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Relation between microbiological quality, metabolite production and sensory quality of equilibrium modified atmosphere packaged fresh-cut produce

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Abstract

The quality of four types of fresh-cut produce, packaged in consumer-sized packages under an equilibrium modified atmosphere and stored at 7 °C, was assessed by establishing the relation between the microbial outgrowth and the corresponding production of nonvolatile compounds and related sensory disorders.

In vitro experiments, performed on a lettuce-juice-agar, demonstrated the production of nonvolatile compounds by spoilage causing lactic acid bacteria and Enterobacteriaceae. *Pseudomonas fluorescens* and yeasts, however, were not able to produce detectable amounts of nonvolatile metabolites.

The type of spoilage and quality deterioration in vivo depended on the type of vegetable. Mixed lettuce and chicory endives, leafy tissues, containing naturally low concentrations of sugars, showed a spoilage dominated by Gram-negative microorganisms, which are not producing nonvolatile compounds. Sensory problems were associated with visual properties and the metabolic activity of the plant tissue. Mixed bell peppers and grated celeriac, on the other hand, demonstrated a fast and intense growth of spoilage microorganisms, dominated by lactic acid bacteria and yeasts. This proliferation resulted in detectable levels of organic acids and the rejection by the trained sensory panel was based on the negative perception of the organoleptical properties (off-flavour, odour and taste).

The applied microbiological criteria corresponded well with detectable changes in sensory properties and measurable concentrations of nonvolatile compounds, surely in the cases where lactic acid bacteria and yeasts were provoking spoilage. Consequently, the freshness of minimally processed vegetables, sensitive for outgrowth of lactic acid bacteria and yeasts (e.g., carrots, celeriac, bell peppers, mixtures with non-leafy vegetables) can be evaluated via analysis of the produced nonvolatile compounds.

Keywords: Microbial quality; Sensory quality; Vegetables; Metabolite production; Packaging

1. Introduction

Deterioration of fresh-cut vegetables is at the same time autolytically due to further respiration, transpiration and enzymatic activity of the living tissue after

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harvest and processing, as from microbiological origin, due to proliferation of spoilage microorganisms on the plant tissue (Nguyen-the and Carlin, 1994).

Brown discolouration of cut surfaces has been recognised as an important defect of fresh-cut vegetables (Bolin and Huxsoll, 1991; Heimdal et al., 1995; Lopez-Galvez et al., 1997; Watada and Qi, 1999; Ahvenainen, 2000). The visual properties of the packaged product are indeed important parameters for consumers in the evaluation of the quality of minimally processed vegetables: absence of discolouration (enzymatic browning of cut surfaces, yellowing of green vegetables and pale colour of bright vegetables) and absence of mechanical damage (i.e., foiled lettuce leaves, absence of cutting damage) are primary demands (Bolin et al., 1977; Bolin and Huxsoll, 1991; Watada and Qi, 1999; Varoquaux, 2001).

In addition to visual defects, some flavour defects may be induced by applying modified atmospheres (Heimdal et al., 1995; Beaudry, 2000) and/or by microbial outgrowth (Carlin et al., 1989; Fleet, 1992; Lopez-Galvez et al., 1997). For example, exposure to elevated CO_2 levels (10–20%) may result in the suppression of various metabolic processes (Kays, 1991; Mathooko, 1996; Watkins, 2000). However, in presence of sufficient O_2, the sensory quality is less affected (Mathooko, 1996; Lopez-Galvez et al., 1997; Watkins, 2000). Off-flavour development in fresh-cut vegetables can also be provoked by a lack of O_2. A shift from an aerobic towards an anaerobic atmosphere will provoke a fermentative metabolism under low O_2 levels (Kader et al., 1989; Kays, 1991; Beaudry, 2000). The severity of off-flavour production will depend on the time of exposure to conditions below the minimum required O_2 concentration and/or above the maximum tolerated CO_2 concentration (Beaudry, 1999; Beaudry, 2000; Watkins, 2000).

Not only the fermentative metabolism of the plant tissue itself but also microorganisms can be responsible for the presence of off-flavours during storage of fresh-cut vegetables. Especially, the outgrowth of lactic acid bacteria can be accompanied with production of organic acids such as lactic acid and acetic acid. Carlin et al. (1989) and Kakiomenou et al. (1996) isolated *Leuconostoc mesenteroides*, as the main spoiler of grated carrots. *Leuconostoc* spp. are heterofermentative and produce, next to lactic acid, also ethanol and CO_2. *Lactobacillus* spp. are responsible for a cheesy, acidic flavour of fresh-cut celery, stored in low O_2 conditions (Robbs et al., 1996a,b). The production of organic acids will cause a drop in the pH of the product (Carlin et al., 1989; Jacxsens et al., 1999a). Also, high amounts of yeasts (>10^5 CFU/g) (*Candida* spp.) can provoke an off-flavour of fresh-cut produce due to the production of CO_2, ethanol, organic acids and volatile esters (Babic et al., 1992; Fleet, 1992). However, little information is generally known about the relation between the outgrowth of spoilage microorganisms, their production of metabolites (volatile and nonvolatile) and the perception of the decay of minimally processed vegetables by consumers.

This research work had the objective to characterise the production of nonvolatile compounds and the consumption of sugars by important spoilage-causing microorganisms (Gram-negative, Gram-positive and yeasts) of fresh-cut produce in vitro on a lettuce-juice-agar under equilibrium modified atmosphere (EMA) conditions. An in vivo study on EMA packaged fresh-cut mixed lettuce, mixed shredded bell peppers, shredded chicory endive and grated celeriac was furthermore performed in order to relate the microbial growth, the sensory quality and the metabolite production as a function of the storage time.

2. Materials and methods

2.1. In vitro experiments

2.1.1. Isolation/identification of spoilage microorganisms on shredded mixed lettuce

Commercially available packaged mixed lettuce (16% radicchio (*Cichorium intybus* var. *foliosum* L.), 10% endive (*Cichorium endiva* L.), 19% curled endive (*C. endiva* L.) and 55% cos lettuce (*Lactuca sativa* var. *longifolia*)) was purchased and stored until the indicated end of shelf life at 7 °C. At that moment, total psychrotrophic count (spread plate on Plate Count Agar, PCA; Oxoid; CM325, Unipath, Basingstoke, Hampshire, UK, incubated for 5 days at 22 °C) and number of yeasts (spread plate on Yeast Glucose Chlorampenicol, YGC; Sanofi Diagnostics Pasteur; 64104, Marnes-La-Coquette, France, incubated for 3 days at 30 °C) was analysed.

From the PCA plates, different typical colonies were isolated and purified via a 4 × 4 plating on Tryptone

Soya Agar (TSA; Oxoid; CM131). After incubation at 30 °C for 24 h, they were characterised by classical identification tests (Gram-straining, oxidase (identification sticks oxidase (Oxoid; BR64A)), catalase (H_2O_2 30% (Vel; 90320; Merck Eurolab, Leuven, Belgium) diluted until 3%)). They were further identified via a biochemical identification kit for Gram-negative bacteria (BBL CristalTM E/NF, Becton Dickinson, Cockeysville, MD, USA). The identification was confirmed by LMG Culture Collection (Ghent University, Ghent, Belgium), applying Biolog GN 2 (biochemical test based on the ability to oxidise 95 different carbon sources) (Biolog, California, USA). Three strains were identified (PR1, PR2 and PR3).

Two lactic acid bacteria were selected for the in vitro study: *Leuconostoc gasicomitatum* (PR4) and *Lactobacillus brevis* (PR5), isolated previously from fresh-cut produce, stored at 4 °C (Jacxsens et al., 2001).

From the YGC plates, colonies were purified via a 4 × 4 plating on YGC, and incubated at 30 °C for 24 h. They were identified with a biochemical kit for yeasts (ID32C, bioMérieux, Marcy l'Etoile, France) and the identification was confirmed by IHEM Culture Collection (Brussels, Belgium), applying the auxanographic method (Vanbreuseghem et al., 1978). Two yeast strains were identified: PR6 and PR7.

The strains PR1, PR2 and PR3 were stored on PCA-slants, PR4 and PR5 on MRS-slants (Oxoid; CM361) and PR6 and PR7 on SAB-slants (Sabouraud Liquid Medium (Oxoid; CM147) + 15 g/l agar (Oxoid; L11)) at 4 °C.

