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ESTIMATION OF DICARBONYL COMPOUNDS INTAKE IN ITALY AND THEIR POTENTIAL ROLE AGAINST FOOD SPOILAGE/PATHOGENIC BACTERIA

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To my Dad,
forever and ever
Your Sparrow

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1 Abstract

Foods, from the earliest times, are subjected to man-made modifications to ensure microbiological safety, enzymatic inactivation, destruction of toxic substances, and optimization of storage time. An undisputable role in making the food softer, tasty and preservable over time is exerted by heat treatment. It is during this phase that a series of chain reactions, known like Maillard's reaction, are triggered. During this reaction, products that affect the flavor, color and aroma of foods are formed. Among these, glyoxal, methylglyoxal and 3-deoxyglucosone are of particular importance.

These compounds are present in food, have antimicrobial activity, are promoters of advanced glycation end products formationand also have toxic effects in in vitro and in vivo studies.

Research activity carried out during Ph.D. study had a common thread: increasing knowledge on 1,2-dicarbonyl compounds.

In this field, the overall aims of this thesis work were: assessment of the content of dicarbonyl compounds in Italian food; assessment of their dietary intake; study of formation/degradation of dicarbonyl compounds using food model systems; evaluation of antimicrobial activity of the main dicarbonyl compounds; study of the interaction between the dicarbonyl compounds and the nutrients present in the microbiological culture media, both in the presence and absence of the microorganism.

The results obtained by the survey on Italian food show that the concentration is variable and the predominant 1,2-

dicarbonyl compounds is 3-deoxyglucosone.
The estimation on dietary intake with a Total Diet Study-like investigation, have brought new evidence to assert that the ingestion with foods is high especially for infants (0-2 years) and children (3-9 years).
The results obtained with the model systems show that time, temperature and ingredients have a strong influence on the formation of the compounds and that it is possible to reduce the level of 1,2-dicarbonyl compounds.
The results of antimicrobial assays lead us to conclude that dicarbonyl compounds, especially GO and MGO, could have a role in the microbial stability of foods, although food composition may strongly influence their availability to act as antimicrobials.
The results obtained by the study of the interaction between the dicarbonyl compounds and the nutrients present in the culture media allow us to assert that the 1,2-dicarbonyl compounds are degraded very quickly when they come into contact with bacteria.
The results obtained outline a framework of knowledge that is a prelude to subsequent important developments.

2 Sommario

Gli alimenti, sin dai tempi più remoti, vengono sottoposti dall'uomo a una serie di modificazioni al fine di garantire un'adeguata sicurezza microbiologica, l'inattivazione enzimatica, la distruzione di sostanze tossiche e l'ottimizzazione del tempo di conservazione. Un ruolo indiscusso, nel rendere gli alimenti conservabili nel tempo, viene esercitato dal trattamento termico. È proprio durante questa fase che si innescano una serie di reazioni a catena, note con il nome di reazione di Maillard. Durante questa reazione si formano dei prodotti che influenzano il sapore, il colore e l'aroma degli alimenti. Tra questi prodotti di particolare importanza sono il gliossale, il metilgliossale e il 3-deossiglucosone.

Questi composti si ritrovano negli alimenti, hanno attività antimicrobica, sono precursori dei prodotti finale della glicosilazione avanzata ed inoltre hanno effetti tossici sia in studi in vitro che in vivo.

L'attività di ricerca svolta nel triennio di dottorato ha avuto un filo conduttore: l'ampliamento della conoscenza sui composti 1,2-dicarbonilici.

Il lavoro svolto durante il dottorato di ricerca, descritto nella presente tesi si propone di valutare il contenuto dei composti 1,2 dicarbonilici negli alimenti e la loro assunzione da parte del consumatore, studiare la formazione/degradazione di questi composti utilizzando sistemi modello, valutare la loro attività antimicrobica e studiare l'interazione di questi composti con i nutrienti presenti nei mezzi di coltura.

I risultati ottenuti sul rilevamento dei livelli negli alimenti

italiani ci permette di dire che la concentrazione è variabile e che il composto 1,2-dicarbonilico predominante è il 3-deossiglucosone.
La valutazione dell'assunzione tramite la dieta, effettuata con il "Total diet study like investigation", ci permettere di asserire che l'ingestione tramite gli alimenti è alta specialmente per gli infanti (0-2 anni) e i bambini (3-9) anni.
I risultati ottenuti con i sistemi modello dimostrano che il tempo, la temperatura e gli ingredienti hanno una forte influenza sulla formazione di questi composti e che è possibile ridurne i livelli.
I risultati ottenuti dall'attività antimicrobica mostrano che i composti dicarbonilici, in particolare gliossale e metilgliossale, potrebbero avere un ruolo nella stabilità microbica degli alimenti, anche se la composizione dell'alimento può influenzare fortemente la loro disponibilità di agire come antimicrobici.
I risultati ottenuti dallo studio dell'interazione tra i composti dicarbonilici e i nutrienti presenti nei mezzi culturali di crescita dimostrano come i composti vengano degradati velocemente nel momento in cui interagiscono con il microrganismo.
I risultati ottenuti delineano un quadro di conoscenze che preludono importanti successivi sviluppi.

3 Introduction

3.1 Degradation of sugar and Maillard reaction

1,2-dicarbonyl compounds are reactive intermediates that are mainly formed from hexoses both during the acid-catalyzed dehydration of hexoses (pH < 3) and, if amino compounds are involved, during the early stage of Maillard reaction, as a degradation product of the Amadori compounds (pH range 4-7) (Yaylayan 1997; Thornalley *et al*., 1999; Belitz *et al*., 2009).

Monosaccharides can react both in the presence and in the absence of amino acids. In a basic medium, enolizations with subsequent retro-aldol reactions and secondary reactions of the fragments predominate. In an acid medium enolizations and subsequent elimination of water with retention of the C-chain predominate (Belitz *et al*., 2009).

For example, glucose can yield via enolitation and dehydration 3-deoxyglucosone (3-DG) and subsequently via C-C-cleavage yield methylglyoxal (MGO) (Figure 1). Instead fructose can yield glyceraldehydes and dihydroxyacetone that, after dehydration yield MGO (Figure 2).

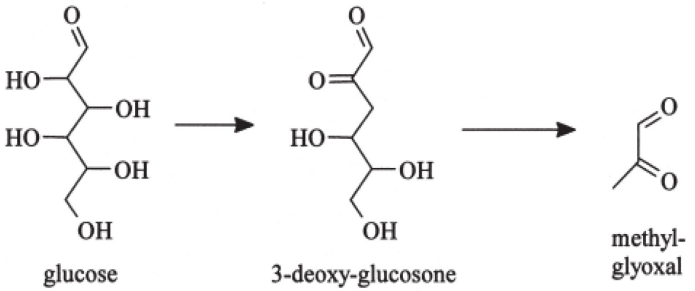

Fig. 1: Enolitation, dehydration and C-C-cleavage of glucose.

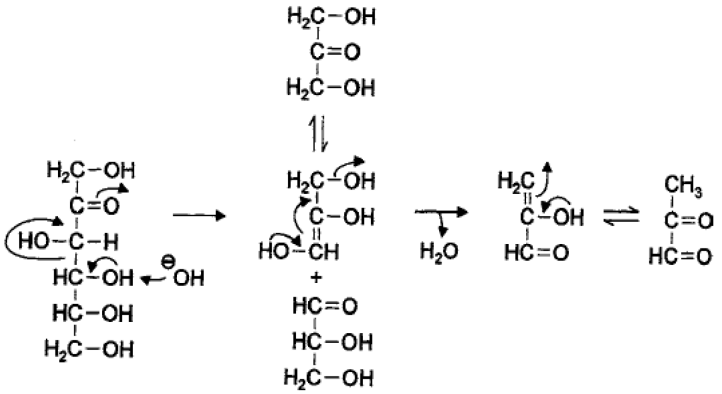

Fig. 2: Degradation of fructose (Belitz *et al*.,2009).

When monosaccharides react in the presence of amino acidsthese reactions are called Maillard reaction.
Louis-Camille Maillard was first reported in 1912 of this reaction that is the result of a several pathway of chemical reactions.
This reaction is produced at medium/low water activity (a_w) and basic pH.
Several factor affect the reaction:

- ✓ Substrates: The main substrates involved are an α-hydroxy carbonyl moiety of a reducing sugar and an amino group. During the reaction there is a greater loss of sugar instead of the amino acid, as parallel chemical reactions occur such as caramelization. The sugars involved are mainly glucose, fructose, maltose, lactose and, to a smaller extent, reducing pentoses. For the amino component a primary amino group are more important than those with a secondary because their concentration in foods is usually higher. Exceptions are, e. g., malt and corn products that have a high proline content (Belitz *et al*., 2009);
- ✓ pH: The initial pH of foods and their buffering capacity plays an important role in the type and intensity of the Maillard reaction. At pH<3 the rate of browning is low and then increases as the pH raise up to a maximum of 10 (Delgado-Andrade and Rufián-Henares, 2009);
- ✓ Temperature: At high temperature the Maillard reaction will be more intense that at low temperatures but, the key factor is also the time

applied. The same degree of browning is obtained if a product is heated at a high temperature for a short time period or if it is heated at a lower temperature for a longer time.

The general scheme of the Maillard reaction is shown in Figure 3.

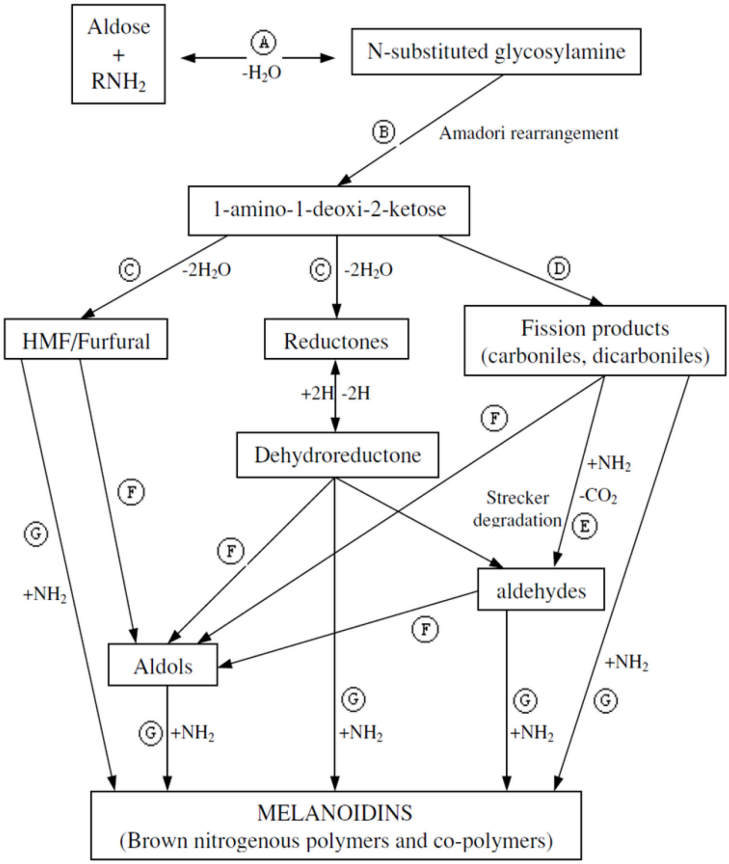

Fig. 3: General scheme of the Maillard reaction (Rufián-Henares *et al*., 2009).

This reaction starts between an amino group and an α-hydroxy carbonyl moiety of a reducing sugar. In this step nucleophilic compounds like amino acids or amines easily add to the carbonyl function of reducing carbohydrates with the formation of imines (*Schiff bases*). (Belitz *et al*., 2009). The imines can rearrange via the 1,2-eneaminols corresponding to the 1,2-enediol.
This initial phase produces two intermediate: Amadori product from aldohexose or Heyn's product from ketohexose (Figure 4).

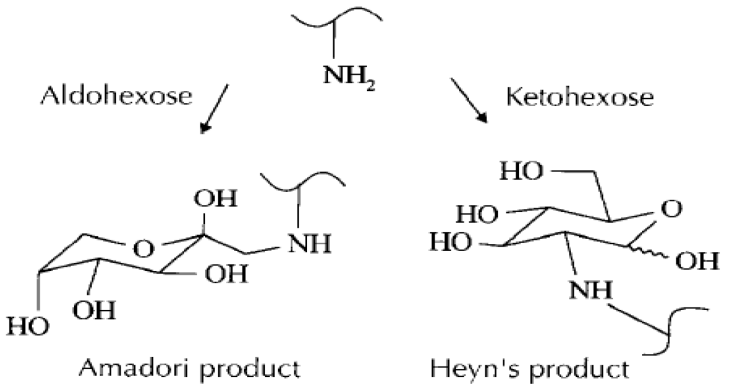

Fig. 4: Amadori or Heyn's product (Yaylayan, 1997).

The Amadori compounds are degraded to 1-, 3-, and 4-deoxydicarbonyl compounds (α-dicarbonyl compounds) in the pH range 4–7 (Figure 5) (Belitz *et al.*, 2009).
These intermediate products can also be produced by autoxidation of glucose or by peroxidation of lipids (glyoxal and methylglyoxal) and yield many secondary products like 5-hydroxymethylfurfural, furfural, pyrrole or pyridine derivatives, compounds with a pyranone structure, maltoxazine, norfuraneol, acetylformoin, maltol, β-galactosyl-isomaltol, β-hydroxypropionic acid, lactic acid ester, furosine, pyrrole and pyridinium betaine (Belitz *et al.*, 2009).
Furthermore, α-dicarbonyl compounds can react with amino acids lead to the formation aldehydes (like methional, phenylacetaldehyde, 3- and 2-methylbutanal and methylpropanal), CO_2 and α-aminoketones. This reaction is the *Strecker* reaction (Figure 6.) and occurs in foods at higher concentrations of free amino acids and at higher temperatures or under pressure (Belitz *et al.*, 2009).
Subsequently, a range of reactions like cyclisations, dehydrations, retroaldolisations, rearrangements, isomerisations and further condensations lead to the formation melanoidins (Martins *et al.,* 2001).

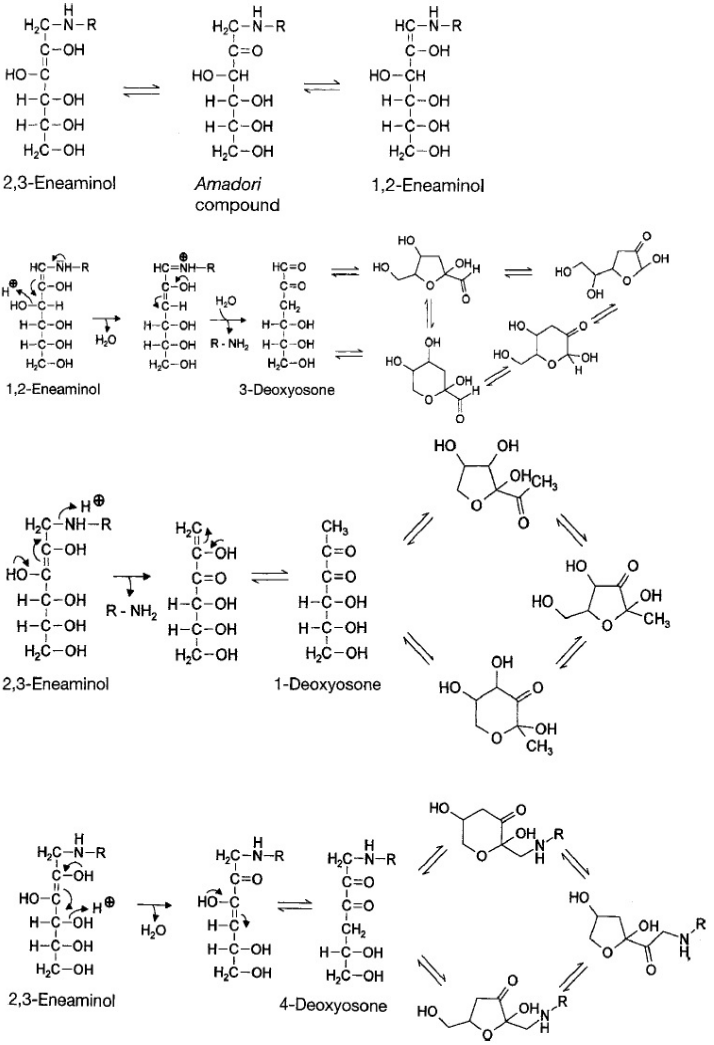

Fig. 5: Degradation of the Amadori compounds (Belitz *et al*., 2009).

