases (17.6, 16.9

Table 1 – Genotoxicity and antigenotoxicity of lyophilised blond Lager beer (LBLB), xanthohumol and folic acid in the *Drosophila* wing spot test.

Compound	Clones per wing (n° spots)[a]						
	Number of wings	Small single clones (1–2 cells) m = 2	Large simple clones (more than 2 cells) m = 5	Twin clones m = 5	Total clones m = 2	Mann–Whitney U-test[b]	Inhibition percentage (%)[c]
H_2O	38	0.157 (6)	0	0	0.157 (6)		
H_2O_2	36	0.305 (11) ns	0.083 (3) ns	0	0.388 (14) +		
Simple treatment							
LBLB (mg/ml)							
[3.125]	45	0.133 (6)	0	0.022 (1)	0.155 (7) i		
[50]	34	0.500 (2)	0.029 (1)	0.059 (2)	0.147 (5) i		
Xanthohumol (mM)							
[0.00125]	28	0.214 (6)	0.036 (1)	0	0.250 (7) i		
[0.02]	39	0.051 (2)	0	0	0.051 (2) −		
Folic acid (mM)							
[0.00141]	31	0.193 (6)	0	0	0.193 (6) i		
[0.0226]	48	0.125 (6)	0.021 (1)	0	0.146 (7) i		
Combined treatment with H_2O_2 (0.12 M)							
LBLB (mg/ml)							
[3.125]	36	0.306 (11)	0.028 (1)	0	0.333 (12) β		14.2
[50]	33	0.091 (3)	0.030 (1)	0	0.121 (4) β		68.8
Xanthohumol (mM)							
[0.00125]	40	0.175 (7)	0	0	0.175 (7) β		54.9
[0.02]	39	0.102 (4)	0.051 (2)	0	0.153 (6) β		60.6
Folic acid (mM)							
[0.00141]	38	0.210 (8)	0	0.053 (2)	0.263 (10) β		32.2
[0.0226]	40	0.125 (5)	0	0	0.125 (5) β		67.8

a Statistical diagnosis according to Frei and Wurgler (1988). + (positive), − (negative) and i (inconclusive) versus negative control; β (signifi-cantly different) versus positive control. m: multiplication factor. Kastenbaum–Bowman Test without Bonferroni correction (Frei & Wurgler, 1995), probability levels $\alpha = \beta = 0.05$.

b Inconclusive results were resolved by Mann–Whitney U-test. Delta marker ( ) means no differences between the treatment and the nega-tive control (H_2O).

c The inhibition percentages for the combined treatments were calculated from the total spots per wing according to Abraham (1994).

and 16.5 days for LBLB, xanthohumol and folic acid, respectively).

Our results are in agreement with Tasset-Cuevas et al. (2013) and Villatoro-Pulido et al. (2012) who showed increases of lifes-pan by adding *Borago* seed oil and *Eruca* and sulforaphane. Although LBLB contains many nutrients including carbohy-drate (Ferreira, 2009) this increase of caloric availability would imply the concurrent intake of nutraceutical polyphenols (Gerhauser, 2005). Studies with xanthohumol in murine model also agree with our results showing a significant increased lifes-pan by delaying neurogical disorders caused by leukaemic cells dissemination (Benelli et al., 2012). Nevertheless, in the case of using another model organism like *Caenorhabditis elegans*, *in vivo* studies showed that the inhibition of folate synthesis led to a dose-dependent increse in the animal lifespan (Virk et al., 2012).

3.5. Healthspan assays

In order to know the quality of life of the *Drosophila* treated in the longevity assays, we studied the 25% of individual sur-vival at the top of the lifespan curves obtained in the previous test for each substance and concentration tested. This part of the lifespan is considered as the healthspan of a curve, characterised by low and more or less constant age-specific mortality rate values (Soh, Hotic, & Arking, 2007). The results are shown in Fig. 3B.

All the assayed compounds induced a significant increase of healthspan in *D. melanogaster* when compared to the control, with the exception of the higher concentration of LBLB (50 mg/ ml) which is similar to the control (Table 2). The values of mean survival time for folic acid was between 15.2 and 20.2 days. On the other hand, LBLB and xanthohumol showed an irregular pattern in their mean survival time values with no dose-dependent effects; being the second-to-higher concentrations assayed of both products, the concentrations that showed the maximum increases were higher than 26 days for LBLB and 27 days for xanthohumol with respect to the control. The rest of concentrations of LBLB and xanthohumol had increasing av-erages of 6.1–10.6 and 10.1–16.4 days, respectively.

No previous results have been reported for the healthspan assay of beer and associated compounds. According to Breinholt, Lauridsen, and Dragsted (1999); Hatch, Lightstone, and Colvin (2000); Noroozi, Angerson, and Lean (1998) only a small per-centage of many dietary polyphenols is absorbed, although human studies suggest that this may be adequate to exert a beneficial effect when there are mixed sources in the diet. We hypothesise that the antioxidant components of beer would

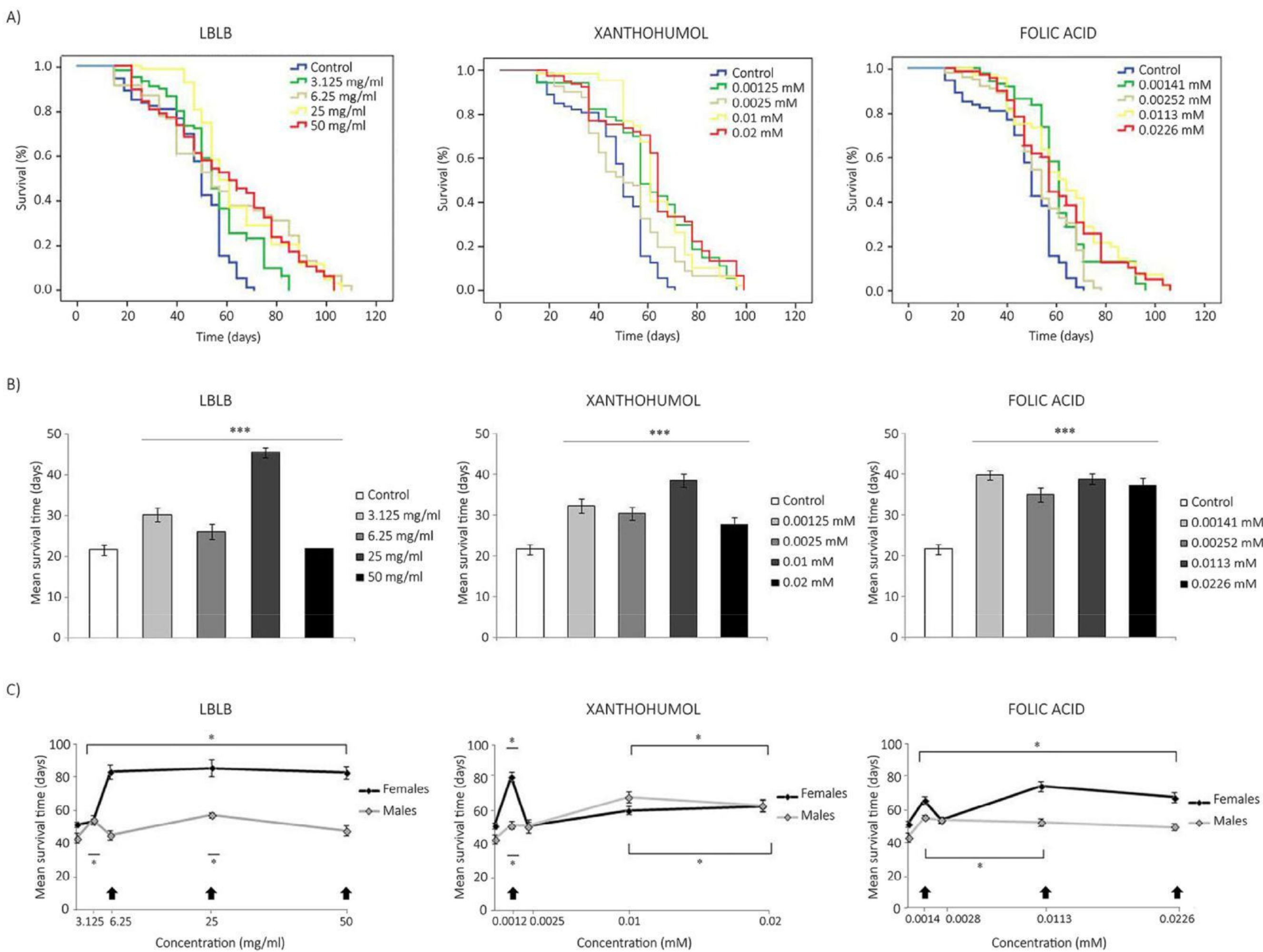


Fig. 3 – Survival parameters of *D. melanogaster* fed with different concentrations of lyophilised blond Lager beer (LBLB), xanthohumol and folic acid over time. (A) Survival curves. (B) Healthspan averages. Mean of survival time in the highest 75% of surviving population. ***Significant ($p < 0.001$) with respect to their concurrent control. (C) Gender comparison. Significances of female and male curves with respect to their controls are symbolised by asterisk (*), meanwhile the significant survival values between females and males are symbolised by arrows (→) in the figure.

counteract the oxidative radicals produced in the metabo-lism of the rest of the diet in the *Drosophila* longevity trials by increasing life and healthspan.

3.6. Gender studies

It is known that gender plays an important role on the human lifespan and has a differential behaviour depending on some-one's lifestyle and specific fitness effects of nutrient intake on the lifespan are common (Maklakov et al., 2008). We analysed the effects of the different concentrations of LBLB and the simple compounds caused by the *Drosophila* survival time curves, taking into account the sex factor (Fig. 3C).

All female and male treatment curves were significantly similar or longer than their corresponding water controls (Fig. 3C). In general, females were more favoured than males in the dif-ferent treatments. From a total of 12 concentrations assayed, females led males by 7 concentrations and were in balance with the male's survival results by the other 5 concentrations left (Fig. 3C and Table 2). The three highest concentrations assayed in females for LBLB produced a significant increase of survival greater than 30 days with respect to their negative control. Con-trarily, males only had a significant increase of survival time in two of the four concentrations assayed with a survival time higher than 12 days with respect to their control. In the xanthohumol assay, both sexes showed significant increases of survival in three of the four concentrations tested with a mean increase of 16 and 18 days for female and male respec-tively. Finally, in the folic acid test, females showed a significant increase of survival time with a range of 14 days with respect to their control while males did not exhibit a significant value for the highest concentration; the rank of survival time in-creased for males had a value of 10.7 days with respect to their control. In general, female increased 23.7, 4.0 and 11.4 days with respect to the male individuals at the treatmets with LBLB, xanthohumol and folic acid, respectively.

In the highest concentration of LBLB assayed, females far exceeded the male's results in an average of 25 days.

Table 2 – Mean and significances of lifespan, healthspan and lifespan curves by gender.