2.1.2. Simulation agar for mixed lettuce: lettuce-juice-agar

The microorganisms (PR1 until PR7) were inoculated on a simulation agar for fresh-cut produce in order to be able to define the relation between their growth characteristics, sugar consumption and production of nonvolatile metabolites. Five different types of lettuce were applied for this: green "lollo bionta", red "lollo rosso" (*L. sativa* var. *crispa*), radicchio, endive and curled endive. From each type of lettuce, 20% was used, because this composition was also utilised in the final in vivo experiments (Section 2.2.1). After washing with cold tap water (60 s), the lettuce was dried for 60 s by means of a manual kitchen centrifuge (Zyliss, Bern, Switzerland) and centrifuged (Braun, type 4290, Kronberg, Germany) to obtain juice and pulp. The juice was heated for 2 h at 80 °C to denature enzymes and proteins. The juice was filtered through a filter (Retsch, 200 μm, Haan, Germany). The yield of the juice was finally 50% (v/w). Agar (Oxoid; L11) was added to the filtrate (15 g/l) even as 0.6% (v/v) oil (colza oil, Vandemoortele, Izegem, Belgium) to prevent excessive foaming during boiling. The solution was autoclaved for 15 min at 121 °C. The pH was measured (electrode (PH 915600, Orion, Boston, USA) and measure unit (model 525A, Ankersmit, Boston, USA)) and was 5.6.

2.1.3. Determination of growth characteristics on the lettuce-juice-agar

Growth curves of the spoilage microorganisms PR1 until PR7 were made under EMA conditions (3% O_2 and 2–5% CO_2 (balanced by N_2)) at 7 °C. The inoculum was prepared from the slants, grafted in broth (for PR1, PR2 and PR3, Brain Heart Infusion broth was used (BHI; Oxoid; CM225); for PR4 and PR5, de Man-Rogosa-Sharp broth (MRS; Oxoid; CM359); for PR6 and PR7, SAB). After 24-h incubation at 30 °C, a subculture was taken (0.1 ml in 5 ml broth), incubated for 16 h at 30 °C followed by 8 h at 7 °C, in order to allow adaptation of the strain to the low temperature. The inoculum size depended on the initial count analysed on the mixed lettuce (10^5–10^6 CFU/plate/56 cm^2/g for PR1, PR2 and PR3, or 10^3–10^4 CFU/plate/56 cm^2/g for PR4, PR5, PR6 and PR7). It was determined that 1 g mixed lettuce was comparable to the surface of a Petri dish (56 cm^2).

Six inoculated plates were packaged inside a bag of 30 × 40 cm (high-barrier film, Euralpack, Wommelgem, Belgium) with an oxygen transmission rate of 2 ml O_2/(m^2 24 h atm) and CO_2 transmission rate of 5.2 ml CO_2/(m^2 24 h atm) at 23 °C and 90% relative humidity. The initial atmosphere (3% O_2, 2–5% CO_2, balanced by N_2) was introduced by using a gas packaging unit (gas mixer, WITT M618-3MSO, Gasetechnik, Germany; vacuum compensation chamber, Multivac A300/42 Hagenmüller, Wolfertschwenden, Germany). Air Products (Vilvoorde, Belgium) supplied the gases (Fresh Line). The gas concentration inside the packages was analysed before opening with a CO_2/O_2 gas analyser (Servomex Food Package Analyser, Series 1400, Crowborough, Sussex, UK). At each analysis point, three plates from each bag were applied to determine the growth curve, and

three were analysed for the consumption of sugars and production of nonvolatile compounds (Section 2.1.4). To enumerate the growth curve, the lettuce-juice-agar of a plate was aseptically transferred to a sterile stomacher bag, diluted with peptone saline solution (PPS, 8.5 g/l NaCl; Vel 8605 + 1 g/l peptone, Oxoid; L34) and homogenised for 60 s with a Colworth Stomacher 400 (Steward Laboratory, London, UK). Tenfold dilution series were made in peptone saline solution for plating on specific media: PCA for the strains PR1, PR2 and PR3, MRS for the strains PR4 and PR5, and YGC for the strains PR6 and PR7. These plates were all incubated for 3 days at 30 °C. Results were expressed as CFU/cm^2. The growth curve was estimated via the Baranyi model at constant temperature with the determination of four parameters: the initial count (N_0), the maximum population density (N_{max}), the lag phase (λ) and the maximum growth rate (μ_{max}) (Grijspeerdt and Vanrolleghem, 1999).

2.1.4. Determination of consumption of sugars/production of metabolites

Three plates of the in vitro test were, on each analysing point, analysed by High Performance Liquid Chromatography (HPLC) for the content of sugars (glucose, fructose, sucrose and oligosaccharides), organic acids (lactic acid, citric acid, acetic acid, succinate, adipic acid and propionic acid) and ethanol. The complete lettuce-juice-agar of a plate was introduced in a stomacher bag and diluted with water (1:1). This was manually homogenised to a fluid solution, which was transferred in an Eppendorf tube. The tubes were centrifuged (20 min/14,000 rpm) (Eppendorf, Centrifuge 5415 C, Hamburg, Germany). The supernatant was filtered (Millipore, 0.22 μm, Waters TCM, Bedford, USA) and this filtrate was injected in the HPLC (100 μl). The HPLC contained a precolumn (Cat. number 125-0129, Hercules, Biorad, Laarne, Belgium), a separation column (Aminex HPX-87H, Biorad) existing out of a polystyrene matrix with sulfonic acid in acid state as functional group, a temperature controller (TCM, Waters, Millipore), a RI-detector (Model 132, Gilson Unipoint, Villiers-Le-Bel, France) and software (Gilson Unipoint). As mobile phase, 5 mM H_2SO_4 (Vel) was applied with a flow rate of 0.6 ml/min and the temperature was set at 35 °C.

2.2. In vivo experiments

2.2.1. Preparation of the vegetables

The produce was industrially prepared in a vegetable processing industry (Allgro, Sint-Lievens-Houtem, Belgium). Mixed lettuce is a commercially available mixture of 20% endive, 20% curled endive, 20% radicchio lettuce, 20% lollo rosso and 20% lollo bionta lettuces (red and green variety). Chopped bell peppers are a mixture of green, red and yellow shredded bell peppers (*Capsicum annuum* L.) (0.4 × 1 cm). The chicory endives (*C. intybus* L.) were manually hand-cut in 0.5-cm slides with a sharp knife. The grated celeriac (*Apium graveolens* var. *rapaceum* L.) was peeled and grated in thin sticks (0.3 × 0.3 × 4 cm) by Compacto Kitchen Cutter (Philips, Eindhoven, the Netherlands). The chicory endives and the grated celeriac were immersed in cold tap water for 60 s and dried for 60 s by means of a manual kitchen centrifuge (Zyliss) to remove the remaining water.

2.2.2. Packaging of the vegetables

For the mixed lettuce, two types of packaging material were applied. A commercial available and nowadays widely applied BOPP film (30 μm), PVdC coated with an oxygen transmission rate of 15 ml O_2/(m^2 24 h atm) at 7 °C and 90% relative humidity (Van der Windt Packaging, Hoogstraten, Belgium). The other film was a prototype of a high permeable packaging film in which equilibrium modified atmosphere conditions (3–5% O_2 and 5–10% CO_2) could sustain (Jacxsens et al., 1999a,b, 2000). The applied packaging configurations for the high permeable films (Hyplast, Hoogstraten, Belgium) were calculated based on a mathematical model (Jacxsens et al., 2000) and are given in Table 1. The initial atmosphere (3% O_2 and 2–5% CO_2) was introduced immediately inside the packages via active modification of the atmosphere. The same packaging technique as in Section 2.1.3 was applied. The packages were stored at 7 °C. Analyses were performed in triplicate on day 0 (production day) and regularly until days 10–13.

2.2.3. Analysis of the headspace gas composition

The O_2 and CO_2 concentration (volume %) of the headspace of the packages were analysed by applying a Servomex gas analyser (Section 2.1.3).

Table 1
Applied packaging configurations and packaging films in order to obtain equilibrium modified atmosphere packages for fresh-cut produce at 7 °C

Type of vegetable	Fill weight (kg)	Package dimensions (length × width)[a] (cm^2)	Required oxygen transmission rate (ml O_2/(m^2 24 h atm))[b]	Applied oxygen transmission rate (ml O_2/(m^2 24 h atm))
Bell peppers (mixed, shredded) (0.4 × 1 cm)	0.15	19 × 15	2838	2897 ± 471
Celeriac (grated)	0.15	19 × 15	3337	3530 ± 286
Chicory endives (0.5 cm)	0.15	19 × 15	3400	3704 ± 574
Lettuce (mixed, shredded)	0.25	20 × 23.5	2270	2270 ± 312

[a] Single-sided.
[b] Calculated based on a mathematical model (Jacxsens et al., 2000) in order to store the EMA packages at 7 °C.

