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Abstract

During the past ten years considerable changes have occurred in the global beverage market. Functional beverages and bottled waters currently constitute the fastest growing sectors. Energy drinks, tooth-friendly beverages, and non-alcoholic malt beverages are also gaining popularity. This literature review aims at providing state-of-the-art knowledge on microbiological spoilage and safety risks in **non-beer beverages produced in a brewery environment** with special emphasis on functional and specialty products.

Many modern beverages have **less antimicrobial hurdles** compared to traditional carbonated soft drinks due to higher level of nutrients for microbial growth, lower acidity and / or milder carbonation level. Thermal processing and the use of chemical preservatives have also been reduced for the production of more “natural” products. These changes in the beverage production are expected to lead to increase in the product spoilage rate unless the gap is filled with improvements in hygiene or with other hurdles.

The **major spoilage microbe types** in the modern beverages will probably remain the same as in the traditional products, but the range of species is expected to increase. Previously innocuous LAB and yeasts common in the brewery environment may be able to grow in the more vulnerable products. Bacteria are expected to gain increasing importance in the product spoilage. New emerging spoilers include e.g. acid-tolerant aerobic bacteria (e.g. *Alicyclobacillus*) in PET-bottled beverages, *Asaia* spp. in flavoured mineral waters, *Propionibacterium cyclohexanicum* in juice-rich drinks, and spore-forming bacteria and enterobacteria in mildly acidic drinks.

The possible **new microbial health risks** in the beverage production may arise from the increasing ingredient import worldwide and from the use of low-acid juices as ingredients. Pathogenic bacteria are not only able to survive but

can also grow in the low acid-fruit and vegetable juices. New ingredients and changes in climate conditions may result in the appearance of new pathogens and spoilage organisms. Moreover, the functional and specialty beverages may allow better survival of pathogens compared to the traditional soft drinks. Therefore, research is needed about the occurrence and behaviour of pathogenic microbes in the new beverages. *Escherichia coli* 0157:H7 is considered the most likely known threat in the acidic products due to its low infective dose and good acid-tolerance.

Whenever new beverages are developed it is important to go through every change made in the recipe, packaging and preservation in order to consider the microbial risks. The modern beverages typically contain several potential growth enhancers and inhibitors, and their microbial stability is difficult to predict. **Predictive microbiology** can help in optimising the preservative systems and in predicting and describing the behaviour of contaminants in non-beer beverages. Microbial adaptation to stress needs to be taken into account in the preservation and quality control of non-beer beverages. Research is needed for optimization of the detection of stressed cells and to develop early warning tools.

The future challenge in the beverage production is to produce safe and acceptably stable products with **minimal processing**. Exploiting the synergistic effect of existing natural antimicrobials together with generally regarded as safe substances and mild physical preservation treatments is a potential approach for controlling harmful microbes in beverages. The future of beverage preservation will be a skilled knowledge-based combination of antimicrobial hurdles to maintain microbial microbiological quality while maintaining maximum sensory and nutritional quality.

Preface

During the past ten years considerable changes have occurred in the global beverage market. Functional beverages and bottled water currently constitute fast growing segments. Energy drinks, still and tooth-friendly beverages as well as malt-based beverages are also gaining popularity. Moreover, alcohol-containing beverage mixes are produced and imported increasingly. In comparison to traditional soft drinks, modern beverages have become compositionally more complex and tend to have less antimicrobial hurdles because of their higher nutrient contents, lower acidity and carbonation level. Moreover, the use of thermal processing and chemical preservatives is being reduced in order to produce more natural products.

This literature review aims at providing state-of-the-art knowledge on microbiological quality and safety risks in non-beer beverages produced in a brewery environment, with special emphasis on functional and specialty beverages. This study was funded by the PBL Brewing Laboratory. The authors thank the members of the PBL Brewing Laboratory for supporting the work and for their valuable comments. The technical editing of the review was kindly supported by VTT Technical Research Centre of Finland.

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Contents

1. Introduction

In recent years, several developments in society have contributed to changes in the global beverage market. Consumers are increasingly aware of the impact of diet on their health and well-being. Beverages are not only consumed to provide refreshment and hydration, but also to increase well-being and to help in preventing nutrition-related disorders (Tenge and Geiger 2001). Moreover, an increasing number of consumers favour minimally processed products from natural ingredients for reducing the intake of chemical additives from food and for obtaining products with improved nutritive and sensory characteristics. For example, studies showing the possible presence of carcinogenic benzene in soft drinks due to the reaction of benzoates (chemical additive) with ascorbic acid and the possible allergenic effects of sulphites and benzoates have naturally contributed to this consumer trend (Ashurst and Hargitt 2009).

Whereas simple carbonated soft drinks still dominate the global beverage market, their market share is decreasing. Functional beverages and bottled water currently constitute the fastest growing beverage sectors (Lawlor et al. 2009). In 2008, functional drinks reached global sales of 26.9 billion dollars, with average growth rates of 15–20% per annum. The energy drinks sector has experienced the greatest volume growth, which is expected to be strongest in 2007–2012 (Heckman et al. 2010). Still and tooth-friendly low-acid beverages are gaining popularity across the product sectors (Lawlor et al. 2009). In addition, non-alcoholic malt beverages provide an excellent alternative to traditional soft drinks. Consumption of these beverages is expected to grow in the coming years (www.euromonitor.com). The Middle East is showing particular potential, as Islamic beliefs limit consumption of alcoholic beverages. In the Western markets, consumers are looking for new, healthier alternatives to conventional soft drinks. From the public health point of view, there is also a need to reduce alcohol consumption.

The beverage industry needs to respond to consumer demands and to develop new product innovations in order to maintain competitiveness in the market. In the soft drink sector, new nutritive and bioactive ingredients are added into the formulations, and the traditional ingredients are being replaced with their lighter, organic or more natural counterparts. New exotic ingredients, such as "superfruits", are used to boost the nutritional value of the products and to find new exotic flavours (Tribst et al. 2009). In the alcoholic beverage sector, breweries increasingly develop new low-alcohol and value-added products, such as fusion drinks mixing alcohol drinks with non-alcoholic beverages, for new and increasingly defined consumer groups (Hutzler et al. 2008). Both soft drinks and alcoholic beverages have become more and more complex in composition. At the same time, consumer demands for more natural, nutritious and tasty products are directing breweries to minimize the use of additives and heat treatments, to increase juice contents in formulations, as well as to reduce the acidity of the products. Possible adverse health effects of benzoic acid have already led many soft drink manufacturers to abandon this additive. Hence, many traditional antimicrobial hurdles present in traditional soft drinks and alcoholic beverages are brought down (Hausman 2009), while product transport time, shelf-life and international trade as well as the use of new ingredients are increasing (Tribst et al. 2009). Recent changes in the product formulation, processing and packaging technologies, transport and trade could expose beverage production to new microbiological risks that require identification in order to maintain acceptable microbiological stability and safety in the future. Moreover, changes in the global climate may have serious impacts on the microbiological quality of foods and beverages. More rigorous control of beverage ingredient quality will be emphasized.

2. Goal

This literature review aims at providing state-of-the-art knowledge on microbiological quality and safety risks in non-beer beverages produced in a brewery environment, with special emphasis on functional and specialty beverages. The study was triggered by the need to evaluate potential microbiological risks related to the changes made in the formulation, preservation and packaging of beverages during the past ten years. In this review, non-beer beverages have been divided into bottled waters, soft drinks and alcoholic beverages (including beer-mixed beverages). Milk- and soya-based drinks, probiotic products, pure fruit and vegetable juices as well as tea and coffee are outside the scope of this review.

3. Bottled waters

3.1 Types of bottled waters

Bottled waters include mineral waters, spring water, or other drinking water (MMM 166/2010). Spring water is defined as water which is intended for human consumption in its natural state, and is bottled at the source (EC 54/2009). Natural mineral water means microbiologically wholesome water, originating from an underground water table or deposit and emerging from a spring tapped at one or more natural or bored exits (EC 54/2009). Natural mineral waters may be very low (< 50 mg/l), low (50–500 mg/l) or rich (> 500 mg/l) in mineral salt content (EC 54/2009). Only carbon dioxide may be added to natural mineral waters.

Mineral waters may also be produced from drinking water by adding Na, Ca, Mg and K salts. According to Finnish legislation, mineral water must contain at least 500 mg/l solids (KTM 1658/1995). Sparkling mineral water is produced by adding 4–8 g/l carbon dioxide (CO_2) (Panimoliitto 2011). If carbon dioxide has not been added, the product must be marked as noncarbonated or still water. In addition to water, minerals and CO_2, both natural and synthetic fruit and berry flavourings (aromas) are nowadays often added to mineral waters. The flavourings include e.g. lemon, grapefruit, apple, cranberry and mandarin. Addition of flavourings is regulated by an EC regulation (EC 1334/2008).

Per capita consumption of bottled water in the European Union varies enormously from one country to another, with an average consumption of 105 l per year (EFBW 2011). Finland has the lowest consumption level, with 16 l a year per inhabitant and Italy the highest at just under 200 l per inhabitant. In Finland, aromatized mineral waters make up one third of the market of mineral waters (Panimoliitto 2011).

3.2 Microbiology of bottled waters

Water always contains microbes, but when it is bottled an open system becomes a closed one. The original microbial community, the amount of dissolved nutrients and oxygen, and temperature affect the microbes after bottling.

The microbiological demands for **water intended for household use** are that it may not contain *Escherichia coli* and enterococci in 100 ml of water (EC 98/1983, STM 461/2000). In addition, it is recommended that in 100 ml water there are no *Clostridium perfringens* or coliformic bacteria, and no marked changes in heterotrophic colony count when incubated at 22 °C. The demands for **bottled water** intended for household use are no *E. coli*, enterococci or *Pseudomonas aeruginosa* in 250 ml, and heterotrophic colony count of < 100 cfu/ml at 22 °C and < 20 cfu/ml at 37 °C. The guidance value for all types of bottled water during marketing is total colony count of 50 000 cfu/ml when incubated 72 h at 20–22 °C or 24 h at 37 °C (MMM 166/2010).

The microbiological quality of bottled waters has been studied in different countries. Natural mineral waters originating from groundwater represent an oligotrophic system with a low level of organic matter and limited bioavailability. The bacteria in these systems are often in a viable but non-culturable state. The viable count usually increases 1–3 weeks after bottling (Moreira et al. 1994, Defives et al. 1999, Leclerc and Moreau 2002). From eight brands of noncarbonated bottled water from UK, France and Belgium, initial counts of up to 10^4 cfu ml^{-1} were detected (Armas and Sutherland 1999). Significant differences occur in microbial numbers between different brands (Tsai and Yu 1997, Armas and Sutherland 1999, Korzeniewska et al. 2005). Bottled waters have been studied in Finland by the National Institute for Health and Welfare (Miettinen and Pursiainen 2009). The heterotrophic colony count varied between 1 and 4 x 10^5 cfu/ml. The microbial numbers were higher in imported bottled waters (on average 4 x 10^3 cfu/ml) compared to domestic ones (average 450 cfu/ml). Tap water always had the lowest microbial counts. *Pseudomonas aeruginosa* was not detected from the samples.

Bottle material influences the number and type of cells adhering to the bottle surface. Higher counts found from plastic bottles compared to glass bottles have been due to the higher surface roughness of plastic (Barbesier 1970, Bischofberger et al. 1990). Jones et al. (1999) reported that cells adhering to HDPE (high density polyethylene bottles) were mainly clumps of coccoid cells. Cells adhering to PET (polyethylene tetraphtalate) bottles were rod-shaped and sparse-

ly distributed on the surface. Biofilm represented 0.03–1.79% of the total viable count in 1.5 l bottles.

Common bacterial species found from bottled waters are shown in Table 1. The predominance of Gram-negative over Gram-positive bacteria is evident. *Pseudomonas* is a common genus in bottled waters (Tsai and Yu 1997, Armas and Sutherland 1999, Leclerc and Moreau 2002). Some moulds, belonging to the genera of *Alternaria, Cladosporium, Paecilomyces* and *Penicillium*, have also been found from bottled waters (Table 1).

Table 1. Examples of identified microorganisms isolated from bottled waters.

