JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Growth Behavior of Off-Flavor-Forming Microorganisms in Apple Juice

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Alicyclobacillus acidoterrestris and *Streptomyces griseus griseus* are two bacteria species that are frequently found in apple juice as spoilage bacteria. They both show thermoacidophilic behavior, adapting to the low pH of the juices and being able to survive high temperatures. They are able to regerminate in the shelf-stable product and spoil the juice by the formation of off-flavor compounds (i.e., guaiacol and 2,6-dibromophenol as metabolites of *A. acidoterrestris* and 2-isopropyl-3-methoxypyrazine, 2-methylisoborneol, 2-isobutyl-3-methoxypyrazine, and geosmin as important metabolites of *S. griseus*). In this study the growth behavior of the strains and the impact on apple juice were investigated under different conditions (i.e., temperature, oxygen supply, and mutual influence of the strains). The off-flavor formation was monitored by GC-MS after headspace SPME and subsequent calculation of the odor activity values. The results showed that *S. griseus* grows and consequently spoils the product even at 4 °C, whereas *A. acidoterrestris* needs at least room temperature to show significant growth. Limited oxygen supply did not significantly reduce off-flavor formation for any of the strains. The simultaneous presence of the strains in the juice reduced the growth of both species; nevertheless, off-flavor was detected.

KEYWORDS: Apple juice; off-flavor; *Alicyclobacillus acidoterrestris*; *Streptomyces griseus griseus*; growth behavior

INTRODUCTION

The rejection of foods due to the occurrence of off-flavor has become one of the most frequent consumers' complaints. Microorganisms not only account for spoilage but may also be responsible for off-flavor formation in the respective food (*1*).

The occurrence of off-flavor in apple juice is a frequently observed problem. Among various different kinds of microorganisms that may be present in apple juice, the occurrence of the so-called "thermoacidophilic" bacteria (TAB) represents a special problem (*2*, *3*). Due to their resistance to high temperatures and low pH values, they may survive thermal treatment such as the pasteurization process and are able to regerminate and grow in the shelf-stable product. Off-flavor compounds are formed as metabolites and may spoil the product via the formation of very unpleasant sensory notes after a certain storage period of the product on the shelf even at room temperature (*4*).

A spore-forming bacterium (*Bacillus acidocaldarius*) responsible for the off-flavor formation in apple juice was first identified and characterized in 1984 (*5*). Due to its frequent occurrence in soils, it was renamed *Bacillus acidoterrestris* in 1987 (6) and, later on, reclassified into a new genus, *Alicyclobacillus* (i.e., *Alicyclobacillus acidoterrestris*) (*7*). From 1984 (*5*), its occurrence in apple juice and the formation of a medicinal−phenolic off-flavor were described in several papers (*8*−*12*). 2,6-Dibromophenol and guaiacol are reported to be the compounds responsible for this distinct off-flavor (*10*, *13*), whereas guaiacol—as a decomposition product of ferulic acid via vanillin—is accepted to be the predominant metabolite (*14*). On the other hand, in many cases moldy, musty, or earthy off-flavors can be detected in apple juice, even though molds cannot be identified in the product. We recently described the occurrence of *Actinomyces* (*Streptomyces griseus griseus*) as a producer of this type of off-flavor in apple juice for the first time (*15*). A rather high number of compounds is responsible for the formation of this type of off-flavor. Geosmin and 2-methylisoborneol as well as 2-isopropyl-3-methoxypyrazine are representatives thereof (*15*, *16*). *S. griseus* is described to be present ubiquitously and is consequently able to spoil water, soil, and various foods (*2*, *3*, *17*, *18*). A contamination of the fruit via contact with soil or (washing) water is likely to occur. The presence of *S. griseus* and the related off-flavor even in pasteurized products (*15*) also indicates thermoacidophilic properties for this strain. **Table 1** gives a summary of the odor descriptors of all compounds of interest as well as the corresponding threshold values in the matrix apple juice (in terms of the recognition value) (*15*).

The characteristics of *A. acidoterrestris* and *S. griseus* as well as their odor-active metabolites are well described in literature.

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10.1021/jf070524j CCC: $37.00
Published on Web 07/07/2007

Table 1. Odor-Active Metabolites of *A. acidoterrestris* and *S. griseus* with Their Sensory Properties and Threshold Values in the Matrix Apple Juice

compound	odor descriptors	odor threshold value in apple juice[c] (μg L^{-1})	LOD[d] (μg L^{-1})	LOQ[d] (μg L^{-1})
m-anisaldehyde[a]	musty, moldy, leather, medicinal, sweet, floral	>250	1.01	3.21
o-anisaldehyde[a]	medicinal, pungent, sweet, chemical, floral	>250	0.45	1.57
p-anisaldehyde[a]	marzipan, sweet, pungent	226	0.41	1.41
α-terpineol[a]	lilac, fragrant, pungent	483	0.31	1.11
2-isobutyl-3-methoxypyrazine[a]	green pepper, green, acrid, parsley, cut grass	0.0033	0.63	2.21
2-isopropyl-3-methoxypyrazine[a]	earthy, potato, green pepper, pea, acrid, green	0.0006	0.66	2.2
2,3-dimethylpyrazine[a]	peanut, nutty, roasty, fatty, aromatic	>140	7.73	25.4
[(1*S*)-endo]-(−)-borneol[a]	sweet, menthol, pungent	67	1.18	3.73
2-methylisoborneol[a]	earthy, humid, moldy, cellar-like, forest-earth	0.0033	0.67	2.24
1-octen-3-ol[a]	mushrooms, varnish, earthy	31	1.09	3.48
3-octanone[a]	stale, moldy, old, slightly fruity, sweet, pear-like, candy-like, 'cooked'	>100	0.93	3.02
fenchyl alcohol[a]	earthy, humid, pungent, menthol-like, pine tree, detergent	3.2	0.52	1.78
geosmin[a]	musty, moldy, cellar-like, sweetish, pungent, red beet	0.027	0.34	1.21
guaiacol[b]	medicinal, sweet, chemical, medical office	2	0.29	1.06
2,6-dibromophenol[b]	smoky, pungent, medicinal, dental office	0.085	0.08	0.27

[a] Compound formed by *S. griseus*. [b] Compound formed by *A. acidoterrestris*. [c] The given odor threshold values correspond to the recognition value, which is the concentration in the matrix apple juice at which the panelists were able to recognize and properly describe the off-flavor of the compound in the juice. [d] LOD, limit of detection; LOQ, limit of quantification (*16*).