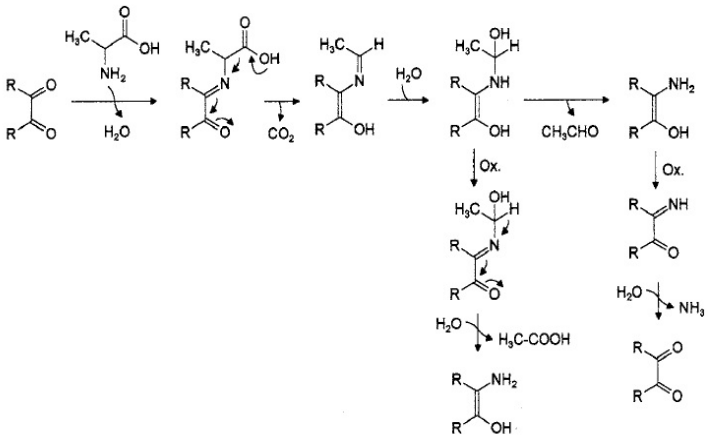

Fig. 6: Strecker reaction (Belitz *et al*., 2009).

Yaylayan (1997), described a conceptual representation of the processes occurring during the Maillard reaction.
The main precursors of Maillard's reaction are sugars (S), amino acids (A) and Amadori and Heyn's compounds (D) (Yaylayan, 1997), from which several intermediate products are formed. Under specific conditions, the nature and relative relationship of these precursors or "parent pool" determines the course of the Maillard reaction.
Each of these three major precursors can:

1. interact with each other to form three 'self-interaction pools' (Figure 7) (Yaylayan, 1997);
2. interact with components of other fragmentation pools to form three 'secondary interaction pools' (Figure 7) (Yaylayan, 1997);

3. The components of all the pools can interact with each other to form complex 'multiple-interaction pools' (Figure 7) (Yaylayan, 1997).

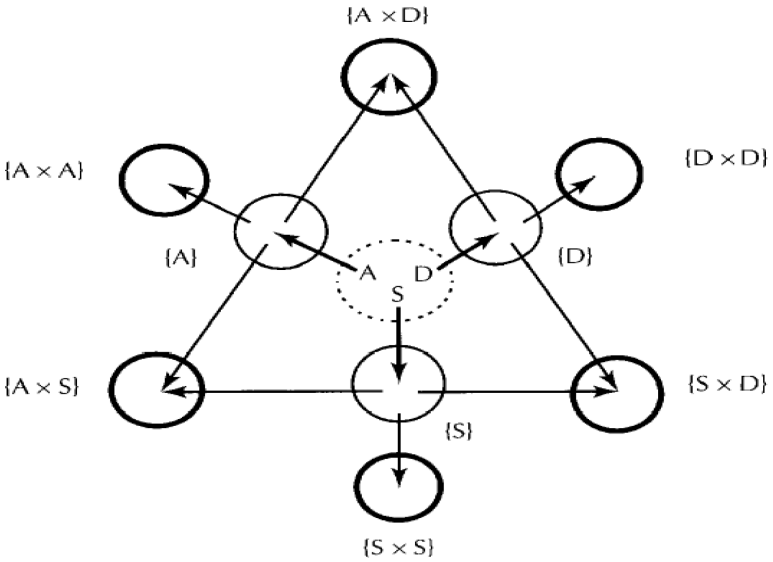

Fig. 7: Primary fragmentation groups of Maillard's reaction (Yaylayan, 1997).

3.2 *Glyoxal, methylglyoxal and 3-deoxyglucosone*

GO, MGO and 3-DG are the main reactive dicarbonyl compounds that can be derived from Maillard reaction and from oxidative glucose degradation.
MGO and 3-DG can be formed by glucose (as reported above in Figure 1).
MGO can be derived by fructose (as reported in above in Figure 2) and starting from 1-deoxy-2,3-hexodiulose, after *retro*-aldol reactions (Figure 8) (Belitz *et al*., 2009).

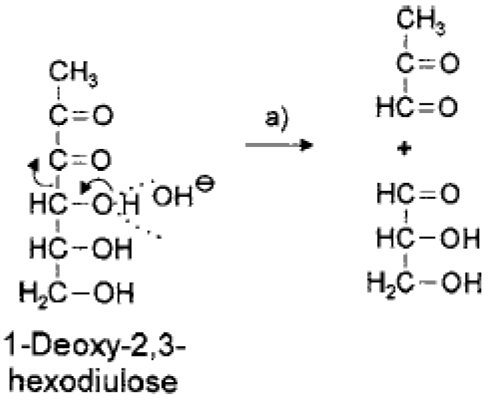

Fig. 8: Formation of MGO starting from 1-deoxy-2,3-hexodiulose by retro-aldol reactions (Belitz *et al.,* 2009).

Thornalley *et al*., (2009), reported a mechanistic interpretation of glyoxal, methylglyoxal, 3-deoxyglucosone and glucosone formation in glucose degradation (Figure 9) and in the early glycation (Figure 10).

In their study the authors claim that glyoxal may be formed in the degradation of glucose by retroaldol condensation reactions activated by deprotonation of the 2- or 3-hydroxy groups (Thornalley *et al*., 2009). The formation of glyoxal is also stimulate by autoxidation of glycoaldehyde to glyoxal, and glucose to glucosone. 3-DG formation occur by a 2,3-enolization. Methylglyoxal may be formed by fragmentation of 3-DG (Figure 9).

Furthermore, they propose that the formation of dicarbonyl compounds in early glycation is mechanistically similarly to that of glucose degradation except for the presence of the the aldimine Schiff's base of the 1,2-dicarbonyl compounds (Figure 10). The presence of the aldimine group accelerates the formation of GO, MGO and 3-DG.

The 1,2-dicarbonyl compounds can be formed from the degradation of fructosyl-lysine.

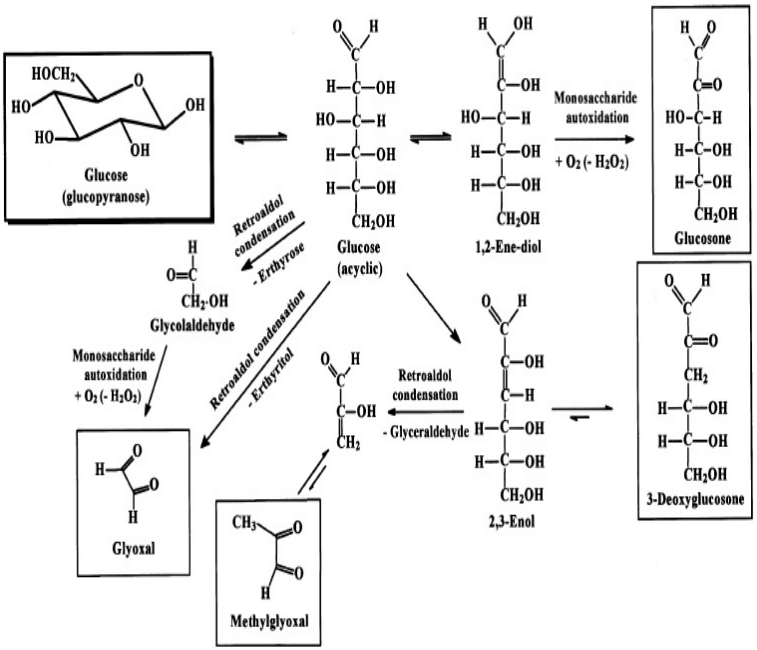

Fig. 9: Mechanistic interpretation of glyoxal, methylglyoxal, 3-deoxyglucosone formation in glucose degradation (Thornalley *et al*., 2009).

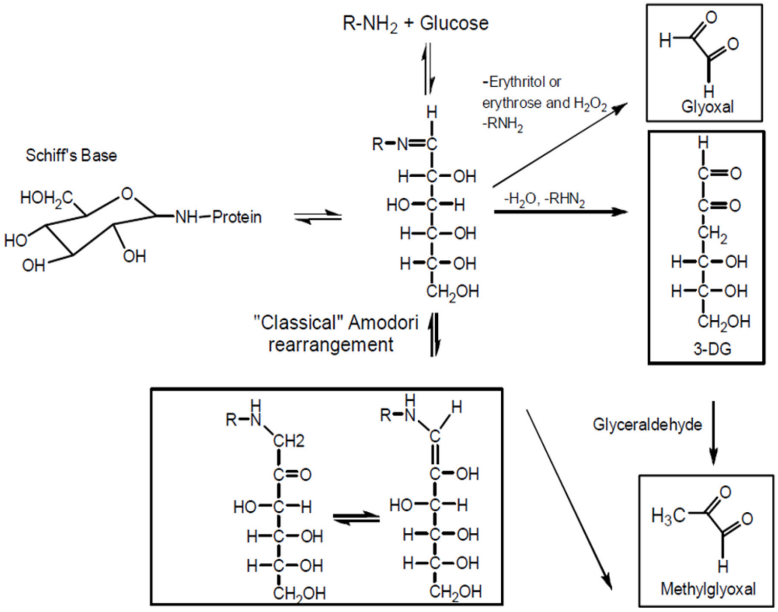

Fig. 10: Mechanistic interpretation of glyoxal, methylglyoxal, 3-deoxyglucosone formation in early glication (Thornalley *et al*., 2009).

3.3 State of the art

3.3.1 1,2-dicarbonyl compounds in food

GO, MGO and the 3-DG are the main 1,2-dicarbonyl compounds generally identified in foods and beverages, particularly in those rich in sugar, with a wide range of concentration and distribution.

Few studies reported levels of α-dicarbonyl in foods. Weigel *et al*., (2004), for the first time, investigated the amounts of α-dicarbonyl compounds in commercial multifloral honeys and during storage; Marceau and Yaylayan (2009), identified the profile of α-dicarbonyl compounds in honeys of different botanical origins. The presence of 3-DG has also been reported in soy sauce as an indicator of the non-enzymatic browning reaction (Kato *et al*., 1961; Kim and Lee 2008), in carbonated beverages (Lo *et al*., 2008) and in beer during aging (Bravo *et al*.,2008).

The distribution of 1,2-dicarbonyl in foods is reported in table 1.

Table 1: The distribution and the corresponding levels of GO, MGO and 3-DG from the literature.

Food stuff	GO	MGO	3-DG	Reference
Alkali-treated pretzel		2.5-16 mg/kg	4.5-34 mg/kg	Degen *et al*., 2012
Apple brandy	0.33 mg/L	0.32 mg/L		Nagao *et al*., 1986
Barley coffee brew	0.92-2.66 mg/L	0.36-2.4 mg/L		Papetti *et al*., 2014
Beer		nd-1.0 mg/L	18.54 mg/L	Hayashi *et al*., 1985; Barros *et al*.,1999; Bravo *et al*.,2008; Degen *et al*., 2012
Bourbon whiskey	0.39 mg/L	1.5 mg/L		Nagao *et al*., 1986
Bread	0.3 mg/kg	nd-28 mg/kg	13-619 mg/kg	Nagao *et al*., 1986; Degen *et al*., 2012
Brewed coffee	0.23-0.87 mg/kg	0.07-25 mg/kg		Hayashi *et al*., 1985; Nagao *et al*., 1986; Papetti *et al*., 2014
Candies		nd-1.1 mg/kg	141-1011 mg/kg	Degen *et al*., 2012
Carbonated soft drink	0.02-1.73 mg/L	nd-1.39 mg/L		Lo *et al.,* 2008; Weerawatanakorn 2013;
Cocoa		1.2 mg/L		Hayashi *et al*., 1985;
Coffee		nd-100 mg/kg;	nd	Daglia *et al.,* 2007; Wang and Chang, 2010; Degen *et al*., 2012
Cola		0.23 mg/L		Hayashi *et al*., 1985
Cookies	4.8-20.5 mg/kg	1.8-78 mg/kg	8.5-385 mg/kg	Arribas-Lorenzo and Morales, 2010; Degen *et al.*, 2012
Decaffeinated brewed coffee		47 mg/L		Hayashi *et al*., 1985
Fried dough twist	nd-11.36 mg/kg	6.98-10.15 mg/kg		Liu and Li, 2014
Fruit beverage		nd-96.7 mg/L	nd-410 mg/L	Hayashi *et al*., 1985; Degen *et al*., 2012; Weerawatanakorn 2013
Honey	0.1-10.9 mg/kg	nd-463 mg/kg	75.9-1641 mg/kg	Arena *et al*., 2011; Degen *et al*., 2012

Instant coffee	0.34 mg/L	1.6-23 mg/L		Hayashi *et al*., 1985; Nagao *et al*., 1986
Jam, jellies, sweeteners		nd-3.9 mg/kg	1.7-1061 mg/kg	Degen *et al*., 2012
Japanase sake	0.29 mg/L			Nagao *et al*., 1986
Liquid condiments/seasonings		nd-3.9 mg/L	nd-212 mg/L	Degen *et al*., 2012
Malt beer		tr.-1.0 mg/L	19-136 mg/L	Degen *et al*., 2012
Manuka honey		139–700 mg/kg		Mavric *et al*, 2008; Adams *et al*., 2008; Atrott *et al*., 2012
Maple syrup		2.5 mg/L		Hayashi *et al*., 1985
Nonfat dry milk		1.4 mg/L		Hayashi *et al*., 1985
Pasta (cooked)		nd	nd-8.8 mg/L	Degen *et al*., 2012
Potatoes (cooked/fries)		nd-tr	nd-18 mg/kg	Degen *et al*., 2012
Rice, millet, mustard		2.2-5.4 mg/kg		Nemet *et al*., 2006
Soft drink	nd	nd-1.4 mg/L	nd-28 mg/L	Nagao *et al*., 1986; Degen *et al*., 2012
Soy sauce	0.62-4.9 mg/L	nd-12 mg/L	32-832 mg/L	Kato *et al*., 1961; Hayashi *et al*., 1985; Nagao *et al*., 1986; Nagao *et al*., 1986a; Kim and Lee 2008; Degen *et al*., 2012; Papetti *et al* 2014
Tea beverage	0.02 mg/L	0.05-253 mg/L		Hayashi *et al*., 1985; Nagao *et al*., 1986; Weerawatanakorn, 2013
Vinegars		1.7-53 mg/L	4.6-2622 mg/L	Degen *et al*., 2012
Wine	0.97 mg/L	nd-4.5 mg/L	2.2-95 mg/L	Hayashi *et al*., 1985; Nagao *et al*., 1986; Barros *et al*.,1999; Degen *et al*., 2012

nd: non detectable; tr: traces

There are less information concerning the levels of 1,2-dicarbonyl compounds on Italian food. Arena *et al*., (2011), reports the level of 1,2-dicarbonyl compounds in 40 commercial honey from different floral origins. The concentration of 3-DG ranged from about 76 to 800 mg/kg, values for GO e MGO were 0.1-10.9 and 0.2-2.9 mg/kg, respectively. Moreover, pH and total phenols are the main chemical characteristics that most influenced the levels of 1,2-dicarbonyl compounds in honey.

3.3.2 Total diet study (TDS) approach

Estimation of food intakes is necessary for risk evaluation, and to determine relationships between adverse effects observed in humans and exposure to particular substances (FAO/WHO 1985). These exposure assessments can be used for regulation of nutritional or chemical products and for safety of food products.
The Total Diet Study (TDS) (EFSA, 2011) is one method used in many countries to address this issue.
TDS is a selection of foods represent a typical diet, whose based on food consumption data that will allow us to estimate population exposure to these substances.
A TDS differs from other chemical monitoring or monitoring programs because it always focuses on chemicals through total diet, food is transformed as actually consumed by the population (cooked or otherwise), so it is believed that the impact of food preparation and cooking on the chemical substance under consideration gives a realistic estimate of exposure and also only the edible part of the

food is analyzed (EFSA, 2011).
The first step is the selection of chemical substances to be analysed. They may influence the food selection, pooling and sampling process. The criteria are:

- ✓ chemical substances for which their presence and level in food are uncertain;
- ✓ use of health data;
- ✓ chemical substances that are recognised as potential health risk to the population, political or socio-economic reasons;
- ✓ budgetary issues.

The TDS survey will provide background information on harmful and beneficial chemical substance levels in the general food supply across the diet, while monitoring and surveillance activities can capture more highly contaminated individual food items. Their complementarities will allow the identification of the relative importance of individual sources of chemical substances in relation to their contribution in the whole diet.
Steps characterizing a TDS include the selection of foods based on food consumption data to represent as best as possible a typical diet, their preparation to food as consumed and the subsequent pooling of related foods before analysis.
The central part of the TDS design is a food list.
The composition of the food list will also be determined by the objective(s) of the study, the allocated funds and available food consumption data.
If many different population groups are to be considered

(e.g. different age and gender groups, ethnic groups or special groups such as vegetarians), more foods need to be analysed individually (because their consumption is different among the population groups), as compared to one population group, e.g. general population, per capita, per adult equivalent.
Once the foods are selected, it-s necessary to determine if it is an international, national or regional/local food. National foods are considered to have a similar distribution for the levels of chemicals throughout the country (e.g. brand name foods produced in a central factory), while regional foods may have different levels depending on the region and/or season (e.g. fruits and vegetables, tap water etc.).
After selecting the foods, it is necessary establish the pooling (creating a unique food sample for analysis by combining various individual food items). To create pooling two types of approach can be considered:

1. individual food approach in which different form of the same food or the food cooking whit different methods merged into one food sample;
2. mixed food approach in which several different foods from the same food group merged into one food sample.