Microorganism	**Source (type of bottled water)**	**Reference**
Bacteria		
Acinetobacter junii	noncarbonated, UK, FR, BE	Armas and Sutherland 1999
Aeromonas sp.	noncarbonated, TW + imported	Tsai and Yu 1997
	noncarbonated, GR	Venieri et al. 2006
A. hydrophila	noncarbonated, TW + imported	Tsai and Yu 1997
Asaia sp.	fruit flavoured, IR	Moore et al. 2002a
Burkholderia cepacia	noncarbonated, UK, FR, BE	Armas and Sutherland 1999
Comamonas sp.	noncarbonated, UK, FR, BE	Armas and Sutherland 1999
Enterococcus	noncarbonated, GR	Venieri et al. 2006
Flavobacterium sp.	noncarbonated, TW + imported	Tsai and Yu 1997
	noncarbonated, GR	Venieri et al. 2006
Gluconacetobacter sacchari	fruit flavoured, IR	Moore et al. 2002b
Moraxella	noncarbonated, UK, FR, BE	Armas and Sutherland 1999
Mycobacterium spp.	noncarbonated	Papapetropoulou et al. 1997
Pasteurella	noncarbonated, TW + imported	Tsai and Yu 1997
	noncarbonated, GR	Venieri et al. 2006
Pseudomonas spp.	noncarbonated, TW + imported	Tsai and Yu 1997
	noncarbonated	Wilkinson and Kerr 1998
	noncarbonated, UK, FR, BE	Armas and Sutherland 1999
P. aeruginosa	noncarbonated, TW + imported	Tsai and Yu 1997
	spring, AR	Tamagnini and Gonzáles 1997
	noncarbonated, GR	Venieri et al. 2006
Providencia	noncarbonated, GR	Venieri et al. 2006
Sphaeromonas paucimobilis	noncarbonated, UK, FR, BE	Armas and Sutherland 1999
Staphylococcus spp.	noncarbonated, UK, FR, BE	Armas and Sutherland 1999
Stenotrophomonas	noncarbonated	Wilkinson and Kerr 1998
maltophilia	noncarbonated, TW + imported	Tsai and Yu 1997
Xanthomonas	noncarbonated, TW + imported	Tsai and Yu 1997

Moulds		
Aureobasidium	mineral, JP + imported	Fujikawa et al. 1997
Acremonium	mineral, JP + imported	Fujikawa et al. 1997
Alternaria	mineral, JP + imported	Fujikawa et al. 1997
A. alternata	noncarbonated mineral, AR	Cabral and Fernandez-Pinto 2002
Cladosporium.	mineral, JP + imported	Fujikawa et al. 1997
C. cladosporioides	noncarbonated mineral, AR	Cabral and Fernandez-Pinto 2002
Moniliella	mineral, JP + imported	Fujikawa et al. 1997
Paecilomyces	mineral, JP + imported	Fujikawa et al. 1997
P. fulvus	carbonated, AR	Ancasi et al. 2006
Penicillium	noncarbonated mineral, AR	Cabral and Fernandez-Pinto 2002
	mineral, JP + imported	Fujikawa et al. 1997
P. citrinum	noncarbonated mineral, AR	Cabral and Fernandez-Pinto 2002
P. glabrum	carbonated, AR	Ancasi et al. 2006
	aromatised mineral, FR	Nevarez et al. 2009

AR = Argentina, BE = Belgium, FR = France, GR = Greece, IR = Ireland, JP = Japan, TW = Taiwan

It should be remembered that the majority of microbiological studies of bottled waters have been made with culturing techniques, and may not include non-culturable cells. The use of DNA-based microbial community detection by e.g. PCR-DGGE (Polymerase Chain Reaction and Denaturing Gradient Gel Electrophoresis) can provide new insights into the species composition of bottled waters (Dewettinck et al. 2001).

Disease outbreaks from bottled water have not been frequent. Historically, bottled water has been the vehicle of transmission of *Vibrio cholerae,* causing an outbreak of cholerae in Portugal (Blake et al. 1974). The bacterial species frequently encountered from bottled waters and causing a risk infection include *Pseudomonas aeruginosa, Aeromonas hydrophila, Stenotrophomonas maltophilia, Burkholderia cepacia and Staphylococcus aureus. P. aeruginosa* is usually regarded as a secondary contaminant, not originating from the source of water, but S. maltophilia and B. cepacia can be found from source waters, and they have the ability to grow with very small concentrations of organic matter (Rosenberg 2003). If pathogenic bacteria are present, they may persist for long periods in bottled waters. Depending on the type of water and level of inoculum, inoculated *Escherichia coli* O157 has been shown to persist even 300 d in bot-

tled water (Kerr et al. 1999, Warburton et al. 1998). Attachment to bottle walls and biofilm formation may help bacteria to survive (Warburton et al. 1998).

Another health risk is the possible mycotoxins produced by moulds. *Alternaria alternata* and *Penicillium citrinum* found from bottled mineral waters are potential producers of mycotoxins, and P. citrinum has been shown to produce citrinin in mineral water (Criado et al. 2005).

3.3 Control of contaminations

3.3.1 Raw material purity

The most important and in some cases the only raw material of bottled water is water. Bottled water may be treated with UV, filtering or ozonation in order to improve its hygienic quality. Exceptions are spring water and natural mineral waters, which may not be disinfected (EC 54/2009). In Finland, drinking water used for mineral water production is usually treated by activated carbon filtration to remove possible faults in the taste or odour of the water.

Flavoured mineral waters are very popular, but little information is available on the possible influence of the aromas on the microbial community of the products. Flavours added to bottled waters may act as sources of microbes, and also as nutrients for indigenous microbes in the water. Moore et al. (2002a, b) found unusual spoilage organisms from aromatized waters and suspected that the natural fruit juices were the source of these organisms. The microbiological quality of the aroma flavours and their potential influence on the product should be taken into account.

3.3.2 Carbonation

Carbonation of water decreases its pH. Carbonated waters generally have less microorganisms than non-carbonated waters (Caroli et al. 1985, Wilkinson and Kerr 1998, Korzeniewska et al. 2005, Pap et al. 2008). In a study of Pap et al. (2008), twelve types of bottled waters with different mineralization and CO_2 levels in PET and glass bottles were inoculated with four different mould species isolated from bottled waters (*Fusarium sp., Cladosporium sp., Penicillium chrysogenum and Aspergillus fumigatus)*. The results showed that fungal growth was mainly determined by the carbonation level and the type of mould strain, whereas neither the inoculation level nor the mineral content had any significant

effect on the survival of the moulds. Results showed decreased numbers in carbonated waters, and slow decreasing, stagnation or even some growth in still waters. *A. fumigatus* was most resistant to carbonation.

3.3.3 Process hygiene

Good manufacturing practices (GMP) and hygienic equipment design help to minimise contaminations from the factory environment, and the Hazard Analysis and Critical Control Points (HACCP) system should be implemented in the bottling process (CAC/RCP 48-2001, Kokkinakis et al. 2008). Beverage process hygiene is discussed in more detail in section 4.8.2.

3.3.4 Storage time and temperature

The quality of bottled water is influenced by storage time – generally the longer the storage the more the microbes proliferate (Criado et al. 2005, Korzeniewska et al. 2005, Miettinen and Pursiainen 2009). Criado et al. (2005) studied the influence of different storage conditions on the germination and growth of mould spores in bottled mineral water, and noted that storage time was the parameter with the greatest influence on mould growth. When mould spores were inoculated into bottles that had just been filled, their growth into visible colonies took 5 months, but when spores were inoculated into bottles that had already been stored for 5 months, they grew into visible colonies in only one month. The authors suggested that moulds used compounds dissolving from PET bottles as nutrients, because a 20% increase of plasticizer additive was measured in 5-month stored bottles compared to recently bottled waters.

Storage temperature also affects microbial growth. Generally temperatures below room temperature are considered beneficial for microbiological quality. However, in the study of Korzeniwska et al. (2005) temperature (5 or 22 °C) had little effect for numbers of studied microorganisms.

In Finland the manufacturer of water may decide the shelf time, and according to a survey made by Finnish Food Safety Authority Evira the shelf time of bottled water is most often one year (Sand 2007).

4. Soft drinks and alcoholic beverages

Soft drink is a general term for a non-alcoholic beverage, differentiating it from an alcoholic beverage. Soft drinks constitute a diverse group of beverages. They can be classified in several ways, for example on the basis of sugar (caloric/diet) and fruit juice content, flavouring, carbonation level (sparkling/still), main non-water ingredient (fruit, malt, tea, soya, milk etc.) and functionality. Functional soft drinks are the trend of today. There is no official definition for a functional beverage in the EU. They can be considered to include enriched and fortified drinks (such as juices and waters with added vitamins and minerals); sports drinks; energy drinks; wellness drinks and nutraceuticals (products with added ingredients targeted at specific medical or health benefits) (Tenge and Geiger 2001). Formulations of functional beverages are increasingly complex and often cross many product categories. There is also a pressure to produce low-acid beverages for improving tooth health, and to minimize the use of chemical additives and synthetic ingredients.

Figure 1. Functional beverages (Tenge and Geiger 2001).

Alcoholic beverages are drinks with more than 2.8% alcohol by volume (abv) (STM 1143/1994). The main alcoholic beverages produced in breweries are ciders, long drinks and alcopops with an alcohol content of 2.9–8% (abv). Alcohol in ciders and in some long drinks is produced by yeast fermentation, whereas alcopops are produced from distilled spirits and soft drinks. In 2009, ciders and long drinks made up 5% of the total alcohol consumption in Finland (Jääskeläinen and Virtanen 2010). The consumption of long drinks has been increasing in Finland since 2004 (Panimoliitto 2011). Beer-mixed beverages (BMBs) are a heterogenic group of products combining beer with soft drinks, aromas and syrups. They are popular especially in Germany, where their sale increased by 17.7% from 2005 to 2006. The market share and product diversity are expected to continue growing (Kelch 2007). Fortification of alcoholic beverages (> 1.2% abv) with vitamins or minerals and nutritional claims about the products are banned in the EU (EC 1925/2006). It is also recommended that stimulating ingredients such as caffeine are not added to alcoholic beverages.

4.1 Ingredients and manufacture of soft drinks

4.1.1 Ingredients in traditional soft drinks

Traditional soft drinks typically contain water (up to 98 vol-%), sweeteners (8–12%, w/v), fruit juice (usually up to 10%), carbon dioxide (0.3–0.6% w/v), acidulants (0.05–0.3%), flavourings (0.1–0.5%), colourings (0–70 ppm), chemical preservatives (legal limits), antioxidants (< 100 ppm), foaming agents (e.g. saponins up to 200 mg/ml), and stabilizers (0.1–0.2% per GMP) (Table 2). Nowadays soft drinks may also contain added vitamins, minerals, proteins, fibres and other functional compounds (Table 2).

Table 2. Categories and typical properties of soft drinks.

Category	Examples	Typical ingredients	Carbonation	pH	Simple sugars
Colas and lemonades	Coca Cola, Pepsi	Sweetener, sugar, acids, flavours (juices), preservatives	Medium to high	2.4–3.2	0–10%
Wellness drinks	Fenix, Hyvää Päivää	Botanical extracts, soluble fibres, vitamins, minerals, preservatives	Low to medium	3.5–4.5	2–7%
Malt-based beverages	Bionade, Naturade	Fermented wort, organic flavours, sweeteners	Low to medium	nd	nd
Energy drinks	Red Bull, Battery	Caffeine, taurine, herbal extracts, L-carnitine, sugar, glucuronolactone, B-vitamins, preservatives	Low to medium	2.5–3.2	1.4–14%
Sports drinks	Gatorade	Salts, simple sugars (caffeine, amino acids), preservatives	None to low	3.2–4.0	5.5–8%
Tooth-friendly beverages	Good for me	Non-nutritive carbohydrates, preservatives	None to low	≥5.0	0%

References: Ashurst and Hargitt 2009, Back 2005, Paquin 2009, Heckman et al. 2010, Mettler et al. 2006, product information from Internet, nd; no published data.

Water as an ingredient. Water is the major ingredient in all soft drinks and should fulfil the criteria for drinking water (EC 98/1983). Soft drinks manufacturers usually use softened water to prevent off-tastes from chlorine residues (Ashurst and Hargitt 2009). This procedure reduces the concentration of metal ions to approx. 50 ppm Mg and Ca (Stratford and James 2003).

Sugar and sweeteners. Soft drinks with the exception of zero caloric formulations contain sugars from 1% to 12% (w/w). Sucrose, glucose and fructose are used as natural carbohydrate sweeteners in various forms (Ashurst and Hargitt 2009). Special carbohydrate sweeteners permitted in the EU are trehalose, isomaltulose (Palatinose^{TM}) and D-tagatose (Ashurst and Hargitt 2009). Isomaltulose is a natural tooth-friendly disaccharide with slow energy release and glycemic index and a mild sweetness (Hausmann 2009). Fruit and vegetable extracts also contain hexose and pentose sugars and polyols (Stratford and James 2003). In the low caloric products, sugars are replaced with non-nutritive intense sweeteners. The most commonly used sweeteners (maximum permitted dosage in the EU) are aspartame (600 mg/l), acesulfame K (350 mg/l), sucralose (300 mg/l) and saccharin (80 mg/l) (94/35 EC). Aspartame can break down in soft drinks to yield phenylalanine amino acid (Stratford and James, 2003). Thaumatin is a naturally sweet plant extract that can be applied as a flavour enhancer (Ashurst and Hargitt 2009).

Fruit juices are a rich source of various nutrients and bioactive compounds, such as sugars, organic acids, phosphates, minerals, vitamins, amino acids and ammonium salts as well as colouring and flavouring compounds and antioxidants (especially polyphenols) (Stratford and James 2003).

Carbonation and acidulants. Carbonation is responsible for the characteristic taste of sparkling beverages. Carbonation of soft drinks is expressed as volumes or grams per litre. One volume equals 1.96 g/l. Carbonation volume varies from 1.5 to 5 and is typically 3–4. A balancing acidity is usually achieved with acidulants or acidity regulators. Organic acids, most commonly citric acid and citrates, are used as acidulants in most formulations. Cola drinks are acidified with phosphoric acid.

Flavourings, colourings, antioxidants and stabilizers. Flavourings are currently classified as natural, nature-identical and artificial. In the EU, their usage is under the control of EU regulations (EC 1334/2008). Several artificial colourings are permitted in soft drinks according to the amount that is needed or at a level of 50–100 mg/l. Their use is becoming less popular due to negative consumer attitudes to chemical additives. Azodyes are banned in Finland. Colour can also be delivered naturally with vegetable and fruit extracts. Carotenoids can be used as a source of natural colours, but they are also added in soft drinks for their antioxidant activity (Gruenwald 2009).