Compound	Concentration	Mean lifespan[a] (days)	Mean healthspan[a] (days)	Lifespan curves by gender (days)		
				Females[a]	Males[a]	Gender sig.[b]
H_2O		47.242	19.769	51.202	49.91	0.386
LBLB	3.125 mg/ml	55.283***	30.351***	52.950*	54.203***	0.280
	6.25 mg/ml	59.111***	25.833**	82.950***	44.774 ns	0.000
	25 mg/ml	64.895***	45.658***	85.398***	56.875**	0.000
	50 mg/ml	60.337***	23.333 ns	82.632***	47.480 ns	0.000
Xanthohumol	0.00125 mM	61.569***	36.168***	80.500***	51.514*	0.000
	0.0025 mM	51.921*	29.842***	51.356 ns	50.958 ns	0.954
	0.01 mM	64.159***	46.686***	60.523***	68.409***	0.051
	0.02 mM	63.490***	32.619***	63.070***	63.416***	0.817
Folic acid	0.00141 mM	61.800***	39.881***	65.630***	54.983***	0.001
	0.00282 mM	53.680***	34.920***	53.618*	53.696**	0.299
	0.0113 mM	63.781***	38.824***	74.251***	52.106*	0.000
	0.0226 mM	60.880***	37.333***	67.434***	49.324 ns	0.002

Means were calculated by the Kaplan-Meier method and significance of the curves were determined by the Log-Rank method (Mantel-cox).
[a] ns: non-significant ($p > 0.05$), *: significant ($p < 0.05$), **: highly significant ($p < 0.01$), ***: very highly significant ($p < 0.001$).
b Significant survival values between females and males caused by the different assayed concentrations symbolised by arrow in Fig. 3C.

Xanthohumol only showed a significant difference between females and males in one concentration. The lowest concen-tration of xanthohumol assayed showed an increase of 20 days of survival time for females comparing to the males' results. Folic acid showed significant differences between females and males in the lowest, medium and highest concentration assayed, with the increase of the survival time for females in relation to males over 8 days.

Genetic differences between males and females have a sig-nificant effect upon aging and lifespan. In general, where the heterogametic sex is male, as in humans and *Drosophila*, females tend to live longer than males (Fenech, Neville, & Rinaldi, 1994; Fox, Dublin, & Pollitt, 2003; Hazzard, 1986; Smith & Warner, 1989; Tower & Arbeitman, 2009). Purine and folate metabolism genes have been argued as potential targets of sex-specific implica-tion for lifespan in *Drosophila* (Bauer et al., 2006). It is important to point out that the findings from studies on *Drosophila*, mice, and *C. elegans* may not fully apply to humans. Gender related differences have already been identified e.g. oestrogens, which are able to increase the expression of genes related to longev-ity (Fenech et al., 1994). We may yet find significant differences in the regulation and action of these pathways as we move from research on lower organisms like *C. elegans* to higher organ-isms like rodents and mammals (Pan & Chang, 2012).

3.7. *Cytotoxicity*

LBLB and xanthohumol showed cytotoxic activity against HL-60 and NIH3T3 cells; folic acid enhanced HL-60 tumour growth and immortal NIH3T3 cells as well (Fig. 4A). LBLB showed a dose dependent response, with an increase of the cytotoxicity level according to increased concentration of LBLB, being the in-hibitory concentration 50 (IC_{50}) of 125 mg/ml in the HL-60 cell line and the same pattern was observed when NIH3T3 cells are treated with lower concentrations of LBLB reaching the IC_{50} at 25 mg/ml. Xanthohumol showed a slight (20%) inhibition at the lowest concentration (0.00125 mM) that is maintained when increasing the concentration, with a maximum inhibition of approximately 30% in HL-60 cells, meanwhile in NIH3T3 cells, a 40% inhibition is achieved at 0.015 mM. In relation to folic acid no inhibition was observed at any concentration tested, but contrarily a slight tendency to increase the cell growth is observed in both cell lines.

According to Gerhauser (2005), chemopreventive activities of beer and xanthohumol relevant to inhibition of carcino-genesis at the initiation, promotion and progression phases, were observed. This fact supports our results in the cytotoxic assays of beer and xanthohumol using promyelocytic HL-60 cells. Xanthohumol results of the present study are in agree-ment with the anti-proliferative activity in different human cancer cell lines (Colgate et al., 2007; Delmulle et al., 2006; Lust et al., 2005; Pan et al., 2005). This biological effect could be related to the anti-inflammatory properties by inhibition of cyclooxygenase activity and its anti-oestrogenic activity (Subbaramaiah, Zakim, Weksler, & Dannenberg, 1997). A wide variety of studies correlated with the chemoprotective activ-ity of xanthohumol with its structure: xanthohumol treatment induced the mitochondrial-mediated pathways of programmed cell death in human colon carcinoma cells and caused a rapid breakdown of the mitochondrial membrane potential, and the release of cytochrome c, leading to apoptosis induction in BPH-1 cells (Strathmann et al., 2010). Some attempts have been made to relate the functional groups of several chalcones to the tox-icity related activities: hydroxyl and prenyl xanthohumol substituents are associated with antioxidant properties, mean-while the thiol reactivity of chalcones is likely to contribute to both cytotoxic and chemoprotective properties of these com-pounds (Go, Wu, & Liu, 2005; Zenger, Dutta, Wolff, Genton, & Kraus, 2015); alpha-methyl chalcones are believed to exhibit greater cytotoxic activity and inhibition of cell growth than unsubstituted analogues (Ducki, 2009; Ducki et al., 1998). Studies about the relationship between cytoxicity and structural-activities of these chalcones are not yet clear: meanwhile a substitution of the A ring drives chalcone activity and cytoxicity, and an OH-group at position 6' of the A-ring instead of a methoxy-group cannot be assigned to any specific activity, but only to the reduced cytoxicity of the resulting chalcone (Zenger et al., 2015). *In vivo* studies by Nozawa et al. (2004) demonstrated

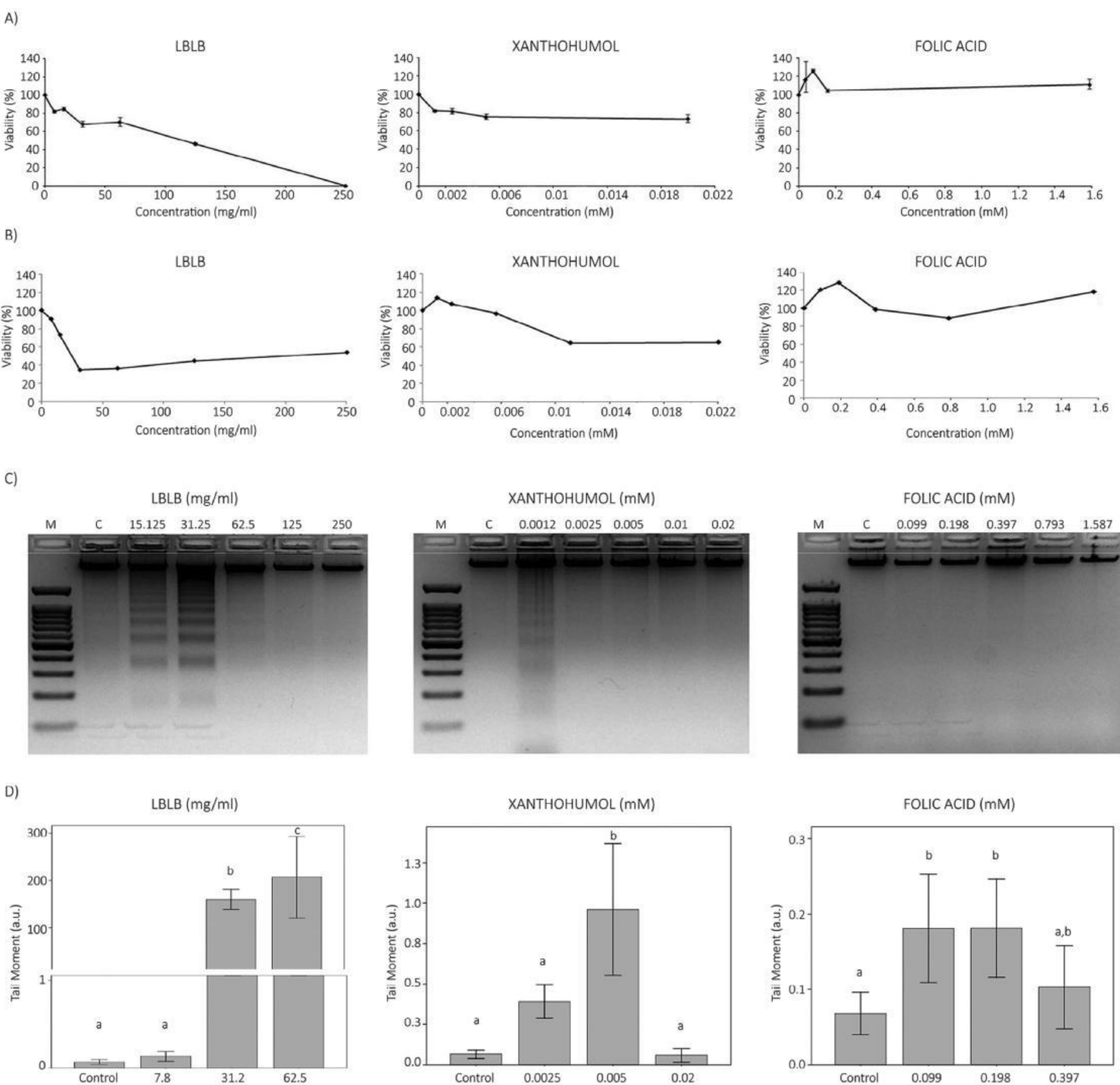


Fig. 4 – Effects of lyophilised blond Lager beer (LBLB), xanthohumol and folic acid on HL-60 cells *in vitro* assays. (A) Viability of HL-60 cells treated with LBLB, xanthohumol or folic acid for 72 hours. (B) Viability of mouse fibroblasts (NIH3T3) in the presence of different concentrations of LBLB and some of its components after 20 hours. Each point represents the growing percentage respect to control mean +/− SD from three independent experiments. (C) Internucleosomal DNA fragmentation in promyelocytic HL-60 cells treated during 5 hours with different concentrations of lyophilised blond Lager beer (LBLB), xanthohumol and folic acid. M indicates DNA size marker. C indicates control treatment. (D) DNA strand breaks induction in promyelocytic HL-60 cells treated during 5 hours with different concentrations of lyophilised blond Lager beer (LBLB), xanthohumol and folic acid. DNA migration is reported as mean TM. The plot shows mean TM values and standard errors. Different letters mean different values after one-way ANOVA and post hoc Tukey's test.

that the intake of beer significantly reduced the formation of colorectal tumours and preneoplastic lesions in Azoxymethane (AOM)-induced experimental carcinogenesis in male Fischer rats. However, considerable variations in inhibitory actions on tumours formation are observed among brands of beer, which may be due to the contents of componets of beer (malt and hops extracts).

On the other hand, chemopreventive activities in LBLB and xanthohumol, relevant to inhibition of carcinogenesis at the initiation, promotion and progression phases, were observed in other studies (Gerhauser, 2005). Xanthohumol has been characterised as a "broad-spectrum" cancer chemopreventive agent *in vitro* studies (Stevens & Page, 2004). One of antican-cer mechanism of xanthohumol is related with inhibition in

the excessive production of prostaglandins, mediators of in-flammation and with the abolishment of the stimulation of proliferation and angiogenesis (Bertl et al., 2004; Gerhauser et al., 2002), as well as the inhibition of the activity of various cyto-chrome P450, which are involved in carcinogen activation (Gerhauser, 2005; Gerhauser et al., 2002; Henderson, Miranda, Stevens, Deinzer, & Buhler, 2000; Miranda et al., 2000). The lack of cytotoxic activity of folic acid is in concordance with the last results which suggest that it could have promoter effect of cancer in humans. The meta-analysis published by Wien et al. (2012) using 10 randomised controlled trials showed a border-line significant increase in frequency of overall cancer in the folic acid group compared to controls. Contrarily, Joshi et al. (2001) proposed that folic acid could prevent cancer due to the free radical scavenging and antioxidant activity, as ubiqui-tous free radical scavengers are believed to be partially responsible for their anticancer activity. On the basis of these epidemiological studies, some doubts about its overuse are arising.

