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PAPER

A TENTATIVE MATHEMATICAL MODEL TO DESCRIBE THE EVOLUTION OF THE AEROBIC AND ANAEROBIC RESPIRATION RATES OF GOLDEN DELICIOUS APPLES WITH TEMPERATURE, PO_2 AND PCO_2

UN MODELLO MATEMATICO PER DESCRIVERE L'EVOLUZIONE DELLA RESPIRAZIONE AEROBIA IN MELE GOLDEN DELICIOUS AL VARIARE DELLA TEMPERATURA E DELLA COMPOSIZIONE GASSOSA (PO_2 E PCO_2)

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ABSTRACT

A mathematical model describing the evolution of aerobic and anaerobic respiration of Golden Delicious apples stored in refrigerated and controlled atmosphere, as a function of temperature, PO_2 and PCO_2 is presented and discussed. This paper reports: the hypothesis utilised to formulate the model; the kinetic equations used; the identification of the best values to be assigned to the functional parameters of the mathematical equations introduced; the model validation carried out

RIASSUNTO

Viene analizzato un modello matematico che descrive l'evoluzione della respirazione aerobia e anaerobia di mele Golden Delicious in funzione della temperatura e della composizione dell'atmosfera di conservazione (PO_2 e PCO_2). Oltre alle ipotesi introdotte nella sua formulazione, viene presentata e discussa la struttura matematica del modello e le equazioni cinetiche impiegate. Mentre per l'identificazione dei migliori valori numerici da attribuire alle costanti funzionali del modello sono sta-

- Key words: aerobic respiration, alcoholic fermentation, kinetics, *Malus domestica*, mathematical model -

by using an independent experimental data set. Despite the low number of functional parameters utilised, this model appears able to suitably describe the evolution of aerobic respiration and alcoholic fermentation rates of Golden Delicious apples stored at different temperatures, PO_2 and PCO_2.

ti utilizzati i valori sperimentali relativi alle velocità della respirazione aerobia ed anaerobia di frutti mantenuti in 80 differenti condizioni di conservazione, nella validazione del modello è stato impiegato un set di dati diverso ottenuto misurando l'evoluzione, provocata dai frutti conservati, della composizione gassosa (PO_2 e PCO_2) dell'atmosfera di conservazione. Sebbene il numero dei parametri previsti dal modello sia relativamente contenuto, i valori calcolati non sembrano discostarsi in modo significativo da quelli sperimentali.

INTRODUCTION

The control of respiratory activity highly contributes to maintaining the quality of the stored product and to prolonging its shelf life (HERTOG *et al.*, 2001; JOHNSTON *et al.*, 2001; DE SMEDT *et al.*, 2002; PETRACEK *et al.,* 2002). Controlled atmosphere (CA) storage and modified atmosphere packaging (MAP) would greatly profit from the application of a mathematical model able to predict the evolution of both aerobic respiration and alcoholic fermentation as a function of storage conditions (SALTVEIT, 2003).

There have been many different approaches used to identify a suitable respiration model and in particular to describe the effect of PO_2 and PCO_2 on aerobic metabolism. Some of these attempts are based on empirical or semi-empirical equations characterised by a limited range of possible utilisations (JURIN and KAREL, 1963; HENIG and GILBERT, 1975; HAYAKAWA *et al.*, 1975; DEILY and RIZVI, 1981; BEAUDRY *et al.*, 1992).

Others have used Langmuir's adsorption theory to describe the O_2 consumption rate in many kinds of fresh products, but the model obtained only describes the aerobic respiration of products maintained in an atmosphere having a negligible amount of CO_2 (MAKINO *et al.*, 1996a). Successively, the same authors improved their model to include the effect of CO_2 on aerobic respiration by the addition of an empirical equation (MAKINO *et al.*, 1996b).

The actual mechanism describing how PO_2 from the surrounding atmosphere acts on the aerobic respiration rate is not yet clear (VAN DONGEN *et al.*, 2003; 2004). Most of the time an enzymatic approach using the Michaelis-Menten equation has been applied. In some cases the Michaelis-Menten equation was modified to improve its applicability (CHEVILLOTTE, 1973; BANKS *et al.*, 1989; ANDRICH *et al.*, 1991; LEE *et al.*, 1991; CAMERON *et al.*, 1994; HERTOG *et al.*, 1998; PEPPELENBOS *et al.*, 1996a). In fact, PEPPELENBOS and VAN'T LEVEN (1996b), changed this equation to describe the inhibitory effect induced by CO_2 on aerobic respiration. Successively, they used the same approach to describe the inhibition due to O_2 on the CO_2 production rate during alcoholic fermentation (PEPPELENBOS and VAN'T LEVEN, 1996b; PEPPELENBOS *et al.*, 1998). Eventually, they succeeded in formulating a mathematical model to describe the ev-

olution of aerobic and anaerobic respiration as a function of temperature, PO_2 and PCO_2 (HERTOG *et al.*, 1998). According to this approach, O_2 would be able to inhibit alcoholic fermentation, while an increase in CO_2 in the surrounding atmosphere would reduce the rates of both aerobic respiration and alcoholic fermentation. So CO_2 would be able to inhibit two different enzymes, both present after glycolysis and, respectively, during the pathway connected with pyruvate transformation to give CO_2 and H_2O, and during alcoholic fermentation.

On the other hand, KERBEL *et al.* (1990) showed that phospho-fructo-kinase (PFK), an enzyme involved in glycolysis, is inhibited by a high value of PCO_2 so as to make the phosphorilation of fructo-6-phosphate a possible rate determining step of glycolysis and perhaps of the whole aerobic respiration process.

On the basis of these considerations, which seem to be consistent with the experimental results obtained during this research, showing that both aerobic respiration and alcoholic fermentation appear to be similarly affected by an increase in PCO_2 (ANDRICH *et al.*, 1994a), a new kinetic approach was adopted and an alternative mathematical model hypothesised.

This model is based on the following hypothesis:

a) the rate determining step of the whole respiratory pathway could be alternatively situated, as a function of the operating conditions adopted (temperature, PO_2 and PCO_2) during glycolysis or during the following oxidation of pyruvate to give CO_2 and H_2O.

In particular an enzyme involved in glycolysis (for example PFK) was assumed to be inhibited by CO_2, so as to make this enzymatic transformation (phosphorilation of fructo-6-phosphate to give fructo-1,6-diphosphate) the rate determining step of the whole respiration process when high values of PCO_2 are present in the storage atmosphere.

In contrast, when an increase in PO_2 in the storage atmosphere increases respiration, the rate determining step would be situated during the last part of aerobic respiration and in particular during pyruvate oxidation (BURTON, 1978; KERBEL *et al.*, 1990).

b) As, from an energetic point of view, pyruvate oxidation is largely more favoured than its transformation to ethanol and CO_2 (18 units of ATP produced per unit of pyruvate transformed by aerobic transformation, versus only one ATP unit which can be obtained by alcoholic fermentation), alcoholic fermentation was supposed to take place only when the PO_2 present in the surrounding atmosphere was not sufficient to ensure the complete transformation, by aerobic respiration, of all the pyruvate produced by glycolysis.

The experimental values of aerobic and anaerobic respiration rates of Golden Delicious apples stored in 80 different conditions (4 temperatures, 4 PO_2 and 5 PCO_2) were then elaborated by a commercial statistical program (BUZZI FERRARIS and MANCA, 1996) to identify the best values to be assigned to the functional parameters involved in the model. A different set of experimental data was successively utilised to validate the model.

MATERIALS AND METHODS

Measuring the gas exchange rates as a function of the working conditions adopted (PO_2, CO_2 and temperature)

The experimental runs were carried out using an automatic cell designed and developed at the Dipartimento di Chimica e Biotecnologie Agrarie of University of Pisa. This apparatus is able to evaluate simultaneously the rates of CO_2 produced (r_{CO_2}) and of O_2 consumed (r_{O_2}) by the fruit stored at the desired temperature (± 1°C) and the gas composition of the storage atmosphere.

To ensure a constant gas composition inside the storage room, the amount of CO_2 produced by the fruit was continuously and automatically monitored by a gas chromatograph. The excess CO_2 produced by the fruit was then removed by traps (aqueous $Ba(OH)_2$ solution) and automatically replaced by an equivalent volume of pure O_2 (ANDRICH *et al.*, 1994b). While the amount of O_2 replenished gave a measure of the aerobic respiration rate ($r_{ar} = r_{O_2}$), the difference between the rates of CO_2 produced and O_2 consumed allowed the calculation of the alcoholic fermentation rate ($r_{af} = r_{CO_2} - r_{O_2}$).

The experimental runs were carried out using commercial Golden Delicious apples. The handling of the fruit, the experimental protocol, the layout of the plant as well as the methodology applied to carry out the automatic monitoring of the experimental runs were reported by ANDRICH *et al.* (1994b). Moreover the experimental data was statistically analyzed according to the procedure reported by ANDRICH *et al.* (1989; 1990).

Estimation of parameters

The identification of the best values to be assigned to model parameters was carried out using a commercial statistical program (BURENL). This statistical program is able to identify in a space of j-dimensions (where j is equal to the number of model parameters) the minimum of function F which is given by the sum of squares of differences occurring among experimental ($Y_{i,exper.}$) and calculated ($Y_{i,calc.}$) values:

$$F = \sum_{i=1}^{N} (Y_{i,calc.} - Y_{i,exper.})^2 \; ; \; N = \text{total number of experimental determinations}$$

The identification of the minimum of this equation is carried out by using different mathematical algorithms and in particular the Gauss-Newton method which utilises the linearization in the Taylor series of the proposed mathematical model. The values assumed by model parameters at the minimum of the "F" function represent the desired best data set (BUZZI FERRARIS and MANCA, 1996).

Model fitness

The mean square error (MSE) connected to the differences occurring between experimental and calculated data related to the PO_2 and PCO_2 evolution inside a cell where the Golden Delicious apples were stored was evaluated according to the following mathematical expression:

$$MSE = \sum_{i=1}^{N} (Y_{i,exper.} - Y_{i,calc.})^2 / N$$

where:

$Y_{i,exper.}$ = a generic experimental measure (i) of PO_2 or PCO_2;
$Y_{i,calc.}$ = corresponding calculated value (i) of PO_2 or PCO_2;
N = total number of experimental determinations.

while the percentage of variance (VAR%) was calculated according to the following expression:

$$VAR\% = (MSE)^{0.5} \cdot 100 \; / \; MEAN$$

$$\text{being } MEAN = \left(\sum_{i=1}^{N} Y_{i,exper}\right) / N$$

MODEL DEVELOPMENT

The evolution of aerobic respiration rate with PO_2

According to the hypothesis introduced, an enzymatic transformation involved in the oxidation of pyruvate (BURTON, 1978) was assumed to be the rate determining step of the respiratory process when PO_2 represents the limiting factor of the whole aerobic respiration (an increase of PO_2 also determines that of

r_{ar}). As extensively reported in the literature (CHEVILLOTTE, 1973; BANKS *et al.*, 1989; ANDRICH *et al.*, 1991; LEE *et al.*, 1991; CAMERON *et al.*, 1994; PEPPELENBOS *et al.*, 1996a; HERTOG *et al.*, 1998), the Henry-Michaelis-Menten equation was used to describe the evolution of aerobic respiration with PO_2:

$$r_{ar} = r_{ar,max} \cdot [O_2] / (K_M + [O_2]) \quad (1)$$

where:

$r_{ar,max}$ = the maximum value of the aerobic respiration rate at the experimental conditions adopted ($mol \cdot kg^{-1} \cdot s^{-1}$);

$[O_2]$ = the amount of O_2 dissolved in the cellular solution ($mol \cdot kg^{-1}$);

K_M = the Michaelis constant ($mol \cdot kg^{-1}$).

As the resistance to O_2 diffusion due to apple skin is very reduced, the O_2 mass transfer rate between surrounding atmosphere and fruit cells appears to be significantly faster than the aerobic respiration rate. So the concentration of O_2 dissolved in the cellular solution can be assumed to be in equilibrium with the PO_2 of the surrounding atmosphere (ANDRICH *et al.*, 1989; PEPPELENBOS *et al.*, 1996a):

$$K_{sat,O_2} = [O_2] / PO_2$$

And the following relationship can be obtained:

$$[O_2] = K_{sat,O_2} \cdot PO_2 \quad (2)$$

The CO_2 inhibition of an enzymatic reaction involved in glycolysis

According to the hypothesis introduced, an enzyme involved in glycolysis is assumed to be inhibited by CO_2, so as to make this enzymatic transformation the rate determining step of glycolysis. To describe the effect induced by an increase in PCO_2 in the surrounding atmosphere, and then also of the CO_2 concentration in the cellular solution, on the rate of glycolysis (hexose metabolisation rate = r_{hm}), the following equation was hypothesised:

$$r_{hm} = r'_{hm} / (1 + K'_{CO_2} \cdot [CO_2]) \quad (3)$$

where:

r_{hm} = the hexose metabolisation rate (rate of glycolysis) when the CO_2 concentration in the cellular solution is greater than zero ($PCO_2 \geq 0$) [$mmol \cdot kg^{-1} \cdot h^{-1}$];

r'_{hm} = the hexose metabolisation rate when the CO_2 concentration in the cellular solution is equal to zero ($PCO_2 = 0$) [$mmol \cdot kg^{-1} \cdot s^{-1}$];

K'_{CO_2} = the constant related to the inhibitory power induced by CO_2 on the hexose metabolisation rate [$kg \cdot mol^{-1}$];

$[CO_2]$ = the concentration of CO_2 dissolved in the cellular solution [$mmol \cdot kg^{-1}$].

Aerobic respiration, alcoholic fermentation, hexose metabolisation and ATP production rates as a function of PO_2 and PCO_2

As, from an energetic point of view, the oxidation of pyruvate is largely more favoured than its transformation to ethanol and CO_2, alcoholic fermentation would take place only when the PO_2 of the surrounding atmosphere is not sufficient to ensure the aerobic respiration of all the pyruvate produced by glycolysis.

If PCO_2 is close to zero and high PO_2 values are present in the storage atmosphere, only aerobic respiration takes place:

$$C_6H_{12}O_6 + 6\ O_2 \longrightarrow 6\ CO_2 + 6\ H_2O$$
(energetic yield = 36 ATP)

Because of the stoichiometric factors connected with this reaction, the rate of hexose (D-glucose) conversion (r_{hm}) will be equal to 1/6 of that of O_2 consumption as well as 1/6 of aerobic respiration ($r_{ar} = r_{O_2}$; see Materials and Methods):

$$r_{hm} = 1/6 \cdot r_{O_2}; \qquad r_{hm} = 1/6 \cdot r_{ar}$$

Moreover, according to this hypothesis and to the results reported in a previous paper (ANDRICH *et al.*, 1994a), in this case, the rate of ATP produced reaches its maximum value ($r_{ATP,max}$) which is related to $r_{ATP,max}$:

$$r_{ATP,max} = 6 \cdot r_{ar,max}$$

If both respiratory gasses are practically absent in the surrounding atmosphere ($PO_2 = PCO_2 \approx 0$), all the pyruvate produced by glycolysis is converted by alcoholic fermentation to produce ethanol and CO_2:

$$C_6H_{12}O_6 \longrightarrow 2\ CO_2 + 2\ C_2H_5OH$$
(energetic yield = 2 ATP)

In these conditions, the rate of ethanol production (r_{af} = alcoholic fermentation rate = $r_{CO_2} - r_{O_2}$; see Materials and Methods), which is double that connected with hexose metabolisation (r_{hm}), reaches its maximum value ($r_{af,max}$). Also the rate of hexose metabolisation (r_{hm}) assumes its maximum value ($r_{hm,max}$), while that related to ATP production (r_{ATP}) is close to its minimum value ($r_{ATP,min}$) (ANDRICH *et al.*, 1994b):

$$r_{af} = 2 \cdot r_{hm} \rightarrow r_{af,max} = 2 \cdot r_{hm,max}$$

$$r_{ATP,min} = r_{af,max} \rightarrow r_{ATP,min} = 2 \cdot r_{hm,max}$$

If higher PCO_2 and reduced PO_2 are utilised, only a part of the reduced amount of pyruvate, which can be produced in these conditions, can be oxidised by aerobic respiration because of the low value of PO_2 employed. The pyruvate which cannot be consumed by O_2, is converted by alcoholic fermentation to produce ethanol and CO_2 (alcoholic fermentation). So, on the basis of this hypothesis and of related considerations, the following relationships can be written:

$$r_{hm} = 1/6 \cdot r_{ar} + 1/2 \cdot r_{af}$$

$$r_{ATP} = 6 \cdot r_{ar} + r_{af}$$

Fig. 1 reports the theoretical evolutions of aerobic respiration, alcoholic fer-

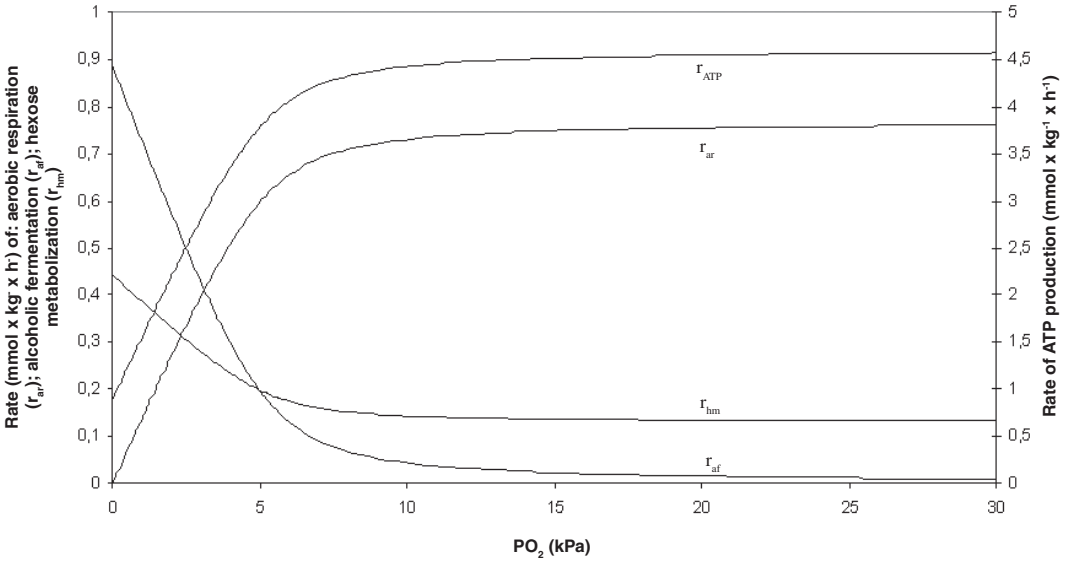


Fig. 1 - Evolution of aerobic respiration (r_{ar}), alcoholic fermentation (r_{af}), ATP production (r_{ATP}) and hexose metabolisation (r_{hm}) rates with PO_2 (PCO_2 = 0 kPa).

mentation, ATP production and hexose metabolisation rates (r_{ar}, r_{af}, r_{ATP}, r_{hm}) with PO_2 when the PCO_2 in the storage atmosphere was maintained close to zero (ANDRICH *et al.*, 1994b). When PO_2 increases also r_{ar} and r_{ATP} increase while, on the contrary, r_{af} and r_{hm} decrease. Moreover the ATP production rate (r_{ATP}) appears to reach an asymptotic maximum value when PO_2 is close to that of air, and in these conditions, the hexose metabolisation rate (r_{hm}) reaches its asymptotic minimum value.

The theoretical evolution of both aerobic respiration and alcoholic fermentation rates as a function of the PCO_2 adopted, is reported in Fig. 2 at four different values of PO_2 (a = 0; b = 3; c = 10; d = 21 kPa), while the temperature is maintained at 21°C. To allow an easier comparison among experimental and calculated values, together with the theoretical curves, the related experimental points (Table 2d) are also reported (Fig. 2 a,b,c,d). If oxygen is not present in the storage atmosphere (Fig. 2a; PO_2 = 0 kPa), aerobic respiration cannot occur, while the alcoholic fermentation rate reaches its maximum value when PCO_2 is equal to zero, and decreases when PCO_2 increases. Because of the more relevant production of ATP, which can be obtained by aerobic respiration compared to that coming from alcoholic fermentation, the reduced amount of O_2 present in the storage atmosphere (PO_2 = 3 kPa; Fig. 2b), would be completely utilised by the fruit to promote aerobic respiration, while the remaining fraction of pyruvate produced in these conditions would be converted by alcoholic

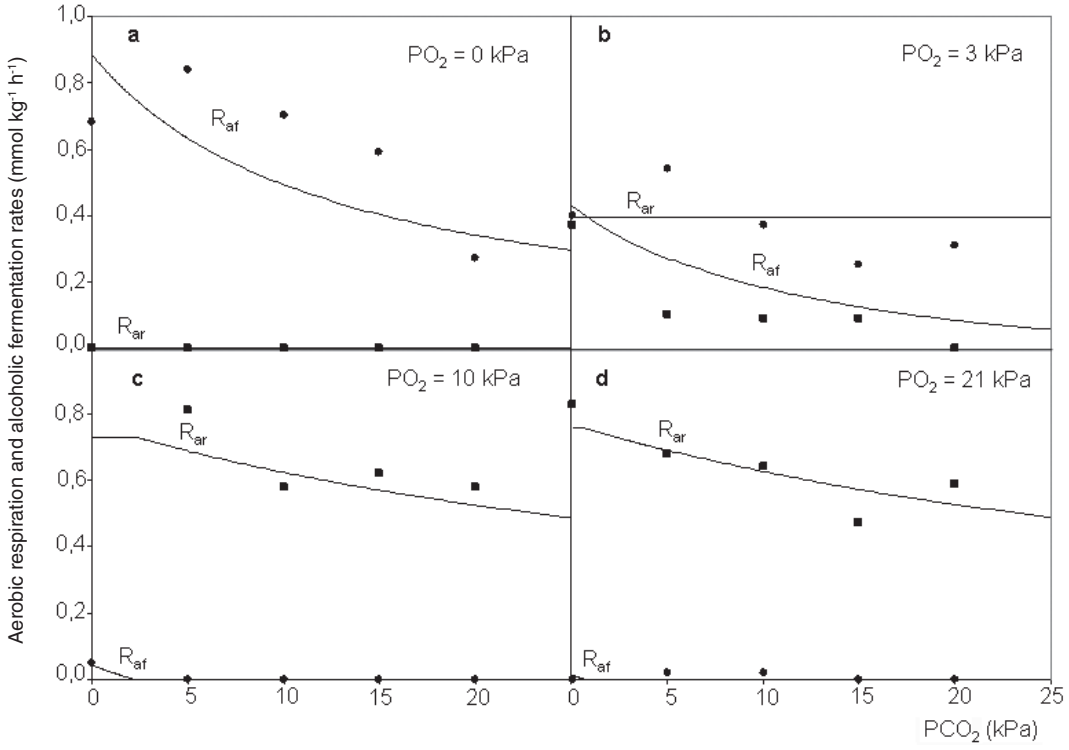


Fig. 2a,b,c,d - Evolution of aerobic respiration (r_{ar}) and alcoholic fermentation (r_{af}) as a function of PCO_2 at four different levels of PO_2 (a = 0, b = 3, c = 10, d = 21 kPa), while temperature was maintained at 21°C. The theoretical curves and the experimental points are reported.

Table 1 - Steps connected with the logical procedure followed by the mathematical model to describe the evolution of aerobic respiration and alcoholic fermentation rates as a function of storage conditions (temperature = T_2, $PO_2 = PO_{2}$ and $PCO_2 = PCO_{2}$).

[Step 1] - Calculation of the values assumed by the model constants as a function of storage temperature (T_2):

1. $K_{sat,O_{2(T=T2)}} = K_{sat,O_{2(T=T1)}} \cdot e^{-\Delta H^{\circ}_{ksat,O2}/R \cdot (1/T2 - 1/T1)}$ <9>
2. $K_{CO_{2(T=T2)}} = K_{CO_{2}(T=T1)} \cdot e^{-\Delta H^{\circ}_{kCO2}/R \cdot (1/T2 - 1/T1)}$ <9>
3. $K_{M(T=T2)} = K_{M(T=T1)} \cdot e^{-\Delta H^{\circ}_{kM}/R \cdot (1/T2 - 1/T1}$ <9>
4. $r_{ar,max(T=T2)} = r_{ar,max\ (T=T1)} \cdot e^{-\Delta Er_{ar,max}/R \cdot (1/T2 - 1/T1)}$ <8>
5. $r_{af,max(T=T2)} = r_{af,max\ (T=T1)} \cdot e^{-\Delta Er_{af,max}/R \cdot (1/T2 - 1/T1)}$ <8>

[Step 2] - Evaluation of the maximum rate assumed by aerobic respiration at the PO_2 and temperature used (PO_2, $T_2 \rightarrow r_{ar}^*$):

1. $[O_2] = K_{sat,O_{2(T=T2)}} \cdot PO_2$ <2>
2. $r_{ar}^* = r_{ar,max\ (T=T2)} \cdot [O_2] / (K_{M(T=T2)} + [O_2])$ <1>
3. the hexose metabolisation rate (r_{hm}^*) connected to this aerobic activity can be then calculated: $r_{hm}^* = 1/6 \cdot r_{ar}^*$

[Step 3] - Evaluation of the hexose metabolization rate (r_{hm}) as a function of temperature, PCO_2 and r_{ar}^* calculated in the previous step (T_2, PCO_2, $r_{ar}^* \rightarrow r_{hm}$):

1. $r_{hm} = 1/(1+K_{CO2(T=T2)} \cdot PCO_2) \cdot \{r_{ar,max_{(T=T2)}}/6 + (1/2 \cdot r_{af,max(T=T2} / r_{ar,max(T=T2)} - 1/6) \cdot (r_{ar,max(T=T2)} - r_{ar}^*)\}$ <7>

[Step 4] - [I° case: $r_{hm} \leq r_{hm}^*$] The metabolized hexose can be completely converted by aerobic respiration:

1. $r_{af} = 0$
2. $r_{hm} = r_{ar}/6 = 1/(1+K_{CO2(T=T2)} \cdot PCO_2) \cdot \{r_{ar,max_{(T=T2)}}/6 + (1/2 \cdot r_{af,max(T=T2)} / r_{ar,max(T=T2)} - 1/6) \cdot (r_{ar,max(T=T2)} - r_{ar})\}$ <7>
3. $r_{ar} = (3 \cdot r_{af,max(T=T2)} \cdot r_{ar,max(T=T2)}) / (K_{CO2(T=T2)>} \cdot PCO_2 \cdot r_{ar,max(T=T2)} + 3 \cdot r_{af,max(T=T2)})$ <10>

[Step 5] - [II° case: $r_{hm} > r_{hm}^*$] In this case, the r_{ar} value is equal to that calculated in Step 1, while r_{af} will be proportional to the difference occurring between r_{hm} and r_{ar}:

1. $r_{ar} = r_{ar}^*$
2. $r_{hm} = r_{ar}/6 + r_{af}/2 = 1/(1+K_{CO2(T=T2)} \cdot PCO_2) \cdot \{r_{ar,max_{(T=T2)}}/6 + (1/2 \cdot r_{af,max(T=T2)} / r_{ar,max(T=T2)} - 1/6) \cdot (r_{ar,max(T=T2)} - r_{ar})\}$ <7>
3. $r_{af} = 2 \cdot r_{hm} - 1/3 \cdot r_{ar} = 2/(1+K_{CO2(T=T2)} \cdot PCO_2) \cdot \{r_{ar,max_{(T=T2)}}/6 + (1/2 \cdot r_{af,max(T=T2)} / r_{ar,max(T=T2)} - 1/6) \cdot (r_{ar,max(T=T2)} - r_{ar})\} - 1/3 \cdot r_{ar}$ <11>

r_{ar}^* = maximum aerobic respiration rate at the PO_2 and temperature used ($PCO_2 = 0$);
K_{sat,O_2} = constant connected with the equilibrium of O_2 partition between the cellular solution of apples and surrounding atmospheres;
T_1 = reference temperature;
T_2 = run temperature;
$\Delta H^{\circ}_{Ksat,O_2}$ = activation energy related to K_{sat};
$r_{ar,max}$ = kinetic constant connected with Michaelis-Menten-like equation;
$\Delta E_{ar,max}$ = activation energy related to $r_{ar,max}$;
K_M = Michaelis constant;
ΔE_M = activation energy related to K_M;
$[O_2]_{cs}$ = concentration of O_2 dissolved in cytosol;
PO_2 = partial pressure of O_2 in the surrounding atmosphere;
k_{mt,O_2} = global mass transfer constant connected with O_2 diffusion;
A = fruit area;
$r_{af,max}$ = maximum alcoholic fermentation rate at the temperature used ($PO_2 = PCO_2 = 0$ kPa);
$\Delta E_{af,max}$ = activation energy related to $r_{af,max}$;
r_{ar} = aerobic respiration rate under the experimental conditions adopted;
r_{hm} = hexose metabolisation rate under the experimental conditions adopted;
r_{af} = alcoholic fermentation rate under the experimental conditions adopted;
K_{CO_2} = equilibrium constant related to the inhibitory effect of PCO_2;
PCO_2 = partial pressure of CO_2 in the surrounding atmosphere.

Table 2a - Aerobic respiration (r_{ar}) and alcoholic fermentation (r_{af}) rates of Golden Delicious apples stored at 1°C as a function of PO_2 and PCO_2 adopted.

PO_2 (kPa)	PCO_2 (kPa)	r_{ar} (mmol · kg^{-1}·h^{-1})	r_{af} (mmol · kg^{-1}·h^{-1})
0	0	0.00	0.33
3	0	0.08	0.08
10	0	0.15	0.00
21	0	0.16	0.03
0	5	0.00	0.07
3	5	0.10	0.00
10	5	0.07	0.00
21	5	0.14	0.00
0	10	0.00	0.05
3	10	0.00	0.00
10	10	0.06	0.00
21	10	0.04	0.00
0	15	0.00	0.04
3	15	0.00	0.00
10	15	0.06	0.00
21	15	0.00	0.00
0	20	0.00	0.00
3	20	0.00	0.00
10	20	0.00	0.00
21	20	0.00	0.00

Table 2b - Aerobic respiration (r_{ar}) and alcoholic fermentation (r_{af}) rates of Golden Delicious apples stored at 6°C as a function of PO_2 and PCO_2 adopted.

PO_2 (kPa)	PCO_2 (kPa)	r_{ar} (mmol · kg^{-1}·h^{-1})	r_{af} (mmol · kg^{-1}·h^{-1})
0	0	0.00	0.43
3	0	0.13	0.07
10	0	0.40	0.00
21	0	0.43	0.00
0	5	0.00	0.16
3	5	0.06	0.00
10	5	0.10	0.00
21	5	0.14	0.00
0	10	0.00	0.08
3	10	0.07	0.00
10	10	0.23	0.00
21	10	0.09	0.00
0	15	0.00	0.05
3	15	0.00	0.00
10	15	0.17	0.00
21	15	0.11	0.00
0	20	0.00	0.00
3	20	0.00	0.00
10	20	0.00	0.00
21	20	0.00	0.00

Table 2c - Aerobic respiration (r_{ar}) and alcoholic fermentation (r_{af}) rates of Golden Delicious apples stored at 16°C as a function of PO_2 and PCO_2 adopted.

PO_2 (kPa)	PCO_2 (kPa)	r_{ar} (mmol · kg^{-1}·h^{-1})	r_{af} (mmol · kg^{-1}·h^{-1})
0	0	0.00	0.59
3	0	0.21	0.26
10	0	0.54	0.00
21	0	0.56	0.03
0	5	0.00	0.59
3	5	0.32	0.03
10	5	0.59	0.00
21	5	0.52	0.00
0	10	0.00	0.52
3	10	0.24	0.11
10	10	0.39	0.02
21	10	0.56	0.00
0	15	0.00	0.47
3	15	0.11	0.05
10	15	0.22	0.00
21	15	0.24	0.00
0	20	0.00	0.05
3	20	0.19	0.00
10	20	0.31	0.00
21	20	0.38	0.00

Table 2d - Aerobic respiration (r_{ar}) and alcoholic fermentation (r_{af}) rates of Golden Delicious apples stored at 21°C as a function of PO_2 and PCO_2 adopted.

PO_2 (kPa)	PCO_2 (kPa)	r_{ar} (mmol · kg^{-1}·h^{-1})	r_{af} (mmol · kg^{-1}·h^{-1})
0	0	0.00	0.68
3	0	0.40	0.37
10	0	1.00	0.05
21	0	0.83	0.00
0	5	0.00	0.84
3	5	0.54	0.00
10	5	0.81	0.00
21	5	0.68	0.02
0	10	0.00	0.70
3	10	0.37	0.09
10	10	0.58	0.00
21	10	0.64	0.02
0	15	0.00	0.59
3	15	0.18	0.09
10	15	0.62	0.00
21	15	0.47	0.00
0	20	0.00	0.27
3	20	0.21	0.00
10	20	0.58	0.00
21	20	0.59	0.00

fermentation. Also in this case, increasing values of PCO_2 reduced the alcoholic fermentation rate.

When increasing values of PO_2 are employed (10, 21 kPa; Fig. 2c and 2d), aerobic respiration takes place and only a very reduced fraction of alcoholic fermentation occurs. But, in any case, it is this process that is initially affected by the increasing values of PCO_2 and only when alcoholic fermentation is reduced to zero, does the aerobic respiration rate begin to be reduced by the increasing values of PCO_2. In these experimental conditions, the two initial maximum values assumed by the aerobic respiration rate at PO_2 equal, respectively, to 10 and 21 kPa (PCO_2 = 0 kPa;) do not substantially differ because in these conditions (high levels of PO_2), the aerobic respiration rate is not so far from its maximum asymptotic value.

A kinetic equation to describe the evolution of the hexose metabolisation rate with PO_2 and PCO_2

According to the considerations previously reported, the hexose metabolisation rate would vary between a minimum and a maximum value:

$$r_{hm,min} \leq r_{hm} \leq r_{hm,max}$$

assuming its minimum value ($r_{hm,min}$) when the fruit is stored at high values of PO_2, while PCO_2 is close to zero. In these conditions the aerobic respiration rate reaches its maximum value, while the hexose metabolisation rate would assume its minimum value ($r_{ar} = r_{ar,max} \rightarrow r_{hm,min} = 1/6 \cdot r_{ar,max}$). The maximum of r_{hm} ($r_{hm,max}$) would be obtained when both respiratory gasses (O_2 and CO_2) are not present in the storage atmosphere and the aerobic respiration rate becomes equal to zero ($r_{ar} = 0 \rightarrow r_{hm,max} = 1/2 \cdot r_{af,max}$).

So the hexose metabolisation rate (r'_{hm}) shown by Golden Delicious apples stored in an atmosphere having variable PO_2, but PCO_2 close to zero, could be evaluated as the sum of two contributions: the minimum value assumed by the hexose metabolisation rate ($r_{hm,min,PCO_2 \approx 0} = r'_{hm,min} = 1/6 \cdot r_{ar,max}$) and a factor supposed to be proportional (K_p) to the difference occurring between the maximum value of the aerobic respiration rate ($r_{ar,max}$) and that (r_{ar}) related to the experimental conditions analysed, and in particular, to the PO_2 utilised:

$$r'_{hm} = 1/6 \cdot r_{ar,max} + K_p \cdot (r_{ar,max} - r_{ar}) \quad (4)$$

As the hexose metabolisation rate reaches its maximum value when both PO_2 (r_{ar}=0) and PCO_2 are close to zero, the following expression can be obtained:

$$r_{hm,max,PCO_2 \approx 0} = r'_{hm,max} = 1/2 \cdot r_{af,max} =$$

$$= 1/6 \cdot r_{ar,max} + K_p \cdot r_{ar,max}$$

and K_p calculated:

$$K_p = 1/2 \cdot r_{af,max}/r_{ar,max} - 1/6$$

so that equation <4> would become:

$$r'_{hm} = 1/6 \cdot r_{ar,max} + (1/2 \cdot r_{af,max}/r_{ar,max} - 1/6) \cdot (r_{ar,max} - r_{ar}) \quad (5)$$

Combining equations <3> and <5> the following expression can be obtained:

$$r_{hm} = r'_{hm} / (1 + K'_{CO_2} \cdot [CO_2]) =$$

$$= 1/(1 + K'_{CO_2} \cdot [CO_2]) \cdot [1/6 \cdot r_{ar,max} +$$

$$+ (1/2 \cdot r_{af,max}/r_{ar,max} - 1/6) \cdot$$

$$(r_{ar,max} - r_{ar})] \quad (6)$$

As, already reported for O_2, also the resistance offered by apple skin to CO_2 diffusion is negligible and the CO_2 mass transfer rate, between the surrounding atmosphere and the fruit cells appears to be largely greater than the respiration rate. In this condition the concen-

tration of CO_2 dissolved in the cellular solution can be assumed to be in equilibrium with the PCO_2 of the surrounding atmosphere (ANDRICH *et al.*, 1990; PEPPELENBOS *et al.*, 1996a):

$$K_{sat,CO_2} = [CO_2] / PCO_2$$

and the following relationship can be obtained:

$$[CO_2] = K_{sat,CO_2} \cdot PCO_2$$

and equation <6> would become:

$$r_{hm} =$$

$$= 1/(1 + K'_{CO_2} \cdot K_{sat,CO_2} \cdot PCO_2) \cdot [1/6 \cdot r_{ar,max} +$$

$$+ (1/2 \cdot r_{af,max}/r_{ar,max} - 1/6) \cdot (r_{ar,max} - r_{ar})]$$

$$= 1/(1 + K_{CO_2} \cdot PCO_2) \cdot [1/6 \cdot r_{ar,max} +$$

$$+ (1/2 \cdot r_{af,max}/r_{ar,max} - 1/6) \cdot$$

$$(r_{ar,max} - r_{ar}))] \qquad (7)$$

where:

$K_{CO_2} = K'_{CO_2} \cdot K_{sat,CO_2}$ = constant related to the inhibitory power due to CO_2 [Pa^{-1}];

PCO_2 = the partial pressure of CO_2 in the surrounding atmosphere [Pa];

$r_{ar,max}$ = the maximum of the aerobic respiration rate [$mol \cdot kg^{-1} \cdot s^{-1}$];

$r_{af,max}$ = the maximum of the alcoholic fermentation rate [$mol \cdot kg^{-1} \cdot s^{-1}$];

r_{ar} = the aerobic respiration rate of apples stored in the adopted experimental conditions [$mol \cdot kg^{-1} \cdot s^{-1}$].