After calculating the concentration in foods of the chemical substanceschosen, it is necessary to calculate the dietary exposure. Population exposure using TDS results is calculated as any other dietary exposure assessment.
The Codex Alimentarius Commission's (CAC) Procedural Manual (FAO/WHO, 2008a) defines exposure assessment

as “the qualitative and/or quantitative evaluation of the likely intake of biological, chemical, and physical agents via food as well as exposures from other sources if relevant”.
The general equation for both acute and chronic dietary exposure (FAO/WHO 2009) is:

$$\text{Dietary exposure} = \frac{\Sigma(concentration\ of\ chemical\ in\ food \times food\ consumption)}{Bodyweight\ (Kg)}$$

To calculate exposure two approaches can be used: the deterministic/point estimates of dietary exposure and the probabilistic distributional analyses.
The first one is a single value that describes some parameter of consumer exposure. For concentration data, the point estimate typically consists of the mean, the median, a high percentile of all observed values point. For food consumption data, the point estimate typically consists of the mean or a high percentile of all the consumption values of a considered food in a population of interest.
In the probabilistic distributional analyses instead of a single value, at least one variable is represented by a distribution function. In this way the model sample from each distribution is a distribution of potential dietary exposures.
Exposure can be expressed as a quantity of ingested chemical substances per capita, by day, by week or by month. Nevertheless, many health-based guidance values for food chemical substances are usually expressed as a quantity of ingested chemical substance per kg of body weight and per period of time. If available, the body weight of each subject should be used for individual exposure. If not, the

international default of a mean weight of 60 kg for adults and 15 kg for children should be used (FAO/WHO, 2009). For nutrients, intakes are expressed per individual per day and compared with reference values also expressed in the same unit.
The exposure can be calculated for the mean as well as for different percentiles for the population.
Sometimes, the chemical substances chosen are only present in particular food groups.
When the TDS approach is used whit only particular food groups known to be major contributors to exposure for the considered chemical substances, these study studies are classified as TDS-like investigations.

3.3.3 Effect of 1,2-dicarbonyl compounds in vitro and in vivo

1,2-dicarbonyl compounds have toxic effects in *in vitro* and *in vivo* studies. These compounds have cytotoxicity properties (Yang *et al*., 2011), inhibit the replication of DNA (Murata-Kamiya and Kamiya, 2001), bind to DNA and form mutations (Kasai *et al*., 1998), promoting tumor growth in the small intestine in Min mice model (Svendsen *et al*., 2016). In vivo studies these compounds have tumor promoting activity (Takahashi *et al*., 1989), increase DNA breaks (Furihata *et al*., 1989; Ueno *et al*., 1991), cause insulin resistance in mice and in muscle cells (Nigro *et al*. 2014; Riboulet-Chavey *et al*., 2006), concur to dysfunction of adipose tissue (Matafome *et al*., 2012), cause microvascular damage and other diabetes-like complications (Berlanga *et al*., 2005).

Furthermore, GO, MGO and 3-DG in physiological systems mediating dicarbonyl stress (Rabbani and Thornalley, 2015) and promote Advanced Glycation End-products (AGEs) (Nagai *et al*., 2012).
However, high level of plasmatic MGO can be derived from exogenous sources, such as food. The effects arising from the assumption of these compounds with food have not yet investigated.

3.3.4 Antimicrobial activity of 1,2-dicarbonyl compounds

Recent studies have also attributed to 1,2-dicarbonyl compounds antimicrobial activity on various microorganisms, including *Listeria monocytogenes*, *Staphylococcus aureus*, *Escherichia coli*, *Staphylococcus epidermidis, Pseudomonas aeruginosa, Proteus mirabilis* and *Streptococcus* mutans (Daglia *et al*., 2007a, Mavric *et al*., 2008). In particular, MGO was identified as the dominant antibacterial component of manuka honey, where it is produced non-enzymatically from dihydroxyacetone (DHA) present in manuka nectar. Although it has been known that MGO has antibacterial activity against Gram-positive bacteria, including methicillin-resistant *S. aureus* and vancomycin-resistant *Enterococcus*, less information is available with regard to its activity against Gram-negative bacteria. Hayashi *et al*., (2014), reported the effect of methylglyoxal against multidrug-resistant *P. aeruginosa* (MDRP) using 53 clinically isolated strains. They also assessed the effect of deleting the five multidrug efflux systems in *P. aeruginosa*, as well as the efflux systems in *E. coli* and *Salmonella enterica* sero var typhimurium, on

MICs of methylglyoxal. The results indicated that MGO inhibits the growth of MDRP at concentrations of 128–512 μg/ml (1.7–7.1 mM) and is not recognized by drug efflux systems. However, MGO possesses instability in air and strong enzyme induced degradation that limit its application. To circumvent this emerging problem, Ghosh *et al*., (2014) have developed a green strategy of using multivalent, biodegradable polymers such as chitosan, and dendrimers for the facile preparation of conjugated nanoformulations (NMG and DMG) of MGO as an antimicrobial agent against resistant bacteria. Interestingly, nanoformulated MGO selectively interferes with the bacterial pathogens while remaining biocompatible to the mammalian cells as reflected in therapeutic index. The functional group, cationic charge and nanosize of MGO allows them to attach to and insert into membrane bilayers of bacteria and could be the defining mechanisms of antimicrobial activity.

Glyoxal, together with methylglyoxal, and diacetyl compounds formed during the roasting process are the main agents responsible for the antibacterial activity of brewed coffee against *S. aureus* and *S. mutans* (Daglia *et al*., 2007a).

Hrynets *et al*., (2016) studied glucosamine browning at 50 °C, with (GlcN/Fe^{2+}) or without iron (GlcN), over time from 0 to 48 h. Generation of α-dicarbonyls (MGO, GO and 3-DG) was evaluated, as well as their antimicrobial activity. The presence of iron significantly increased the concentration of α-dicarbonyls at an early incubation time (3 h). GlcN/ Fe^{2+} (48 h) exhibited a MIC50 against highly heat-resistant *E. coli* AW 1.7 at pH 5, but not at pH 7. Among α-dicarbonyls, MGO was the most effective

followed by diacetyl (DA), GO, 3-DG, and glucosone (G). The MIC50 at pH 5 was 0.05 (MGO), 0.1 (DA), 0.4 (GO), 1.0 (3-DG), and 23.5 g/L (G). Increasing the pH caused an increase in MIC50 by 4.3, 3.0, 2.0, 1.7, and 1.3 times for MGO, DA, GO, 3-DG, and G, respectively. GlcN caramel solutions have, therefore, the potential to serve as both flavoring compounds and antimicrobial agents in formulated food systems.
Further to be present in the foods, 1,2-dicarbonyl compounds have antimicrobial activity.
However, the mechanism of action of these molecules has not yet been investigated.

3.4 Objectives of the thesis work

The aim of this Ph.D. project is to clarify the safety of the main dicarbonyl compounds generally present in food (glyoxal, methylglyoxal and the 3-deoxyglucosone) and their antimicrobial activity, with the purpose to suggest a possible use of this substances as preservatives.
Within the overall objective mentioned above, this Ph.D. thesis project can be subdivided into the following activities:

- ✓ Assessment of the content of dicarbonyl compounds in Italian food with the purpose of acquiring a complete picture of the distribution of these compounds in food;
- ✓ Assessment of the dietary theoretical intake;
- ✓ Evaluation of formation/degradation of dicarbonyl compounds using food model systems;

- ✓ Evaluation of antimicrobial activity against food spoilage and human-pathogenic bacterial strains;
- ✓ Assessment of the interference of microbiological culture media and nutrients on the levels of 1,2-dicarbonyl compounds.

4 Materials and methods

4.1 Chemical

High purity (P >98%) sucrose, fructose, glucose, o-phenylenediamine (OPD) and glyoxal (GO) and methylglyoxal (MGO) solutions were purchased from Sigma-Aldrich (St. Louis, Mo., USA). 3-deoxyglucosone (3-DG) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Methanol, acetic acid and water were of HPLC grade and obtained from JT Baker (Deventer, Holland). Gemini NX C18 (150 mm × 4.6 mm, 5 μm) columns were obtained from Phenomenex (Torrance, CA, USA). Syringe filters (0.45 μm) were supplied by Albet.
Tryptone Soya Agar, Tryptone Soya Broth were purchased from Oxoid (Basingstoke, UK).

4.2 Microorganisms and culture conditions

The bacterial strains used in this study were five reference strains and three clinical strains. The clinical isolates, provided by the Laboratory of Microbiology of Azienda Ospedaliero-Universitaria "Policlinico-Vittorio Emanuele" (Catania, Italy), were *Escherichia coli*, *Bacillus cereus* and *Staphylococcus aureus*. These strains were isolated from human faeces and blood.
The reference strains, provided by DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany), were: *Escherichia coli* 10198, *Pseudomonas fluorescens* 50091, *Staphylococcus aureus* 1104, *Listeria innocua* 20649 and *Salmonella typhimurium* 14028.

Bacterial strains were stored at 4°C on Petri dishes containing on Tryptone Soya Agar.

4.3 Statistical analysis

Data were analyzed separately by using the Statistical software (Minitab 16 Statistical Software Minitab Inc., State College, Pennsylvania, USA). The arithmetic means were calculated and analyzed by using one-way analysis of variance (ANOVA) submitted to evaluate the significant differences ($p < 0.05$) according to Fisher's method.

4.4 Sampling of Italian foods

A total of 53 food items belonging to different food categories was selected (Table 2). Food samples were purchased in different local supermarkets. Wheat bread and durum wheat bread samples were both from local industrial and artisanal bakery.

Table 2: Sampling food.

Sample	Code	Type of product	Producer
(n>1)			
Biscuit	1	No butter and eggs	A
Biscuit	2	No butter and eggs	B
Biscuit	3	No butter and eggs	C
Shortcake	4	With butter and eggs	A
Shortcake	5	With butter and eggs	D
Shortcake	6	With butter and eggs	E
White bread	7	White flour	F
White bread	8	White flour	G
White bread	9	White flour	H
White bread	10	White flour	I

Durum wheat bread	11	Semolina	J
Durum wheat bread	12	Semolina	K
Durum wheat bread	13	Semolina	M
Industrial durum wheat bread	14	Semolina	N
Industrial durum wheat bread	15	Semolina	N
Industrial durum wheat bread	16	Semolina	O
Pasta	17	Spaghetti	P
Pasta	18	Spaghetti	P
Pasta	19	Spaghetti	P
Fruit juice	20	Grapefruit	Q
Fruit juice	21	Orange	Q
Fruit juice	22	Pineapple	R
Fruit juice	23	Grapefruit	R
Fruit juice based drink	24	Pear	S
Fruit juice based drink	25	Peach	S
Fruit juice based drink	26	Pear	S
Fruit juice based drink	27	Pear	T
Fruit juice based drink	28	Pear	Q
Fruit juice based drink	29	Peach	R
Fruit juice	30	Pineapple	Q
Fruit juice based drink	31	Peach	R
Fruit juice based drink (n=1)	32	Pear	R
Jam	33	Strawberry	U
Jam	36	Peach	U
Jam	37	Williams pear	U
Jam	38	Cherry	U
Jam	39	Apricot	U
Marmalade	40	Orange	U
Honey	41	Multifloral	V
Honey	42	Multifloral	w
Honey	43	Multifloral	X
Honey	44	Multifloral	Y
Honey	45	Eucalyptus	Z
Honey	46	Eucalyptus	X
Honey	47	Thistle	X

Coffee	48	Arabica	AA
Coffee	49	Arabica	AA
Coffee	50	Arabica	AA
Whole Milk UHT	51		BB
Whole Milk UHT	52		BB
Whole Milk UHT	53		CC

n: number of sample

4.4.1 Sample preparation and 1,2-dicabonyl compounds extraction

Pasta: Preparation of pasta was carried out following the instructions given by the manufacturer on the packaging. 100 g of pasta were cooked into a suitable Pyrex glass container with boiling water (1 L) and 7 g of salt. After 8 minutes recommended, the sample was placed in a sieve to remove the cooking water and immediately cooled with cold tap water.

Coffee: Coffee samples were prepared with a moka coffee maker. To obtain coffee, 180 mL of distilled water was placed into the boiler and 18.68 g of coffee powder was placed into the filter dispenser. The moka was placed on a hot plate and the coffee was obtained in 5 minutes and 44 seconds.

Bread, shortcake and biscuits: All the samples were ground in a home grinder (La Moulinette, Moulinex, 2002).

Jam and marmalade: All the samples were mechanically homogenized in water by Ultra-Turrax and then diluted.

After samples preparation, 1,2dicarbonyl compounds were extracted and derivatized as follow.

5 g of the ground or liquid sample (except for fruit juices, fruit juices based drinks and milk) was transferred into a volumetric flask (50 ml), and 25 ml of water was added (JT. Baker, Deventer, Holland). The solution was stirred for 10 min, and then the sample was diluted up to 50 ml with distilled water (JT. Baker, Deventer, Holland) and centrifuged for 45 min at 5000 rpm.

16 mL of fruit juices, fruit juices based drinks and milk, after centrifugation for 20 min at 5000 rpm, was transferred

into a volumetric flask and diluted up to 10 ml with distilled water. The solution was stirred for 10 min.
The remaining samples did not need preliminary preparations.

4.4.2 Derivatisation and HPLC analysis

An aliquot of the supernatant was filtered through a 0.45 μm filter (Albet). An aliquot (1 mL) of the filtered supernatant was mixed with a 0.2% (w/v) aqueous solution of OPD and were allowed to react at room temperature for 12 h in the absence of light. After 12 h the derivatised mixture was analyzed by measuring their corresponding quinoxalines according to Arena *et al*., (2011).
The samples were injected into an HPLC (Spectra System) equipped with a diode array detector (DAD – UV 6000 LP) and an autosampler (AS 3000) (Thermo Electron, San Jose, Calif., USA). The HPLC column used was a Phenomenex NX C18 (250 mm× 4.6 mm, 5 μm).
The detector wavelength was set to 312 nm and HPLC conditions were: eluent A was 0.1% (v/v) acetic acid in water and eluent B was methanol; flow rate, 0.7 mL/min; injection volume, 20 μL. The gradient program was: t0 85% A and 15% B; t10 65% A and 35% B; t15 35% A and 65% B; t25 100% B; t30 85% A and 15% B.
All compounds were identified by comparing retention times and UV spectra with those of standards and by splitting each sample with standards. Quantification of each compounds was performed using external calibration curves.
The HPLC-DAD method for the analysis of dicarbonyl

compounds, was validated by the determination of linearity, limits of detection (LODs), limits of quantification (LOQs) and precision. Linearity was checked for 3-DG between 3.25 and 40.62 mg/kg; for GO between 1.66 and 25.2 mg/Kg and for MGO between 0.83 and 36.00 mg/kg. Good linearity of the HPLC method was established over 2 orders of magnitude (R^2 = 0.999–1.000) for all 1,2-dicarbonyl compounds. Figure 11, 12 and 13 reported the calibration curve for 3-DG, GO, and MGO, respectively.

LOD and LOQ were calculated as 3σ/slope and 10σ/slope, respectively. The precision of the method was expressed as the repeatability relative standard deviation (RSDr).

The extraction procedure and the analyses were performed in duplicate for each sample, the reported concentration of each dicarbonyl compound was therefore the average of four values. The results were expressed as mg of dicarbonyl compound/kg.

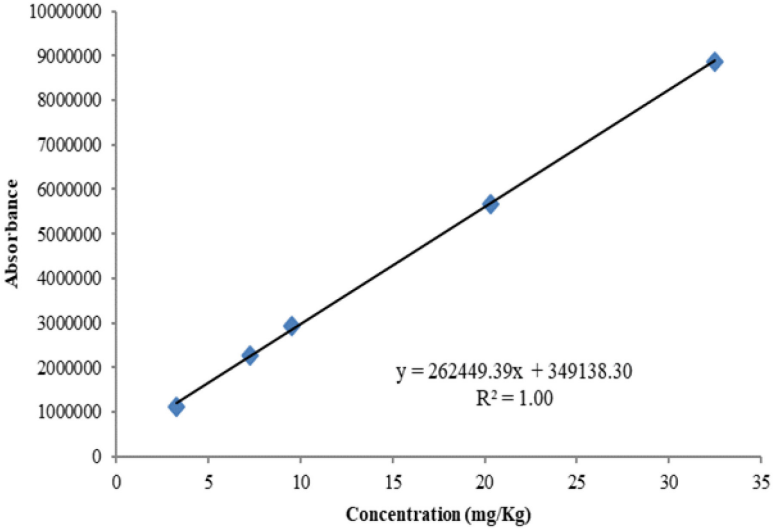

Fig. 11: Calibration curve of 3-DG.