Various hydrocolloids, such as guar and locust gum, pectin and xanthan, are used as stabilizers and thickeners especially in diet drinks (improve mouth-feel) and fruit juice drinks (reduce phase separation). Antioxidants, usually ascorbic acid, are used to prevent flavour deterioration especially in oxygen permeable packages.

Chemical preservatives are used to improve the microbiological stability of soft drinks. Sorbates, benzoates and dimethydicarbonate are permitted in ready-to-drink beverages in Europe (EC 1333/2008). The use of SO_2 (250 mg/l) is limited to juice concentrates. Benzoates can react with ascorbic acid (vitamin C) to form benzene, and are nowadays omitted from increasing numbers of formulations. These additives and sulphites may also cause allergic reactions in sensitive individuals (Ashurst and Hargitt 2009).

4.1.2 Ingredients in functional soft drinks

Fortification of soft drinks with vitamins (especially A, B, C, E) and minerals (Ca, Zn, Mg, and Na) has a long history. For nutrition claims in the label, they

must provide 15% of the Recommended Daily Allowance per package or per 100 ml (Ashurst and Hargitt 2009). Functional beverages contain an ever-increasing variety of unconventional ingredients. Product labelling is under the control of Nutrition and Health Claim Regulations (EC 1924/2006). European Food Safety Authority (EFSA 2011) maintains a list of approved health and nutrition claims for food and beverages.

Nutraceuticals and wellness drinks usually contain a mixture of bioactive compounds including "superfruits" (e.g. pomegranate, acai, acerola, noni, mangosteen), berries (e.g. cranberries), and botanical extracts (e.g. ginger, ginko, melissa) (Gruenwald 2009). Plant sterols and omega-3 fatty acids are used for heart health. Many products also include dietary fibres for a nutrition claim. Many dietary fibres, such as inulin and maltodextrin, are prebiotics that selectively modulate host microbiota, conferring a health benefit (Fallourd and Viscione 2009). Cereal-based high-fibre ingredients have also attracted interest. β-Glucans and cereal fibres in general have well recognised health effects. EFSA (2009) recently issued a scientific opinion confirming the relationship between consuming β-glucans and a healthy blood cholesterol level. On the basis of the data available, the Panel concluded that a cause and effect relationship has been established between consumption of β-glucans and the reduction of blood cholesterol concentrations. In order to bear the claim, foods should provide at least 3 g/d of β-glucans from oats, oat bran, barley, barley bran, or from mixtures of non-processed or minimally processed beta-glucans in one or more servings. The target population is adults with normal or mildly elevated blood cholesterol concentrations.

Energy drinks contain caffeine (360–630 mg/l), taurine (average 3 180 mg/l), caffeine-rich plant extracts (e.g. tea, ginseng, guarana, yerba mate) as typical energizing components, and B-vitamins (Heckman et al. 2010). The main constituents of **sport drinks** are carbohydrates in the form of glucose, fructose and maltodextrin (5.5–8.2%), salts and water (Mettler et al. 2006). Sodium and potassium concentrations are 20–30 and 5 mM, respectively (Maughan 2009). There is also a growing trend to incorporate other functional ingredients in sports drinks.

4.1.3 Soft drink manufacturing processes

Soft drink manufacture is essentially a simple process consisting of dissolving flavourings, juices, acids, antioxidants and sugars into water to form a beverage

(Stratford and James 2003). All non-sugar ingredients are usually first dissolved in water and then added to a sugar syrup. The final syrup is directed to proportioning pumps where it is mixed with water and filled into PET or glass bottles or aluminium cans. Many simple carbonated soft drinks, such as colas and lemonades, are only preserved with chemical additives (Lawlor et al. 2009). Sensitive preservative-free products including organic sodas, energy drinks and sparkling juices are usually tunnel pasteurised in glass bottles or aluminium cans (Lawlor et al. 2009). Normal PET bottles are not suitable for in pack pasteurisation. Still beverages are typically thermally and chemically preserved (Lawlor et al. 2009).

4.2 Ingredients and manufacture of alcoholic beverages

Some general characteristics of non-beer alcoholic beverages produced in a brewery environment are shown in Table 3.

Table 3. Examples of alcoholic beverages produced in a brewery environment.

Category	pH	Ethanol (abv)	Carbonation (vol)	Simple sugars (%)	Pasteurization	Permitted preservatives
Ciders:						
- Scandinavian	3.2–3.5	4.4–4.7	2.5–3.5	0.5–8.5	Flash	Sorbates, SO_2
- Traditional	3.3–4.3	4.5–6.0		2.5–4.4	None/flash	SO_2
BMBs	3.1–4.8	0.15–6	2.5	0.1–7.2	Flash	From non-beer portion
Long drinks:						
- Fermented	2.9–3.2	4.4–4.7	3	< 0.5–8	Flash	Sorbates, benzoates
- Blended	2.9–3.2	2.6–7.5	3	0.5–8.5	None/flash	Sorbates, benzoates

BMB; beer mixed beverage, abv; alcohol by volume.

4.2.1 Blended products involving a yeast fermentation

In Finland, ciders are defined as fruit wines that are produced from fresh or dried apples or pears or from juice or juice concentrates thereof and that do not contain more than 8.5% alcohol by volume (STM 1143/1994b). Scandinavian-type

blended ciders are produced in breweries by fermenting the fruit juice and sugar syrup with brewer´s yeast for a few days at ambient temperature to produce alcohol. The resulting wine is sweetened and diluted to 4.4–4.7% (abv) with fresh juice and water. The final sugar content varies from 1% to 12% (w/w) in caloric products. Sweetness in diet products is achieved with aspartame and acesulfame K. Natural aromas from berries, spices and fruits are preferred as flavourings. Chemical oxidation is normally minimised with ascorbic acid, whereas acidity control is achieved with citric acid. Natural vegetable and fruit extracts, or less commonly artificial E-coded colours such as antocyanins are used for colour modification. The desired texture is achieved using stabilizers such as arabic gum and maltodextrin. Scandinavian-type ciders are essentially carbonated soft drinks containing alcohol. Beverages in this category are usually flash – pasteurized before cold filling. They may be additionally chemically preserved. Up to 20 mg/l residual sulphites from the fruit juice ingredients is permitted in the final product without declaration (KTM 752/755).

4.2.2 Blended products not involving a yeast fermentation

Alcopops are prepared by mixing distilled spirits (such as vodka, rum or gin) or readily fermented beverages (wine, beer) with fruit juices or lemonades and flavourings. They are normally carbonated and chemically preserved. Examples of products in this category are Bacardi Breezer, Smirnoff Ice and Gin Long Drink (from distilled gin). Alcopops made from wine and non-alcoholic beverages are specifically called wine coolers.

Beer mixed beverages (BMBs) combine various types of beers with lemonade, cola drinks, energy drinks, spirits, flavourings and flavoured syrups. Hutzler et al. (2008) analyzed intrinsic properties of 20 commercial BMBs sold in Germany. They contained 0.15–6% (abv). Wide variation was also observed in acidity (pH 3.1-4.8), bitterness (3.10–20.50 BU), fermentable sugars (0.13–7.23%, w/v) and free amino nitrogen (1.5–17.9%, w/v). Carbonation level was around 0.5% (w/w) and protein content was 0.07–0.6% (w/v).

4.2.3 Traditional-type ciders

Traditional cider is an alcoholic beverage produced through controlled or natural fermentation of apple or pear juice or their mixture. Many producers of traditional ciders rely on **controlled fermentation**. Indigenous and adventitious mi-

crobes are largely inhibited by the addition sulphur dioxide (100–200 mg/l) into the juice (pH ~3.0), followed by inoculation with a cider yeast (*Saccharomyces cerevisiae* or *S. uvarum*). The yeast is propagated in the cider factories, or alternatively a dried or frozen starter is used. The fruit juice is fresh or reconstituted from a concentrate (about 72 °Brix) and may be enriched with fermentable sugars and yeast nutrients. Alcoholic fermentation is allowed to proceed at 15–25 °C until dryness, which takes from 10 days to 12 weeks. The raw cider is separated from the lees and transferred to sealed vats for maturation. It may be filtered or centrifuged at this stage. Significant changes occur in the aroma and flavour of the ciders during maturation. Some processes include malolactic fermentation, which is accomplished by starters or back-slopping. This secondary fermentation converts malic acid to lactic acid, reducing the acidity, and to other flavour-modifying metabolites. The alcohol concentration of pure juice ciders is up to 6.5% (abv), whereas sweetened ciders may reach 12% (abv). Many ciders are finally carbonated and blended to obtain the desired alcohol content, sweetness and flavour.

Natural cider fermentation is still practised in many countries, especially in England, France, Ireland, and Spain. Fermentation of the pressed juice from the specialty cider apples is carried out by the indigenous yeasts and acetic acid bacteria and heterofermentative lactic acid bacteria present in the fresh apple musts and in the cider factories. The apple juice may or may not be sulphite-treated to restrict the growth of undesired microbes. *Kloeckera*/*Hanseniaspora* type yeasts typically dominate during the initial phases, followed by more alcohol-tolerant *S. cerevisiae* in the fermentation phase (Morrissey et al. 2004, Duenas et al. 2006). *Brettanomyces*/*Dekkera* may become prevalent in the maturation step (Morrissey et al. 2004). Fermentation temperatures greatly affect microbial population dynamics. Malolactic fermentation is carried out by bacteria naturally present in vats. The ingress of air into the vats is limited in order to restrict the growth of aerobic microbes, which can be detrimental to the cider quality (see spoilage 4.3). *Lactobacillus brevis*, *Lb. mali, Lb. plantarum,* and *Leuconostoc mesenteroides* are common species during this step (Jarvis 2003, Hammes and Hertel 2006). Natural ciders may be maturated for more than one year (Morrissey et al. 2004). Natural ciders from Spain had a pH value of 3.3–4.5 (typically > 3.7) (Coton et al. 2006, Garai-Ibabe et al. 2008). The most abundant residual carbohydrates were fructose (0–5 g/l), glycerol (3–6 g/l), lactic acid (3–5 g/l) and acetic acid (0.5–3 g/l) (Coton et al. 2006). Natural products are not normally preserved before bottling.

4.3 Spoilage microbes in soft drinks

A range of microbes can be associated with soft drink manufacture, but only a few are able to cause spoilage. Microbiological spoilage leads to deterioration of the sensory quality and typically appears as off-flavours, odours and visual changes in the product (Table 4). Spoilage will require a certain critical cell number (10^5 –10^6 cells/ml) and therefore microbial growth (Stratford 2006). In addition to direct spoilage mediated by viable cells, carry-over of microbial metabolites from raw materials and the production process can lead to indirect spoilage. Microbial contamination of raw materials can cause off-flavours, overfoaming (gushing) and production failures and product spoilage even if no viable cells are left.

Spoilage microbes must tolerate an acidic environment that is low in oxygen and nutrients and usually rich in CO_2. As microbes differ in their growth requirements, different beverages support different spoilage microbes (Back 2005, Stratford 2006, Lawlor 2009, Tribst et al. 2009). So-called specific spoilage microbes can grow even in products produced under good manufacturing practices. In case of production failures, less specialised opportunistic species are often involved, as they are more common in the production environment. New ingredients or new applications of established ingredients could introduce new spoilage species and growth factors in beverages, thereby expanding the spoilage microbe range beyond the well-known species. Important properties of common soft drink spoilage organisms are presented in Appendix A.

Table 4. Examples of metabolites and quality changes associated with common spoilage microbes.

Spoilage microbe	Off-flavours / odours	Visual spoilage	Metabolites
Yeasts	Bad beer, vinegar, sweet pineapple note, sweet butter, yeasty, aldehyde off-flavour, petroleum-like odour	Swollen packages, tainting, haze, clouds, particulates, surface films	CO_2, ethanol, acetic acid, diacetyl, acetaldehyde, acetoin, esters, 1,3-pentadiene, exocellular polysaccharides
Lactic acid bacteria	Cheesy notes, sour, green apple	Loss of CO_2, ropiness, turbidity	Lactic acid, CO_2, ethanol, acetic acid, diacetyl, formic acid, exocellular polysaccharides
Acetic acid bacteria	Sour, vinegar	Haze, swollen packages, ropiness	CO_2, gluconic acid, acetic acid, ethyl acetate, acetoin
Alicyclobacillus spp.	Antiseptic and smoky taints	Difficult to detect	2,6-dibromophenol, guaiacol (from vanillic acid)
Moulds	Musty, stale	Mycelial mats, discoloration, swollen packages	Pectin degradation, formic acid, increase in pH due to metabolism of acids, gas production, gluconic acid

References: Back 2005, Bevilacqua et al. 2008, Lawlor et al. 2009, Stratford 2006, Tribst et al. 2009, Wareing and Davenport 2005.

4.3.1 Yeasts and soft drink spoilage

Yeasts are typical contaminants in soft drinks. They are common in the brewery environment and in the ingredients (Stratford 2006). Yeasts are considered as the primary spoilage microbes in carbonated products mainly due to their ability to withstand carbonation levels exceeding 3.0 vol. They also tolerate acidic conditions well. Most species grow in the pH range 1.5–8.5 (Sperber 2009) and have their growth optimum in the pH range 3.0–6.5 (Lawlor et al. 2009). Yeasts that form heat-resistant ascospores are also the principal spoilers in thermally processed carbonated soft drinks (Lawlor et al. 2009).