The dependence of the kinetic constants introduced from temperature

To describe the temperature dependence of all the kinetic constants introduced ($r_{ar,max}$, $r_{af,max}$) the Arrhenius equation was utilised (ANDRICH *et al.*, 1998):

$$k_{T=T2} = k_{T=T1} \cdot e^{\Delta Ea/R \cdot (1/T1 - 1/T2)} \qquad (8)$$

where:

$k_{T=T2}$ = kinetic constant at the employed temperature $T=T_2$;

$k_{T=T1}$ = kinetic constant at the reference temperature $T=T_1$;

$\Delta Ea/R$ = the ratio between the activation energy and ideal gas constant.

While a close Vant'Hoff expression has been introduced to describe the temperature dependence of the equilibrium constants (K_{sat,O_2}, K_{CO_2}, K_M):

$$K_{T=T2} = K_{T=T1} \cdot e^{\Delta H°r/R \cdot (1/T1 - 1/T2)} \qquad (9)$$

where:

$K_{T=T2}$ = equilibrium constant at the employed temperature $T=T_2$;

$K_{T=T1}$ = equilibrium constant at the reference temperature $T=T_1$;

$\Delta H°r/R$ = the ratio between the standard enthalpy of reaction and the ideal gas constant.

Aerobic respiration and alcoholic fermentation rates as a function of working conditions

The logical procedure followed by the model to evaluate the aerobic respiration and alcoholic fermentation rates as a function of the working conditions adopted (temperature, PO_2 and PCO_2), can be explained by the five steps reported in Table 1.

On the basis of the values calculated for the constants (K_{sat,O_2}; K_{CO_2}; $r_{ar,max}$; $r_{af,max}$; K_M) at the storage temperature (T_2) (step 1), the model determines the maximum possible rate (r_{ar}*) which can be assumed by aerobic respiration at the PO_2 tested (step 2), together with the related hexose metabolisation rate (r_{hm}*). Then as a function of PCO_2 of the surrounding atmosphere and of the aerobic respiration rate (r_{ar}*) previously determined, a preliminary tentative value for the hexose metabolisation rate ($r°_{hm}$) is then calculated to be compared with the r_{hm}* found in step 2.

If the $r°_{hm}$ value is lower than (or equal

to) r_{hm}* ($r°_{hm} \leq r_{hm}$*) [step 4, I° case], it means that all the pyruvate produced by glycolysis can be converted by aerobic respiration and no alcoholic fermentation occurs ($r_{af} = 0$).

In this case:

$$r_{hm} = 1/6 \cdot r_{ar} = 1/(1 + K_{CO_2} \cdot PCO_2) \cdot$$

$$[1/6 \cdot r_{ar,max} +$$

$$+ (1/2 \cdot r_{af,max}/r_{ar,max} - 1/6) \cdot (r_{ar,max} - r_{ar})]$$

so both r_{ar} and r_{hm} can be calculated:

$$r_{ar} = (3 \cdot r_{af,max\ (T=T2)} \cdot r_{ar,max\ (T=T2)})/$$

$$(K_{CO_2(T=T2)} \cdot PCO_2 \cdot r_{ar,max\ (T=T2)} +$$

$$+ 3 \cdot r_{af,max\ (T=T2)}) \qquad (10)$$

$$r_{hm} = 1/6 \cdot (3 \cdot r_{af,max\ (T=T2)} \cdot r_{ar,max\ (T=T2)})/$$

$$(K_{CO_2(T=T2)} \cdot PCO_2 \cdot r_{ar,max\ (T=T2)} + 3 \cdot r_{af,max\ (T=T2)}).$$

If, on the contrary, the $r°_{hm}$ value is greater than r_{hm}* ($r°_{hm} > r_{hm}$*) [step 5, II° case], it means that only a part of the pyruvate produced by glycolysis can be converted by aerobic respiration and, in this case, the aerobic respiration rate would assume the value calculated in step 2 ($r_{ar} = r_{ar}$* and then also $r_{hm} = r_{hm}$*), while the rate of alcoholic fermentation can be so evaluated:

$$r_{hm} = 1/6 \cdot r_{ar} + 1/2 \cdot r_{af} \rightarrow$$
$$r_{af} = 2 \cdot r_{hm} - 1/3 \cdot r_{ar}$$

$$r_{af} = 2/(1 + K_{CO_2} \cdot PCO_2) \cdot [1/6 \cdot r_{ar,max} +$$
$$+ (1/2 \cdot r_{af,max}/r_{ar,max} - 1/6) \cdot (r_{ar,max} - r_{ar}))]$$
$$- 1/3 \cdot r_{ar}) \qquad (11)$$

IDENTIFICATION OF THE BEST VALUES TO BE ASSIGNED TO THE MODEL PARAMETERS

The best values to be assigned to the model parameters were identified by a commercial statistical program "BURENL" (BUZZI FERRARIS and MANCA, 1996), which utilised the experimental data of the aerobic respiration (r_{ar}) and alcoholic fermentation (r_{af}) rates reported in Table 2 a-d. These experimental points were measured at 80 different experimental conditions (4 temperatures, 4 PO_2 and 5 PCO_2) (see Materials and Methods).

Operating at high values of PCO_2 (15, 20 kPa) and at reduced temperature (1, 6°C), it was not possible to obtain significant values for aerobic respiration and alcoholic fermentation rates because of the very reduced activities to be measured.

The experimental data obtained were then compared with those calculated according to the equations and the logical procedure reported in Table 1, assigning different values to the model parameters introduced (K_{sat,O_2}; $\Delta H°_{Ksat,O2}$; K_{CO_2}; $\Delta H°_{KCO_2}$; $r_{ar,max}$; $\Delta Er_{ar,max}$; $r_{af,max}$; $\Delta Er_{af,max}$; K_M; $\Delta H°_{KM}$).

The minimum of function "F", obtained as the sum of the differences occurring between estimated and experimental data, allowed the identification of the best values to be assigned to the model parameters, to obtain good overlapping between calculated and experimental values (Table 3).

After 80304 loops, the initial value assumed by the F function (3501) was reduced to 185 when further reduction of the F function could be not obtained. Moreover the correlation coefficients connected with aerobic respiration ($r_{rar} = 0.90$) and alcoholic fermentation ($r_{raf} = 0.86$) rates, calculated for the model linearised in the Taylor's series, give a measure of suitability of the model introduced to describe their evolution with the temperature, PO_2 and PCO_2 adopted. The minimum of function F occurred when the values reported in Table 3 were assigned to the functional parameters introduced by the adopted mathematical model.

Table 3 - Values and related confidence intervals (C.I.; p = 0.05), calculated for the parameters by the mathematical optimisation of the model introduced to describe the evolution of aerobic respiration and alcoholic fermentation rates of apples stored in refrigerated and controlled atmospheres, as a function of temperature, PO_2 and PCO_2.

Parameter	Symbol Adopted	Numerical value coming from optimisation mean ± C.I. (p=0.05)	Units
Equilibrium constant related to O_2 partition	$K_{sat,O2\ (T=21°C)}$	$(1.08 \pm 25.15) \cdot 10^{-5}$	$mol \cdot kg^{-1} \cdot Pa^{-1}$
Standard enthalpy connected to O_2 partition	$-\Delta H°_{Ksat,O2}/R$	$(6.93 \pm 2.97) \cdot 10^{1}$	°K
Constant related to the inhibitory power of CO_2	$K_{CO2\ (T=21°C)}$	$(1.24 \pm 2.06) \cdot 10^{-4}$	Pa^{-1}
Standard enthalpy connected to the inhibitory power of CO_2	$-\Delta H°_{KCO2}/R$	$(8.17 \pm 5.13) \cdot 10^{3}$	°K
The maximum rate of aerobic respiration at 21°C	$r_{ar,max(T=21°C)}$	$(7.29 \pm 1.00) \cdot 10^{-4}$	$mol \cdot kg^{-1} \cdot h^{-1}$
The activation energy related to the maximum rate of aerobic respiration $r_{ar,max}$	$\Delta Er_{ar,max}/R$	$(5.42 \pm 2.30) \cdot 10^{3}$	°K
The maximum rate of alcoholic fermentation at 21°C	$r_{af,max\ (T=21°C)}$	$(6.27 \pm 1.11) \cdot 10^{-4}$	$mol \cdot kg^{-1} \cdot h^{-1}$
The activation energy related to the maximum rate of alcoholic fermentation $r_{af,max}$	$\Delta Er_{af,max}/R$	$(7.33 \pm 3.10) \cdot 10^{3}$	°K
Michaelis constant related to aerobic respiration	$K_{M\ (T=21°C)}$	$(2.00 \pm 23.48) \cdot 10^{-8}$	$mol \cdot kg^{-1}$
Standard enthalpy connected to the Michaelis constant	$-\Delta H°_{KM}/R$	$(6.03 \pm 1.19) \cdot 10^{4}$	°K
Correlation coefficient related to aerobic respiration	r_{rar} (*)	0.90	--
Correlation coefficient related to alcoholic fermentation	r_{raf} (*)	0.86	--
Number of experimental values used in the optimisation		80	--

(*) evaluated for the model linearised in the Taylor's series.

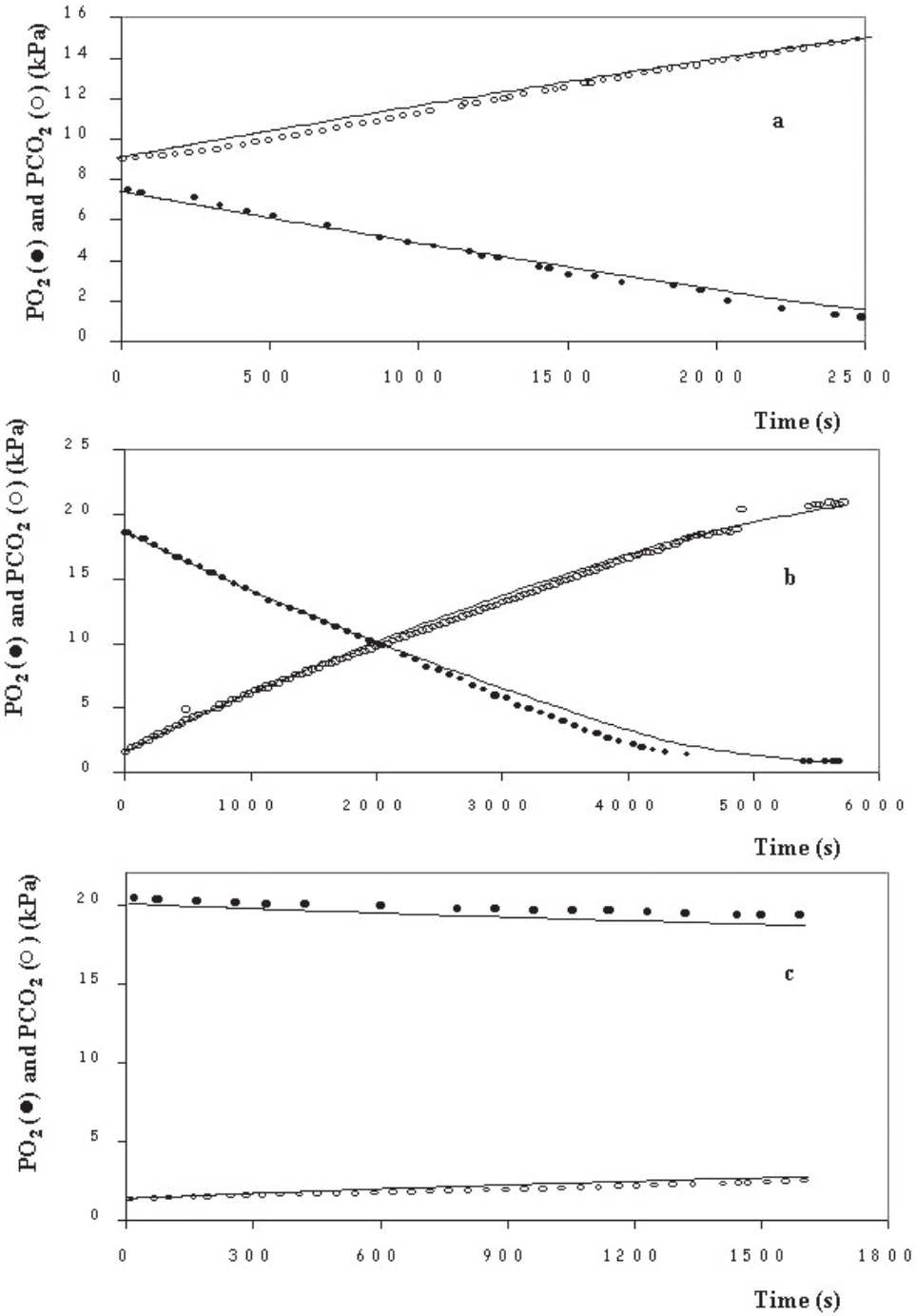


Fig. 3a,b,c - Time evolution of PO_2 and PCO_2 (theoretical lines and experimental points) in the atmosphere of a cell where Golden Delicious apples were stored (a) temperature = 21°C, $PO_{2,t=0}$ = 7.5 kPa and $PCO_{2,t=0}$ = 9.0 kPa; (b) temperature = 21°C, $PO_{2,t=0}$ = 18.6 kPa and $PCO_{2,t=0}$ = 1.5 kPa; (c) temperature = 6°C, $PO_{2,t=0}$ = 20.5 kPa and $PCO_{2,t=0}$ = 0.3 kPa).

MODEL VALIDATION

To validate the model an experimental data set completely different from that previously employed to identify the functional parameters of the proposed model was utilised. To test this model in a dynamic situation (SALTVEIT, 2003), the experimental values related to the evolution of PO_2 and PCO_2 in the atmosphere of a storage room refilled with Golden Delicious apples and stored at different temperatures were utilised (Fig. 3,a-c). To compare experimental and calculated values, the mathematical integration of the differential equations related to the mathematical model was carried out (WORTING and GEFFNER, 1965).

The initial experimental conditions are reported in Table 4 and the experimental points and the corresponding theoretical curves which describe PO_2 and PCO_2 evolution inside a glass jar are reported in Fig. 2,a-c.

The reduced values assumed by the mean square error and by the percentage of variance (Table 4), together with the good degree of overlapping occurring between the theoretical and experimental points (Fig. 3,a-c), seem to test the suitability of the mathematical model introduced. Only when very low values of PCO_2 (0.92 kPa) are used, does the corresponding value of VAR% becomes too high (24.75%), even if, in these conditions, a reduced MSE value is obtained (0.052).

Although the composition of the storage atmosphere did not differ significantly (compare initial conditions related to Fig. 3b with those of Fig. 3c), the evolution of PO_2 and of PCO_2 and then also of r_{ar} and of r_{af} change significantly with temperature indicating the strong effect induced by this variable on both aerobic respiration and alcoholic fermentation (Fig. 3b,c).

CONCLUSION

This model does not take into account the effect produced on the kinetics of aerobic respiration and alcoholic fermentation by ethylene and water loss. In fact, the experimental data utilised to identify the best values for model parameters were obtained in the absence of ethylene (absorption by an aqueous solution of $KMnO_4$) and using a gaseous mixture close to saturation (Relative Humidity ≈ 100%). The dependence of aerobic respiration and alcoholic fermentation on water transpiration and ethylene accumulation should be successively evaluated to increase the validity and the usefulness of the model.

Although the number of parameters involved is not particularly high (10 constants, 5 of which were utilised to describe temperature evolution), this model appears to be able to suitably describe the evolution of aerobic respiration and alcoholic fermentation rates of stored

Table 4 - The initial conditions experimentally adopted to evaluate the time evolution of PO_2 and PCO_2 in the atmosphere of a glass jar (total volume $9.5 \cdot 10^{-3}$ m^3) refilled with Golden Delicious apples together with the statistical analysis of the results obtained.

Run	Temp. (°C)	$PO_{2\ t=0}$ (kPa)	$PCO_{2\ t=0}$ (kPa)	MSE[a] PO_2	VAR%[a] PO_2	MSE[a] PCO_2	VAR%[a] PCO_2
A	21	11.2	7.2	0.147	8.60	0.193	3.40
B	21	18.6	1.5	0.240	5.70	0.140	3.22
C	6	20.5	0.3	0.047	1.09	0.052	24.75

a) see Materials and Methods

fruit with temperature, PO_2 and PCO_2. Moreover each constant introduced has a well – defined chemical – physical meaning, so their modifications can be directly connected to the alteration of a specific characteristic of the fruit. In particular, the maximum rates of alcoholic fermentation ($r_{af,max}$) and aerobic respiration ($r_{ar,max}$) appear to be strongly related to the ability of the fruit to produce the enzymes involved in the corresponding biochemical pathways, and give a measure of the physiological status of the stored fruit.

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PAPER

PHYSICO-CHEMICAL CHARACTERISTICS OF GREEK FIR HONEYDEW HONEY FROM *MARCHALINA HELLENICA* (GEN.) IN COMPARISON TO OTHER MEDITERRANEAN HONEYDEW HONEYS

CARATTERISTICHE FISICO-CHIMICHE DEL MIELE GRECO DI MELATA DI ABETE PRODOTTA DA *MARCHALINA HELLENICA* (GEN.) CONFRONTATE CON ALTRI MIELI DI MELATA MEDITERRANEI

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ABSTRACT

The first samples of recently produced fir honeydew honey, obtained from *Marchalina hellenica* (Gennadius) (Homoptera, Coccoidea, Margarodidae), originating from Mount Helmos in Greece were analysed and examined on the basis of physical and chemical parameters including humidity, acidity, sugar profile, hydroxymethylfurfural (HMF) and diastase activity. These data

RIASSUNTO

I primi campioni di miele di melata di abete, recentemente prodotti sul Monte Helmos in Grecia, derivante da *Marchalina hellenica* (Gennadius) (Homoptera, Coccoidea, Margarodidae) sono stati analizzati dal punto di vista delle caratteristiche fisico-chimiche, con riguardo ai parametri umidità, acidità, profilo zuccherino, idrossimetilfurfurale (HMF) e diastasi. I dati ottenuti

- Key words: chemical characteristics, fir honeydew honey, *Marchalina hellenica*, physical characteristics, pine honeydew honey -

were compared with five other Mediterranean honeydew honeys of different origin obtained from the literature. Analytical data of the Greek fir honey from *M. hellenica* honeydew complied with the E.U. legal limits. The sensorial analysis indicated that the organoleptic characteristics of this type of honeydew honey were very similar to the Greek fir honey derived from other coccids with respect to colour, taste and smell. The statistical analysis of the physico-chemical parameters of the six honey types revealed two major groups. One group consisted of three Greek honeys and the other included one Spanish and two Italian honeys. It also showed a marked similarity between the two Greek honeys that came from *M. hellenica* honeydew on either fir or pine trees.

dalle analisi sono risultati rispondenti ai limiti previsti dalla normativa europea. Le analisi sensoriali indicano che le caratteristiche organolettiche di questo tipo di miele sono molto simili per colore, sapore e odore, al miele Greco di abete prodotto da altri insetti appartenenti alla super-famiglia Coccoidea. Essi sono stati inoltre confrontati con quelli relativi ad altri 5 tipi di miele di melata di diversa origine, prodotti in area mediterranea. L'elaborazione statistica di tali dati ha messo in evidenza due principali gruppi di miele. Del primo fanno parte tre tipi di miele prodotti in Grecia, del secondo due mieli prodotti in Italia e uno in Spagna. È stata anche rilevata un'elevata analogia tra i due mieli greci di melata derivanti entrambi da *M. hellenica*, l'uno su abete e l'altro su pino.

INTRODUCTION

In Greece there are approximately 1,250,000 beehives (ANONYMOUS, 1998), kept by about 25,000 beekeepers, giving an annual honey production of approximately 15,000 tons. Sixty-five percent of the Greek honey is honeydew honey (SANTAS, 1983; THRASYVOULOU and MANIKIS, 1995; BACANDRITSOS, 1998, 2002; NATIONAL STATISTIC SERVICES, 1998; BACANDRITSOS and PAPADOPOULOU, 1999). Sixty per cent of the Greek honeydew honeys is produced by insects that parasite pine trees (*Pinus halepensis* Miller, *P. silvestris* Miller, *P. pinea* L., *P. brutia* Ten) and five per cent that parasitize fir trees (*Abies cephalonica* Loud., *A. borisii-regis* Mattf.) (SANTAS, 1983; THRASYVOULOU and MANIKIS, 1995; NATIONAL STATISTICS SERVICE, 1998; BACANDRITSOS and PAPADOPOULOU, 1999; BACANDRITSOS *et al.,* 2002). The remaining 35% is nectar honey. Nectar honeys include those produced from *Thymus capitatus*, *Satureja* spp., *Origanum* spp., *Calluna vulgaris*, *Citrus* sp. (*C. auratium, C. reticulata, C. limon)* and *Gossypium hirsutum* and *Heliantus annuus* L..

The honeydew from Greek pine forests is produced mainly by the scale insect *Marchalina hellenica* (Gennadius) (Homoptera, Coccoidea, Margarodidae) (SANTAS, 1979) which is endemic in pine forests in Greece and other Mediterranean countries as well as by the insects *Cinara palaestinensis* H.R.L. and *Cinara* close *pini*. In Greek fir forests, this type of honey is produced by the scale insects *Physokermes hemicryphus* (Dalman), *Eulecanium sericeum* (Lindinger) and by the aphids, *Mindarus abietinus* (Koch), *Cinara juniperi* (De Geer), *Cinara confinis* (Koch) and *Cinara pectinatae* (Nördl.), with *P. hemicryphus* being the most important (SANTAS, 1983; THRASYVOULOU and MANIKIS, 1995; BACANDRITSOS, 2002).

The study of the physical and chemical characteristics of Greek honeys was pioneered by EMMANOUEL (1932) and continued by KODOUNIS (1962), MAVRICOS *et al.* (1978), THRASYVOULOU and BLADENOPOULOU (1984), BACANDRITSOS *et al.* (1993, 2002), THRASYVOULOU and MANIKIS (1995), BACANDRITSOS and PAPADOPOULOU (1999), PASALOGLOU and KOKKORAS (1999).

The insect *M. hellenica* was successfully established, for first time, in the Greek fir forest of Mt. Helmos (latitude 22°11'30"E, longitude 37°59'30"N, altitude 2000 m) after an anthropogenic introduction in 1997 (BACANDRITSOS, 2002). Two years later a new type of fir honey from *M. hellenica* honeydews was collected from one apiary that was placed near the infested fir trees on Mt. Helmos.

The Greek fir honey produced by the scale insect *M. hellenica* constitutes a new type of honeydew honey. The main purpose of this study was to obtain some preliminary data concerning some of the major physical and chemical characteristics of this new type of fir honeydew (*M. hellenica*) honey. In addition the data obtained in this study were compared with the literature data of five other honeydew honeys from Greece, Italy and Spain.

MATERIALS AND METHODS

Samples

A total of 24 samples of fir honeydew *(M. hellenica)* honey (Type-I), collected during 1999 and 2001 from 24 beehives of an apiary situated near the Mt. Helmos ski resort (longitude 22°11'33"E, latitude 37°59'30"N, altitude 2000 m) were analysed. Empty frames were put in the hive box and the honey samples were gathered each year during August when the other coccids and aphids of the Greek fir trees did not produce honeydew (SANTAS, 1983; THRASYVOULOU, 2001). The samples were stored at 4°-6°C in the dark until the end of the analysis. All the samples were subjected to microscopical, physico-chemical and sensorial analysis.

Sensorial analysis

Sensorial analysis was carried out to define the organoleptic characteristics according to the accredited protocol developed by INA (Istituto Nazionale di Apicoltura) (ISO 6564/1985 and ISO 6658/1985).

Pollen analysis

The microscopical analysis of the sediment was conducted according to the methods of melissopalinology described by LOUVEAUX *et al.* (1978). In addition, the HDE/P criterion was examined. (HDE: Honeydew elements such as fungi spores, algae, etc., P = total frequency of pollen grains).

Physico-chemical analysis

Water content, hydroxymethylfurfural content (HMF), diastase activity, pH, total acidity and electrical conductivity were determined according to methods of the CODEX ALIMENTARIUS COMMISSION (1989) and BOGDANOV *et al.* (1997).

The sugar profile was determined by gas chromatography following the technique of SABATINI *et al.* (2001) in which a flame ionization detector is used with honey solutions derived with Pierce-Pourtallier reagent [oxime reagent (pyridine solution containing hydroxylamine hydrochloride); hexamethyldisilazane; trifluoroacetic acid] and with INA method reagent [silanising solution (five parts pyridine + two parts hexamethyldisilazane + one part trimethylchlorosilane)]. Identification and quantification of the single carbohydrates were obtained by comparison with a reference mixture of known concentration. The chromato-

graphic conditions used were: oven temperature cycle: initial temperature 70°C, increased by 5°C/min until a temperature of 140°C, increased by 6°C/min up to 300°C, maintained for 15 min; Carrier gas: hydrogen, flow velocity 47.3 cm/s; apolar capillary column model SUPELCO (CCFS MEGA Labocest, Bologna, Italy): SE52, 25m, 0.32 mm id, 0.1-0.15 µm film thickness.

Other honeys

The other honey types used for comparison purposes were:

- Type-II: Greek fir honeydew honey (n=10). The insects involved are: *Physokermes hemicryphus* (Dalman), *Eulecanium sericeum* (Lindinger), *Mindarus abietinus* (Koch) and *Cinara juniperi* (De Geer) for *Abies chephalonica* (LAZARIDOU *et al.*, 2003).
- Type-III: Greek pine honeydew honey (n=14). The insect involved is *M. hellenica* (LAZARIDOU *et al.*, 2003).
- Type-IV: Italian fir honeydew honey (n=67). The insects involved are*: Cinara cofinis* (Koch), *C. pectinatae* (Nordlinger), *C. pilicornis* (Hortig), *Mindarus abietinus* (Koch), *Physokermes piceae* (Schrank) for *Abies alba* (Miller) and *Cinara costata* (Zetterstent), *C. pilicornis* (Hortig), *C. pruinosa* (Hortig), *C. piceae* (Panzer), *Physokermes hemicryphus* (Dalman), *P. piceae* (Schrank) for *Picea excelsa* (Link) (PERSANO ODDO *et al.*, 2000).
- Type-V: Italian honeydew honey (n=53). The insect involved is *Metcalfa pruinosa,* a flatid that infects both wild and cultivated plants. Despite the notable economical damage caused to the plants, its honeydew provides food for bees that produce a large amount of honey (SABATINI and BARBATTINI, 2003).
- Type-VI: Spanish forest honeydew honey (n=16). The honeydew is produced by several insects that parasitize the *Quercus* spp. (MATEO and BOSCH-REIG 1997, 1998).

Statistical analysis

Cluster analysis of 11 parameters (fructose, glucose, sucrose, maltose, isomaltose, raffinose, erlose, melezitose, water content, electrical conductivity and pH) was applied to all of the honeydew honey types in order to determine the similarity among the six honeys of different origin; for the analysis, each honey type was represented by its mean value. The other parameters (trehalose, panose, isomalto-triose, maltotriose malto-tetraose, total acidity, diastase and HMF) were excluded from the analysis because their mean values were not available for all of the honeydew honey types. A dendrogram was constructed using the complete Linkage method with Euclidean distances. For data analysis, the Statistica v.6 (StatSoft Inc.) software was used.

RESULTS AND DISCUSSION

Sensorial analysis

The Greek fir honeydew (*M. hellenica*) honey (type-I) was liquid for a long time (up to three years kept at temperatures between 12° and 25°C) and very viscous. It was light amber in colour with hints of a milky pearl-like opaqueness. It had a typical, decisive and persistent taste that was pleasant, not excessively sweet, with an intense smell and resinous aroma.

The organoleptic characteristics of the Greek fir-honeydew (other coccids) honey (type-II) was similar to those of the type-I honey (BACANDRITSOS *et al.*, 2003).

The type-IV honey was liquid for a long time, very viscous and cloudy due to moderate crystallization. It was dark amber to almost black in colour with greenish hues. It had a characteristic smell of wood, resin and balm. It was not very sweet, normally acidic with persistent flavour (PERSANO *et al.*, 2000).

The type-V honey was liquid for a long time and viscous. It was dark amber to almost black in colour and was brown when crystallized. It had an intense, not very sweet, herbaceous smell. It had a persistent flavour of dried fruit, preserved tomato, molasses, fig jam and malt (SABATINI and BARBATTINI, 2003).

Considering the characteristics described above, it seems that the type-I honey is similar to the type-II honey. Compared with the other types, type-I honey is similar to the type IV and V honey in viscosity but different in colour.

Pollen analysis

The samples contained high quantities of honeydew elements (HDE). The mean value of the HDE/P ratio was equal to 4. These data agree with LOUVEAUX *et al.* (1978) who proposed values above 3 for honeydew honeys. No single pollen was dominant but the following were present in percentages between 16% and 3%: *Trifolium hybridum*, Rosaceae, Brassicaceae, *Rubus f.*, *Trifolium repens gr.*, *Smilax*, Umbelliferae and *Castanea sativa*.

Some isolated pollens included *Citrus*, Compositae T, *Amorpha*, Liliaceae, *Trifolium pratense gr.*, *Galega*, *Ailanthus*, *Melilotus*, *Alkanna*, *Gleditsia*, *Mentha f.*, *Clematis*, *Salix*, *Erica f.*, *Polygonum aviculare*, *Hedera*, Labiatae S, *Lotus*, *Helianthus f.*, *Vicia*, *Polygonum lapathifolium*, *Echium*, *Xanthium*, *Arbutus*, Compositae A and Scrophulariaceae. The diversity of the pollen is representative of the summertime flowering plants in the area investigated.

Physico-chemical characteristics

The results of the analysis of the physico-chemical characteristics of the type-I fir honeydew (*M. hellenica*) honey are summarized in Table 1. Relevant data concerning the other five Mediterranean honeydew honeys (types II-VI) are included in the same table for compar-

Table 1 - Physico-chemical characteristics of the fir honey from *Marchalina hellenica* honeydew (type-I) and five other honeydew honeys (types II-VI). The data are reported as average values±standard deviations. Minimum and maximum values are given in parentheses. Values followed by the same letter are not statistically significant (t-test), p≤0.05. NA: data not available.

Honey type		Water (%)	Electrical conductivity (mS/cm)	Total acidity (meq/kg)	Diastase (DN)	HMF (mg/kg)	pH
I	Fir- honeydew-GR, n=24. (Present research)	16.3±0.4 ^{a} (15.7-17.0)	1.31±0.29 ^{c} (0.85-1.72)	34.1±2.3 ^{b} (30.1-38.3)	27.3±0.8 ^{b} (24.1-29.1)	4.8±0.8 ^{a} (3.1-6.5)	4.6±0.3 ^{a} (4.1-4.9)
II	Fir-honeydew-GR, n=10. (LAZARIDOU *et al.*, 2003)	14.0±0.7 ^{d} (13.0-15.2)	1.40±0.30 ^{bc} (0.90-1.80)	29.4±1.8 ^{c} (26.0-32.0)	14.2±1.6 ^{e} (11.6-16.8)	1.8±1.5 ^{b} (0.3-4.3)	4.8±0.1 ^{b} (4.6-5.0)
III	Pine-honeydew-GR, n=14. (LAZARIDOU *et al.*, 2003)	15.8±1.4 ^{abc} (13.9-18.9)	1.30±0.20 ^{c} (1.00-1.70)	35.3±3.0 ^{b} (22.0-62.0)	20.0±3.7 ^{d} (12.3-25.8)	2.4±2.7 ^{b} (0.5-9.7)	4.7±0.2 ^{ab} (4.4-4.9)
IV	Fir-honeydew-IT, n=67. (PERSANO ODDO *et al.*, 2000)	15.5±0.9 ^{c}	1.45±0.26 ^{b}	28.4±6.7 ^{c}	23.4±5.8 ^{c}	1.7±2.2 ^{b}	5.2±0.3 ^{c}
V	Honeydew-IT, n=53. (SABATINI and BARBATTINI, 2003)	15.8±0.7 ^{b}	1.74±0.19 ^{a}	41.3±6.5 ^{a}	34.2±7.5 ^{a}	1.7±2.1 ^{b}	5.1±0.4 ^{c}
VI	Forest (*Quercus* spp.)-honeydew-SP, n=16. (MATEO and BOSCH-REIG, 1997, 1998)	15.8±0.5 ^{bc} (14.6-16.4)	0.97±0.11 ^{d} (0.82-1.21)	N.A.	N.A.	N.A.	4.6±0.2 ^{a} (4.4-5.0)
	EU Limits (Council Directive, 2001)	<20.0	>0.8	<50.0	>8.0	<40.0	not determined

ison purposes. Table 2 shows the sugar composition of the honeys. Other parameters that determine the liquid state of the sample, such as fructose and glucose content, fructose/glucose and glucose/water ratio, are summarized for all the honey types in Table 3. All the data are reported as average values ± standard deviations; the minimum and maximum values are given in parentheses.

Water content, a critical characteristic of honeys because it affects shelf life, depends on the type of honey and on the harvesting technique. A high water content helps keep honey liquid but it also increases the risk of fermentation (BACANDRITSOS *et al.*, 2002). The maximum water content value permitted by the European Directive (COUNCIL DIRECTIVE, 2001) is 20% (with the exception of heather (Calluna) and baker's honey that can reach 23%). According to a National Greek norm, Mainalou-Vanilia fir honeydew honey (which takes its name from the place of origin) must have a water content between 14.0 and 15.5% (HELLENIC MINISTRY OF AGRICULTURE, 1994). In the samples examined (type-I) the mean water content value (Table 1) was 16.3±0.4%. This value was not statistically different from the type-III honey (p=0.2000) and was within the limits reported by THRASYVOULOU and MANIKIS (1993, 1995) (<18.0%) and the COUNCIL DIRECTIVE (2001) (<20%). These two honey types were produced from the same insect honeydew (*M. hellenica*). Among the six types of honeydew honeys, the fir honey (type-II) had the lowest mean water content value (14.0%). For fir honeydew honey, THRASYVOULOU and MANIKIS (1993) and BACANDRITOS *et al.* (1993) reported mean water content values of 15.2%; BACANDRITSOS *et al.* (2002, 2003) reported 15.1% and THRASYVOULOU *et al.* (2002) reported 15.7%. For the pine honeydew honey BACANDRITSOS *et al.* (1993, 1999, 2003) reported a mean water content value of 16.6%, while THRASYVOULOU *et*

Table 2 - Sugar content (%) of the fir honey from *Marchalina hellenica* honeydew (type I) and five other honeydew honeys (types II-VI). The data are reported as average values±standard deviations. Minimum and maximum values are given in parentheses. ND indicates values below the detection limit. Values followed by the same letter are not statistically significant (t-test), p≤0.05. NA: data not available.