The occurrence of off-flavor caused by the presence of *A. acidoterrestris*, when the final product was stored on the shelf at common room temperatures, was described previously several times (*10*, *11*, *13*). Neverthless, rather little information can be found about the growth behavior of the two strains in any food matrix. Pettipher et al. (*21*) investigated cell growth and sensory defects based on the presence of *A. acidoterrestris*. Furthermore, the influence of oxygen and temperature on the growth of *A. acidoterrestris* was described recently (*19*, *20*). On the contrary, no information can be found about the dependence of the behavior of *S. griseus* on any external parameters, especially in the matrix apple juice. As a consequence, we systemically investigated the growth behavior of both strains in apple juice as well as the consequences for the sensory properties of the product. The influence of temperature as well as different amounts of disposable oxygen was the subject of the investigations. Furthermore, the mutual influence of the strains was studied in systems in which both bacteria were present simultaneously. For this purpose, microbial techniques were used to monitor the growth behavior as well as gas chromatography−mass spectrometry to determine the off-flavor compounds. The interpretation on the impact of the metabolites on the sensory properties of the apple juice was performed by applying the odor activity value (OAV) concept (OAV = concentration of the compound/odor threshold in the matrix) (*24*). On the basis of this concept, only compounds with an OAV ≥ 1 influence the flavor of the product.

With the results from these investigations we aim to contribute to a better understanding and handling of off-flavor occurrence in apple juice.

MATERIALS AND METHODS

Chemicals and Solvents Used. 2,3-Dimethylpyrazine (95%+), [(1*S*)-endo]-(−)-borneol (99%), *m*-anisaldehyde (97%), *p*-anisaldehyde (>98% purum), *o*-anisaldehyde (>98% purum), 1-octen-3-ol (98%), 3-octanone (>97% purum), fenchyl alcohol (97%), 3-isopropyl-2-methoxypyrazine (97%), α-terpineol (96%+), guaiacol (98%), 2,6-dibromophenol (99%), cinnamaldehyde (98%), 2-ethyl-3-methoxypyrazine (99%), D-camphor (97%), 5-octen-1-ol (98%), 5-methyl-3-heptanone (97%), and 2-chlorophenol (99%) were purchased from Sigma-Aldrich, Steinheim, Germany. Geosmin (98.7%), 2-methylisoborneol (99%), and 2-isobutyl-3-methoxypyrazine (99%) were purchased from Supelco, Bellefonte, PA. Ethanol (p.a. quality) was purchased from Merck, Vienna, Austria. Methanol (for residue analysis) and sodium sulfate (granular, anhydrous resin for residue analysis) were purchased from Promochem, Wesel, Germany. NaCl (≥99.5%) was purchased from Fluka, Germany. For the preparation of the media yeast extract, D-(+)-glucose (anhydrous) and glycerin (p.a. quality) were purchased from Merck−Microbiology, Vienna, Austria. Malt extract and $CaCO_3$ were bought from Fluka, Germany. For the trace element solution $CaCl_2 \cdot 2H_2O$, $MgSO_4 \cdot 7H_2O$, $(NH_4)_2SO_4$, KH_2PO_4, $ZnSO_4 \cdot 7H_2O$, and $MnCl_2 \cdot 4H_2O$ (all p.a. quality) were purchased from Merck, Vienna, Austria. H_3BO_3, $CoCl_2 \cdot 6H_2O$, $CuCl_2 \cdot 2H_2O$, $NiCl_2 \cdot 6H_2O$, and $Na_2MoO_4 \cdot 2H_2O$ (all p.a. quality) were bought from Sigma-Aldrich. Agar Bacteriological was received from Gibco BRL Life Technologies, Eggenstein, Germany.

Cultivation and Characterization of the Bacteria Strains. *A. acidoterrestris* (DSMZ 2498) and *S. griseus griseus* (DSMZ 40236) were obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) in lyophilized form. According to the recommendations given by DSMZ, the bacteria strains were cultivated in two different media. After preparation, both media were stored at 4 °C until use.

For the preparation of the media for *A. acidoterrestris* the following compounds were diluted in double-distilled water: 0.25 g L^{-1} $CaCl_2 \cdot 2H_2O$, 0.5 g L^{-1} $MgSO_4 \cdot 7H_2O$, 0.2 g L^{-1} $(NH_4)_2SO_4$, 2 g L^{-1} yeast extract, 5 g L^{-1} glucose, 3 g L^{-1} KH_2PO_4. After the pH had been adjusted to 4.0 with 0.1 mol L^{-1} HCl, the following trace element solution (1 mL L^{-1}) was added: 0.1 g L^{-1} $ZnSO_4 \cdot 7H_2O$, 0.03 g L^{-1} $MnCl_2 \cdot 4H_2O$, 0.3 g L^{-1} H_3BO_3, 0.2 g L^{-1} $CoCl_2 \cdot 6H_2O$, 0.01 g L^{-1} $CuCl_2 \cdot 2H_2O$, 0.02 g L^{-1} $NiCl_2 \cdot 6H_2O$, and 0.03 g L^{-1} $Na_2MoO_4 \cdot 2H_2O$. For the preparation of agar plates 15 g L^{-1} agar was added. Growth of *A. acidoterrestris* is described between 20 and 55 °C depending upon the strain and composition of the medium (*22*), with best growth at 45 °C at pH 4.0−4.5 (*10*, *11*, *13*, *21*). To cultivate *A. acidoterrestris* from the lyophilized form, 45 °C was used for the cultivation on agar plates. Samples in liquid media were incubated at 37 °C, which is slightly below the growth optimum and which was the highest temperature available with a shaking device.