2.2.4. Analysis of the microbiological quality

Growth of the most important groups of micro-organisms, associated with the spoilage of minimally processed vegetables, was followed during the in vivo storage experiment. From each package, 30 g was taken with 270 ml peptone saline solution (PPS, 8.5 g/l NaCl; Vel 8605 + 1 g/l peptone, Oxoid; L34) in a sterile stomacher bag and homogenised for 60 s with a Colworth Stomacher 400 (Steward Laboratory). Ten-fold dilution series were made in peptone saline solution for plating. To enumerate the total aerobic psychrotrophic count (TPC), pour plates were made on PCA and incubated for 5 days at 22 °C. The number of lactic acid bacteria (LAB) was followed on a pour plate with top layer on MRS, incubated for 3 days at 30 °C. Yeasts were followed on a spread plate on YGC, incubated for 3 days (yeasts) at 30 °C. The outgrowth of the TPC, LAB and yeasts on the mixed lettuce was as well fitted by the Baranyi equation in order to be able to compare the obtained growth characteristics with the ones obtained via the in vitro growth curves (Section 2.1.3).

2.2.5. Analysis of pH

During the storage experiments, the pH of the vegetables was followed as well. A sample of 50 g was homogenised by a mixer (Commercial blender 8010, Waring, New Hartford, CT, USA). The pH was measured with the same instrument as in Section 2.1.2.

2.2.6. Analysis of the sensory quality

The sensory quality was evaluated via a taste panel. The panel contained 8–10 trained members. The first part (organoleptical characteristics as taste, crispness (texture evaluation), odour/off-flavour) was judged under red light to exclude interference of visual judgement. Under daylight, the visual characteristics such as colour, general appearance and general freshness were evaluated. Scores were given from 1 (= excellent fresh) until 10 (= completely deteriorated). The sample was considered as unacceptable for a sensory characteristic when the score was higher than 5 (= just acceptable).

2.2.7. Analysis of the consumed sugars and produced nonvolatile metabolites

A sample of 50 g fresh-cut vegetables was centrifuged (Braun, type 4290) and the juice was filtered over a paper filter (5971/2, Schleicher and Schuell, Dassel, Germany). The filtrate was further centrifuged (20 min/14,000 rpm) (Eppendorf, Centrifuge 5415 C). The supernatant was again filtered (0.22 μm, Millipore) and this filtrate was injected in the HPLC column (100 μl) (Section 2.1.4).

2.3. Statistics

The in vitro experiments were conducted in triplicate. Results of the growth curves and metabolite concentrations are given by the mean and the 95%

confidence interval. The parameters of the Baranyi model were estimated by SPSS 9.0 for Windows, applying the Levenberg–Maranquardt algorithm and minimising the residual sum of squared errors of the biomass and optimising R^2 towards 1. Each in vivo storage experiment was conducted in triplicate. The enumeration of the spoilage microorganisms as well as the HPLC analysis, the headspace gas composition and the pH are given by the mean and 95% confidence interval. The mean and the 95% confidence interval of the scores of the individual sensory properties were calculated.

3. Results and discussion

3.1. In vitro growth of spoilage microorganisms, metabolite production/sugar consumption

3.1.1. In vitro growth of spoilage microorganisms on lettuce-juice-agar

Gram-negative microorganisms, isolated from the mixed lettuce on the indicated end of shelf life, were respectively *Pantoea agglomerans* (PR1), *Rahnella aquatilis* (PR2) and *Pseudomonas fluorescens* (PR3). The isolated yeasts were respectively *Candida humicola* (PR6) and *Cryptococcus laurentii* (PR7). Growth characteristics of the microorganisms PR1 until PR7 are summarised in Table 2. The gas composition stayed stable until the end of the growth curve (stationary phase) when the O_2 concentration started to decrease. The lag phases of the Gram-negative microorganisms are not significantly different from each other. *C. laurentii* had a longer lag phase compared to *Candida* spp. and both lactic acid bacteria. The maximum specific growth rate of *C. humicola* is significantly lower than the one of *P. agglomerans* and *C. laurentii*. Magnuson et al. (1990) reported a

Table 3
In vitro acid production (mg/ml lettuce-juice-agar ± 95% confidence interval) of *Pantoea agglomerans* and *Rahnella aquatilis* on lettuce-juice-agar, stored under 3% O_2/2–5% CO_2 (balanced by N_2) at 7 °C

Rahnella aquatilis

Time (h)	Number of microorganisms (log CFU/cm^2)	Succinate (mg/ml)
0	4.59	–[a]
133.3	7.82	–
138.8	7.97	0.196 (0.100–0.292)
162.1	8.14	0.466 (0.272–0.660)
186.3	8.19	0.333 (0.299–0.367)

Qualitative detection of adipic acid and propionic acid after 138.8 h

Pantoea agglomerans

Time (h)	Number of microorganisms (log CFU/cm^2)	Succinate (mg/ml)	Acetic acid (mg/ml)
0	4.25	–	–
135.5	8.38	–	–
144.5	8.36	0.607 (0.466–0.748)	0.605 (0.482–0.728)
160.5	8.43	0.604 (0.370–0.838)	0.622 (0.455–0.789)
167.5	8.44	0.828 (0.738–0.918)	0.835 (0.683–0.987)

Qualitative detection of adipic acid and propionic acid after 144.5 h

[a] No detection of the metabolites.

Table 2
Growth characteristics of spoilage microorganisms of fresh-cut produce on lettuce-juice-agar, stored under 3% O_2/2–5% CO_2 (balanced by N_2) at 7 °C: lag phase λ (h), maximum specific growth rate μ_{max} (1/h), initial count N_0 (log CFU/cm^2) and maximum population density N_{max} (log CFU/cm^2) (± 95% confidence interval), estimated via the Baranyi model

Spoilage microorganism	λ (h)	μ_{max} (1/h)	N_0 (log CFU/cm^2)	N_{max} (log CFU/cm^2)	R^2
Pantoea agglomerans	47.5 (32.3–62.6)	0.066 (0.047–0.085)	4.25 (3.88–4.61)	8.47 (8.12–8.82)	0.982
Rahnella aquatilis	48.7 (25.0–72.4)	0.054 (0.030–0.079)	4.59 (4.18–5.00)	8.22 (7.77–8.67)	0.969
Pseudomonas fluorescens	45.7 (28.5–62.9)	0.068 (0.043–0.093)	3.85 (3.44–4.26)	7.84 (7.36–8.31)	0.976
Leuconostoc gasicomitatum	1.5 (–35.5–38.5)	0.042 (0.029–0.055)	2.24 (1.46–3.02)	8.02 (7.37–8.67)	0.967
Lactobacillus brevis	13.7 (–12.2–39.6)	0.055 (0.040–0.070)	2.30 (1.57–3.03)	8.23 (7.77–8.70)	0.975
Candida humicola	15.4 (–8.3–39.2)	0.038 (0.030–0.045)	1.87 (1.48–2.27)	7.21 (6.85–7.56)	0.988
Cryptococcus laurentii	73.8 (61.0–86.6)	0.067 (0.050–0.085)	2.03 (1.72–2.35)	6.85 (6.50–7.20)	0.980

lower μ_{max} of yeasts compared to bacteria on lettuce. Bennik et al. (1998) published for *R. aquatilis* and *P. fluorescens* a μ_{max} of 0.13/h and 0.11/h, respectively, on BHI agar under 1.5% O_2 and 5% CO_2 at 8 °C. These values are the double of the values summarised in Table 2. A possible explanation is that the BHI agar is a richer growth medium compared to the applied lettuce-juice-agar for the microorganisms. However, the composition of the lettuce-juice-agar is more similar to the composition of minimally processed vegetables. Also the temperature is slightly higher, which can also provoke an increase of the growth rate. The applied O_2 concentration is as well different but the decreased O_2 concentration has no inhibiting effect on the growth in these ranges (Bennik et al., 1995, 1998).