Honey type		Fructose	Glucose	Sucrose	Trehalose	Maltose	Isomaltose	Raffinose	Erlose	Melezitose	Panose	Isomalto-triose	Malto-triose	Malto-tetraose
I	Fir- honeydew-GR, n=24. (Present research)	27.6±1.4 ^{c} (23.0-29.1)	19.3±1.6 ^{c} (16.1-21.1)	0.3±0.1 ^{ce} (0.1-0.6)	1.7±0.6 ^{a} (0.9-3.0)	1.0±0.2 ^{d} (0.8-1.3)	2.0±0.2 ^{bd} (1.4-2.4)	0.42±0.23 ^{b} (0.10-0.90)	0.31±0.20 ^{b} (0.10-0.80)	0.18±0.12 ^{c} (ND-0.40)	0.76±0.20 ^{a} (0.30-1.20)	0.20±0.07 ^{a} (0.10-0.30)	0.28±0.11 ^{b} (0.10-0.50)	0.32±0.15 ^{b} (0.10-0.80)
II	Fir-honeydew-GR, n=10. (LAZARIDOU *et al.*, 2003)	26.4±3.8 ^{c} (22.1-32.5)	18.1±4.2 ^{c} (13.5-25.0)	0.1±0.1 ^{bf} (0.1-0.2)	2.0±0.9 ^{a} (0.8-3.1)	3.3±1.3 ^{b} (1.9-5.4)	1.0±0.2 ^{e} (0.7-1.2)	0.76±0.23 ^{a} (0.30-1.00)	0.85±0.67 ^{a} (0.40-2.10)	9.52±4.66 ^{a} (1.30-14.40)	0.65±0.36 ^{a} (0.10-1.10)	0.09±0.02 ^{c} (0.02-0.05)	0.35±0.08 ^{a} (0.20-0.50)	0.31±0.09 ^{ab} (0.20-0.05)
III	Pine-honeydew-GR, n=14. (LAZARIDOU *et al.*, 2003)	27.3±3.7 ^{c} (22.2-32.3)	23.2±3.2 ^{b} (18.4-28.7)	0.4±0.4 ^{ace} (0.1-1.4)	0.2±0.2 ^{c} (0.1-0.6)	4.7±0.9 ^{a} (3.9-6.7)	2.0±0.7 ^{abc} (1.0-3.2)	0.49±0.15 ^{b} (0.20-0.60)	1.49±1.09 ^{a} (0.60-3.30)	0.11±0.03 ^{d} (0.10-0.20)	0.32±0.13 ^{b} (0.20 -0.50)	0.13±0.05 ^{b} (0.08-0.20)	0.24±0.06 ^{b} (0.10-0.30)	2.04±3.16 ^{a} (0.20-8.00)
IV	Fir-honeydew-IT, n=67. (PERSANO ODDO *et al.*, 2000)	31.5±2.6 ^{b}	24.1±1.8 ^{b}	0.4±0.4 ^{ac}	2.4	0.8±0.3 ^{e}	2.3±0.9 ^{a}	2.20	0.90	5.00	N.A.	N.A.	<0.10	N.A.
V	Honeydew-IT, n=53. (SABATINI and BARBATTINI, 2003)	31.7±3.3 ^{b}	23.7±2.7 ^{b}	0.1±0.1 ^{bf}	0.2	1.4±0.5 ^{c}	2.0±0.6 ^{bcd}	0.50	1.00	<0,10	N.A.	N.A.	1.00	N.A.
VI	Forest (*Quercus spp*)-honeydew- SP, n=16. (MATEO and BOSCH-REIG, 1997, 1998)	34.3±1.1 ^{a} (32.6-35.9)	25.8±1.5 ^{a} (22.7-28.5)	0.2±0.2 ^{ef} (tr - 0.7)	N.A.	4.9±0.8 ^{a} (3.4-6.2)	1.8±0.9 ^{ad} (0.4-4.5)	0.58±0.35 ^{ab} (0.00-1.26)	(ND-0.52)	0.80±0.69 ^{b} (0.15-3.40)	N.A.	N.A.	N.A.	N.A.

Table 3 - Glucose and fructose content, fructose / glucose ratio, glucose / water ratio of the fir honey from *Marchalina hellenica* (type-I) honeydew and five other honeydew honeys (types II-VI). The data are reported as average values±standard deviations. Minimum and maximum values are given in parentheses. Values followed by the same letter are not statistically significant (t-test), p≤0.05.

Honey type		Fructose + Glucose (%)	Fructose / Glucose ratio	Glucose / Water ratio
I	Fir- honeydew-GR, n=24.	46.9±2.2 ^{c}	1.44±0.14 ^{a}	1.18±0.11 ^{e}
	(Present research)	(43.1-49.7)	(1.14-1.80)	(0.99-1.32)
II	Fir-honeydew-GR, n=10.	44.5±7.9 ^{c}	1.49±0.12 ^{a}	1.28±0.26 ^{de}
	(LAZARIDOU *et al.*, 2003)	(35.7-57.0)	(1.24-1.64)	(0.96-1.68)
III	Pine-honeydew-GR, n=14.	50.5±6.8 ^{c}	1.18±0.06 ^{c}	1.47±0.18 ^{bcd}
	(LAZARIDOU *et al.*, 2003)	(40.9-61.0)	(1.13-1.29)	(1.23-1.76)
IV	Fir-honeydew-IT, n=67.	55.7±3.8 ^{b}	1.31±0.11 ^{b}	1.55±0.13 ^{b}
	(PERSANO ODDO *et al.*, 2000)			
V	Honeydew-IT, n=53.	55.3±4.7 ^{b}	1.35±0.19 ^{b}	1.49±0.16 ^{c}
	(SABATINI and BARBATTINI, 2003)			
VI	Forest (*Quercus spp*)-honeydew- SP, n=16.	60.1±2.6 ^{a}	1.33±0.06 ^{b}	1.63±0.10 ^{a}
	(MATEO and BOSCH-REIG, 1997, 1998)	(55.3-64.4)	(1.22-1.77)	(1.45-1.77)
	EU Limits (Council Directive, 2001)	> 45	not determined	not determined

al. (2002) reported 16.7%. In Greece, the EU limit is considered to be too high because the majority of honeys have a water content around 16% (BACANDRITSOS *et al.*, 2002). POURTALLIER and TALIERCO (1970) reported a value of 17.6% for the French honeys, while DONER (1977) and WHITE (1993) reported a value of 17.2% for American honeys.

In the COUNCIL DIRECTIVE (2001), electrical conductivity was added as a criterion for determining the quality of honeys. This measurement depends on the ash and acid content of the honey. The higher the ash and acid content, the higher the resulting conductivity (VORWOHL, 1964). Honeydew honeys are characterized by their high ash and electrical conductivity values (PERSANO ODDO *et al.*, 1986; CAMPOS *et al.*, 2001; DIEZ *et al.*, 2004). The mean value of the fir honeydew (*M. hellenica*) honey (Table 1, type-I) was 1.31±0.29 mS/cm. In comparison with the other five honey types, the type-I honey did not statistically differ from types-II (p=0.4268) and III (p=0.9009). All the honey types were within the EU limit (>0.8 mS/cm). THRASYVOULOU *et al.* (2002) reported mean conductivity values of 1.34 mS/cm and 1.23 mS/cm for the Greek fir and pine honeydew honeys, respectively.

Honeys have an acidic reaction due to the organic acids they contain. The mean value of the total acidity of the fir honeydew (*M. hellenica*) honey (type-I) was 34.1±2.3 meq/kg. This mean value was not statistically different from the value of the type-III honey (p=0.2047), which was produced from the same coccid honeydew. With regard to the European directive (COUNCIL DIRECTIVE, 2001), the type-I honey examined and the other types (when the data were available) had mean values, which were within the limit (<50 meq/kg). BACANDRITSOS *et al.* (1993, 2002, 2003), BACANDRITSOS and PAPADOPOULOU (1999) and THRASYVOULOU *et al.* (2002) reported mean conductivity values from 30.2 to 31.4 meq/kg

for Greek fir honeydew honey and mean values from 26.1 to 28.9 meq/kg for the Greek pine honeydew honey.

Concerning diastase activity, type-I honey had a mean value of 27.3±0.8 DN. All the honey types met the requirements defined by European legislation (>8 DN). BACANDRITSOS *et al.* (1993, 2002, 2003), THRASYVOULOU and MANIKIS (1993), BACANDRITSOS and PAPADOPOULOU (1999) and THRASYVOULOU *et al.* (2002) reported mean diastase activity values from 18.5 to 21.6 DN for greek fir honeydew honey and mean values from 28.4 to 31.7 DN for greek pine honeydew honey.

Hydroxymethylfurfural (HMF) is one of the criteria upon which the stage of freshness and preservation of honey is defined. Its percentage increases with time and temperature and it is therefore a quality control criterion. HMF is produced following the hydrolysis of fructose in an acidic environment (THRASYVOULOU and MANIKIS 1993). ROGERS (1978) reported that fresh honey rarely exceeds the 10 mg/kg limit. The mean value of the type-I samples examined was 4.8±0.8 mg/kg, which was the highest and only statistically significant value among the other five honeys. All of the mean values of the honey types were within the EU limit (<40 mg/kg). THRASYVOULOU and MANIKIS (1993), THRASYVOULOU *et al.* (2002) and BACANDRITSOS *et al.* (2002, 2003) reported mean HMF values from 2.0 to 3.6 mg/kg for fir honeydew honey and mean values from 2.4 to 5.3 mg/kg for pine honeydew honey.

The mean pH value for the type-I honey was 4.6±0.3, which was not statistically different from the types III (p=0.2266) and VI (p=1.0) honeys. The type-I and type-VI (Spanish honeydew) honeys had the lowest mean values among the six honey types. In contrast, the type-IV (Italian fir honey) had the highest mean value. BACANDRITSOS *et al.* (1993, 2002, 2003), BACANDRITSOS and PAPADOPOULOU (1999), THRASYVOULOU and MANIKIS (1993) and THRASYVOULOU *et al.* (2002) reported mean pH values from 4.7 to 5.3 for fir honeydew honey and mean values from 4.2 to 4.6 for the greek pine honeydew honey.

In honey, fructose and glucose are the predominant monosaccharides, sucrose and maltose are the most important disaccharides, and raffinose, erlose and melezitose are the main trisaccharides, with a high content of melezitose and some other low-molecular oligosaccharides (DONER 1977; DONER and HICKS 1982). The type-I [fir honeydew (*M. hellenica*)] honey had a sugar composition of 27.6±1.4% fructose, 19.3±1.6% glucose, 0.3±0.1% sucrose, 1.0±0.2% maltose, 0.42±0.23% raffinose, 0.31±0.20% erlose and 0.18±0.12% melezitose content. The comparison of the type-I honey with the other honey types revealed some similarities. Firstly, the fructose content was not statistically different from the types II (p=0.3386) and III (p=0.7724) honeys; secondly the glucose content was not statistically different from type II (p=0.3868) and thirdly the sucrose content did not differ greatly from types-III (p=0.3643), -IV (p=0.0623), -VI (p=0.0719) honeys. In reference to the European directive for sucrose content, all of the honey types had mean values within the EU limit (<5.0%). Melezitose, a basic trisaccharide, is found in honeydew honeys. In some honey types the melezitose content is high [type-IV (5.0%), type-II (9.52%)], while in others it is low [type-V (0.1%), type-I (0.18%)]. It seems that the melezitose content could be a criterion for separating the honeydew honey types.

The sum of the fructose and glucose content in the type-I honey had a mean value of 46.9±2.2% (Table 3); it was not statistically different from types -II (p=0.3515) and –III (p=0.0624). The value was greater than 45 g/100 g which is the limit for honeydew honey according to the EU legislation (COUNCIL DIRECTIVE, 2001). The fructose/glucose ratio (Table 3) of the fir honey (type-I) was

1.44±0.14%; which did not differ from the type-II honey (p=0.3004).

In addition, the glucose/water ratio was estimated. MANIKIS and THRASYVOULOU (2001) suggest that the glucose/water ratio is a very useful indicator for predicting honey crystallization. This process, commonly called granulation, is undesirable in liquid honey because it makes the honey less appealing to the consumer. The mean value of the fir honey (type-I) was 1.18±0.11%. This value was in agreement with our observation that the fir honey (type-I) has a low tendency to granulate. Honey crystallises rapidly when the glucose/water ratio is 2.1 and higher (AUSTIN, 1953; JAMIESON, 1954; WHITE *et al.*, 1962; WHITE and JONATHAN, 1975; KODOUNIS, 1962). There are some other ways to predict granulation such as the glucose content (PHILLIPS, 1929; KODOUNIS, 1962; BOGDANOV, 1993), the melezitose content (BOGDANOV, 1993) and the fructose/glucose ratio (JAMIESON, 1954; KODOUNIS, 1962; WHITE and JONATHAN, 1975).

Cluster analysis (Complete Linkage - Euclidean distances) (Fig. 1) of the data revealed two major groups. The first group is comprised of the Greek honeys (I, II and III) while the second group is made up of the two Italian honeys (IV, V) and the Spanish honey (VI). It is interesting to note that within the Greek honey group, those that came from *M. hellenica* honeydew (I and III) were quite similar even though one came from fir tree and the other came from pine tree; the type-II honey which came from the honeydew of the other insects was quite different. It should also be noted that the

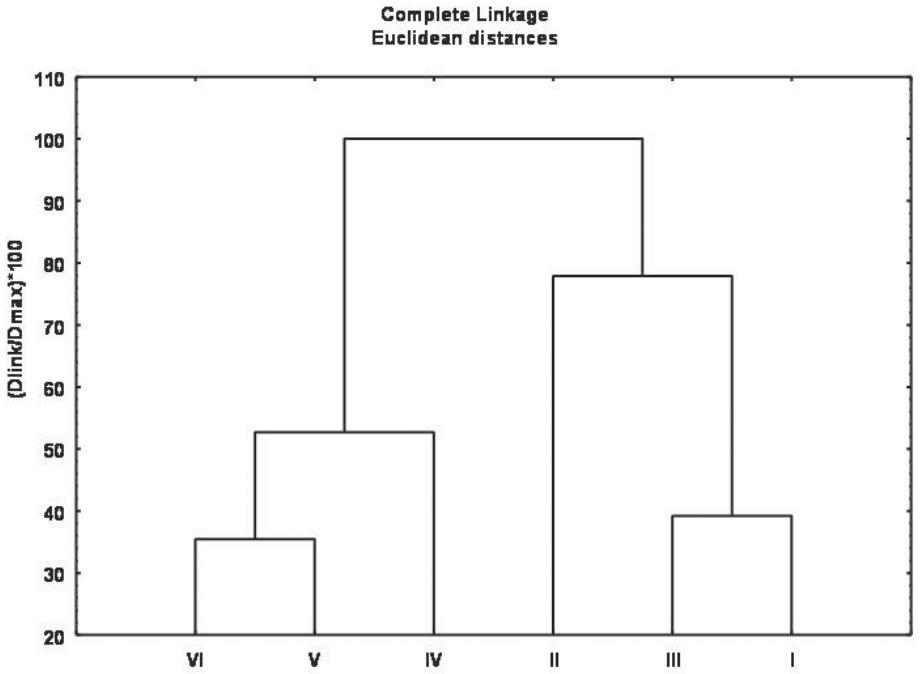


Fig. 1 - Dendrogram (Complete Linkage - Euclidean distances) indicating the similarity of six honeys of different origin: Honey type I (Fir-honeydew-Greece); II (Fir-honeydew-Greece); III (Pine-honeydew-Greece); IV (Fir-insect-Italy); V (various plants-honeydew-Italy); VI (*Quercus* spp-honeydew-Spain).

two Greek fir honeydew honey types (I and II) are quite similar in colour, smell and flavour.

All of the above observations demonstrate the important role that the honeydew honey insect plays in determining the physico-chemical characteristics of the honey. Honeys derived from *M. hellenica* from either fir or pine forests showed similar qualitative characteristics. The spread of *M. hellenica* to other areas, including fir and pine forests, could provide a honey with as standardized quality and low crystallisation tendency in a period that is different from the other fir coccids (fir forest).

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PAPER

INVESTIGATION ON THE MICROFLORA OF CAPRINO LOMBARDO CHEESE FROM RAW GOAT MILK

INDAGINE SULLA MICROFLORA DEL FORMAGGIO CAPRINO LOMBARDO

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ABSTRACT

Samples of raw milk, whey culture and cheese from seven farms located in different areas of Lombardy (Italy) were analysed to investigate the microflora present in the production chain of Caprino Lombardo, a fresh goat cheese traditionally curdled using artisan starters. Lactic acid bacteria, coliforms and enterococci proved to be the main bacterial groups in the raw milk, while lactococci predominated in the artisan whey cultures. The 1-day-old and 15-day-old cheese samples showed, re-

RIASSUNTO

Campioni di latte, innesto naturale e formaggio, raccolti presso sette produttori dislocati in differenti valli della Lombardia, sono stati sottoposti ad analisi al fine di determinare la microflora presente lungo la filiera produttiva del Caprino Lombardo, un formaggio fresco tradizionalmente preparato a partire da latte crudo di capra. Nei campioni di latte i principali gruppi microbici riscontrati sono stati batteri lattici, coliformi ed enterococchi, mentre lattococchi e lieviti sono risultati dominanti

- Key words: Caprino Lombardo cheese, goat milk, *Lactococcus lactis*, *Lactococcus garvieae*, yeast -

spectively, mean log counts: 9.27 and 8.58 CFU/g for lactococci, 5.95 and 7.21 CFU/g for yeasts and 4.41 and 4.07 CFU/g for enterococci. *Lactococcus lactis* subsp. *lactis* was the predominant species in cheese, whereas *L. garvieae*, *Streptococcus thermophilus* and *L. raffinolactis* were present as minor components. The most detected yeast species in cheese samples were *Galactomyces geotrichum*, *Kluyveromyces lactis* and *Yarrowia lipolytica*.

negli innesti naturali. Nei campioni di formaggio appena prodotto e dopo 15 giorni i valori medi di conta erano rispettivamente: lattococchi, 9,27 e 8,58 log UFC/g; lieviti, 5,95 e 7,21 log UFC/g; enterococchi, 4,41 e 4,07 log UFC/g. *Lactococcus lactis* subsp. *lactis* è risultata la specie dominante nel formaggio, mentre *L. garvieae*, *S. thermophilus* e *L. raffinolactis* erano presenti come specie accessorie. *Galactomyces geotrichum*, *Kluyveromyces lactis* e *Yarrowia lipolytica* sono state le specie più frequentemente riscontrate.

INTRODUCTION

In the mountainous regions of Italy, cheese-making from goat milk is one way of improving the economics of farmers living on small farms in remote areas. This, in turn, could help to preserve the human-social web as well as the environment, in territories that could, otherwise, soon become abandoned. Moreover, because of the renewed consumer interest in typical traditional foods and efforts by the authorities to prevent the loss of both the biodiversity and the heritage of rural communities, artisanal cheeses produced without selected starter cultures have come under greater scrutiny, and there has been a marked increase in the scientific knowledge about such cheeses (COGAN *et al.*, 1997; COPPOLA *et al.*, 2001; RANDAZZO *et al.*, 2002).

Caprino Lombardo cheese is one such artisan cheese; its manufacture requires only a small productive unit, no particular technological investment and the use of cultures selected through local cheesemaking practices. Traditional Caprino is a fresh, soft, Northern Italian cheese made from raw goat milk (FOSCHINO *et al.*, 1999). However, there is also a Caprino cheese made from pasteurised cow milk. The traditional Caprino cheese production makes use of artisanal starters derived from the cultured whey of a previous production. These starters allow a slow, acidic curdling of the milk incubated overnight at room temperature. Rennet is added at a very low concentration. After curdling, cloths are used to gently extract the curd that is drained and then salted by mixing with a final NaCl concentration of 0.8-1% (w/w). No preservatives are added. The finished product is typically cylindrical in shape, small in size and chalk white; it is stored under refrigeration and consumed fresh within two weeks. In the year 2000, the Lombardy Region officially recognised Caprino Lombardo cheese as a traditional product. So it is now protected and promoted as such (REGIONE LOMBARDIA, 2000). Moreover, the Italian Ministry of Agriculture has funded a research project oriented at protecting the microbial biodiversity in 44 different Italian cheeses, most of which are produced from raw milk. This has led to numerous studies on the indigenous microbial population present in the Italian dairy environments (GIRAFFA *et al.*, 2003).

The aim of our work was to characterise the autochthonous microflora

present throughout the production chain of Caprino Lombardo cheese. This was done by analysing samples of raw milk, artisan whey starters and cheese collected from artisan cheese-makers located in different areas of the Lombardy region.

MATERIALS AND METHODS

Farms

Seven goat farms, labelled from A to G, were selected on the basis of herd size (from 10 to 100 milking head), type of breed (Alpine for B, D; Saanen for A, C, and E; both for F and G) and geographical area (mountainous districts of Bergamo for A, B and C; Lecco for D, E and F; Varese for G). Farms A, C, D and F milked manually twice a day, whereas farms B, E and G used milking machines; after milking, the raw milk was refrigerated, except on farm A. None of the cheese-makers used selected starter cultures or preservatives in the manufacturing of Caprino Lombardo cheese.

Sampling

Samples (150 mL or g) of raw milk, whey culture starter, 1-day-old cheese and cheese at the end of its shelf life (15-day-old cheese) stored at 5°C, were aseptically collected from each farm in April, July and September and chilled at 5°C until analysis, which was carried out within 24 h. The samples were prepared for microbiological examination according to the International Dairy Federation Standard 122B:1992 (IDF, 1992). The pH of the undiluted samples was determined with a combined glass-calomel electrode connected to a pH-meter (Gibertini DP 100-NE, Italy).

Microbiological counts

After suitable dilutions, the following microbiological counts were made:

Lactic acid bacteria, plated by spreading on HHD agar (Biolife, Italia) (MC DONALD *et al.*, 1987) and modified in this work by adding 0.5% lactose, were incubated at 30°C for 72 h in anaerobic conditions (GasPack® Anaerobic System, BBL, Cockeysville, MD, USA). This medium was used to enhance discrimination among the colonies formed by different strains.

Enterococci, plated by spreading on Kanamycin Aesculin Azide agar (Merck, Darmstadt, Germany) (MOSSEL *et al.*, 1978), were incubated at 44°C for 24 h. The incubation temperature was increased with respect to the original description to improve medium selectivity.

Coliforms, plated on Violet Red Bile Lactose agar (Difco, Kansas City, MO, USA), were incubated at 30°C for 24h according to the International Dairy Federation Standard 73B:1998 (IDF, 1998).

Yeasts and moulds, plated by spreading on Yeast Glucose Chloramphenicol agar (Merck, Darmstadt, Germany), were incubated at 25°C for 5 days according to International Dairy Federation Standard 94B:1990 (IDF, 1990).

Coagulase-positive staphylococci were plated by spreading on Baird-Parker agar (Oxoid, Basingstoke, UK) and then incubated at 37°C for 48 h according to the International Dairy Federation Standard 145A:1997 (IDF, 1997).

Isolation and preliminary classification of bacteria

To investigate the microflora composition, a representative collection of dominant bacterial forms was isolated from modified HHD plates at high dilutions of samples of 1-day-old cheese. The colonies were selected by morphology, counted separately, and purified by two subsequent subcultures in the same medium. The isolates were then observed microscopically and a preliminary classification was made on the basis of pheno-

type, analysed by: Gram staining, catalase reaction, CO_2 production from D-glucose and sodium D-gluconate, NH_3 production from arginine, hydrolysis of aesculine, growth at 10°, 40° and 45°C, growth at 4 and 6.5% (w/v) NaCl, coagulation of reconstituted 10% (p/v) skim milk after 24 h at 30°C. Isolates were maintained at -30°C in modified HHD broth with 20% (v/v) glycerol.

Isolation and preliminary classification of yeasts

As already described above, representative colonies were isolated from YGC plates at high dilutions of 1-day-old cheese samples. Isolates were purified by two subsequent subcultures in YEPD agar (ANDRIGHETTO *et al.*, 2000) and then a preliminary classification was done on the basis of phenotype, by using: the microscopic appearance of the cell, urease reaction, nitrate assimilation, erythritol, cellobiose and mannitol assimilation, growth with cycloheximide, glucose and maltose fermentation according to DEAK and BEUCHAT (1996). Assimilation capability on different carbohydrates was investigated by the API ID 32 C system (BioMerieux, Marcy l'Etoile, France). Isolates were maintained at -30°C in YEPD broth with 20% v/v glycerol.

DNA extraction

Bacterial cells were grown overnight at 30°C in modified HHD broth; 1 mL of cell suspension was then centrifuged and washed in distilled water. The pellet was resuspended in 100 µL of TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA) containing 40 µg/mL of mutanolysine and 25 mg/mL of lysozyme and incubated at 37°C for 1 h. After centrifugation at 8,000 *g* for 10 min, the pellets were resuspended in 500 µL of 50 mM Tris-HCl, pH 7.4 and 20 mM EDTA, mixed with 50 µL 10% w/v SDS and incubated at 65°C for 30 min. Samples were chilled in ice. Two-hundred microlitres of 5 M potassium acetate were added to the samples to precipitate proteins, which were then centrifuged at 14,000 g for 5 min. The supernatants were mixed gently with an equal volume of cold propanol to precipitate nucleic acids. After centrifugation and washing with 70% v/v ethanol, the DNA was resuspended in 100 µL of TE buffer.

Yeast cells were grown overnight at 25°C in YEPD broth under agitation, centrifuged and washed in distilled water. The pellets were resuspended in 100 µL TE buffer and the nucleic acids were extracted according to the protocol of QUEROL *et al.* (1992), modified by using 25 mg/mL of lytic enzyme from *Rhizoctonia solani* (in 1M sorbitol, 0.1 M EDTA, pH 7.5), and incubating at 45°C for 2 h in order to digest the cell wall. The DNA was then precipitated as described above.

PCR protocols for bacterial isolates

DNA amplifications were performed in a 25 µL reaction mixture containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM each of dATP, dCTP, dGTP and dTTP, 1 U of *Taq* polymerase (Applied Biosystem) and 80 ng of DNA. For the 16S-23S Spacer Region Analysis, 0.5 µM of forward primer G1 (5'-GAAGTCGTAACAAGG-3') and 0.5 µM of reverse primer L1 (5'-CAAGGCATCCACCGT-3') were used (JENSEN *et al.*, 1993); the temperature profile repeated for 30 cycles was: denaturation step at 94°C for 45 s; annealing step at 58°C for 45 s; extension step at 72°C for 2 min. As confirmation tests for some species, specific PCR assay were performed according to the authors; DNA sequences of the primers and relevant gene targets were His1 (5'-CTTCGTTATGATTTTACA-3') and His2 (5'-CAATATCAACAATTCCAT-3') for histidine biosynthesis operon in *L. lactis* (CORROLER *et al.*, 1999), pLG-

1 (5'-CATAACAATGAGAATCGC-3') and pLG-2 (5'-GCACCCTCGCGGGTTG-3') for 16S rRNA in *L. garvieae* (ZLOTKIN *et al.*, 1998), St1 (5'-CACTATGCTCAGAATACA-3') and St2 (5'-CGAACAGCATTGATGTTA-3') for lacZ gene in *S. thermophilus* (LICK *et al.*, 1996). A T-Gradient Biometra Thermocycler (Biometra, Göttingen, Germany) was used.

PCR protocols for yeast isolates

DNA amplifications were performed in a 25 µL reaction mixture as reported above. For RAPD PCR, two separate primers were used: 1 µM primer M13 with the following temperature profile repeated for 35 cycles: denaturation step at 94°C for 1 min, annealing step at 40°C for 20 s, ramp to 72° at 0.6°C/s and extension step at 72°C for 2 min; 1 µM primer RF2 (5' CGGCCCCTGT 3') (PAFFETTI *et al.*, 1995) with the following temperature profile repeated for 35 cycles: denaturation step at 95°C for 30 s, annealing step at 36°C for 1 min and extension step at 72°C for 1 min and 30 s. Amplification products were resolved by electrophoresis in 2% (w/v) agarose TAE (40 mM Tris-HCl, pH 8.2; 1 mM EDTA) gels, stained with ethidium bromide and photographed under UV illumination.

DNA sequence analysis

A portion of the 16S rDNA gene was sequenced for 23 representative bacterial isolates. The PCR amplification was performed in a 100 µL reaction mixture, with 0.5 µM forward primer (5' CTACGGCTACCTTGTTACGA 3') and 0.5 µM reverse primer (5' AGAGTTTGATCCTGGCTCAG 3'); the temperature profile repeated for 35 cycles was: denaturation step at 94°C for 45 s; annealing step at 50°C for the first five cycles and at 55°C for the remaining for 45 s; extension step at 72°C for 2 min.

A portion of the internal transcribed spacers between 18S and 26S rDNA genes (ITS1-5.8S-ITS2) was sequenced for 14 representative yeast isolates. The PCR amplification was performed in a 100 µL reaction mixture, as reported above, with 0.1 µM of forward primer 2234C (5' GTTTCCGTAGGTGAACCTGC 3') and 0.1 µM of reverse primer 3126T (5' ATATGCTTAAGTTCAGCGGGT 3') as described by MONTROCHER *et al.* (1998). The temperature profile repeated for 35 cycles was: denaturation step at 95°C for 1 min, annealing step at 50°C for 1 min and extension step at 72°C for 1 min. A T Gradient Biometra Thermocycler (Biometra, Göttingen, Germany) was used.

The amplified products were purified by QIAquick spin columns (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions and sequenced with ABI Prism® BigDye™ Terminators v 3.0 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) in an ABI Prism® 310 Genetic Analyser. For the 16S rDNA bacterial fragment and the ITS1-5.8S-ITS2 yeast region, the relevant forward primers were used. The BLAST program was employed to search the GenBank database for homologous sequences.

RESULTS

Monitoring of raw milk, artisanal whey culture and cheese

All the farmers made Caprino Lombardo cheese from goat milk, milked from the farmer's own herd. Table 1 shows the log counts and pH values of the dairy samples from each farm; the data are the means of three separate tests done at different times (April, July and September).

The lactic acid bacteria proved to be the predominant component of the microflora in the raw milk samples (mean log count = 4.69 CFU/mL, SD =1.07) with the samples from farm A having

Table 1 - Mean log counts (CFU/mL or CFU/g) of microorganisms and pH values in raw goat milk, artisanal whey culture and Caprino Lombardo cheese samples according to farm.

Sample	Farm	Lactic Acid Bacteria (CFU/mL or CFU/g)	Enterococci (CFU/mL or CFU/g)	Yeasts (CFU/mL or CFU/g)	Coliforms (CFU/mL or CFU/g)	Coagulase-positive Staphylococci (CFU/mL or CFU/g)	pH
Raw	A	5.75±0.62	3.10±0.71	4.20±0.84	3.84±0.32	< 3.00	6.49±0.05
milk	B	4.09±0.12	< 3.00	< 3.00	< 3.00	< 3.00	6.74±0.04
	C	4.50±0.71	< 3.00	< 3.00	4.04±1.65	3.54±1.74	6.80±0.57
	D	5.01±1.11	3.00±1.18	< 3.00	< 3.00	3.80±0.15	6.76±0.09
	E	3.83±0.75	< 3.00	3.44±0.20	3.68±0.11	< 3.00	6.83±0.04
	F	4.93±0.42	< 3.00	< 3.00	4.22±1.38	< 3.00	6.73±0.03
	G	4.56±1.92	3.13±1.17	3.69±1.96	3.74±0.62	< 3.00	6.77±0.06
Natural	B	9.32±0.35	< 3.00	3.54±0.95	3.95±2.76	< 3.00	4.52±0.14
whey	C	8.77±0.37	3.02±0.03	4.35±0.90	< 3.00	< 3.00	4.49±0.16
culture	D	9.40±0.13	3.60±0.42	4.00±1.44	< 3.00	< 3.00	4.66±0.08
	E	9.17±0.45	< 3.00	4.93±1.63	< 3.00	< 3.00	4.53±0.38
	F	9.30±0.27	< 3.00	3.82±1.74	< 3.00	< 3.00	4.40±0.14
	G	9.23±0.56	3.65±0.49	4.76±0.39	5.11±0.66	< 3.00	4.63±0.18
1-day-old	A	8.96±1.16	5.15±0.21	6.72±0.25	7.79±0.25	4.90±0.89	5.03±0.52
cheese	B	9.43±0.48	< 3.00	4.33±0.58	< 3.00	3.47±0.82	4.09±0.07
	C	9.21±0.41	4.63±1.49	6.23±1.30	3.67±1.15	< 3.00	4.15±0.12
	D	9.28±0.48	3.13±1.59	5.89±1.25	< 3.00	4.54±0.09	4.08±0.22
	E	9.26±0.40	4.35±2.05	6.20±1.42	3.15±0.21	< 3.00	3.95±0.15
	F	9.51±0.48	4.46±1.78	5.93±0.95	< 3.00	< 3.00	3.96±0.06
	G	9.19±0.44	4.55±0.38	6.34±1.23	5.12±1.46	3.10±0.15	4.17±0.12
15-day- old	A	9.06±0.49	5.49±0.16	7.73±0.56	6.67±1.89	< 3.00	4.84±0.25
cheese	B	7.97±0.96	< 3.00	6.57±1.03	< 3.00	< 3.00	4.19±0.16
	C	8.79±0.12	4.15±1.03	8.13±0.21	< 3.00	< 3.00	4.10±0.34
	D	8.60±0.69	3.06±0.08	6.96±0.99	< 3.00	< 3.00	3.95±0.22
	E	8.92±0.27	3.73±0.18	7.23±0.75	< 3.00	< 3.00	4.27±0.34
	F	8.33±0.48	4.39±1.70	7.11±0.70	< 3.00	< 3.00	4.13±0.37
	G	8.40±0.28	3.73±0.66	6.50±0.65	4.51±1.83	< 3.00	4.09±0.05

Table 2 - Phenotypic and genotypic characteristics of bacterial isolates from Caprino Lombardo cheese samples according to farm.

Farm	Isolate	% distribution of strains for sample			NH_3 from ARG	Growth at			Growth at 6.5% NaCl	Aesculine fermen-tation	Milk coagulation after 24 h	Band (bp) in RSA profile	Identification by 16S rDNA sequence
		April	July	September		10°C	40°C	45°C					
A	123	86			+	+	+	-	+	-	-	420	
	5	8			+	+	+	-	+	-	+	420	
	45	3			+	+	+	-	+	-	+	380	
	46	3			-	+	+	-	-	+	-	450	*L. raffinolactis*
	47		84		+	+	+	-	+	-	+	380	
	6		15		+	+	-	-	+	-	+	380	
	302		1		+	+	+	-	-	-	+	380	
	7			56	+	+	+	-	+	+	+	420	*L. garvieae*
	49			31	+	+	+	-	-	+	+	380	*L. lactis* subsp. *lactis*
	48			6	+	+	+	-	+	-	+	420	
	50			5	+	+	+	-	-	-	+	380	
	8			2	+	+	+	-	+	+	+	380	*L. lactis* subsp. *lactis*
B	67	48			+	+	+	-	-	-	+	380	
	15	43			+	+	+	-	+	-	+	380	
	16	7			+	+	+	-	+	-	+	380	
	65	1			+	+	+	-	+	-	+	380	
	66	1			+	+	+	-	-	-	+	380	
	69		30		+	+	+	-	+	-	+	380	
	17		26		+	+	+	-	+	+	+	380	*L. lactis* subsp. *lactis*
	28		23		+	+	+	-	+	+	+	380	*L. lactis* subsp. *lactis*
	304		18		+	+	+	-	+	-	+	380	
	68		3		+	+	+	-	-	+	+	380	*L. lactis* subsp. *lactis*
	29			58	+	+	+	-	+	+	+	420	
	95			22	+	+	+	-	+	-	-	380	
	31			20	+	+	+	-	+	+	+	420	
C	76b	54			+	+	+	-	+	-	+	380	*L. lactis* subsp. *lactis*
	160	42			+	+	+	-	+	+	+	380	
	129	4			+	+	+	-	-	+	+	380	*L. lactis* subsp. *lactis*
	120		39		+	+	+	-	+	-	+	380	
	112		30		+	+	+	-	+	+	+	380	*L. lactis* subsp. *lactis*
	161		30		+	+	+	-	-	-	+	380	
	119		1		+	+	+	-	+	-	+	380	
	113			51	+	+	+	-	+	-	+	380	
	103			47	+	+	+	-	+	-	+	380	
	104			2	-	-	+	+	-	-	+	350	*S. thermophilus*

Farm	Isolate	% distribution of strains for sample			NH_3 from ARG	Growth at			Growth at 6.5% NaCl	Aesculine fermentation	Milk coagulation after 24 h	Band (bp) in RSA profile	Identification by 16S rDNA sequence
		April	July	September		10°C	40°C	45°C					
D	142b	46			+	+	+	-	+	-	-	380	
	83b	33			+	+	+	-	+	-	+	380	
	143	18			+	+	+	-	-	+	+	380	*L. lactis* subsp. *lactis*
	84b	3			+	+	+	-	+	-	+	380	
	146		47		+	+	+	-	-	+	+	380	*L. lactis* subsp. lactis
	85b		43		+	+	-	-	+	-	+	380	
	86b		6		+	+	+	-	+	-	+	380	
	144b		3		+	+	+	-	+	+	+	380	*L. lactis* subsp. *lactis*
	145b		1		+	+	+	-	+	+	+	380	*L. lactis* subsp. *lactis*
	163			64	+	+	+	-	+	+	+	380	*L. lactis* subsp. *lactis*
	164			23	+	+	+	-	+	+	+	380	*L. lactis* subsp. *lactis*
	147			8	+	+	+	-	+	-	+	380	
	87b			5	+	+	-	-	+	+	+	380	
E	174	50			+	+	+	-	+	-	+	380	
	301	50			+	+	+	-	+	-	+	380	*L. lactis* subsp. *lactis*
	185		32		+	+	-	-	-	-	+	380	
	176		25		+	+	+	-	+	-	+	380	
	175		20		+	-	+	+	-	-	+	350	
	177		18		-	-	+	+	-	-	+	350	*S. thermophilus*
	184		5		-	+	+	-	-	-	+	380	
	178			54	+	+	+	-	-	-	+	380	
	220			27	+	+	+	-	-	+	+	380	*L. lactis* subsp. *lactis*
	186			19	+	+	+	-	-	-	+	380	
F	243	100			+	+	+	-	+	-	+	380	
	212		38		+	+	+	-	+	-	+	380	
	257		31		+	+	+	-	+	-	+	380	
	211b		13		+	+	+	-	+	-	+	380	
	256		8		+	+	+	-	+	-	+	380	
	254		7		+	+	-	-	-	-	-	380	
	255		3		+	+	+	-	-	-	+	380	
	210			27	+	+	+	-	+	+	-	420	
	213			23	+	+	+	-	+	+	-	420	*L. garvieae*
	204			23	+	+	+	-	+	+	+	380	*L. lactis* subsp. *lactis*
	226			17	+	+	+	-	+	-	+	380	
	227			10	+	+	+	-	+	-	+	380	

Farm	Isolate	% distribution of strains for sample			NH_3 from	Growth at			Growth at 6.5%	Aesculine fermen-	Milk coagulation	Band (bp) in RSA	Identification by 16S rDNA sequence
		April	July	September	ARG	10°C	40°C	45°C	NaCl	tation	after 24 h	profile	
G	281	33			+	+	+	-	+	-	+	380	
	282	29			+	+	+	-	-	-	-	380	
	280	27			+	+	+	-	-	-	-	380	
	284	10			+	+	+	-	-	-	+	380	
	129b	1			+	+	+	-	-	-	+	380	
	286		64		+	+	+	-	+	-	-	380	
	287		23		+	+	+	-	+	-	+	380	
	264		7		+	+	+	-	+	-	-	380	
	285		6		+	+	+	-	+	-	-	380	
	300			62	+	+	-	-	+	+	+	420	*L. garvieae*
	299			38	+	+	-	-	+	-	+	380	

the highest concentration. On this farm, the milk was ripened at room temperature prior to adding the rennet because the farm was not equipped with a refrigerated tank, and no culture was used as starter. The findings varied with regard to enterococci. The samples from four farms had log counts < 3.00 CFU/mL, while the samples from the other farms showed, on average, a log count of 3.17 CFU/mL (SD = 0.82). The log count of moulds was always < 3.00 CFU/mL, while on three farms yeasts were present in the samples at a mean log count of 3.78 CFU/mL (SD = 1.02). Coliforms were detected at a mean log count of 3.93 CFU/mL (SD = 0.93) in samples from five of the seven farms. Samples from farms C and D showed coagulase-positive staphylococci counts that were over the limit fixed by European regulations (E.C.D.,1992). The pH values of raw milk ranged from 6.49 to 6.83, with an overall mean of 6.75 (SD = 0.22).