3.8. *DNA internucleosomal fragmentation*

Fig. 4B shows the electrophoresis of the genomic DNA of HL-60 cells treated with different concentrations of LBLB, xanthohumol and folic acid. DNA internucleosomal fragmen-tation is represented by a DNA laddering and it is associated to the activation of the apoptotic way in cancer cells being a hallmark of the genomic integrity (Wyllie, Kerr, & Currie, 1980). Fragmentation was observed in the two lowest concentra-tions of LBLB (15.125 and 31.25 mg/ml) and slightly in the intermediate concentration (62.5 mg/ml). On the other hand, the lowest concentration of xanthohumol (0.0012 mM) also induced the characteristic laddering as an apoptotic activity.

According to Dorn et al. (2010), xanthohumol treatment leads to apoptosis in activated human hepatic stellate cells (HSC) that is mediated by activation of caspase 3. Other studies re-ported that xanthohumol treatment induced the mitochondrial-mediated pathways of programmed cell death in human colon carcinoma cells and caused a rapid breakdown of the mito-chondrial membrane potential, and the release of cytochrome c, leading to apoptosis induction in BPH-1 cells (Strathmann et al., 2010). Finally, folic acid did not induce internucleosomal fragmentation. To our knowledge, this is the first time that LBLB, and folic acid have been assayed in the *in vitro* DNA fragmen-tation. The results obtained in the internucleosomal fragmentation assays suggest that xanthohumol could account for most of the induction of proapoptotic DNA fragmentation of LBLB.

3.9. *Comet assay*

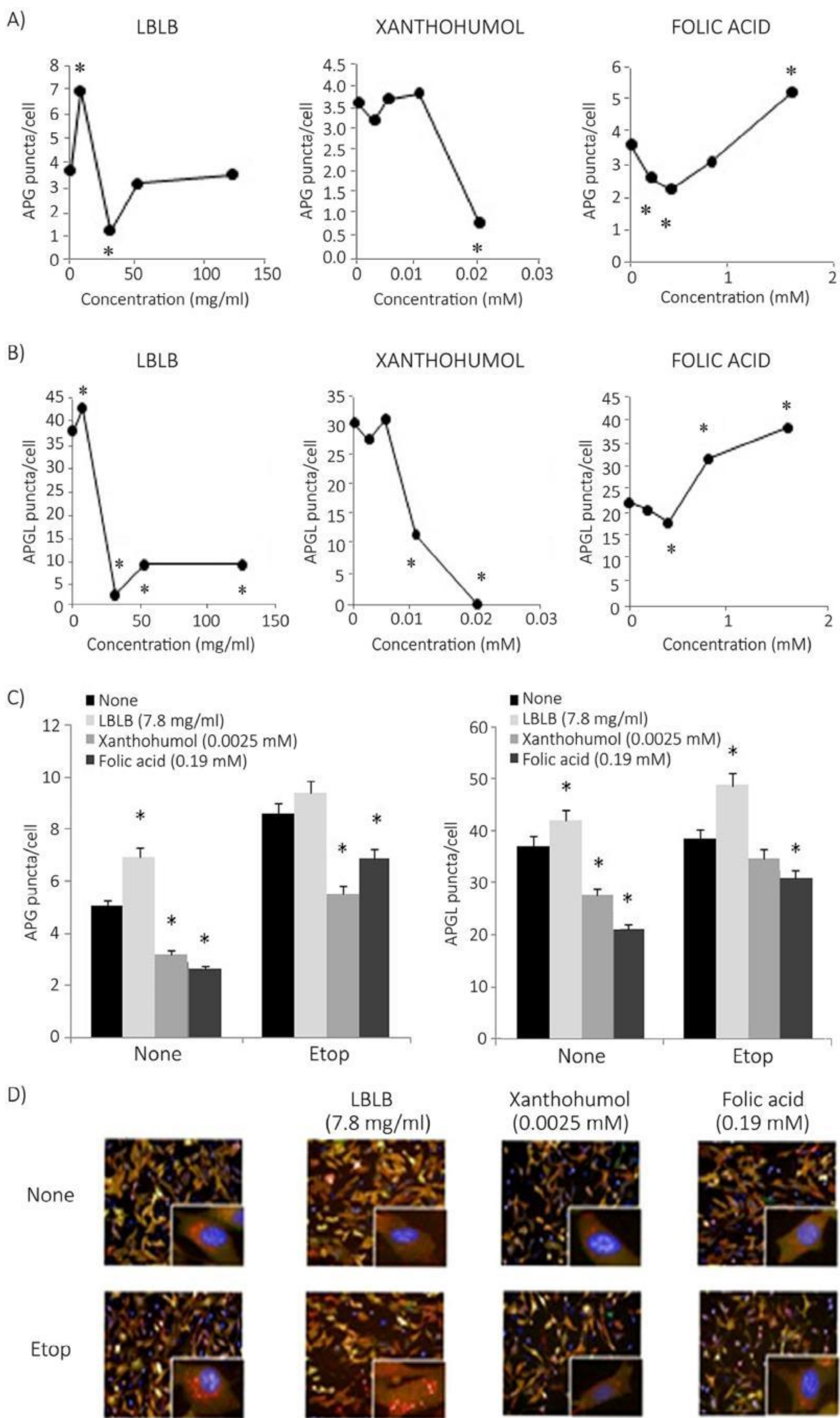
Results of the different tail moment (TM) for each treated cells are shown in Fig. 4C. By using the comet assay we were able to readily identify apoptotic nuclei based on the characteris-tic morphological changes attributable to extensive DNA damage (Poe & O'Neill, 1997). The comet assay tail moment measurements have been shown to correlate directly to the percentage of viable cells as monitored using trypan blue ex-clusion for several human lymphoblast cell lines. Our results agree with the above correlations mentioned showing significant TM values correlated with the cytotoxicity ones that have been observed in the single cell gel electrophoresis. This fact could be caused by the double and/or the single strand breaks induced by the two highest concentrations assayed of LBLB, the 0.005 mM concentration of xanthohumol and the two lowest tested folic acid concentrations. According to Fairbairn, Walburger, Fairbairn, and O'Neill (1996) the TM measure-ments are correlated with the cytotoxicity ones. Taking the DNA fragmentation into account, we could assume that the comet assay shows single strand breaks in the xanthohumol and folic acid treatments because of the lack of DNA internuclear frag-mentations showed in the genomic DNA electrophoresis of these compounds. On the other hand, double and single strand breaks are induced by LBLB as both DNA internuclosomal frag-mentation and single cell gel electrophoresis comet assay show significant results. Studies with xanthohumol supported the significantly inhibited menadione-induced DNA single-trand breaks in Hepa1c1c7 cells pretreated (Dietz et al., 2005). Folic acid is essential for the synthesis and repair of DNA. Duthie and Hawdon (1998) studied the effects of folate depletion on DNA stability in normal human lymphocytes *in vitro*. They ob-served that lymphocytes cultured under follate-deficient conditions failed to grow normally compared with control cells. Morever, cells deprived of folate were unable to efficiently repair oxidative DNA damage induced by hydrogen peroxide.

3.10. *Macroautophagy activity*

Results of LBLB, xanthohumol and folic acid effect on MA are shown in Fig. 5. MA is an important cellular pathway for main-taining homeostasis, development, differentiation and survival of cells, where damaged organelles, proteins and intracellu-lar pathogens are degraded. Defects in MA lead to altered metabolic homeostasis, protein quality control, among others. This effect varies, depending on the site of autophagic block-age (Schneider & Cuervo, 2014). It is known that MA decreases with age in almost all the organisms studied and diet or spe-cific nutrients affect MA activity (Hannigan & Gorski, 2009).

Our results show that LBLB at low concentration tested (7.8 mg/ml) induces an increase of MA activity; on the basis of these findings, moderate intake of beer could be helpful for restoring the deficit on MA activity found in aging (Cuervo, 2008; Cuervo et al., 2005). Limited dietary intake decreases the in-cidence of age-related disorders and increases lifespan in numerous animal experimental models from invertebrates to mammals (Masoro, 2006). In the present research, the effects on *Drosophila* lifespan of low to moderate doses of LBLB are similar to a limited dietary intake as an increase of the lifes-pan is observed. These data fit to the MA significant activation at low to moderate LBLB doses in NIH3T3 cells. On the other hand, high-lipid content diets have an inhibitory effect on MA and chaperone mediated autophagy that explains how obesity and fatty liver disease disrupt this pathway (Rodriguez-Navarro & Cuervo, 2012), so the daily intake of LBLB could protect against lipotoxicity.

Only one of the components tested, folic acid acts as an ac-tivator of MA in immortal cells at high concentrations assayed. The intake of folic acid is controversial during the years as it was recommended for its effects on DNA synthesis and repair,



A)
LBLB
XANTHOHUMOL
FOLIC ACID
APG puncta/cell
Concentration (mg/ml)
Concentration (mM)
B)
LBLB
XANTHOHUMOL
FOLIC ACID
APGL puncta/cell
Concentration (mg/ml)
Concentration (mM)
C)
None
LBLB (7.8 mg/ml)
Xanthohumol (0.0025 mM)
Folic acid (0.19 mM)
APG puncta/cell
APGL puncta/cell
None
Etop
D)
LBLB (7.8 mg/ml)
Xanthohumol (0.0025 mM)
Folic acid (0.19 mM)
None
Etop

Fig. 5 – Effect of lyophilised blond Lager beer (LBLB), xanthohumol and folic acid on Macroautophagy activity. Macroautophagy activity was measured in NIH3T3 cells stably expressing the tandem reporter mCherry-GFP-LC3-IL. Analysis was done using high content microscopy in 9 different fields per condition and well (approximately 3500 total cells per condition). (A) Quantification of the number of Autophagosomes (APG) and (B) Autophagolysosomes (APGL) was performed. *Significant ($p < 0.05$). (C) Effect of Etoposide on Macroautophagy induced by the tested compounds. Cells were grown in absence or presence of the stressor (Etopo: 25 M). Concentrations were selected on the basis of the results obtained in single treatments shown in Fig. 5A–B. Quantification of the number of autophagyc vacuoles (AV: total red puncta), Autophagosomes (APG: total yellow puncta) and Autophagolysosomes (APGL: red but not green puncta) was performed. *Significant ($p < 0.05$). (D) Representative merged images of these cells and high magnification insets. Autophagosomes are shown as yellow puncta and Autophagolysosomes are shown as red puncta. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

but recently (Wien et al., 2012), a relationship between folate intake and the frequency of overall cancer has been suggested.