3.1.2. In vitro production of metabolites/consumption of sugars by spoilage microorganisms

No metabolite production was detected by *P. fluorescens*. This bacterium shows no fermentative character, in contrast to the Enterobacteriaceae and the lactic acid bacteria (ICMSF, 1996). In the stationary phase, the O_2 concentration dropped towards 0% and from that moment, acetic acid and succinate were developed by *P. agglomerans* and only succinate by *R. aquatilis* (Table 3). These Enterobacteriaceae are able to ferment sugars to acids under anaerobic conditions (ICMSF, 1996). Also adipic acid and propionic acid were detected, but no quantification was possible. The metabolites, produced by *L. gasicomitatum* and *L. brevis*, are different from the ones produced by the Enterobacteriaceae and are produced earlier in the growth curve (late exponential phase) (Table 4). Lactic acid bacteria are micro-aerophilic and will grow faster under the low O_2 concentrations (ICMSF, 1996). *L. brevis* produced more lactic acid and also ethanol compared to *L. gasicomitatum*. Both lactic acid bacteria are heterofermentative and next to lactic acid, also acetic acid and ethanol was produced (ICMSF, 1996; Björkroth et al., 2000).

The yeasts *C. humicola* and *C. laurentii* produced no detectable nonvolatile compounds during the in vitro growth curve analysis. Fleet (1992) reported that some species of *Cryptococcus* and *Candida*, but also *Rhodotorula* spp. and *Pichia* spp., are able to con-

Table 4
In vitro metabolite production (mg/ml ± 95% confidence interval) of *Leuconostoc gasicomitatum* and *Lactobacillus brevis* on lettuce-juice-agar, stored under 3% O_2/2–5% CO_2 (balanced by N_2) at 7 °C

Leuconostoc gasicomitatum

Time (h)	Number of microorganisms (log CFU/cm^2)	Lactic acid (mg/ml)	Acetic acid (mg/ml)
0	2.24	–[a]	–
133.0	7.18	–	–
138.0	7.30	0.185 (−0.036–0.406)	0.184 (0.045–0.323)
161.5	7.69	0.602 (0.322–0.882)	0.495 (0.264–0.726)
210.0	7.97	2.466 (2.261–2.671)	2.175 (1.996–2.354)

Lactobacillus brevis

Time (h)	Number of microorganisms (log CFU/cm^2)	Lactic acid (mg/ml)	Acetic acid (mg/ml)	Ethanol (mg/ml)
0	2.30	–	–	–
70.0	5.37	–	–	–
86.5	6.18	0.063 (0.049–0.078)	0.037 (−0.016–0.090)	–
120.0	7.49	1.071 (1.065–1.077)	0.304 (0.245–0.363)	–
138.0	7.89	1.643 (1.268–2.018)	0.613 (0.397–0.829)	–
185.0	8.20	3.211 (2.918–3.504)	1.764 (1.154–2.374)	–
210.0	8.22	4.428 (3.881–4.975)	2.207 (1.975–2.439)	0.246 (−0.044–0.536)

[a] No detection of the metabolites.

sume sugars under aerobic conditions, but not under the anoxic conditions, which were created at the end of the growth curves. However, most yeasts are able to ferment sugars under facultative anaerobic conditions accompanied with the release of CO_2, ethanol, acids and volatile esters (Fleet, 1992; ICMSF, 1996).

Initially, oligosaccharides, which could be assimilated by the inoculated microorganisms as a function of their needs, were present in the lettuce-juice-agar. The detected oligosaccharides could be stachyose (a tetrasaccharide existing out of 2 U galactose, 1 U glucose and 1 U fructose). In the case of *R. aquatilis* (Fig. 1), oligosaccharides were converted at the beginning of the exponential phase to glucose and fructose (decrease in concentration of oligosaccharides and slight increase in concentration of glucose and fructose). At the end of the exponential phase and during the stationary phase, the concentration of oligosaccharides decreased again, but this was accompanied with a sharp decrease in the concentration of fructose and glucose. At that moment, the population density and the consumption of nutrients were high and monosaccharides, formed from the hydrolysis of the oligosaccharides, were immediately fermented with the production of acids as a consequence (Table 3). The sucrose concentration stayed constant while the concentration of citric acid changed during the time. This pattern in the evolution of the concentration of sugars, citric acid and oligosaccharides was as well found for *P. agglomerans*, although there was no decrease in the concentration of the oligosaccharides at the beginning of the exponential phase (data not shown).

Fig. 2 illustrates this evolution for the lettuce-juice-agar, inoculated with *L. gasicomitatum*. Both the glucose and the sucrose concentration decreased at the beginning of the stationary phase and evolved towards zero at the end of this phase. At that moment, also the concentration of fructose, oligosaccharides and citric acid decreased. Out of the fermentable sugars, lactic acid, acetic acid and ethanol were produced (Table 4) (ICMSF, 1996; Batt, 2000). These changes were as well found with the growth of *L. brevis* (data not shown). Björkroth et al. (2000) published that out of sucrose slime was produced by *L. gasicomitatum*. Carlin et al. (1989) detected a decrease in the sucrose concentration of 55% in packaged, grated carrots after 14 days storage at 10

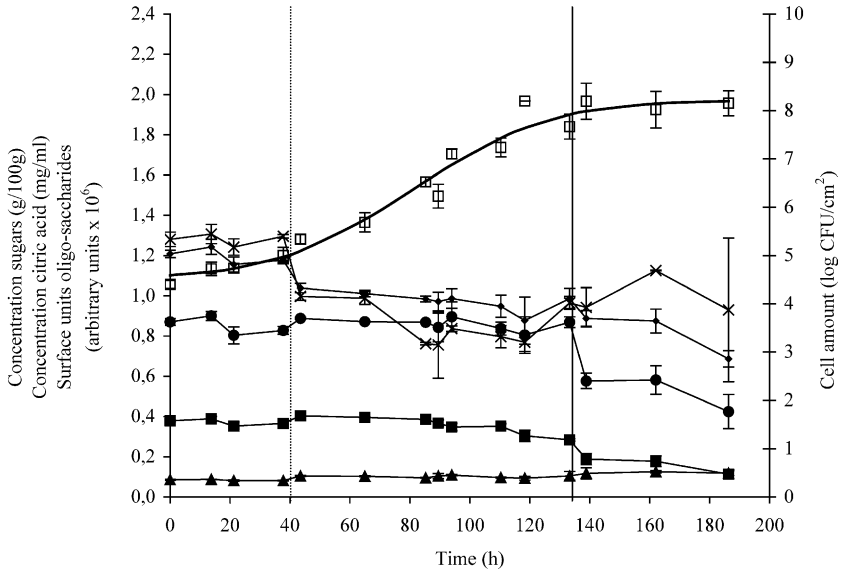

Fig. 1. Evolution of concentration (± 95% confidence interval) of sugars ((■) glucose, (▲) sucrose, (●) fructose, (♦) oligosaccharides), concentration of citric acid (×), growth curve (□) and estimated growth curve via Baranyi model (—) on the lettuce-juice-agar, inoculated with *R. aquatilis*, stored under 3% O_2 and 2–5% CO_2 (balanced by N_2) at 7 °C. Dashed line indicates the begin of the exponential phase, full line indicates the end of the exponential phase.

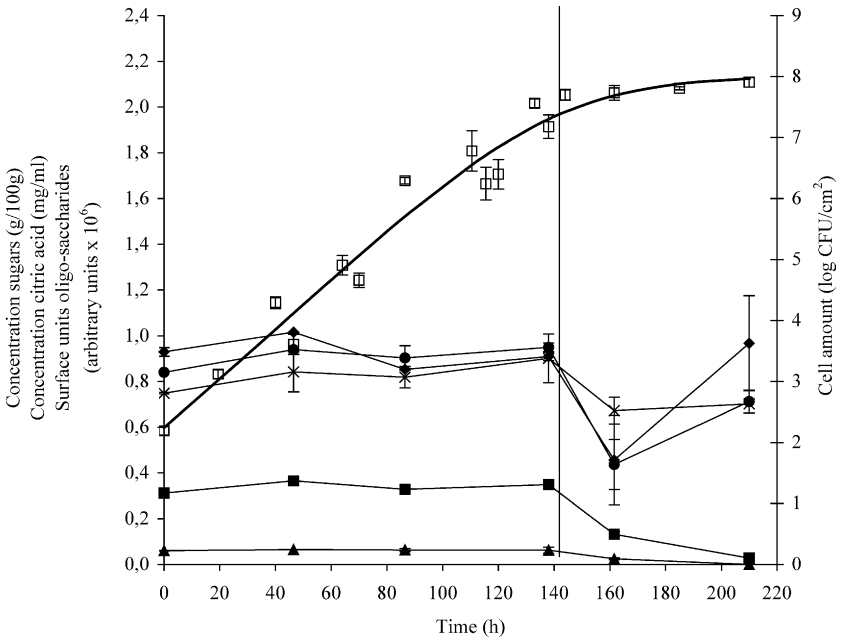

Fig. 2. Evolution of concentration (± 95% confidence interval) of sugars ((■) glucose, (▲) sucrose, (●) fructose, (♦) oligosaccharides), concentration of citric acid (×), growth curve (□) and estimated growth curve via Baranyi model (—) on the lettuce-juice-agar, inoculated with *L. gasicomitatum*, stored under 3% O_2 and 2–5% CO_2 (balanced by N_2) at 7 °C. Full line indicates the end of the exponential phase.