For the cultivation of *S. griseus* the media were prepared in double-distilled water using 4 g L^{-1} glucose, 4 g L^{-1} yeast extract, and 10 g L^{-1} malt extract. For solid media agar (12 g L^{-1}) and $CaCO_3$ (2 g L^{-1}) were added. Both liquid and solid media were inoculated at 30 °C due to the lower optimum growth temperature of this organism.

For the storage of the strains, 4 mL liquid cultures were put into sterile tubes. The tubes were stored at 4 °C for about 1 week to avoid rapid growth of the cells and to keep the cultures unchanged. Reference cultures were stored at −20 °C in sterile tubes with the addition of glycerin (30%) to avoid destruction of the cell membranes.

Each growth experiment was followed and controlled by light optical microscopy, whereas the appearance and size of bacteria cells were checked. Additionally, the results were documented by digital photography.

Determination of Colony-Forming Units. Liquid media were inoculated with one colony using a sterile platinum eyelet and incubated at the respective temperature for 5 days. From this solution the colony-forming units (cfu mL^{-1}) were determined as a measure for living and reproducing cells. After the incubation, the liquid media were diluted with sterile NaCl solution (0.9%) in the ratios 1:10, 1:100, 1:1.000, and 1:10.000, respectively. One hundred microliters of each dilution was plated on solid media and incubated for 3 days at 45 °C for *A. acidoterrestris* and at 30 °C for *S. griseus*. Afterward, the colonies were counted and the cfu mL^{-1} values were calculated according to method of ref *23*.

Juice Samples. The apple juice used for the investigations was commercially available juice made from concentrate. For all experiments the same brand was used. pH was controlled (pH 3.6−4.0). The used apple juices were tested by sensory evaluation by three well-trained panelists prior to the experiments. Only juices without any detectable off-flavor were used. To avoid the presence of undesired microorganisms in the juice, each sample was filtered through sterile filters (pore size = 45 μm; Nalgene filtration systems) prior to use.

Investigation of the Growth Behavior. *Influence of Available Oxygen.* Twenty milliliters of sterile filtered apple juice was filled into 50 mL test tubes. Each sample was inoculated from one colony picked from the master agar plate with the inoculating loop under sterile conditions. One part of the tubes was closed tightly so that only the oxygen remaining in the tube was available for the bacteria strains. The other part was closed with cotton plugs to allow free oxygen admittance. The samples were incubated at 37 °C (*A. acidoterrestris*) and 30 °C (*S. griseus*) on a shaking device for a period of 4 weeks. One milliliter samples for the determination of colony-forming units and quantification of off-flavor compounds were taken in weekly intervals.

Influence of Growth Temperature. These experiments were carried out using 200 mL cardboard packages to simulate real-life situations. Storage of the samples was carried out at 4 °C, room temperature (i.e., average = 21.5 °C), and 30 °C. For each strain and storage temperature at least two samples were inoculated. For each condition a control sample was treated the same way. Each sample was inoculated with 500 μL of the inoculum containing 4×10^5 cfu mL^{-1} of either *A. acidoterrestris* or *S. griseus*, corresponding to an inoculation level of 1×10^3 cfu mL^{-1} apple juice for each strain. Inoculation was performed under sterile conditions at the perforation for the drinking straw. The package was prepierced at the perforation with a sterile platinum eyelet. The sample was inoculated and afterward closed again using Parafilm. Samples were stored for 30 days. Every fifth day samples were taken from each sample and analyzed for the presence of the strains and the off-flavor compounds of interest.

Mutual Influence of the Two Strains. Two hundred and fifty milliliters of sterile filtered apple juice were inoculated with 500 μL of each inoculum containing 4×10^5 cfu mL^{-1} of either *A. acidoterrestris* or *S. griseus* under sterile conditions, corresponding to an inoculation level of 0.8×10^3 cfu mL^{-1} apple juice for each strain. The samples were incubated at 30 °C on the shaking device for 4 weeks. As references, samples containing only one strain each were treated the same way. Oxygen was not limited in the experiment. One milliliter samples were taken in weekly intervals for the determination of cfu mL^{-1} and quantification of the off-flavor compounds.

All growth experiments were carried out in duplicate. Given cell numbers are mean values thereof. When large deviations were observed between the duplicates, the experiments were repeated.

Determination of Off-Flavor Compounds. The instrumental determination of the off-flavor compounds was performed by gas chromatography−mass spectrometry (GC-MS) after headspace solid-phase microextraction (SPME). For the SPME, a 50/30 μm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) Stable Flex fiber (length = 2 cm, Supelco, Bellefonte, PA) was used. Two and a half grams of Na_2SO_4 was added to 0.5 mL of apple juice diluted with 4.5 mL of water. The samples were equilibrated for 5 or 10 min at 60 °C while the sample was thoroughly stirred. The fiber was then exposed to the headspace of the sample at 60 °C for 10 or 30 min, respectively. Afterward, the fiber was transferred immediately into the injection port of the GC for thermal desorption.

For the GC-MS measurements a Hewlett-Packard system (HP G1800A GCD System) was used. SPME sampling was performed using a CombiPAL Multi sampler (CTC Analytics, Zwingen, Switzerland). The capillary column used was an HP5 (cross-linked 5% phenyl methyl siloxane; column length = 30 m, inner diameter = 0.25 mm, film thickness = 1 μm). Helium with a purity of 99.999% (Air Liquide, Austria) was used as carrier gas. The conditions were as follows: column head pressure, 0.54 bar; starting temperature, 10 °C (hold time = 1 min); constant pressure (gas flow at 10 °C, 39.8 cm s^{-1}); different temperature rates (8 °C min^{-1} or 10 °C min^{-1}), final temperature 250 °C. Temperatures below 45 °C were controlled by blowing liquid nitrogen into the GC-oven. Splitless injection mode was used, the split valve being opened after 2 min. A special SPME glass liner with a constant inner diameter of 0.75 mm (Supelco, Bellefonte, PA) was used. Injector temperature was 270 °C, and detector temperature was 280 °C. Electron impact ionization was used (70 eV), and data were acquired in the selected ion mode. Quantification of the compounds was performed using the following internal standards: 2-ethyl-3-methoxypyrazine for 2,3-dimethylpyrazine, 2-isopropyl-3-methoxypyrazine, 2-isobutyl-3-methoxy-pyrazine; cinnamaldehyde for *o*-, *m*-, and *p*-anisadehyde; D-camphor for fenchyl alcohol, [(1*S*)-endo]-(−)-borneol, α-terpineol, 2-methylisoborneol, and geosmin; 5-octen-3-ol for 1-octen-3-ol; 5-methyl-3-heptanone for 3-octanone; and 2-chlorophenol for guaiacol and 2,6-dibromophenol. The analytical procedures were fully validated. Limits of detection (LOD) and limits of quantification (LOQ) can be seen in **Table 1**. A detailed description of the method and the ions that were selected for the identification and quantification as well as method validation are given in ref *16*.