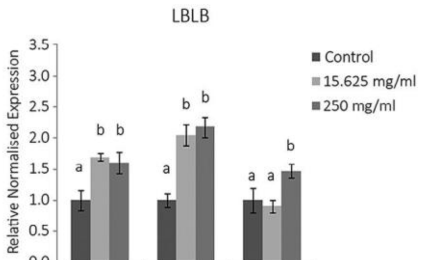
Additionally we also analysed the effect of xanthohumol on MA activity and we found that xanthohumol is an inhibi-tor of MA at any concentration assayed; we could define xanthohumol as responsible for the inhibitory effect of LBLB at higher concentrations; with findings and confirmation from Delmulle, Berghe, Keukeleire, and Vandenabeele (2008) who could not detect any typical apoptotic morphological fea-tures like vacuoles. Once again, the importance of doses– effect is revealed when food or beverages are intended to be used as a nutraceutic. For that reason, moderate concentra-tions of folic acid and the other phenols, like those contained in beer, are acceptable. This is the first time that xanthohumol is proposed to be considered as a new family of autophagy in-hibitors. Besides, it could be used in cancer therapy as a pharmacological inhibitor of MA. Moreover, the folic acid results allow to propose it as an activator of MA, so it could be used in aging as a pharmacological upregulator of MA. In this context more studies are needed to clarify the doses and time point of folic acid administration.

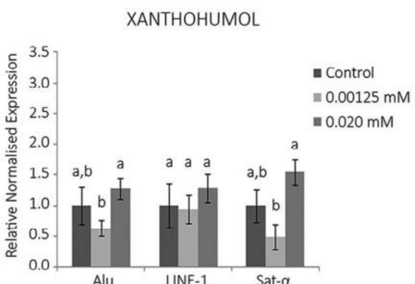
Furthermore, the DNA damaging topoisomerase inhibitor (etoposide) in control cells induces an increase on MA. This re-sponse is still maintained in the presence of all treatments analysed at a lower concentration (Fig. 5).

3.11. Methylation status

The relative normalised expressions of the three repetitive se-quences (Alu M1, LINE-1 and Sat-α) studied in HL-60 cells treated with different concentrations of LBLB, xanthohumol and folic acid are shown in Fig. 6. LBLB showed a significant hypermethylated status in all the tested concentrations except in the lowest concentration (15.625 mg/ml) for the Sat-α re-petitive sequence, with respect to the concurrent control. Xanthohumol did not exhibit a highlight diversity in the modu-lation of the methylation status as both concentrations assayed showed methylation levels similar to the normalised control except the lowest concentration (0.00125 mM) in the Alu regions and the highest (0.020 mM) concentration in Sat-α regions that showed a significant demethylation and methylation status, respectively. Folic acid exhibited a methylation status in the lowest concentration tested (0.099 mM) in Alu and LINE-1 regions and a demethylation status for that concentration in the Sat-α sequences, with respect to the normalised control.

Overall, all the assayed substances act as a preventive by blocking repetitive sequences of HL-60 tumoural cells as it changes the usual hypomethylation status of these elements in cancer cells to a methylated status. This fact agrees with the assays about methylation levels in the Alu, LINE-1 and Sat-ellite repetitive DNA sequences in normal and tumoural cells obtained by Chalitchagorn et al. (2004); Ehrlich et al. (2003); Roman-Gomez et al. (2008); Schmid (1991). Studies about the effect of folic acid on genomic DNA methylation suggest that folic acid plays a critical role in the prevention of chromo-some breakage and hypomethylation of DNA (the hypomethylation can be reversed by folic acid) (Fenech, 2001; Pufulete et al., 2005). Furthermore, the biochemical roles of folate in DNA synthesis, repair, and methylation are well estab-lished (Joshi et al., 2001). No previous studies about the properties that beer and xanthohumol have on the modifica-tion on the DNA methylation status have been reported. Studies have been carried out using diet components as possible modu-lator agents of DNA methylation in cancer cells. Not only methyl donor agents are able to modify epigenetic patterns of DNA





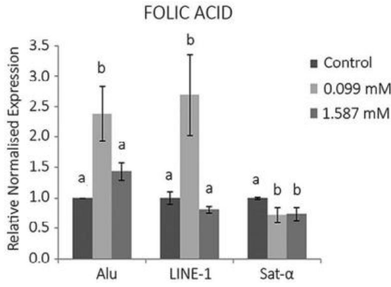


Fig. 6 – Relative normalised expression data of each repetitive element (Alu, LINE-1 and Sat-α). Different letters mean different values after one-way ANOVA and post hoc Tukey's test.

but also some phenolics. The modulation of CB_1 tumour sup-pressor gene expression by extra virgin olive oil phenolic compounds via epigenetic mechanism, both in vitro and in vivo, is proposed to provide a new therapeutic avenue for treat-ment and/or prevention of colon cancer (Di Francesco et al., 2015). Further studies on other repetitive sequences could be useful for better understanding the methylating role of xanthohumol on promyelocytic cells found in the present re-search. Epigenetic therapy against harmful effects of diet components could be a potential tool in chemotherapy because epigenetic alterations are reversible in contrast to genetic defects (Miyamoto & Ushijima, 2005). Diet components could act in a dual way by increasing or decreasing the methylation levels depending on the specific gene promoter or the genomic region.

4. Conclusions

In the present study, we evaluated the effects of LBLB and some of its components at the individual, cellular and DNA level. All data indicated that: (i) the intake of LBLB at moderate doses could be recommended because it is neither toxic nor genotoxic, exerts genomic protection against hydrogen peroxide, in-creases lifespan, improves healthspan, inhibits tumour cells growth, induces DNA proapoptotic fragmentation and acti-vates MA (only at low concentration); (ii) Xanthohumol protects against genotoxicant, improves the extension and quality of life of *D. melanogaster* and it is an inhibitor of MA activity; (iii) Folic acid is not toxic, improves extension and quality of life and is an activator of MA. Despite the relations between the structure and the activities that chalcone and folate exibit, the effect of isolated components of LBLB on the degenerative checkpoints studied has been demonstrated not to have the same beneficial effect as the whole complex mixture of beer, suggesting that most of the beneficial properties are exerted when there are mixed sources in the diet.

All the *in vivo* and *in vitro* assays carried out in the present paper of the role of LBLB suggest an important role on the modulation of degenerative processes. The safety (non-toxicity, non genotoxicity), the protection against the oxidative genotoxine H_2O_2 (antitoxicity, antigenotoxicity), the induc-tion of life and healthspan extension, the cytotoxicity against leukaemia HL-60 cells and the induction of the proapoptotic DNA fragmentation and DNA damage in individual cells of LBLB have been established. The phenol xanthohumol and folic acid contained in beer showed most of the activities of beer as a complex mixture. Some interesting exceptions have to be pointed out: folic acid is not antitoxic, but it exerts a syner-gistic activity with H_2O_2 and has no cytotoxic activity but contrarily, it seems to increase the growth of tumour cells HL-60. In conclusion, results suggest that daily moderate consumption of beer may be healthy due to the properties that the full beverage shows.

Conflict of interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.jff.2016.09.014.

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FULL COMMUNICATION

Role of Choline in the Modulation of Degenerative Processes: In Vivo and In Vitro Studies

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ABSTRACT The purpose of the present study was to examine the nutraceutical potential of choline as an added value to its well-known brain nutrient role. Several toxicity, antitoxicity, genotoxicity, antigenotoxicity, and longevity endpoints were checked in the somatic mutation and recombination test in in vivo Drosophila animal model. Cytotoxicity in human leukemia-60 cell line (HL-60) promyelocytic and NIH3T3 mouse fibroblast cells, proapoptotic DNA fragmentation, comet assay, methylation status, and macroautophagy (MA) activity were tested in in vitro assays. Choline is not only safe but it is also able to protect against the DNA damage caused by an oxidative genotoxin. Moreover, it improves the life extension in the animal model. The in vitro results show that it is able to exhibit genetic damage against leukemia HL-60 cells. Single-strand breaks in DNA are observed at the molecular level in treatments with choline, although only a significant hypermethylation on the long interspersed elements-1 and a hypomethylation on the satellite-alpha DNA repetitive DNA sequences of HL-60 cells at the lowest concentration (0.447 mM) were observed. Besides, choline decreased MA at the lower assayed concentration and the MA response to topoisomerase inhibitor (etoposide) is maintained in the presence of treatment with 0.22 mM choline. Taking into account the hopeful results obtained in the in vivo and in vitro assays, choline could be proposed as a substance with an important nutraceutical value for different purposes.

KEYWORDS: antigenotoxicity antitoxicity choline cytotoxicity DNA damage Drosophila melanogaster HL-60 and NIH3T3 cell lines longevity macroautophagy methylation status

INTRODUCTION

Choline is a natural amine classified in the vitamin B complex and is a precursor for many other biologically important molecules. It was officially recognized as an es-sential nutrient important in the diet by the Institute of Med-icine in 1998,[1] its requirement extending into adulthood and old age.[2] Choline is synthesized in the liver. It is present in several foods, with liver, egg, and wheat germ among the best dietary sources of choline.[3]

Choline is important for brain development as it is a precursor for the neurotransmitter acetylcholine, which is essential for the activity of cholinergic neurons.[4,5] Choline is important for reducing the risk of neural tube defects[6] for memory development[7] and it has also been demonstrated to be an essential element for survival and normal growth of cultured cells.[8–11] In primary neuronal cortical cell cultures, inhibition of phosphatidyl choline synthesis through N-methyl-d-aspartate receptor is a key early event in the process of cellular excitotoxicity that leads to necrotic cell death[12] due to cytoplasmic membrane damage caused by increased hydrolysis of membrane phospholipids.[13] Furthermore, similar to folic acid, genomic stability has been attributed to choline[14] because both are methyl donors and a high choline diet has been associated with a decreased risk of breast cancer.[15] On the other hand, studies have shown that choline deficiency may have adverse effects such as increased DNA damage and apoptosis in lymphocytes,[16] increased plasma homocysteine levels, which have been associated with cardiovascular disease,[17] cognitive decline,[18] bone fractures,[19] development of fatty liver and liver muscle damage,[20] and a reduced secretion of liver triglyceride, resulting in accumulation of liver triglycerides,[21] among other pathologies. Despite the beneficial effects of a daily intake of choline, a greater intake than the recommended dosage has been associated with harmful effects such as nausea and diarrhea,[22] body odor, sweating and salivation,[23–25] hypotension,[26] hepatotoxicity,[27] and dermatitis.[28] In rare cases, a large amount of choline consumption has been associated with depression[29] and with exacerbated symptoms of parkinsonian patients.[30]

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Wide range of factors affect the dietary choline requirements, such as the work of choline in concert and interaction with other nutrients,[5] the gender,[31] the physical activity of individuals,[32,33] the bioavailability of choline,[34] and the genetic polymorphisms,[35] among others.

Many nutrients can be considered as a nutraceutic because of their additional biological activities mostly related to delaying degenerative processes. A nutraceutical substance should be able to prevent genetic oxidative damage, increase life span in normal cells/organisms, and induce clastogenic injuries in tumor cells. In this study, we have tested the nutraceutical potential of choline. Several points related to degenerative processes have been checked: (i) in vivo assays using the Drosophila model, as 70% of human disease genes are conserved in this organism,[36] to evaluate the toxicity, antitoxicity, genotoxicity, antigenotoxicity, and longevity induction and (ii) in vitro assays to evaluate the cytotoxic, DNA-induced damage activity, methylation status, and macroautophagy activity in the human leukemia-60 cell line (HL-60) promyelocytic and immortalized NIH3T3 cell models.