Yeasts can be classified into four groups based on their ability to spoil soft drinks (Table 5) (Davenport 1996). The most troublesome ones are fermentative and preservative-resistant species that can cause spoilage at almost any step of the manufacturing process. These extremophilic species are relatively rare in the manufacturing environment and typically present in very low numbers (Stratford and James 2003). The second group consists of yeasts that cause spoilage when something goes wrong in manufacturing. Most spoilage incidents are caused by these species, which are normally controlled by the preservative systems (Strat-

ford and James 2003). The yeasts in the third group serve as indicators of poor hygiene of the manufacturing plant, but they do not cause spoilage of the final product. The fourth group consists of species that are not normally associated with soft drink environments.

Table 5. Examples of yeast species found in soft drink factory environments.

Group 1 – Fermentative and preservative-resistant	**Group 2 – Spoilage and hygiene indicators**	**Group 3 – Hygiene indicators**	**Group 4 – Aliens**
Dekkera anomala	*Candida davenportii*	*Aureobasidium pullulans*	*Kluyveromyces lactis*
D. bruxellensis	*C. parapsilopsis*	*Candida sake*	*K. marxianus*
D. naardenensis	*Debaryomyces hansenii*	*C. solani*	
Saccharomyces cerevisiae (atypical)	*Hanseniaspora uvarum*	*C. tropicalis*	
S. exiguus	*Issatchenkia orientalis*	*Clavispora lusitaniae*	
Schizosaccharomyces pombe	*Lodderomyces elongisporus*	*Cryptococcus albidus*	
Zygosaccharomyces bailii	*Pichia anomala*	*Cryptococcus laurentii*	
Z. bisporus	*P. membranifaciens*	*Debaryomyces etchellsii*	
Z. lentus	*Saccharomyces bayanus*	*Rhodotorula glutinis*	
Z. rouxii	*S. cerevisiae*		

Reference: Davenport 2006.

Yeast spoilage of soft drinks is often observed as off-flavours and -odours caused by fermentation products as well as turbidity (Lawlor et al. 2009) (Table 4). The production of CO_2 may cause package swelling or even explosion. Gas formation by fermentative spoilage yeasts, measured after 2 weeks of growth in a soft drink containing 1 M glucose, generated 2 to 7 bars gas pressure (Stratford 2006). By degrading weak acid preservatives, yeasts may also help other microbes to grow in soft drinks. Yeasts produce ethanol as an end-product of fermentative metabolism and the ethanol level in spoiled soft drink may exceed the legal limit for non-alcoholic products.

Saccharomyces cerevisiae is the most frequent spoiler of lemonades and fruit juices (Back 2005). It is highly fermentative and produces large quantities of CO_2 (Stratford and James 2003). Some strains also tolerate benzoates, sorbates and sulphates (Mollapour and Piper 2008). Many *S. cerevisiae* strains possess pectinolytic activity, which can lead to clarification of hazy products (Back 2005). The soft drink spoilage strains have usually better acid-tolerance than

brewer´s yeast strains. However, brewer´s yeasts which are ubiquitous contaminants in a brewery environment may also cause spoilage (Back 2005).

Candida davenportii is a relatively new spoilage species. It grows well in fruit-containing soft drinks, cola beverages and synthetic soft drinks (Stratford and James 2003). *C. davenportii* causes spoilage relatively rarely and is regarded as a group 2 spoiler.

Zygosaccharomyces bailii is notorious for its extreme resistance to weak organic acids including common preservatives, good osmotolerance and vigorous fermentation of sugars, particularly fructose (Steels et al. 2002, Martorell et al. 2007). It is a good example of the group 1 spoilage yeasts. *Z. bailii* is common in fruit concentrates and syrups (Back 2005). This species is also able to catalyse oxidative degradation of sorbates and benzoates, which may lead to better growth rates of other spoiling organisms (Mollapour and Piper 2008). Only a few cells in a package may lead to quality defects (Van Esch 1987). *Z. lentus* closely resembles *Z. bailii* in its physiological properties. However, it is also able to grow in refrigerator temperatures and grows poorly under aerobic conditions (Steels et al. 1999).

Ascospore-forming *Dekkera* yeasts (teleomorph of *Brettanomyces*) are among the most common soft drink spoilage yeasts (Stratford and James 2003). *Dekkera* species are slow-growing, and the development of spoilage symptoms may take several weeks. They have an extreme carbonation tolerance, but are only moderately resistant to sorbates and benzoates. They usually produce dense clouds and sediments and may oxidize sugars to acetic acid. They are only weakly fermentative in low-oxygen conditions. *D. anomala* appears to be particularly common in soft drinks. It is less fastidious in vitamin requirements than *D. bruxellensis* or *D. naardenensis.*

4.3.2 Bacteria and soft drink spoilage

Lactic and acetic acid bacteria are the most common spoilage bacteria found in soft drinks. Their ability to tolerate environments with low pH is essential for growth in soft drinks.

Lactic acid bacteria (LAB) are microaerophilic, Gram-positive bacilli or cocci. They can grow in properly sealed bottles and cans low in oxygen, causing spoilage of beverages. LAB typically enter breweries from raw materials, juice ingredients and packaging materials (Lawlor et al. 2009). The most frequent spoilage species are *Lactobacillus paracasei* and *Leuconostoc mesenteroides*

(Back 2005). In addition, *Lactobacillus brevis, Lactobacillus buchneri, Lactobacillus plantarum, Lactobacillus perolens* and *Weissella confusa* are commonly found in contaminated products. Many of these species are also potential or obligate beer spoilers (Back 2005). LAB ferment sugars predominantly to lactate. Depending on the species and growth conditions, sugar catabolism can also lead to formation of ethanol, acetate, formate or succinate (Hammes and Hertel, 2009). Some strains produce diacetyl, which tastes and smells buttery, and is an unwanted metabolite in soft drinks. Formic acid formation has been detected in apple juice and proposed as a spoilage indicator (Gökmen and Acar, 2004). LAB can also cause a loss of carbonation and astringency (Lawlor et al. 2009). Furthermore, *L. mesenteroides* and *W. confusa* strains can produce extracellular fructose or glucose polymers from sucrose, which causes ropiness of the final product (Back 2005).

The most common spoiling **acetic acid bacteria** (AAB) belong to the genera *Acetobacter* and *Gluconobacter*. In addition, *Gluconacetobacter* and *Asaia* spp. have been associated with soft drinks. The genus *Asaia* was described in 2000 and currently comprises eight species (Yamada and Yukphan 2008, Suzuki et al. 2010). AAB are aerobic Gram-negative short or coccoid motile or non-motile rods. They are widespread in nature particularly in sugar- and ethanol-enriched habitats (Back 2005, Suzuki et al. 2010). Their high number in process environments is considered to indicate poor hygiene (Back 2005, Raspor and Goranovic 2008). Many species share the ability to form biofilm on the production surfaces (Back 2005, Horsáková et al. 2009). AAB acquire energy from the oxidation of sugars, organic acids, sugar alcohols and alcohols with the production of acetic, gluconic, lactic and succinic acids, acetaldehyde and ketone compounds. The end products depend on the species and growth conditions. AAB do not have amino acid requirements and ammonia can serve as sole source of nitrogen. B vitamins may be needed in certain conditions. AAB are acid-tolerant bacteria. Most species grow at pH 3.6–3.8, and some even at pH 3.0 (Raspor and Goranovic 2008, Lawlor et al. 2009, Suzuki et al. 2010). The optimum temperature for growth lies at 25–30 °C (Back 2005). The growth in soft drinks may cause flavour changes, package swelling, ropiness, haze and sediments (Raspor and Goranovic 2008, Horsáková et al. 2009). Ropiness is characterized by an increase in the viscosity of the beverage. *Gluconobacter* spp. are the most frequent spoilers in soft drinks. AAB are not as common in soft drinks as LAB, since they are strictly aerobic and demand at least some oxygen for growth (Lawlor et al. 2009). They are mainly a problem in beverages packed in oxygen-permeable

containers, e.g. in certain types of PET bottles. Many AAB tolerate commonly used preservatives (benzoates, sorbates, dimethyldicarbonate) rather well (Raspor and Goranovic 2008). *Asaia* spp. are emerging spoilers of still fruit drinks, ice teas and fruit-flavoured bottled waters (Moore et al. 2002a, Horsáková et al. 2009).

Propionibacterium *cyclohexanicum* was isolated from a spoiled pasteurized orange juice with off-flavour, but it is also capable of growing in other juices even at refrigerator temperatures (Kusano et al. 1997). It is a Gram-positive pleomorphic rod that produces propionic acid as the main product of sugar fermentation. Acetic and lactic acids are also formed. Amino acids stimulate growth but are not necessary. All strains require the vitamins pantothenate and biotin (Kusano et al. 1997). Growth occurs at 20–40 °C. High concentrations of potassium sorbate (500 mg/l) and natrium benzoate (1 000 mg/l) inhibited their growth in orange juice (Walker and Phillips 2008). The minimum pH for growth in juice was around 3.6 (Walker and Phillips 2007). The organism was able to survive heat treatment at 95 °C for 10 min and thus is not killed in regular juice pasteurization procedures (Walker and Phillips 2007).

Enterobacteria (e.g. *Klebsiella*, *Citrobacter*, *Serratia*) are a heterogenic group of facultatively anaerobic Gram-negative bacteria that carry out mixed acid fermentation resulting in unclean aroma and flavour as well as gas formation. They are not highly acid-tolerant but have been reported to multiply in citrus juices with pH values below 4.3 (Lawlor et al. 2009). Exocellular polymers and sulphuric compounds may also be produced.

Spore-forming bacteria of the genera *Bacillus* and *Clostridium* are usually inhibited in soft drinks due to low pH. However, spores may remain viable in these products. *Bacillus* and *Clostridium* species are typical spoilage organisms in vegetable juices that are less acidic (pH > 4) than fruit juices (Back 2005, Tribst et al. 2009). With the development of mixed beverages containing cereal fibres and vegetable or fruit juices their importance as beverage spoilers is expected to increase (Tribst et al. 2009). Anaerobic butyrate-forming clostridia such as *Clostridium butyricum* and *Clostridium sporogenes* can spoil sugar syrups used in the beverage industry during syrup manufacture or storage, causing a rancid off-flavour in the final products. These bacteria were active even at pH values of 3.6–3.8 (Hawthorne et al. 1991, Stenius et al. 1991). Elimination of spore-forming bacteria is difficult due to their inherent resistance to many physical and chemical factors.

The genus *Alicyclobacillus* is mainly associated with spoiling of fruit juices. Spoilage incidents have also been reported in carbonated fruit juice drinks, lemonade, isotonic water and ice-tea (Yamazaki et al. 1996, Smit et al. 2011). *A. acidoterrestris* is the primary spoilage species but *A. acidiphilus, A. acidocaldarius, A. cycloheptanicus A. hesperidum, A. herbarius* and *A. pomorum* have also been implicated. The classification of endospore-forming bacteria has recently undergone major changes and it is possible that some alicyclobacilli were misidentified in the past. Alicyclobacilli are thermo-acidophilic Gram-positive endospore-forming rods. The endospores withstand normal juice pasteurization procedures and can germinate and grow even at pH 2–3 (Smit et al. 2011). The temperature growth range for the juice-associated species is 20–70 °C. Alicyclobacilli are aerobic organisms capable of growing even with a limited oxygen supply (Smit et al. 2011). Spoilage is manifested by medicinal or antiseptic off-odour deriving from guaiacol and halophenols produced during ferulic acid metabolism (Smit et al. 2011). Sediment, haze and discoloration may also be formed, although they are usually hard to detect (Bevilacqua et al. 2008). Alicyclobacilli contamination does not always lead to quality deterioration. *A. acidocaldarius* uses hexoses (glucose, fructose), pentoses (ribose, xylose, rhamnose) and sugar alcohols as growth substrates (Bevilacqua et al. 2008).

Streptomyces *griseus* has been found to form musty, mouldy or earthy off-flavour in pasteurized apple juices. It is Gram-positive actinomycete forming branched filaments and spores that can be resistant to high temperatures. Growth in ambient products is possible with limited oxygen supply even at 4 °C. Several metabolites such as geosmin 2-methylisoborneol and 2-isopropyl-3-methoxypyrazine are responsible for the off-odours (Siegmund and Pöllinger-Ziegler 2007, Tribst et al. 2009).

4.3.3 Filamentous fungi (moulds) and soft drink spoilage

Raw materials, semi-manufactured and final products can be contaminated with fungal spores or conidia and mycelium fragments from the environment (Filtenborg et al. 2004). Soil is considered as the main source of heat-resistant moulds related to fruit juice contamination (Tribst et al. 2009). Moulds can also enter the soft drink manufacturing factory due to poor process hygiene or contaminated packages.

Like yeasts, several moulds tolerate low pH, and high acidity is considered to be the single most important factor in fungal spoilage of fruit, berry and acid

products. In contrast to many bacteria and yeasts, oxygen is usually necessary for the growth of moulds. However, some species can also grow under anaerobic conditions with fermentative metabolism (Filtenborg et al. 2004). In addition, several species (*Fusarium* and *Rhizopus* spp.) can grow at low oxygen concentration (0.01% v/v) (Scholte et al. 2004). If oxygen cannot be decreased to a sufficiently low level, increasing the headspace concentration of CO_2 is often effective in inhibiting mould growth and mycotoxin production (Scholte et al. 2004). In general, the germination of fungal conidia is more susceptible to carbon dioxide inhibition than mycelial growth.