All concentrations given in **Tables 2−4** are average values from at least quadruplicate determination (i.e., each solution from any microbial experiment was analyzed by GC-MS twice). Standard deviations for the concentrations from quadruplicate determination ranged from ±2 to ±15%.

RESULTS AND DISCUSSION

Both investigated strains—*A. acidoterrestris* as well as *S. griseus griseus*—are described to be aerob (*4*, *5*, *25*). Two previous studies describe that *A. acidoterrestris* shows a distinct decrease of the growth rate when lower amounts of oxygen were supplied (*19*, *20*); no data can be found concerning the behavior of *S. griseus*. Regarding the growth of *A. acidoterrestris* when different amounts of oxygen were available for the strain, the results of our experiments confirmed these findings. Nevertheless, under both conditions (free oxygen and limited oxygen supply) rather high cell numbers could be observed after 4 weeks (free oxygen supply, 2×10^4 cfu mL^{-1}; limited oxygen supply, 1.3×10^4 cfu mL^{-1}). *S. griseus* showed a similar behavior; the growth rate was decreased significantly when lower amounts of oxygen were supplied, but, nevertheless, again high cell numbers were found at the end of the growth experiment under both conditions (free oxygen supply, 1.7×10^4 cfu mL^{-1}; limited oxygen supply, 1×10^4 cfu mL^{-1}). The concentrations and the OAVs of the 15 relevant off-flavor compounds were followed over the whole experiment (**Table 2**). It can be seen that guaiacol and 2,6-dibromophenol were formed by *A. acidoterrestris* in detectable amounts under both conditions. For both compounds the OAVs were >1 and consequently had a negative impact on the flavor of the juice. It is interesting to see that especially for guaiacol the concentrations were already very high after 1 week. This fact corresponds well with the data published in ref *14*, which showed a quick conversion of vanillin as a decomposition product of ferulic acid in apple juice to guaiacol when *A. acidoterrestris* was present. For guaiacol as

Table 2. Off-Flavor Compound Formation When Oxygen Is Supplied in Different Amounts (either *A. acidoterrestris* or *S. griseus* Was Present per Sample)

		7 days		14 days		21 days		28 days	
compound		concn (μg L^{-1})	OAV[d]	concn (μg L^{-1})	OAV	concn (μg L^{-1})	OAV	concn (μg L^{-1})	OAV
m-anisaldehyde	free O_2 access	4.58	0.02[c]	6.26	0.03[c]	8.94	0.04[c]	12.0	0.05[c]
	limited O_2 supply	2.11[b]	0.01[c]	4.25	0.02[c]	7.21	0.03[c]	10.1	0.04[c]
o-anisaldehyde	free O_2 access	1.01[b]	0.00	1.01[b]	0.00[c]	5.16	0.02[c]	9.56	0.04[c]
	limited O_2 supply	1.01[b]	0.00	1.01[b]	0.00[c]	1.01[b]	0.00[c]	2.36	0.01[c]
p-anisaldehyde	free O_2 access	4.36	0.02	15.8	0.07	35.5	0.16	48.1	0.21
	limited O_2 supply	3.35	0.01	11.6	0.05	21.4	0.09	34.1	0.15
α-terpineol	free O_2 access	3.56	0.01	5.63	0.01	12.4	0.03	32.2	0.07
	limited O_2 supply	5.26	0.01	7.63	0.02	15.5	0.03	42.6	0.09
2-isobutyl-3-methoxy-pyrazines	free O_2 access	1.42[b]	430	1.42[b]	**430**	1.42[b]	**430**	1.42[b]	**430**
	limited O_2 supply	1.42[b]	430	1.42[b]	**430**	1.42[b]	**430**	1.42[b]	**430**
2-isopropyl-3-methoxy-pyrazines	free O_2 access	3.46	**5770**	4.56	**7600**	4.98	**8300**	5.02	**83670**
	limited O_2 supply	3.39	**5650**	4.21	**7020**	4.36	**7270**	4.98	**8300**
2,3-dimethylpyrazine	free O_2 access	nd[a]		nd		nd		16.6[b]	0.12
	limited O_2 supply	nd		nd		nd		16.6[b]	0.12
[(1*S*)-endo]-(−)-borneol	free O_2 access	nd		2.46[b]	0.04	2.46[b]	0.04	2.46[b]	0.04
	limited O_2 supply	nd		2.46[b]	0.04	2.46[b]	0.04	4.56	0.07
2-methylisoborneol	free O_2 access	nd		1.46[b]	**440**	1.46[b]	**440**	1.46[b]	**440**
	limited O_2 supply	1.46[b]	**440**	1.46[b]	**440**	2.63	**800**	3.15	**960**
1-octen-3-ol	free O_2 access	2.29[b]	0.07	2.29[b]	0.07	9.23	0.30	10.1	0.33
	limited O_2 supply	nd		nd		2.3[b]	0.07	4.36	0.14
3-octanone	free O_2 access	1.98[b]	0.02[c]	3.15	0.03[c]	12.7	0.13[c]	16.0	0.16[c]
	limited O_2 supply	1.98[b]	0.02[c]	1.98[b]	0.02[c]	5.36	0.05[c]	12.2	0.12[c]
fenchyl alcohol	free O_2 access	nd		1.15	0.36	1.98	0.62	3.53	**1.10**
	limited O_2 supply	nd		3.56	**1.11**	8.65	**2.70**	9.12	**2.85**
geosmin	free O_2 access	1.26	**46.7**	6.45	**240**	9.12	**340**	14.0	**520**
	limited O_2 supply	0.78	**28.7**	3.09	**110**	7.12	**260**	10.4	**380**
guaiacol	free O_2 access	3.34	**1.67**	4.03	**2.02**	5.25	**2.63**	5.87	**2.94**
	limited O_2 supply	7.57	**3.79**	8.29	**4.15**	8.86	**4.43**	10.9	**5.47**
2,6-dibromophenol	free O_2 access	nd		0.18[b]	**2.06**	0.18[b]	**2.06**	0.33	**3.88**
	limited O_2 supply	0.18[b]	**2.06**	0.18[b]	**2.06**	0.28	**3.29**	0.44	**5.18**