The main aim of our study is to identify the possible nutraceutical potential of choline on the degenerative process to propose it as a substance with therapeutic potential and consider it not only as an essential element for daily intake. For that purpose, we fed Drosophila melanogaster with different concentrations of choline and we analyzed the survival percentage, the mutagenic effect, the protection against H_2O_2, and the extension of life span. Moreover, using in vitro models of human leukemia cells (HL-60) and mouse fibroblast cells (NIH3T3), we studied the effect of choline on growth inhibition of tumoral cells, DNA damage (internucleosomal fragmentation as double-strand breaks DNA laddering associated with activation of the apoptotic pathway or single/double-strand breaks in cells), the modulation of methylation status, and the macroautophagy (MA) activity.

MATERIALS AND METHODS

Simple compound

Different concentrations of choline (Fluka; 26980-50G) were tested (0.447, 0.895, 1.791, 3.581, and 7.163 mM) taking into account the average daily food intake of D. melanogaster (1 mg/day) and the average body weight of D. melanogaster (1 mg).[37] The concentration range was cal-culated to make it comparable with the daily tolerable upper intake for humans (3.5 g/day)[38,39] and higher concentrations were also assayed to study the possible nutraceutical po-tential of choline.

In vivo assays

Drosophila melanogaster strains. Two Drosophila strains were used, each with a hair marker in the third chromosome:

flare genetic marker (flr^3)/In (3LR) TM3, $rip^p sep bx^{34e} e^s Bd^S$, where the flr^3 (flare) marker is a homozygous lethal recessive mutation that produces deformed tricomas, but is viable in homozygous somatic cells once larvae start the development.[41]

Strains were maintained at 25LC and 80% humidity in glass tubes with a homemade meal (0.5 g NaCl, 12 g agar-agar, 100 g yeast, 25 g sucrose, 5 mL propionic acid, 3.5 mL of 0.2% streptomycin sulfate solution, and 1 L of water), making changes three times per week.

Virgin females were obtained from these tubes and were crossed to perform the crosses for the different assays of toxicity, antitoxicity, genotoxicity, antigenotoxicity, and longevity.

Toxicity and antitoxicity assays. Survival percentages of treated Drosophila were studied in the toxicity assays [(number of individuals born in each treatment/number of individuals born in the negative control) · 100]. We assayed five concentrations of choline ranged between 0.447 and 7.16 mM. The antitoxicity tests consisted of combined treat-ments of the same concentrations as in the toxicity assays by adding the H_2O_2 at 0.12 M (Sigma; H1009).[42] The negative controls were prepared with medium and distilled water and positive controls with medium and H_2O_2.[43] Three indepen-dent experiments were carried out for each assay. Chi-square test in a Microsoft Office Excel 2007 was used to determine if the tested compounds significantly inhibited the survival of flies, with respect to the control and among the concentra-tions. In the toxicity assay, statistical chi-square values ($P < .05$) for the different concentrations tested were obtained by comparing the different concentrations with respect to the negative control, whereas statistical chi-square values of antitoxicity assays were obtained by comparing the different concentration values with respect to the positive control.

Genotoxicity and antigenotoxicity assays (somatic mutation and recombination test). The genotoxicity assays were carried out following the method described by Graf et al. by crossing transheterozygous larvae for mwh and flr^3 genes.[44] The treatment tubes contained 0.85 g of Drosophila Instant Medium (Formula 4–24; Carolina Biological Sup-ply) and 4 mL of solutions with 0.447 and 7.16 mM of choline. The antigenotoxicity tests were performed by combining 0.12 M H_2O_2 and the same concentrations used in genotoxicity assays of choline.

Wings of emerging transheterozygous individuals (mwh flr^+/mwh^+ flr^3) for each control and concentration were mounted on slides using Faure's solution (30 g gum Arabic, 20 mL glycerol, 50 g chloral hydrate, and 50 mL water) and scored under a photonic microscope at 400 · magnification. Similar numbers of male and female wings were mounted and wing hair mutations were scored from a total of 24,400 monotricoma wild-type cells per wing.[45] In the balancer-heterozygous genotypes (mwh/TM3, Bd^S), mwh spot phenotypes are produced predominantly by somatic point mutation and chromosome aberrations since mitotic

recombination between the balancer chromosome and its structurally normal homolog is a lethal event. Wing hair spots were grouped into three different categories: a small single spot corresponding to one or two cells exhibiting the mwh phenotype and occur in the late stages of mitotic division; a large single spot with three or more cells showing mwh or flr[3] phenotypes and occur in the early stages of larval development; or a twin spot corresponding to two juxtapo-sitioning clones, one showing the mwh phenotype and other the flr[3] phenotype. Small and large spots are originated from somatic point mutation, chromosome aberration, and so-matic recombination, while twin spots are produced exclu-sively by somatic recombination between the flr[3] locus and the centromere.

A total of 25–44 wings were mounted and analyzed for the different treatments. The total number of spots was determined and a multiple decision procedure was applied to determine whether a result is positive, inconclusive, or negative.[46] The frequencies of each type of mutant clone per wing were compared with the concurrent control and ana-lyzed applying the binomial Kastenbaum and Bowman test,[47] without Bonferroni correction and at 5% level of significance. All inconclusive results were analyzed with the nonparametric U-test of Mann–Whitney and Wilcoxon (a = b = 0.05). The inhibition percentages (IPs) for combined treatments were calculated from total spots per wing with the following formula[48]:

IP ¼ [(single genotoxin combined treatment)=
single genotoxin] · 100

Life span assays. All experiments were carried out at 25LC according to the procedure described by Tasset-Cuevas et al.[49] Sets of 10 adult individuals of the same gender were selected and placed into sterile vials containing 0.21 g of Drosophila Instant Medium and 1 mL of different concentrations of choline solution (0.447, 0.895, 3.581, and 7.16 mM). Four replicates were followed during the com-plete life span for each control and the established concen-trations. Alive animals were counted and the respective media renewed twice a week.

To know the quality of life of treated Drosophila in longevity trials, the upper 25% of life span survival curves was studied. This part of the life span is considered as the health span of a curve, characterized by low and more or less constant age-specific mortality rate values.[50]

The statistical treatment of survival data for each control and concentration was assessed with the SPSS Statistics 17.0 software (SPSS, Inc.), applying the Kaplan–Meier method. The significance of curves was determined using the log-rank method (Mantel-Cox).

In vitro assays

Cell culture conditions. The promyelocytic human leukemia cell line HL-60 was grown in RPMI-1640 medium (Sigma; R5886) supplemented with heat-inactivated fetal bovine serum (Linus; S01805), l-glutamine (Sigma; G7513), and antibiotic–antimycotic solution (Sigma; A5955). Cells were incubated at 37LC in a humidified atmosphere of 5% CO_2. The cultures were plated at 2.5 · 10^4 cells/mL density and passed every 2 days.

The NIH3T3 cell line, derived from mouse fibroblast cells, was cultured at 37LC, 95% humidity, and 5% CO_2 in complete Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% (v/v) heat-inactivated newborn calf serum (Gibco) and 1% penicillin/streptomycin/Fungizone mix. The cell cultures were passaged every 3–4 days by trypsinization (0.05%). Only passages 10–25 were used.

The use of these two types of cells will allow us to study the effect of choline on promyelocytic leukemia and on an immortalized fibroblast cell model line. Although they belong to different organisms (human and mouse), results complemented each other and cytotoxicity assays will be comparable.

Cytotoxicity assay. HL-60 cells were placed in 96-well culture plates (2 · 10^4 cells/mL) and treated for 72 h with choline 0.447–7.16 mM. Cell viability was determined by the trypan blue dye (Sigma; T8154) exclusion test in a Neubauer chamber at 100 · magnification (AE30/31; Motic). Curves were plotted as survival percentage of three independent experiments with respect to the control growing for 72 h.

Table 1. Primer Information

Reaction ID	GenBank number	Amplicon start	Amplicon end	Forward primer sequence 5^0 to 3^0 (N)	Reverse primer sequence 5^0 to 3^0 (N)	GC content (%)	
						Forward	Reverse
ALU-C4	Consensus sequence	1	98	GGTTAGGTATAGTGGTTTA TATTTGTAATTTTAGTA (36)	ATTAACTAAACTAATCT TAAACTCCTAACCTCA (33)	25	27.3
ALU-M1	Y07755	5059	5164	ATTATGTTAGTTAGGATGGTTTC GATTTT (29)	CAATCGACCGAACGCGA (17)	27.6	58.8
LINE-1	X52235	251	331	GGACGTATTTGGAAAATCGGG (21)	AATCTCGCGATACGCCGTT (19)	47.6	52.6
SAT-a	M38468	139	260	TGATGGAGTATTTTTAAAATA TACGTTTTGTAGT (34)	AATTCTAAAAATATTCCTCTT CAATTACGTAAA (33)	23.5	21.2

Source from Weisenberger et al.[60]
Sat-a, satellite-alpha DNA; LINE, long interspersed elements.

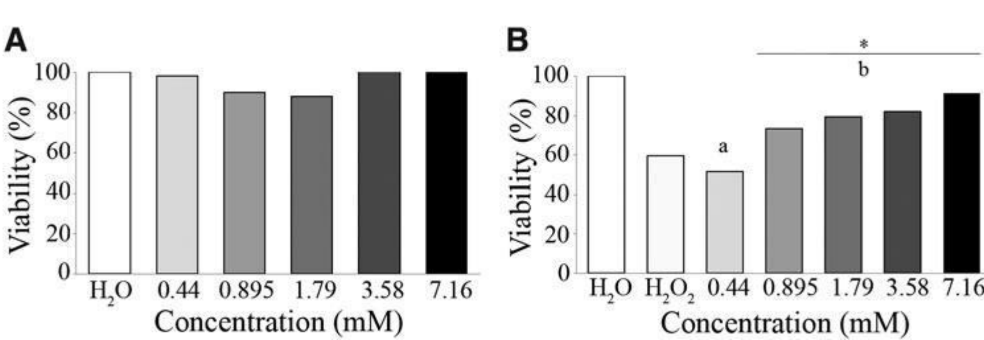

FIG. 1. Toxicity (A) and antitoxicity (B) levels of choline in Drosophila melanogaster. Data are expressed as percentage of surviving adults with respect to 300 untreated 72-h-old larvae from three independent experiments treated with different concentrations of choline (A) and combined treatments with 0.12 M H_2O_2 (B). *Significant differences with respect to the positive control; [ab]Pairwise comparison among concentrations.

NIH3T3 cells were plated in 96-well flat-bottom plates (BD Biosystems) at $1 \cdot 10^4$ cells per well and treated for 24 h with choline 0.223–7.16 mM. Cell viability was measured using the CellTiter-Blue Cell Viability Assay (Promega). After treatments, 20 lL of CellTiter-Blue Reagent was added to each well and cells were cultured for 2 h as changes in fluorescence at 540 nm excitation and 590 nm emission wavelengths were measured using a Tecan, Infinite M200Pro microplate reader. Fluorescence intensity values were nor-malized to values of untreated cells. Curves were plotted as survival percentage of three independent experiments with respect to the control growing for 24 h.