The growth of fungi in raw materials, ingredients and in a final product may result in several kinds of spoilage. Moulds can produce a vast number of enzymes such as lipases, proteases, and carbohydrases, and uncontrolled fungal activity may lead to production of off-odours and -flavours. Production of volatiles such as dimethylsulphide and geosmin (an earthy musty compound) may be indicators of mould activity (Filtenborg et al. 2004). In addition, fungal contamination may lead to discolouration of the products, formation of allergens and production of toxigenic compounds.

In the beverage industry heat-resistant moulds, such as species belonging to the genera of *Byssochlamys, Neosartorya* and *Talaromyces*, are the primary spoilage agents of heat-processed fruit-based products, including canned fruits and fruit juices, fruit purees (used as ingredients), flavoured mineral waters, jellied fruit and baby fruit gels (Hocking and Pitt 2001). Fungi that cause damages amounting to millions of dollars in the fruit-juice branch include *Byssochlamys nivea* (or *fulva*), *Talaromyces flavus* (or *macrosporus*), *Neosartorya fischeri* and *Eupenicillium brefeldianum* (Scholte et al 2004). They are able to grow fermentatively at low oxygen levels. They may produce enzymes such as pectinases resulting in the degradation of fruit structure, and changes in taste and flavour, visible spoilage, and less frequently gas formation (Scholte et al. 2004). Furthermore, they are capable of producing mycotoxins. Other common spoiling moulds in the soft drink and juice industry belong to the genera *Penicillium* and *Cladosporum* (Wareing and Davenport 2005).

Heavy fungal infection of raw material may also lead to production of gushing inducers. Gushing is a term used to describe spontaneous overfoaming of packaged beer immediately on opening. Gushing is a very complex phenomenon, and it can at least partially be explained by the secretion of specific gushing factors by fungi which are present in malt or in other cereal-based raw materials applied in brewing (Amaha and Kitabatake 1981, Munar and Sebree 1997, Sarlin et al.

2005). It has been demonstrated that fungal hydrophobins isolated from strains of the genera *Fusarium*, *Nigrospora* and *Trichoderma* cause gushing of beer (Sarlin et al. 2005 and 2007). Hydrophobins are surface active fungal proteins which are assumed to stabilize carbon dioxide bubbles in beer by forming a layer around the microbubbles (Draeger 1996, Pellaud 2002). This layer may prevent breakdown of the bubbles, leading to overfoaming. Alternatively, hydrophobins may form aggregates in beer which provoke overfoaming. Very small amounts of hydrophobins, as low as 0.003 ppm, have been reported to induce beer gushing (Sarlin et al. 2005). In addition to beer gushing, fungal gushing inducers may be present in apple- and grape-based raw material applied in cider and sparkling wine production. Some wine and cider producers have indicated that extensive overfoaming of ciders and sparkling wines has been due to heavy fungal contamination of raw materials (Laitila 2010, personal discussions). Thus, gushing inducers originating from *Penicillium, Fusarium* or other moulds may be the causative agents of intensive gushing of ciders and sparkling wines. However, this is still only a hypothesis and studies are needed to link gushing of grape and apple-based products with hydrophobins.

4.4 Spoilage microbes in alcoholic beverages

The production of alcoholic beverages is not an aseptic process. However, only relatively few types are able to spoil the final products which are typically very acidic (pH 2.4–3.5), strongly carbonated (2.5–3 vol), contain up to 8.5% (abv), and have a low level of oxygen and a high carbon to nitrogen ratio. Therefore, the major spoilage microbes in alcoholic beverages are fermentative yeasts and acid-tolerant LAB as in the case of carbonated soft drinks. This review focuses on the spoilage microbes of non-beer alcoholic beverages. Microbiological spoilage problems in beer production have been recently reviewed elsewhere (e.g. Suzuki et al. 2008a). Microbiological risks of Scandinavian-type ciders have been evaluated based on their properties and production processes due to lack of published studies.

4.4.1 Yeasts and spoilage of alcoholic beverages

Fermentative yeasts are the most probable spoilers in alcoholic beverages produced in a brewery environment, since they are able to ferment sugars in acidic and anaerobic conditions at alcohol levels present in these products. Moreover,

many spoilage strains are preservative-resistant. In traditional ciders, *Saccharomycodes ludwigii* is the most dangerous spoiler due to its sulphite tolerance (1 000–1 500 mg/l SO_2). It can grow at all stages of the manufacture, but the spoilage usually occurs in bottled products which normally become contaminated during filling operations (Jarvis 2003). *Sc. ludwigii* forms "snowflake" particles and high levels of acetaldehyde and acetoin with pungent apple and sweet butter off-flavour, respectively. Fermentation of residual sugars by sulphite-tolerant *Saccharomyces* and *Zygosaccharomyces* strains (*Z. bailii, Z. lentus*) can lead to explosion of bottles due to the high pressure formed (up to 9 bars) (Jarvis 2003). Excess growth of *Kloeckera apiculata* during the initial stages of fermentation can lead to excess levels of esters and volatile acids (Stratford and James 2003). During maturation, film-forming *Brettanomyces/Dekkera* and *Pichia* can cause "mousy" off-flavour deriving from tetrahydropyridines (Jarvis 2003). Ethanol and L-lysine are precursors of the synthesis of tetrahydropyridines (Malfeito-Ferreira et al. 2009). Several spoilage species such as *Z. bailii, D. bruxellensis, Sz. pombe* and *S. cerevisiae* can also produce volatile phenols from phenolic acids present in juice ingredients. Volatile phenols are harmful to flavour in high concentrations (Harris et al. 2008, Malfeito-Ferreira et al. 2009). Metabolism of phenolic acids appears to be a part of their detoxification and may protect cells from their inhibitory effects.

Sensitivity of BMBs to yeast spoilage varies depending on the formulation. In general *S. cerevisiae* wild yeasts and the brewer´s yeast strains (especially top-fermenting) have the highest spoilage potential (Hutzler et al. 2008). Hutzler et al. (2008) analyzed 20 commercial BMBs sold in Germany, and *S. cerevisiae* wild yeasts could grow in all of them, leading to sensory changes and extreme internal bottle pressures. The brewer´s yeast strains caused haze in all products, and other sensory changes and pressure increased in most products. In addition, some *D. bruxellensis* strains showed similar spoilage potential to that of *S. cerevisiae* wild yeasts. *Sz. pombe* caused haze formation and organoleptic changes in half of the products. Interestingly, *Z. bailii* was not able to cause major quality defects in any of the products.

4.4.2 Bacteria and spoilage of alcoholic beverages

Spoilage LAB in alcoholic beverages mainly belong to the genera *Lactobacillus* and *Pediococcus*.

Acidophilic alcohol-resistant ***Lactobacillus*** **spp.** can form off-flavours and decrease the alcohol yield at all stages of traditional cider manufacture (Hammes and Hertel 2006). Lactobacilli are also potential spoilers of Scandinavian-type ciders. Heterofermentative species can produce excessive amounts of acetic acid, destroying the product flavour (Jarvis 2003). They can also cause bitterness by converting glycerol to 3-hydroxypropionaldehyde, which can chemically transform to acrolein and bind with polyphenols creating bitter compounds. (Sauvageot et al. 2000, Garai-Ibabe et al. 2008). *Lb. collinoides* is the most common species in bitter ciders, but *Lb. diolivorans* and *Lb. reuteri* are also able to cause this defect. Some lactobacilli form diacetyl from citric acid in the fruit juice. Diacetyl gives a buttery flavour with a taste threshold of 0.6 mg l^{-1} in cider (Jarvis 2003). Heterofermenters may also produce tetrahydropyridines with a mousy off-flavour. Ropiness is a common defect in natural ciders (Ibarburu et al. 2010). It is caused by exopolysaccharide-producing strains. The polysaccharides are typically β-glucans, which are preferentially produced from glucose. *Lb. collinoides*, *Lb. diolivorans* and *Lb. suebicus* are the most common rope-forming species (Ibarburu et al. 2010). Many lactobacilli are also able to form volatile phenols with phenolic and medicinal off-flavours (Barthelmebs et al. 2000a, Hammond et al. 1999, Couto et al. 2006).

Pediococci form uniform spherical cells that typically occur in tetrads. They grow under facultatively aerobic to microaerophilic conditions, preferably under CO_2 atmosphere. Metabolism is facultatively homofermentative, i.e. lactic acid is mainly produced from glucose. Pediococci require complex growth factors, including several amino acids and vitamins. Manganese is required by all strains and most strains also need calcium (Holzapfel et al. 2006). Spoilage symptoms in alcoholic beverages include haze and buttery and sour off-flavours from diacetyl and lactic acid formation. Pediococci may produce volatile phenols (Barthelmebs et al. 2000b, Couto et al. 2006) and ropiness. *P. parvulus* is a species found in ropy French ciders (Ibarburu et al. 2010). Ropy pediococci are also found in the brewery environment, and are typically strains of *P. damnosus* and *P. claussenii* (Dobson et al. 2002). Two new ethanol-tolerant pediococci, *P. cellicola* and *P. ethanolidurans*, were described in a distilled spirit production environment (Zhang et al. 2005, Zhang and Dong 2006). They are potential spoilers of alcoholic beverages as they tolerate 6.5% or more ethanol at pH 3.5.

AAB of the genera *Acetobacter* and *Gluconobacter* can cause quality defects at any stage of cider production where oxygen is available. They are indigenous in apple musts and can survive long periods in microaerophilic conditions

(Bartowsky and Henschke 2008). Common species in traditional cider manufacture and in the brewery environment include *A. pasteurianus* and *G. oxydans* (Jarvis 2003, Back 2005). Most AAB oxidize ethanol to acetic acid. *Acetobacter* and *Gluconacetobacter* are able to catabolise acetic acid further to CO_2 and water (Bartwosky and Henschke 2008). *Acetobacter* are more alcohol-tolerant than *Gluconobacter* spp., which prefer sugar-enriched environments. AAB may also oxidise glycerol to CO_2 and water. They tolerate the levels of SO_2 used in normal cider manufacture (Jarvis 2003). During the maturation step, excess growth of AAB can result in a vinegary note from acetic acid and other volatiles. AAB are a problem in packaged ciders only when the seal integrity is compromised or the package is oxygen permeable (Bartwosky and Henschke 2008). The spoiled product has typically a vinegary flavour with a lowered pH and ethanol content (Raspor and Goranovic 2008). AAB are also involved in so-called cider sickness (see below).

Zymomonas mobilis is one of the major spoilage microbes in natural ciders, causing so called cider sickness ("framboisé") (Coton et al. 2006). It is a Gram-negative aerotolerant anaerobe that ferments fructose and glucose almost quantitatively to ethanol and CO_2. Traces of acetaldehyde and H_2S and lactic acid are also produced. Sweet ciders with pH above 3.7 are particularly susceptible to spoilage. Cider sickness is recognized by frothing and abundant gas formation, rotten lemon skin or grassy notes in the aroma and flavour, reduction of sweetness, and development of a marked turbidity with a heavy deposit. *Z. mobilis* currently comprises three subspecies that have all been found in ciders. *Z. mobilis* cannot grow in standard lager beers, but priming with glucose makes beer more susceptible to spoilage. Hence, this organism has potential to spoil BMBs that usually contain glucose or fructose. *Z. mobilis* is a beneficial organism in natural plant fermentations and has also been intensively studied for fuel ethanol production.

Spore-forming bacteria are not usually of concern in alcoholic beverages due to high acidity and carbonation level.

4.5 Microbiological health risks associated with beverages

Soft drinks are traditionally perceived as safe beverages with no significant associated food-borne illnesses. However, health risks cannot entirely be ruled out. There have been 32 documented outbreaks of food-borne illness due to con-

sumption of beverages, especially unpasteurized fruit juices (apple cider is a term used in the USA for unfiltered fresh apple juice), since 1922 (Parish 2009). Many of the outbreaks were considered or confirmed to have resulted from poor manufacturing practices and hygiene in the plant area or at retail points (Vojdani et al. 2008). Since the implementation of HACCP regulations for juice production in USA, juice-associated outbreaks appear to have decreased (Vojdani et al. 2008). Based on recent expert elicitation, the contribution of non-dairy beverages to US food-borne illness incidences was estimated to be 3.5% (Hoffman et al. 2007). The value includes all non-dairy beverages, not only soft drinks.

Beverage-related outbreaks were caused by various enteric pathogens including bacteria, viruses and protozoans. However, in many cases the causative agent remained unknown (Parish 2009). Other health risks associated with beverages are fungal mycotoxins (McCallum et al. 2002). Examples of possible microbial hazards related to beverage production are shown in Table 6. EFSA is the EU risk assessment body for food safety that provides independent scientific advice to risk managers

Table 6. Examples of health hazards associated with microorganisms in soft drinks.

Microbes	**Hazards**	**Influence on human**	**Species**	**Disease vehicle**	**Special notes**
Moulds	Mycotoxins	Severe chronic and acute toxicity	*Penicillium, Aspergillus, Byssochlamys*	Fruit juices, cereals	Mycotoxins can end up in product even if no viable cells are left.
Bacteria	Food-borne infections and intoxications, allergic reactions	Variable	*Salmonella, Escherichia coli* O157:H7, *Listeria monocytogenes*	Fruit juices and concentrates, water	
Viruses	Human infections	Liver inflammation, gastroenteritis	Hepatitis A, norovirus, rotavirus	Fruit juices, water	
Protozoa	Human infections	Gastrointestinal illness	*Cryptosporidium parvum, Cr. hominis, Cyclospora cayatenensis*	Water, fruit juices and concentrates	
Yeasts	Fermentation products	Emetic responses	Unknown	Fruit juices	Scientific proof missing.