[a] Not detectable. [b] The compound was identified, but the concentration was lower than the limit of quantification. As a consequence, the value was set to (LOD + LOQ)/2. [c] No definite recognition value was determined for those compounds. To simulate a worst-case scenario, the highest concentration that was used for the threshold determination was used to calculate the OAV in these experiments. [d] OAV values >1 are shown in bold.

well as for 2,6-dibromophenol the concentrations and OAVs were higher in the case of limited oxygen supply. We suppose that this fact is based on a stress response of the aerobic strain under limited oxygen supply. Following the 13 investigated odor-active metabolites of *S. griseus* only 4 (i.e., 2-isopropyl-3-methoxypyrazine, 2-methylisoborneol, 2-isobutyl-3-methoxypyrazine, and geosmin; in decreasing order of the impact on the off-flavor formation) contributed significantly to the off-flavor of the juice with OAVs up to >8.000. A further small impact can also be expected by fenchyl alcohol. It must be noted that no general correlation between the available oxygen and the concentrations of the compounds could be found. The very potent compounds 2-isopropyl-3-methoxypyrazine and 2-isobutyl-3-methoxypyrazine did not show any dependence on the oxygen supply. *o*-, *m*-, and *p*-anisaldehydes as well as 1-octen-3-ol and 3-octanone were formed in higher concentrations when more oxygen was available. It should be noted that the production of *o*-anisaldehyde almost stopped when less oxygen was available. Geosmin—a very potent off-flavor compound with very high OAVs—was also formed in higher concentrations when more oxygen was at the cells' disposal. High increases in geosmin concentration were observed versus the end of the experiments in the stationary growth period. This behavior might be indicative of the fact that mainly older cells produce high amounts of geosmin. The fact that the typical note of geosmin can be perceived very intensely from solid media with old *S. griseus* colonies emphasizes this assumption. α-Terpineol, fenchyl alcohol, [(1*S*)-endo]-(−)-borneol, and 2-methylisoborneol were synthesized in higher concentrations when lower oxygen amounts were supplied, which might be a kind of stress response of the cells. The different formation rates for the metabolites of *S. griseus* show that obviously the available oxygen has a significant influence on the cells' metabolic process and consequently on the formation rates of the off-flavor compounds. The higher concentrations of the latter four compounds in the stress situation, for example, might be a hint that the isoprenoid pathway (*26*) is stimulated under these conditions. Further on, the results indicate that the compounds, on the one hand, are formed via different formation pathways in the cell and, on the other hand, might be synthesized in the cell for a different purpose in the cell's period of life.

To gain information about the development of the juice at storage temperatures that are commonly used by the consumer, we investigated the growth and off-flavor formation at 4 °C, room temperature (i.e., 21.5 °C on average), and 30 °C as a common storage temperature in the summer period. To simulate a "real-life scenario" the experiments were performed using 200

Table 3. Off-Flavor Compound Formation at Different Growth Temperatures (either *A. acidoterrestris* or *S. griseus* Was Present per Sample)