°C. All Gram-positive bacteria were identified as *L. mesenteroides*. A decrease in citric acid can be due to the breakdown to diacetyl by lactic acid bacteria (Bozogen and Yurdugül, 2000).

Changes in sugar concentrations were not found for the yeasts and *P. fluorescens*, possibly because no fermentative character could be established for these strains.

3.2. In vivo growth of spoilage microorganisms and their metabolite production

3.2.1. Headspace gas composition

The gas composition in packages from BOPP film with mixed lettuce changed fast towards anoxic conditions (day 3). The film is not sufficiently permeable to balance the produce respiration by diffusion of O_2 (Exama et al., 1993). The most difficult task at this moment in the technology of minimally processed vegetables is to reach the optimal EMA conditions inside the packages. The main problem is that only a few packaging materials on the market are permeable enough to compensate the respiration of fresh-cut vegetables. Most films do not result in optimal O_2 and CO_2 conditions, especially when the produce has a high respiration rate (Ahvenainen, 2000; Lange, 2000). In the permeable packaging film, the O_2 level dropped towards 1% but stayed constant during further storage. The minimal O_2 concentration for lettuce is reported as 0.5% (Beaudry, 2000). The gas composition stayed stable inside the packages filled with shredded chicory endive and grated celeriac, but changed towards anoxic conditions at the end of the shelf life for the mixed bell peppers (days 6–7). The shift from a steady state to an anoxic atmosphere occurred when the microbial contamination became high on the minimally processed vegetables (Figs. 3–5). In the case of chicory endives and grated celeriac, an increase of the O_2 concentration was measured, respectively from days 9 and 7, probably because of a decreased vitality and respiration of the plant tissue at the end of the storage period (Kays, 1991).

Carbon dioxide could not accumulate to levels above 10% due to the high permeability of the applied packaging films. However, in the BOPP packaging film, concentrations were build up until 20% CO_2 at

day 6, because of the lower permeability of this type of packaging film (Exama et al., 1993; Lange, 2000).

3.2.2. Microbial spoilage of the EMA packaged minimally processed vegetables

The initial contamination of TPC was compromised between the goal and the tolerance criteria, proposed by CNERNA-CNRS (1996) and Debevere (1996), for fresh-cut produce on the production day (10^5–10^6 CFU/g TPC) except for the mixed bell peppers (Fig. 3). Consequently, the mixed bell peppers reached the limiting criterium of 10^8 CFU/g of TPC very fast (between days 3 and 4), followed by the grated celeriac (between days 5 and 6). Shredded chicory endives contained only after day 8 more than 10^8 CFU/g TPC. A difference was found between the outgrowth on the mixed lettuce, stored in the two atmospheres: the accumulation of CO_2 inside BOPP packages and also the created anoxic conditions prevented a fast outgrowth of the TPC (Church and Parsons, 1995). The experiment was interrupted at day 6 because of unacceptable sensory quality. The EMA packaged lettuce reached the limit of TPC at day 6 and no further outgrowth was detected (Fig. 3).

The goal and tolerance limit for lactic acid bacteria of fresh-cut produce at the day of production (LAB) is set between 10^3 and 10^4 CFU/g (Debevere, 1996). Apparently, some problems existed in reaching these limits for the industrially prepared mixed lettuce and mixed bell peppers (Fig. 4). High initial counts of lactic acid bacteria on minimally processed vegetables indicate a bad disinfection of cutting machines, a temperature abuse or a too long storage period of the product (Brocklehurst et al., 1987; Dijk et al., 1999). The growth of the LAB on the shredded chicory endives was slow. Grated celeriac, on the other hand, showed a fast growth pattern, comparable with the growth of LAB on grated carrots (both are root vegetables, containing a high concentration of sugars) (Carlin et al., 1989; Kakiomenou et al., 1996; Jacxsens et al., 1999a). The fastest growth was detected on the mixed bell peppers, where the limit of 10^7 CFU/g was exceeded after 3 days of storage at 7 °C. The limit of 10^7 CFU/g for LAB on fresh-cut vegetables is only valid when at the same time organoleptical tests show that the sensory quality is still acceptable. The low pervasive character of the metabolites of the LAB, compared to the ones produced by Gram-negative microorganisms, allows often higher amounts of LAB (until 10^8 CFU/g) (Carlin et al., 1989; Kakiomenou et al., 1996).

Also for yeasts, an initial count between 10^3 and 10^4 CFU/g is defined as goal and tolerance limit, respectively, at the day of production (Debevere, 1996). The end of the microbiological shelf life is reached, when the limit of 10^5 CFU/g is exceeded.

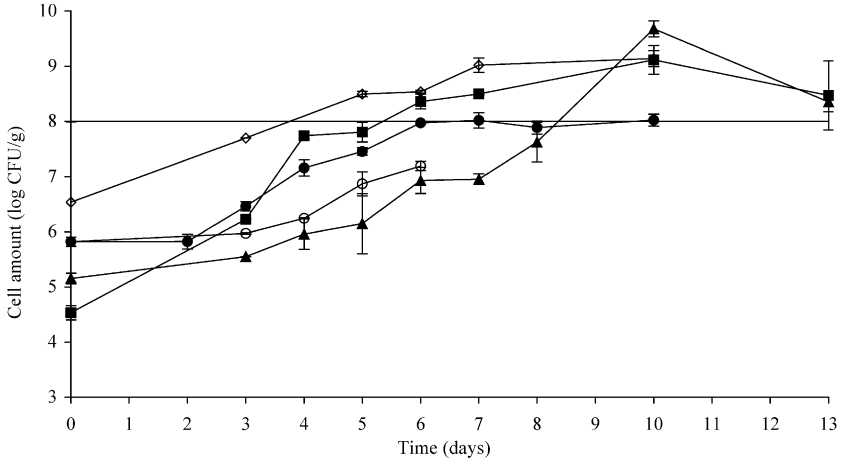

Fig. 3. Growth of total psychrotrophic count (log CFU/g ± 95% confidence interval) during storage at 7 °C under equilibrium modified atmosphere (EMA) ((■) grated celeriac, (●) mixed lettuce, (▲) shredded chicory endive, (◇) mixed bell peppers) or in packaged in BOPP film ((○) mixed lettuce). Full line indicates the shelf life limiting number of 10^8 CFU/g TPC.

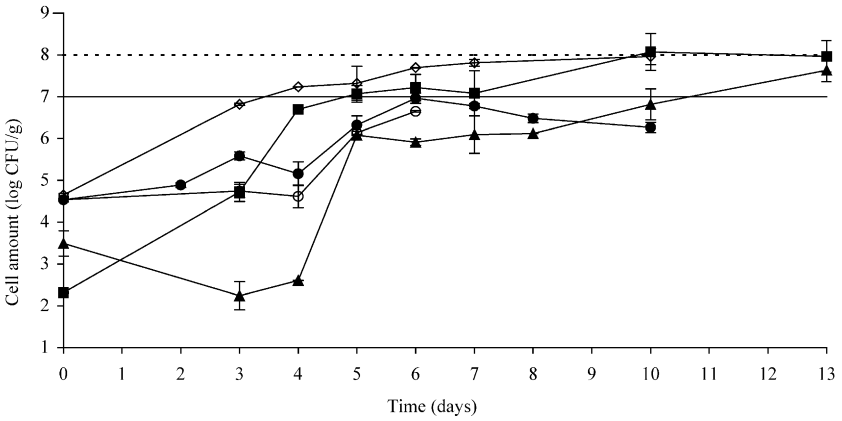

Fig. 4. Growth of lactic acid bacteria (log CFU/g ± 95% confidence interval) during storage at 7°C under equilibrium modified atmosphere (EMA) ((■) grated celeriac, (●) mixed lettuce, (▲) shredded chicory endive, (◇) mixed bell peppers) or in packaged in BOPP film ((○) mixed lettuce). Full line indicates the shelf life limiting number of LAB of 10^7 CFU/g. Dashed line indicates the shelf life limiting number of LAB if no detrimental sensory properties are detected.