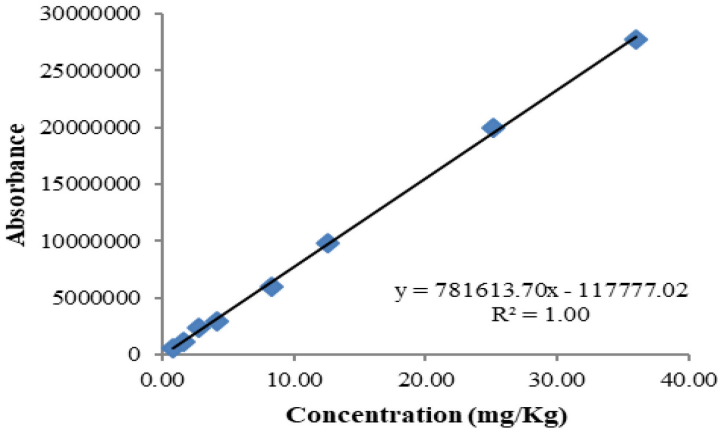

Fig. 12: Calibration curve of GO.

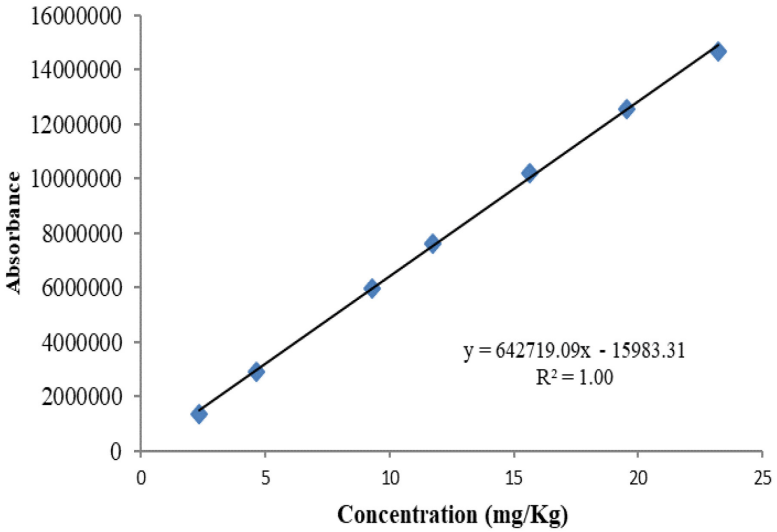

Fig. 13: Calibration curve of MGO.

4.4.3 Assessment of the dietary theoretical intake

A TDS-like investigations was applied because only some food groups are known to be major contributors to exposure for the 1,2-dicarbonyl compounds and not the total diet.
In order to evaluate the intake of 1,2-dicarbonyl compounds whit food, the average values obtained were correlated whit the average consumption expressed in mg/kg body weight/day for each food categories, and for the whole food intake. The average value obtained has been multiplied for 2 and 3 times standard deviation and then was correlated with the high percentiles (95^{th}, 99^{th}) respectively.
The theoretical intake was evaluated also for age for total population and for age for south & islands.
Table 3 and 4 reports mean, median, high percentiles (95^{th}, 99^{th}) of individual consumption (g/kg body weight/day) by food category and age for total population and for south & islands respectively.
For consumption data, survey on food consumption in Italy (INRAN SCAI 2005-06) was utilize. The food samples were regrouped into the category showed in table 5.

Table 3: Mean, high percentiles (95^{th}, 99^{th}) of individual consumption (g/kg body weight/day) by food category and age for total population**.**

Sample	Age category	Mean	95^{th}	99^{th}
Biscuits	Total Population	0.26	1.13	2.48
Bread		1.66	3.99	6.01
Fruit and vegetable juices		0.78	3.92	10.98
Jam		0.05	0.31	0.61
Honey		0.27	0.73	1.21
Coffee		2.03	5.48	9.75
Milk		2.73	7.20	20.83
Biscuits	Infants (0–2 years)	1.34	3.64	4.01
Bread		1.46	7.04	7.88
Fruit and vegetable juices		5.45	22.11	28.57
Jam		0.03	0.29	0.80
Honey		0.50	2.28	3.05
Coffee		1.12	9.86	19.23
Milk		40.25	183.33	280.00
Biscuits	Children (3–9 years)	0.80	2.91	4.50
Bread		2.95	7.63	13.18
Fruit and vegetable juices		3.46	12.3	21.33
Jam		0.08	0.55	0.88
Honey		0.42	1.46	3.00
Coffee		1.45	7.81	21.88
Milk		8.53	20.26	35.78
Biscuits	Teenagers (10–17 years)	0.46	1.54	3.16
Bread		1.97	4.80	6.60
Fruit and vegetable juices		1.75	8.17	12.24
Jam		0.04	0.26	0.43
Honey		0.24	0.73	1.15
Coffee		1.29	5.56	9.44
Milk		3.07	6.77	10.44
Biscuits	Adults (18–64 years)	0.19	0.78	1.44
Bread		1.52	3.57	4.80
Fruit and vegetable juices		0.45	2.56	4.75
Jam		0.05	0.30	0.57
Honey		0.26	0.69	1.07
Coffee		2.05	5.33	9.22
Milk		1.55	4.35	5.56
Biscuits		0.18	0.73	1.17

Bread	Elderly	1.68	3.84	5.17
Fruit and vegetable juices	(65 years and	0.33	2.30	4.40
Jam	above)	0.05	0.33	0.59
Honey		0.26	0.69	0.98
Coffee		1.91	5.40	7.69
Milk		1.88	5.00	6.12

Adapted from INRAN-SCAI 2005-06 appendices 1-B4, 8-B4, 14-B4, 11-B4

Table 4: Mean, median, high percentiles (95th, 99th) of individual consumption (g/kg body weight/day) by food category and age for south & islands.

Sample	Age category	Mean	95th	99th
Biscuits	Total Population	0.19	0.81	1.79
Bread		1.67	4.00	6.21
Fruit and vegetable juices		0.76	4.18	11.86
Jam		0.03	0.20	0.51
Honey		0.22	0.61	0.95
Coffee		1.92	5.48	9.75
Milk		3.07	8.20	25.64
Biscuits	Infants (0–2 years)	0.99	2.46	2.56
Bread		1.19	5.62	7.04
Fruit and vegetable juices		5.53	20.83	28.57
Jam		0.06	0.37	0.80
Honey		0.44	1.00	30.5
Coffee		0.47	3.13	6.67
Milk		49.72	256.67	280.00
Biscuits	Children (3–9 years)	0.54	2.00	3.54
Bread		3.24	9.17	16.47
Fruit and vegetable juices		3.83	14.81	22.22
Jam		0.06	0.44	0.73
Honey		0.38	0.8	3.00
Coffee		0.91	5.96	21.88
Milk		9.29	18.67	59.10
Biscuits	Teenagers (10–17 years)	0.33	1.23	3.16
Bread		2.09	4.93	7.57
Fruit and vegetable juices		1.90	9.13	14.63
Jam		0.04	0.21	0.43
Honey		0.18	0.68	1.21
Coffee		0.49	2.02	7.14
Milk		3.36	6.78	10.44
Biscuits	Adults (18–64 years)	0.13	0.52	1.13
Bread		1.47	3.40	4.49
Fruit and vegetable juices		0.30	2.00	3.92
Jam		0.03	0.20	0.49
Honey		0.21	0.56	0.79

Coffee		1.44	3.43	5.73
Milk		1.46	4.17	5.55
Biscuits		0.12	0.46	1.05
Bread	Elderly	1.71	3.50	4.63
Fruit and vegetable juices	(65 years and	0.15	1.09	2.22
Jam	above)	0.01	0.16	0.28
Honey		0.19	0.58	0.83
Coffee		1.28	3.46	3.87
Milk		1.88	5.00	6.12

Adapted from INRAN-SCAI 2005-06 appendices 1-B4, 8-B4, 14-B4, 11-B4

Table 5: Food categories used.

Food categories	Single food items
Cereals, cereal products and substitutes	
Bread	Wheat bread, durum wheat bread, industrial and artisanal durum wheat bread
Pasta	Industrial pasta
Biscuits	Biscuits and shortcake
Water and other non-alcoholic beverages	
Fruit, fresh and processed	Fruit juice and fruit juice based drink
Coffee, tea, herbal tea and substitutes	Coffee
Sweet products and substitutes	
Candies, jam and other sweet products	Jam and marmalade
Sugar, fructose, honey and other nutritious sweeteners	Honey and Honey of Sicilian black bee
Milk, milk products and substitutes	Milk

4.5 Study of formation/degradation of dicarbonyl compounds using food model systems

Five recipes were prepared by varying the types of fat and sugar. All ingredients were purchased from local supermarkets. Model biscuits were prepared according to a recipe described in the American Association of Cereal Chemists (AACC) Method 10-54 (American Association of Cereal Chemists (AACC) International, 2000) with some modifications. The recipe for the control biscuits was as follows: 240 g of wheat flour (100 units), 100.8 g of sugar (sucrose, fructose or glucose, 42 units), 96 g of butter (40 units), 3 g of salt (1.3 units), 2.4 g of sodium bicarbonate (1.0 units), 1.2 g of ammonium bicarbonate (0.5 units) and 52.8 mL of deionised water (22 units). According to recipe, all of the ingredients were thoroughly mixed using a dough mixer (model 1596, Ariete, Italy) for 7 min. Afterwards, the dough was laminated three times using a manual laminator (Imperia, Lusso, sp 150 model, Bologna, Italy) and was formed into discs with a diameter of 5 cm and a thickness of 0.2 cm. Seventy-five biscuits were obtained from each batch of dough. The biscuits were baked in a laboratory oven (Thermo Scientific, Heratherm oven, Italy) at three different temperatures, 150°C, 170°C and 190°C, for different time (5, 10, 15, 20 and 25 min) to monitor chemical changes in biscuits composition in terms of GO, MGO, and 3-DG. Baking temperatures used in this study were in the range suggested for short doughs (Manley, 2001). Fifteen biscuits were sampled for each time point. They were immediately cooled in a fridge at 4°C for 2 h and then ground in a home

grinder (La Moulinette, Moulinex, France) before chemical analyses.
An aliquot of the milled sample (1 g) was transferred to a volumetric flask (10 mL) and 5 mL of deionised water was added. The solution was stirred for 10 min, then the sample was diluted to 10 mL with deionised water and centrifuged at 10°C for 15 min at 8500 rpm (ALC 4128, Italy).
The derivatization and the HPLC analyses were conducted as reported on section 4.4.2.
The extraction procedure and the analyses were performed in duplicate, the reported concentration of each dicarbonyl compound was therefore the average of four values. The results were expressed as mg of dicarbonyl compound/kg of biscuit dry matter.

4.6 Antimicrobial assay and determination of minimum inhibitory concentrations (MIC)

Twenty-four-hour bacterial cultures grown on TSA at 37°C were suspended in sterile physiologic solution (0.9% NaCl). The suspensions were adjusted to 10^7 cells/mL. One mL was placed into a sterile Petri dish and then 20 mL of sterilised melted TSA (~45°C) was poured into the plate and mixed gently and thoroughly. After solidification, wells that were 10 mm in diameter were bored into the medium, and 0.4 mL of the 1,2-dicarbonyl compound solutions were placed into the wells. The ranges for GO, MGO and 3-DG were 126 to 3060 mg/kg, 66 to 3488 mg/kg and 400 to 10000 mg/kg, respectively.
The plates were incubated at 37°C until there was evidence of antimicrobial activity (24-72h). The antimicrobial effect

was considered positive when a clear zone of inhibition surrounded the well. The minimum inhibitory concentrations (MIC) corresponds to the lowest concentration for which a zone of inhibition zone was detected. The assays were performed in triplicate.

4.7 Influence of media and nutrients on the levels of 1,2-dicarbonyl compounds

Twenty-four-hour bacterial cultures of two target microorganism (*S. aureus* DSMZ 1104 and *E. coli* DSMZ 10198) were suspended in sterile physiologic saline solution and in Tryptic Soy Broth (TSB). The suspensions were adjusted to 5×10^8 cells/mL and then 100 µL of the 1,2-dicarbonyl compounds at three different concentrations [MIC (Minimum Inhibitory Concentration), 1/2 and 1/4 MIC], were placed into the test tubes.

As control, test tubes containing bacterial cultures without 1,2-dicarbonyl compounds solution were used. The test tubes were incubated at 37°C. The bacterial growth (CFU/mL) into the test tubes was analyzed every 10 minutes up to one hour. One hundred µL of each tube solution were plated onto TSA using a spiral Plater Eddy Jet. The means of colonies growth ($\log_{10}$ CFU/mL) was evaluated after 24 h at 37°C. After this preliminary test, the next step was to determine the stability and interaction between the dicarbonyl compounds and the nutrients present in the culture media.

GO and MGO, were chosen, at three different starting concentrations [MIC (Minimum Inhibitory Concentration), 1/2 and 2 MIC], in the following culture conditions:

1. Distilled water;
2. TSB;
3. TSB+ *E. coli* DSMZ 10198
4. TSB+ *E. coli* clinical strain;
5. TSB+ *S. aureus* DSMZ 1104;
6. TSB+ *S. aureus* clinical strain.

The following reaction times (minutes) were selected: T0, T30, T60, T90, T150, T210, T240, T250, and T270.
For 3-DG, experiments were designed to evaluate the residual concentration up to 24 h after it was placed in the culture conditions reported above, with the following concentrations: 537 mg/kg, 1240 mg/kg and 1930 mg/kg. The two target microorganism were *S. aureus* DSMZ 1104 and *E. coli* DSMZ 10198.
Test tubes containing bacterial strains and dicarbonyl compounds were centrifuged at 9000 rpm for 5 minutes. The derivatization and the HPLC analyses of samples were conducted as reported on section 4.4.2.

5 Results

5.1 1,2-dicarbonyl compounds in Italian food

An HPLC method was used to quantify 3-DG, GO, and MGO, by measuring their quinoxaline derivatives, which were obtained after derivatization with OPD (ortho-phenylenediamine).
LOD values were 0.03, 0.01, and 0.04 mg/kg, while LOQ values were 0.08, 0.03, and 0.12 mg/kg for 3-DG, GO, and MGO, respectively. The precision of the method, calculated as RSDr, was 0.09, 0.12, and 0.13 for 3-DG, GO, and MGO, respectively (Table 6).

Table 6: Limits of detection (LODs), limits of quantitation (LOQs), and repeatability relative standard deviations (RSDr) for 1,2-dicarbonyl compounds.

	LODs (mg/kg)[a]	**LOQs (mg/kg)[b]**	**RDSr (%)**
3-DG	0.01	0.02	0.07
GO	0.03	0.01	0.09
MGO	0.008	0.009	0.09

[a] Calculated as 3σ/slope of the calibration curve.
[b] Calculated as 10σ/slope of the calibration curve.

As shown in Table 7, all sample showed high 3-DG levels, ranging from 4.10 to 703.12 mg/kg. The honeys sample had the highest 3-DG levels. Bread and fruit juice and fruit based drink samples had the lowest mean 3-DG contents (approximately 28.84 and 29.87 mg/kg respectively); average value for biscuit, jam and marmalade samples was about 35.82 mg/kg and 48.33 mg/kg respectively, instead the average value for honey samples was about 320.2 mg/kg.

GO was present at low concentrations in all samples ranging from 3.36 to 23.38 mg/kg.

Honey samples had the lowest mean value for GO (4.27±0.63 mg/kg), while jam and marmalade samples had the highest average value (9.62±1.72 mg/kg).

MGO was predominant in marmalade with concentration up to 195.38 mg/kg, for the other samples the MGO levels ranging from about 1.07 to 76.98 mg/kg.

Pasta, coffee and milk samples had a content of 1,2-dicarbonyl compounds lower than the limit of detection.

Table 7: Distributions (mg/kg±s. d.) of 1,2-dicarbonyl compounds in the food items.