		5 days		10 days		15 days		20 days		25 days		30 days	
compound	temp	concn (μg L^{-1})	OAV[e]	concn (μg L^{-1})	OAV	concn (μg L^{-1})	OAV	concn (μg L^{-1})	OAV	concn (μg L^{-1})	OAV	concn (μg L^{-1})	OAV
m-anisaldehyde	4 °C	nd[a]		2.11[b]	0.01[c]	2.11[b]	0.01[c]	2.11[b]	0.01[c]	2.11[b]	0.01[c]	2.11[b]	0.01[c]
	RT[d]	2.11[b]	0.01[c]	2.11[b]	0.01[c]	2.11[b]	0.01[c]	3.25	0.01[c]	3.78	0.02[c]	4.19	0.02[c]
	30 °C	2.11[b]	0.01[c]	2.11[b]	0.01[c]	3.48	0.01[c]	5.86	0.02[c]	7.48	0.03[c]	12.5	0.05[c]
o-anisaldehyde	4 °C	nd		nd		nd		nd		nd		nd	
	RT	nd		nd		nd		1.01[b]	0.00[c]	1.01[b]	0.00[c]	1.01[b]	0.00[c]
	30 °C	nd		nd		nd		1.01[b]	0.00[c]	2.15	0.01[c]	2.48	0.01[c]
p-anisaldehyde	4 °C	nd		1.79	0.01	2.44	0.01	2.54	0.01	2.85	0.01	8.53	0.04
	RT	nd		2.52	0.01	3.45	0.02	5.48	0.02	6.48	0.03	9.48	0.04
	30 °C	2.53	0.01	3.59	0.02	4.58	0.02	7.64	0.03	8.49	0.04	12.5	0.06
α-terpineol	4 °C	nd		nd		2.63	0.01	5.45	0.01	6.48	0.01	7.04	0.01
	RT	2.96	0.01	3.48	0.01	9.45	0.02	12.9	0.03	15.7	0.03	18.6	0.04
	30 °C	3.63	0.01	5.48	0.01	12.7	0.03	18.7	0.04	21.5	0.04	28.5	0.06
2-isobutyl-3-methoxy-pyrazines	4 °C	1.38[b]	**420**	1.38[b]	**420**	1.38[b]	**420**	1.38[b]	**420**	1.38[b]	**420**	1.38[b]	**420**
	RT	1.38[b]	**420**	1.38[b]	**420**	3.41	**1030**	3.86	**1170**	4.02	**1220**	4.52	**1370**
	30 °C	2.65	**800**	3.25	**990**	3.48	**1060**	4.15	**1260**	4.98	**1510**	5.69	**1720**
2-isopropyl-3-methoxy-pyrazine	4 °C	4.54	**7570**	5.69	**9480**	5.72	**9530**	5.89	**9820**	11.9	**19800**	13.3	**22100**
	RT	4.57	**7620**	6.48	**10800**	6.98	**11600**	7.58	**12600**	12.6	**20900**	16.3	**27100**
	30 °C	6.98	**11600**	9.15	**15300**	13.3	**22100**	15.2	**25400**	16.1	**26900**	17.0	**28300**
2,3-dimethylpyrazine	4 °C	16.6[b]	0.12[c]	16.6[b]	0.12[c]	16.6[b]	0.12[c]	16.6[b]	0.12[c]	16.6[b]	0.12[c]	16.6[b]	0.12[c]
	RT	16.6[b]	0.12[c]	16.6[b]	0.12[c]	16.6[b]	0.12[c]	16.6[b]	0.12[c]	16.6[b]	0.12[c]	16.6[b]	0.12[c]
	30 °C	16.6[b]	0.12[c]	16.6[b]	0.12[c]	16.6[b]	0.12[c]	16.6[b]	0.12[c]	16.6[b]	0.12[c]	16.6[b]	0.12[c]
[(1*S*)-endo]-(−)-borneol	4 °C	nd		nd		nd		nd		nd		nd	
	RT	nd		nd		2.46[b]	0.04	2.46[b]	0.04	2.46[b]	0.04	2.46[b]	0.04
	30 °C	nd		nd		2.46[b]	0.04	2.46[b]	0.04	2.46[b]	0.04	2.46[b]	0.04
2-methylisoborneol	4 °C	nd		nd		nd		1.46[b]	**440**	3.47	**1050**	3.49	**1060**
	RT	nd		nd		2.67	**810**	3.86	**1170**	4.12	**1250**	4.56	**1380**
	30 °C	3.14	**950**	4.15	**1260**	4.98	**1510**	5.12	**1550**	5.45	**1650**	5.69	**1720**
1-octen-3-ol	4 °C	nd		nd		nd		nd		nd		nd	
	RT	2.29[b]	0.07	2.29[b]	0.07	2.29[b]	0.07	4.15	0.13	5.36	0.17	7.48	0.24
	30 °C	3.59	0.12	4.58	0.15	6.78	0.22	8.01	0.26	9.48	0.31	14.0	0.45
3-octanone	4 °C	1.98[b]	0.02[c]	6.09	0.06[c]	7.15	0.07[c]	8.69	0.09[c]	8.75	0.09[c]	8.89	0.09[c]
	RT	6.11	0.06[c]	7.45	0.07[c]	8.79	0.09[c]	8.99	0.09[c]	9.16	0.09[c]	10.8	0.11[c]
	30 °C	5.12	0.05[c]	6.45	0.06[c]	6.79	0.07[c]	9.56	0.10[c]	12.4	0.12[c]	18.9	0.19[c]
fenchyl alcohol	4 °C	nd		nd		nd		3.65	**1.14**	3.69	**1.15**	3.77	**1.18**
	RT	3.63	**1.13**	3.79	**1.18**	3.81	**1.19**	4.15	**1.30**	4.59	**1.43**	4.89	**1.53**
	30 °C	3.12	0.98	3.82	**1.19**	4.14	**1.29**	4.57	**1.43**	4.83	**1.51**	5.03	**1.57**
geosmin	4 °C	nd		nd		nd		nd		1.48	**54.8**	1.56	**57.8**
	RT	0.78[b]	**28.7**	0.78[b]	**28.7**	0.78[b]	**28.7**	2.59	**95.9**	3.09	**110**	4.26	**160**
	30 °C	0.78[b]	**28.7**	0.78[b]	**28.7**	1.25	**46.3**	4.56	**170**	6.59	**240**	9.00	**330**
guaiacol	4 °C	nd		0.68[b]	0.34	0.68[b]	0.34	0.68[b]	0.34	0.68[b]	0.34	0.68[b]	0.34
	RT	nd		0.68[b]	0.34	1.08	0.54	3.98	**1.99**	4.05	**2.03**	4.87	**2.44**
	30 °C	0.68[b]	0.34	1.36	0.68	2.49	**1.25**	2.98	**1.49**	4.09	**2.05**	5.76	**2.88**
2,6-dibromophenol	4 °C	nd		nd		nd		nd		0.18[b]	**2.06**	0.18[b]	**2.06**
	RT	nd		nd		nd		0.18[b]	**2.06**	0.46	**5.41**	0.86	**10.1**
	30 °C	nd		nd		0.18[b]	**2.06**	0.50	**5.88**	0.84	**9.88**	1.05	**12.4**

[a] Not detectable. [b] The compound was identified, but the concentration was lower than the limit of quantification. As a consequence, the value was set to (LOD + LOQ)/2. [c] No definite recognition value was determined for those compounds. To simulate a worst-case scenario, the highest concentration that was used for the threshold determination was used to calculate the OAV in these experiments. [d] RT, room temperature, average = 21.5 °C, minimum = 20 °C, maximum = 23 °C. [e] OAV values >1 are shown in bold.

mL cardboard boxes. This experimental setup also implied very little oxygen at the cells' disposal.

Figure 1 shows the growth curves for the two strains at the investigated temperature levels. From **Figure 1a** it can be seen very clearly that *A. acidoterrestris* favors higher temperatures for growth. Nevertheless, even though the optimum growth temperature of this strain is described to be 45 °C, a small increase in cell numbers could be observed at 4 °C as well as decent growth at room temperature. At 30 °C, approaching the optimum growth temperature, *A. acidoterrestris* merged very quickly into the exponential growth phase. **Figure 1b** gives a picture about the growth rates of *S. griseus* in apple juice at the investigated temperatures. As may be expected for a strain with an optimum growth temperature of 28 °C, very little growth could again be observed at 4 °C, whereas at room temperature and 30 °C fairly quick growth was observed.