Artisanal whey cultures were dominated by lactic acid bacteria with a mean log count of 9.19 CFU/mL (SD = 0.39), whereas half the samples showed the presence of enterococci, but at low levels (mean log count = 3.42 CFU/mL, SD = 0.43). While moulds were never observed at the dilutions tested, yeasts were well evident; they were a constant component of the starter population with a mean log count of 4.20 CFU/mL (SD = 1.10). Low pH values (mean 4.54, SD = 0.19) inhibited the growth of coagulase-positive staphylococci and kept the coliforms under control, since the log count was often < 3.00 CFU/mL. Farmer A did not use any starter.

In the 1-day old cheese samples, lactic acid bacteria reached, on average, a log count of 9.27 CFU/g (SD = 0.53) and the pH values dropped to a mean value of 4.20 (SD = 0.40), confirming regular fermentation during the overnight incubation at room temperature. Enterococci were found at a mean log count of 4.41 CFU/g (SD = 1.27) with notable differences among the samples from the different farms. Yeasts were present at a significant level (mean log count 5.95 CFU/g, SD 1.15), whereas the mould log count was < 3.00 CFU/g. Coliforms were unevenly detected (mean log count = 5.09 CFU/g, SD = 2.06), except for chees-

es from farms A and G, where the counts were always high. The coagulase-positive staphylococci had a log count < 3.00 CFU/g for samples from farms C, E and F, while the samples from the other farms showed a mean log count of 3.95 CFU/g (SD = 1.09). The cheeses made on farms A and D aroused concern because the coagulase-positive staphylococci count exceeded the maximum limit set by European Council Directive 92/46/ECC (E.C.D., 1992) for dairy products made from raw milk.

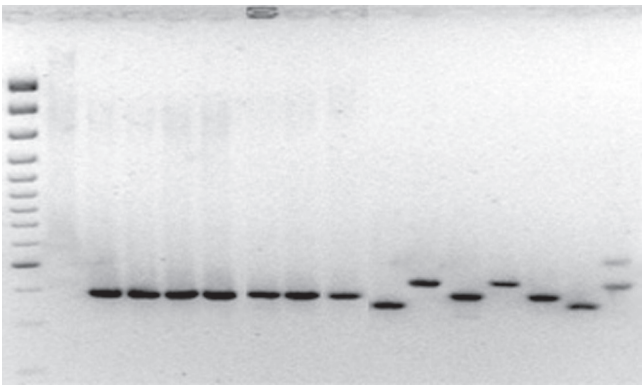


Fig. 1 - RSA profiles of some representative bacterial isolates and relevant type strains. Lane 1: marker 100 bp DNA Ladder Plus (Fermentas, Lithuania); lane 2: negative control; lane 3, isolate 49; lane 4, isolate 8; lane 5, isolate 17; lane 6, isolate 54; lane 7, isolate 18; lane 8, isolate 112; lane 9, isolate 143; lane 10, isolate 177; lane 11, isolate 7; lane 12, *L. lactis* subsp. *lactis* DSM 20481^T; lane 13, *L. garvieae* DSM 20684^T; lane 14, *L. lactis* subsp. *cremoris* DSM 20069^T; lane 15, *St. thermophilus* DSM 20617^T; lane 16, *Enterococcus faecium* ATCC 19434^T.

With regard to cheese samples maintained at 5°C for 15 days, the concentration of lactic acid bacteria and enterococci decreased, with respective mean log counts of 8.58 CFU/g (SD = 0.58) and 4.07 CFU/g (SD = 1.15). Conversely the yeasts reached, on average, a log count of 7.21 CFU/g (SD = 0.82) thus proving that they are significant components of the microflora of Caprino Lombardo cheese. Moulds were not observed at the tested dilutions (log count < 5.00 CFU/g). Maintenance in an acidic environment (mean value of pH 4.22, SD = 0.36) probably caused the decrease in the coliform counts, that were detected in samples from A and G, and the coagulase-positive staphylococci counts, where the log count was < 3.00 CFU/g in all samples.

Classification of bacterial isolates from 1-day-old cheese samples

Eighty-one isolates were selected from modified HHD plates that came from high dilutions and were counted separately on the basis of colony morphology. The aim was to determine the percent distribution of the various strains in the lactic acid bacteria population. All the isolates were paired or short-chain cocci, Gram positive, catalase negative, had a homofermentative metabolism and were able to grow in 4% NaCl; 95% of the isolates produced NH_3 from arginine, 96% were able to grow at 10°C, 93% at 40°C but only 4% at 45°C; 69% grew at 6.5% NaCl, 30% hydrolysed aesculine and 21% did not coagulate the milk within 24 h (Table 2). On the basis of the 16S-23S rDNA PCR patterns, the isolates were first ascribed at the species level. Nine isolates were recognized as *Lactococcus garvieae* since they produced a unique band migrating at approximately 420 bp. This identification was confirmed by the species-specific PCR assay (ZLOTKIN *et al.*, 1998). Sixty-eight isolates were attributed to *Lactococcus lactis* as they exhibited a single band located at 380 bp; the application of a PCR targeted for the histidine biosynthesis operon (CORROLER *et al.*, 1999) proved that all 68 *L. lactis* isolates belonged to the subspecies *lactis*, since they amplified only the expected fragment of 933 bp (data not shown). Three isolates showed an amplification fragment of 350 bp that is indicative for *Streptococcus thermophilus*, *S. macedon-*

icus and *S. salivarius*; the specific PCR assay attributed the strains to the first species (LICK *et al.*, 1996). Only one isolate revealed a band of 450 bp, which is indicative of *Lactococcus raffinolactis* (BLAIOTTA *et al.*, 2002). Figure 1 shows the RSA profiles of some representative strains.

Classification of yeast isolates from 1-day-old cheese samples

In order to assess the incidence of the yeast species in samples of 1-day-old cheese, 51 isolates were selected from YGC plates at high dilution. Microscopic observations revealed 65% of them had at an oval shape with buds, while the remaining 35% were cylindrical cells that often had a pseudo-mycelium. None of the isolates hydrolysed urea or assimilated nitrate, whereas 16% assimilated erythritol, 33% cellobiose and 71% mannitol. Ninety-two percent of the isolates were able to grow with cycloheximide; 82% assimilated galactose or D-L lactate, but only 37% grew with lactose as the sole carbon source. The identification by phenotypic analyses attributed 18 isolates to *Candida spherical*, 18 to *Geothrichum* spp., 8 to *C. lipolytica*, 2 to *C. zeylanoides*, and 1 to each of *C. rugosa*, *C. lusitaniae* and *C. kefir*; 2 isolates were not identifiable (Table 3). The dendrogram based on RAPD-PCR pattern analysis revealed that the yeast strains grouped into nine clusters at a similarity level of 65% (Fig. 2). For some representative isolates of each cluster, a portion of the Internal Transcribed Spacers was sequenced in order to confirm the classification. The first (cluster 1) gathered the patterns of eight isolates belonging to *Y. lipolytica*, whereas cluster 2 was made up of two isolates from cheese from farm D that were identified as *C. zeylanoides*. Clusters 3 and 4 were formed by only one strain, ascribed respectively to *Clavispora lusitaniae* and *C. zeylanoides*. The fifth group (cluster 5) gath-

Table 3 - Phenotypic and genotypic characteristics of yeast isolates from Caprino Lombardo cheese samples according to farm.

Farm	Isolate	Assimilation of						Growth with cycloheximide	Fermentation of glucose	Identification by API ID 32C	RAPD profile (N° cluster)	Identification by ITS sequence
		cellobiose	erythritol	galactose	lactate	lactose	mannitol					
A	AFF2b	-	-	+	-	-	+	-	+	*C. rugosa*	4	*C. zeylanoides*
	AFF3b	+	-	+	+	+	+	+	-	*C. sphaerica*	5	
	AF2a	+	-	+	+	+	+	+	+	*C. sphaerica*	5	
	AFF2a1	+	-	+	+	+	+	+	-	*C. sphaerica*	5	
	AFF2a2	-	-	+	+	-	+	+	-	*Geotrichum* spp.	9	
	AFF3a	-	-	+	-	-	-	+	-	*Geotrichum* spp.	9	
	AF3c	-	-	+	+	-	+	+	-	*Geotrichum* spp.	9	
	AF3b	+	-	+	+	+	+	+	+	*C. sphaerica*	5	*K. lactis*
	AF3a	-	-	+	+	+	-	+	+	*C. sphaerica*	5	
B	BF3a	-	-	+	+	-	-	+	-	*Geotrichum* spp.	9	
	BFF3a	-	-	+	+	-	-	+	-	*Geotrichum* spp.	9	
C	CFF2a	+	-	+	+	+	+	+	-	*C. sphaerica*	5	*K. lactis*
	CF3b	+	-	+	+	-	+	+	-	*C. sphaerica*	5	
	CF2b	+	-	+	+	+	+	+	-	*C. sphaerica*	5	

Farm	Isolate	Assimilation of						Growth with cycloheximide	Fermentation of glucose	Identification by API ID 32C	RAPD profile (N° cluster)	Identification by ITS sequence
		cellobiose	erytrhritol	galactose	lactate	lactose	mannitol					
	CFF3b	-	+	-	+	-	+	+	-	*C. lipolytica*	1	
	CF3a	-	+	-	-	-	+	+	-	*C. lipolytica*	1	
	CF3c	-	-	+	+	+	+	+	-	*C. sphaerica*	5	
	CFF3a	+	-	+	+	+	+	+	-	*C. sphaerica*	5	
	CF2a	-	+	+	+	-	+	+	-	*C. lipolytica*	1	
D	DF2a	+	-	+	-	+	+	-	+	*C. lusitaniae*	3	*Clav. lusitaniae*
	DFF2b	-	+	-	+	-	+	+	-	*C. lipolytica*	1	*Y. lipolytica*
	DFF3a	+	+	-	+	-	+	+	-	*C. lipolytica*	1	
	DF3a	-	-	+	+	+	+	+	-	*C. sphaerica*	5	
	DF3b	-	-	-	-	-	+	+	-	*C. zeylanoides*	2	
	DFF3b	-	-	-	-	-	+	+	-	*C. zeylanoides*	2	*C. zeylanoides*
	DFF2a	-	-	+	+	-	-	+	-	*Geotrichum* spp.	9	
	DFF2c	-	-	+	+	+	+	+	+	*C. sphaerica*	5	
E	EF2a	-	-	+	+	-	-	+	-	*Geotrichum* spp.	9	
	EFF3b	+	-	+	+	+	+	+	-	*C. sphaerica*	5	*K. lactis*
	EFF2a	-	-	+	+	-	-	+	-	*Geotrichum* spp.	9	
	EF3b	+	-	+	+	+	+	+	-	*C. sphaerica*	5	
	EF3a	-	-	+	+	-	-	+	-	*Geotrichum* spp.	9	
	EFF2c	+	-	+	+	+	+	+	-	*C. sphaerica*	5	
	EFF2b	-	+	-	+	-	-	+	-	*C. lipolytica*	1	
	EFF3a	-	-	+	+	-	+	+	-	*Geotrichum* spp.	9	*G. geotrichum*
F	FF3a	-	-	+	-	-	+	+	-	*Geotrichum* spp.	9	
	FFF2a1	-	+	-	+	-	+	+	-	*C. lipolytica*	1	
	FFF2a2	-	-	+	+	+	+	+	-	*Geotrichum* spp.	9	
	FFF3b	+	-	+	+	-	+	+	-	*C. sphaerica*	5	
	FFF3a	-	+	-	+	-	+	+	-	*C. lipolytica*	1	*Y. lipolytica*
G	GFF3a	-	-	+	+	-	+	+	-	*Geotrichum* spp.	9	
	GF2a	-	-	+	+	-	-	+	-	*Geotrichum* spp.	9	*G. geotrichum*
	GFF3c	-	-	+	-	-	-	-		inacceptable	7	*S. cerevisiae*
	GFF2a	+	-	+	+	-	+	+	-	*Geotrichum* spp.	9	
	GFF3b	-	-	+	+	+	-	+	+	*C. kefir*	6	*K. marxianus*
	GF3b	-	-	+	+	-	-	+	-	*Geotrichum* spp.	9	
	GF3c	+	-	+	+	+	+	+	+	*C. sphaerica*	5	*K. lactis*
	GF3d	-	-	+	-	-	-	-		inacceptable	8	
	GF3a	-	-	+	+	-	+	+	-	*Geotrichum* spp.	9	*G. geotrichum*
	GFF2b	+	-	+	+	+	+	+	-	*C. sphaerica*	5	
	GFF2c	-	-	+	+	-	+	+	-	Geotrichum spp.	9	

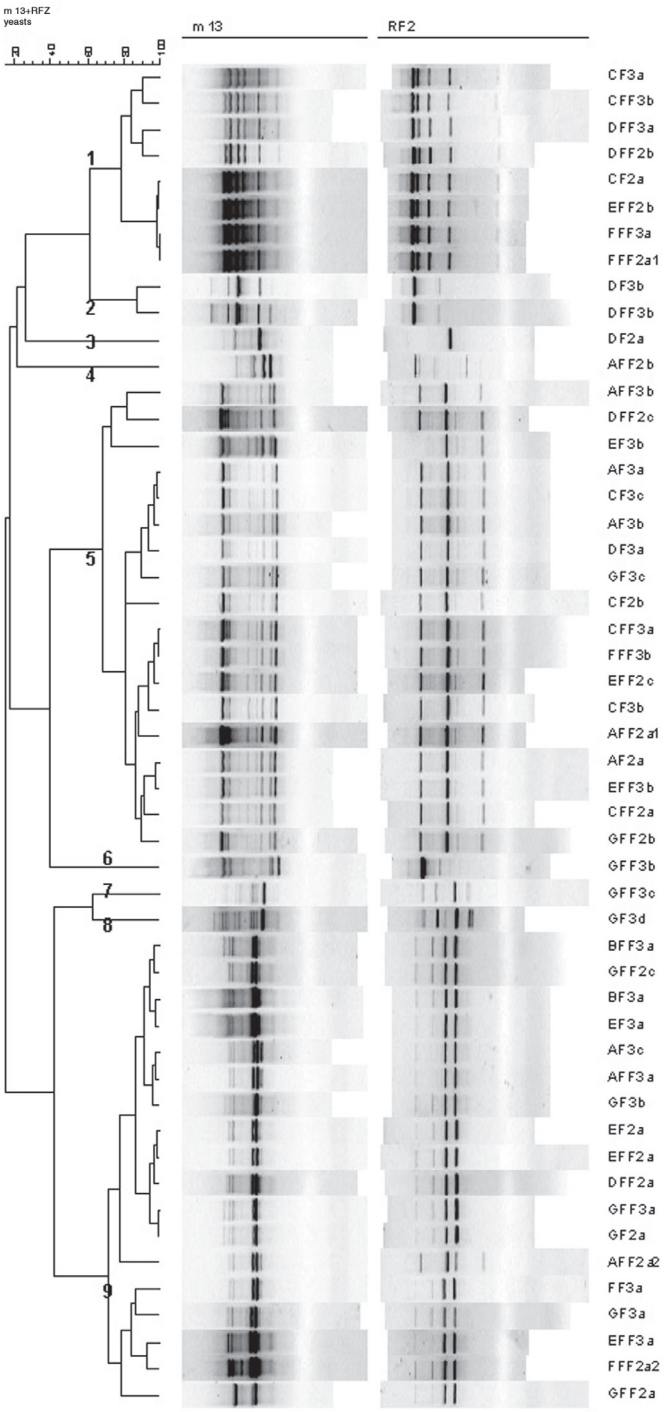


Fig. 2 - Cluster analysis of RAPD-PCR patterns from yeast strains obtained with primers M13 and RF2. Numbers indicate cluster names. Distance values between branches in the dendrogram are reported as percentage of similarity (0-100%).

ered RAPD profiles of 18 isolates attributed to *K. lactis*; a further differentiation into sub-clusters at a level of more than 80% similarity was observable without any evident link with farm or time of isolation. Cluster 6 was composed of a single strain of *K. marxianus*, whereas two strains of *S. cerevisiae* isolated from farm G were positioned in different clusters (7 and 8). The ninth cluster grouped the patterns of 18 isolates ascribed to *Galactomyces geotrichum* collected from samples from most of the farms.

DISCUSSION

Raw goat milk is rich in lactic acid bacteria; thus it is naturally apt for cheesemaking in accordance with what has been described for Tenerife (ZARATE *et al.*, 1997), Avenato (HATZIKAMARI *et al.*, 1999) and Ibores (MAS *et al.*, 2002) cheeses in which the predominant species is *Lactococcus lactis* subsp. *lactis*, and which are traditionally produced without the addition of starter cultures. On the other hand, hygienic markers such as coliforms and staphylococci are secondary, but constant, components of the microflora found in goat milk (LODI 1993; ZARATE *et al.*, 1997; MAS *et al.*, 2002). Therefore, it cannot be excluded that potentially hazardous germs are present in the cheese production chain (BIELASZEWSKA *et al.*, 1997; FOSCHINO *et al.*, 1999; 2002). Artisan cheese-makers however, reject the idea of subjecting the raw milk to thermal treatment for economic reasons but also because they believe that such treatment has a negative effect on the sensorial quality of the end product. Thus the safety of traditional Caprino Lombardo cheese has to be achieved by improving the microbiological quality of the milk, and by the controlled use of starters for the rapid and safe acidification of the curd. It was not by chance that the highest coliforms and coagulase-positive staphylococci levels were found in the cheese samples collected from farm A where artisanal whey cultures were not used. The cheese from this farm also had the highest pH values. Top priority must be given to insure that hygiene measures are implemented, and compulsory, with regard to the equipment and practices used in animal care, milking, and chilling of milk during storage and cheese processing. Artisan whey cultures were confirmed to be the functional source of lactic acid bacteria for regular milk fermentation processes. Nevertheless, the yeasts detected in all the whey samples were responsible for a significant microbial presence that marked the typicity of the product. The microflora of Caprino Lombardo cheese consists mainly of lactococci and yeasts, whose counts increase during storage at 5°C. It should also be noted that, despite the absence of preservatives, no moulds were visible in any of the samples. This observation and data, already reported in the literature (SUZZI *et al.*, 2001; VILJOEN, 2001), suggest that yeasts play a positive role in both the sensorial characterisation and shelf-life extension of the product by causing the formation of aromatic compounds or relevant precursors due to proteolytic and lipolytic activities and ethanol production. Though remaining at low pH leads to a decrease in coagulase-positive staphylococci of $<10^3$ CFU/g after 15 days, it does not appear sufficient enough to reduce the coliform numbers, as already reported for other fresh goat cheeses (ZARATE *et al.*, 1997; MAS *et al.*, 2002).

Investigations of the dominant microflora of dairy products are often carried out by collecting and identifying a few randomly selected colonies from high dilution plates; this approach does not always lead to accurate knowledge of the species distribution in the matrix. In the present work, the actual incidence of the species in the bacterial population of cheese samples was investigated by monitoring the phenotyp-

ic and genotypic characteristics of each strain, taking into account the frequency of the colony morphology during the isolation from modified HHD plates. As regards the identification of the bacterial isolates, *Lactococcus lactis* subsp. *lactis* was dominant in 81% of all the samples collected, whereas, surprisingly, *Lactococcus garvieae* was the main species in the remaining 19% of the samples were obtained from three of the seven farms. As already described by other authors (CORROLER *et al.*, 1998; 1999; MANNU *et al.*, 2000; FORTINA *et al.*, 2003) physiological tests commonly used to differentiate the species among the genus *Lactococcus* failed to identify wild strains: for instance, in this work, 69% of the *L. lactis* subsp. *lactis* isolates were able to grow at 6.5% NaCl. Even the real occurrence of *L. garvieae* can be underestimated if molecular identification techniques are not used, because the phenotypic traits of this species are very similar to the *L. lactis* group. Till now, *L. garvieae* has occasionally been isolated from dairy sources as a minor milk component (AN *et al.*, 2004) and curd (MOREA *et al.*, 1999) or as an agent of mastitis in cow and water buffalo (TEIXEIRA *et al.*, 1996; VELA *et al.*, 2000). Nevertheless, when a genetic approach to strain classification is adopted, the incidence of this species in dairy samples increases, as FORTINA *et al.* (2003) recently reported in samples of Toma Piemontese, a traditional Italian PDO cheese made from raw cow milk without the addition of any starter culture. The presence of *L. garvieae* in the dairy chain should be further investigated in order to determine a possible technological role, or potential pathogenicity, since this species has frequently been associated with episodes of septicaemia in fish (ELDAR *et al.*, 1996; 1999; VELA *et al.*, 2000).

As for the yeasts, *Galactomyces geotrichum* (anamorph *Geotrichum candidum*), *Kluyveromyces lactis* (anamorph *Candida sphaerica*) and *Yarrowia lipolytica* (anamorph *Candida lipolytica*) were mainly isolated from Caprino Lombardo cheese; they were found in 85, 71 and 43% of the samples respectively; these yeasts are psycrotrophs that can metabolise lactate. They have proteolytic and, for *G. geotrichum* and *Y. lypolytica*, lipolytic activities that contribute to the organoleptic features of the cheese. Different authors have also shown evidence of a meaningful incidence of the same species in fresh and short-ripened cheeses such as Caprino (ANDRIGHETTO *et al.*, 2000), water-buffalo Mozzarella (ROMANO *et al.*, 2001, SUZZI *et al.*, 2000), Feta (COSENTINO *et al.*, 2001; FADDA *et al.*, 2001; PSOMAS *et al.*, 2001) and Rokpol cheeses (WOJTATOWICZ *et al.*, 2001). The API 32 ID test has proven to be a suitable tool for the classification of yeasts in the dairy environment since between 48 to 51 isolates (96%) were identified properly when the results of the phenotypic analysis were compared with those obtained with RAPD finger-printing and ITS sequencing (Table 3). Moreover, RAPD-PCR using primers M13 and RF2 has been confirmed to be a useful technique for discriminating yeast at the species level, as previously stated by various authors (ANDRIGHETTO *et al.*; 2000, SUZZI *et al.*; 2000, PSOMAS *et al.*, 2001). Yeast development in traditional Caprino Lombardo cheese is expected, given that the product's high acidity, medium moisture content, lack of preservatives and low storage temperature are factors that support the proliferation of fungi. Furthermore, the constant presence of yeasts in artisan whey cultures and cheeses is beneficial. They are a major component of the microflora that contributes to ripening and flavour development; therefore such yeasts should be considered a distinctive trait of the typicity of the product. No relationships between the prevalence of species in cheese and the period of isolation or territory were observed.

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PAPER

FATE OF *LISTERIA MONOCYTOGENES* DURING THE MANUFACTURE AND STORAGE OF CHEVRE METSOVO AND PICHTOGALO CHANION CHEESES

EVOLUZIONE DI *LISTERIA MONOCYTOGENES* DURANTE LA PRODUZIONE E LO STOCCAGGIO DEI FORMAGGI CHEVRE METSOVO E PICHTOGALO CHANION

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ABSTRACT

A mixture of pasteurized and raw goat's milk inoculated with different populations of *Listeria monocytogenes* was used in the manufacture of Chevre Metsovo cheese (pH>5.0). *L. monocytogenes* grew rapidly with maximum populations up to 8.38-8.78 $\log_{10}$ CFU/g after 5-10 d (strain Scott A) or 7.60-7.78 $\log_{10}$ CFU/g after 30 d (strain CA). In separate experiments a pasteurized mixture of ewe's and goat's milk inocu-

RIASSUNTO

Nella produzione di formaggio Chevre Metsovo (pH>5) è stata impiegata una miscela di latte di capra, pastorizzato e non, inoculata con differenti popolazioni di *Listeria monocytogenes*. La *L. monocytogenes* cresceva rapidamente fino ad un massimo di carica batterica di 8.38-8.78 $\log_{10}$ CFU/g dopo 5-10 giorni (ceppo Scott A), o 7.60-7.78 $\log_{10}$ CFU/g dopo 30 giorni (ceppo CA). In altre sperimentazioni, latti di capra e di

- Key words: Chevre Metsovo cheese, *Listeria monocytogenes,* Pichtogalo Chanion cheese -

lated with *L. monocytogenes* was made into Pichtogalo Chanion cheese (pH at 4.43). The population of *L. monocytogenes* decreased rapidly and listeriae were not countable after 2-10 days. Depending on the number of cells in the initial inoculum, *L. monocytogenes* was not detected between 5 and 20 days after cheesemaking using cold enrichment. *L. monocytogenes* survived in frozen 48 h cheese at -20°C for one to more than three months.

pecora sono stati inoculati con *L. monocytogenes*, per la produzione del formaggio Pichtogalo Chanion (pH 4.43). In quest'ambiente sfavorevole, la popolazione di *L. monocytogenes* diminuiva rapidamente e non risultava possibile effettuare una conta delle Listeriae dopo 2-10 giorni. In funzione del numero di cellule nell'inoculo iniziale, la *L. monocytogenes* non era presente fra i 5-20 giorni dopo il processo di caseificazione effettuata con il metodo di arricchimento a freddo. La *L. monocytogenes* sopravviveva da uno fino a più di tre mesi nel formaggio di 48 ore congelato a -20°C.

INTRODUCTION

Soft cheeses, contaminated with *Listeria monocytogenes* have often been incriminated as the causative agents of human foodborne listeriosis (LINNAN *et al.*, 1988; BILLE *et al.*, 1988; BULA *et al.*, 1995). *L. monocytogenes* has often been found in raw ewe's and goat's milk, obtained from clinically healthy or diseased herds. The microorganism has been recovered from 0 to 43% of the milk samples examined (HIRD and GENIGEORGIS, 1990; GREENWOOD *et al.*, 1991; RODRIGUEZ *et al.*, 1994). In Greece, *Listeria* spp. have been found in 3.75% of raw cow's milk, 6% of raw goat's milk, 6% of mixtures of raw ewe's and goat's milk samples and 20% of environmental samples taken from 8 cheese–processing plants (GENIGEORGIS *et al.*, 1991b). The exact number of *L. monocytogenes* in raw milk is not known, but GREENWOOD *et al.* (1991) estimated fewer than 200 CFU/mL of milk. According to WHO (1988), this number is expected to be much lower in bulk tanks, because the various sources from which the milk has been collected are not all contaminated.

L. monocytogenes behaves differently in different types of cheese depending on the different physico-chemical conditions during the manufacture, ripening and storage of the cheese [e.g. starter cultures, acidity, temperature and duration of ripening, moisture (a_w), salt and salt in moisture phase]. It has been reported that *L. monocytogenes* grows modestly during the early stages of Cheddar, Feta and Blue cheese production and then ceases to grow when the pH drops below 5.0, with *L monocytogenes* populations decreasing significantly during ripening and storage (RYSER and MARTH, 1987a; PAPAGEORGIOU and MARTH, 1989a;b). Substantial growth of *L. monocytogenes* occurs in Camembert and Brick cheese during the ripening process when the pH is above 5.75 (RYSER and MARTH, 1987b; 1989). The growth of *L. monocytogenes* in market cheeses was correlated with high pH (>5.5) and the absence of starter cultures during cheesemaking (GENIGEORGIS *et al.*, 1991a). The pathogen grows in Greek whey cheeses with pH values ranging from 6.0 to 6.5, even at refrigeration temperatures (PAPAGEORGIOU *et*

al., 1996). *L. monocytogenes* survives for more than 40 d in Touloumotyri cheese at a pH of 4.53, but it is inactivated within 16-27 d in Galotyri cheese at a pH of 4.35 (PAPAGEORGIOU *et al.*, 1998a).

L. monocytogenes is likely to be present in the environment of small, traditional cheese-processing plants, or in raw ewe's and goat's milk, which is usually not pasteurized during traditional cheesemaking. In this study, the behavior of *L. monocytogenes* during the manufacture, ripening and storage of Chevre Metsovo (CM) and Pichtogalo Chanion (PC) cheeses was investigated. CM, a hard cheese with a pH of ca. 5.5, is traditionally produced in the area of Epirus, from a mixture of raw and pasteurized goat's milk. PC is a soft white spreading cheese with Protected Designation of Origin, with a pH of ca 4.5. It is traditionally produced on the island of Crete from ewe's and/or goat's milk (raw or pasteurized). *L. monocytogenes* was found in 4 out of 62 samples of PC cheese analyzed by PAPAGEORGIOU *et al.* (1998b). This study was undertaken to determine whether the conditions during manufacture, ripening and storage of these two cheeses support the growth and/or survival of *L. monocytogenes*.

MATERIALS AND METHODS

Cultures

L. monocytogenes Scott A and California (CA) were used in this study. Cultures were maintained at 4°C on tryptose agar (TA) (Difco/Becton Dickinson, Sparks, MD) slants and were transferred monthly. Working cultures used to inoculate milk for the manufacture of PC and CM cheeses were prepared as described by PAPAGEORGIOU and MARTH (1989c). The final culture contained ca. $1.75x10^8$ CFU of *L. monocytogenes*/mL. A sufficient volume of this culture was dispersed in 100 mL of pasteurized milk to yield the desirable concentration of *L. monocytogenes* when added to the milk used for cheesemaking.

Lyophilized *Lactococcus lactis* subsp. *lactis, Lactobacillus casei* subsp. *pseudoplantarum, Lactobacillus casei* subsp. *casei* and *Lactococcus lactis biovar diacetylactis* strains isolated from traditionally manufactured PC cheese (PAPAGEORGIOU *et al.*, 1998b) were regenerated in sterile skimmed cow's milk, which was incubated for 24 h at 23°C. To manufacture PC cheese, a sufficient volume of each of these cultures was added to the cheese milk, so as to contain 1.3, 1.3, 1 and 0.4%, of each culture, respectively. *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* were grown in sterile skimmed cow's milk for 24 h at 42°C, and then used (0.2% v/v) for the manufacture of CM cheese.

Cheesemaking

Chevre Metsovo cheesemaking

Six liters of pasteurized (63°C/30 min) goat's milk and 6 L of raw goat's milk were placed in a small stainless steel cheese-vat placed in a larger pilot-plant-sized cheese-vat filled with water. The milk was inoculated with *L. monocytogenes* (at different levels ranging from ca. $2x10^1$ to $4x10^3$ CFU/mL); after warming the milk to 30°C, 0.2% (v/v) of the starter culture was added. After 15 min 50 mg of rennet diluted in 50 mL tap water was added. After 1 h the curd was cut into 0.5-inch cubes and was left undisturbed for 10 min. The cut curd was then transferred into two round metal hoops (18 cm diameter), 0.3% ground black pepper sterilized in a dry-air oven (1 h/180°C) was sprinkled onto the curd using a salt shaker and the curd was allowed to drain for 24 h at 17°C. The hoops were turned twice at 8 h intervals. After draining, the cheese was placed in a 19% sterile salt-brine for 24 h at 17°C. The cheese was ripened for 60 d at 17°C at a relative humidity of 80-85% and

subsequently stored at 4°C for 60 d. The 30-d cheeses were then coated with paraffin to minimize moisture loss.

Pichtogalo Chanion cheesemaking

A mixture of equal volumes of whole raw ewe's and goat's milk was obtained from a local dairy plant and was pasteurized in an open vat at 63°C for 30 min. Eight liters of pasteurized milk were placed in the same small cheese-vat described earlier and inoculated with *L. monocytogenes* at different levels ranging from ca. $2x10^2$ to $2x10^5$ CFU/mL. After warming the milk to 23°C, 4% (v/v) of the starter culture was added. Fifteen min later, 34 mg of rennet diluted in 50 mL tap water was added; coagulation took place within ca. 2 h. After standing at 23°C for 24 h for souring, the curd was transferred without cutting into hoops and was allowed to drain for 8 h. After draining, 1% salt was added and the cheese was refrigerated. Forty-eight hours later, half of the cheese was frozen at -20°C in ca. 150 g portions placed in sterile stomacher bags, and the rest was stored at 4°C.

Microbiological analyses of cheeses

Sampling

Milk samples of 25 mL were used to determine the presence of, or to count the *L. monocytogenes* cells from: a) the milk of the cheese-vat before inoculation and b) the inoculated milk, immediately after inoculation. Also, milk samples of 11 mL were used to count the lactic acid bacteria (LAB) cells immediately after the starter cultures were added. For the CM cheese trials, the samples for counting the *L. monocytogenes* and LAB cells and determining pH were taken at the following stages: a) curd in hoops before draining b) whey after hooping c) curd in hoops after 24 h of draining d) fresh 48 h cheese just after salting in brine e) 5, 10, 20, 30, 40, 60, 90 and 120 d cheese during ripening and storage. For the PC cheese trials, the samples for counting the *L. monocytogenes* and LAB cells and determining the pH were taken at the following stages: a) 2, 4, 6, 8, 10, 12, 14, 22 and 24 h curd b) 32 h curd after draining and before the addition of salt c) drained whey d) refrigerated cheese at 2, 5, 10, 15, 20, 25 and 30 d and e) frozen cheese at 15, 30, 45, 60, 75 and 90 d. At each sampling, a frozen sample was thawed at 35±1°C (within 1 h) and duplicate 10 g cheese samples (one from the outer 1.5-2.0 cm of the cheese and the second from the center of the cheese) were tested. Samples in which *L. monocytogenes* was not detectable by plating were re-examined after cold enrichment.

Enumeration of microorganisms

Twenty-five milliliters or grams of milk, whey or cheese were homogenized with 225 mL of warm (42±1°C) sterile 1% tryptose broth (TB) supplemented with 2% sodium citrate (Sigma-Aldrich Co. Ltd., Gillingham, UK) in sterile stomacher bags (Stomacher 400, Techmar Co. Inc., Cincinnati, OH) for 3 min. Appropriate serial decimal dilutions were made using warm sterile 2% sodium citrate solution.

L. monocytogenes was counted by surface-plating 0.2 mL of milk or appropriate curd- or cheese-dilutions on lithium chloride phenylethanol moxalactam (LPM) agar (LEE and MCCLAIN, 1986). Plates were incubated aerobically at 35°C for 48 h. Typical, blue-green colonies on LPM agar were counted and a number of them, equal to their square root, were confirmed as *L. monocytogenes* by Gram staining, observation of tumbling motility under wet mount, catalase reaction, esculin hydrolysis, production of acid from rhamnose, xylose, mannitol and a-methyl-D-mannoside and CAMP test, as described by LOVETT (1987).

Cold-enrichment was performed by incubating the primary dilution (10^{-1}) in sterile TB at 4°C for up to 8 weeks. Every

2 weeks, a drop of the culture was placed on the surface of 3 LPM agar plates. The drop was surface-plated with a bent rod, and the same rod was used to consecutively spread the remaining 2 Petri dishes. Plates were incubated aerobically at 35°C for 48 h.

The LAB were counted in MRS and M17 agar (OXOID, Hampshire, U.K.). One-mL of the appropriate dilutions was pour-plated in 15 mL of agar and plates were overlaid and incubated anaerobically at 35°C for 72 h (Gaspak system, BBL Microbiology Systems, Cockeysville, MD.).

Physico-chemical analyses of cheeses

Duplicate fat and moisture determinations were made for each trial according to the Standard Methods for the Examination of Dairy Products (BRADLEY *et al.*, 1993). Duplicate NaCl determinations were made on 10 g cheese samples using the Quantab Chloride Titrator test strip method (Miles Laboratories Inc., Elkhart, IN).

At the time of testing for *L. monocytogenes*, the pH values were measured using a pH meter (type S22, Wissenschatflich-Technische Werkstätten, Germany). For CM cheese, 10 g were homogenized with 20 mL of distilled water, the electrode was immersed into the emulsion and when a stable measurement was obtained the pH value was recorded. For PC cheese, the pH measurement was obtained by directly inserting the pH-electrode into the soft cheese mass.

Calculation of growth rate and statistical methods

Generation times for *L. monocytogenes* during CM cheese manufacture were calculated (for all 6 trials) using the following formula: generation time = $1/\log P_2 - \log P_1/0.301\ (T_2-T_1)$, where T_1 = time of P_1, T_2 = time of P_2, P_1 = CFU/g at T_1 and P_2 = CFU/g at T_2.

For statistical analysis of the results obtained in all 8 trials after the first day of PC cheesemaking, a reliability and survival analysis was carried out with the MINITAB package (release 11/96).