	Sample	3-DG	GO	MGO
Biscuit	1	nd	6.61±0.44	19.16±1.77
	2	57.38±8.87	13.76±0.99	12.54±2.22
	3	113.83±5.15	7.12±0.25	39.64±2.74
	4	nd	nd	nd
	5	nd	4.74±0.21	76.98±7.33
	6	43.72±1.62	5.01±0.91	2.26±0.04
	Mean value	**35.82±43.73**	**6.21±4.29**	**25.1±27.90**
Bread	7	11.26±2.62	3.52±0.05	2.74±0.42
	8	24.86±1.96	3.67±0.12	1.07±0.04
	9	12.81±0.76	3.73±0.01	1.26±0.03
	10	24.83±2.40	4.42±0.19	5.11±1.45
	11	nd	4.46±0.13	1.98±0.07
	12	18.53±1.72	5.10±0.07	4.54±0.62
	13	4.10±2.77	6.93±1.62	4.06±0.71
	14	39.02±3.27	12.24±2.33	11.50±0.81
	15	26.03±0.71	15.31±1.06	9.34±0.66
	16	56.95±8.76	23.38±1.63	17.82±2.04
	Mean value	**21.84±16.60**	**8.28±6.53**	**5.94±5.29**
Pasta	17	nd	nd	nd
	18	nd	nd	nd
	19	nd	nd	nd
Fruit juice and fruit juice based drink	20	36.65±1.21	6.95±0.01	19.17±0.39
	21	26.17±1.48	6.89±0.23	24.42±0.89
	22	39.25±0.42	3.36±0.23	13.20±0.32
	23	37.76±0.02	7.70±0.01	21.60±0.01
	24	34.94±0.65	5.22±0.04	12.62±0.47
	25	25.49±0.03	4.58±0.37	10.52±1.82
	26	49.23±1.63	7.28±0.19	17.19±0.32
	27	13.16±1.05	6.71±0.72	7.74±0.65
	28	20.42±3.79	5.35±0.69	26.29±2.13
	29	17.15±2.28	5.03±0.19	9.01±1.39

	30	19.57±2.79	5.73±0.28	13.02±0.13
	31	33.80±2.39	6.88±0.56	13.93±1.22
	32	33.40±0.61	7.51±0.40	13.74±0.33
	Mean value	**29.77±10.26**	**6.09±1.32**	**15.57±5.72**
Jam and Marmalade	33	40.69±5.14	8.80±0.40	4.97±0.19
	36	47.45±2.48	10.57±0.23	9.64±1.74
	37	39.01±3.12	6.70±0.90	8.82±0.32
	38	86.94±2.29	10.74±0.48	8.80±0.28
	39	47.01±2.00	11.52±0.39	6.91±0.21
	40	28.86±5.50	9.41±1.04	195.38±13.85
	Mean value	**48.33±19.35**	**9.62±1.72**	**39.11±73.13**
Honey	41	394.05±44.12	4.84±0.18	4.62±0.24
	42	158.28±3.84	3.74±0.16	5.58±0.25
	43	331.46±23.13	4.02±0.09	5.25±0.56
	44	156.65±5.89	3.94±0.02	3.85±0.07
	45	703.12±33.57	4.93±0.23	5.19±0.08
	46	421.60±25.58	5.05±0.14	7.61±0.60
	47	76.18±2.32	3.39±0.02	3.98±0.11
	Mean value	**320.19±203.02**	**4.27±0.63**	**5.15±1.29**
Coffee	48	nd	nd	nd
	49	nd	nd	nd
	50	nd	nd	nd
Milk	51	nd	nd	nd
	52	nd	nd	nd
	53	nd	nd	nd

nd: non detectable

5.2 Dietary theoretical intake

Table 8 reports the medium and the high percentiles (95th, 99th) intake (mg/kg body weight/day) for GO, MGO and 3-DG for food category and age for total Italian population. Table 9 reports the medium and the high percentiles (95th, 99th) intake (mg/kg body weight/day) for GO, MGO and 3-DG for food category and age for south & islands Italian population.

The medium intake for total population was of 0.022 mg/kg body weight/day, for 95th was of 0.144 mg/kg body weight/day and for 99th was of 0.341 mg/kg body weight/day of GO.

About the intake by age, data show that the category who ingest more GO with the diet are the infants (0-2 years) and the children (3-9 years). The intake for 99th was of 0.615 and 0.699 mg/kg body weight/day respectively.

Teenagers category (10–17 years) assumes with diet 0.031 mg/kg body weight/day of GO.

The medium intake for adults (18–64 years) and elderly (65 years and above) was of 0.018 mg/kg body weight/day of GO.

The medium intake of MGO for total population was very low (0.032 mg/kg body weight/day), instead the intake for 99th was very high (0.929 mg/kg body weight/day).

Infants (0-2 years) category assumes the highest level of MGO for medium and both high percentiles.

The intake for this category was of 0.132 mg/kg body weight/day for medium, 1.079 mg/kg body weight/day for 95th and 1.777 mg/kg body weight/day for 99th.

The lowest intake was for teenagers (10–17 years) category.

As concerns 3-DG the medium intake for total population was of 0.156 mg/kg body weight/day and the intake for 99th was of 2.699 mg/Kg body weight/day.
Even in the case of 3-DG the categories that assumes the highest level of 3-DG were infants (0-2 years) and children (3–9 years).
The medium intake for teenagers (10-17 years) was of 0.190 mg/kg body weight/day, for adults (18–64 years) and elderly (65 years and above) was of 0.138 mg/kg body weight/day.
The medium intake for total population of south & islands was of 0.021 mg/kg body weight/day, for 95th was of 0.140 mg/kg body weight/day and for 99th was of 0.340 mg/kg body weight/day of GO.
About the intake by age, data show that the category who ingest more GO with the diet are the children (3-9 years) and the infants (0-2 years). The intake for 99th was of 0.78 and 0.733 mg/kg body weight/day respectively. Teenagers category (10–17 years) assumes whit diet 0.032 mg/kg body weight/day of GO. The medium intake for adults (18–64 years) and elderly (65 years and above) was of 0.016 mg/kg body weight/day of GO.
The medium intake of MGO for total population of south & islands was of 0.029 mg/kg body weight/day, and the intake for 99th was of 0.859 mg/kg body weight/day.
Infants (0-2 years) category assumes the highest level of MGO for medium and both high percentiles.
The intake for this category was of 0.122 mg/kg body weight/day for medium, 0.932 mg/kg body weight/day for 95th and 1.844 mg/kg body weight/day for 99th.

Also the children have a high intake both for medium and high percentiles (0.097, 0.801 and 1.687 mg/kg body weight/day respectively).
The lowest intake was for adults (18–64 years) and elderly (65 years and above).
The medium intake of 3-DG for total population was of 0.138 mg/kg body weight/day and the intake for 99^{th} was of 2.399 mg/kg body weight/day.
Even in the case of 3-DG the category that assumes the highest level of 3-DG was infants (0-2 years) specially for 99^{th} (31.089 mg/kg body weight/day).
The medium intake for children (3–9 years) was of 0.329 mg/kg body weight/day.
The medium intake for teenagers (10-17 years) was of 0.175 mg/kg body weight/day, for adults (18–64 years) and elderly (65 years and above) was about of 0.110 mg/kg body weight/day.

Table 8: Intake of GO, MGO and 3-DG (medium, high percentiles 95th, 99th) for food category and age of total Italian population.

Sample/dicarbonyl compound	GO[a]			MGO[a]			3-DG[a]		
Total population									
	Medium	95th	99th	Medium	95th	99th	Medium	95th	99th
Biscuits	0.002	0.017	0.047	0.007	0.091	0.270	0.009	0.139	0.414
Bread	0.014	0.085	0.167	0.010	0.066	0.131	0.036	0.220	0.431
Fruit and vegetable juices	0.005	0.034	0.110	0.012	0.106	0.359	0.023	0.197	0.665
Jam	0.000	0.004	0.009	0.002	0.057	0.158	0.002	0.027	0.065
Honey	0.001	0.004	0.008	0.001	0.006	0.011	0.086	0.530	1.124
∑ Intake	0.022	0.144	0.341	0.032	0.326	0.929	0.156	1.113	2.699
Infants (0-2 years)									
	Medium	95th	99th	Medium	95th	99th	Medium	95th	99th
Biscuits	0.008	0.054	0.077	0.034	0.295	0.436	0.048	0.449	0.670
Bread	0.012	0.150	0.220	0.009	0.116	0.172	0.032	0.387	0.564
Fruit and vegetable juices	0.033	0.193	0.287	0.085	0.597	0.935	0.162	1.112	1.729
Jam	0.000	0.004	0.012	0.001	0.054	0.207	0.001	0.025	0.085
Honey	0.002	0.013	0.019	0.003	0.017	0.027	0.160	1.656	2.834
∑ Intake	0.055	0.414	0.615	0.132	1.079	1.777	0.403	3.629	5.882
Children (3–9 years)									
	Medium	95th	99th	Medium	95th	99th	Medium	95th	99th
Biscuits	0.005	0.449	0.086	0.020	0.235	0.490	0.029	0.359	0.751
Bread	0.024	0.387	0.367	0.018	0.126	0.287	0.064	0.420	0.944
Fruit and vegetable juices	0.021	0.107	0.214	0.054	0.332	0.698	0.103	0.618	1.291
Jam	0.001	0.007	0.013	0.003	0.102	0.227	0.004	0.048	0.094
Honey	0.002	0.008	0.019	0.002	0.011	0.027	0.134	1.060	2.788
∑ Intake	0.053	0.328	0.699	0.097	0.806	1.729	0.334	2.505	5.868

	Teenagers (10–17 years)								
	Medium	95th	99th	Medium	95th	99th	Medium	95th	99th
Biscuits	0.003	0.023	0.060	0.012	0.125	0.344	0.016	0.190	0.528
Bread	0.016	0.102	0.184	0.012	0.079	0.144	0.043	0.264	0.473
Fruit and vegetable juices	0.011	0.071	0.123	0.027	0.221	0.401	0.052	0.411	0.741
Jam	0.000	0.003	0.006	0.002	0.048	0.111	0.002	0.023	0.046
Honey	0.001	0.004	0.007	0.001	0.006	0.010	0.077	0.530	1.069
∑ Intake	0.031	0.203	0.380	0.054	0.479	1.010	0.190	1.418	2.857
	Adults (18–64 years)								
	Medium	95th	99th	Medium	95th	99th	Medium	95th	99th
Biscuits	0.001	0.012	0.027	0.005	0.063	0.157	0.007	0.096	0.240
Bread	0.013	0.076	0.134	0.009	0.059	0.105	0.033	0.196	0.344
Fruit and vegetable juices	0.003	0.022	0.048	0.007	0.069	0.155	0.013	0.129	0.288
Jam	0.000	0.004	0.008	0.002	0.056	0.147	0.002	0.026	0.061
Honey	0.001	0.004	0.007	0.001	0.005	0.009	0.083	0.501	0.994
∑ Intake	0.018	0.118	0.224	0.024	0.252	0.573	0.138	0.948	1.927
	Elderly (65 years and above)								
	Medium	95th	99th	Medium	95th	99th	Medium	95th	99th
Biscuits	0.001	0.011	0.022	0.005	0.059	0.127	0.006	0.090	0.195
Bread	0.014	0.082	0.144	0.010	0.063	0.113	0.037	0.211	0.370
Fruit and vegetable juices	0.002	0.020	00.044	0.005	0.062	0.144	0.010	0.116	0.266
Jam	0.000	0.004	0.009	0.002	0.061	0.153	0.002	0.029	0.063
Honey	0.001	0.004	0.006	0.001	0.005	0.009	0.083	0.501	0.911
∑ Intake	0.018	0.121	0.225	0.023	0.250	0.546	0.138	0.947	1.805

[a](mg/kg body weight/day)

Table 9: Intake of GO, MGO and 3-DG (medium, high percentiles 95th, 99th) for food category and age for south & islands.

Sample/dicarbonyl compound	GO[a]			MGO[a]			3-DG[a]		
				Total population					
	Medium	95th	99th	Medium	95th	99th	Medium	95th	99th
Biscuits	0.001	0.012	0.034	0.005	0.066	0.195	0.007	0.100	0.299
Bread	0.014	0.085	0.173	0.010	0.066	0.135	0.036	0.220	0.445
Fruit and vegetable juices	0.005	0.036	0.119	0.012	0.113	0.388	0.023	0.210	0.718
Jam	0.000	0.003	0.008	0.001	0.037	0.132	0.001	0.017;	0.054
Honey	0.001	0.003	0.006	0.001	0.005	0.008	0.070	0.443	0.883
∑ Intake	0.021	0.140	0.340	0.029	0.286	0.859	0.138	0.991	2.399
				Infants (0-2 years)					
	Medium	95th	99th	Medium	95th	99th	Medium	95th	99th
Biscuits	0.006	0.036;	0.049	0.025	0.199	0.279	0.035	0.303	0.428
Bread	0.010	0.120	0.196	0.007	0.093	0.153	0.026	0.309	0.504
Fruit and vegetable juices	0.034	0.182	0.287	0.086	0.563	0.935	0.165	1.047	1.729
Jam	0.001	0.005	0.012	0.002	0.069	0.207	0.003	0.032	0.085
Honey	0.002	0.006	0.189	0.002	0.008	0.270	0.141	0.726	28.343
∑ Intake	0.053	0.349	0.733	0.122	0.932	1.844	0.370	2.417	31.089
				Children (3–9 years)					
	Medium	95th	99th	Medium	95th	99th	Medium	95th	99th
Biscuits	0.003	0.030	0.068	0.014	0.162	0.385	0.019	0.247	0.591
Bread	0.027	0.196	0.459	0.019	0.151	0.359	0.071	0.505	1.180
Fruit and vegetable juices	0.023	0.129	0.223	0.060	0.400	0.727	0.114	0.745	1.345
Jam	0.001	0.006	0.011	0.002	0.082	0.189	0.003	0.038	0.078
Honey	0.002	0.004	0.019	0.002	0.006	0.027	0.122	0.581	2.788
∑ Intake	0.056	0.365	0.780	0.097	0.801	1.687	0.329	2.116	5.982

	Teenagers (10–17 years)								
	Medium	95th	99th	Medium	95th	99th	Medium	95th	99th
Biscuits	0.002	0.018	0.060	0.008	0.100	0.344	0.012	0.152	0.528
Bread	0.017	0.105	0.211	0.012	0.081	0.165	0.046	0.271	0.542
Fruit and vegetable juices	0.012	0.080	0.147	0.030	0.247	0.479	0.057	0.459	0.886
Jam	0.000	0.003	0.006	0.002	0.039	0.111	0.002	0.018	0.046
Honey	0.001	0.004	0.008	0.001	0.005	0.011	0.058	0.494	1.124
∑ Intake	0.032	0.210	0.432	0.053	0.472	1.110	0.175	1.394	3.126
	Adults (18–64 years)								
	Medium	95th	99th	Medium	95th	99th	Medium	95th	99th
Biscuits	0.001	0.008	0.022	0.003	0.042	0.123	0.005	0.064	0.189
Bread	0.012	0.073	0.125	0.009	0.056	0.098	0.032	0.187	0.322
Fruit and vegetable juices	0.002	0.017	0.039	0.005	0.054	0.128	0.009	0.101	0.237
Jam	0.000	0.003	0.007	0.001	0.037	0.127	0.001	0.017	0.052
Honey	0.001	0.003	0.005	0.001	0.004	0.007	0.067	0.407	0.734
∑ Intake	0.016	0.104	0.198	0.019	0.025	0.050	0.114	0.776	1.534
	Elderly (65 years and above)								
	Medium	95th	99th	Medium	95th	99th	Medium	95th	99th
Biscuits	0.001	0.007	0.020	0.003	0.037	0.114	0.004	0.057	0.175
Bread	0.014	0.075	0.129	0.010	0.058	0.101	0.037	0.193	0.332
Fruit and vegetable juices	0.001	0.010	0.022	0.002	0.029	0.073	0.004	0.055	0.134
Jam	0.000	0.002	0.004	0.000	0.030	0.072	0.000	0.014	0.030
Honey	0.001	0.003	0.005	0.001	0.004	0.007	0.061	0.421	0.771
∑ Intake	0.017	0.097	0.180	0.016	0.158	0.367	0.106	0.740	1.442

[a](mg/kg body weight/day)

5.3 Study of formation/degradation of dicarbonyl compounds using food model systems

Role of lipids

Table 10 summarized the level of 1,2-dicabonyl compounds in biscuits prepared with different fat.

The concentration of 1,2-dicabonyl compounds increased during baking, especially at the highest temperature.

The highest concentration of 3-DG was found in the sample with olive oil (1007.05 mg/kg of dry matter), followed by the sample with seeds oil (557.91 mg/kg of dry matter). Lowest concentration of 3-DG was found in samples with margarine and in control sample (72.61 and 68.80 mg/kg of dry matter respectively).