Table 3 shows the concentrations and the OAVs of the investigated off-flavor compounds. It can be noted that for all compounds the formation rate increased with increasing storage temperature. Guaiacol as one metabolite of *A. acidoterrestris* could be observed at all three temperature levels. At 4 °C the concentrations were very low and did not influence the flavor of the juice, whereas at room temperature the OAV was >1 after a storage period of 20 days and at 30 °C already after 15 days. For 2,6-dibromophenol even at 4 °C the OAV was >1 after 25 days; at higher temperatures the influence of this

Table 4. Off-Flavor Compound Formation by either a Mixed Culture or One Individual Strain (Growth Temperature = 30 °C)

		7 days		14 days		21 days		28 days	
compound	bacteria strain/s present	concn (μg L^{-1})	OAV[g]	concn (μg L^{-1})	OAV	concn (μg L^{-1})	OAV	concn (μg L^{-1})	OAV
m-anisaldehyde	*S. griseus*[d]	2.11[b]	0.01[c]	2.11[b]	0.01[c]	3.56	0.01[c]	4.12	0.02[c]
	both strains[f]	nd[a]		nd		nd		nd	
o-anisaldehyde	*S. griseus*	nd		1.01[b]	0.00[c]	1.01[b]	0.00[c]	1.01[b]	0.00[c]
	both strains	nd		nd		1.01[b]	0.00[c]	1.01[b]	0.00[c]
p-anisaldehyde	*S. griseus*	4.99	0.02	8.45	0.04	9.12	0.04	10.2	0.05
	both strains	nd		0.91[b]	0.00	2.23	0.01	4.12	0.02
α-terpineol	*S. griseus*	1.63	0.00	3.45	0.01	4.36	0.01	16.3	0.03
	both strains	4.69	0.01	7.15	0.01	10.3	0.02	22.4	0.05
2-isobutyl-3-methoxy-pyrazines	*S. griseus*	1.38[b]	**420**	3.56	**1080**	4.15	**1260**	6.12	**1860**
	both strains	1.38[b]	**420**	1.38 [b]	**420**	1.38 [b]	**420**	1.38[b]	**420**
2-isopropyl-3-methoxy-pyrazine	*S. griseus*	2.36	**3930**	2.89	**4820**	5.01	**8350**	7.25	**12100**
	both strains	nd		nd		nd		1.43[b]	**2380**
2,3-dimethylpyrazine	*S. griseus*	nd		nd		nd		nd	
	both strains	nd		nd		nd		nd	
[(1S)-endo]-(–)-borneol	*S. griseus*	nd		nd		nd		nd	
	both strains	nd		nd		nd		nd	
2-methylisoborneol	*S. griseus*	nd		nd		nd		1.46[b]	**440**
	both strains	1.46[b]	**440**	1.46[b]	**440**	1.46[b]	**440**	3.02	**920**
1-octen-3-ol	*S. griseus*	3.56	0.11	4.15	0.13	6.84	0.22	7.15	0.23
	both strains	nd		2.29[b]	0.07	8.12	0.26	9.15	0.30
3-octanone	*S. griseus*	1.98[b]	0.02[c]	1.98[b]	0.02[c]	4.59	0.05[c]	12.2	0.12[c]
	both strains	1.98[b]	0.02[c]	1.98[b]	0.02[c]	6.45	0.06[c]	14.6	0.15[c]
fenchyl alcohol	*S. griseus*	1.15[b]	0.36	1.15[b]	0.36	1.15[b]	0.36	3.65	**1.14**
	both strains	1.15[b]	0.36	1.15[b]	0.36	2.56	0.80	4.15	**1.30**
geosmin	*S. griseus*	0.73[b]	**26.9**	1.23	**45.6**	5.12	**190**	6.45	**240**
	both strains	0.73[b]	**26.9**	2.65	**98.1**	5.98	**220**	13.1	**490**
guaiacol	*A. acidoterrestris*[e]	nd		1.65	0.83	4.45	**2.23**	4.97	**2.49**
	both strains	0.68[b]	0.34	1.12	0.56	2.63	**1.32**	3.15	**1.58**
2,6-dibromophenol	*A. acidoterrestris*	nd		nd		0.34	**4.00**	0.75	**8.82**
	both strains	nd		nd		nd		0.18[b]	**2.06**

[a] Not detectable. [b] The compound was identified, but the concentration was lower than the limit of quantification. As a consequence, the value was set to (LOD + LOQ)/2. [c] No definite recognition value was determined for those compounds. To simulate a worst-case scenario, the highest concentration that was used for the threshold determination was used to calculate the OAV in these experiments. [d] Only *S. griseus* was present in the juice. [e] Only *A. acidoterrestris* was present in the juice.. [f] *S. griseus* and *A. acidoterrestris* were present in the juice simultaneously. [g] OAV values >1 are shown in bold.

compound on the off-flavor could be observed after shorter storage periods. Among the odor-active metabolites of *S. griseus* the most potent odorants were found to be 2-isopropyl-3-methoxypyrazine, 2-methylisoborneol, 2-isopropyl-3-methoxy-pyrazine, geosmin, and fenchyl alcohol (in decreasing order according to the OAV values). For all other compounds, an increase in concentration was observed, although the OAVs were <1, and consequently no influence on the off-flavor has to be expected. It is important to note that for 2-isopropyl-3-methoxypyrazine, 2-isobutyl-3-methoxypyrazine, 2-methyl-isoborneol, and geosmin, even temperatures of 4 °C were sufficient to cause the formation of very high concentrations and to spoil the product. The results from this experiment show that when apple juice is contaminated with either one of the two strains, storage at temperatures of >20 °C will lead to off-flavor formation. Storage at 4 °C can prevent off-flavor formation when the product is contaminated with *A. acidoterrestris*, but will not prevent the off-flavor formation caused by *S. griseus*.