References: Akond et al. 2008, Enache and Chen 2007, García and Heredia 2009, McCallum et al. 2002, Parish 2006, Sheth et al. 1988, Tribst et al. 2009, Wareing and Davenport 2005.

4.5.1 Pathogenic bacteria

Some pathogenic bacteria can survive in acidic and carbonated soft drinks, although they are not able to grow during storage. *Escherichia coli* and *Salmonella* survived 48 h in inoculated cola-type soft drink (Sheth et al. 1988). *E. coli, Salmonella enterica, Listeria monocytogenes* and *Staphylococcus aureus* were not killed in Coca Cola during 5 min incubation despite the high acidity of the drink (pH 2.7) (Medina et al. 2007). *Yersinia enterocolitica* was found to survive in an inoculated commercial orange soft drink (pH 3.5) for 3 days at 30 °C. A recent study of microbiological quality of carbonated soft drinks sold in Bangladesh showed indirectly that 1) the number of pathogenic bacteria in soft drinks can be high as a result of poor hygiene and 2) bacterial pathogens can remain viable in carbonated soft drinks for extended periods (Akond et al. 2009). Of the 225 samples, 95% were contaminated with *Pseudomonas aeruginosa* and 54% with *Salmonella*, presumably originating from contaminated raw materials or from water, and surviving because of poor manufacturing processes. In the developed countries, there are no reports of the occurrence of bacterial pathogens in commercial soft drinks.

Pathogenic bacteria most commonly encountered in fruit juice outbreaks were enterohemorrhagic or Shiga-toxin-producing *E. coli*, especially the serotype 0157:H7, and *Salmonella* (several serotypes) (Parish 2009). Moderately acidic apple and orange juices were the most common disease vehicles (Parish 2009, Tribst et al. 2009). The outbreak investigations and laboratory studies have shown that these bacteria are able to survive in acidic juices long enough to transmit diseases (Oyrzabal et al., 2003; Vojdani et al., 2008; Parish, 2009). Enteric pathogens do not belong to indigenous microbes of fruits, and the contamination comes from direct or indirect contact with faeces (Tribst et al. 2009). *Listeria monocytogenes* has not yet been implicated in juice-related outbreaks, but it has been shown to be capable of long-term survival in various frozen juice concentrates (Oyarzabal et al. 2003). A recent study also demonstrated a prolonged survival of *Y. enterocolitica* in freshly pressed orange juice (pH 6.3) (Estrada et al. 2010).

Many exotic juices used in modern beverage formulations (e.g. acai, melon, persimmon, papaya) have low acidity (pH 4.8–6.2). These juices provide suitable conditions not only for survival, but also for the growth of pathogenic bacteria (Tribst et al. 2009). Sweet wort also allows the growth of several pathogenic bacteria (Mentz et al. 2010). Modern soft drinks without preservatives, with low

carbonation and high fruit juice volumes resemble more and more natural fruit juices with known association with food-borne illness. Hence, the modern soft drinks and new exotic ingredients could pose an increased risk to public health caused by pathogenic microbes. *E. coli* 0157:H7 is the most dangerous bacterial pathogen associated with beverages due to its low infective dose and the severity of the illness (Garcia and Heredia 2009).

There are no records of illness outbreaks caused by pathogenic microbes in alcoholic beverages. The survival and growth of pathogenic bacteria in alcoholic beverages has only been studied in beer. *E. coli* H157:07, *Salmonella typhimurium* and *L. monocytogenes* have been shown to rapidly inactivate during fermentation of typical beer wort (20 EBU, pH 5.5, original gravity 1.040) (Mentz et al. 2010). However, pathogen survival is enhanced with increasing pH and decreasing ethanol concentration and original gravity (Menz et al. 2010).

4.5.2 Parasites and viruses

Parasites and viruses have also been associated with disease outbreaks from consumption of fruit juices. Protozoa do not replicate outside their hosts, but they can survive for long periods in the environment in a resting stage, i.e. in oocysts, which are secreted into the faeces of the infected hosts (Dawson 2005). Contamination of food and beverages occurs via the faecal-oral route. Even a few oocysts can lead to gastroenteritis (Erickson and Ortega 2006). Water has been identified as the most important vehicle.

The protozoan *Cryptosporidium parvum* was isolated from apple cider and juice outbreaks in the 1990s and 2000s (Parish 2009). The outbreaks were mainly associated with under- or unprocessed products. *C. parvum* oocysts have been shown to lose > 85% of viability during 24 h incubation in beer (pH 3.81–3.85) and a cola drink (pH 2.46) at 4 °C and 22 °C (Friedman et al. 1997). Inactivation was higher at room temperature. Low pH and carbonation were considered to be major factors in the inactivation of the oocysts held in the cola drink, whereas low pH and alcohol were the factors ascribed to decreased viability in beers (Friedman et al. 1997). The loss of viability in orange juice (pH 3.87) was < 35%. The survival of the oocysts in natural mineral waters was variable. Waters with high mineral contents had higher inactivation rates at 20 °C compared to waters with low mineral content (Erickson and Ortega 2006). *Cyclospora cayatensis* is another protozoan transmissible through fresh produce, especially

through raspberries. The risk of food-related protozoa infections is presumed to be low in the developed countries (Newell et al. 2010).

Viruses do not grow in foods as they need living cells for replication. However, only a few virus particles may result in a high probability of infection (Newell et al. 2010). Hepatitis A, norovirus and rotavirus could potentially transmit disease via improperly produced beverages. Hepatitis A virus was transmitted via orange juice in the 1960s (Parish 2009). Norovirus has been associated with outbreaks from raspberries irrigated with contaminated water (Newell et al. 2010). There are some studies concerning the survival of pathogenic viruses in beverages. Leong et al. (2007) showed that SA11 rotavirus was able to survive for 3 h at 28 °C in fresh papaya (pH 5.1) and honeydew melon (pH 6.3) juices, but not in pineapple juice (pH 3.6). In cold-stored fruit juice (pH 3.01) rotavirus survived 3 d (Mahony et al. 2000). Thus, contaminated juices could potentially also transmit rotavirus. Recent expert advice on food-borne viruses for Codex Alimentarius concluded that prevention and control measures should be considered for noroviruses and hepatitis A in fresh produce, and for rotavirus in water for food use (WHO 2011).

4.5.3 Mycotoxins

Growth of filamentous fungi is normally not expected in beverage production. However, in the field or during storage many filamentous fungi are capable of producing toxic secondary metabolites in response to stressful conditions. Mycotoxins are fungal metabolites that cause sickness or death in people and other animals when ingested, inhaled and/or absorbed (Paterson and Lima 2010). Mycotoxins include a very large, heterogeneous group of substances, and toxigenic species can be found in all major taxonomic groups (Drusch and Ragab 2003). Thousands of mycotoxins exist, but only a few present significant food safety challenges (Murphy et al. 2006). The relevant mycotoxins related to foods and beverages are produced by species in the genera *Aspergillus, Penicillium, Fusarium* and *Alternaria,* and include aflatoxins, ochratoxin A, patulin and *Fusarium* toxins such as trichothecenes and zearalenone. When present in high levels, mycotoxins can have toxic effects ranging from acute (for example kidney or liver damages) to chronic symptoms (increased cancer risk and suppressed immune system). Production of a particular mycotoxin is a species- or strain-specific property, and usually a toxigenic fungus can produce several tox-

ins. Therefore, several different toxins are often present in the contaminated raw materials, and they have poorly understood synergistic effects.

In addition to human health hazards, mycotoxins may have an impact on plant tissues and may cause loss of viability and reduced quality of plant seed. Mycotoxins also have adverse effects on animal health if they are transmitted to side-streams used as animal feed. Some of the most common mycotoxins associated with foods and beverages are presented in Table 7.

Table 7. Some mycotoxins most commonly associated with particular fungi.

Mycotoxin	**Major producer fungi**	**Common food and Beverage source**
Aflatoxins B_1 (AFB_1), AFB_2 AFB_1, AFB_2 AFG_1, AFG_2	*A. flavus* *A. paracitus*	Cereals, nuts, seeds, dried fruits, spices
Ochratoxin A (OTA)	*A. carbonarius , P. verrucosum*	Dried fruits, cereals, grape juice, wine, coffee
Patulin[1)]	*Byssochlamys fulva, B. nivea P. expansum, A. terreus, A. clavatus*	Apricots, grapes, peaches, pears, apples, berries, olives, cereals and low acid fruit juices
Trichothecenes [2)] (such as DON, DAS, T-2)	*F. acuminatum, F. cerealis, F. culmorum, F. graminearum, F. langsethiae, F. sporotrichoides,*	Cereals and cereals products
Zearalenone (ZEA)	*F. crookwellense, F. culmorum, F. equiseti, F. graminearum, F. semitectum*	Cereals and cereal products , other food commodities
Fumonisin B_1 (FB_1)	*F. verticillioides, F. proliferatum, F. nygamai*	Corn, corn meal and grits

[1)] 60 species belonging to over 30 genera are capable of producing patulin.

[2)] Over 170 compounds are included in the trichothecenes. In addition to *Fusarium* fungi, species belonging to the genera *Myrothecium*, *Phomopsis*, *Stachybotrys*, *Trichoderma*, *Trichothecium* can also produce trichothecenes.

References: Bennet and Klich 2003, Frisvad and Thrane 2004, Drusch and Ragab 2003, Murphy et al. 2006, Paterson and Lima 2010.

Mycotoxins are stable compounds and can therefore survive throughout the process and enter the final product. Raw materials (such as fruit juices and cereal fractions and extracts) used in beverage production can potentially be contaminated with mycotoxins. Mycotoxins are also known to cause process failures in beverage production. They are known to disturb yeast metabolism during fermentation (Boeira et al. 1999a, 1999b, 2000, Whitehead and Flannigan

1989), and the presence of mycotoxins may be one of the reasons for unex-unexplained unfinished fermentations (Kelsall and Lyons 2003, see Table 8). The degree of growth inhibition has been shown to depend on the toxin concentration, the type of yeast strain and the length of fermentation (Boeira et al. 1999 a, b). AFB_1 and trichothecenes are known to inhibit the alcohol hydrogenase activity resulting decreased fermentation activity and lower CO_2 liberation (Klosowski et al. 2010). Boeira et al. (1999a, b) reported that DON, ZEA and FB_1 inhibited the growth of brewer's yeasts at concentrations of 50–200, 50–75 and 10–100 μg/ml, respectively. Mycotoxin contamination of raw materials has also led to significant reduction of ethanol yield (Klosowski et al. 2010).

Table 8. Effects of mycotoxins on yeast growth (Kelsall and Lyons 2003).

Mycotoxin	Level required to inhibit yeast growth (ppm)
Zearalenone	50
Deoxynivalenol	100
Fumonisin	10

The European Commission has set a regulation (EC 1881/2006) for maximum levels of mycotoxins in foods and beverages (Appendix B, Tables 1–2). Intake estimates have indicated that the presence of T-2 and HT-2 toxins can be of concern to public health, particularly in oats and oat products. However, the maximum levels for T-2 and HT-2 are still under consideration. The proposals for the sum of T-2 and HT-2 are currently 500 μg/kg for oats and oat products, 200 μg/kg for other cereals, 100 μg/kg for bread, and 50 μg/kg baby foods.

Patulin has attracted considerable attention during recent years, and is perhaps the most important mycotoxin in fruit and berry juices (Delage et al. 2003). Patulin is a mycotoxin produced by *Penicillium* spp. and is associated especially with apple and pear juices and apple ciders. *P. expansum* is the major producer and a causative agent of blue mould rot in post-harvest fruits. Patulin induces DNA-DNA cross-links, and mutation of cells by patulin might be an indirect mutagenic mechanism. Finally, direct reactivity with DNA has been demonstrated (Paterson and Lima 2010). Therefore, patulin is considered as a mycotoxin with potential carcinogenic effects. Although direct patulin-induced toxicity in humans is still poorly understood, the potential hazard has led U.S. Food and Drug Administration and the EC to set a maximum tolerance limit of 50 ppm in apple-derived products. Other DNA-damaging mycotoxins include

aflatoxins, sterigmatocystin, OTA, zearalenone, citrinin, luteoskyrin and penicil-penicillic acid (Paterson and Lima 2010).

Recently, several surveys have been carried out to determine patulin levels in marketed apple juice and cider products (Harris et al. 2009, Murillo-Arabizu et al. 2009). Harris et al. (2009) reported that most of the apple cider and juice samples from Michigan were below the maximum limits. However, 23% of samples contained detectable amounts patulin with 11% having patulin over 50 µl/l. Similarly Murillo-Arabizu et al. (2009) reported that 11% of the apple juice samples in Spain exceeded the permitted maximum level. In a survey carried out in Northeast China (Yuan et al. 2010), 16% of the apple products, including apple juice, baby food, apple juice concentrates and mixed juices, contained > 50 µl/l patulin. Children are often heavy consumers of apple beverages, and decreasing the maximum value from 50 µg/l in order to protect them has been proposed (Tangni et al 2003). Murillo-Arbizu et al. (2009) reported that estimated daily intakes of patulin for adults, children and babies in Spain were well below the provisional maximum tolerable daily intake (PMTDI) (400 ng kg^{-1} bw day^{-1}), although for babies and children they may represent approximately 50% of the total PMTDI or even more. Thus, given that other fruits and vegetables contribute to patulin intake throughout the world, means to reduce human exposure to patulin, especially for babies and children, are needed.