In control biscuits at a temperature of 150°C there was no formation of 3-DG until 25 minutes of baking, while at 170°C the presence of 3-DG was found after 20 minutes of baking. In the sample baked at 190 °C the formation of 3-DG began after 15 minutes of baking. The concentration ranged from approximately 3.25 mg/kg to 67.80 mg/kg of dry matter.

In biscuits with olive oil, the formation of 3-DG appeared after 20 minutes at the temperatures of 150 and 190°C, but at the end of baking the levels were very different (10.95 mg/kg and 1007.10 mg/kg respectively). At 170°C, the presence of 3-DG was found after 15 minutes of baking, ranging from approximately 0.20 mg/kg to 92.95 mg/kg of dry matter.

A different behaviour, in terms of 3-DG formation, was found in biscuits samples in which the butter was replaced with seeds oil. In fact, the trend of formation of the 3-DG

was similar to that found for control samples, but the formation of 3-DG started after 10 minutes at 150°C, after 5 minutes at the temperature of 170°C and immediately at the temperature of 190°C. The concentration ranged from approximately 2.50 mg/kg to 557.91 mg/kg of dry matter.
In the sample biscuits with margarine, the formation of 3-DG proportionally increases with baking temperature and time. At 150°C there was no formation of 3-DG until 25 min of baking, while at 170°C the presence of 3-DG was found after 20 min of baking. In the sample baked at 190°C instead the formation of 3-DG starts after 10 minutes of baking.
The formation of GO and MGO started immediately in all samples. The levels of GO remained stable ranging from approximately 9.47 to 16.54 mg/kg of dry matter for all samples at the baking temperature of 170°C. At temperature of 190°C the levels of GO increased with time of baking for all the samples. The highest concentration was found in the control sample at the end of baking (approximately 243.53 mg/kg of dry matter).
A different behaviour was found for MGO. Its levels in control samples baked at 150°C ranged from approximately 7.59 to 9.29 mg/kg of dry matter, at 170°C the concentration ranged from approximately 7.04 to 12.49 mg/kg of dry matter and at 190°C the concentration ranged from approximately 7.37 to 132.37 mg/kg of dry matter. In the biscuits with olive oil, the levels of MGO remained similar at the temperature of 170°C and until 20 minutes of baking. After 25 minutes of baking, at 150°C and 170°C the concentration was found approximately of 33.42 and 73.80 mg/kg of dry matter respectively. At the end of baking at

190°C the concentration of MGO was the highest reaching up to 305.35 mg/kg of dry matter.
The levels of MGO in biscuits with seeds oil at 150°C ranged from approximately 10.08 to 51.49 mg/kg of dry matter, at 170°C from approximately 7.98 to 73.89 mg/kg of dry matter, and at 190°C from approximately 25.30 to 229.98 mg/kg of dry matter.
The levels of MGO in biscuits with margarine at 150°C ranged from approximately 17.29 to 32.09 mg/kg of dry matter, at 170°C from approximately 11.58 to 44.62 mg/kg of dry matter and at 190°C from approximately 35.81 to 178.95 mg/kg of dry matter.

Table 10: 1,2-dicarbonyl compounds concentration (mean ± s.d.) in biscuits prepared with different type of fat.

Temperature (°C)	Samples	Time (min)	3-DG (mg/kg dry matter)	GO (mg/kg dry matter)	MGO (mg/kg dry matter)
150°C	Control	5	nd	9.47±0.06b	7.59±0.29c
		10	nd	9.57±0.34b	7.35±0.35c
		15	nd	9.40±0.22c	8.16±0.54c
		20	nd	9.86±0.22b	8.47±0.14d
		25	5.95±0.90b	10.54±0.45b	9.29±0.30c
	Olive oil	5	nd	8.91±0.01c	8.26±0.28c
		10	nd	9.15±0.04b	8.35±0.79c
		15	nd	9.37±0.01c	8.16±0.29c
		20	0.30±0.07b	9.48±0.01b	13.12±1.24c
		25	10.95±1.33a	9.25±0.36c	33.42±1.14b
	Seeds oil	5	nd	9.71±0.17a	10.08±0.28b
		10	nd	10.52±0.32a	15.51±0.73a
		15	2.50±0.05	13.74±0.05a	15.90±0.81b
		20	4.50±0.30a	11.37±0.63a	25.30±0.42b
		25	25.50±6.65bc	10.43±0.35b	51.49±2.19a
	Margarine	5	nd	9.05±0.12c	17.29±0.91a
		10	nd	11.04±0.45a	10.94±0.73b
		15	nd	11.12±0.80b	19.69±0.81a
		20	nd	10.88±0.02a	27.22±0.42a
		25	3.20±0.42c	13.04±1.01a	32.09±2.19b
170°C	Control	5	nd	9.34±0.12b	7.04±0.30c
		10	nd	9.56±0.32b	7.33±0.36d
		15	nd	9.69±0.42ab	8.62±0.24c
		20	6.09±0.86c	9.84±0.12c	8.48±0.44b
		25	37.7±1.48d	11.15±0.62b	12.49±0.52c
	Olive oil	5	nd	9.34±0.03b	8.65±0.62b
		10	nd	9.82±0.90b	8.40±0.28c
		15	0.20±0.02b	9.21±0.01b	9.89±2.69bc
		20	20.25±4.37b	9.47±0.02c	17.47±1.89a
		25	92.95±7.01a	11.01±0.18b	73.80±4.41a
	Seeds oil	5	nd	10.32±0.15a	7.98±0.15b
		10	14.10±1.84a	11.60±0.13a	11.51±0.62b
		15	16.50±4.60a	11.16±1.42a	97.13±1.91a
		20	7.55±0.55c	13.52±1.30a	17.95±4.74a
		25	48.86±2.02c	16.54±1.07a	73.89±12.36a
	Margarine	5	nd	10.65±0.90a	11.58±0.15a
		10	nd	9.31±0.14b	22.27±0.62a
		15	nd	10.64±0.65ab	12.75±0.65b
		20	25.80±0.86a	11.42±0.46b	17.95±0.46a
		25	68.45±3.22b	9.35±0.49c	44.62±0.79b

190°C	Control	5	nd	9.17±0.15c	7.37±0.11c
		10	nd	9.32±0.03c	8.17±0.20b
		15	3.25±0.47bc	10.40±1.07c	10.03±0.52c
		20	45.9±0.93c	12.37±0.89b	16.97±0.39d
		25	67.80±2.05c	243.53±11.55a	132.37±13.07d
	Olive oil	5	nd	10.83±0.81bc	20.41±3.51b
		10	nd	11.13 ±0.35b	11.25±1.58b
		15	nd	51.14±4.00a	10.69±0.80c
		20	3.50±0.22d	55.34±7.70a	65.40±5.01b
		25	1007.10±37.4 9d	64.33±0.77c	305.35±8.91a
	Seeds oil	5	5.70±2.09a	12.62±2.00ab	25.30±3.51b
		10	2.80±0.52a	12.08±0.20a	41.61±2.87a
		15	9.50±2.55a	17.15±0.80b	57.09±3.09a
		20	63.14±1.92a	16.05±4.35b	162.83±5.01a
		25	557.91±18.15a	160.34±3.55b	229.98±11.36b
	Margarine	5	nd	13.24±0.45a	35.81±3.51a
		10	1.75±0.20b	11.75±0.65ab	39.23±2.87a
		15	6.34±2.57ab	14.04±0.25bc	42.08±3.09b
		20	57.32±5.45b	15.36±0.72b	48.31±5.01c
		25	72.61±7.60b	51.70±1.20d	178.95±11.36c

nd: not detected. Different letters indicate significant differences at $p < 0.05$ within temperature and time of baking.

Role of sugars

The levels of dicarbonyl compounds in biscuits prepared with different sugars are reported in table 11.

The concentration of 1,2-dicabonyl compounds increased during baking, especially at the highest baking temperatures. Substitution of sucrose with fructose or glucose affected the production of the three dicarbonyl compounds particularly 3-DG.

In control biscuits baked at temperatures of 150 and 170°C there was no formation of 3-DG until 20 minutes of baking, while at 190°C the presence of 3-DG was found after 15 minutes of baking.

After 25 min of baking at 150°C, the control sample

developed about 7 mg/kg of dry matter 3-DG, instead at 170°C the level of 3-Dg was about 70.6mg/kg of dry matter and at 190°C the level was about 1197.2 mg/kg of dry matter.
In samples with fructose and glucose the formation of 3-DG started immediately.
For biscuit with fructose after 25 min of baking at 150°C, level of 3-DG was about 1038.8 mg/kg of dry matter, at 170°C the level of 3-Dg was about 2324.0 mg/kg of dry matter and at 190°C the level was about 934.6 mg/kg of dry matter.
For biscuit with glucose after 25 min of baking at 150°C, level of 3-DG was about 3941.7 mg/kg of dry matter, at 170°C the level of 3-Dg was about 3204.5 mg/kg of dry matter and at 190°C the level was about 2060.0 mg/kg of dry matter.
The substitution of sucrose with other sugars led to an increase in both GO and MGO compared to control biscuits.
Overall, GO was high in the glucose system and MGO was high in the fructose one.
For GO in control biscuits baked at a temperature of 150°C there was no formation until 15 minutes, while at 170°C the formation of GO started after 10minutes of baking. Instead, in the control samples baked at 190°C the formation of GO began after 5 minutes of baking. The concentration ranged from approximately 8.70 to 362.6 mg/kg of dry matter.
In the biscuits with fructose and glucose the formation of GO started immediately and the concentration, at the end of baking, was higher for the sample with fructose, ranging from approximately 9.00 to 3014 mg/kg of dry matter; the only exception was observed at the temperature of 150°C.

The formation of MGO started immediately also in the control for all the baking temperatures. The highest level was found in the sample with fructose ranging approximately from 9.42 to 503 mg/kg of dry matter.

Table 11: 1,2-dicarbonyl compounds concentration (mean ± s.d.) in biscuits prepared with different sugar.

Temperature (°C)	Samples	Time (min)	3-DG (mg/kg dry matter)	GO (mg/kg dry matter)	MGO (mg/kg dry matter)
150°C	Control	5	nd	nd	9.30±4.60a
		10	nd	4.40±0.60b	7.90±0.40b
		15	0.80±0.20c	9.40±0.20b	7.80±0.60b
		20	4.10±5.40c	9.72±1.00a	7.80±1.30b
		25	6.70±1.70c	10.70±0.40b	8.40±0.30c
	Fructose	5	34.50±3.40a	10.50±0.30a	9.40±0.30a
		10	101.10±7.00ab	10.50±0.60a	12.70±0.40a
		15	241.90±23.80b	11.00±0.70a	21.80±0.50a
		20	523.90±47.50b	11.40±0.47a	30.50±2.80a
		25	1038.80±133.00b	14.50±1.80b	33.5±0.900a
	Glucose	5	35.90±22.40a	11.10±0.50a	9.40±1.00a
		10	207.50±50.30a	10.40±0.60a	8.40±0.70b
		15	810.00±160.30a	11.10±1.10a	9.30±0.90b
		20	2113.90±384.60a	14.30±3.20a	11.80±0.80b
		25	3941.70±169.00a	26.10±7.70a	17.90±1.90b
170°C	Control	5	nd	nd	8.20±0.50b
		10	0.50±0.30c	5.50±0.40b	7.6±0.400b
		15	1.00±0.60c	9.10±0.40b	7.51±0.50c
		20	6.10±1.3c	10.300±0.60a	8.30±0.40c
		25	70.60±9.20c	11.20±0.60c	14.90±1.70c
	Fructose	5	69.20±7.80a	11.00±0.30a	11.20±0.70a
		10	208.80±20.30b	10.30±0.70a	20.80±0.80a
		15	481.30±79.90b	11.10±0.40a	31.50±1.80a
		20	1162.30±188.50b	16.00±2.30a	39.80±2.10a
		25	2324.00±516.90ab	105.00±82.80a	83.00±17.50a
	Glucose	5	100.40±42.70a	10.80±0.70a	8.70±0.50b
		10	591.50±32.60a	10.00±0.60a	9.60±0.90b
		15	1846.20±120.30a	11.40±1.20a	12.50±0.70b
		20	3517.90±206.00a	15.40±4.00a	17.30±1.30b
		25	3204.50±348.10a	36.90±15.20ab	47.10±1.70ab
190°C	Control	5	nd	nd	7.80±0.40b
		10	1.50±0.50c	8.70±0.90a	7.20±0.50b
		15	5.30±1.30c	10.70±0.40a	8.40±0.30c
		20	77.40±5.90b	11.60±0.40b	14.70±1.60b
		25	1197.20±27.70b	362.60±260.90b	293.00±162.70ab
	Fructose	5	100.30±7.10b	9.00±0.40a	13.20±0.80a

	10	331.20±41.90b	11.50±2.10a	28.90±2.60a
	15	960.90±119.40b	14.10±1.05a	37.50±2.10a
	20	2144.40±243.90a	45.50±5.80a	118.20±22.10a
	25	934.60±302.60b	3014.20±265.40a	503.00±134.50a
Glucose	5	211.60±17.10a	10.00±0.70a	8.80±0.70b
	10	1251.40±111.50a	10.80±1.100a	12.10±2.00b
	15	3250.30±54.70a	15.60±4.10a	18.70±0.80b
	20	2913.60±659.70a	46.70±15.40a	105.30±30.40a
	25	2060.00±81.20a	249.10±126.10b	314.10±72.70ab

nd: not detected. Different letters indicate significant differences at $p < 0.05$ within temperature and time of baking.

5.4 Antimicrobial assays and determination of minimum inhibitory concentrations (MIC)

Table 12 contains the antibacterial activities (MIC values) of the three studied chemical compounds (3-DG, GO and MGO) against each of the eight tested strains. These data demonstrate antibacterial activity for GO and MGO. 3-DG did not exhibit any antibacterial activity.

For MGO the lowest MIC value (488 mg/kg) was measured for *E. coli* DSMZ 10198, *P. fluorescens* DSMZ 50091 and *S. aureus* clinical strain. A value of 585 mg/kg was recorded for *L. innocua* DSMZ 20649, a value of 1642.2 was recorded for *E. coli* clinical strain, while an MIC value of 975 mg/kg was obtained both for *B. cereus* clinical strain and *S. aureus* DSMZ 1104. The highest MIC value (3722.32 mg/kg) was measured for *S. typhimurium* DSMZ 14028.

For GO the lowest MIC value (553 mg/kg) was measured for *S. aureus* clinical strain. A value of 765 mg/kg was recorded for *L. innocua* DSMZ 20649, a value of 1076 mg/kg was obtained for *S. aureus* DSMZ 1104, a value of 1652 mg/kg was obtained for *E. coli* DSMZ 10198, *P.*

fluorescens DSMZ 50091 and *B. cereus* clinical strain, while a value of 4224 mg/kg was detected for *E. coli* clinical strain. The highest MIC value (4480 mg/kg) was measured, similarly to MGO, for *S. typhimurium* DSMZ 14028.
The antimicrobial activity in plate assay of MGO against *E. coli* DSMZ 10198 is reported in figure 14.

Table 12: Minimum Inhibitory Concentration (MIC) of 1,2-dicarbonyl compounds.

Compound/Targeted microorganism	**GO (mg/kg)**	**MGO (mg/kg)**	**3-DG (mg/kg)**
E. coli DSMZ 10198	1652	488	No inhibition observed up to 10000 mg/kg
E. coli clinical strain	4224	1642.2	
S. typhimurium DSMZ 14028	4480	3722.32	
P. fluorescens DSMZ 50091	1652	488	
B. cereus clinical strain	1652	975	
S. aureus DSMZ 1104	1076	975	
S. aureus clinical strain	553	488	
L. innocua DSMZ 20649	765	585	

Fig. 14: Antimicrobial activity of MGO against *E. coli* DSMZ 10198.

5.5 *Influence of media and nutrients on the levels of 1,2-dicarbonyl compounds*

In view of the fact that the dicarbonyl compounds are unstable compounds, they can react with the culture medium used in antimicrobial assays, therefore affecting the MIC values. With the aim to verify this hypothesis, the residual levels of 1,2-dicarbonyl compounds were carried out in liquid antibacterial assays.

The culture medium used to determine the MICs (Tryptic Soy Agar), is rich in the peptone fraction and free amino acids. Additionally, the pH is 7.3, which ensures that all necessary conditions for a reaction between the dicarbonyl compound and an amino group (Maillard reaction) are satisfied. These reactions could compete with the interaction between the dicarbonyl compounds and the bacteria. It was also hypothesised that the agar had a strong propensity to trap dicarbonyl compounds.

For these reasons, liquid assay experiments were designed to evaluate the residual concentration of the studied antimicrobials.