RESULTS AND CONCLUSIONS

Composition of Chevre Metsovo and Pichtogalo Chanion cheeses

The moisture content of CM cheese was 35.51% (S.D. ± 1.31) and the fat in the dry matter (FDM) was 47.03% (S.D. ± 0.54). The salt content was 3.01% (S.D. ± 0.13) and the pH of the 90 and 120 d ripened cheeses was 5.18 (S.D. ± 0.29) and 5.16 (S.D. ± 0.29), respectively. The same parameters for the PC cheese were as follows: moisture content 64.45% (S.D. ± 2.82); FDM 55.33% (S.D. ± 4.41) and salt 1.25% (S.D. ± 0.4). The pH of the 2 d cheese was 4.23 (S.D. ± 0.19). The Greek regulatory standards for PC cheese specify that it must not contain more than 65% moisture or less than 50% FDM (Greek Codex Alimentarius, 1989); these two criteria were met in all the trials. No standards are specified for CM cheese.

Growth of *L. monocytogenes* during the manufacture and ripening of Chevre Metsovo cheese

The milk used for all 6 trials was free of *L. monocytogenes* (measured by direct plating and cold-enrichment for up to 8 weeks) prior to inoculation with the pathogen. The milk was inoculated at 4 different levels so as to contain ca. $2x10^1$, $2x10^2$, $4x10^2$ and $4x10^3$ CFU/mL of strain Scott A and at 2 different levels of ca. 10^2 and 10^3 CFU/mL of strain CA. The behavior of *L. monocytogenes* strains Scott A and CA during the manufacture and ripening of CM cheese is shown in Fig. 1. No growth of *L. monocytogenes* was observed during the first 2 h prior to, and during curdling. *L. monocytogenes* populations increased during the whey drainage phase and the day following cheesemaking, the pathogen pop-

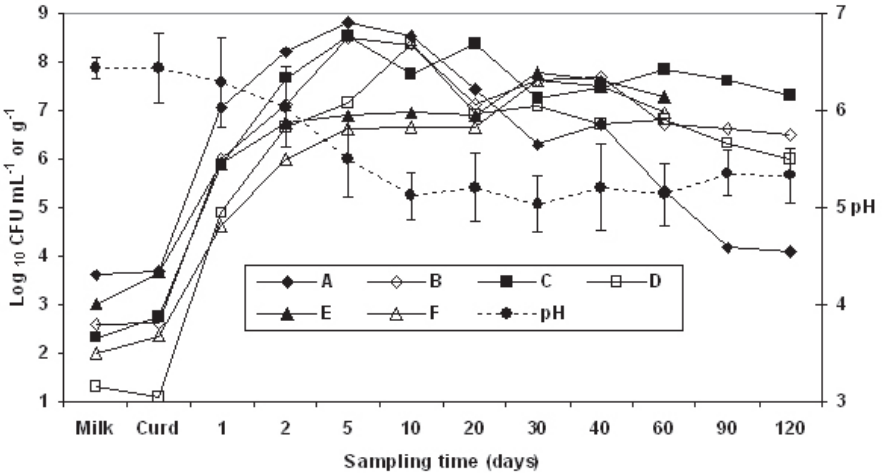


Fig. 1 - Growth of *L. monocytogenes* (strain Scott A, trials A-D; strain CA trials E-F) and changes in pH during the manufacture and ripening of Chevre Metsovo cheese. Temperatures were 17°C (ripening, days 1-60) and 4°C (storage, days 61-120).

ulations reached levels of 4.88 to 7.07 and 4.60 to 5.91 $\log_{10}$CFU/g for strains Scott A and CA, respectively. The generation times of *L. monocytogenes* in the first day (from the time of curd formation) were 2.11 h (S.D. ± 0.13) and 3.24 h (S.D. ± 0.04) for strains Scott A and CA, respectively. The pathogen continued to grow during cheese-ripening and the generation times of the second day were 5.23 h (S.D. ± 1.12) and 6.96 h (S.D. ± 1.73) for strains Scott A and CA, respectively (Fig. 1).

The maximum populations of *L. monocytogenes* Scott A were reached after 5-10 d and ranged from 8.38 to 8.78 $\log_{10}$ CFU/g. The maximum populations of strain CA were only reached after 30 d and ranged from 7.60 to 7.78 $\log_{10}$ CFU/g but were already at high levels (6.60 to 6.88 $\log_{10}$ CFU/g) by day 5. The *L. monocytogenes* populations in CM cheese during the 2-month ripening period at 17°C and after 2 months of storage at 4°C remained high (6.00 to 7.30 $\log_{10}$CFU/g) except for one batch inoculated with strain Scott A in which the pathogen count decreased to 4.07 $\log_{10}$CFU/g. It is important to note that low levels of thermophilic starter were used [*L. delbrueckii* subsp. *bulgaricus* 2.69 (S.D. ± 0.61) $\log_{10}$CFU/mL and *S. thermophilus* 4.70 (S.D. ± 0.70) $\log_{10}$CFU/mL of milk], but these increased slowly during cheesemaking and after 5 d of ripening the populations of lactobacilli (MRS agar counts) and streptococci (M17 agar counts) were 8.74 (S.D. ± 0.20) and 9.03 (S.D. ± 0.43) $\log_{10}$CFU/g, respectively (data not shown). Later, the LAB population declined slightly during ripening, with MRS and M17 agar counts in the 60 d ripened cheeses ranging from 7.00 to 8.34, and from 7.74 to 8.59 $\log_{10}$CFU/g, respectively. The LAB levels remained relatively constant during the subsequent 60 d storage of CM cheese at 4°C.

As shown in Fig. 1 the pH of CM cheese decreased slowly during the first 5 d and remained above 5.0 for all trials. Market samples of this cheese manufactured by traditional cheesemakers also had pH values around 5.5. The high pH and ripening temperature of 17°C are very favorable for *L. monocytogenes* multiplication. The 3.01% salt content in CM cheese (calculated salt in water phase 7.83 ± 0.50) permitted the

growth of *L. monocytogenes*. Other studies have shown that the same *L. monocytogenes* strains did not grow and their populations gradually became inactive in whey or skim milk containing 12% NaCl. However, these strains grew in the same batches of whey and skim milk containing 6% NaCl, even at 4°C (PAPAGEORGIOU and MARTH, 1989c). Modest growth of *L. monocytogenes* was also reported during the first stages of production of Cheddar cheese (RYSER and MARTH, 1987a), Feta cheese (PAPAGEORGIOU and MARTH, 1989a) and Blue cheese (PAPAGEORGIOU and MARTH, 1989b). In these experiments, growth ceased when the pH in the cheeses dropped below 5.0 and the populations decreased markedly during ripening and storage. *L. monocytogenes* can also grow in market cheeses at pH values above 5.5 (GENIGEORGIS *et al.*, 1991a) and in the whey cheeses, Myzithira, Anthotyros and Manouri, with high pH values of ca. 6.4 (PAPAGEORGIOU *et al.*, 1996). The results of this work are in agreement with those reported in the literature and confirm that CM cheese, having a high pH value (>5.0) and ripened at 17°C, supports the growth of *L. monocytogenes* (Fig. 1).

Fate of *L. monocytogenes* during the manufacture and storage of Pichtogalo Chanion cheese

The milk used for all the trials was free of *L. monocytogenes* (measured by direct plating and cold-enrichment for up to 8 weeks) prior to inoculation with the pathogen. A pasteurized mixture of ewe's and goat's milk was inoculated with *L. monocytogenes* Scott A to contain ca. $2x10^2$ CFU/mL in trials A, C and E, and ca. $2x10^3$ CFU/mL in trials B, D and F. A higher inoculum of ca. $2x10^5$ CFU/mL was used in trials G and H. In trial G, raw milk was used and no starter culture was added, while in the other seven trials 4% mesophilic starter culture was added (consisting of 1.3% *Lactococcus lactis* subsp. *lactis*, 1.3% *Lactobacillus casei* subsp. *pseudoplantarum*, 1% *Lactobacillus casei* subsp. *casei* and 0.4% *Lactococcus lactis biovar diacetylactis*). A high level of mesophilic starter culture is also used by traditional cheesemakers on the island of Crete for the production of PC cheese (PAPAGEORGIOU *et al.*, 1998b).

The behavior of *L. monocytogenes*, strain Scott A during the manufacture of PC cheese is shown in Fig. 2. The *L. monocytogenes* populations remained stable during the first 8-12 h of cheesemaking, except for trial B, in which a modest growth of ca. 0.45 $\log_{10}$CFU/g of curd was noted. *L. monocytogenes* populations decreased rapidly by 1.23 and 1.25 $\log_{10}$CFU/g in trials A and B, respectively during the subsequent 12 h of cheesemaking, whereas the populations in the other six trials remained relatively stable. This difference in the fate of *L. monocytogenes* in trials A and B could be attributable to the earlier decrease in the pH of these cheeses.

After souring the curd for 24 h, *L. monocytogenes* populations decreased significantly ($P<0.001$) in all the trials and the pathogen was finally inactivated after 5-10 d of cheese storage at 4°C in trials A through F and after 20 d of storage in trials G and H (Fig. 3). It is important to note that trials G and H were conducted after the results of trials A through F had been obtained, and the inoculum of the pathogen in these two trials was intentionally higher. As a result, the pathogen survived for an additional 10 d in trials G and H even though the most substantial loss of *L. monocytogenes* viability occurred during the first 10 d of cheese storage at 4°C. *L. monocytogenes* decreased by 2.74 and 3.90 $\log_{10}$CFU/g after 5-10 d (trials A through F) and by 5.59 and 5.49 $\log_{10}$CFU/g after 10 d (trials G and H) and then dropped to non-detectable levels. In addition, the results of cold-enrichment were negative for the PC cheese samples stored at 4°C beyond the above-mentioned survival

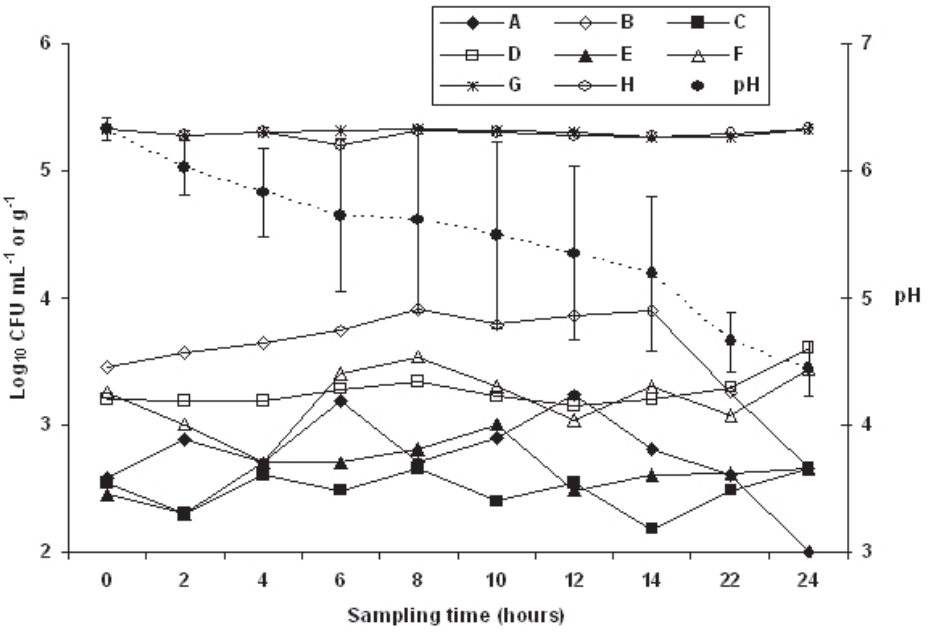


Fig. 2 - Behavior of *L. monocytogenes* Scott A and changes in pH during the manufacture of Pichtogalo Chanion cheese.

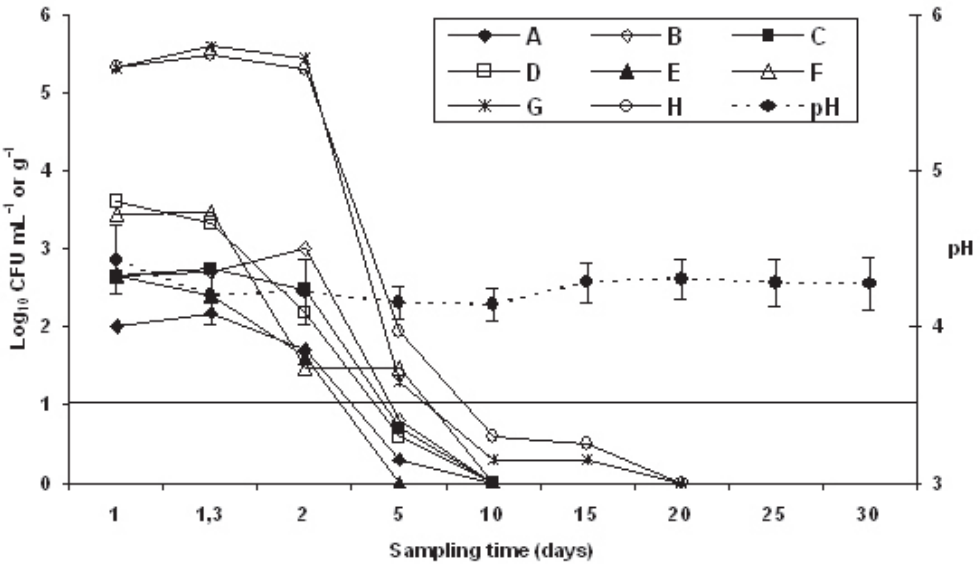


Fig. 3 - Loss of viability of *L. monocytogenes* Scott A and changes in pH during storage of Pichtogalo Chanion cheese at 4°C. Values below 1 $\log_{10}$CFU/g represent positive results obtained after cold enrichment. Values at 0 $\log_{10}$CFU/g represent negative results obtained after cold-enrichment.

times of *L. monocytogenes*. The statistical analysis of the results obtained after the first day of PC cheesemaking in all the trials indicated that the average estimated time required for one decimal reduction of the *L. monocytogenes* population (estimated D-value) during this sharp inactivation phase in PC cheese was 1.81 (S.D. ± 0.40) d. Results from previous work in our laboratory (PAPAGEORGIOU *et al.*, 1998a) indicated that the death rate of *L. monocytogenes* was the same during the storage of Galotyri cheese (pH = 4.35) at 5°C (estimated D-value 1.24 to 2.97 d).

The pH during PC cheesemaking decreased rapidly and after souring the curd at 23°C for 24 h, the pH values in all the trials was ca. 4.43 (S.D. ± 0.20), which was unfavorable for the subsequent survival of *L. monocytogenes*. It is also important to note that after the addition of 4% mesophilic starter culture, the average populations of lactobacilli and lactococci in the milk were equal to 7.55 (S.D. ± 0.45) and 7.51 (S.D. ± 0.52) $\log_{10}$CFU/g, respectively (data not shown). Also, in trial G the initial populations (at time 0) of lactobacilli and streptococci (MRS and M17 agar counts) were high (7.90 $\log_{10}$CFU/g each). The LAB population increased during curd souring at 23°C for 24 h with MRS and M17 agar counts for all trials reaching 8.70 (S.D. ± 0.74) and 8.85 (S.D. ± 0.58) $\log_{10}$CFU/g, respectively. These high LAB levels remained relatively constant during the storage of PC cheese at 4°C for 30 d.

Survival of *L. monocytogenes* during frozen storage of Pichtogalo Chanion cheese

Ewe's and goat's milk production is seasonal in Greece and culminates in April and May. Since most of this milk is used for cheesemaking, it is common to freeze and store the ewe's and goat's milk or cheese curd after drainage to ensure year-round manufacture of the cheeses (ALICHANIDIS *et al.*, 1981; PAPAGEORGIOU *et al.*, 1997). PC cheese is mainly consumed fresh during the time of its seasonal production. However, because freezing of this cheese does not considerably affect its texture or organoleptic properties, it is a common consumer practice to purchase fresh cheese immediately after it is produced and freeze a portion of it for future consumption. Therefore, it was important to determine the survival of *L. monocytogenes* in PC during short periods of refrigeration at 4°C or after freezing at -20°C.

The results concerning the survival of *L. monocytogenes* in PC during frozen storage are shown in Table 1. Populations of *L. monocytogenes* in 2 d cheese prior to freezing at -20°C ranged from 1.48 to 3.00 $\log_{10}$CFU/g in trials A through F and were equal to 5.43 and 5.30 $\log_{10}$CFU/g in trials G and H, respectively. For trials G and H, where a high initial *L. monocytogenes* inoculum was used, there was a substantial reduction in the pathogen populations within the first 15 d of frozen storage, as shown by a reduction of 1.55 (trial G) and 1.76 (trial H) $\log_{10}$CFU/g by day 15. However, additional cold storage of the cheese up to 90 d did not result in any further substantial reduction in the numbers of viable cells. In contrast, in trials A through F, where the *L. monocytogenes* populations prior to freezing the 2 d cheese were low (1.48-3.00 $\log_{10}$CFU/g), the pathogen counts decreased rapidly and reached non-detectable levels by day 15. Nevertheless, the pathogen was viable in all cheese samples at day 30, and in 1 out of 6 trials at day 75 of frozen storage at -20°C. Cold-enrichment results were negative for all samples stored at -20°C for 90 d and 5 out of 6 trials were negative even at 60 d (Table 1). The formation of larger intracellular and extracellular ice crystals during slow freezing at -20°C injures and kills bacterial cells. The damage that bacterial cells undergo during slow freezing

Table 1 - Results of the survival of *L. monocytogenes* (log_{10}CFU/g and positive or negative results after cold-enrichment) during frozen storage of Pichtogalo Chanion cheese at -20°C.

Frozen storage time	Trial*							
(days)	A	B	C	D	E	F	G	H
2[a]	1.70	3.00	2.48	2.18	1.60	1.48	5.43	5.30
15	+	+	+	+	+	+	3.88	3.54
30	+	+	+	+	+	+	3.40	3.95
45	+	+	+	-	-	+	3.48	3.78
60	-	-	+	-	-	-	3.18	3.40
75	-	-	+	-	-	-	3.48	3.65
90	-	-	-	-	-	-	3.40	3.51

[a] Counts at day 2 are from cheese samples just before freezing.
* See sub-section "Fate of *L. monocytogenes* during the manufacture and storage of Pichtogalo Chanion cheese" under the "Results and Conclusions" section for explanation of trials.

and frozen storage at -18°C, the particularly lethal effect of the freeze-thaw cycle on *L. monocytogenes*, and the ability of strain Scott A to survive at subzero temperatures are well-established facts (EL KEST and Marth, 1992; PAPAGEORGIOU *et al.*, 1997).

In conclusion, the results of this work indicate that, if *L. monocytogenes* is present in milk, even at a low count, it can grow during the traditional manufacture and ripening of CM cheese. These results emphasize that products like CM cheese with high pH values (>5.0) ripened at relatively elevated temperatures (17°C) support the growth of *L. monocytogenes*. The addition of 3.0% salt and 0.3% black pepper did not prevent *L. monocytogenes* growth. Some authors have recommended the use of inhibitory substances such as nisin to reduce or prevent the growth of *L. monocytogenes* in certain dairy foods (BENKERROUM and SANDINE, 1988; JUNG *et al.*, 1992). Regarding PC cheese, our results indicate that, even if *L. monocytogenes* is present in the milk in high numbers, the pathogen cannot grow during cheesemaking due to the rapid drop in pH during the first 24 h and the microorganism is inactivated after 5 to 20 d of storage at 4°C. *L. monocytogenes* populations declined substantially in PC cheese during the first 15 d of frozen storage, but some cells (ca. 1% or less) remain viable or sublethally injured up to 90 d. Even though the conditions are unfavourable for the growth and survival of *L. monocytogenes* in PC cheese, it is important to note that this unripened cheese is ready for consumption 2 d after manufacture. Therefore, every effort must be made to keep this cheese pathogen-free. Thus, to minimize *L. monocytogenes* and/or other milkborne pathogen contamination of CM and PC cheeses, precautions must be taken during the collection of raw milk and there should be proper pasteurisation. To achieve this Good Manufacturing Practices and Hazard Analysis Critical Control Point programs must be followed.

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PAPER

USE OF COLOUR MEASUREMENT TO EVALUATE YOGHURT QUALITY DURING STORAGE

MISURAZIONE STRUMENTALE DEL COLORE PER LA VALUTAZIONE DELLA QUALITÀ DELLO YOGURT DURANTE LA CONSERVAZIONE

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ABSTRACT

The aim of the investigation was to evaluate the relationship between colour parameters (L*, a*, b*) and the syneresis and titratable acidity of yoghurt. After manufacture, the pH values of the yoghurts were adjusted to 4.45, 4.40 and 4.35. The susceptibility of the yoghurts to syneresis immediately after manufacture was identical, but it increased during storage. After 3 weeks of storage, the yoghurt with an initial pH value of 4.35 was the most prone to syneresis. The lower the initial

RIASSUNTO

È stata studiata la relazione tra i parametri di colore (L*, a*, b*), la sineresi e l'acidità titolabile dello yogurt. Gli yogurt presi in esame avevano valore di pH di 4.45, 4.40 e 4.35. Mentre la suscettibilità degli yogurt alla sineresi immediatamente dopo la produzione non subiva variazioni, essa aumentava nel corso della conservazione. Dopo 3 settimane di conservazione, lo yogurt con un pH iniziale di 4,35 è stato quello più soggetto a sineresi. Più basso risultava il valore iniziale di pH dello yogurt mag-

- Key words: colour, quality, syneresis, titratable acidity, yoghurts -

pH of the yoghurt, the greater the increase of titratable acidity during storage. As the storage time increased, the degree to which the L*, a* and b* colour parameters decreased, depended on the initial pH of the yoghurts. The lowest values were recorded in yoghurt with an initial pH of 4.35. A high correlation coefficient was found between the L*, a* and b* colour parameter values and the syneresis and titratable acidity values, irrespective of the initial pH.

giore era l'incremento di acidità titolabile durante la conservazione. All'aumentare del tempo di conservazione il grado in cui i parametri di colore, L*, a* e b*, decrescevano dipendeva dal valore iniziale di pH degli yogurt. I valori più bassi sono stati registrati in yogurt con valore di pH iniziale di 4.35. Un elevato coefficiente di correlazione è stato riscontrato tra i valori dei parametri di colore L*, a* e b*, la sineresi ed i valori di acidità titolabile, indipendentemente dai valori di pH iniziale.

INTRODUCTION

Yoghurt quality and hence its consumer acceptability is determined primarily by the degree of coagulation of the curd. Any change in acidity of the yoghurt, resulting from the activity of the microflora deliberately introduced in the course of the manufacturing process, causes changes in the viscosity and structure of the product and alters its susceptibility to syneresis (LUCEY, 2002; PUVANENTHIRAN *et al.*, 2002; SHAKER *et al.*, 2003; SANDOVAL-CASTILLA *et al.*, 2004). The values of these parameters not only influence the initial quality of the yoghurt, but can also be used to determine the suitability of the product for further storage (BIROLLO *et al.*, 2000; CAIS-SOKOLIŃSKA *et al.*, 2004). Therefore, these parameters (e.g. syneresis), or instrumental measurements (e.g. colour parameter L*, a*, b*) are essential for a rapid and objective determination of the extent of changes that have occurred in the product (DELLO STAFFOLO *et al.*, 2004; KUMAR and MISHRA, 2004; YAZICI and AKGUN, 2004).

The objective of the investigation was to establish correlations between the syneresis and titratable acidity of the yoghurt examined in relationship to colour measurements.

MATERIALS AND METHODS

Natural stirred yoghurt with a 2% fat content, manufactured under industrial conditions was used. It was manufactured from skimmed milk that had been pasteurised for 10 min at 75°C and then thickened by adding powdered skimmed milk (5%), which increased the average dry matter content from 12.67 to 17.02%. Fat content standardisation to reach 2% in the processed milk was achieved using cream with a 30% fat content, which was pasteurised at 90°C for 5 s. After heating the processed milk at 50°C, it was homogenised under 15 MPa pressure and then pasteurised at 95°C for 3 min. After cooling the milk to 40°C, a freeze-dried culture containing *Lactobacillus delbrueckii subsp. bulgaricus* and *Streptococcus thermophilus* (1:1) was added (50 u/250 L milk). The inocula were obtained from the Chr. Hansen's Laboratory A/S Denmark. The milk was incubated at 42°C until the following pH values were reached: 4.45, 4.40 and 4.35. The curd obtained was then cooled

to 20°C, mixed and poured into 0.2 L containers, which were then cooled to 6°C. Six batches of 2,000 L of each different pH value were manufactured for each yoghurt. Assays and measurements were performed directly after manufacture as well as after 7, 14 and 21 days of storage at 6°C. The analyses were carried out on the six batches and three replications of each assay or measurement were conducted.

The pH of the yoghurts was determined using a pH-meter (CP-315, Elmetron Co., Zabrze, Poland), which was equipped with a combined ESAgP-301W type electrode (Eurosensor Co., Gliwice Poland) consisting of a glass half-cell and a saturated chloro-silver half-cell. The method proposed by DANNENBERG and KESSLER (1988) was applied to measure the susceptibility of the yoghurt to syneresis at 10°C. The titratable acidity of the yoghurt was assessed in accordance with conditions specified in the appropriate standard (IDF/ISO STANDARD, 1991). The colour of the yoghurt was measured reflectionally using a spectrophotometer (Hitachi U-3000, Tokyo, Japan) with a 1 cm thick layer and the C light source. The evaluation of colour in reflected light was performed using the CIELab colour system L*, a* and b* recommended by the Commission Internationale de l`Eclairage, where L* designates brightness, and a* and b* are chromaticity coordinates (CIE BULLETIN, 1974). The temperature of the samples ranged from 12° to 10°C. In addition, the colour difference value (ΔE) was calculated between the colour of each sample and the model of ideal whiteness, using the formula (FRANCIS and CLYDESDALE, 1975):

$$\Delta E=[(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2]^{0,5}$$

for which the following assumptions were made: L*=100, a*=0 and b*=0.

The results of the measurements and assays were then subjected to statistical analysis using the Excel calculation sheet of Statistica 6.0. The results were then used to determine the standard error (±SE), linear and polynomial regressions of different degrees using the least squares method, the square of the correlation coefficient and verification of the hypotheses at the p=0.05 level of significance (BRANDT, 1997).

RESULTS AND DISCUSSION

The results of the investigations showed that the pH values of the yoghurt immediately after manufacture significantly influenced the range of selected physico-chemical traits. Differences in the pH values of the yoghurts directly after manufacture did not significantly affect their susceptability to syneresis measured at time 0 (Table 1). Nevertheless, differences were observed on the 7th day of storage and these differences remained until the end of the storage period. No significant differences were found between yoghurts with initial pH values of 4.45 and 4.40, while yoghurts with an initial pH of 4.35 were more susceptable to syneresis. Irrespective of the differences, all of the yoghurts become more susceptable to syneresis with increasing storage time ($0.98<r<0.99$). After three weeks of storage, yoghurts with initial pH values of 4.45 and 4.40 were 73.2% more susceptable to syneresis than at the beginning, whereas yogurts with an initial pH of 4.35 were 88.4% more susceptable. When carrying out investigations on natural yoghurts with a pH value of 4.6 containing traditional, high-aromatic cultures, DANNENBERG and KESSLER (1988) showed that their susceptability to syneresis ranged from 38 to 58%, depending on the extent of the denaturation of β-lactoglobulin in the processed milk. Similar relationships were also demonstrated by SAVELLO and DARGAN (1997).

The lower the yoghurt pH after its manufacture, the higher was its titrat-

Table 1 - Changes in the syneresis of the yoghurts during storage (%).

pH of yoghurt after production	Storage time (days)				Standard error ±SE
	0	7	14	21	
4.45	4.3 ^{Aa}	4.9 ^{Ab}	6.2 ^{Ac}	7.4 ^{Ad}	0.25
4.40	4.3 ^{Aa}	5.1 ^{Ab}	6.5 ^{Ac}	7.5 ^{Ad}	0.17
4.35	4.3 ^{Aa}	5.6 ^{Bb}	6.9 ^{Bc}	8.1 ^{Bd}	0.04

A, B: different capital letter superscripts within the same column indicate significant differences (p=0.05) between the means of yoghurts with distinct initial pH; a, b, c, d: different small letter superscripts within the same row indicate significant differences (p=0.05) between the means of a yoghurt with a particular initial pH on the storage days.

able acidity (Table 2). This trend was maintained throughout the storage period. With increase in storage time, there was a significant increase in the titratable acidity values ($0.94<r<0.99$). The smallest increase in titratable acidity (10.2%) was in yoghurts with the highest pH value (pH 4.45), while the highest one (13.2%) was found in yoghurts with the lowest initial pH value (pH 4.35).

The range of changes in the quality parameters of the yoghurts was determined by using the instrumental colour measurements. Regardless of storage time, yoghurts with an initial pH of 4.35 had the lowest L* values (Fig. 1), while the yoghurt with an initial pH of 4.45 had the highest. Storage time exerted a significant influence on the decrease of the colour lightness value ($0.88<r<0.96$). The lightness values of the yoghurts with initial pH values of 4.45 and 4.40 were lower than the values measured directly after their manufacture by 3.97 and 4.71 units, respectively, while those of the yoghurt with pH 4.35 were up to 15.60 units less.

The a* index values of the yoghurts with initial pH values of 4.45 and 4.40 differed significantly from the values determined in the yoghurts with a pH of 4.35 (Fig. 2). This difference remained throughout the storage period. The a* index values declined significantly towards an intense green colour (on average by 2.19 units), in proportion to the length of storage time ($0.95<r<0.99$).

The results of the measurements showed the absence of a significant influence of the initial pH value of the yoghurts on the b* index value at 0 days of storage time (Fig. 3). The mean b* index

Table 2 - Changes in the titratable acidity of the yoghurts during storage (°SH).

pH of yoghurt after production	Storage time (days)				Standard error ±SE
	0	7	14	21	
4.45	38.30 ^{Aa}	39.06 ^{Aa}	40.20 ^{Aa}	42.21 ^{Ab}	0.45
4.40	42.30 ^{Ba}	43.01 ^{Ba}	44.05 ^{Bb}	47.17 ^{Bc}	0.89
4.35	46.60 ^{Ca}	49.15 ^{Cb}	50.88 ^{Cc}	52.75 ^{Cd}	0.28

A, B, C: different capital letter superscripts within the same column indicate significant differences (p=0.05) between the means of yoghurts with distinct initial pH; a, b, c, d: different small letter superscripts within the same row indicate significant differences (p=0.05) between the means of a yoghurt with a particular initial pH on the storage days.

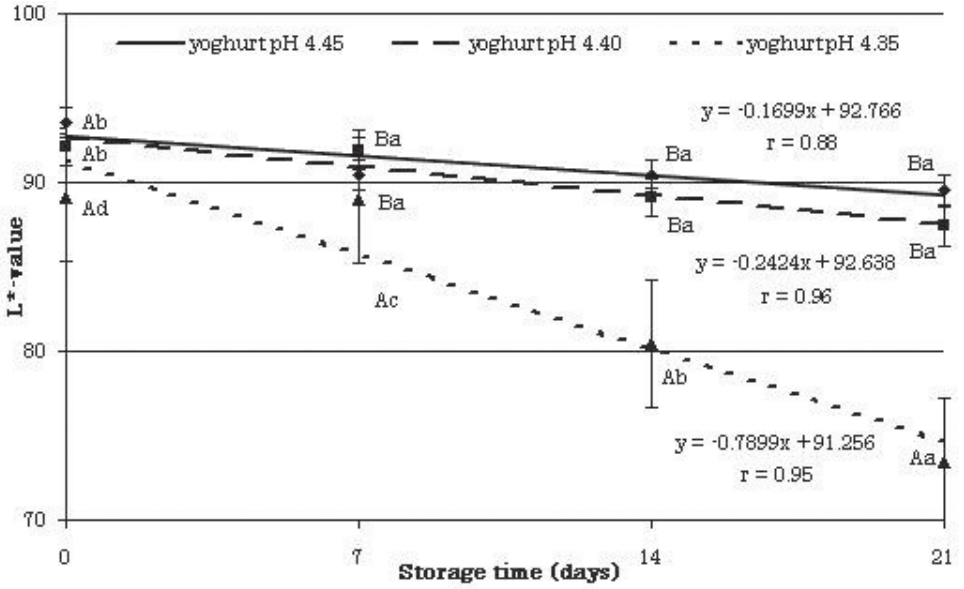


Fig. 1 - Changes in the L* value (±SE) of the yoghurts during storage.
A-B; a-d - different capital letters with mean values for the same storage time indicate statistically significant differences between yoghurts with different initial pH values; different small letters with mean values for the yoghurt with the same initial pH indicate statistically significant differences between different storage times, at the level of p=0.05.

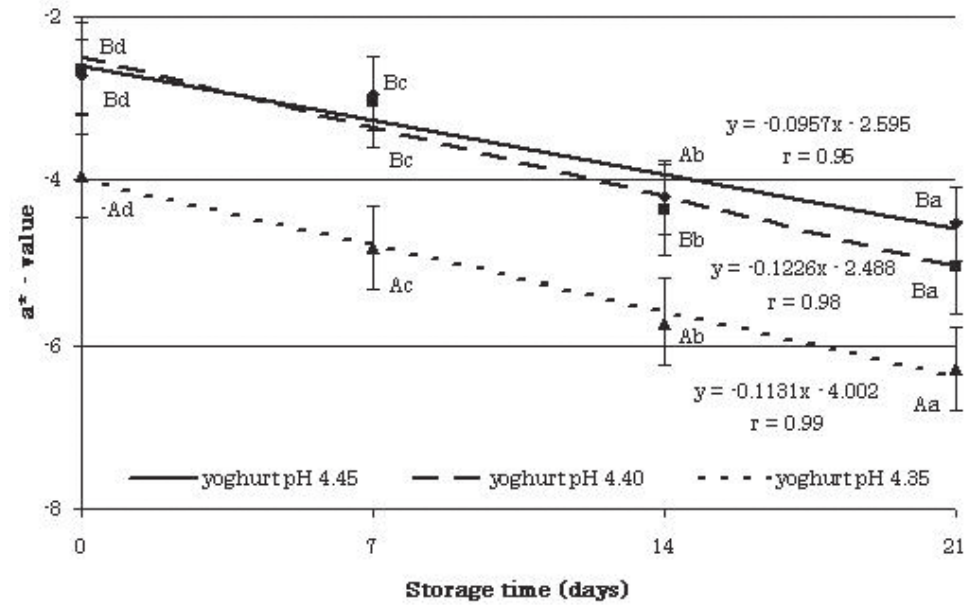


Fig. 2 - Changes in the a*-value (±SE) of the yoghurts during storage.
A-B; a-d - designations as in Fig. 1.

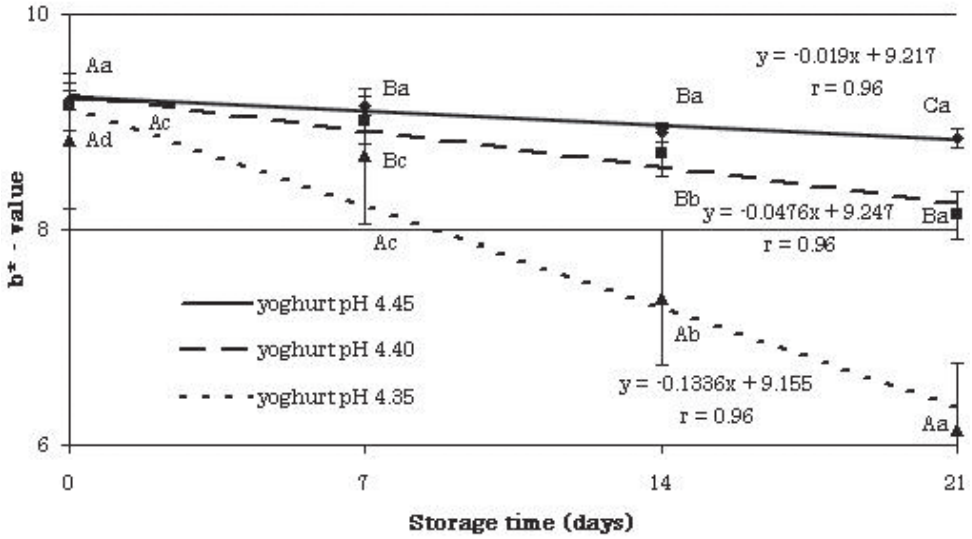


Fig. 3 - Changes in the b*-value (±SE) of the yoghurts during storage.
A-C; a-d - designations as in Fig. 1.

value of the yoghurts directly after manufacture was 9.05. There were significant differences between the b* values of the yoghurts with initial pH values of 4.45 and 4.40 at 21 days of storage, with that with an initial pH of 4.45 being significantly higher. After 21 days of storage the b* index values in the yoghurts with an initial pH of 4.35 decreased by 2.68 units and was equal to 6.14.

The lack of significant differences between the yoghurts with initial pH values of 4.45 and 4.40 during the entire period of storage became apparent when coefficient values of the white remoteness ΔE were calculated (Fig. 4). Immediately after manufacture, the distance from white of all the yoghurts was identical. However, 7 days later, the yoghurts with an initial pH of 4.35 were the most distant from the white standard. This difference continued to increase during storage time and after three weeks of storage, the ΔE index value had doubled.

In comparing the results of the colour instrumental measurements of the yoghurts with different initial pH values with the results of their susceptibility to syneresis, there was a different level of correlation (Table 3). The highest values of the determination coefficient ($94.64<R<99.34$) were calculated for the linear function determining the correlation between the a* index value and the susceptability to syneresis of the same samples. A similar, high correlation was determined between the results of the susceptability of the yoghurt to syneresis and the b* index values measured in the course of 3 weeks for the same yoghurt samples. On the other hand, the low correlation, which was determined between the susceptability of the yoghurts to syneresis and their lightness index L* values, affected the results of the comparative analysis with values that indicate the distance from the white standard (ΔE).