4.5.4 Other harmful microbial metabolites

Biogenic amines (BA) such as heterocyclic amines (histamine, tryptamine), aromatic amines (tyramine, phenyletylamine), diamines (putrescine, cadaverine), and polyamines (spermine and spermidine), are commonly found in a variety of foods and beverages such as cheese, meat, fish products, wine, beer and other fermented products (Silla Santos 1996). BAs in foods are generated either as the result of endogenous amino acid decarboxylase activity in raw food materials, or by the growth of decarboxylase-positive microbes such as species of *Bacillus, Citrobacter, Clostridium, Klebsiella, Escherichia, Proteus, Pseudomonas, Shigella, Photobacterium* and LAB (Silla Santos 1996). BA amounts are usually increased during microbial fermentation of food or in the course of spoilage (Karovicova and Kohajdova 2005). Higher amounts of certain amines may be found in products as a consequence of the use of poor quality raw materials, microbial contamination and inappropriate food processing and storage.

BA do not usually cause health hazards to individuals unless large amounts are ingested (Donhauser et al. 1993, Halasz et al. 1999). Hypertensive crises have been observed after beer consumption in patients taking monoamine oxidase inhibitors (MAOI) (Shulman et al 1997, Tailor et al. 1994). The adverse effects were found both in tap and non-alcoholic beers and were caused by tyramine. Tyramine intakes exceeding 6 mg within a 4-hour period or from beers containing > 10 mg/l tyramine have been considered as dangerous for such patients (Tailor et al. 1994). Health risks have not been reported for healthy consumers. Biogenic amines are also potential precursors for the formation of carcinogenic nitrosoamines (Shalaby 1996). Recent studies have showed that LAB may also produce carcinogenic amines from some azo dyes (Pérez-Díaz and McFeeters 2009). Azo dyes are used widely in the soft drink industry, but in Finland they are in the list of banned additives.

Production of BA is a signal of unwanted microbial activity and can be used as a freshness indicator in packaged products (Rokka et al. 2004). Increased amounts of BA such as tryptamine, cadaverine and histamine in beers or other fermented beverages could be used as an indicator of poor hygiene during brewing (Slomkowska and Ambroziak 2002). Halasz et al. (1999) also reported that the histamine content of beer is a good indicator for hygienic conditions of barley storage, malting and brewing, as the histamine content of the product does not originate from barley or from the malt (Halasz et al 1999).

LABs are often associated with amine build-up in beers and fermented beverages (Izquierdo-Pulido et al. 1997). Some LAB are capable of producing biogenic amines such as putrescine and agmatine from a common amino acid, arginine, in fruit juices (Arena and Manca de Nadra 2001). Kalac et al. (2002) reported that considerable levels of tyramine and histamine can be formed in bottled beers by LAB contaminants, mainly by lactobacilli surviving insufficient pasteurization. Concerns have also been raised on the impacts of biological acidification on the production of BA. Biological acidification plays an important role in brewhouse technology and is also applied in the production of functional drinks. Donhauser et al. (1993) used 11 LAB strains in their study, and reported that biological acidification did not result in notably higher BA content compared to the original wort. Our studies with *Lactobacillus plantarum* starter culture showed that addition of LAB into the pilot or industrial malting process did not increase the amount of BA in final beer (unpublished data).

In natural cider production, alcoholic and malolactic fermentation occurs spontaneously with indigenous yeast and LAB (Garai et al. 2006). Thus, the

production of BA is possible. Histamine, tyramine and putrescine were found in various natural ciders (Garai et al. 2006). Amine levels were variable, ranging from not detected to 23 mg/l. Garai et al. (2007) demonstrated that LAB microbiota associated with cider production had the ability to produce BAs, particularly histamine and tyramine (Garai et al. 2007). *Lb. diolivorans* was the most intensive histamine producer. They highlighted the importance of controlling LAB in cider fermentations.

Another compound of public health concern is **ethylcarbamate**, a potential human carcinogen formed naturally during fermentation of food and beverages. Ethylcarbamate is produced from the reaction between ethanol and urea, which is accelerated at high temperatures. Urea in beverages derives from the metabolism of arginine and citrulline by yeast and bacteria such as LAB. The major dietary sources of ethyl carbamate are spirit drinks, especially whisky. A recent risk assessment concluded that the margin of exposure uptake from daily food and alcoholic beverages combined are of concern (EFSA 2007).

Acetaldehyde (ethanal, CH_3CHO) is an aldehyde of acetic acid. It is a potent volatile flavouring compound found in many beverages and foods. Furthermore, it is naturally found in low levels in some foods prepared by fermentation, such as milk products, soy products, canned vegetables and non-alcoholic beverages. It is also found in fruit and fruit juices, especially apple products. During fermentation small amounts of acetaldehyde are formed as a metabolite of alcohol. Lachenmeier and Sohnius (2008) reported that beer had significantly lower acetaldehyde contents (0–63 mg/l) than wine (0–221 mg/l) or spirits (0–1 159 mg/l). The highest acetaldehyde concentrations (12–800 mg/l) were generally found in fortified wines. High consumption of alcoholic drinks is a major source of exposure. In addition, the microbes of the intestinal tract can produce acetaldehyde in saliva, gastric juices and in the contents of the colon from the intake of alcohol. People with a certain genotype – found especially in East Asian populations – suffer especially from the adverse effects of acetaldehyde. The role and impact of acetaldehyde are under dispute and require further studies.

4.6 Microbial contamination sources

Microbial contamination may originate from any step along the beverage manufacturing process. Raw materials, factory environment, dirty packages and

unhygienic process equipment are all possible contamination sources (Stratford 2006).

4.6.1 Raw materials

Controlling raw material quality is necessary in order to produce microbiologically safe and stable beverages. Potential pre-harvest contamination sources include soil, faeces, irrigation water, dust, insects and animals. Post-harvest sources for fresh produce include harvesting equipment, human handling, rinse water, transport vehicles and processing equipment (Burnett and Beuchat 2000). Enteric pathogens contaminate fresh produce via direct or indirect contact with faeces e.g. through bird droppings, insects, soil and contaminated irrigation water. Soil is a rich reservoir for harmful spore-forming (e.g. *Alicyclobacillus*, *Clostridium*) and non-sporeforming bacteria (e.g. *Zymomonas*, *Listeria monocytogenes*) and heat-resistant fungi (e.g. *Byssochlamus*) (Coton et al. 2006, Bevilacqua et al. 2008, Parish 2009, Tribst et al. 2009).

Water is the main component of soft drinks. The quality of beverage water and process water is usually ensured with various chemical and physical treatments (Lawlor et al. 2009). If treated improperly, water may bring and spread food-borne pathogens and other spoilage microbes to the process areas and to the final product. Process waters, especially contaminated cooling and rinsing waters, are common sources of yeasts in soft drinks (Stratford 2006). Supplying sufficient safe water for food production and processing will probably be one of the greatest issues facing agriculture and food production in the future. Water supplies will almost certainly not keep up with demand in many parts of the world, making irrigation and reclaimed water usage more common. Both of these practices can introduce microbiological hazards to food and beverage production (Buckley and Reid 2010).

Sweeteners and sugar are common sources of spoilage organisms (Davenport 1996). Sweeteners used in the soft drink industry are typically syrups. They contain on average 67 ºBrix and have low water activity. Especially osmophilic yeasts such as *Z. rouxii* may grow in these syrups (Wareing and Davenport 2005). Low water activity controls the growth of yeasts, and therefore it is important to prevent condensate formation in syrup storage tanks and containers. Drops of condensate water may establish microenvironments with higher water activity and lead to rapid increase in yeast growth rate (Lawlor et al. 2009).

Fruit juices are increasingly used in beverage production. Fruits represent natural habitats for spoilage microbes associated with soft drink and alcoholic beverages. They may also carry pathogens if not properly handled and processed (Parish 2009). Fruits growing in close contact with soil are more likely to be contaminated with heat-resistant microbes than fruits with greater distance to soil (Tribst et al. 2009). Dried ingredients, such as spices, can contain pathogenic and spoilage microbes as well as mycotoxins (Ito et al. 2009). Other ingredients such as CO_2, minerals, acids, natural and artificial flavourings, colourings, chemical preservatives, foaming agents and stabilizers are not common contamination sources (Lawlor et al. 2009). The increasing trend of using new and often exotic ingredients may cause unexpected problems in ensuring microbiological quality of beverages. The microbial ecology of exotic fruits is poorly known, and they are a potential source of new harmful microbes or metabolites (Tribst et al. 2009). Furthermore, addition of new ingredients can also be considered to increase potential contamination sources.

Pitching yeast can contaminate cider and long drink bases produced in breweries in addition to other sources shared with soft drink production. The practice of yeast recycling in the beer production process may enrich yeast and bacterial contaminants in the yeast slurries. If the brewer´s yeast is subsequently used for fruit juice fermentation, contaminants and their metabolites may end up in the non-beer products that may be more sensitive to spoilage than beer.

4.6.2 Factory environment and production process

It has been estimated that poor factory hygiene accounts for 95% of soft drink spoilage incidences caused by yeasts (Van Esch 1987).

Secondary contaminations may arise from the factory environment and dirty processing equipment such as packaging, filling and capping machines, conveyors, soap, lubrication systems, meters and proportioning pumps and valve seals (Stratford 2006). Returnable glass bottles can also be a significant source of spoilage microbes (especially yeasts) in the factory environment. Non-returnable glass and PET bottles usually have very low microbial counts (< 10 CFU/pack) (Lawlor et al. 2009).

Poor sanitary design and improper cleaning and sanitation procedures favour the build-up of spoilage microbes within the factory and increase the contamination and spoilage risk of final products (Stratford 2006). Microbes attach easily on the manufacturing surfaces (e.g. processing pipes, feeding lines),

forming biofilms which are difficult to clean. The formation of biofilms starts from the initial attachment of the bacteria to a solid surface, the formation of microcolonies, and differentiation of these colonies into a form of mature biofilm encased in exopolysaccharides (Cloete et al. 2009). The matrix protects the microorganism from the effects of detergents and disinfectants. Members of the *Enterobacteriaceae* family and *Pseudomonas* spp. are often isolated from brewery biofilms (Storgårds and Priha 2009). Although bacteria usually outnumber yeasts in brewery biofilms, yeasts are frequently isolated. Soft drink spoiling *Candida* spp., *Debaryomyces* spp. and *Saccharomyces* spp. are the most common yeasts isolated from brewery samples (Storgårds and Priha 2009). Cross-contaminations occur when microbes slough-off from biofilms and contaminate bottles during the rinsing process (Lawlor et al. 2009). More detailed information about brewery biofilms can be obtained from Timke (2004) and Storgårds and Priha (2009).

Raw material and product spills may spread microbes in the factory environment. Microbes may drift in the air within aerosols of employees' coughing, as well as with aerosols developed in the course of factory cleaning with high pressure hoses (Sperber 2009). Basidiomycetous pigmented yeasts (*Rhodotorula, Cryptococcus*), moulds and spore-forming bacteria spread easily in air. Fruit flies and other insects are also potential sources of contamination (Lawlor et al. 2009). Spoilage microbes in biofilms and in product spills may become adapted and enriched for growth in the finished product (Sperber 2009).

In the production of traditional type ciders, the fruits at harvest, the press house, fermentation vats and general factory environment can be sources of both beneficial and detrimental microbes in the process and in the final products (Jarvis 2003).

4.7 Factors affecting microbial survival and growth in beverages

Several intrinsic and extrinsic factors influence the microbial stability and safety of soft drinks and alcoholic beverages. Intrinsic factors relate to the recipe of the soft drink and include acidity, carbonation, nutrients and antimicrobials present in the system (such as those coming from flavours and plant extracts). In addition, the quality of ingredients, manufacturing hygiene, processing, packaging and storage conditions contribute to the microbiological quality of the

products (Sperber 2009). This section reviews the impact of intrinsic product factors on beverage-associated microbes.

4.7.1 pH and acidity

Acidity and pH are the most important antimicrobial hurdles in beverages (Mentz et al. 2010). In general, the risk of spoilage and growth of pathogens increases with increasing pH. Food-borne pathogens do not generally grow at **pH 4.6** or below, which has been set as an official borderline between acid and low-acid foods (Lawlor et al. 2009). Therefore, the pH of soft drinks and alcoholic beverages is usually adjusted below this value (Sperber 2009). Low pH alone does not ensure the product safety and stability. The minimum pH for growth and the rate of inactivation depend on the nature of the acidulant, the presence of other inhibitors and the acid resistance mechanisms of an organism (Lücke 2003). The presence of weak acids affects greatly the survival of microbes in low pH environments (Stratford 2006, Sperber 2009). Weak acids inhibit microbial growth in their undissociated forms, which are predominant in low pH values (Sperber 2009).

The acid-tolerance of beverage-related microbes generally decreases in the order: moulds and yeasts, alicyclobacilli, lactobacilli, AAB, and leuconostocs. The product sensitivity to bacterial spoilage is expected to greatly increase at around pH 3.5–4. Butyric acid-forming clostridia may grow in the pH range 4.0–4.5 at ambient or higher temperatures (Lücke 2003). Pathogenic *E. coli* H157:07 and *Salmonella typhimurium* grew in unhopped sweet wort at pH 4.5 but not at pH 4.0 (Mentz et al. 2010). pH values of many exotic low-acid fruit juices such as acai, cantaloupe, melon, papaya, persimmon and watermelon are also in the growth range of beverage-associated pathogens (Tribst et al. 2009). It is noteworthy that many food pathogens can survive for long periods in acidities that do not permit their multiplication. Prior acid adaptation can further enhance the survival and may also provide cross-resistance to other preservative factors such as thermal processing (Leyer et al. 1995, Chung et al. 2006, Vojdani et al. 2008). Inactivation of protozoans by low pH treatments has produced variable results (Erickson and Ortega, 2006). Many food-borne viruses are extremely acid tolerant and will probably survive acidification or fermentation as food preservation methods (Baert et al. 2009).