Results of microbial growth (log_{10} CFU/mL) at three different concentration of GO and MGO are reported in table 13. Test tubes containing targeted microorganisms, TSB and GO at MIC concentration showed no microbial growth (nd) after 30 min. At concentration of ½MIC there was no microbial growth after 50 min. Concentration of ¼MIC showed no effect on microbial growth.

Test tubes containing *E. coli*, TSB and MGO at MIC concentration showed no microbial growth after 20 min followed by a temporary increase between 40 and 60

minutes. At concentration of ½MIC and ¼MIC there was no microbial growth after 50 and 60 min, respectively.
In test tubes containing *S. aureus*, TSB and MGO at MIC concentration there was no microbial growth after 50 min. Concentration of ½MIC and ¼MIC showed no effect on microbial growth.

Table 13: Microbial growth (log10 CFU/ml) at three different concentrations of GO and MGO.

Dicarbonyl compound	Sample	Time (min)					
		T10	**T20**	**T30**	**T40**	**T50**	**T60**
GO	*E. coli* control	7.00±0.01d	7.08±0.01d	7.00±0.01b	7.26±0.01a	7.30±0.01b	7.48±0.01a
	E. coli MIC	6.20±0.01g	4.68±0.01h	nd	nd	nd	nd
	E. coli ½ MIC	6.48±0.01f	5.70±0.01g	4.74±0.01e	4.20±0.01e	nd	nd
	E. coli ¼ MIC	6.58±0.01e	6.11±0.01f	5.98±0.01d	5.90±0.01d	5.30±0.01d	4.60±0.01c
	S. aureus control	7.00±0.01d	7.18±0.01b	7.00±0.01b	7.00±0.01b	7.40±0.01a	7.48±0.01a
	S. aureus MIC	7.11±0.01c	6.81±0.01e	nd	nd.	nd.	nd
	S. aureus ½ MIC	7.14±0.01b	7.11±0.01c	6.82±0.01c	6.67±0.01c	nd.	nd
	S. aureus ¼ MIC	7.30±0.01a	7.40±0.01a	7.18±0.01a	7.00±0.01b	6.38±0.01c	6.45±0.01b
MGO	*E. coli* control	7.00±0.01d	7.92±0.01a	7.26±0.01a	7.28±0.01a	7.30±0.01b	7.78±0.01b
	E. coli MIC	3.85±0.01g	nd	nd	3.00±0.01h	3.60±0.01e	nd
	E. coli ½ MIC	5.30±0.01f	3.30±0.01g	4.48±0.01g	3.30±0.01g	nd	nd
	E. coli ¼ MIC	6.26±0.01e	6.30±0.01f	4.81±0.01f	3.90±0.01f	3.00±0.01f	nd
	S. aureus control	7.00±0.01d	7.00±0.01c	7.00±0.01c	7.00±0.01b	7.48±0.01a	7.85±0.01a
	S. aureus MIC	7.23±0.01c	6.63±0.01e	5.11±0.01e	4.20±0.01e	nd	nd
	S. aureus ½ MIC	7.36±0.01a	6.94±0.01d	6.64±0.01d	6.11±0.01d	5.32±0.01d	4.62±0.01d
	S. aureus ¼ MIC	7.30±0.01b	7.23±0.01b	7.08±0.01b	6.98±0.01c	6.75±0.01c	6.46±0.01c

nd: below the detection limit of plating count method; Within time and dicarbonyl compound different letters indicate significant differences at $p < 0.05$ according to Fisher's method.

The residual levels (%) of GO, MGO and 3-DG are reported in table 14, 15 and 16 respectively.
Independently of the presence of bacteria, the degradation of GO in the sterile distilled water seemed to depend on the starting concentration. With concentrations up to 1500 mg/kg there was no degradation. Starting from 2600 mg/kg the GO degraded approximately by 30% after 150 min. When TSB was added to the system, the GO degraded faster but in inverse proportion to the concentration.
In fact, at 270 min, there was 21% of residual GO referred to the starting concentration of 350 mg/kg, 53.51% of the starting concentration of 650 mg/kg, 57.17% of the starting concentration of 1500 mg/kg, 66.95% of the starting concentration of 2600 mg/kg, 72.58% of the starting concentration of 4224 mg/kg and 61.08% of the starting concentration of 8448 mg/kg.
When *S. aureus* DSMZ 1104 or *S. aureus* clinical strain was added to the system, the degradation of GO seemed to depend on time. At the end of the considered exposure time intervals, the residual GO was about 60% for all the three tested concentrations.
Instead, when *E. coli* DSMZ 10198 was added to the system, the degradation of GO seemed to depend on concentration. In fact, at the lowest concentration the degradation of GO started after 60 min, at concentration of 1500 and 2600 mg/kg the degradation started after 150 min. At the end of the considered exposure time intervals the residual GO was about 77% for all the three tested concentrations.
When *E. coli* clinical strain was added to the system, there was no degradation of GO.

Table 14: Residual percentage of GO (mean ± s.d) in different culture conditions and concentrations.

Sample /Time (minutes)	Initial concentration	T0	T30	T60	T90	T150	T210	T240	T270
		% Residual							
H_2O+GO	350 mg/kg	100.0±2.8a	99.7±5.6a	101.0±0.8a	96.9±4.5a	99.0±6.4a	99.3±3.9a	95.5±3.0a	92.6±0.9a
TSB+GO		95.4±3.8a	92.8±7.6b	75.0±1.5b	74.2±0.9b	66.3±1.6b	67.3±1.6b	55.0±2.5b	53.8±1.3b
TSB+GO+*S. aureus* clinical		81.9±2.3b	81.3±1.6c	71.3±4.9b	67.7±1.9b	56.3±4.6c	52.8±7.9b	46.8±4.5c	44.2±6.7c
H_2O+GO	650 mg/kg	100.0±0.8a	98.1±2.7a	101.5±7.3a	97.6±1.6a	85.5±1.6a	93.8±2.8a	89.7±1.6a	92.8±5.2a
TSB+GO		90.7±0.9b	86.2±2.6b	80.6±1.1b	78.3±1.9b	71.1±1.3b	65.3±4.6c	58.0±0.4c	53.5±1.0c
TSB+GO+*S. aureus*1104		87.4±7.4b	82.1±6.6b	84.9±5.2b	78.6±2.9b	68.4±3.3b	63.0±1.7cd	60.2±1.2c	59.0±0.8c
TSB+GO+*S. aureus* clinical		86.6±3.3b	85.1±2.4b	70.6±5.2b	71.9±4.7b	62.2±1.5c	55.8±5.4d	49.4±5.6c	51.1±0.5c
TSB+GO+*E. coli* 10198		100.0±0.6a	94.2±4.6a	78.3±16.0b	79.8±12.5b	69.8±5.4b	78.6±5.0b	74.9±12.1b	78.3±12.3b
H_2O+GO	1500 mg/kg	100.0±13.5a	85.9±7.1ab	118.6±46.5a	83.2±1.9b	83.5±2.6ab	71.0±1.4a	83.3±10.6a	68.3±1.4b
TSB+GO		94.2±1.3ab	86.9±2.0ab	95.1±14.7a	99.9±1.4a	94.8±16.0a	55.3±0.2b	85.9±5.3a	57.2±0.5c
TSB+GO+*S. aureus*1104		101.9±3.7a	97.5±3.4b	131.6±74.4a	82.4±4.8b	79.1±1.2ab	79.1±4.0a	70.4±3.1a	70.5±1.1b
TSB+GO+*S. aureus* clinical		87.1±6.6b	103.3±9.6b	75.3±3.5a	86.4±20.0c	78.0±12.5b	77.7±27.0b	67.2±10.1b	66.0±9.7c

TSB+GO+*E. coli* 10198		100.0±0.90a	78.7±14.9b	92.6±8.4a	85.9±5.8b	71.1±27.0ab	73.0±10.6a	78.3±13.9a	87.7±8.6a
H_2O+GO	2600 mg/kg	100.0±2.8a	101.9±5.0a	106.0±3.0a	84.8±6.1a	76.3±3.2a	78.0±3.4b	75.6±0.4a	80.9±3.2b
TSB+GO		92.6±2.2b	86.2±2.7a	96.1±10.3a	83.7±3.2a	72.0±0.6a	90.5±9.5a	71.4±8.5a	66.9±7.3b
TSB+GO+*S. aureus*1104		79.2±6.4c	81.2 ±0.7a	77.6±2.0a	77.6±0.1a	68.4±4.9a	68.8±2.8b	68.4±2.0a	63.7±1.8b
TSB+GO+*E. coli* 10198		100.0±0.9a	87.8±18.3a	89.65±15.0a	80.0±17.0a	78.0±12.0a	49.7±4.3c	76.3±23.4a	77.6±13.6b
TSB+GO+*E. coli* clinical		100.0±0.9a	91.2±18.5a	92.8±34.7a	100.0±17.7a	106.1±46.5a	94.7±5.6a	75.6±17.7a	101.7±15.7a
H_2O+GO	4224 mg/kg	100.0±5.5a	94.6±1.3a	73.3±3.4b	69.7±2.9a	81.6±7.4b	76.6±2.8ab	73.7±0.5a	77.8±0.2b
TSB+GO		82.6±2.2b	82.1±0.3a	95.1±14.6ab	87.3±9.7a	65.3±0.7c	66.0±2.9a	66.6±3.5a	72.6±3.1b
TSB+GO+*E. coli* clinical		100.0±0.0a	100.4±27.5a	109.8±12.3a	88.7±24.0a	99.1±8.9a	84.1±10.2a	81.7±13.0a	93.9±9.0a
H_2O+GO	8448 mg/kg	100.0±8.2a	77.5±5.3b	84.9±3.3b	59.8±8.5c	57.4±5.7c	58.4±1.7c	62.4±2.3b	67.3±0.5b
TSB+GO		87.9±7.0b	89.5±6.9a	74.4±4.6c	71.2±3.4b	71.8±1.3b	65.2±2.5b	61.6±2.6b	61.1±3.3c
TSB+GO+*E. coli* clinical		100.0±0.0a	92.0±5.2a	96.8±1.9a	95.2±1.2a	93.9±2.0a	94.9±1.3a	97.9±1.4a	90.3±2.1a

Different letters indicate significant differences at $p \leq 0.05$ within sample and time according to Fisher's method.

Although MGO was stable in sterile distilled water, it degraded when TSB was added to the system, independently of the starting concentration. The residual level after270 min was approximately 21% for all tested concentrations, except when MGO was added at 2000 and 3443 mg/kg for which the residual levels were approximately 35% and 43% respectively.

A similar behavior was observed when *E. coli* DSMZ 10198 was added to the system except for concentration of 500 and 100 mg/kg where degradation was greater. At the end of the considered exposure time intervals the residual MGO was about 23% for all the three tested concentrations.

When *E. coli* clinical strain was added to the system, the degradation seemed to depend on concentration. In fact, for both lowest and highest concentrations, the degradation of MGO started after 150 min; at concentration of 2000 mg/kg the degradation of MGO started after 30 min. At the end of the considered exposure time intervals the residual MGO was about 45%, 28% and 39% for the three tested concentrations, respectively.

When *S. aureus* DSMZ 1104 or *S. aureus* clinical strain was added to the system, the degradation of MGO was independent of time and concentration. The degradation of MGO started immediately, similarly to TSB alone. At the end of the considered exposure time intervals the residual MGO was about 22% for all the three tested concentrations.

Table 15: Residual percentage of MGO (mean ± s.d) in different culture conditions and concentrations.

Sample /Time (minutes)	Initial concentration	T0	T30	T60	T90	T150	T210	T240	T270
		% Residual							
H_2O+MGO	250 mg/kg	100.0±2.8b	99.7±5.6a	101.0±0.8a	97.0±4.5a	99.0±6.4a	99.3±4.0a	100.5±2.3a	104.3±2.7a
TSB+MGO		111.6±2.1a	92.8±7.6a	75.0±1.5b	60.2±0.9b	42.7±2.5b	27.3±0.6b	22.9±0.7b	21.2±0.5b
TSB+MGO+*S. aureus* clinical		86.8±5.8c	70.2±6.8b	58.2±4.9c	43.5±2.1c	30.1±3.1c	20.68±2.9c	19.2±4.1b	17.2±3.4b
TSB+MGO+*E. coli* 10198		100.0±0.0b	96.6±10.4a	67.73±6.7b	56.5±6.9b	40.5±5.9b	16.5±0.4c	21.4±1.8b	22.3±3.7b
H_2O+MGO	500 mg/kg	100.0±1.7a	101.8±0.6a	102.5±0.9a	80.8±0.5a	83.2±4.6a	79.4±2.0a	85.3±3.8a	86.3±8.0a
TSB+MGO		94.2±1.2a	88.0±2.8b	69.8±0.6b	55.7±4.0b	40.2±1.0bc	24.5±0.3b	20.0±0.4c	16.3±0.4b
TSB+MGO+*S. aureus*1104		87.0±3.8b	72.6±7.7c	57.0±5.5c	44.8±5.2c	29.7±5.8d	22.6±1.6b	18.9±1.0c	18.0±0.7b
TSB+MGO+ *S. aureus* clinical		83.9±7.4b	75.0±3.6c	61.6±4.0c	51.7±7.5bc	32.8±6.1cd	22.6±3.9b	18.5±2.1c	16.2±2.2b
TSB+MGO+*E. coli* 10198		100.0±0.0a	82.7±10.6bc	49.0±33.8b	23.4±2.2d	47.2±6.4b	15.3±4.0c	26.8±1.8b	23.4±7.9b
H_2O+MGO	850 mg/kg	100.0±5.5a	95.4±2.0a	101.1±0.3a	99.4±4.5a	93.6±2.8a	102.3±0.4a	95.2±1.8a	98.2±3.5a
TSB+MGO		85.2±2.2b	83.2±3.7a	77.2±9.6a	62.7±3.1b	45.7±7.3b	39.5±6.8b	27.20±0.2c	21.9±0.3c
TSB+MGO+*E. coli* clinical		100.0±0.1a	105.8±35.8a	89.8±23.9a	79.4±26.3ab	51.2±18.4b	51.9±16.4b	54.1±13.6b	45.3±14.7b

H_2O+MGO	1000 mg/kg	100.0±5.5a	95.054±2.0a	101.1±0.3a	99.4±4.5a	93.6±2.8a	102.3±0.3a	95.2±1.8a	98.2±3.5a
TSB+MGO		85.2±2.2b	83.2±3.7b	77.2±9.6b	62.7±3.1b	45.7±7.3b	39.5±6.8b	27.2±0.2bc	21.9±0.3b
TSB+MGO+*S. aureus*1104		75.5±7.9c	62.1±10.0c	55.3±4.1c	44.9±1.5d	32.9±3.7c	25.9±2.1d	22.2±2.2d	21.3±1.2b
TSB+MGO+ *S. aureus* clinical		84.2±5.7bc	79.0±1.0b	56.5±5.2c	54.6±3.3c	35.4±1.5c	25.4±4.0d	24.5±3.1cd	20.6±5.3b
TSB+MGO+*E. coli* 10198		100.0±0.0a	88.5±8.1ab	65.5±13.8c	63.5±2.6b	32.7±2.1c	32.6±0.6c	28.5±0.6b	23.0±2.1b
H_2O+MGO	2000 mg/kg	92.6±0.7ab	87.8±0.6a	91.8±6.2a	86.8±1.4a	80.5±4.2a	80.0±6.5a	92.4±6.0a	83.9±1.9a
TSB+MGO		91.3±10.5ab	87.2±1.6a	70.9±4.9b	65.1±0.2b	45.7±5.4b	36.3±0.9b	37.2±2.6b	35.0±0.0b
TSB+MGO+*S. aureus*1104		83.9±7.4b	77.4±2.0a	64.8±9.5b	46.1±14.8c	47.6±4.4b	35.3±1.4b	30.6±1.7bc	30.9±1.4c
TSB+MGO+*E. coli* clinical		100.0±0.0a	105.3±72.5a	60.4±6.4b	55.7±7.6bc	42. 6±5.9b	35.5±2.8b	27.8±2.1c	28.0±2.4c
H_2O+MGO	3443 mg/kg	100.0±3.6a	77.5±2.0a	94.8±2.0a	86.17±0.1a	94.4±6.3a	88.2±6.2a	86.0±2.7a	88.6±6.5a
TSB+MGO		77.6±8.2b	86.6±0.6a	63.9±3.4c	61.7±5.1b	51.7±3.5b	46.6±2.3b	44.6±2.6b	43.1±0.1b
TSB+MGO+*E. coli* clinical		100.0±0.0a	61.5±36.5a	79.7±2.1b	66.7±8.5a	47.6±9.4b	46.7±7.0b	52.9±6.4b	39.3±4.9b

Different letters indicate significant differences at $p \leq 0.05$ within sample and time according to Fisher's method.