Determination of DNA fragmentation. DNA inter-nucleosomal fragmentation is observed by DNA laddering and it is associated with activation of the apoptotic pathway in cancer cells as a hallmark of apoptosis affecting genomic integrity.[51] HL-60 cells ($1 \cdot 10^6$ cells/mL) were treated with different concentrations of choline for 5 h. Treated cells were collected and centrifuged at 3000 rpm for 5 min, and DNA was extracted as follows: the cell pellet was re-suspended in 900 lL of cell lysis buffer pH 8.0 (10 mM Tris-HCl, 5 mM ethylenediaminetetraacetic acid [EDTA], 100 mM NaCl) and 100 lL of sodium dodecyl sulfate 10% and 25 lL of proteinase K solution (20 mg/mL) were added and incubated with shaking for 5 h at 55LC. After this, 432 lL of 5 M NaCl was added and the samples were centrifuged at 13,000 rpm for 15 min. Supernatant was re-covered into a fresh tube and 750 lL of cold isopropanol was added to precipitate DNA. Then, samples were centri-fuged at 13,000 rpm for 10 min, washed with 1 mL of 70% ethanol, and DNA dried and resuspended in 20 lL of deio-nized water. Finally, 0.6 lL of 0.4 mg/mL RNase was added and incubated at 37LC (300 rpm) overnight. Total extracted DNA was quantified in a spectrophotometer (Nanodrop ND-1000) and 1200 ng of DNA was subjected to 2% agarose gel electrophoresis at 80 mA for 25 min, stained with ethidium bromide, and visualized under UV light.

Comet assay. The DNA strand break induction was determined by the alkaline comet assay (pH <13), following the method previously described[52–54] with some modifica-tions. Cells were treated with choline (0.895, 1.791, and 7.16 mM) for 5 h. After washing steps in phosphate-buffered saline, a concentration of $6.25 \cdot 10^5$ cells/mL was mixed with 0.75% low-melting point agarose (Sigma; A4018) and transferred to frosted-end slides. The slides were bathed in lysis solution (2.5 M NaCl, 100 mM Na-EDTA, 10 mM Tris,

Table 2. Genotoxicity and Antigenotoxicity of Choline in the Drosophila Wing Spot Test

	Clones per wing (no. spots)[a]						
Compound	Number of wings	Small single clones (1–2 cells) m = 2	Large simple clones (more than two cells) m = 5	Twin clones m = 5	Total clones m = 2	Mann–Whitney U-test[b]	Inhibition percentage (%)[c]
H_2O	38	0.157 (6)	0	0	0.157 (6)		
H_2O_2	36	0.305 (11)	0.083 (3)	0	0.388 (14) +		
Simple treatment							
Choline (mM)							
[0.447]	41	0.097 (4)	0.073 (3)	0	0.171 (7) i	D	
[7.16]	25	0.200 (5)	0	0	0.200 (5) i	D	
Combined treatment with H_2O_2 (0.12 M)							
Choline (mM)							
[0.447]	44	0.182 (8)	0.045 (2)	0	0.227 (10) k		41.5
[7.16]	38	0.079 (3)	0.052 (2)	0	0.131 (5) k		66.2

+ (positive) and i (inconclusive) versus negative control; k (significantly different) versus positive control.
Levels of significance a = b = 0.05, tail test without Bonferroni correction. Inconclusive results were resolved by U-test of Mann–Whitney and Wilconxon.
[a]Statistical diagnosis according to Frei and Wurgler.[46]
[b]Inconclusive results were resolved by Mann–Whitney U-test. Delta marker (D) means no differences between the treatment and the negative control.
[c]The inhibition percentages for the combined treatments were calculated from total spots per wing according to Abraham.[48]

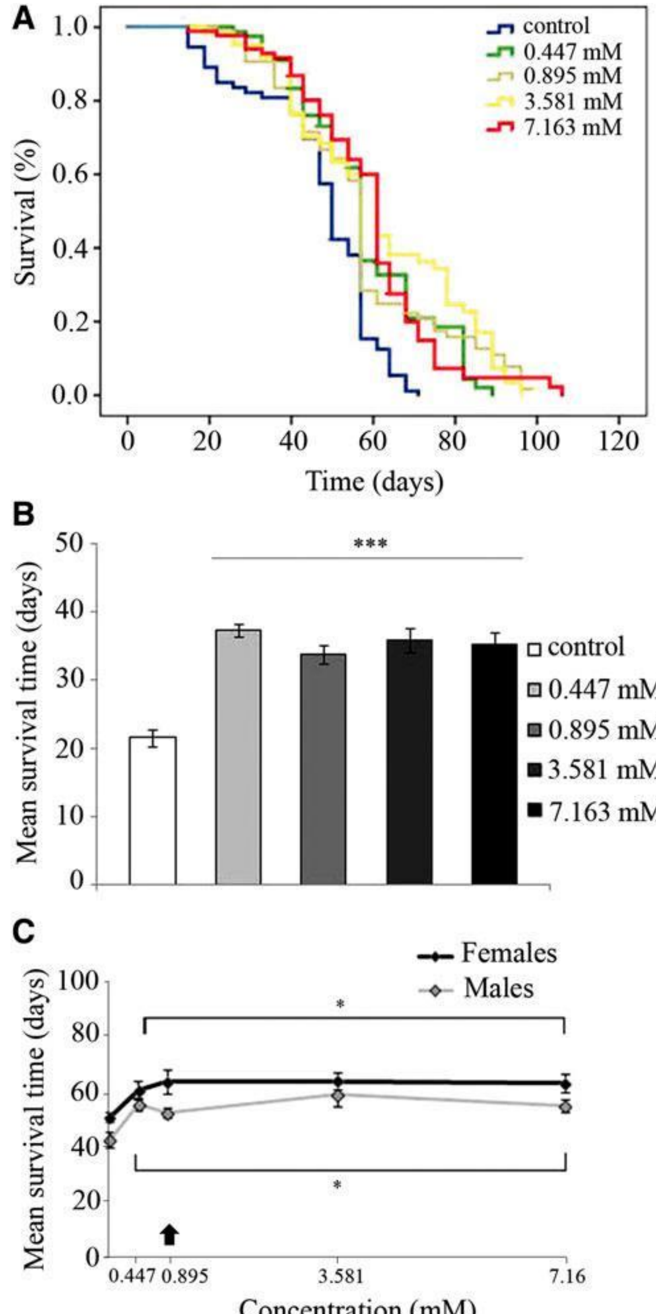

FIG. 2. Survival parameters of Drosophila melanogaster fed with different concentrations of choline. (A) Survival curves, (B) health span averages, and (C) gender comparison. (A) Survival curves of Drosophila melanogaster. (B) Mean of survival time in the highest 75% of surviving population. ***Indicates significant differences (P < .001) with respect to the control. (C) Significance between fe-male and male curves with respect to their controls is symbolized by asterisk (*); significant survival values between females and males at the different treatments are symbolized by arrows (/).

250 mM NaOH, 10% dimethyl sulfoxide, and 1% Triton X-100; pH 13) for 1 h at 4LC and immersed into alkaline electrophoresis buffer (300 mM NaOH and 1 mM Na-EDTA; pH 13) for 20 min at 4LC. Then, electrophoresis (20 V, 400 mM for 15 min) was performed and slides were submerged into neutralization buffer (0.4 M Tris-HCl buff-er; pH 7.5). Finally, slides were dried overnight at room temperature in the dark. DNA of 50–100 single cells was visualized by treating slides with 7 lL of a 10 lg/mL stock solution of propidium iodide (Sigma; P4170).[53] Comet im-ages were analyzed at 400 · magnification using a Leica DM 2500 fluorescence microscope with green filter and an at-tached camera (JAI CV-M4CL). The OpenComet plugging from ImageJ (NIH) was used to score individual parameters for each cell.

Statistical analysis of data was performed using SPSS Statistics 17.0 software. The main parameters of comet imaging are total DNA, % of DNA in the comet and tail, tail length, and tail moment (TM). The latter is the single most relevant index of DNA damage, which is calculated as the percent DNA in the tail multiplied by the distance between the means of the head and tail distributions.[52,53] Moreover, TM is considered appropriate for regulatory or interlaboratory comparison studies.[55] The TM data were analyzed applying a one-way analysis of variance (ANOVA) and post hoc Tukey's test to determine the effect of choline on HL-60 cell DNA integrity. P £ .05 was considered statistically significant.

Methylation status. Genomic DNA was isolated in the same way as described in the DNA fragmentation section. Bisulfite-modified DNA from the 0.447 and 7.163 mM choline treatments, using the EZ DNA Methylation-Gold Kit, was used as a template for fluorescence-based, real-time, quantitative methylation-specific PCR (qMSP). The final reaction mixture with a total volume of 10 lL consisted of 2 lL of deionized water, 5 lM each of forward and re-verse primers, 2 lL of iTaq Universal SYBR Green Super-mix (Bio-Rad; containing antibody-mediated hot-start iTaq DNA polymerase, dNTPs, $MgCl_2$, SYBR Green I dye, en-hancers, stabilizers, and a blend of passive reference dyes, including ROX and fluorescein), and 25 ng of bisulfite-converted genomic DNA.

qMSP conditions were as follows: one step at 95LC for 3 min, 45 cycles at 95LC for 10 s, 60LC for 15 s, 72LC for 15 s, another step at 95LC for 30 s, followed by a 65LC step during a period of 30 s, and finally a boost step from 65LC to 95LC for 95 s increasing 0.5LC per 0.05 s. qMSP was carried out in 48-well plates in MiniOpticon Real-Time PCR System (MJ Mini Personal Thermal Cycler; Bio-Rad) and was analyzed by Bio-Rad CFX Manager 3.1 software.

We selected repetitive elements to analyze a wide range of human genomic DNA. While Alu and long interspersed el-ement (LINE) sequences are interspersed throughout the ge-nome, satellite DNA is confined to the centromere areas.[56–59] All sequences had been obtained from Isogen Life Science. Alu M1, LINE-1, and satellite-alpha DNA (Sat-a) sequences were used (see Table 1 for detailed information[60]). The relative yielded results were normalized with the housekeeping sequence Alu C4 using the Nikolaidis et al.[61] and Liloglou et al.[62] comparative CT method. Each sample was analyzed in triplicate. One-way ANOVA and post hoc Tukey's tests were used to evaluate the differences between the tested compound, repetitive elements, and concentrations.

Table 3. Mean and Significances Of Life Span, Health Span, and Life Span Curves by Gender

Compound	Concentration (mM)	Mean life span[a] (days)	Mean health span[b] (days)	Life span curves by gender (days)		
				Females[a]	Males[a]	Gender sig.[b]
H_2O		47.242	19.769	51.202	49.914	0.386
Choline	0.447	58.430***	38.826***	60.829**	55.926**	0.131
	0.895	57.270***	33.810***	63.973**	52.663*	0.004
	3.581	62.120***	35.800***	64.225***	59.327***	0.449
	7.163	59.213***	37.098***	63.534***	55.501***	0.161

Means were calculated by the Kaplan–Meier method and significance of the curves was determined by the log-rank method (Mantel-cox).
[a]ns, nonsignificant (P > .05), *significant (P < .05), **highly significant (P < .01), ***very highly significant (P < .001).
[b]Significant survival values between females and males caused by the different assayed concentrations symbolized by arrow in Figure 2C.

MA activity evaluation. MA activity in NIH3T3 cells was evaluated as follows: for mCherry-GFP-LC3 conversion, NIH3T3 fibroblasts were transduced with a lentivirus carrying the tandem construct and cells were analyzed at least 1 week after transduction to assure stable expression; $1 \cdot 10^4$ cells were plated per well in glass-bottom 96-well plates and fluorescence after the indicated treatments was read in both channels using a high-content microscope (Operetta; Perkin Elmer). Moreover, the cytotoxic anticancer stressor, etoposide (Etopo; 25 lM), was added to NIH3T3 cells to analyze the growing behavior of cells treated with choline. Images of nine different fields per well were captured, which rendered an average of 2000–3000 cells counted. Nuclei, cell perimeter, and puncta were identified using the manufacturer's software Columbus 2.4.2. Puncta positive for both fluorophores correspond to autophagosomes, whereas those only positive for the red fluorophore correspond to autolysosomes. Autophagic flux was determined as the conversion of autophagosomes to au-tolysosomes (red-only puncta).