Above this number, a negative effect on the sensory properties can occur, characterised by gas production, off-flavour development and visible colony formation (Fleet, 1992). Yeasts exceeded the limit for mixed lettuce packaged under EMA on day 6, grated celeriac on day 5, mixed bell peppers on day 7 (Fig. 5). No important outgrowth was enumerated on the mixed lettuce, packaged in the BOPP film, due to the unfavourable CO_2 concentration for yeasts (Church and Parsons, 1995). Previous work demonstrated as well the slow growth of yeasts on shredded chicory endives at 7 °C under EMA (Jacxsens et al., 1999a) but not in Jacxsens et al. (2001) at 4 °C. At that temperature, no outgrowth of competitive LAB was noticed, which was the case at 7 °C (Fig. 4; Jacxsens et al., 1999a). At 4 °C, grated celeriac was rejected after 3 days storage under EMA because of too high concentrations of yeasts (Jacxsens et al., 2001).

3.2.3. Evolution of type of spoilage microorganisms as a function of storage time

P. fluorescens was initially the most dominating microorganism, and remained important during the further storage of mixed lettuce, stored under EMA or

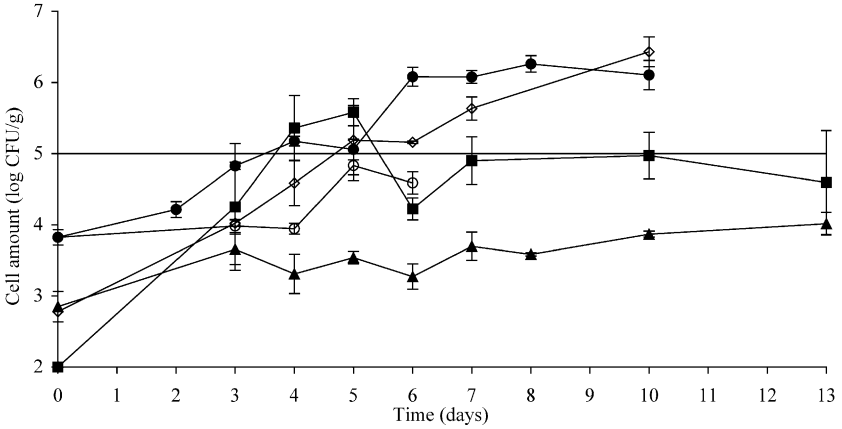

Fig. 5. Growth of yeasts (log CFU/g ± 95% confidence interval) during storage at 7 °C under equilibrium modified atmosphere (EMA) ((■) grated celeriac, (●) mixed lettuce, (▲) shredded chicory endive, (◇) mixed bell peppers) or in packaged in BOPP film ((○) mixed lettuce). Full line indicates the shelf life limiting number of yeasts of 10^5 CFU/g.

anoxic conditions at 7 °C (Table 5). No difference was found between the two atmospheres: in anoxic conditions, a slightly lower percentage *P. fluorescens* was detected in favour of facultative anaerobic *P. agglomerans* and *R. aquatilis*.

In the case of grated celeriac, the Gram-positive microorganisms, and more specifically, *Lactobacillus plantarum*, dominated the spoilage flora. They counted about 70% of the total flora and this number remained constant during storage. *R. aquatilis* became important at the end of the storage period, with 32% at day 13. Next to this, *Pseudomonas* spp. are frequently isolated, commonly associated with the spoilage of celery (Robbs et al., 1996a,b). Only one type of yeast was identified on the grated celeriac, *Candida curvata*. These yeasts are responsible for a softening of the product as they are able to produce pectinolytic enzymes (Fleet, 1992).

Gram-negative *Pseudomonas* spp. dominated the spoilage flora of shredded chicory endives. The lactic acid bacteria *L. plantarum* and yeasts *Cryptococcus humicola* were identified as well. The microbial flora, present on the shredded chicory endives, is rather uniform. Bennik et al. (1998) detected a shift from *P. viridiflava* to *P. fluorescens* after 7 days storage at 8 °C under 1.5% O_2–20% CO_2–78.5% N_2 on shredded chicory endives.

No important role of Gram-negative bacteria was found in the spoilage of mixed bell peppers, only *P. agglomerans* became important at the end of the storage period (days 7 and 10). They are able to produce lactic acid under 10% O_2 (Huber, 2000). Lactic acid bacteria were the most important spoilers (80% of the total flora). *L. brevis* has been identified as dominating the lactic acid bacteria, capable of producing lactic acid in a heterofermentative way (Teixeira, 2000). An almost uniform yeast flora was detected on the mixed bell peppers consisting out of *Kloeckera* spp.

3.2.4. Change in pH during storage

The pH of the shredded chicory endives stayed stable around 6, during the whole storage period of 13 days. The same pH was measured for the grated celeriac, until at day 13, the pH of the celeriac then dropped from 6.0 to 5.5 (outgrowth of LAB, Fig. 4). The pH of the mixed bell peppers, on the other hand, decreased from pH 5.0 at day 6 until pH 4.0 at day 10. This pH drop can be explained by the outgrowth of LAB, which exceeded an amount of 10^7 CFU/g between days 3 and 4 on the mixed bell peppers. The pH of the mixed lettuce increased during storage from 5.9 until maximum 6.2. The pH of the lettuce, packaged under anoxic conditions, increased slightly faster. This pH increase is typical for vegetables in which Gram-negative microorganisms play an important role in the spoilage. King et al. (1991) measured a pH increase of minimally processed lettuce from 5.8 until 6.3 in 24 days storage at 2 °C. Also Hao et al. (1999) found an increase in the pH of broccoli florets stored at 4 °C in low O_2 (1–3%). This pH increase can result from the breakdown of proteins with the release of basic compounds as a result. Lettuces contain in average 1.4 g/100 g proteins (Nubel, 1999). This breakdown is apparently more important than the production of acids during spoilage (Section 3.2.6).

3.2.5. Comparison of in vitro and in vivo growth of spoilage microorganisms on mixed lettuce

The R^2 of the Baranyi equation for the LAB was lower compared to the other groups of microorgan-

Table 5
Predominant Gram-negative spoilage microorganisms (expressed in % suspected colonies on the selective media) on mixed lettuce, stored under equilibrium modified atmosphere (EMA) or anoxic conditions (BOPP film) at 7 °C

Type of microorganism	Day 0	Day 3		Day 6		Day 10
		EMA	Anoxic	EMA	Anoxic	EMA
Pantoea agglomerans[a]	27.3	16.1	19.7	20.5	18.9	11.3
Rahnella aquatilis[a]	26.7	20.8	17.5	15.2	28.5	20.8
Pseudomonas fluorescens[a]	42.2	57.6	51.3	44.3	39.7	55.4

[a] Isolated from PCA plates (% is balanced by non identified colonies).

isms (Table 6) because no pronounced outgrowth of LAB was detected on the mixed lettuce. Comparing Tables 2 and 6, it can be noticed that the lag phase of the Gram-negative microorganisms, counted on TPC, showed a trend to be slightly longer during the in vivo experiment than in the in vitro experiment, although not significant, due to large confidence intervals of the estimated lag phases. The same result was found for the LAB, where the difference was more pronounced but still not significant. The microorganisms on the vegetables started to grow slightly later compared to the ones on the lettuce-juice-agar. This result could be expected because the cell wall of the vegetables forms a barrier and this rigid structure is not present anymore in the case of the in vitro experiments. Only pectinolytic microorganisms, such as *Pseudomonas* spp., *Erwinia* spp., and yeasts, such as *Candida* spp., can develop fast on vegetables. Once these microorganisms have broken the barrier of the cell wall, also the other spoilage causing microorganisms have enough nutrients available to grow (Robbs et al., 1996a,b; Liao et al., 1997; Heard, 2000). Nevertheless, it can be concluded that the applied simulation medium (lettuce-juice-agar) was a good substitute for fresh-cut produce. Growth rates were also very similar between the in vitro and in vivo experiments. Bennik et al. (1998) found a μ_{max} for Enterobacteriaceae and *Pseudomonas* spp. of 0.059/h and 0.053/h, respectively, on shredded chicory endive, stored under 1.5% O_2–20% CO_2 (balance N_2) at 8 °C. This was close to the μ_{max} of the TPC (Table 6) and the Gram-negative microorganisms (Table 2). The yeasts have a lower growth rate compared to bacteria, which was also demonstrated by Magnuson et al. (1990).