3-DG was stable in the sterile distilled water, independently of concentration. When TSB was added to the system, the 3-DG degraded faster but in inverse proportion to the starting concentration. In fact, at the concentrations of 537 and 1240 mg/kg there was approximately 22% residual concentration after 24 h, instead at concentration of 1930 mg/kg there was approximately 47%. The addition of bacteria to the system decreased the consumption of 3-DG except for the concentration of 1930 mg/kg.

Table 16: Residual percentage of 3-DG (mean ± s.d) in different culture conditions and concentrations.

Sample/ Time (hours)	Initial concentration	H_2O+3-DG	TSB+3-DG	TSB+3-DG+*S. aureus* 11044	TSB+3-DG+*E. coli* 10198
T0		100.0±4.5a	100.0±0.5a	100.0±2.1a	100.0±18.8a
T2		97.7±3.0ab	87.0±191b	98.0±1.6ab	107.0±2.5b
T4		95.6±5.3a	78.7±4.5b	103.7±1.7a	106.5±9.7a
T6		95.6±6.5a	75.7±7.1b	94.2±15.3a	93.8±3.2a
T8		91.1±7.2ab	76.6±2.2c	95.8±3.3a	86.8±2.6b
T10		93.2±5.2a	74.5±2.8b	95.2±2.5a	80.3±2.9b
T12	537 mg/kg	91.1±6.9a	73.6±3.7b	86.7±0.7a	76.0±3.1b
T14		67.8±6.6a	35.6±5.4b	61.1±15.7a	71.6±1.6a
T16		62.1±9.0a	33.3±7.4b	49.4±2.4ab	59.2±15.4a
T18		74.4±11.9a	29.5±6.4c	48.0±0.6b	44.9±0.5b
T20		72.9±10.1a	29.1±8.1c	42.8±0.3b	41.7±1.3b
T22		71.0±10.6a	40.5±19.8b	65.5±18.7ab	43.6±6.6ab
T24		69.7±11.3a	22.2±5.7c	42.1±1.3b	58.7±0.4a
T0		100.0±0.9a	100.0±9.8a	100.0±6.8a	100.0±4.7a
T2		98.4±1.9a	92.5±4.3b	99.0±0.9a	95.9±2.2ab
T4		95.5±4.7a	83.6±2.0b	100.0±1.9a	94.7±3.1a
T6		95.4±6.4a	78.7±5.3b	94.9±2.8a	79.5±2.9b
T8		94.7±5.8a	77.6±2.3b	87.6±2.4a	68.5±4.0c
T10		108.4±29.7a	74.2±2.1b	89.8±2.1ab	64.1±2.0b
T12	1240 mg/kg	93.1±6.1a	72.0±1.7c	82.4±0.2b	62.4±1.9d
T14		78.4±9.3a	54.7±5.4b	52.4±2.4b	53.9±0.9b
T16		77.4±10.6a	38.5±6.2c	57.7±14.5b	42.0±3.8bc
T18		92.0±12.2a	35.0±9.5b	56.2±19.9b	34.2±0.3b
T20		90.9±13.4a	33.1±6.9b	49.2±11.8b	31.8±5.0b
T22		87.7±12.1a	30.0±7.8c	54.6±16.0b	32.7±2.6c
T24		89.5±9.1a	24.1±7.5b	32.3±12.3b	36.8±0.7b
T0		100.0±2.3a	100.0±12.9a	100.0±1.8a	100.0±2.7a
T2		97.7±1.5a	89.0±4.8b	91.4±0.8b	92.5±2.8ab
T4		96.6±1.1a	86.3±3.0b	98.6±1.6a	89.5±1.5b
T6		96.1±3.5a	83.9±4.1ab	83.3±19.1ab	74.1±3.0b
T8		94.9±3.4a	79.5±3.2b	84.5±2.6b	63.3±0.3c
T10		96.8±1.5a	79.3±2.3b	82.0±3.0b	63.4±1.3c
T12	1930mg/kg	92.9±4.2a	77.2±3.3b	79.0±1.3b	60.3±2.0c
T14		83.0±12.0a	64.8±23.4a	81.0±43.5a	54.9±1.1a
T16		88.4±18.8a	56.4±3.2b	53.3±1.0bc	37.9±0.9c
T18		97.5±5.8a	52.5±9.7b	64.5±14.0b	34.3±4.2c
T20		98.4±13.0a	48.6±4.5b	46.6±2.0b	32.2±1.0c
T22		95.1±14.0a	46.9±6.8b	48.5±0.9b	29.9±1.4c
T24		93.9±14.4a	47.5±8.9b	39.0±3.8b	33.8±1.4b

Different letters indicate significant differences at $p \leq 0.05$ within sample and time according to Fisher's method.

These data confirmed the hypothesis that the dicarbonyl compounds are degraded very quickly upon the introduction of bacteria and, especially, of TSB. This is particularly true for MGO. In fact, while MGO and GO are more stable in sterile distilled water, MGO is consumed much faster than GO. This is also evident in the presence of TSB and TSB plus bacteria.

6 Discussion

6.1 1,2-dicarbonyl compounds in Italian food

In this study, the levels of 1,2-dicarbonyl compounds in commercial Italian food were determined. As reported in the introduction, few studies have been conducted on 1,2-dicarbonyls on commercial Italian food.

In this work, commercial biscuits contained GO in the range of nd-13.76 mg/kg, MGO in the range of nd–76.98 mg/kg and 3-DG in the range of nd-113.83. Degen *et al*., (2012) found in commercial cookies 8.5–385 mg/kg of 3-DG and 1.6–68 of MGO. Arribas-Lorenzo and Morales (2010), found a range of 4.8–26.0 mg/kg of GO and a range of 3.7–81.4 mg/kg of MGO.

The data above demonstrates that the ingredients deeply modifies the levels of dicarbonyl compounds. Biscuit samples with the highest concentration of 3-DG, reported the presence of glucose−fructose syrup as ingredients in the label, while biscuit samples with the lowest concentration did not have glucose−fructose syrup as ingredients.

The different levels of GO and MGO can be explained by the different types of sugar used. An important factor is the temperature and the cooking times. These, being commercial samples, are not known.

Nagao *et al*., (1986), found in bread 0.79 and 0.3 mg/kg of MGO and GO respectively. Degen *et al*., (2012), found a range of 13–619 mg/kg of 3-DG and a range of nd-28 mg/kg for MGO.

The average levels found in this study were about 21.8

mg/kg for 3-DG, 8.3 mg/kg for GO and 5.9 mg/kg for MGO. The bread samples with the highest amount of dicarbonyl compounds were the industrial one. This means that the key factor in this case is only the temperature and the time of baking. In fact, the recipes of these samples do not contain glucose or invert sugar syrup.

For jam and marmalade, the mean level of 3-DG was about 47 mg/kg except in two samples in which the levels of 3-DG were about 87 and 29 mg/kg. For MGO, only one sample had a high level (195.38 mg/kg); the level of GO is about 9 mg/kg. The differences between these value could result from a different heat treatment and a different percentage of sugar used during processing.

The average values of dicarbonyl compounds in honey samples found in this study, were slightly higher compared with reported literature data (Weigel *et al*., 2004; Arena *et al*., 2011).

For fruit juice and fruit based drink samples, the average value is higher than those reported in other studies (Degen *et al*., 2012; Weerawatanakorn, 2013). The concentrations of dicarbonyl compounds, found in this study, were similar in all samples even if the composition of fruit juice was different. 3-DG was the main dicarbonyl compounds, followed by MGO and GO. Contrary to the literature, (Hayashi and Shibamoto, 1985; Daglia *et al*., 2007; Nagao *et al*, 1986; Wang *et al*., 2010; Papetti *et al*., 2014) no 1,2-dicarbonyl compounds were detected in coffee prepared with moka.

Also in pasta and milk samples no 1,2-dicarbonyl compounds were detected.

6.2 Dietary theoretical intake

In this study, for the first time, the dietary theoretical intake with a tds-like investigations of dicarbonyl compounds was assessed.

The data demonstrated that infant's category and children's category assumes the highest level of all three dicarbonyl compounds.

For these categories, the medium intake of MGO was of 0.132 mg/kg body weight/day, for 95th was of 1.079 mg/kg body weight/day and for 99th was of 1.777 mg/kg body weight/day. For south & islands the intake for this category was of 0.122 mg/kg body weight/day for medium, 0.932 mg/kg body weight/day for 95th and 1.844 mg/kg body weight/day for 99th.

Committee on toxicity of chemicals in food, consumer products and the environment (COT, 2009), reports that no overt signs of toxicity were seen in short-term studies in adult mice, rats and dogs dosed orally by gavage with MGO at 1000 mg/kg body weight/day. An 18-week study in rats indicated effects on blood pressure and clinical chemistry at doses in the region of 500 mg/kg body weight/day.

Furthermore, using the occurrence literature data, COT (2009) has estimated an intake of 1.3 mg/kg body weight/day and 3.9 mg/kg body weight/day for mean and high level adult consumers, respectively. For infants and children COT (2009) has estimated an intake of 7.7 mg/kg body weight/day and 22.8 mg/kg body weight/day, for mean and high level consumers respectively.

The intake calculated for infants and children in this study is lower of intake estimated by COT (2009).

For infant (0-2 years) and children (3-9 years), the medium intake of GO was very low (about 0.054 mg/kg body weight/day), for 95th was of 0.414 and 0.328 mg/kg body weight/day respectively, and for 99th was of 0.615 and 0.699 mg/kg body weight/day respectively.About the intake by age, for south & islands data show that the intake for 99th was of 0.78 and 0.733 mg/kg body weight/day for the same age category.

WHO (2004) presupposes on the basis of studies in rats, a tolerable intake of about 0.2 mg/kg body weight/day for lifetime oral exposure to GO.

In this case only 95th and 99th ingest twice and three times more than suggested tolerable intake.

Even in the case of 3-DG the category that assumes the highest level of 3-DG was infants (0-2 years) specially for 99th (31.089 mg/kg body weight/day).

The medium intake for children (3–9 years) was of 0.329 mg/kg body weight/day.

Even for south & island the category that assumes the highest level of 3-DG was infants (0-2 years) specially for 99th (31.089 mg/kg body weight/day).

No data are present in literature about the tolerable intake of 3-DG.

However, a further intake of all three dicarbonyl compounds might come from other fermented products (dairy products or vegetables), from other popular roasted or fried products (meat, fish, mushrooms, sausages), or from additional bakery products.

The dietary theoretical intake assessed in this study has not been previously reported.

6.3 Study of formation/degradation of dicarbonyl compounds using food model systems

In this study, for the first time, the role of single sugars (sucrose, glucose and fructose) and single fat (margarine, olive oil and seeds oil) on the development of 3-DG, GO and MGO was assessed.

Time, temperature and sugar type had strong effects on the measured parameters. Substitution of sucrose with fructose or glucose affected the production of the three dicarbonyl compounds particularly 3-DG.

In the glucose samples, at 150°C, 3-DG reached its highest level at 3942 mg/kg after 25 min of baking. In the fructose samples, at 190°C after 25 min of baking, GO an MGO reached its highest level at 3014 mg/kg and 503 mg/kg respectively.

Also the substitution of butter with margarine, olive oil and seeds oil affected the production of the three dicarbonyl compounds: at the lowest baking temperatures for 3-DG and MGO, for all the dicarbonyl compounds at 190°C. Moreover, olive oil and seeds oil mostly increased the level of dicarbonyl compounds. In the olive oil samples, at 170°C, both 3-DG and MGO reached their highest level at 1007 mg/kg and 305 mg/kg respectively, after 25 min of baking.

For GO the temperature affected its formation. Until 170°C there are no substantial differences between the sample. At 190°C the highest levels were found for control sample (243 mg/kg).

In the fructose samples, at 190°C after 25 min of baking, GO an MGO reached its highest level at 3014 mg/kg and

503 mg/kg respectively.
There are no data available in the literature on the role of single sugars and single fat on formation of dicarbonyl compounds.

6.4 *Antimicrobial assay and determination of minimum inhibitory concentrations (MIC)*

In this study, the antibacterial activity of dicarbonyl compounds against pathogenic and food spoilage bacteria was assessed.
The data demonstrated antibacterial activity for GO and MGO. 3-DG did not exhibit any antibacterial activity. The activity was dependent upon the bacterial species and strain tested.
In agreement with data in the literature, MGO exhibited higher antibacterial activity than GO. For MGO the lowest MIC value (488 mg/kg) was measured for: *E. coli* DSMZ 10198, *P. fluorescens* DSMZ 50091 and *S. aureus* clinical strain. The highest MIC value (3722.32 mg/kg) was recorded for *S. typhimurium* DSMZ 14028.
For GO the lowest MIC value (553 mg/kg) was measured for *S. aureus* clinical strain, while the highest MIC value (4480 mg/kg) was measured for *E. coli* clinical strain.
Although there is not much information describing the activity of MGO against *Pseudomonas* spp., very recently it has been found that MGO inhibits the growth of multidrug-resistant *Pseudomonas aeruginosa* at concentrations of 1.7–7.1 mM (Hayashi *et al*., 2014).
The level of antibacterial activity, in terms of MIC values, of both GO and MGO is strongly dependent on the bacterial

species and strain, as well as on the nutrients present in the assay system.
The antibacterial activity of GO and MGO *against L. innocua, P. fluorescens, S. typhimurium* and *B. cereus* demonstrated in this study has not been previously reported. The data in Table 12 demonstrate that these compounds can effectively inhibit the growth of these bacterial strains.

6.5 Influence of media and nutrients on the levels of 1,2-dicarbonyl compounds

In this study, for the first time, the influence of media and nutrients on the level of 1,2-dicarbonyl compounds was assessed.
The results highlighted that the nutrient medium reacts quickly with both GO and MGO. In any case, regardless of the type of dicarbonyl compound, its initial concentration and the presence of bacteria, the residual levels of dicarbonyl compounds were below or near the detection limit.
These data confirmed the hypothesis that the dicarbonyl compounds are degraded very quickly upon the introduction of bacteria, and especially of TSB. This is particularly true for MGO. In fact, while all 1,2-dicarbonyl compounds studied are more stable in sterile distilled water, MGO is consumed much faster. This is also true in the presence of TSB and TSB with bacteria.
The influence of culture media demonstrated in this study has not yet been reported.

7 Conclusion

Research activity carried out during the present Ph.D. study had a common thread: to increase knowledge on 1,2-dicarbonyl compounds.

In this work, for the first time, a survey of 1,2-dicarbonyl compounds in Italian food with a TDS-like investigation was carried out. The results obtained by such survey showed that the predominant 1,2-dicarbonyl compound is 3-deoxyglucosone with a wide range of concentration.

The food item with the highest level of 3-DG was honey (703.12 mg/kg), the food item with the highest level of GO was bread (23.38 mg/kg), the food item with the highest level of MGO (195.38 mg/kg) was marmalade.

In addition, the results obtained from the survey demonstrated that the ingredients and processing deeply modify the levels of dicarbonyl compounds.

The estimation on dietary intake, with a Total Diet Study-like investigation, highlight that the ingestion with foods, for all three 1,2-dicarbonyl compounds studied, is high especially for infants (0-2 years) and children (3-9 years) both for 95th and 99th percentiles.

For MGO the intake is lower than suggested tolerable intake by COT (2009).

The intake of GO at 95th and 99th percentiles is twice and three times more than the suggested tolerable intake.

For 3-DG no data are present in literature about the tolerable intake of 3-DG.

However, a further intake of all three dicarbonyl compounds might come from other fermented products (dairy products or vegetables), from other popular roasted or fried products

(meat, fish, mushrooms, sausages), or from additional bakery products. So the intake may be higher than that calculated in this study.
The results obtained with the model systems show that time, temperature and ingredients have a strong influence on the formation of the compounds and that it is possible reduce the level of 1,2-dicarbonyl compounds.
The results of antimicrobial assays lead us to conclude that dicarbonyl compounds, especially GO and MGO, could have a role in the microbial stability of foods, although food composition may strongly influence their availability to act as antimicrobials.
In addition, when the targeted bacteria are introduced into the antimicrobial assay system, the 1,2-dicarbonyl compounds are degraded very quickly.
The results obtained outline a framework of knowledge that is a prelude to subsequent developments.
In fact, the data obtained in this research work will constitute a starting point for: designing an experimental study with food of other countries to know their dietary intake, with the purpose, if necessary, to regulate the levels of 1,2-dicarbonyl compounds in food; designing an experimental study on the effects of dicarbonyl compounds on the human gut microbiota with the purpose to monitor the evolution of the tested compounds after digestion; designing an experimental study to know the mechanism of action of these molecules to act as antimicrobials with the purpose to suggest their possible use as food preservatives.

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