The analysis comparing the results of titratable acidity with the results of the colour measurement showed that the highest degree of correlation occurred with the a* and b* index values (Table 4). High coefficient R values indicate the possibility of evaluating changes in yo-

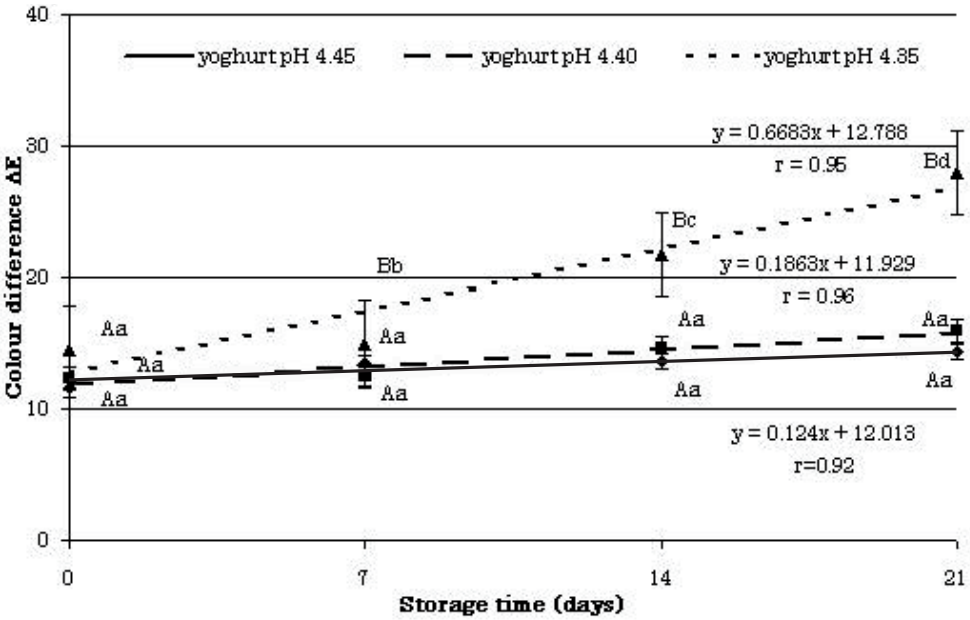


Fig. 4 - Changes in the colour difference ΔE (±SE) of the yoghurts during storage.
A-B; a-d - designations as in Fig. 1.

ghurt titratable acidity on the basis of changes in the a* index (86.32<R<99.12) and b* index values (85.21<R<99.31). It was not possible to demonstrate a high degree of correlation between the value of titratable acidity of the samples during 3 weeks of storage and the results of the lightness index L* and, hence, the values of the distance from the white standard ΔE.

Table 3 - Parameters of yoghurt colour assessment (*y*) as a function of syneresis (*x*) during 3 weeks of storage.

Colour parameters	pH of yoghurt after production	*y(x)*	Determination coefficient R	Correlation coefficient r
L*	4.45	-1.01x + 96.76	65.04	0.81
	4.40	-1.57x + 99.28	96.09	0.98
	4.35	-4.34x + 109.96	89.54	0.94
a*	4.45	-0.64x + 0.02	94.64	0.97
	4.40	-0.79x + 0.83	99.12	0.99
	4.35	-0.62x + 1.30	99.34	0.99
b*	4.45	-0.12x + 9.73	93.74	0.97
	4.40	-0.30x + 10.52	92.33	0.96
	4.35	-0.73x + 12.32	91.41	0.95
colour difference ΔE	4.45	0.75x + 9.03	73.08	0.85
	4.40	1.21x + 6.82	95.88	0.98
	4.35	3.67x - 3.03	90.16	0.95

Table 4 - Parameters of yoghurt colour assessment (*y*) as a function of titratable acidity (*x*) during 3 weeks of storage.

Colour parameters	pH of yoghurt after production	*y(x)*	Determination coefficient R	Correlation coefficient r
L*	4.45	-0.81x + 123.51	63.58	0.79
	4.40	-1.00x + 134.24	88.31	0.94
	4.35	-2.64x + 214.48	84.31	0.92
a*	4.45	-0.49x + 16.15	86.75	0.93
	4.40	-0.49x + 17.77	86.32	0.93
	4.35	-0.39x + 14.31	99.12	0.99
b*	4.45	-0.09x + 12.89	85.21	0.92
	4.40	-0.21x + 17.94	99.31	0.99
	4.35	-0.45x + 30.07	86.68	0.93
colour difference				
ΔE	4.45	0.60x - 10.83	71.43	0.84
	4.40	0.77x - 20.22	89.10	0.94
	4.35	2.23x - 91.61	85.17	0.92

CONCLUSION

The results indicate that the pH of yoghurts immediately after their manufacture significantly influenced the results of the colour measurements. Despite their different initial pH values, the yoghurts, immediately after manufacture, had the same propensity to syneresis. However, after 3 weeks of storage, yoghurts with the highest titratable acidity values and whose initial pH value was 4.35, were the most susceptable to syneresis. Furthermore, directly after manufacture, yoghurts with a pH value of 4.45 and 4.40 were characterised by the same lightness index L* values as well as the same a* and b* index values. At the same time, yoghurts with an initial pH of 4.35 had the lowest L* values and were characterised by the lowest a* and b* index values. With increase in storage time, the lightness index L* value declined significantly. In addition, the a* index value decreased towards an intense green colour, while that of the b* index decreased toward an intense yellow colour. Changes in the a* and b* index values were highly correlated with yoghurt titratable acidity values and their susceptability to syneresis, which is one of the most important parameters of yoghurt quality.

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PAPER

IN VITRO ANTIMICROBIAL AND ANTIOXIDANT ACTIVITIES OF THE ESSENTIAL OILS AND METHANOL EXTRACTS OF *HYSSOPUS OFFICINALIS* L. SSP. *ANGUSTIFOLIUS*

ATTIVITÀ ANTIMICROBICA ED ANTIOSSIDANTE *IN VITRO* DI OLI ESSENZIALI E DI ESTRATTI METANOLICI DI *HYSSOPUS OFFICINALIS* L. SSP. *ANGUSTIFOLIUS*

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- Key words: antimicrobial activity, antioxidant activity, essential oil, hyssop, *Hyssopus officinalis* -

ABSTRACT

The in vitro antimicrobial and antioxidant activities of the essential oil and methanol extract from *Hyssopus officinalis* L. ssp. *angustifolius* were investigated. The essential oil, which has pinocarvone, pinocamphone, and β-pinene as main components, exhibited activity against eight bacteria, and ten fungi and a yeast, *C. albicans*, with Minimum Inhibitory Concentration values (MIC) ranging from 15.625 to 250 µL/mL; the methanol extract was inactive. With regard to antioxidant activity, the essential oil was inactive, while the methanol extract provided 50% inhibition at a concentration of 117.0 µg/mL in DPPH assay and 40% inhibition at the concentration of 2 g/L in linoleic acid test system.

RIASSUNTO

È stata studiata l'attività antimicrobica ed antiossidante in vitro di olio essenziale e di estratto metanolico di *Hyssopus officinalis* L. ssp. *angustifolius*. L'olio essenziale, che ha come principali costituenti il pinocarvone, pinocamphone e β-pinene, presentava attività antimicrobica su otto tipologie batteriche, dieci fungine ed un lievito, *C. albicans*, con una Concentrazione Minima Inibente (MIC) fra 15,625 e 250 µL/mL; l'estratto metanolico risultava inattivo. Per quanto riguarda l'attività antiossidante, l'olio essenziale risultava inattivo, mentre l'estratto metanolico presentava un'attività inibente del 50% ad una concentrazione di 117,0 µg/mL nel test de DPPH e del 40% ad una concentrazione di 2 g/L nel test dell'acido linoleico.

INTRODUCTION

Hyssop (*Hyssopus officinalis* (L.), Lamiaceae), a wild perennial undershrub, is cultivated in temperate regions of Asia, Europe and America. It includes about 5 species, all found around the Northern Mediterranean coast and in Asia Minor (KÖNEMANN, 1999). In the flora of Turkey, this genus is represented by one taxon, *Hyssopus officinalis* L. ssp. *angustifolius* (DAVIS, 1982). This plant is called "Zulfa otu" by the local people around Erzurum Turkey and the leaves of this species are mainly used as a condiment for soups as a replacement for mint (BAYTOP, 1997). In its native area, hyssop is found on dry banks and among rocks and ruins (LE STRANGE, 1977) and grows to a height ranging from 20 to 70 cm.

H. officinalis L. has been known as a culinary and medicinal herb for hundreds of years. It is used for cough, bronchitis, asthma, and chronic catarrh, rheumatism, sore throat, wounds, ulcers, and tumors (SIMON *et al.*, 1984; BOWN, 1995). In modern medicine hyssop is considered a reasonably effective treatment for mild irritations of the respiratory tract that accompany the common cold (TYLER, 1993). Furthermore, its essential oil is widely used in the food, pharmaceutical and cosmetic industries. Many reports have been published concerning the composition of hyssop essential oil (SHAH, 1991; SCHULZ and STAHL-BISKUP, 1991; TSANKOVA *et al.*, 1993; KERROLA *et al.*, 1994 ; GORUNOVIC *et al.*, 1995; SALVATORE *et al.*, 1998; GARG *et al.*, 1999; PICCAGLIA *et al.*, 1999; CHALCHAT *et al.*, 2001; FRATERNALE *et al.*, 2004). The main components of *H. officinalis* L. are pinocamphone, isopinocamphone, β-pinene, 1,8-cineole, myrtenol, linalool, pinocarvone, methyl euge-

nol, and limonene. These studies have also emphasized the existence of marked chemical differences in the oils extracted from plants from different locations or genotypes.

Plant extracts and essential oils have been known to possess antibacterial and antifungal activity against a wide range of microorganisms (OUATTARA *et al.*, 1997; HAMMER *et al.*, 1999; CHAO *et al.*, 2000). This property of herbs has been attributed to the presence of some antimicrobial constituents contained in their oils. However, there have been a relatively limited number of reports in the literature dealing with the effect of *H. officinalis* extract and/or essential oils against various bacterial and fungal species (MAZZANTI *et al.*, 1998; RENZINI *et al.*, 1999; LETESSIER *et al.*, 2001; MARINO *et al.*, 2001; FRATERNALE *et al.*, 2004).

There is increasing interest in the antioxidant compounds of plants because they improve the flavour of food, retard the oxidative degradation of lipids and play an important role in disease prevention. There are only a few reports concerning the antioxidant activity of *H. officinalis* L. in the literature. While DAPKEVICIUS *et al.* (1998) reported no activity from the extracts prepared using different extraction techniques, DJARMATI *et al.* (1991) found high activity related to rosmanol-9-ethyl ether which was much greater than the synthetic antioxidant agent, butylated hydroxy toluene (BHT). FERNANDEZ-LOPEZ *et al.* (2003) also reported that hyssop inhibited lipid oxidation and degradation of heme pigments caused by cooking and storage. In another report, hyssop extracts were found to exhibit prooxidative action and increase conjugated diene formation in sunflower oil (ABDALLA and ROOZEN, 1999).

ÖZER *et al.* (2005) examined the essential oil composition of *H. officinalis* L. ssp. *angustifolius*. However, to our knowledge, the biological activity of *H. officinalis* L. ssp. *angustifolius* from Turkey has not been studied previously. Therefore, in this paper, we report the antimicrobial and antioxidant activities of essential oil and methanol extracts from *H. officinalis* plants collected from the eastern Anatolia region of Turkey.

MATERIALS AND METHODS

Plant material

H. officinalis L. ssp. *angustifolius* plants at the full flowering stage were collected in August 2002 in Köse, Gümüşhane, Turkey. The taxonomic identification of the plant material was confirmed by a senior plant taxonomist, Meryem Şengül, in the Department of Biology, Atatürk University, Erzurum, Turkey. The plant material was dried in the shade, and the plant leaves were separated from the stems, and ground in a grinder with a 2 mm diameter mesh. The voucher specimen was deposited at the Herbarium of the Department of Biology, Ataturk University, Erzurum-Turkey (ATA. HERB 9734).

Isolation of the essential oil and GC-MS analysis

The air-dried and ground aerial plant parts were water-distilled for 3 hours using a Clevenger-type apparatus (yield 1.13% v/w). The essential oil (EO) obtained was dried over anhydrous sodium sulphate and, after filtration, stored at +4°C until tested and analyzed. GC-MS conditions were reported in a previous study (ÖZER *et al.*, 2005).

Preparation of the methanolic extract

The dried and powdered leaves (500 g) were extracted with 1 L of methanol using a Soxhlet extractor for 72 h at a temperature not exceeding the boiling point of the solvent (LIN *et al.*, 1999). The extract was filtered using What-

man filter paper (N. 1) and then concentrated in vacuo at 40°C using a Rotary Evaporator. The residues obtained were stored in a freezer at -80°C until further tests.

Microbial strains

The methanolic extracts and the essential oil were tested individually against a range of 40 microorganisms, including 24 bacteria, 15 fungi, and a yeast species. The microorganisms used are listed in Tables 1 and 2. Microorganisms were provided by the Department of Clinical Microbiology, Faculty of Medicine, and Plant Diagnostic Laboratory, Faculty of Agriculture at Ataturk University, Erzurum, Turkey. The identity of the microorganisms used in this study was confirmed by the Microbial Identification System in the Biotechnology Application and Research Center at Ataturk University.

Antimicrobial activity

Disc-diffusion assay

The dried plant extracts were dissolved in methanol to a final concentration of 30 mg/mL and sterilized by filtration through 0.45 µm Millipore filters. Antimicrobial tests were then carried out by the disc diffusion method (MURRAY *et al.*, 1995) using 100 µL of a suspension containing 10^8 CFU/mL of bacteria, 10^6 CFU/mL of yeast and 10^4 spore/mL of fungi spread on nutrient agar (NA), sabouraud dextrose agar (SDA) and potato dextrose agar (PDA) medium, respectively. The discs (6 mm in diameter), impregnated with 10 µL of essential oil or 30 mg/mL extracts (300 µg/disc), were placed on the inoculated agar. Negative controls were prepared with the same solvents used to dissolve the plant extracts. Ofloxacin (10 µg/disc), sulbactam (30 µg)+cefoperazona (75 µg) (105 µg/disc) and/or netilmicin (30 µg/disc) were used as positive reference standards to determine the sensitivity of one strain/isolate in each microbial species tested. The inoculated plates were incubated at 37°C for 24 h for clinical bacterial strains, 48 h for the yeast and 72 h for fungi isolates. Plant-associated microorganisms were incubated at 27°C. Antimicrobial activity was evaluated by measuring the zone of inhibition against the test organisms. Each assay was repeated twice.

Micro-well dilution assay

The minimal inhibition concentration (MIC) values were determined for the bacterial strains which were sensitive to the essential oil in the disc diffusion assay. The inocula of the bacterial strains were prepared from 12 h broth cultures and suspensions were adjusted to 0.5 McFarland standard turbidity. The essential oils and extracts of *H. officinalis*, dissolved in 10% dimethylsulfoxide (DMSO), were first diluted to the highest concentration (500 µg/mL) to be tested, and then serial two-fold dilutions were made in order to obtain a concentration range from 7.8 to 500 µg/mL in 10 mL sterile test tubes containing nutrient broth. The MIC values of the *H. officinalis* extracts against bacterial strains and *Candida albicans* isolates were determined based on a micro-well dilution method (ZGODA and PORTER, 2001; GÜLLÜCE *et al.*, 2004) with some modifications.

The 96-well plates were prepared by dispensing 95 µL of nutrient broth and 5 µL of the inoculum into each well. One-hundred microlitres from the stock solutions of *H. officinalis* essential oil prepared at a concentration of 500 µg/mL was added to the first wells. Then, 100 µL from the serial dilutions was transferred to the six consecutive wells. The last well containing 195 µL of nutrient broth without compound and 5 µL of the inoculum on each strip was used as a negative control. The final

volume in each well was 200 µL. Maxipime (Bristol-Myers Squibb, New York, USA) at a concentration range of 500-7.8 µg/mL was prepared in nutrient broth and used as a standard drug for positive control. The plate was covered with a sterile plate sealer. Contents of each well were mixed on a plate shaker at 300 rpm for 20 s and then incubated at appropriate temperatures for 24 h. Microbial growth in each medium was determined by reading the respective absorbance (Abs) at 600 nm using the ELx 800 universal microplate reader (Biotek Instrument Inc, Highland Park, VT, USA) and confirmed by plating 5 µL samples from clear wells on nutrient agar medium. The oil tested in this study was screened twice against each organism. The MIC was defined as the lowest concentration of the compounds that inhibited the growth of the microorganisms.

MIC agar dilution assay

The agar dilution method, as described previously by GUL *et al.* (2002), was used to determine the MIC values of the fungi isolates. The essential oils of *Hyssopus officinalis* were added aseptically to sterile molten PDA medium, containing Tween 20 (Sigma 0.5%, v/v), at the appropriate volume to produce the concentration range of 7.8-500 µg/mL. The resulting PDA solutions were immediately poured into Petri plates after vortexing. The plates were spot inoculated with 5 µL (10^4 spore/mL) of each fungal isolate. Amphotericin B (Sigma A 4888) was used as a reference antifungal drug. The inoculated plates were incubated at 27° and 37°C for 72 h for plant and clinical fungi isolates, respectively. At the end of the incubation period, the plates were evaluated for the presence or absence of growth. MIC values were determined as the lowest concentration of the essential oil where absence of growth was recorded. Each test was repeated at least twice.

Antioxidant activity

DPPH assay

The hydrogen atoms or electron donating ability of the corresponding extracts and some pure compounds was determined from the bleaching of purple-coloured methanol solution of DPPH. This spectrophotometric assay uses the stable radical 2,2'-diphenylpicrylhydrazyl (DPPH) as a reagent (CUENDET *et al.*, 1997; DAPKEVICIUS *et al.*, 1998; BURITS and BUCAR, 2000). Fifty microliters of various concentrations of the extracts in methanol were added to 5 mL of a 0.004% methanol solution of DPPH. After a 30 min incubation period at room temperature the absorbance was read against a blank at 517 nm. Inhibition free radical DPPH in percent (I%) was calculated in the following way:

$$I\ \% = (A_{blank} - A_{sample} / A_{blank}) \times 100$$

Where A_{blank} is the absorbance of the control reaction (containing all the reagents except the test compound), and A_{sample} is the absorbance of the test compound. Extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotted as inhibition percentage against extract concentration. Tests were carried out in triplicate.

β-Carotene-linoleic acid assay

In this assay antioxidant capacity is determined by measuring the inhibition of the volatile organic compounds and the conjugated diene hydroperoxide formation from linoleic acid oxidation (DAPKEVICIUS *et al.*, 1998). A stock solution of β-carotene-linoleic acid mixture was prepared as follows: 0.5 mg β-carotene was dissolved in 1 mL of chloroform (HPLC grade), 25 µL linoleic acid and 200 mg Tween 40 were added. Chloroform was completely evaporated using a vacuum evaporator. Then, 100 mL of distilled water saturated with oxygen (30 min at a flow rate of 100 mL/min) were

added with vigorous shaking. This reaction mixture (2.500 µL) was dispersed in test tubes and 350 µL portions of the extracts, prepared in 2 g/L concentrations, were added and the emulsion system was incubated up to 48 hours at room temperature. The same procedure was repeated with a positive control BHT and a blank. After this incubation time, the absorbance of the mixtures was measured at 490 nm. Antioxidative capacities of the extracts were compared with those of the BHT and the blank.

Assay for total phenolics

The total phenolic constituents of the *H. officinalis* extracts were determined using the literature methods involving Folin-Ciocalteu reagent and gallic acid as standard (CHANDLER and DODDS, 1983; SLINKARD and SINGLETON, 1977). The extract solution (0.1 mL) containing 1000 µg extract was put in a volumetric flask; 46 mL of distilled water and 1 mL of Folin-Ciocalteu reagent were added and the flask was shaken thoroughly. After 3 min, 3 mL of a 2% Na_2CO_3 solution were added and the mixture was allowed to stand for 2 h with intermittent shaking. The absorbance was measured at 760 nm. The same procedure was repeated for all the standard gallic acid solutions (0-1.000 g 0.1 mL^{-1}) and the standard curve was determined using the equation given below:

Absorbance:
0.0012 x Gallic acid (µg) + 0.0033

RESULTS AND DISCUSSION

Antimicrobial activity of the essential oil

The composition of the essential oil of *H. officinalis* was examined previously by ÖZER *et al.* (2005). The main constituents of the essential oil were pinocarvone (36.3%), isopinocamphone (19.6%), β-pinene (10.6%), 1,8-cineole (7.2%), and pinocamphone (5.3%). In the literature, the results obtained for hyssop (SCHULZ and STAHL-BISKUP, 1991; TSANKOVA *et al.*, 1993; GORUNOVIC *et al.*, 1995; SALVATORE *et al.*, 1998; GARG *et al.*, 1999; PICCAGLIA *et al.*, 1999: CHALCHAT *et al.*, 2001) show pinocamphone and isopinocamphone to be the main components. In the present study the oil also contained pinocamphone and isopinocamphone, but had much higher levels of pinocarvone (36.3%).

A part of the essential oil obtained from the previous study (ÖZER *et al.*, 2005) was used in the current investigation to determine its antimicrobial and antioxidant activity. The antimicrobial activities of *H. officinalis* essential oil and extracts against microorganisms were examined and their potency was qualitatively and quantitatively assessed by the presence or absence of inhibition zones and zone diameter, and MIC values. The results show that the essential oil of *H. officinalis* L. ssp. *angustifolius* had antimicrobial activities against eight bacteria (Table 1) and ten fungi and one yeast species (Table 2). The data clearly indicate that the essential oil is more effective against fungi than bacteria. In contrast, the methanol extract from the aerial parts of *H. officinalis* L. showed no antimicrobial activity. These observations support the hypothesis that the essential oil of plants contains more antimicrobial substances than their extracts, including water, methanol, ethanol and hexane (AHMAD *et al.*, 1998; ELOFF, 1998).

The maximal inhibition zones and MIC values for the bacterial strains that were sensitive to *H. officinalis* essential oil were in the 8-24 mm, and 31.25-250 µL/mL ranges (Table 1). In general, the gram-positive bacterial strains tested seemed to be affected by the essential oil to the same extent as the gram-negative bacteria strains, while

Table 1 - Antimicrobial activity of *Hyssopus officinalis* L. ssp. a*ngustifolius* essential oil against the bacterial strains tested.

Test microorganisms	*Hyssopus officinalis*		Antibiotics	
Bacteria	Inhibition zone [a] (mm)	Minimal Inhibitory concentration (MIC)	Inhibition zone [b] (mm)	Minimal Inhibitory concentrations [c] (MIC) (µg/mL)
Acinetobacter baumanii-A8	16	62.50	18 (OFX)	31.25
Bacillus macerans-A199	24	62.50	19 (OFX)	15.62
Bacillus megaterium -A59	-	-	9 (SCF)	15.62
Bacillus subtilis-ATCC-6633	-	-	28 (OFX)	62.50
Bacillus subtilis-A57	-	-	28 (OFX)	125
Brucella abortus-A77	-	-	12 (SCF)	62.50
Burkholdria cepacia-A225	-	-	22 (SCF)	125
Clavibacter michiganense-A227	-	-	25 (SCF)	16.62
Enterobacter cloacae-A135	-	-	20 (NET	31.25
Enterococcus faecalis-ATCC-29122	8	250	18 (SCF)	31.25
Escherichia coli-A1	18	31.25	- (OFX)	62.50
Klebsiella pneumoniae-A137	-	-	12 (OFX)	125
Proteus vulgaris-A161	-	-	12 (OFX)	125
Proteus vulgaris-KUKEM1329	12	125	13 (OFX)	125
Pseudomonas aeruginosa-ATCC9027	-	-	22 (NET)	31.25
Pseudomonas aeruginosa ATCC27859	-	-	22 (NET)	15.62
Pseudomonas syringae pv.*tomato* A35	-	-	24 (OFX)	125
Salmonella enteritidis-ATCC-13076	-		27 (SCF)	62.50
Staphylococcus aureus-A215	14	125	22 (SCF)	31.25
Staphylococcus aureus-ATCC-29213	12	125	22 (SCF)	62.50
Staphylococcus epidermis-A233	-	-	- (SCF)	15.62
Streptococcus pyogenes-ATCC-176	10	125	10 (OFX)	62.50
Streptococcus pyogenes-KUKEM-676	-	-	13 (OFX)	31.25
Xanthomonas campestris-A235	-	-	20 (SCF)	31.25

[a] Inhibition zone in diameter (mm) around the discs impregnated with 300 µg/disc of methanol extract.
[b] OFX=Ofloxacin (10 µg/disc); SCF=sulbactam (30 µg)+cefoperazona (75 µg) (105 µg/disc) and NET=Netilmicin, (30 µg/disc) were used as positive reference standard antibiotic discs (Oxoid).
[c] Maxipine (µg/mL) was used as reference antibiotic in micro well dilution assay (Sigma).

previous studies reported that gram-positive bacteria were more sensitive to plant oil and extracts than gram-negative bacteria (COSENTINO *et al.*, 1999; KARAMAN *et al.*, 2003). As with antifungal and anticandidal efficacy, the maximal inhibition zones and MIC values of the yeast and fungi species that were sensitive to the *H. officinalis* essential oil were 7-35 mm and 15.62-250 µL/mL, respectively (Table 2). These findings confirm the observations reported by HOVADIK and CHLADEK (1974), MAZZANTI *et al.* (1988) and MARINO *et al.* (2001). They also suggest that *H. officinalis* essential oil has some antimicrobial effect.

While it is well known that the antimicrobial effect of hyssop oil is due to its chemical constituents, we have failed to find the evidence that the main component of this hyssop oil, pinocarvone (36.30%), has an antimicrobial effect. However, VILJOEN *et al.* (2002) reported the antimicrobial and antifungal effect of essential oil containing

pinocarvone and *trans*-pinocarveol as the main components in *Myrothamnus flabellifolius*. Furthermore, it is highly likely that the antimicrobial activity of the oil is also associated with the contribution of pinocamphone (19.64%) and the monoterpenes β-pinene and 1,8-cineole (10.62 and 7.22%, respectively), which are known to possess antibacterial activity (MAGIATIS *et al.*, 1999; MARTINS *et al.*, 2000; LETESSIER *et al.*, 2001; TZAKOU *et al.*, 2001; FRATERNALE *et al.*, 2004). ABOU-JAWDAH *et al.* (2002) also reported that antimicrobial activity may be associated with a compound that is present in a low quantity but possesses a high biological activity. To better understand the reason for its antimicrobial effect, further studies involving individual components and different combinations of the main components of hyssop oil are needed.

Antioxidant activity

Methanol extract and the essential oil were subjected to a screening for their possible antioxidant activity by using two complementary test systems, namely, DPPH radical and beta carotene/linoleic acid. Free radical scavenging activity was measured in the DPPH assay and the reaction followed a concentration-dependent pattern. As shown in Fig. 1, the inhibition percentage (I %) rapidly

Table 2 - Anticandidal and antifungal activities of *Hyssopus officinalis* ssp. *angustifolius* essential oil against the yeast and fungi isolates.

Test yeast and fungi	*Hyssopus officinalis*		Antibiotics	
	Inhibition zone [a] (mm)	Minimal Inhibitory concentrations (MIC) (µg/mL)	Inhibition zone [b] (mm)	Minimal Inhibitory concentrations[c] (MIC) (Amp B)
Yeast				
Candida albicans-A117	7	250	- (NET)	31.25
Fungi				
Alternaria alternate	35	62.50	- (NET)	31.25
Aspergillus flavus	35	62.50	- (NET)	15.62
Aspergillus variecolor	20	62.50	- (NET)	15.62
Fusarium acuminatum	-	-	- (NET)	62.50
Fusarium oxysporum	35	62.50	- (NET)	62.50
Fusarium solani	24	31.25	- (NET)	62.50
Fusarium tabacinum	-	-	- (NET)	62.50
Moniliania fructicola	-	-	- (NET)	15.62
Penicillum spp.	35	31.25	- (NET)	31.25
Rhizopus spp.	-	-	- (NET)	125
Rhizoctonia solani	35	31.25	- (NET)	31.25
Sclorotinia minor	35	15.62	- (NET)	125
Sclorotinia sclerotiorum	34	31.25	- (NET)	62.50
Trichophyton rubrum	35	-	- (NET)	31.25
Trichophyton mentagrophytes	-	-	- (NET)	15.62

[a] Inhibition zone in diameter (mm) around the discs impregnated with 300 µg/disc of methanol extract.
[b] NET=Netilmicin (30 µg/disc) was used as positive reference standard antibiotic discs (Oxoid).
[c] AmpB = Amphotericin B (µg/mL) was used as reference antibiotic in micro well dilution (Sigma).

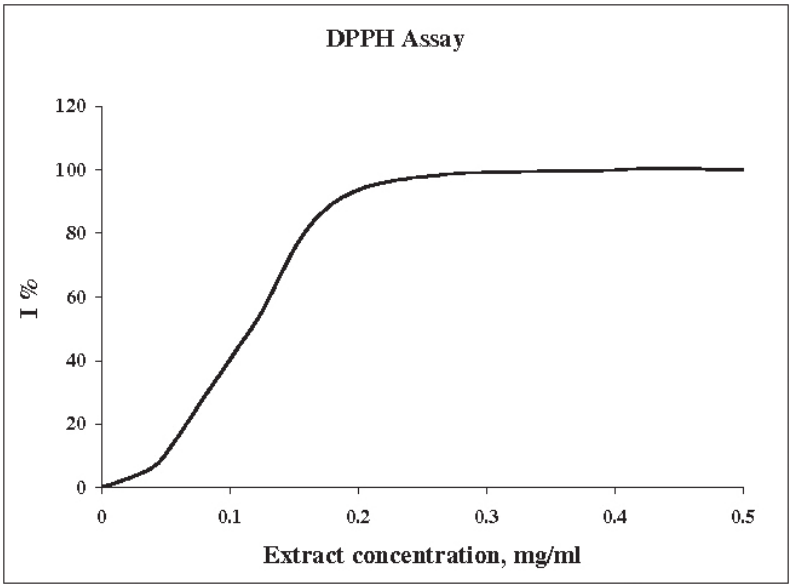


Fig. 1 - Inhibition of DPPH stable free radical by methanol extract of *Hyssopus officinalis* ssp. *Angustifolius.*

increased with increasing extract concentration.

Extract concentrations providing 50% inhibition (IC_{50}) were determined from the corresponding concentration-inhibition percentage graphs. In the β-carotene-linoleic acid assay *H. officinalis* exhibited moderate activity.

Only the methanol extract seems to be promising with 50% inhibition at a concentration of 117.0 µg/mL in DPPH assay and 40% inhibition at the concentration of 2 g/L in the linoleic acid test system. In contrast to ABDALLA and ROOZEN (1999) methanol extracts of *H. officinalis* exhibited good free radical scavenging activity. The methanol extract also consisted of 75 µg gallic acid equivalent phenolics in one mg of extract (7.5%) which are thought to be mainly responsible for the activity. Polyphenols, especially phenolic acids, are always considered to be strong antioxidants. High amounts of protocatechuic, chlorogenic, syringic, ferulic and rosmarinic acid were found in methanol extracts of *H. officinalis* L. by ZGORKA and GLOWNIAK (2001). Rosmarinic acid, a well known antioxidant agent in this family, could be responsible for the activity observed in this study. The phenolic acids need to be isolated and determined in a future investigation.

To our knowledge, this is the first report of the antimicrobial and antioxidant properties of *H. officinalis* L. ssp. *angustifolius* oil and extract. It is apparent that the essential oil possesses compounds with antimicrobial properties, which could be used as antimicrobial agents in new drugs for therapy against infectious diseases in humans and plants. Furthermore, the study also revealed that the hyssop extracts tested had antioxidant activity.

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SHORT COMMUNICATION

FLAVONOIDS AND TERPENOIDS IN GOAT MILK IN RELATION TO FORAGE INTAKE

FLAVONOIDI E TERPENOIDI NEL LATTE DI CAPRA
IN RELAZIONE AL FORAGGIO

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ABSTRACT

Milk from goats fed borage or hawthorn shows the presence of flavonoids and terpenoids. A comparison between the flavonoid content in the plants and in the milk of the goats seems to prove that the mammary route is one of the excretory pathways. Rutin and quercetin are excreted in part without modifications, while other compounds appear to have less structural complexity, indicating a metabolism that is probably mediated by the gastrointestinal microflora. The presence of phenolic

RIASSUNTO

Il latte di capre alimentate con borragine o biancospino rivela la presenza di flavonoidi e terpenoidi provenienti dalla dieta. Il confronto fra il contenuto flavonoidico delle piante e del latte degli animali studiati sembra provare che l'escrezione mammaria sia una delle vie metaboliche possibili per i composti flavonoidici. La rutina e la quercetina sono parzialmente escrete senza modificazioni, mentre altri composti presenti nel latte presentano minore complessità strutturale rispetto ai me-

- Key Words: *Borago officinalis*, *Crataegus oxyacantha,* flavonoids, goat milk, mammary excretion -

compounds in the milk could affect the quality and sensory traits of the milk and milk products.

tabolici presenti nelle piante, a riprova di una probabile mediazione metabolica da parte delle microflora gastrointestinale. La presenza di composti fenolici nel latte può essere importante in relazione alla qualità sensoriale del latte e dei suoi derivati.

INTRODUCTION

Mediterranean pastures in southern Italy are highly variable depending on the season. The proportion of graminaceous plants decreases from 80-85% to 55-60% from winter to spring, while the proportion of forbs increases from 25-30% to 60-65%, from late spring to early summer (FEDELE *et al.*, 1993). In early summer, goats graze predominantly on forbs (FEDELE *et al.*, 1993), some of which are used as medicinal plants by man.

In recent years, several studies have shown that some plant compounds (e. g. vitamins, volatile organic compounds) are directly transferred from the grazed herbage to the milk (FEDELE *et al.*, 2000; PIZZOFERRATO *et al.*, 2000a; VIALLON *et al.*, 2000). In fact, the physico-chemical and organoleptic features of milk and cheese can be greatly affected by the diet of the grazing animals since a large variety of the organic components present in the plant tissues can accumulate in the lipid and water-soluble fraction of the milk. In particular, monoterpene compounds can greatly influence the aroma of the milk. Terpenes could be used as biochemical indicators of the composition of the forage grazed and could be used to determine the origin of cheese (FEDELE *et al.*, 2000).

The impact of animal diet on the monoterpene and sesquiterpene content in cheese has been studied by many authors. DUMONT and ADDA (1978), DUMONT *et al.* (1981), BOSSET *et al.* (1994, 1997, 1999) and BUCHIN *et al.* (1998) have shown that these aromatic compounds are more frequent in dairy products when animals are fed mountain forages. Other authors (VIALLON *et al.*, 1999; COULON *et al.*, 2000; VERDIER-METZ *et al.*, 2000) have reported that highland grass with different botanical composition strongly affects the flavour of milk and cheese.

Little information is available on the fate of other non-volatile phyto-constituents and the possibility of finding these compounds and/or their metabolites in milk. O'CONNEL and FOX (2001) reported that the majority of phenolic compounds found in cow milk are derived from the feed and when the cows are fed large quantities of particular crops, other phenolic compounds may also be detected in ruminant milk. Morevoer, some alkylphenols present in ruminant milk are derived from phenolics ingested through the animal feed (KILIC and LINDSAY, 2005).

The aim of this study was to investigate the effect of some secondary metabolites of two plants (*Crataegus oxyacantha* L. and *Borago officinalis* L.), whose chemical composition is well known (ZHANG *et al.*, 2001; PEIRETTI *et al.*, 2004), on the chemical composition of milk. These plants are normally preferred for grazing by goats in the spring and summer (FEDELE *et al.*, 1993).

MATERIALS AND METHODS

Plant material

Borage (*Borago officinalis* L.) and hawthorn (*Crataegus oxyacantha* L.) were collected at the Bella farm of the Istituto Sperimentale per la Zootecnia, Potenza, Italy. The plants were identified using PIGNATTI's "Flora d'Italia" (1976). A voucher specimen of the plants is stored in the Herbarium of the Medical Botany Chair at the State University of Salerno.

Animal experiment

Nine lactating Maltese goats were divided into three equal groups and each group was housed in a box (3x2 m). Group C (control) was fed *ad libitum* with a diet of natural hay *ad libitum* and 300 g/per head day^{-1} of concentrates (barley 200 g and maize 100 g).

Groups H (hawthorn) and B (borage) were fed *ad libitum* with green plants of *C. oxyacantha* and *B. officinalis*, respectively. A sufficient quantity of borage and hawthorn in the bud-flowering stage was harvested daily from a native pasture on the farm of the Istituto Sperimentale per la Zootecnia. No vitamins, minerals or concentrates were added to diet of these animals.

During ten days of adaptation to the diet, the feeding behaviour was evaluated. Thereafter, data were collected regarding the measurements of daily plant intake and milk production for each group for seven days. Cumulative milk samples for each group were collected daily and stored in bottles at -20°C.

Plant samples made up of the plant parts usually grazed by the goats were harvested from the same pasture. The samples were air dried and preserved in canvas sacks until used for chemical analysis.