The statistical significance of MA activity for each concentration was determined using Student's t-test with the SPSS Statistics 17.0 software (SPSS, Inc.).

RESULTS

Toxicity and antitoxicity

Figure 1A shows the relative percentage of emerging adults after toxicity treatments with larvae with different concentrations of choline. A nonsignificant survival rate compared with the control is shown.

The antitoxicity assays revealed the ability of choline to protect individuals against oxidative stress at concentrations of 0.895–7.16 mM. Figure 1B shows that the positive con-trol H_2O_2 was toxic at 0.12 M with an average survival rate of 62% with respect to the water control. On the other hand, choline showed a positive dose–response effect in the combined treatments at high concentrations.

Genotoxicity and antigenotoxicity

Table 2 shows the results of genotoxicity and antigenotoxicity assays in the somatic mutation and recombination test. Negative control showed a frequency of mutations per wing of 0.157, which falls into the historical range for the wing spot test.[63,64] The concentration of H_2O_2 used (0.12 M) has been demonstrated to exert a potent genotoxic effect capable to induce somatic mutations and mitotic recombination in D. melanogaster[65] at a rate of 0.388 spots/wing. Choline seems to have no genotoxic effect at the tested concentrations with mutation rates not significantly different from that of water control (0.200 and 0.171 spots/wing for the highest and lowest concentrations, respectively). Moreover, choline was able to inhibit the genotoxic activity of H_2O_2 in a dose-dependent manner (41.5% and 66.2% for the lowest and highest concentrations, respectively).

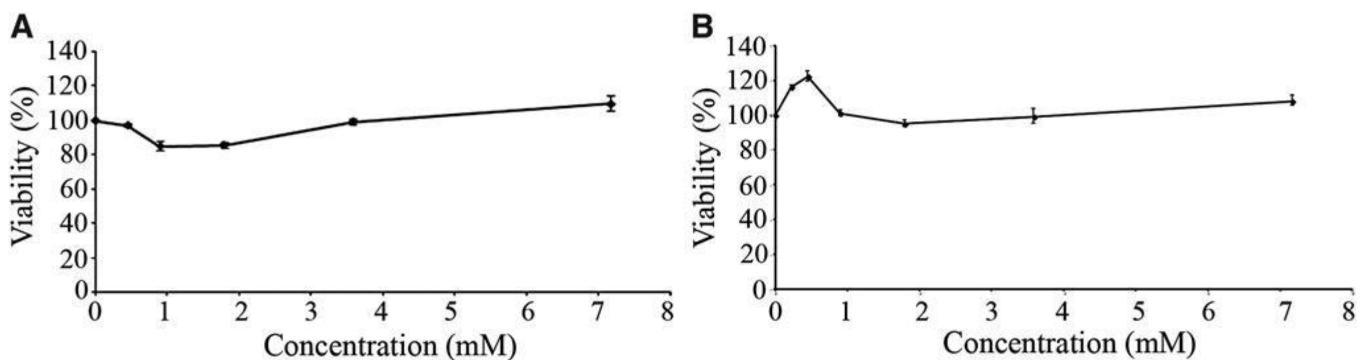

FIG. 3. Effect of choline on cell viability. (A) Viability of promyelocytic human leukemia cells (HL-60) treated with different concentrations of choline for 72 h. (B) Viability of mouse fibroblasts (NIH3T3) in the presence of different concentrations of choline after 20 h. Each point represents the growing percentage with respect to its control. Values are mean – SE from three independent experiments. SE, standard error.

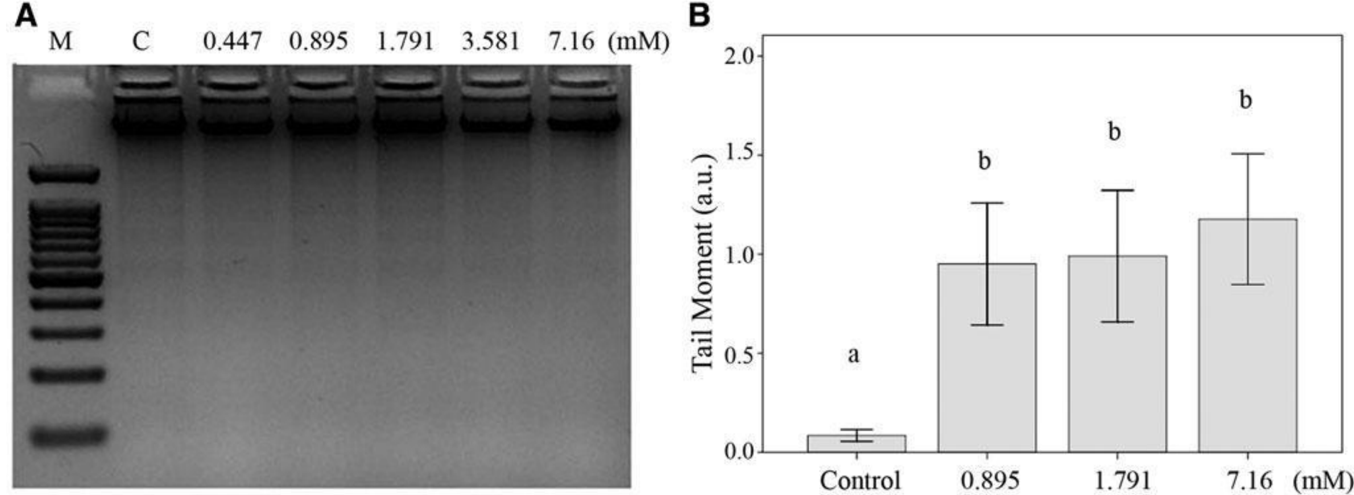

FIG. 4. DNA-induced damage in promyelocytic HL-60 cells treated with different concentrations of choline for 5 h. (A) Internucleosomal DNA fragmentation. (B) DNA strand break induction. (A) M indicates DNA size marker, C indicates Control treatment. (B) Alkaline comet assay (pH <13) of HL-60 cells treated with 0.895, 1.791, and 7.16 mM of choline. DNA migration is reported as mean TM. Values are mean – SE. Different letters in treatments mean differences with respect to the negative control after one-way ANOVA and post hoc Tukey's test. TM, tail moment; ANOVA, analysis of variance.

Longevity assays

The entire life span curves obtained by the Kaplan–Meier method for each substance and concentration are shown in Figure 2A. Choline induced a life span extension in D. melanogaster with an average range of 57.2–62.1 days for the different concentrations of choline with respect to the control (47 days on average) (Table 3).

Health span assays. Figure 2B shows that choline induced a significant increase of health span in D. melanogaster when compared with the control (Table 3). The different concentrations of choline (0.447–7.16 mM) showed similar mean survival time values among them with an increased range of 14.1–19.1 days with respect to the control.

Gender studies. All the assayed choline concentrations showed significantly increased survival time, with respect to their control, with an average of 11.9 and 5.9 days for fe-males and males, respectively (Fig. 2C; Table 3). Moreover, comparing female and male life span curves, there was a significant difference in longevity at the choline concen-tration of 0.895 mM with an increase in 11 days for females with respect to males.

Cytotoxicity

Choline did not exert any cytotoxic effect in the in vitro assays in HL-60 and NIH3T3 cells (Fig. 3A).

DNA internucleosomal fragmentation

Figure 4A shows electrophoresis of the genomic DNA of HL-60 cells treated with different concentrations of choline. Results show that choline did not induce internucleosomal fragmentation.

Comet assay

Results of the TM for different concentrations of choline-treated HL-60 cells are shown in Figure 4B. Choline in-duced significant genomic DNA damage, increasing the TM of comets in HL-60 promyelocytic cell line with respect to the concurrent control.

Methylation status

The relative normalized expression of three repetitive sequences (Alu M1, LINE-1, and Sat-a) studied in HL-60 cells treated with different concentrations of choline is shown in Figure 5. Choline did not exhibit modulation of the

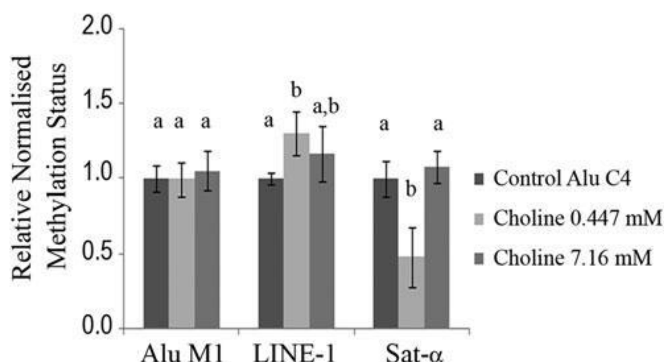

FIG. 5. Relative normalized expression data of each repetitive element. Relative normalized expression data of each repetitive element (Alu M1, LINE-1, and Sat-a). Different letters mean different values after one-way ANOVA and post hoc Tukey's test. Sat-a, satellite-alpha DNA; LINE, long interspersed elements.

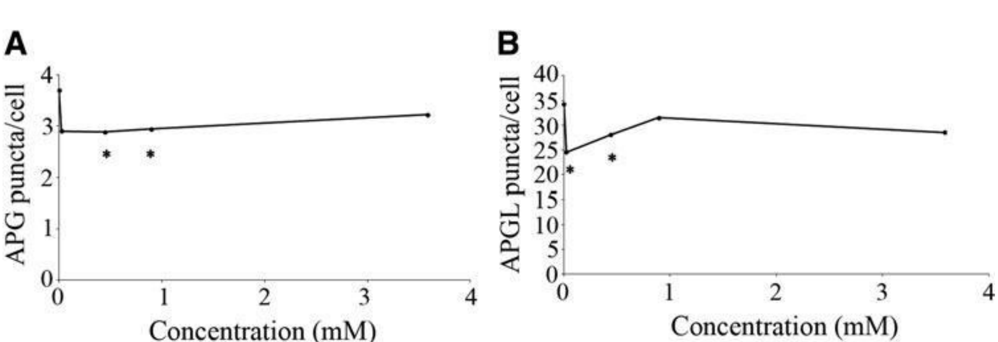

FIG. 6. Effect of choline on macroautophagy activity. Macroautophagy activity was measured in NIH3T3 cells stably expressing the tandem reporter mCherry-GFP-LC3. Analysis was done using high-content microscopy in nine different fields per condition and well (approximately total of 3500 cells per condition). (A) Quantification of the number of autophagosomes (APG) and (B) autophagolysosomes (APGL). *Significant (P < .05).

methylation status as both concentrations assayed showed methylation levels similar to that of the normalized control. The only exception was for the lowest concentration of choline in the LINE-1 and Sat-a regions that showed a significant hypermethylation and demethylation status, respectively (Fig. 5).

MA activity

Choline decreased MA in NIH3T3 cells at the lower concentrations assayed (Fig. 6). However, the MA response (of increased MA) to topoisomerase inhibitor (etoposide) is still maintained in the presence of the lowest concentration of choline analyzed (Fig. 7).