As contamination with only one strain is very unlikely to occur in reality, we investigated the mutual influence of *A. acidoterrestris* and *S. griseus* on the growth and the off-flavor formation. To create acceptable growth conditions for both strains, the experiments were performed at a temperature of 30 °C. To avoid further stress on the strains, the experiments were performed under free oxygen access. **Figure 2** shows the behavior (i) when only one strain was present in the juice and (ii) when both strains were present simultaneously. In the forefront of the experiment we supposed that the growth of *A. acidoterrestris* would be reduced due to the fact that *S. griseus* is a known producer of antibiotics (*23*, *25*). However, the results show that both strains were influenced significantly in their growth behavior. Not only was the growth rate of *A. acidoterrestris* reduced—as we would have expected—but also a significantly lower growth rate of *S. griseus* was observed. A competition about certain nutrients or inhibition with metabolites specific for each of the strains could be the reason for this behavior.

The lower growth rates are well reflected in the concentrations of the odor-active metabolites of *A. acidoterrestris*. Both compounds—guaiacol and 2,6-dibromophenol—were found in smaller concentrations when both strains were present. Nevertheless, after periods of 21 and 28 days, respectively, the OAVs were >1, even when both strains were present. In the case of the metabolites of *S. griseus* the compounds can again be divided into two groups. On the one hand, the compounds *o*-, *m*-, and *p*-anisaldehyde, 2-isopropyl-3-methoxypyrazine, and 2-isobutyl-3-methoxypyrazine were formed in significantly lower amounts

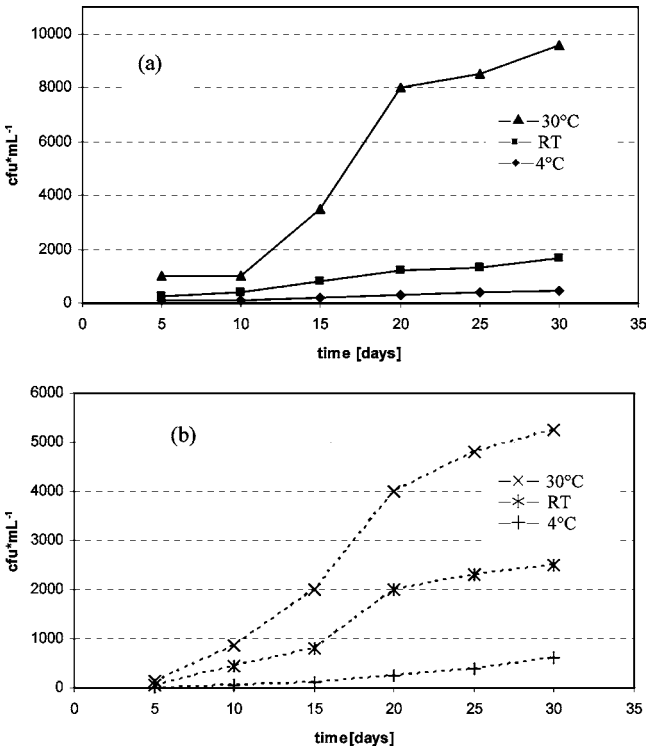

Figure 1. Growth rates depending on the temperature: (**a**) *A. acidoterrestris*; (**b**) *S. griseus*.

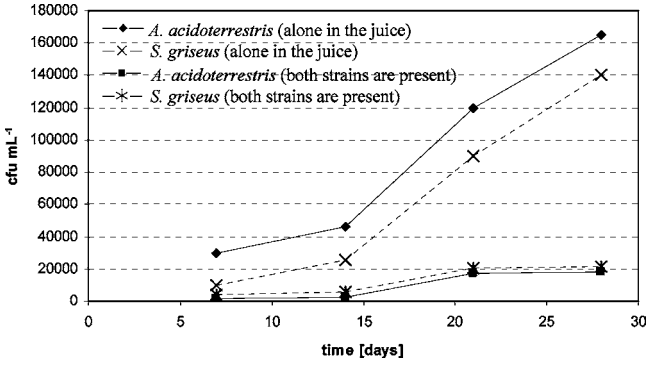

Figure 2. Mutual influence of the two strains. Growth temperature in all cases was 30 °C.

when both strains were present simultaneously. The explicit lower production rates of the two pyrazines must be noted, even though at the end of the experiment concentrations were sufficient to negatively influence the flavor of the juice. On the other hand, for the compounds α-terpineol, fenchyl alcohol, 1-octen-3-ol, 3-octanone, 2-methylisoborneol, and geosmin higher formation rates were observed when both strains were present. The metabolites 2-methylisoborneol and geosmin are again of importance as they showed very high OAVs in the experiment with both strains. The higher concentrations of α-terpineol, fenchyl alcohol, 2-methylisoborneol, and geosmin in the experiment with both strains in the system could again be indicators for a stress response with stimulated isoprenoid pathway in the cell.

The results of our experiments proved that the contamination of apple juice with either *A. acidoterrestris* or *S. griseus griseus* leads to rather quick off-flavor formation in the product. Both strains proved to be very tolerant of the external conditions. The storage of the juice at very low temperatures may prevent the spoilage of the product only by metabolites of *A. acidoterrestris*, whereas odor-active metabolites of *S. griseus* were also formed in high concentrations at 4 °C. Limitations of oxygen in the product led to lower formation rates, but nevertheless the off-flavor compounds were formed in sufficient amounts to negatively influence the product. The simultaneous presence of the two strains may lower the growth rates and consequently the formations rates of the off-flavor compounds, but, nevertheless, negative impacts on the sensory properties have to be expected. These results show that the only way to guarantee an off-flavor-free juice is to produce "TAB-free" juices, because the presence of either of the investigated bacteria strains will sooner or later lead to the formation of an undesired flavor of the product.

ABBREVIATIONS USED

GC-MS, gas chromatography−mass spectrometry; LOD, limit of detection; LOQ, limit of quantification; nd, not detectable; OAV, odor activity value; RT, room temperature; SPME, solid-phase microextraction.

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Received for review February 22, 2007. Revised manuscript received May 30, 2007. Accepted May 31, 2007. This project was funded in part by a DOC-FFORTE scholarship (doctoral scholarship program of the Austrian Academy of Sciences, Women in Research and Technology).

JF070524J