Plant extraction and analysis

Five-hundred grams of air-dried borage stems, flower buds and flowers were successively extracted with petroleum ether, chloroform and methanol at room temperature at a concentration of 10% w/v to give 2.2, 2.8 and 3.7 g of residues, respectively. Air-dried leaves, flowers and terminal sprouts of hawthorn (500 g) were extracted in the same way, obtaining 2.6 g of petroleum ether, 4.7 of chloroform and 5.8 g of methanol residues.

Both chloroform extracts were fractionated on a silica-gel (Merck, Milan, Italy) column (80x4 cm) eluting with chloroform and mixtures of $CHCl_3$ and MeOH of increasing polarity.

Fractionation of the borage chloroform extract gave 467 fractions that were pooled into 17 main fractions (BI-BXVII) on the basis of their thin layer chromatography (TLC) similarity. The hawthorn chloroform extract was fractionated into 576 fractions, and then pooled into 15 main fractions (H1-HXV).

Fractions containing flavonoidic compounds (BVII and HVIII) were purified by RP-HPLC on a µ-Bondapack column (30 cm x 7.8 mm) eluting with a 1:1 mixture of H_2O-MeOH.

Quercetin (12.3 mg) and 5,7-dihydroxyflavone (3.5 mg) were recovered from fraction BVII. Fraction BIX yielded pure oleanolic acid (2.5 mg).

Fraction HVIII afforded pure quercetin (12.7 mg) and vitexin-7-glucoside (2.5 mg).

Methanol extracts of borage and hawthorn were both fractionated in the same way. Methanol extracts were fractionated in a H_2O-BuOH system. BuOH extracts dissolved in methanol were purified on a Sephadex LH-20 (Amersham, Milan, Italy) column, eluting with MeOH.

Ninety-four fractions were obtained from the borage methanol extracts (BME), which were then grouped into 8 main fractions (BMEI-BMEVIII) on the basis of their TLC similarity. Fraction BMEV was purified by RP-HPLC under the same conditions as reported above; rutin (4.5 mg), kaempferol (4.6 mg) and

kaempferol 3-O-β-D-glucopyranoside (1.9 mg) were recovered.

Hawthorn methanol extract (HME) gave 87 fractions, that were pooled into 8 main fractions (HMEI-HMEVIII) on the basis of their TLC similarity in BuOH: AcOH:H_2O (12:3:5) and $CHCl_3$:MeOH: H_2O (70:30:3). Rutin (8.9 mg) and apigenin (2.3 mg) were obtained as pure constituents by purifying the HMEVI fraction by means of RP-HPLC, on a μ-Bondapack column (30 cm x 7.8 mm) eluting with a 1:1 mixture of H_2O-MeOH.

Five-hundred grams of the diet fed to group C (Control) were extracted and fractionated as reported for borage and hawthorn; no flavonoids were found.

Milk extraction and analysis

One litre of each milk ($D_{20°}$ = 1.021) sample was lyophilized and the residue was extracted successively at room temperature and at a concentration of 10% w/v with petroleum ether, chloroform, methanol. Milk from group H gave 8.9 g, 465.3 mg and 2.6 g of petroleum ether, chloroform and methanol residues, respectively. The milk from group B gave 9.9 g of petroleum ether, 768.9 mg of chloroform and 2.8 g of methanol residues. The milk from group C (control) gave 7.5 g, 879.3 mg and 1.8 g of petroleum ether, chloroform and methanol residues, respectively. Like the chloroform plant extracts, the chloroform milk extracts were fractionated on a silica-gel column eluting with chloroform and mixtures of chloroform and methanol of increasing polarity.

Chloroform extract fractions of milk B were pooled into 9 main fractions (MBI-MBIX). Fraction MBVI was further purified by RP-HPLC on a Bondapack column (30 cm x 7.8 mm) eluting with a mixture of H_2O-MeOH 1:1. Pure compounds of 5,7-dihydroxyflavone (1.4 mg), flavone (2.7 mg) and 5,7,4'-tri-hydroxyflavonol (2.7 mg) were recovered from this fraction. Fraction MBIII, purified in the same way, contained pure β-amyrin (1.5 mg). Methanol extract, treated in the same manner as the plant methanol extracts, was fractionated on a Sephadex LH-20 column. Seventy-five fractions were collected and pooled into seven main fractions MBMEI-MBMEVII. Fraction MBMEIV, purified by RP-HPLC revealed the presence of rutin (1.7 mg).

Fractions of the chloroform extract of milk H were pooled into 10 main fractions (MHI-MHX). Fraction MHV was purified by RP-HPLC, under the same conditions as reported for the H milk, and revealed pure 5,7-dihyroxyflavonol (2.3 mg), flavone (3.5 mg), and quercetin (1.2 mg). Pure β-sitosterol (1.8 mg) was purified from fraction MHIII. The methanol extract did not show the presence of flavonoids.

The control milk did not reveal the presence of flavonoid derivatives in the $CHCl_3$ or MeOH extracts.

Structural determination of compounds

The structure of the pure compounds that were isolated from both plants and milk was determined by accurate analysis of ^{1}H, ^{13}C and ^{13}C DEPT NMR data and by comparison with literature data (AGRAWAL, 1989). NMR spectra were obtained on a Bruker DRX 600 Spectrometer (Bruker, Karlsruhe, Germany). The identification of the flavonoid compounds isolated from the plants and milk was also confirmed, when possible, by HPLC analyses by comparing the retention times with those of standard compounds (Sigma-Aldrich Co., Milan) (FICARRA *et al.*, 1984, 1990).

Statistical analysis

Determinations of compounds from both plants and milk were repeated three times and the data are reported as mols/kg of dry plant or milk, and represent means of these three determinations.

RESULTS AND DISCUSSION

The feeding behaviour and food intake of goats changed in relation to plant species and feeding regimes. The goats preferred to eat the apex of the stems, the flower buds and flowers of borage, while they preferred the leaves, flowers and terminal sprouts of hawthorn. The goats browsed 85.3 and 51.8% of borage and hawthorn, respectively, in relation to the total amount available for the animals.

The level of borage dry matter intake (DMI) varied from 50 to 220 g/head day^{-1} (115±33 g); for hawthorn it was from 364 to 955 g/head day^{-1} (516±44). The DMI of the control group was 860 g/head day^{-1} (755±105).

The metabolites of plant origin (terpenoids and flavonoids) found in the plant and milk, respectively, are listed in Table 1. The triterpenoids are only represented by oleanolic acid, found in the borage chloroform extract and by β-amyrin, present in the milk (chloroform extract) of the goats fed borage. β-sitosterol was isolated in the chloroform extract of group H.

In contrast, flavonoids were present in all the plant and milk extracts. The chloroform extract of milk from goats fed borage contained 5,7,4'-tri-OH-flavonol, 5,7-di-OH-flavone, and flavone; the methanol extract contained rutin. Milk of the hawthorn-fed group contained quercetin, 5,7-di-OH-flavone, and flavone (chloroform extract). Only the methanol extract of milk from goats fed hawthorn did not contain any of these compounds. The control milk did not contain triterpenoid or flavonoid derivatives. No other phenolics were found in the milk. The chemical composition of the milk from each group (B, H, and control) was homogeneous for the entire experiment.

The milk of both groups B and H, contained compounds that probably came from the diet; flavonoids and terpenoids, in fact, are metabolites that are wide-

Table 1 - Plant metabolites found in plants and milk. Data are expressed as mols/1,000 g of dry plant or milk. Data are means of three determinations.

Extract	Plants			Milk	
	Borage	Hawthorn	Control	Borage fed	Hawthorn fed
Chloroform					
	Quercetin $8.1\pm0.3\times10^{-5}$ 5,7-di-OH-Flavone $2.7\pm0.3\times10^{-5}$ Oleanolic acid $1.1\pm0.1\times10^{-5}$	Quercetin $8.4\pm0.6\times10^{-5}$ Vitexin-7-glucoside $0.8\pm0.1\times10^{-5}$	None	5,7,4'-tri-OH-Flavonol $9.2\pm0.3\times10^{-6}$ 5,7-di-OH-Flavone $5.4\pm0.3\times10^{-6}$ Flavone $12\pm0.4\times10^{-6}$ β-Amyrin $3.4\pm0.2\times10^{-6}$	Quercetin $3.9\pm0.1\times10^{-6}$ 5,7-di-OH-Flavonol $8.3\pm0.3\times10^{-6}$ Flavone $16\pm0.2\times10^{-6}$ β-Sitosterol $4.2\pm0.1\times10^{-6}$
Methanol					
	Rutin $1.5\pm0.3\times10^{-5}$ Kaempferol $2.0\pm0.2\times10^{-5}$ Kaempferol-3-O-β-D-glucopyranoside $0.9\pm0.2\times10^{-5}$	Rutin $2.9\pm0.2\times10^{-5}$ Apigenin $1.7\pm0.2\times10^{-5}$	None	Rutin $2.7\pm0.1\times10^{-6}$	None

spread in higher plants. The goats in group B ingested about 435 mg/week of total flavonoidic compounds, excreting about 105 mg/week of these compounds in the milk. The ratio 435/105 is similar to that found for rutin, 80 mg ingested/23 mg excreted. The goats of group H showed a greater intake of dry matter and, consequently, had greater quantities of total flavonoids. The presence of flavonoids in the milk is not unexpected, considering that the mammary route is one of the drug excretion pathways. On the other hand, the literature reports the presence of phenolics in ruminant milk that came from the plants ingested by the animals (O'CONNELL and FOX, 2001; KILIC and LINDSAY, 2005) and flavonoid transport by mammalian endothelial cells has been proposed (SCHRAMM *et al.*, 1999).

The concentration of the total flavonoid fraction found in the milk of both groups is low in comparison to the corresponding fractions found in the plants. This finding is in agreement with the literature which indicates that urinary and biliar excretion of flavonoids and/or their metabolites are the main metabolic pathways for such compounds (BABA *et al.*, 1981; SAWAI *et al.*, 1987; HUTCHINS *et al.*, 1995; LEE CHAO *et al.*, 2002). The concentration of flavonoids in plants ranges from 0.8 to $8.4x10^{-5}$ mols/kg, whereas the concentration recovered in the milk was about 10-times less, ranging from 2.7 to $16x10^{-6}$ mols/kg.

The qualitative analysis of the flavonoidic fraction present in the milk provided some insights: 1) two flavonoids (rutin and quercetin) are present both in plants and in the milk; however, their concentration in the milk is low if compared with the concentration found in the plants. GEE *et al.* (2000) and MORAND *et al.* (2000) reported that rutin is hydrolysed during absorption in the monogastric digestive tract. The presence of rutin and quercetin in milk may constitute a non-hydrolysed aliquot or may indicate a difference between monogastrics and ruminants. 2) Other flavonoids that were purified from the milk were different from those found in each plant, but generally had a chemical structure that was less substituted than that of the plant compounds.

In the literature, controversies abound concerning which form of flavonoids are absorbed (LEE CHAO *et al.*, 2002). Some animal studies have shown that some flavonoids should be considered non-absorbable because they are bound to sugars such as β-glycosides (DI CARLO *et al.*, 1999). On the other hand, other animal models have shown that gastrointestinal metabolism of flavonoids is dependent on the intestinal microflora (GRIFFITHS *et al.*, 1981; BOKKENHEUSER *et al.*, 1987; WINTER *et al.*, 1989; SCHNEIDER *et al.*, 1999; JUSTESEN *et al.*, 2000; LEE CHAO *et al.*, 2002). Our hypothesis is that the gastrointestinal microflora of the goat can structurally modify feed flavonoids through hydrolyses and/or other interactions that result in molecules that are structurally less complex.

The data presented in this study raise new questions: 1) for how long a time can flavonoids be recovered from milk? 2) What is the concentration of such compounds in the milk in relation to time? 3) Does the presence of such compounds in milk have nutriceutical applications that could improve health benefits of milk for humans?

Flavonoids are well known for their biological activities (DI CARLO *et al.*, 1999) and, in some modern strategies for preparing fortified foods, these compounds are added to milk and other food (NINFALI *et al.*, 2002). Our data demonstrate that the presence of phenolics and terpenoids in milk depends on the animal feed. This finding has also been confirmed for other classes of compounds, g. e. tocopherol and retinol (PIZZOFERRATO *et al.*, 2000b) and volatile terpenes (COULON *et al.*, 2000). Moreover, the consumption of phenolic-rich foods can

affect both the animal's health and the sensory aspects of the milk and their products (O'CONNELL and FOX, 2001).

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SHORT COMMUNICATION

EFFECT OF *BIFIDOBACTERIUM LACTIS* 420 ON MICROBIOTA AND IMMUNE FUNCTION

EFFETTO DEL *BIFIDOBACTERIUM LACTIS* 420 SUL MICROBIOTA E SUL SISTEMA IMMUNITARIO

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ABSTRACT

A pilot feeding trial was performed for four weeks to study the influence of *Bifidobacterium lactis* 420 on healthy adult volunteers. *B. lactis* levels increased by more than 1,000-fold. Total bifidobacteria increased only in subjects who had low initial levels. *B. lactis* 420 appeared to have some influence on the metabolism of the intestinal microbiota and immune markers were only marginally influenced. Interestingly, trends for inverse correlations between faecal

RIASSUNTO

È stata condotta una prova di alimentazione per quattro settimane a livello pilota allo scopo di studiare l'influenza del *Bifidobacterium lactis* 420 sulla salute di volontari adulti sani. I livelli di *B. lactis* aumentavano più di mille volte. I bifidobatteri totali aumentavano solo in soggetti che avevano bassi livelli iniziali. *B. lactis* 420 sembrava avere influenza sul metabolismo di microbioti intestinali e i markers immunitari venivano solo marginalmen-

- Key words: *Bifidobacterium lactis*, immune function, intestinal microbiota, microbiota activity, probiotic -

TNF-α and IgA concentrations and *B. lactis* were noted. Further investigation with more subjects is needed to confirm these influences on the microbial metabolic activity.

te influenzati. Saranno stati osservati andamenti interessanti di correlazioni inverse fra le concentrazioni di TNF-α fecale e IgA e *B. lactis.* Sono necessari ulteriori approfondimenti su un numero superiore di soggetti per confermare queste influenze sull'attività metabolica microbica.

INTRODUCTION

Selected strains of probiotics have specific health benefits. Probiotics often, though not always, seem to exert their health benefits by modulating the composition and/or activity of the intestinal microbiota. Markers for the beneficial effects of probiotics can be measured by assessing the composition of the intestinal microbial community, as well as microbial metabolites (BENNO *et al.,* 1996) and markers of immune function (ACHESON and LUCCIOLI, 2004).

The aim of the current pilot study was to investigate the influence of consuming *Bifidobacterium lactis* 420 on markers of intestinal health in healthy adults.

MATERIALS AND METHODS

The study was approved by the Ethics Committee of the Department of Surgery, Hospital District of Helsinki and Uusimaa and an informed written consent was obtained from each of the subjects. Eight healthy adults (4 men, 4 women; 28-48 years old, mean age 40) were recruited for the study.

The subjects were enrolled for a baseline period of one week, during which time they were instructed to refrain from consuming probiotic-containing products. During the following four-week period, the subjects consumed daily one sachet containing 10^9 CFU *B. lactis* 420 and 1 g saccharose. The subjects were followed for another week of washout. Faecal samples were collected at the baseline (0), during the administration (1, 2, 3 and 4 weeks) and two and seven days after probiotic consumption had been stopped. The samples were stored at -20°C 3-4 h after defecation.

The total number of microbes (both dead and viable) was determined by flow cytometry as described by PEURANEN *et al.* (2004), using SYTO® 24 (Molecular Probes Inc. Eugene, OR, USA).

Faecal levels of total bifidobacteria and *B. lactis* were determined with quantitative real-time PCR as described by GUEIMONDE *et al.* (2004).

Microbial activity was determined by measuring the short-chain fatty acids (SCFA), acetic, butyric and propionic acids, and branched-chain fatty acids, valeric, iso-butyric, 2-methyl butyric and iso-valeric acids. These were determined by gas chromatography as described by HOLBEN *et al.* (2002).

IgA and tumour necrosis factor (TNF)-α were measured from faecal samples by ELISA as described by PEURANEN *et al.* (2004). For the measurements, commercial kits were used according to the manufacturers' instructions; the IgA, E80-102, was from Bethyl Laboratories, Inc. (Montgomery, TX, USA), and the TNF-α, Quantikine HS Human TNF-α, SSTA00C, was obtained from R&D Systems Inc. (Minneapolis, MN, USA).

Faecal dry matter was determined by drying weighed samples in an oven at 105°C for 18 h.

Differences between time points were calculated according to Wilcoxon signed rank test. Correlations were calculated using Spearman rank correlation tests. Results were considered significant with p-values less then 0.05.

RESULTS AND DISCUSSION

Neither the total number of microbes, nor the total number of bifidobacteria were influenced by the administration. This result may be related to the relatively high initial levels of bifidobacteria (mean $4x10^9$/g) in the subjects. However, four subjects who had low initial bifidobacteria levels ($<10^9$/g) exhibited a significant increase ($p=0.027$) in the level of faecal *Bifidobacterium* after the four-week period with probiotic consumption. The results of previous studies in which other probiotics (BENNO *et al.,* 1996) and also prebiotics (TUOHY *et al.*, 2001) were used showed that bifidogenic effects were mainly observed in subjects who had low initial *Bifidobacterium* levels.

Compared to the baseline numbers of faecal *B. lactis* ($1.2x10^5$/g faeces), there was a significant increase ($p<0.03$) in these values at each of the time points during the intervention and two days after probiotic consumption was stopped (10^8/g). The faecal *B. lactis* count returned to pre-intervention levels seven days after probiotic consumption had been stopped (Fig. 1). These results in-

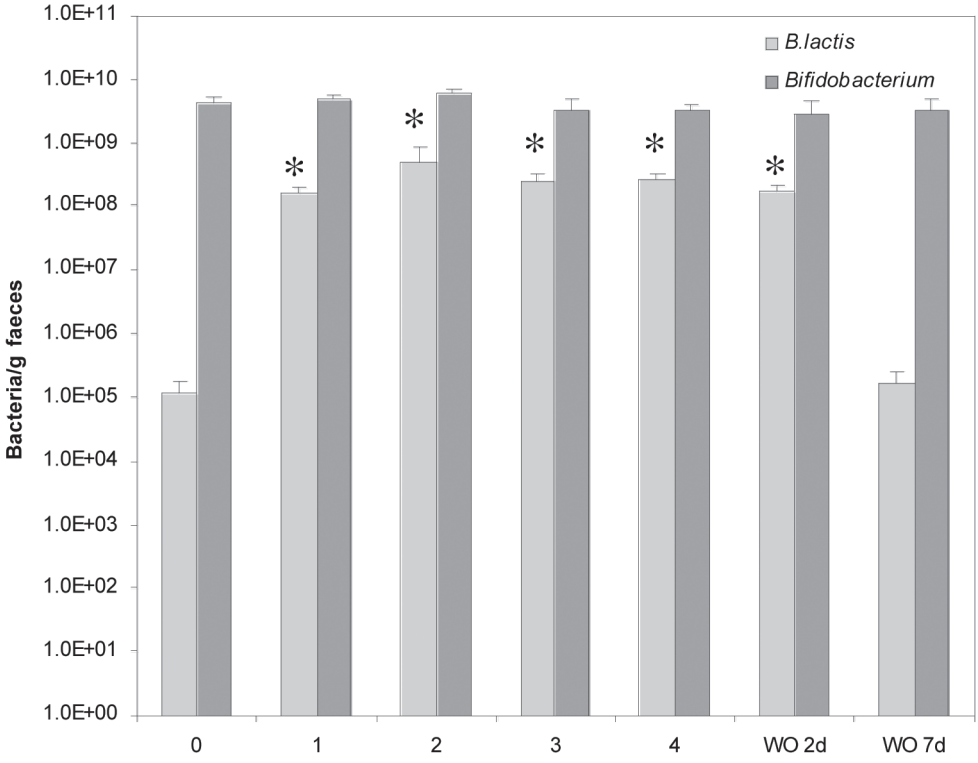


Fig. 1 - *Bifidobacterium lactis* and total *Bifidobacterium* levels in faeces of volunteers consuming probiotic *Bifidobacterium lactis* 420 as determined by quantitative real-time PCR. Bars indicate the average of eight subjects and standard deviation. * Significantly different from baseline (0). WO; washout 2 and 7 days.

dicate that there is only a transient colonisation by *B. lactis* 420.

The intervention had some effect on the levels of metabolic end-products of the intestinal microbiota, which indicated that the consumption of *B. lactis* 420 influenced the metabolism of the microbiota. The percentage of acetic acid within the total short-chain fatty acid composition increased slightly (p=0.0357) from the baseline (57%) to the end of the intervention (61%). The values returned back to the pre-intervention levels (58%) 2 days after probiotic consumption had stopped. No significant changes over time were observed for total the short-chain fatty acids (82 mmol/kg faeces), propionic and butyric acids accounted for, 17 and 13% of the total short-chain fatty acids, respectively.

Inverse correlations were observed between the change in total bifidobacteria and the change in faecal propionic and butyric acid content from the baseline to week 4 (p=0.0376 and 0.1015, respectively and rho=-0.786 and -0.619, respectively). The decrease in propionic acid maybe related to the fact that it can be metabolised by bifidobacteria (ROY, 2001). Furthermore, a positive trend (p=0.1015) was observed in the correlation (rho=0.619) of faecal acetic acid content from the baseline to the end of the intervention. Thus, increases in bifidobacteria were associated with lower propionic and butyric acid levels and a higher level of acetic acid in the faeces.

Of the branched-chain fatty acids tested, only valeric acid decreased at 2 and 4 weeks (p=0.0117 and 0.0357, respectively) compared to the baseline. These values returned to pre-intervention levels 2 days after probiotic consumption had stopped. Lower valeric acid values indicate that amino acid fermentation was reduced. This is desirable, because this translates into a concomitant reduction in phenols and indoles. A significant (p=0.0322) inverse correlation (rho=-0.810) was observed between the change in bifidobacteria and the change in the level of faecal valeric acid from the baseline to the end of the intervention. An increase in bifidobacteria was associated with lower levels of valeric acid in the faeces.

The faecal TNF-α levels were low and ranged from below the detection limit (0.5 pg/g) to 12 pg/g, which are normal levels for healthy adults (QADRI *et al.*, 2002). The faecal IgA concentrations ranged from 4 to 336 μg/g faeces (mean 102 μg/g), which are also within the range reported for healthy adults (PESSI *et al.*, 2000). The consumption of probiotics did not influence the faecal TNF-α or IgA levels. The lack of an immune response upon the consumption of the probiotic may have been related to the fact that the volunteers were healthy adults. The lack of an immunological response was previously reported in a similar study population (MÄKELÄINEN *et al.*, 2003). Considering all the pooled data, a trend (p=0.067) for an inverse correlation (rho=-0.69) between faecal *B. lactis* and the TNF-α and IgA levels (p=0.0508; rho=-0.724) was observed. Thus, an increase in *B. lactis* was associated with a decrease in the TNF-α and IgA levels in the faeces. While the significance of these inverse correlations is not known, it should be noted that most of the IgA was bound to the faecal bacteria (VAN DER WAAIJ *et al.*, 1996), while the assay measured free IgA. Another potential explanation is that *B. lactis* 420 may enhance cellular immune responses, which, in turn would suppress humoral responses (CROSS *et al.*, 2001).

CONCLUSION

The consumption of *B. lactis* 420 seems to influence the metabolic activity of the intestinal microbiota. Although the changes associated with the consumption of *B. lactis* 420 were statistically significant, they were in gener-

al small. Their biological relevance has yet to be determined. The limited effects may have been related to the small size of the study group which limits the statistical power of the intervention. Furthermore, all of the subjects were healthy adults, which may also have limited the possibility of observing physiological effects, such as changes in the immune response (MÄKELÄINEN *et al.*, 2002). Nevertheless changes were observed and a larger clinical intervention that will focus on the intestinal microbiota activity is warranted. In order to observe changes in immune function, a subject group should be selected that has reduced immune function or inflammation (GILL *et al.*, 2001).

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OCCURRENCE OF OCHRATOXIN A IN RAW HAM MUSCLES AND IN PORK PRODUCTS FROM NORTHERN ITALY

PRESENZA DI OCRATOSSINA A IN CAMPIONI DI MUSCOLO DI COSCIE FRESCHE E IN PRODOTTI A BASE DI CARNE SUINA PROVENIENTI DAL NORD ITALIA

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ABSTRACT

The occurrence of ochratoxin A (OTA) in different pork products was surveyed. Twenty-two samples of raw ham muscle were obtained from industrial plants for ham production and 84 samples of dry-cured ham (n=30), cooked ham (n=12), salami (n=12), coppa (n=18) and würstel (n=12) were collected from retail outlets located in the Emilia region (northern Italy). After extraction and immunoaffinity clean-up steps, OTA was quantified using HPLC-fluorescence detection. The limits of de-

RIASSUNTO

È stata effettuata un'indagine sulla contaminazione da ocratossina A (OTA) di carne suina fresca e conservata. Sono stati raccolti 22 campioni di carne fresca (rifilature di prosciutto) presso stabilimenti industriali e sono stati prelevati complessivamente 84 campioni di prosciutto crudo (n=30), prosciutto cotto (n=12), salame (n=12), coppa (n=18) e würstel (n=12), in punti vendita dell'Emilia. Dopo estrazione e purificazione su colonna di immunoaffinità, l'OTA è stata quantificata mediante HPLC con

- Key words: occurrence, ochratoxin A, pork meat products -

tection and quantification (LOQ) for the method used were 0.01 and 0.03 µg/kg, respectively. OTA was detected in 47% of the samples, but only 24% contained OTA above the LOQ. High levels of OTA were only found in some dry-cured ham samples: 5 (17%) exceeded 1.0 µg/kg, the limit set by the Italian Ministry of Health guideline and 2 (7%) exceeded 10 µg/kg; however, the median was only 0.02 µg/kg. The other pork products showed lower OTA contamination that was always below 0.30 µg/kg. The contribution of pork products to OTA intake in Italy appears to be negligible.

rivelazione fluorimetrica. I limiti di rivelazione e di quantificazione (LQ) per il metodo usato erano di 0,01 e 0,03 µg/kg, rispettivamente. La tossina è risultata presente nel 47% dei campioni, ma solo nel 24% sopra al LQ. Alti livelli di OTA sono stati trovati solo in alcuni campioni di prosciutto crudo: in 5 (17%) di questi la contaminazione trovata ha superato il livello di 1,0 µg/kg, limite fissato dal Ministero della Sanità, e in 2 era superiore a 10 µg/kg; tuttavia, la mediana è risultata solamente di 0,02 µg/kg. Gli altri tipi di salumi hanno mostrato una bassa contaminazione da OTA, sempre inferiore a 0,3 µg/kg. Il contributo delle carni suine all'assunzione di OTA in Italia sembra essere trascurabile.

INTRODUCTION

Ochratoxin A (OTA) is a mycotoxin produced by various *Aspergillus* and *Penicillium* species. Several studies have shown that the toxin has carcinogenic, nephrotoxic, immunotoxic, teratogenic and possibly neurotoxic and genotoxic properties. It has also been associated with Balkan Endemic Nephropathy and the development of urinary tract tumours in humans (MARQUARDT and FROHLICH, 1992; PLEŠTINA, 1996; SCHLATTER *et al.*, 1996). The toxicology and human health risk of OTA have been assessed at both the European and international level. OTA has been classified by the International Agency for Research on Cancer (IARC) into Group 2B as a possible human carcinogen (CASTEGNARO and WILD, 1995). The Scientific Committee on Food (SCF) of the European Commission (COMMISSION OF THE EUROPEAN COMMUNITIES, 1998) noted that tolerable daily intake (TDI) estimates from other organizations (Joint FAO/WHO Expert Committee on Food Additives [JECFA], Canadian authorities, Nordic Working Group on Food Toxicology and Risk Evaluation) ranged from 1.2-14 ng/kg b.w./day. Due to concerns about the potential genotoxicity of OTA, the SCF recommended that exposure should be kept towards the lower end of the range, e.g. below 5 ng/kg b.w./day.

The worldwide occurrence of OTA contamination of raw agricultural products has been amply documented. Cereals and derived products are assumed to be the major dietary source of OTA, even if the occurrence of the toxin in other plant products such as dried fruit, coffee, cocoa, spices and wine has been recently demonstrated (BENFORD *et al.*, 2001; TRUCKSESS *et al.*, 1999; THIRUMALA *et al.*, 2001, JØRGENSEN, 1998). Products of animal origin can also contribute to the OTA intake in humans; OTA can occur in meat and meat products as a result of either: (1) indirect transmission from animals exposed to naturally contaminated feed, or (2) direct contamina-

tion with moulds or with naturally contaminated spice mixtures used as ingredients (GAREIS, 1996). Among farm animals, pigs are particularly sensitive to the toxin, while ruminants are able to degrade the toxin in the rumen. OTA is absorbed in the small intestine and then bound to serum proteins; the serum half-life of the toxin in pigs was calculated to be in the range of 72 to 120 h. The highest amounts of OTA are found in the blood, while distribution within the tissues follows the pattern kidney > liver > muscle > fat (KROGH *et al.*, 1974; PATTERSON *et al.*, 1976; GALTIER *et al.*, 1981; CURTUI *et al.*, 2001). OTA can also be produced by moulds growing on pork products during ripening: *Penicillium verrucosum* and OTA were found in hams sampled during the curing time (SPOTTI *et al.*, 2001). Several studies have been carried out in different countries on the occurrence of OTA in edible tissues of pigs and in pork products. All the available data were gathered and a weighed mean concentration of 0.12 and 0.052 µg/kg was calculated for pig kidney and products of animal origin (mainly pork products), respectively. The contribution of this food category and the total OTA intake in Europe were calculated to be about 1.5 and 45 µg/kg b.w. per week, respectively (JECFA, 2001 and references therein). Few surveys have been published on the contamination by OTA of pork products produced in Italy. In 2001, a retail survey of different cured pork products (ZANNOTTI *et al.*, 2001) found that 56% of 159 samples contained OTA at a level >1.0 µg/kg, the limit set by the Italian Ministry of Health guideline (MINISTERO DELLA SANITÀ, 1999). CHIAVARO *et al.* (2002) reported on the occurrence of OTA in 42 dry-cured ham samples, in the middle and at the end of the ripening period. The OTA level was >1.0 µg/kg on 15 samples and the maximum level found was 2.3 µg/kg. No detectable OTA (>0.1 µg/kg) was found in a survey on 15 salami and 5 mortadella samples (CANTONI *et al.*, 2004).

This paper reports the results of a survey on the incidence and levels of OTA in fresh pork and pork products produced in northern Italy, in order to evaluate the contribution of these products to the OTA intake in humans.

MATERIALS AND METHODS

Samples

During the years 2001 and 2002, 22 samples of raw ham muscle were obtained from industrial plants for ham production and 84 samples of pork products (30 dry-cured ham, 12 cooked ham, 12 salami, 18 coppa and 12 würstel samples) were collected from retail outlets in the Emilia region (northern Italy). The products were sold in consumer-size packages and the minimum sample size was 100 g. All sampled products were produced in northern Italy. After collection, the samples were frozen and kept at -20°C until the time of analysis.

Reagents and standards

The immunoaffinity columns (Ochraprep) were purchased from Rhône Diagnostics Technologies (Glasgow, UK). Chemicals and solvents used for the extraction and clean-up solutions were ACS grade or equivalent. Phosphate buffered saline (PBS) was prepared as per Rhône (NaCl 8 g/L, KCl 0.2 g/L, Na_2HPO_4 1.15 g/L, KH_2PO_4 0.2 g/L; pH 7.4). Acetonitrile and acetic acid used for HPLC mobile phases were LC grade.

OTA standard was obtained from Sigma-Aldrich (St. Louis, MO, USA). A solution of OTA (40 µg/mL in benzene:acetic acid 99:1) was calibrated spectrophotometrically at 333 nm using the value 5550 for the extinction coefficient (AOAC International, 1995) and stored at -20°C when not in use; after calibration of the

OTA solution, working standards were prepared by evaporating an exact volume under a stream of nitrogen and redissolving the residue in the mobile phase. Ten OTA standards of between 2 and 60 pg were injected.

Analysis for ochratoxin A

Extraction and immunoaffinity clean-up

OTA in meat products was extracted and purified, using an immunoaffinity column, as in the method reported by DRAGACCI *et al.* (1999), with the following modifications. Samples were homogenized in a Moulinex mill and aliquots of 10 g were weighed in a 250 mL plastic centrifuge bottle; 100 mL of chloroform acidified with 0.8 mL 85% orthophosphoric acid were added and the resulting mixture was extracted for 2 min using an Ultra-Turrax T25 homogenizer at 12,000 rpm and subsequently for 15 min with a wrist-action shaker. After centrifugation at 6,400 *g* for 10 min at 4°C and filtration through a folded filterpaper, an aliquot of 50 mL was transferred into a separatory funnel; then a liquid-liquid extraction was performed by adding 50 mL of 0.5 M $NaHCO_3$ solution. Extraction was repeated with 25 mL $NaHCO_3$, the aqueous phases were recombined, adjusted to pH 7.5 with 3N HCl and the final volume was measured. The immunoaffinity column was placed on an SPE vacuum manifold (Visiprep, Supelco, Bellefonte, Pennsylvania, USA), then 30 mL of the aqueous extract was applied to the column (flow rate: 0.5 mL/min), followed by a washing with PBS (10 mL). The OTA was then slowly eluted (0.5 mL/min) from the column with methanol acidified with acetic acid (98:2, 2 mL) into a graduated glass vial; the eluate was concentrated under nitrogen, brought to 1 mL with the mobile phase (acetonitrile:2% acetic acid 41:59) and vortex-mixed for a few seconds. The extract was filtered (Millipore Corporation, Bedford, MA, USA, HV 0.45 µm) before HPLC analysis.

Chromatography

The HPLC system consisted of a Perkin Elmer 200 (Perkin Elmer, Norwalk, Connecticut, USA), equipped with a Jasco AS 1555 sampling system and a FP 1520 fluorescence detector (Jasco Corporation, Tokyo, Japan) set at 333 nm excitation and 470 nm emission. The system was controlled by Borwin 1.5 software (Jasco). A RP-18 column (4 µm particle size, 125x4 mm i.d., Merck) was used at ambient temperature, with a mobile phase of acetonitrile:2% acetic acid (41:59) for OTA and (55:45) for OTA methyl ester, at 1.0 mL/min. The retention time stabilities were checked by injecting a standard every 5 samples. The injection volume for both standards and sample extracts was 30 µL, corresponding to 60 mg of meat sample. Quantification was on the basis of peak areas using the Borwin software. Derivatization of OTA through methylation (GAREIS, 1999) with subsequent HPLC analysis was used for the qualitative confirmation of positive samples.

Statistics

The normal distribution of OTA content was checked using the "Proc. Univariate Normal" (SAS Institute, Inc., 1999, Release 8). Since the OTA content was not always normally distributed, the statistical significance of differences was examined by a two sample t-test, equivalent to a Wilcoxon rank sum test, using the t-approximation for the level of significance.

RESULTS AND DISCUSSION

The calibration curve showed good linearity over the range 2-60 ng ($r>0.998$). The limits of detection (LOD) and quan-

tification (LOQ) were determined by the signal-to-noise approach. The analyte response and the chromatographic noise were both measured from the chromatogram of a blank sample extract to which between 0.2 and 1.5 mL of an OTA solution (0.222 ng/mL) had been added. The LOD (0.01 µg/kg) and LOQ (0.03 µg/kg) were defined at those levels resulting in signal-to-noise ratios of 3 and 10, respectively. The recovery values were estimated by spiking known blank matrices with OTA at levels ranging from 0.2 to 1.0 µg/kg. Average recoveries for four replicate samples of each pork product ranged from 84 to 91% (Table 1). Results were not corrected for recovery. The identity of OTA was confirmed in a selection of 12 positive samples by preparing its methyl ester and re-injecting the sample into the HPLC. The disappearance of the OTA peak, and the corresponding appearance of a methyl-OTA peak was considered positive identification. Similarly, 8 samples with no detectable OTA were derivatized to check against the occurrence of false negatives: none were detected.

Figures 1 and 2 show typical chromatograms from a sample of naturally-contaminated dry-cured ham, as well as of OTA and OTA methyl ester standard solution.

The results for the 106 samples are shown in Table 2. OTA was detected in 50 samples (47%), but only 25 (24%) contained OTA concentrations above the LOQ.

Levels of OTA >1.0 µg/kg, the limit set by the Italian Ministry of Health guideline, were found in 5 (17%) out of 30 dry-cured ham samples (28.42, 12.50, 2.65, 1.83, 1.03 µg/kg). The mean value (1.62 µg/kg) was significantly higher when compared with those of the other pork products, that showed a very limited OTA contamination.

Comparing our data on dry-cured ham with those obtained in a previous Italian survey (CHIAVARO *et al.*, 2002), we found a lower incidence of samples exceeding 1 µg/kg (17 *vs.* 36%), but higher maximum values. Different results were obtained by ZANNOTTI *et al.* (2001). In this survey, the mean OTA level in dry-cured ham samples was 1.29 µg/kg, but 65.5%

Table 1 - Recovery of ochratoxin A from artificially contaminated pork products[a].

	Spike level (µg/kg)	Number of analyses	Recovery (%)	
			Mean±standard deviation	Range
Dry-cured ham	0.2	4	87±3	85-91
	1.0	4	89±2	87-92
Cooked ham	0.2	4	90±3	86-93
	1.0	4	91±3	89-94
Salami	0.2	4	85±4	81-88
	1.0	4	88±4	85-91
Coppa	0.2	4	84±3	82-86
	1.0	4	87±4	84-90
Würstel	0.2	4	86±4	82-90
	1.0	4	88±2	86-91
Muscle (for ham)	0.2	4	85±4	81-88
	1.0	4	86±3	84-89

[a] OTA concentration in the original matrix <0.010 µg/kg.