DISCUSSION

Recent work points out the critical role of choline in brain development.[66] Besides, getting adequate choline in the diet is important throughout life for optimal health in pregnancy and lactation,[67] neural tube defects,[68] memory development,[69] heart disease,[70] inflammation[16,71] and cancers.[15,72] The antitoxic activity of choline against H_2O_2 found in our in vivo experiments would be in agreement with the well-known biological effects in humans showing that choline-deficient diets are related to liver and kidney de-generative diseases[73] and a dietary supplementation with choline induces a reduction of tissue injury and mortality in rat[74] and juvenile carp,[75] although an excess of choline in-take could be toxic in humans.[38] Oxidative species, partic-ularly hydrogen peroxide and derived hydroxyl radicals, are known genotoxic carcinogens.[76] Our antitoxicity and anti-genotoxicity results showed that choline is able to protect against genomic damage induced by this genotoxin. Our results of genotoxic inhibitory ability of choline in vivo due to its antioxidant activity against peroxide caused by its hy-droxy amine groups[77] are in agreement with previous in vitro studies on the genomic impact of choline deficiencies.[14,35] Longevity is a trait influenced by many factors, including

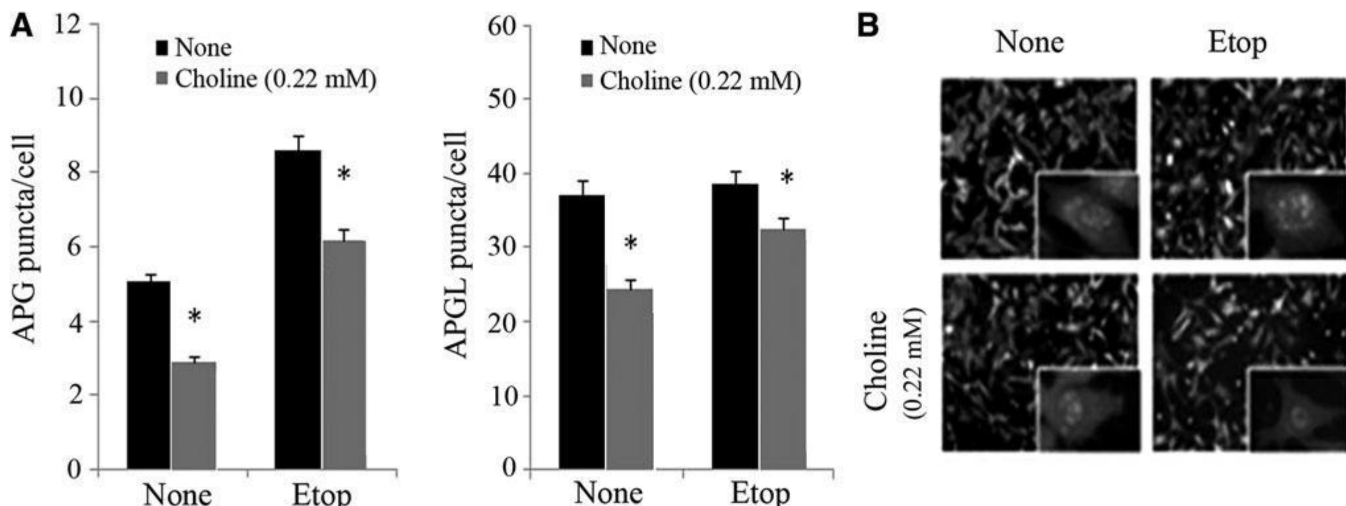

FIG. 7. Effect of etoposide on macroautophagy induced by choline. Macroautophagy activity was measured in NIH3T3 cells stably expressing the tandem reporter mCherry-GFP-LC3; cells were grown in the absence or presence of the stressor (Etopo: 25 lM). Concentration was selected on the basis of results obtained in single treatments shown in Figure 5. Images and analysis were done using high-content microscopy in nine different fields per condition and well (approximately total of 3500 cells per condition). (A) Quantification of the number of autophagic vacuoles (AV: total red puncta), autophagosomes (APG: total yellow puncta), and autophagolysosomes (APGL: red, but not green, puncta) was performed. *Significant (P < .05). (B) Representative merged images of these cells and high-magnification insets. Autophagosomes and autophagolysosomes are shown as yellow and red puncta, respectively.

gender, genetic variation, environmental influences such as diet, lifestyle, access to healthcare, and cultural influences.[78] Choline at lower concentrations, like other methyl donors such as purine and folate,[79] could be argued as a potential target of sex-specific implication for Drosophila life span. Besides, methyl-deficient diets cause fatty livers and promote liver carcinogenesis in rodents.[80] On the other hand, supplementation with choline improves hepatic steatosis.[81] Our results suggest that lower concentrations of choline could be an essential nutrient to prevent and protect the degenerative processes like aging and DNA damage.

Our results on genotoxicity of choline against HL-60 tumor cells are consistent with the conclusively demonstrated fact that choline metabolism is altered in a wide variety of cancers,[82] hence proposing novel anticancer therapies targeting choline metabolism. Experimental studies in mice established dose–effect relationship between choline deficiency and carcinogenic activity of diethanolamine,[83] which is related with greater membrane fragility than cells grown in a control medium.[84] The use of two types of model cell lines allowed us to obtain complementary conclusions on the mode of action of choline. Methodologies for the dif-ferent cells are used following the standard protocols de-scribed in the manufacturer's manuals as they belong to different suspension and adherence types of cultures, hence conditions are different, but still comparable.

Although we did not obtain significant results for the DNA damage assays (DNA internucleosomal fragmentation and comet assay), studies suggest that choline deficiency in humans is associated with significant DNA damage and with apoptosis in lymphocytes.[16] Moreover, studies affirm that choline has a significant impact on genomic stability and cell death, depending on its availability.[14]

Chemopreventive molecules induce demethylation in tumoral cells, contrary to the general hypermethylation that tumoral cells exhibit.[85] Nevertheless, several studies have identified a usual hypomethylation status of specific Alu, LINE-1, and Sat-a repetitive sequences in cancer cells.[86–88] The present pilot study on the epigenetic effects of choline treatments in tumoral cells did not show any significant blocking effect of choline on repetitive sequences on HL-60 tumoral cells, except for hypermethylation in LINE-1 sequences and hypomethylation in Sat-a sequences caused by 0.447 mM choline. According to Zeisel[35] and Locker et al.,[89] DNA methylation is influenced by the availability of choline: an excess of choline used to cause hypermethylation, which is associated with gene silencing or reduction of gene expression, and furthermore common genetic polymorphisms have effects on the dietary requirements; whereas a choline deficiency is associated with DNA hypomethylation, which is related with health consequences in brain development and birth defects, fatty liver, muscle damage, elevation of plasma homocysteine, and others problems in adults. LINE sequences are moderately dispersed repetitive DNA, mainly transposable elements, which may be involved in regulation of gene expression. LINE-1 methylation status has been shown to be a good indicator of genome-wide methylation. On the other hand, alpha-satellite DNA are highly repetitive sequences located in heterochromatic regions around the centromere/telomere, with structural or organizational roles, role in chromosome pairing, involvement in cross-over or recombination, and junk. Taking into account the importance of choline requirements and functions of the different repetitive sequences studied, for LINE sequences, either a deficit or an excess of choline in treatments of HL-60 cells leads to a hypermethylation status in these repetitive sequences. The major fact observed on the DNA demethylation status of alpha-satellite sequences of HL-60 cells treated with 0.447 mM choline (lower concentration than the recommend one) should be considered as the most important effect due to their main consequences on health. More research should be carried out on the epigenetic modifications induced by some dietary molecules, until clear response of the entire methylation status of genome is known.

MA is considered an inducible type of autophagy, with an essential role in the maintenance of cellular homeostasis and as the forefront of the response to cellular stress.[90] It is known that MA decreases with age in almost all organisms studied and diet or specific nutrients affect MA activity.[91] We have also observed that dietary choline inhibits MA activation. Further studies are needed to understand this apparent paradox as choline exerts benefits on longevity and at the same time inhibits MA activity.

In the present study, a general overview of the biological activity at individual, cellular, and molecular levels drawn from the in vivo and in vitro assays could be obtained to evaluate the role that choline has in the modulation of degenerative processes. Our data indicate that choline is nei-ther toxic nor genotoxic, protects from oxidative genetic damage, and increases life span and health span in the ani-mal model. Nevertheless, choline does not inhibit tumor growth, increase MA, and induce DNA damage and it rarely modifies the methylation status of repetitive sequences of tumoral cells. Taking into account the overall studies carried out in the present work, dietary choline requirements are shown as an excellent example of nutrigenomics due to the important health implications of choline. The hopeful results of our in vivo assays about protection against oxidative agents and longevity support this fact.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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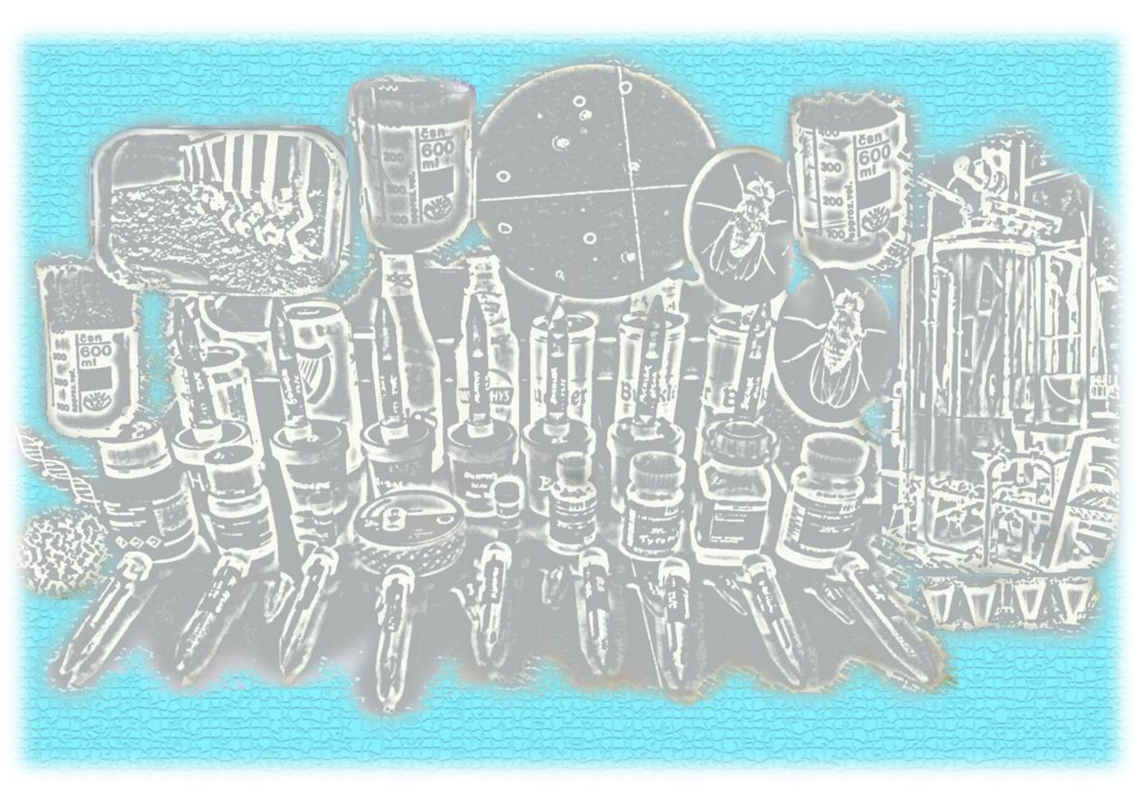

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