3.2.6. Production of metabolites/consumption of sugars during storage of fresh-cut vegetables

The pattern of the consumption of sugars and production of metabolites during the storage of grated celeriac is demonstrated in Fig. 6. Fast outgrowth of LAB and yeasts in the first 3 days of storage (Figs. 4 and 5) was accompanied with the consumption of glucose, fructose, sucrose and conversion of oligosaccharides into these molecules. Oligosaccharides are faster converted into sucrose, fructose and glucose from day 3 as an increase is detected in their concentration. The oligosaccharides, on the other hand, are produced from the enzymatic breakdown (enzymes from the plant tissue and microorganisms) of polysaccharides such as pectines (from day 4). The produced sugars are consumed faster by microorganisms from day 7 than they are produced. From day 10, the microbial contamination is high (Figs. 3–5), the available sugars are consumed in a high rate and the conversion of polysaccharides into oligosaccharides is slowed down, possibly due to the lower general metabolic activity of the plant tissue at the end of the storage period. Lactic acid and acetic acid could be quantified from day 5. Concentrations of lactic acid and acetic acid increased respectively from 0.046 ± 0.012 mg/ml lettuce juice at day 5 (>10^7 CFU LAB/g) until 5.660 ± 0.016 mg/ml at day 13 (10^8 CFU LAB/g), and 0.189 ± 0.047 mg/ml at day 5 until 1.283 ± 0.655 mg/ml at day 13. The chemical composition of grated celeriac is comparable to grated carrots. Spoilage of grated carrots, under EMA systems, is characterised by the occurrence of an acid taste and odour due to the production of lactic acid and/or acetic acid accompanied with a slime

Table 6
Growth characteristics of spoilage microorganisms (total psychrotrophic count (TPC), lactic acid bacteria (LAB) and yeasts) on mixed lettuce, stored under equilibrium modified atmosphere at 7 °C: lag phase λ (h), maximum specific growth rate μ_{max} (1/h), initial count N_0 (log CFU/g) and maximum population density N_{max} (log CFU/g) (± 95% confidence interval), estimated via Baranyi model

Microorganism	λ (h)	μ_{max} (1/h)	N_0 (log CFU/g)	N_{max} (log CFU/g)	N_0 (log CFU/cm^2)[a]	N_{max} (log CFU/cm^2)[a]	R^2
TPC	68.9 (50.7–87.1)	0.057 (0.03–0.08)	5.74 (5.43–6.05)	7.99 (7.79–8.18)	3.99 (3.68–4.30)	6.24 (6.04–6.43)	0.987
LAB	56.2 (14.1–98.4)	0.066 (−0.06–0.19)	4.53 (3.75–5.32)	6.59 (6.18–7.00)	2.78 (2.00–3.57)	4.84 (4.43–5.25)	0.939
Yeasts	56.2 (33.6–78.9)	0.043 (0.02–0.07)	3.83 (3.53–4.13)	6.20 (5.98–6.41)	2.08 (1.78–2.38)	4.45 (4.23–4.66)	0.992

[a] N_0 and N_{max} were converted to CFU/cm^2 taking into consideration that 1 g mixed lettuce is ≈ 56 cm^2 lettuce, to make comparison with Table 2 possible.

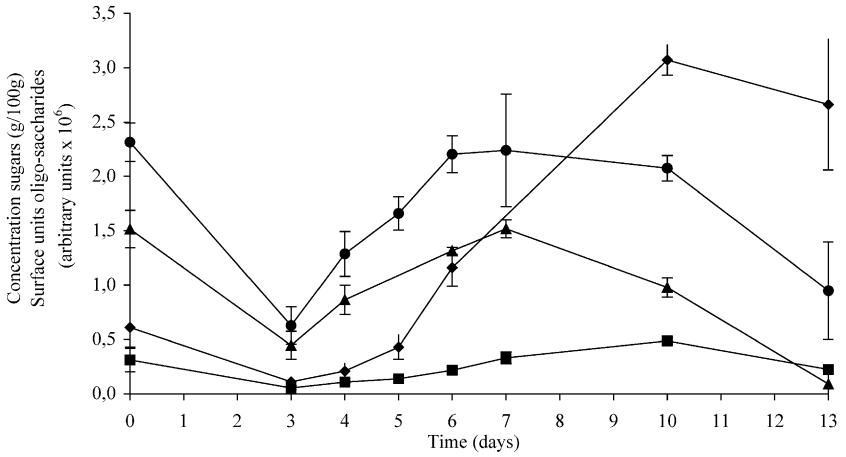

Fig. 6. Evolution of concentration (± 95% confidence interval) of sugars ((■) glucose, (▲) sucrose, (●) fructose, (♦) oligosaccharides) of grated celeriac during storage under equilibrium modified atmosphere (EMA) at 7 °C.

production and loss of texture (Carlin et al., 1989; Jacxsens et al., 1999a).

In the case of shredded chicory endives, a more stable pattern was found (Fig. 7). The concentration of sucrose, oligosaccharides and citric acid stayed constant during storage, but the glucose and fructose are consumed by the dominating Gram-negative micro-organisms (Figs. 3–5). Also the production of metabolites was very limited: only lactic acid was detected on day 13 (1.842 ± 0.046 mg/ml) (7.63 ± 0.27 log CFU LAB/g). There was no decrease in pH measured as well (Section 3.2.4). The predominant spoilers were Gram-negative bacteria, which are causing a soft rotting without producing nonvolatile metabolites.

The concentration of fructose and sucrose was constant while glucose was consumed from day 5 on for the mixed bell peppers (Fig. 8). At that moment, the concentration in oligosaccharides started to increase, because of the turn over from polysaccharides to oligosaccharides, possibly related to the high amount

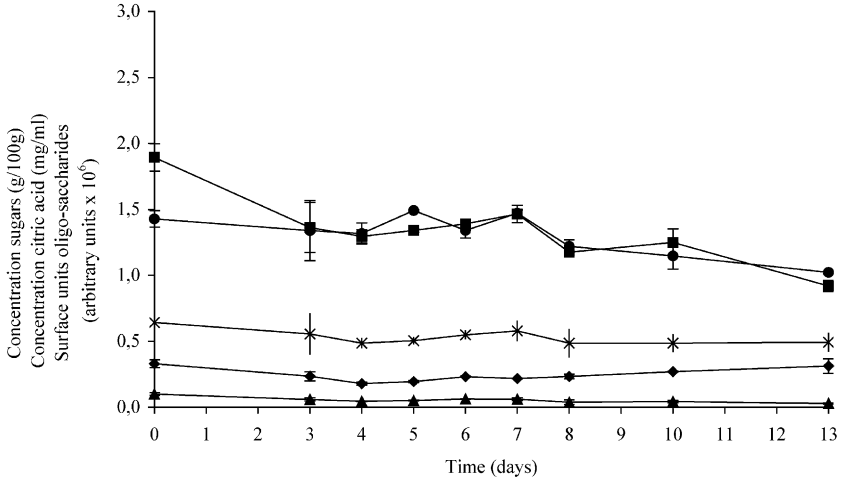

Fig. 7. Evolution of concentration (± 95% confidence interval) of sugars ((■) glucose, (▲) sucrose, (●) fructose, (♦) oligosaccharides), concentration of citric acid (×) of shredded chicory endives during storage under equilibrium modified atmosphere (EMA) at 7 °C.