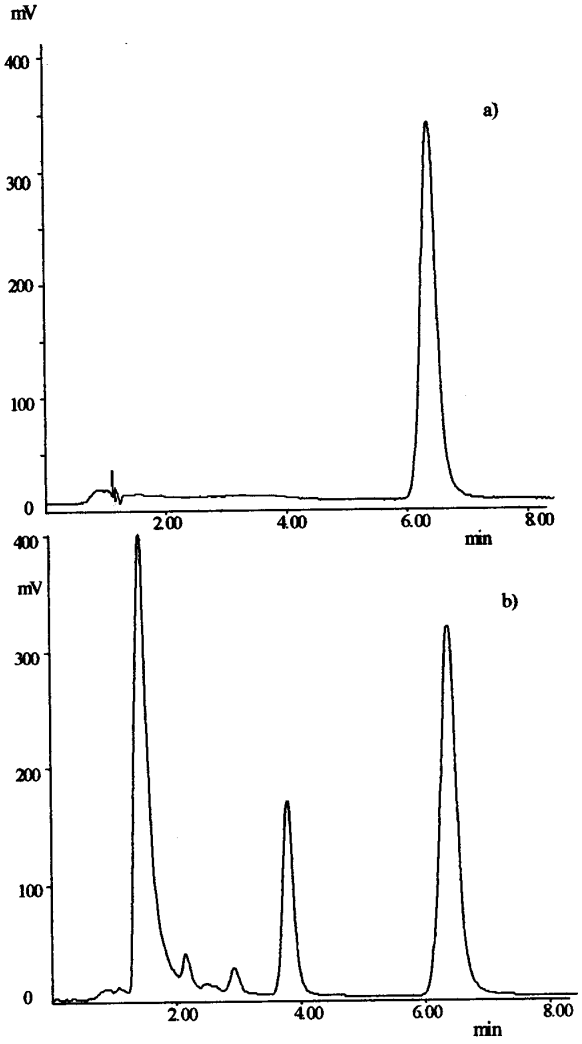


Fig. 1 - Chromatogram (HPLC; fluorescence detection) of: a) an OTA standard solution (5.55 µg/L), equivalent to 166.5 pg of OTA injected; b) a naturally-contaminated dry-cured ham sample containing 2.64 µg/kg (equivalent to 158 pg of OTA injected). Injection volume 30 µL each.

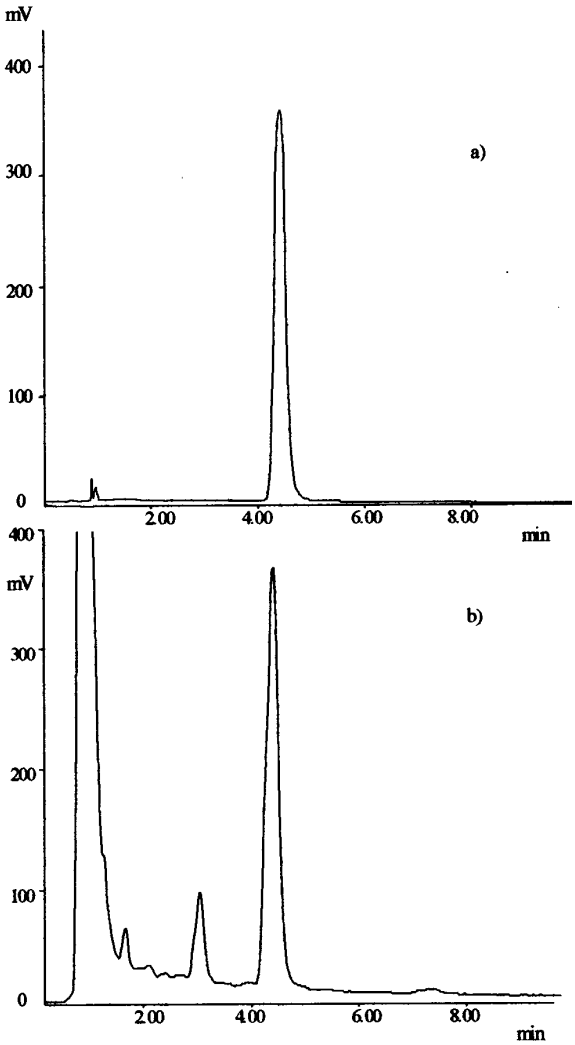


Fig. 2 - Chromatogram (HPLC; fluorescence detection) of: a) an OTA methyl ester standard solution (4.44 µg/L), equivalent to 133 pg of OTA injected; b) a naturally-contaminated dry-cured ham sample (extract derivatized) containing 1.83 µg/kg (equivalent to 110 pg of OTA injected). Injection volume 30 µL each.

of them exceeded 1 µg/kg. Moreover, in the same survey a diffuse contamination in some pork products was observed, with mean levels of 1.22, 1.05 and 1.54 µg/kg in cooked ham, salami and würstel samples, respectively.

In Italy, consumption of salt pork products is about 18.8 kg per person/year; dry-cured ham consumption is 21.3% of the total, corresponding to 11 g per person/day (ISTITUTO PER LA VALORIZZAZIONE DEI SALUMI ITALIANI, 2003).

Table 2 - Occurrence of ochratoxin A in pork and pork products produced in northern Italy.

	n	Positives (>LOQ)	Incidence of positives (%)	Mean of positives (µg/kg)	Mean of all (µg/kg)*	Median (µg/kg)	Maximum (µg/kg)
Muscle (for ham)	22	2	9	0.05	0.003[a]	<0.01	0.06
Dry-cured ham	30	12	40	4.06	1.62[b]	0.02	28.42
Cooked ham	12	1	8	0.05	0.004[a]	<0.01	0.05
Salami	12	4	33	0.06	0.02[a]	<0.01	0.08
Coppa	18	5	28	0.12	0.03[a]	<0.01	0.24
Würstel	12	1	8	0.06	0.005[a]	<0.01	0.06

[a,b] mean values with different superscripts (a-b) differ at P<0.001.
* assumes values <LOQ = 0 µg/kg.

In terms of public health, the potential impact of the presence of OTA in dry-cured ham can be evaluated considering consumption data and the median OTA level that we found (0.02 µg/kg). An exposure of 0.22 ng per person/day or 0.004 ng/kg b.w./day can be calculated, which is about 0.07% of the TDI of 5 ng/kg b.w./day fixed by the SCF of the European Commission. In Europe, cereals and wine contribute about 3.6 and 1.3 ng/kg b.w./day, respectively, to the average OTA intake (JECFA, 2001). If compared with these products, the contribution of dry-cured ham is therefore negligible.

CONCLUSIONS

This survey indicates that OTA contamination of pork products is a phenomenon which particularly concerns dry-cured ham. Both raw ham muscle samples and other Italian pork products, like salami, coppa, cooked ham and würstel show a very low occurrence and low levels of OTA. This finding suggests that indirect transmission from animals exposed to contaminated feed to the presence of OTA in pork and pork products occurs very rarely. Only direct contamination with OTA-producing moulds during the ripening of dry-cured hams can explain the high levels found in some of these samples. This conclusion is supported by a study carried out by an interdisciplinary group at our university, that showed the presence of *P. verrucosum* in the air of an industrial ripening room and on the surface of hams (BATTILANI, 2005).

In conclusion, the presence of OTA in dry-cured ham is not a cause for alarm, but should be monitored. Further research is necessary to study conditions that favour the growth of OTA-producing fungi and the preventive action that can be undertaken to reduce this contamination.

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CHEMICAL AND NUTRITIONAL CHARACTERIZATION OF SOME ITALIAN NICHE MEAT PRODUCTS, RAW AND COOKED

CARATTERIZZAZIONE CHIMICA E NUTRIZIONALE DI ALCUNI PRODOTTI DI NICCHIA ITALIANI A BASE DI CARNE, CRUDI E COTTI

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ABSTRACT

The nutritional variations in some typical semi-cured Italian meats after the cooking process were investigated. Three raw and three cooked samples of *Bondiola*, *Cotechino (Modena and Cremona)*, *Salama da sugo (Ferrara)* and *Lingua salmistrata (Veneto)* were studied. For *Lingua salmistrata* the root, centre and tip were analysed separately. Water, NaCl and protein %, total lipid content and its characterization, nitrate and nitrite concentration and the energy supply were determined.

RIASSUNTO

Questo lavoro riguarda lo studio delle variazioni nutrizionali in alcuni prodotti semiconservati a base di carne. Sono stati esaminati alcuni prodotti italiani tipici; per ciascun tipo sono stati analizzati tre campioni crudi e tre cotti, provenienti dallo stesso lotto. Della lingua salmistrata sono state analizzate tre porzioni: radice, centro e punta. Sono stati determinati il contenuto di nitrati e nitriti, le percentuali di acqua, NaCl, e proteine, il contenuto e la caratterizzazione dei lipidi totali e

- Key words: chemical composition, curing additives, niche meat products, nutritional value -

The nutrient composition and energy supply did not change due to cooking, while the NaCl, nitrite and nitrate concentration decreased.

l'apporto energetico. La concentrazione di NaCl, nitriti e nitrati diminuisce, mentre la composizione dei nutrienti e l'apporto energetico non variano molto dopo la cottura.

INTRODUCTION

Cured meats destined to be cooked include some salt-cured meats and some other products such as *lingua salmistrata* (corned tongue). Such products, obtained through different procedures, are usually boiled and more rarely grilled. With cooking there is a variation in the water content and the level of soluble proteins, low melting lipids, sodium chloride and nitrites and nitrates which are usually present as additives. These latter are of particular importance and their respective percent content may undergo positive or negative variations.

As far as food additives are concerned, there are legal limits. Therefore, it is necessary to determine if products comply with the law. It is also important to calculate the total daily intake of food additives (MARIANI COSTANTINI *et al.*, 1999).

Nitrates are ingested as part of our daily diet through products such as water, vegetables, fruit and their by-products, some kinds of cheese and salt-cured meats. If nitrate intake surpasses the acceptable daily dose, they are potentially hazardous, because they are transformed into nitrites by bacterial microflora. The European Commission's Scientific Committee on Food has fixed the maximum daily dose of nitrites and nitrates at 0.06 mg/kg of body weight and 3.7 mg/kg of body weight, respectively.

Nitrates and nitrites are used as additives to salt-cured meats for different purposes. One is to keep the deep crimson colour of the meat, through the reaction of nitrite with myoglobin. In raw meat the reaction gives rise to red nitrosylmyoglobin, while in cooked meat, where globin has been denatured, the reaction gives a pink colour, due to nitrosomyochromogen (PÖHNL, 2005). Another purpose is to prevent the growth of *Clostridium botulinum* strains that are sensitive to acidic pHs. As far as consumer health is concerned, most conscientious food industries add ascorbic or erythorbic acid to cured meats to reduce the nitrite concentration (MIRVISH *et al.*, 1995; COVELLI *et al.*, 1998). The starter bacteria used in the preparation of salt-cured meats may also reduce the pH. Moreover, nitrites preserve flavour and retard rancidity development (CAPPELLI and VANNUCCHI 2005).

The toxicity of nitrites is particularly associated with the formation of carcinogenic nitrosamines. These compounds may develop in the stomach, especially in persons who take antacids and in smokers, or may already be present in food (POGODA and PRESTON-MARTIN 2001). Infantile methemoglobinemia, less frequent than in the past, is mainly caused by the consumption of nitrate-rich vegetables, by persons whose enzymatic heme-protection systems are not completely efficient (CAPPELLI and VANNUCCHI 2005).

The UE Directive (1995) concerning salt-cured meats allows a maximum content for both sodium nitrite (E250) and potassium nitrite (E249) of 100 parts (175 for bacon) per million (ppm) in the finished product. Moreover, it allows a maximum of 250 ppm for both so-

dium nitrate (E251) and potassium nitrate (E252), which function as nitrite reserves. It has been acknowledged that these limits need to be lowered.

This study is a part of a broader research program to determine the nitrates and nitrites in foods of animal and plant origin (CENGARLE *et al.*, 2001; TILLOCA *et al.*, 2004). In this study the raw materials were some salt-cured meats and *lingua salmistrata* (corned tongue), that is a kind of beef by-product, rich in proteins with fat spread in non homogeneous way, that has been treated with saltpetre for preservation. The nitrate and nitrite content present in these products and their possible variation due to the cooking process were investigated. The data obtained, together with the lipid and the protein content, may be used to assign a correct nutritional value to the cooked products.

MATERIALS

Samples of *bondiola veneta, cotechino, lingua salmistrata and salama da sugo*, obtained from retail outlets, were used.

Bondiola veneta is a salt-cured meat stuffed into a pig bladder that gives it its typical round shape; it weighs about 800 grams and consists of lean pork meat, pork fat, pigskin, lard, garlic, red wine, salt, pepper and natural flavourings. Saltpetre is used as an additive and it is aging from twenty days to three months.

Salama da sugo is prepared with pork meat (53% neck, 32% bacon, 10% throat lard, 3% liver, 2% tongue), salt, pepper and red wine. No additives were declared. It is stuffed into a pig bladder that is suitable for aging since it has a solid, uniform thickness; it is tied up with a string that divides them it into eight parts. Aging is between six and twelve months at 10°-12°C with 75% water content and it is protected from the light.

Cotechino is also stuffed into pig's or beef's intestine. It has a variable weight and consists of equal amounts of processed lean pork meat, pork fat and pigskin, red wine with natural flavourings, salt, pepper, sugar and spices. Additives are saltpetre and sometimes soy lecithin (in the Cremona-type); it is seasoned outdoors for at least 15-20 days, according to the pigskin content; cooking time depends on the seasoning.

Lingua salmistrata is an ox tongue that is carefully cleaned, washed and beaten vigorously in order to breakdown the fibers. Additives are saltpetre, vinegar, pepper and spices and it weights 800-1,200 grams.

METHODS

Samples of each type were taken from the same lot in order to have reasonably homogeneous products to analyse and that would allow evaluation of the alterations in the composition that occurred as a result of the cooking process with a certain level of accuracy. Samples were taken from whole cooked products, so as to avoid breaking the casing, which would affect the reliability of the determinations.

Three raw samples and three cooked samples of each product were used; each portion was homogenised before being analysed. *Lingua salmistrata* samples were separated into the root, centre and tip, and the different parts were analysed separately.

Before cooking, the casing was lightly pricked with a needle, to avoid skin breaking during cooking. For boiling, the label instructions were followed.

The following measurements were made: water content and total lipids %, characterization of the total lipid fraction, by thin layer chromatography (TLC) and proton nuclear magnetic resonance (^{1}H-NMR), % protein, % NaCl and nitrite and nitrate (ppm). The caloric value was calculated for both raw and

cooked products. The water content, was determined according to the official method (BALESTRIERI and MARINI, 1996), while total lipids were extracted according to the BLIGH and DYER method (1959).

The distribution of the glyceridic forms was analysed using TLC, on Silica gel 60 plates, 20x20 cm, 0.25 mm thick (SIL-25 MACHEREY-NAGEL, Düren, Germany, cat n. 809013). The eluent mixture: hexane: diethyl ether: formic acid = 70 : 30 : 1.5 (C. Erba); detector: iodine vapours. Reading with TotalLab software (NONLINEAR DYNAMICS; Newcastle upon Tyne, UK) after scanning.

The ratio of polyunsaturated/unsaturated fatty acids was calculated by ^{1}H-NMR spectra: Spectrometer Varian XL 200 (Palo Alto, California, USA), solvent $CDCl_3$, solute/solvent ratio = 1:5, power 200 MHz. Two other parameters were determined, the number of vinyl protons (NV) and the number of methylene protons in α at two double bonds (NM), which are proportional to the total unsaturation and polyunsaturation levels, respectively. The ratio NM/NV was also determined.

$$NV = (A - B/4) / B \qquad NM = C/B$$

where A = area concerning the methinic protons of the double bonds + methinic proton at 2 of the esterified glycerol, at around 5.2 ppm; B = multiplet area at 4.22 ppm, concerning methylenic protons of esterified glycerol; C = area of methylenic protons included between two double bonds, at 2.77 ppm (CENGARLE *et al.*, 1992).

Total nitrogen was determined by the Kjeldahl method (BIFFOLI, 1990); the values for the proteins were obtained by multiplying the percentage of organic nitrogen by 6.25.

The NaCl content was estimated through differential argentometric titration (TATEO, 1978), while the number of nitrate and nitrite ions was estimated through colorimetric analysis using the method of BARBIERI *et al.* (1983).

RESULTS AND DISCUSSION

Table 1 reports the water content and total lipid % values. The variations are evident in the range of standard devia-

Table 1 - Mean values of the water content and total lipids (%/edible portion) in the samples before and after cooking.

Samples		part	Water content		Total lipids	
			mean	S.D.	mean	S.D.
Bondiola veneta	raw		47.40	0.28	27.1	1.27
	boiled		45.70	2.45	27.81	6.95
Cotechino Modena	raw		50.43	2.84	32.25	2.33
	boiled		36.90	3.58	38.47	7.47
Cotechino Cremona	raw		49.80	2.1	27.23	0.32
	boiled		49.20	1.8	26.68	0.18
Lingua salmistrata veneta	raw	root	55.13	0.9	15.80	2.47
		center	52.83	1.0	18.63	0.53
		tip	62.23	2.89	3.05	1.34
	boiled	root	55.38	0.52	12.30	2.10
		center	53.52	2.40	19.96	1.00
		tip	60.72	2.18	3.65	0.47
Salama da sugo Ferrara	raw		26.00	1.22	37.75	0.42
	boiled		27.82	3.11	16.59	0.60

tion (S.D.). The values decreased in every sample except for the *salama da sugo,* where the values increased slightly.

After cooking the edible part (e.p.) contained less water, therefore the related percentage lipid composition increased; the marked reduction recorded for the *salama da sugo* is certainly due to the loss of fat that occurred when the cap of the cured cooked meat was cut.

Regarding the composition of the lipid fraction, the results of the ^{1}H-NMR (Table 2) reveal that there are no significant variations in the unsaturated acids, in general, and in the polyunsaturated acids, in particular. In examining the results concerning the glyceridic forms (Table 3), it should be taken into consideration that, since they are relative percentages, the apparent increase of some components may be due to the decrease of others. For instance, while the increase of fatty acids might be ascribed to some hydrolysis phenomena, a real increase in triacylglycerols cannot be assumed. In this way the apparent increase of triacylglycerols and free fatty acids in the *salama da sugo* could be explained by a simultaneous reduction of polar compounds that passed through the casing.

In *lingua salmistrata,* besides the variability in the individual samples, that obviously originated from the different specimens, the results are rather difficult to explain as most of the root lacks casing.

The protein content (Table 4) was quite constant; the solubilization of proteins is evidently prevented by both the casing and the denaturation that occurred following the preparation processes.

The NaCl content (Table 4) decreased slightly in each sample with cooking: the minimum loss was 40 mg/100 g edible part (*bondiola* and *salama da sugo*), while the maximum loss was 580 and 560 mg/100 g edible part on the center and tip parts of the tongue, respectively.

The nitrite and nitrate content values (Table 5) in each cooked sample were within the legal limits. It should be noted that these ions were also present in the *salama da sugo* even though they were not declared among the ingredients. The highest nitrate content was found in the *lingua salmistrata*, due to

Table 2 - Mean values of the ^{1}H NMR parameters in the samples before and after cooking.
NV = (A - B/4) / B; NM = C/B, where: A = area of the methinic protons at around 5.2 ppm; B = multiplet area at 4.22 ppm, area of the methylenic protons of esterified glycerol; C = area of methylenic protons included between two double bonds, at 2.77 ppm.

Samples		part	NV	NM	NM/NV
Bondiola	raw		1.05	0.22	0.21
	boiled		1.09	0.27	0.25
Cotechino Modena	raw		1.04	0.24	0.23
	boiled		1.07	0.27	0.26
Cotechino Cremona	raw		1.11	0.24	0.22
	vapour cooked		1.12	0.22	0.19
Lingua salmistrata	raw	root	0.93	0.10	0.11
		center	1.00	0.35	0.35
		tip	0.68	0.21	0.31
	boiled	root	0.87	0.12	0.14
		center	0.70	0.77	1.10
		tip	0.73	0.10	0.63
Salama da sugo	raw		1.08	0.23	0.21
	boiled		1.13	0.33	0.30

Table 3 - Ratios between different glyceridic forms by TLC: MG, DG and TG = mono-, di- and triacylglycerols; ox = oxidated products; FFA = free fatty acids.

	Bondiola		Cotechino		Salama da sugo	
	raw	boiled	raw	boiled	raw	boiled
Polars	20.23	33.95	16.7	4.6	8.71	8.52
MG			0.0	0.0	1.55	0.41
1.2 D	5.44	9.52	21.9	6.6	3.61	8.63
1.3 D	7.29	24.68	13.4	14.9	4.34	7.07
ox			-	-	5.75	2.13
FFA	1.49	7.17	21.1	29.7	17.22	15.41
TG	64.92	16.40	20.1	44.2	49.48	54.83
Sterol esters	0.63	8.28	6.8	-	9.33	3.00
			Tongue			
	root raw	root boiled	center raw	center boiled	tip raw	tip boiled
Polars	6.9	15.8	11.4	17.7	15.6	7.8
MG	0.0	0.0	0.0	0.0	1.6	4.3
1.2 D	3.5	2.6	5.8	1.7	2.2	3.9
1.3 D	3.5	3.0	6.9	1.9	9.9	8.5
FFA	11.3	7.2	14.8	9.6	25.6	16.5
TG	74.7	71.4	61.1	69.2	45.1	59.0

Table 4 - Mean values of the protein and NaCl content (%/edible portion) in the samples before and after cooking.

			Protein		NaCl	
Samples		part	mean	S.D.	mean	S.D.
Bondiola	raw		24.37	0.9	2.8	0.02
	boiled		25.19	0.04	2.8	0.01
Cotechino di Modena	raw		24.07	2.50	1.9	0.10
	boiled		23.01	1.83	1.6	0.06
Cotechino	raw		14.2	0.25	1.1	0.09
	vapour cooked		15.99	0.11	1.0	0.10
Lingua salmistrata	raw	root	20.09	0.23	2.8	0.02
		center	22.00	0.22	2.8	0.10
		tip	21.89	0.45	2.7	0.16
	boiled	root	25.99	5.85	2.5	0.00
		center	23.55	0.98	2.2	0.13
		tip	25.98	1.20	2.3	0.14
Salama da sugo	raw		22.73	3.51	2.8	0.02
	boiled		26.51	1.57	2.8	0.01

Table 5 - Mean values of the content of nitrite and nitrate ppm/edible portion in the samples before and after cooking. Standard deviation <5%.

Samples		part	$NaNO_2$ ppm/e.p. mean	$NaNO_3$ ppm/e.p. mean
Bondiola	raw		2.6	5.5
	boiled		1.6	3.5
Cotechino di Modena	raw		20.3	110.5
	boiled		14.5	80.5
Cotechino	raw		6.8	74.5
	vapour cooked		6.1	73.0
Lingua salmistrata	raw	root	24.2	176.0
		center	23.6	174.0
		tip	21.9	119.5
	boiled	root	22.3	116.0
		center	21.6	113.5
		tip	18.1	98.0
Salama da sugo	raw		7.42	49.0
	boiled		5.16	26.0

the type of preparation and the partial lack of epithelial protection. During the cooking process 25 to 33% of the nitrate content was lost.

The caloric value of each sample (Table 6) remained practically unchanged after cooking, except for the *salama da sugo* in which there was a 40% decrease, due to the large loss of lipids. In these products, the lipid content and the related chemical composition changes from one stock to another and is a determining factor of the nutritional property of the cooked samples.

From these results, it is concluded that in the specific preparation and cooking operations adopted, there is little variation in the nutritional and energetic

Table 6 - Mean caloric supply of 100 g of the edible part before and after cooking.

Samples		part	Energy supply Kcal/100 g e.p.
Bondiola veneta	raw		341
	boiled		351
Cotechino di Modena	raw		387
	boiled		438
Cotechino locale	raw		302
	vapour cooked		304
Lingua salmistrata	raw	root	223
		center	255
		tip	115
	boiled	root	215
		center	274
		tip	137
Salama da sugo	raw		431
	boiled		255

characteristics of the product, which is a very remarkable positive effect; there is a slight loss of sodium chloride and greater reduction in the concentration of nitrite and nitrate.

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1st ANNOUNCEMENT

9th International Symposium on Kinetics in Analytical Chemistry (KAC-2006) Marrakech, Morocco 4-6 November 2006

International Scientific Committee
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Important Deadlines:

June 1st, 2006: 2nd Announcement
September 16th, 2006: Submission of the abstracts
September 23rd, 2006: Information to authors about acceptance of abstracts
September 30th, 2006: Early registration with reduced fee

The Scientific programme will include plenary lectures, oral presentations and poster sessions to cover the following topics.

Analytical chemistry applications based on the kinetics of:
- Electrochemical reactions
- Spectrometric systems
- Biorecognition systems
- Enzyme systems
- Flow injection systems

Further details, including registration form, scientific programme, travel and accommodation will be sent with second announcement and will soon be available on the KAC-2006 web site.

Communication should be directed to Professor Aziz Amine

Association Biocap, Faculté des Sciences et Techniques de Mohammedia, B.P. 146. 20800 Mohammedia, Morocco - Fax +212 23 315353 - e-mail: azizamine@yhaoo.fr

Farber Assumes Presidency of the International Association for Food Protection

Jeffrey M. Farber, Research Director at Health Canada in Ottawa, Ontario, Canada, assumed the presidency of IAFP at the conclusion of IAFP 2005. He is currently responsible for the management of research and policy development in the area of microbiological food safety. He is also Adjunct Professor at the University of Ottawa and a member of the ICMSF. Dr. Farber obtained his B.Sc. and M.Sc. (A) degrees in Applied Microbiology and Immunology from McGill University in Montreal and his Ph.D. in Food Microbiology from McGill University in Quebec.

During his career, he has published over 100 papers in refereed journals, book chapters, and is an invited lecturer, internationally. Since joining

IAFP in 1986, Dr. Farber has served on many committees and Professional Development Groups, organized symposia and served as Program Committee Chair.

The Italian Journal of Food Science congratulates Dr. Farber, who has published in IJFS and has served as a referee on many occasions.

IDF highlights cheese as a star dairy product

Cheese production is growing all around the world, and is at record levels in some EU countries. This is one of the positive facts to emerge from the Sixth edition of The World Market for Cheese, published by the International Dairy Federation.

The report presents the latest trends and structural changes in total world cheese production, trade and consumption. Thirty countries representing approximately 80% of total world cheese production from cow's milk participated in the survey.

As far as international cheese trade is concerned, world cheese trade increased by approximately 32% from 1995 to 2004. Oceania accounted for the largest rise in world cheese market shares, up from 25% in 1995 to 37% in 2004. Another remarkable growth comes from the new EU 10, whose exports almost doubled from 1995 to 2003, reaching 172,000 tonnes in 2003. As to US, they experienced a growth of almost 100%. According to the survey, the major suppliers of cheese to the world market in 2004 were Australia, New Zealand and Germany.

The new Bulletin of IDF n. 402/2005 **The World Market for Cheese 1995-2004, Sixth Edition** is a reference tool for those who need the facts on cheese (marketers, officials, policy makers). It can be ordered in electronic form on the IDF Website at www.fil-idf.org.

For further information, please contact Marylène Tucci, IDF Communications and Public Affairs Assistant at IDF on + 32 2 7068644.

The World Dairy Situation 2005

The International World Dairy Federation announces the release of its annual report on the World Dairy Situation. This major tool including statistics on production, consumption and trade assist researchers, policymakers, and economists in providing a tool for strategy formulation. The new IDF publication presents a thorough and broad overview on trends in production, milk processing, consumption, trade and prices in major parts of the world. Detailed statistics by world, region and individual countries are also included.

Data are also available from countries which are not IDF members. Contributions from the ZMP which, on behalf of IDF, has continued its networking with other institutions working on the economy of the world dairy sector, have significantly added to the publication. Milk production, industrial milk processing, trade in dairy products, development of demand and consumption and prices for 2004 are presented, along with some dairy product information and forecasts for 2005.

Country reports provided by experts from member states, describing developments and sometimes subjective prognosis, are presented.

The WDS Forum section comprises three papers: OECD review of the potential effects of liberalization of world dairy trade; Comparison of the world dairy trade projections produced by various authoritative bodies; effects of accession to the EU of the dairy sector of the new member states.

See Bulletin 399/2005 to read the abstract and to order. - Pages: 86 - Price: 70 Euro - http://www.fil-idf.org.

For further information, please contact: Marylène Tucci, IDF Communications and Public Affairs, Tel.: +32 2 7068644, Fax: +32 2 7330413, E-mail: MTucci@fil-idf.org - http://www.fil-idf.org.

Probiotics to improve health and quality of life

Experts in probiotics gathered from 13 to 14 October 2005 at the International Symposium on "Understanding the role of probiotics in health" in Gent, Belgium to review latest research demonstrating the positive health benefits of probiotics.

Latest scientific studies revealed new evidence that probiotics play a major role regarding the reduction of complaints of constipation and several types of diarrhea, decrease of urinal infections, alleviation of abdominal pain in patients suffering from Irritable Bowel Syndrome (IBS), effective treatment of pouchitis and reduction of the occurrence of inflammatory symptoms. The positive influence of probiotics on the immune system including reduction of the risk of colon cancer is a main focus of current research.

Probiotics are living microorganisms that confer positive health benefits to the host if ingested regularly and in large numbers, for instance with fermented milk products. Since their commercialization by the dairy industry in the first half of the 20th century, probiotics play an increasingly important role in maintaining and improving human health and quality of life for people all over the world.

For more information, contact: Marylène Tucci, IDF Communications and Public Affairs, MTucci@fil-idf.org, Tel.: +32 2 7068844, http://www.fil-idf.org.

MILK BENEFITS TO HUMAN HEALTH Scientific reviews published in the Supplement to the Journal of the American College of Nutrition

Seven independent scientific review papers recording the views of some top nutrition scientists on milk and its role on the diet have been published as a supplement to the December issue of the Journal of the American College of Nutrition.

Science shows that consuming dairy foods each day helps people meet their nutrient recommendations and may help to reduce the risk of chronic diseases such as osteoporosis, hypertension and obesity.

These review papers address key health topics such as dairy and breast cancer, weight, osteoporosis, asthma, allergy and lactose intolerance. "This information will help health professionals respond to questions and confront misinformation about the role of milk and milk products in a healthy diet", commented Jill Nicholls, nutrition expert for the International Dairy Federation (IDF), which commissioned the work, and Director, Scientific Affairs at the U.S.-based National Dairy Council.

"Specialists in several key areas of dairy and health contributed to this supplement which provides a comprehensive overview of the nutritional value of dairy foods. This work reinforces the public perception of milk and milk products as wholesome and highly nutritious foods. It is an important reference tool to which health professionals and others interested in the health benefits of dairy can refer, especially when facing conflicting viewpoints". said Christian Robert, IDF Director General.

Dairy Food Consumption and Health: State of the Science on Current Topics, Supplement to the December issue of

the Journal of the American College of Nutrition is available for consultation on the IDF Website.

Glanbia unveils innovation centre

Glanbia Nutritionals has announced the official opening of the Glanbia Group Innovation Centre (GIC) in Kilkenny, by the Irish Minister for Enterprise, Trade and Employment, Mr Michéal Martin. The centre, which will involve an investment of over €15 over the next four years, is supported by Enterprise Ireland and has been in operation since September 2004. The facility currently stands at 1,265 sq metres and includes specialist laboratories, a pilot plant, sensory analysis facilities and a research library. Forty-two people are employed there, comprising 16 development professionals and 26 highly skilled research scientists. This number is set to increase in October 2005 when a 700 sq metre expansion will be completed.

Glanbia Nutritionals, a division of Glanbia plc, is one of the world's leading suppliers of science-based nutritional solutions to a broad range of industries. These include functional foods, clinical, infant and sports nutrition, and dietary supplements.

For more information about Glanbia Nutritionals and its ingredients, please contact: Avril Twomey, Glanbia Nutritionals, Glanbia Innovation Centre, Leggetsrath Business Park, Carlow Road, Kilkenny, Ireland. Tel.: +353 (056) 7796029, Fax: + 353 (056) 7796001, E-mail: atwomey@glanbia.ie, Or visit: www.glanbianutritionals.com.

Paying farmers for their milk Latest IDF survey presents results in 29 countries

Devising payment systems is a complex exercise. Modifying existing systems is just as difficult. New research published by the International Dairy Federation reveals the variety of solutions found around the world to meet the variety of demands made on farmers and the dairy industry.

In order to meet consumers' expectations, farmers pay attention to the composition of their milk, its hygienic quality, absence of contaminants and antibiotics and also environmental issues, farm assurance programmes. All these feature in payment systems in some countries but there is no universal system. Differences from country to country – climate, market structure, and role of government, export orientation – mean that the aims of the payment systems also differ. Frequency and method of payment, how the actual price paid is determined, what markets are served, what premiums are paid, and how cost factors such as cooling and transportation are dealt with this new IDF publication.

This new IDF survey covers all parameters currently taken into account to calculate how much a farmer gets paid for the milk. Practices in 29 countries are presented, from the most sophisticated dairy sectors to the least and covering cows milk, buffaloes, sheep and goats. IDF does not seek to recommend any one system, however to ensure that it provides a source of ideas for those who establish or adapt payment systems.

"Payment systems for ex-farm milk" is the new Bulletin of IDF n° 403/2006. It can be ordered in electronic form via the IDF website at http: www.fil-idf.org

GUIDE FOR AUTHORS

ITALIAN JOURNAL OF FOOD SCIENCE - IJFS

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(1) Manuscripts must be typed, double-spaced and **two** copies submitted along **with** the computer disk. There should be liberal margins on top, bottom and sides (2.5 cm). English is the official language. Authors who are not fluent in written English must seek help from a person fluent in scientific English. The Assistant Editor reserves the right to make literary corrections and to make suggestions to improve brevity, but **the paper must be revised by a native English speaker** before submission.

Pages and lines on all pages, including those pages for "References" and figure legends, **must be electronically numbered in the left margin**, beginning with number one at the top of the page.

The paper must also be submitted by e-mail or on a digital support (cd-rom or floppy disk). Indicate which word processor was used to generate the file and save the file also in format "Text only", DCA-RTF or ASCII, if you do not have programs for Macintosh; **graphics, pictures and diagrams must be saved at 300 dpi in TIF, JPEG, EPS or PICT formats** (not included in MsWord documents).

(2) Every paper should be divided under the following headings in this order:

Title. Informative of the content of the article (<50 characters + spaces). Author(s). Initials and Surname, omit professional and official titles. The Institute and address where the research was carried out and the current address of each author should be given as a footnote on the title page.

Abstract. Clearly state the objective of the study, give a concise description of experiment(s), observations, results and conclusions. No references should be cited. DO NOT EXCEED 100 WORDS. An abstract and title in Italian (corresponding to the English) must also be included.

Keywords. Up to six words, in alphabetical order, which describe the document must be given to aid data retrieval and indexing.

Introduction. Review pertinent previous work and cite appropriate references. State the purpose of the investigation.

Materials and Methods. Indicate apparatus, instruments, reagents, etc., giving sufficient detail to allow the work to be repeated.

Results and Conclusions. Results and Conclusions may be presented together or separately. Concisely present results using tables and figures to help justify conclusions (do not present the same information in both forms). Use statistical analysis when appropriate. Unsupported hypotheses should be avoided. Conclusions should point out the significance of the findings and, if possible, relate the new findings to some problem in Food Science and Technology.

Acknowledgments. Acknowledgments of assistance are appropriate provided they are not related to analyses, or other services performed for a fee. Financial support, thanks for assistance, article number or thesis fulfillment may be included.

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parentheses (only the initials in capital letters). If there are more than two authors, mention the first author and add *et al.*

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(5) Standard Usage, Abbreviations and Units. The Concise Oxford and Webster's English Dictionaries are the references for spelling and hyphenation. Statistics and measurements should always be given in figures, e.g. 10 min, except when the number begins a sentence. When the number does not refer to a unit of measurement it is spelled out unless it is 100 or greater. Abbreviations should be used sparingly, only when long or unwieldy names occur frequently, and never in the title; they should be given at the first mention of the name. International Standard abbreviations should generally be used except where they conflict with current practice or are confusing. For example, 3 mm rather than $3x10^{-3}$ m. Abbreviations should be defined the first time that they are used in the text and they should be used consistently thereafter. Temperatures should be expressed in the Celsius (centigrade) scale. Chemical formulae and solutions must specify the form used, e.g. anhydrous or hydrated, and the concentration must be in clearly defined units. Common species names should be followed by the Latin binomial (italics) at the first mention. For subsequent use, the generic name should be contracted to a single letter if it is unambiguous.

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Scientific contributions in one of the following forms may be submitted:

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Short Communications, Surveys and Papers will be subjected to critical review by the referees. Upon receiving papers from authors, the Advisory Board with the Editor-in-Chief will select papers in relationship to innovation and originality and send copies to the referees. A letter stating that the paper has been accepted for refereeing will be sent to the authors. Papers needing revision will be returned to the author, and the author must return the revised manuscript to the Editor-in-Chief, otherwise the paper will be considered as withdrawn. Papers not suitable for publication will be returned to the author with a statement of reasons for rejection.

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