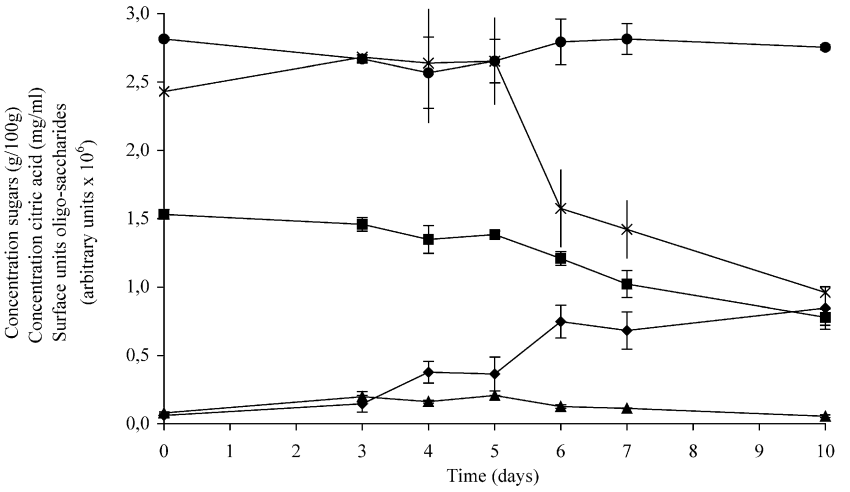

Fig. 8. Evolution of concentration (± 95% confidence interval) of sugars ((■) glucose, (▲) sucrose, (●) fructose, (♦) oligosaccharides), concentration of citric acid (×) of mixed bell peppers during storage under equilibrium modified atmosphere (EMA) at 7 °C.

of microorganisms and autolytic activity of the plant tissue. The microbial spoilage of the mixed bell peppers was going very fast and was dominated by LAB and yeasts. Consequently, a fast and intense metabolite production was measured. Acetic acid was detectable from day 5 (0.307 ± 0.276 mg/ml lettuce juice) (7.32 ± 0.41 log CFU LAB/g and 5.19 ± 0.49 log CFU yeasts/g) until day 10 (3.602 ± 0.417 mg/ml) (7.96 ± 0.19 log CFU LAB/g and 6.43 ± 0.21 log CFU yeasts/g). Lactic acid was formed 1 day later, at day 6 (1.192 ± 0.237 mg/ml) (7.69 ± 0.01 log CFU LAB/g and 5.16 ± 0.02 log CFU yeasts/g) until day 10 (2.421 ± 0.354 mg/ml). However, propionic acid could also be detected from day 7 (0.073 ± 0.035 mg/ml) until day 10 (0.349 ± 0.097 mg/ml). The acids are probably produced by *L. brevis*, the dominating LAB (3.2.3.). Again, the breakdown of citric acid can be attributed to the production of diacetyl, producing an off-flavour, by LAB (Bozogen and Yurdugül, 2000).

The mixed lettuce, packaged under EMA conditions, showed no metabolite production during the whole storage period at 7 °C (10 days). The dominating microorganisms were the same as for the shredded chicory endives (Gram-negative bacteria) although the yeasts were the limiting microorganisms (Figs. 3 and 5). Because no acid production was detected, the pH of the mixed lettuce could increase (Section 3.2.4). Also, the concentration of sugars remained constant over the storage period. Only sucrose was consumed after 5 days of storage (data not shown), at that moment, a high amount of TPC and yeasts was counted (Figs. 3 and 5).

However, for the mixed lettuce, stored in the commercial BOPP film, metabolite production was detected. This was probably more related to the anaerobic respiration (anoxic conditions inside the packages) than to growth of microorganisms (Figs. 3–5), because only ethanol was detected. After 3 days of storage at 7 °C, the packages became anoxic (Fig. 3) (0% O_2–13.8% CO_2) and the ethanol production started (0.045 ± 0.063 mg/ml) until day 6 (0.235 ±0.018 mg/ml). No change in sugars (fructose, glucose, sucrose and oligosaccharides) was detected during the 6 days of storage of the mixed lettuce in the BOPP type of packaging film. Lopez-Galvez et al. (1997) related the development of off-flavours, judged by a trained taste panel, to the production of ethanol and acetaldehyde in the lettuce tissue when stored under 0.2–1.5% O_2 and 5–30% CO_2 at 5 °C, conditions which are comparable to those created in the BOPP packages.

3.2.7. Sensory quality

The evolution of the sensory properties of the mixed lettuce differed for the specific lettuce components: endive and lollo bionta suffered faster from enzymatic discolouration and soft rotting compared to the other components (Artes and Martinez, 1996). At day 6, the

Table 7
Overview of the shelf life (days) of packaged fresh-cut vegetables, stored at 7 °C, based on the microbiological quality (exceeding limiting number of total psychrotrophic count (TPC), lactic acid bacteria (LAB) or yeasts (Y)), the sensory quality and production of nonvolatile metabolites

Type of fresh-cut vegetable	Shelf life based on microbial count (days)	Shelf life based on sensory quality (days)	Beginning of metabolite production (days)
Mixed lettuce (EMA)	6–10 TPC, –[a] LAB, 6 Y	6 (musty taste and odour, colour)	–[b]
Mixed lettuce (BOPP)	–[a] TPC, –[a] LAB, –[a] Y	4 (fermented, alcoholic taste and odour)	3 (ethanol)
Grated celeriac	6 TPC, 5–7 LAB, 4–5 Y	5 (colour), 6 (acid taste and odour)	5 (lactic acid and acetic acid)
Mixed bell peppers	5 TPC, 4–7 LAB, 7 Y	6 (acid taste and odour)	5 (acetic acid), 6 (lactic acid)
Shredded chicory endives	10 TPC, 13 LAB, –[a] Y	6 (colour), 13 (acid taste and odour)	13 (lactic acid)

[a] Limit not exceeded.
[b] No metabolite production.

mixed lettuce stored under EMA conditions was found to be unacceptable by the trained panel. The main property was the odour, which was described as ‘‘musty’’. The taste followed the same pattern as the odour. This musty odour and taste was probably provoked by production of volatile compounds by plant metabolism and/or microbial metabolism. However, no nonvolatile compounds were detected via HPLC analysis (Section 3.2.6).

The mixed lettuce, stored in the BOPP film, was becoming unconsumable on day 4. Again, the odour and the taste were the limiting properties, which were described as alcoholic and fermented. In the anoxic conditions, a high concentration of ethanol was detected, because of the anaerobic respiration (Kays, 1991; Peppelenbos, 1996).

The mixed bell peppers are rejected at day 6 based on their organoleptical properties (acid odour and taste) but also a massive water loss was found, and consequently, a loss of the crispness (texture). Shredded endive, on the other hand, is very sensitive for enzymatic discolouration (Jacxsens et al., 2001) and the colour was the limiting sensory property at day 6. Taste and odour became a problem at day 13, when the microbial contamination was high (Figs. 3–5) and lactic acid was detected (Section 3.2.6). Also the grated celeriac is very sensitive to enzymatic browning (Jacxsens et al., 2001) and it was rejected based on the colour at day 5, while the organoleptical properties were unacceptable from day 6.

3.2.8. Microbial growth–metabolite production–sensory quality interaction

It was not possible to define a clear relation between the growth of microorganisms/evaluation of the organoleptical properties/metabolite production for the tested leafy vegetables packaged under EMA (Table 7). Their microbial spoilage was dominated by Gram-negative microorganisms, not producing nonvolatile compounds in measurable concentrations for analysis by HPLC. Their shelf life was limited by the visual properties (enzymatic browning) or volatile compounds. It indicates that the sensory approach to determine the shelf life of leafy vegetables, next to microbial analysis, is the best. An agreement could be found between the development of the off-flavours inside the BOPP packages, filled with mixed lettuce, and the production of ethanol, inherent to the anaerobic respiration of plant tissue.

Sugar-rich product, such as the mixed bell peppers and grated celeriac, showed a different type of spoilage: dominated by lactic acid bacteria and yeasts. From the moment that acids were detectable by the HPLC analysis, the trained sensory panel rejected the minimally processed vegetables, based on their acid taste and odour. In these cases, metabolic studies can predict the shelf life period.

4. Conclusions

The applied lettuce-juice-agar was found to be a good simulation medium for fresh-cut produce. Estimated lag phase and growth rate both in vitro and in vivo corresponded well.

The applied microbiological criteria for the most important spoilage-causing microorganisms (>10^8 CFU/g for TPC, >10^5 CFU/g yeasts, >10^7–10^8 CFU/g LAB) corresponded well with the detectable changes in sensory properties and measurable metab-

olite production. This was most obvious when LAB and yeasts are provoking the spoilage. The limit of 10^7 CFU/g LAB has always to be evaluated together with the sensory properties because only after 2–3 days storage at levels between 10^7 and 10^8 CFU/g LAB, detectable amounts of lactic acid and acetic acid were found and spoilage became notable for the trained sensory panel.

Spoilage of leafy tissues, containing naturally low quantities of sugars, is mainly attributed to the proliferation of Gram-negative microorganisms, which are not producing nonvolatile metabolites. A sensory approach can be proposed as a fast evaluation of their shelf life. Sugar-rich produce, on the other hand, demonstrated a fast and intense growth of LAB and yeasts. Consequently, metabolic studies can be sufficient to evaluate the freshness of these products. The obtained results demonstrate as well the necessity of judging the quality of fresh-cut vegetables not solely on bacterial counts but also on sensory quality aspects.

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