4.7.2 Carbonation and oxygen

Carbonated soft drinks are generally less prone to microbial spoilage than noncarbonated soft drinks (Back 2005). Carbonation may inhibit the growth of spoilage microbes by inhibition of cell division, inhibition of amino acid uptake, perturbation of cytoplasmic buffering, induction of sporulation, and lowering cytoplasmic pH (Stratford 2006). The typical carbonation level of soft drinks is around 3 volumes. However, there are many spoilage yeasts which are resistant to carbonation in standard volumes (Stratford 2006). The most resistant yeasts belong to the genera *Dekkera* and *Saccharomyces* (Stratford 2006) (Appendix A). Many new drinks are more lightly carbonated than traditional soft drinks and could allow less carbonation resistant spoilage species to grow. Viruses have been shown to survive well in modified atmosphere packaging with high CO_2 levels (Baert et al. 2009).

Many spoilage microbes need oxygen to grow, and in carbonated soft drinks the levels of oxygen are usually very low. Lack of oxygen in food is the major factor in preventing microbial spoilage (Stratford 2006). In oxygen-impermeable packages the major spoilage microbes are LAB and yeasts. Stratford (2006) speculated that the ability of *Z. bailii* to grow in anaerobic conditions may depend on yet unidentified micronutrients which do not occur in fully synthetic media. Oxygen permeability of plastics and other packaging materials varies greatly. The oxygen content in PET bottles increases with time, whereas glass bottles are impermeable to oxygen (Stratford 2006). Hence, the species that are most likely to spoil the product in PET bottles are different from those which spoil the product in glass bottles (Appendix A). It has been speculated that the incidence of fruit juice spoilage by alicyclobacilli could have been accelerated due to increased use of PET bottles (Lawlor et al. 2009). This type of spoilage could in some cases be prevented by the use of antioxidants.

4.7.3 Nutritional status

Nutritional composition of beverages impacts the type and rate of microbial spoilage. In general, all ingredients that provide nutrients for microbes increase the product susceptibility to spoilage. Beverages differ greatly in their nutritional status. Synthetic soft drinks represent the poorest environment, whereas high juice fortified drinks are among the richest environments. *Dekkera* species

tolerate citric acid and utilize nitrates, which may enhance their capability to spoil nutrient-poor soft drinks (Stratford 2006).

The addition of sugars to sugar-poor formulations is likely to increase their sensitivity to yeast spoilage. In BMBs, clear correlation was found between the concentration of fermentable sugars and the susceptibility to spoilage (Hutzler et al. 2008). Decreasing the sugar concentration of sugar-rich soft drinks did not significantly reduce the spoilage risk by fermentative yeasts (Stratford 2006). Soft drinks containing 5% or 10% sugar were equally likely to spoil. A herbal drink formulation lacking sugar was found to support the growth of a variety of moulds, but none of 150 yeast species could grow in the product (Stratford 2006).

Mixing beer with soft drinks or sugar syrups can increase the vulnerability of the product to yeast spoilage, as it increases the fermentable sugar content and decreases the level of chemical preservatives in the product. BMBs could have an increased risk of spoilage by potential beer-spoiling LAB, since natural antimicrobial hurdles, such as hop compounds, are diluted with the addition of new growth factors. The potential beer-spoiling species are not as resistant to hop compounds and utilize fructose and glucose more readily compared to the obligate spoilers (Back 2005).

The use of artificial sweeteners instead of sugars tends to reduce the sensitivity of beverages to yeast spoilage (Stratford and James 2003, Stratford 2006, Hutzler et al. 2008). The non-nutritive sweetener palatinose is becoming increasingly popular in sport and energy drinks and alcohol-free malt-based beverages (Pahl et al. 2010). The ability of brewer´s yeast strains and common beer-spoilage bacteria to utilize palatinose has recently been studied (Pahl et al. 2010). It was found out that only 3/60 brewer´s yeast strains and a *Sz. pombe* strain were able to ferment this compound. BMBs sweetened with palatinose were less susceptible to spoilage by *S. cerevisiae* var. *diastaticus* compared to the products sweetened with sucrose or high-intensity sweeteners. Metabolism of aspartame during yoghurt fermentation by LAB has been documented (Keller et al. 1991). Some LAB and yeasts have also been reported to metabolise artificial food colourings (Turtura and Minguzzi 1992, Pérez-Diaz and McFeeters 2009).

Nowadays, fruit juices are increasingly added to soft drinks and are essential ingredients of ciders and many alcopops. The addition of fruit juice greatly improves the nutritional status of beverages. Fruit juices contain sugars, vitamins, organic acids, nitrogen and minerals. Bacteria, yeasts and moulds all

benefit from the nutritional value of juices (Back 2005, Wesley 2009). Yeasts and LAB can grow with the sugars and organic acids present in juices. For example, lactobacilli are able to utilise citric, tartaric, succinic and malic acids (Hammes and Hertel 2009). Fruit juices also contain precursors of vanillic acid for guiacol production by alicyclobacilli (Bevilacqua et al. 2008).

Simple soft drinks are often poor in nitrogen. Organic flavourings contribute low levels of nitrogen to the beverages and can be the sole nitrogen source in juice-free drinks. Yeasts are able to thrive with low levels of nitrogen (0.2–0.5 mg/l) and the addition of nitrogenous compounds does not greatly increase the risk posed by these organisms (Hutzler et al. 2008, Lawlor et al. 2009). Yeasts do not usually degrade proteins, and amino acids and ammonium are their preferred N sources (Stratford and James 2003). Yeasts can also scavenge nitrogen for growth by degrading their cellular constituents (so called macroautophagy) (Lawlor et al. 2009). Amino acid-containing ingredients can improve the growth of fastidious bacteria such as LAB in the product (Wareing and Davenport 2005). In nutritionally stressed conditions, *Lb. plantarum* can also efficiently utilize dipeptides as nitrogen sources (Saguir et al. 2008). AAB may thrive with inorganic nitrogen sources (Bartowsky and Henschke 2008).

Metal salts and trace elements in soft drinks mainly originate from “hard water” used in the manufacturing process (Stratford and James 2003). Metal salts such as magnesium and calcium are essential nutrients for spoilage yeasts and increase the product sensitivity to spoilage (Stratford 2006). Organic acids may chelate metal ions, making them unavailable for microbes and improving the product stability (Lawlor et al. 2008). Phosphates usually derive from water or fruit components. Cola-types beverages contain high phosphate levels as they are acidified with phosphoric acid (Stratford and James 2003). Phosphates are typical yeast nutrients (Stratford 2006).

Yeast-fermented beverages tend to be more complex in composition compared to their non-fermented counterparts. Yeast fermentation of fruit juices can increase the amount of growth factors in ciders and long drinks if the yeast is allowed to undergo autolysis (Jarvis 2003). Antimicrobial yeast metabolites (e.g. ethanol, organic acids and SO_2) are well known for their protective effects against spoilage and pathogenic microbes (Mentz et al. 2010).

4.7.4 Functional ingredients

New functional drinks are increasingly being developed. The formulations contain a growing list of non-traditional ingredients which includes e.g. exotic fruit concentrates, herbal and other plant extracts, spices, proteins and amino acids and dietary fibres (Table 9). Some of these ingredients, such as herbs and antioxidants, may inhibit microbial growth, whereas others may increase the product sensitivity to spoilage or pathogen survival. New ingredients, especially fruits from different exotic locations throughout the world, may also harbour new potential spoilage and pathogenic microbes (Stratford and James 2003).

The addition of **vitamins and minerals** to soft drinks that lack these compounds may boost microbial growth in the products. For example, the supplementation of soymilk with B-vitamins greatly enhanced the growth of lactobacilli (Ewe et al. 2010). B-Vitamins function as enzyme cofactors and they are essential for many cellular functions, and thereby may increase the survival of harmful microbes in soft drinks. Some important food-spoilage yeasts, such as *Z. bailii*, also require B-group vitamins for growth (Stratford 2006).

Some LABs such as *Lb. casei* and *Lb. paracasei* are able to use soluble **dietary fibres** such as fructo-oligosaccharides (FOS), inulin and other oligosaccharides as energy sources for growth (Kaplan and Hutkins 2003, Makras et al. 2005). The optimal concentrations of FOS and maltodextrin for the growth of *L. casei* were 4.8% and 6.9% (w/v) (Liong and Shah 2005). The incorporation of these ingredients into functional drinks may increase the product sensitivity to LAB spoilage. *Lb. paracasei* is a common soft drink spoiler that is also found in brewery environments. Oligosaccharides and cereal fibres are used in many probiotic drinks to improve the stability of probiotic bacteria (Charalampopoulos et al. 2003). However, they could also enhance the survival of contaminating microbes in the products.

Fortification with **calcium lactate** may increase the spoilage potential of the product due to increased pH and buffering capacity (Lawlor et al. 2009). Moreover, AAB can oxidize calcium lactate to calcium carbonate. On the other hand, the ability of some spoilage and pathogenic bacteria to survive in orange juice has been reported to decrease in high calcium lactate concentrations (Yeh et al. 2004). The effects of calcium also appear to depend on the type of supplement.

Energy drinks typically contain an amino acid **taurine** (3–4 g/l). A *Klebsiella* isolate from soil has been shown to be able to use taurine as sole N source for

aerobic and anaerobic growth (Chien 2008). In theory, taurine could facilitate enterobacterial growth in low acid (pH > 4) soft drinks (Sperber 2009). Plant sterols and omega-3 fatty acids added as functional ingredients could potentially enhance the growth of spoilage yeasts in the products.

Plant extracts are complex ingredients that contain various phenolic compounds, vitamins, minerals and amino acids. In functional beverages, plant extracts such as yerba mate, guarana, coffee and tea are used as natural sources of **caffeine** (phenolic acid) and other bioactive compounds. Caffeine is a standard ingredient in energy drinks and also in traditional cola-type soft drinks. Guarana and tea extracts have shown antimicrobial activity against food-borne pathogens and spoilage fungi in laboratory media (Majhenic et al. 2007). BMBs with caffeine-containing lemonades suppressed the growth of several yeasts that could grow in other BMBs (Hutzler et al. 2008). The suppression of spoilage *Dekkera/Brettanomyces* with phenolic acids has also been demonstrated in a wine model medium (Harris et al. 2008). High natural caffeine content in apples was thought to reduce the viability of *E. coli* H157:07 in natural apple juices (Reinders et al. 2001). Interestingly, it was also shown that the consumption of caffeinated soft drink reduced bacterial prevalence in voice prosthetic biofilms (Free et al. 2000). Many potential beverage-spoiling microbes such as *S. cerevisiae*, *Lb. plantarum* and *P. pentosaceus* may also form off-flavours from phenolic beverage constituents (Hammond et al. 1999, Barthelmebs 2000a, b, Harris et al. 2008). This is thought to confer a selective advantage to microbes growing on plants. Several studies have shown that essential oils and their active constituents extracted from fruits, herbs and spices have natural antimicrobial properties. For example, essential oils from oranges were shown to protect lemonade from spoilage by *S. cerevisiae* (Ndagijimana et al. 2004). Cinnamaldehyde and eugenol could be used to inhibit the germination of *A. acidoterrestris* spores (Bevilacqua 2008).

Table 9. Examples of components used in beverages and their effects on microbial growth.

Component	Growth promotion	Growth inhibition	References
Allium (e.g. garlic and onion)		Various food-borne pathogens	Davidson and Zivanovic 2003
Arrowroot tea extract		Various Gram-negative and Gram-positive bacteria	Kim and Fung 2004

B-vitamins	LAB, some spoilage yeasts		Ewe et al. 2010, Stratford 2006
Calcium lactate	*A. acidoterrestris, Lb. plantarum, Lb. sakei*, *Salmonella*, spoilage yeasts		Yeh et al. 2004
Cereal extract	*Lb. plantarum*		Charalampopoulos & Pandiella 2010
Dietary fruit and vegetable juices		Food-borne pathogens	Lee et al. 2003, O'Mahony 2009
Essential oils		Food-borne pathogens and spoilage yeasts	Elgayyar 2001, Belletti et al. 2007, Burt 2004, Gutierrez et al. 2009, Carovic-Stanko et al. 2009
Essential oils of orange		*Saccharomyces cerevisiae*	Ndagijimana et al. 2004
Essential oils of *Cunila* taxa		Food-borne pathogens	Sandri et al. 2007
Green tea polyphenols		Thermophilic sporeforming bacteria	Sakanaka et al. 2000
Guar meal extract	*Lactobacillus*	*Escherichia coli, Staphylococcus aureus* and *Salmonella* Typhimurium	Hassan et al. 2010
Guarana		Food-borne pathogens and spoilage fungi	Majhenic et al. 2007
Hydroxycinnamic acids		Food-borne pathogens, yeasts and moulds	Davidson et al. 2003
Isothiocyanates		Fungi, yeasts and bacteria	Davidson et al. 2003
Soluble fibres (e.g. β-glucan, inulin)	*Lactobacillus*, *Bifidobacterium, P. pentosaceus*		Kaplan & Hutkins 2003, Makras et al. 2005, Yeo & Lion 2009
Spice and herbal extracts		Various food-borne pathogens and moulds	Davidson et al. 2003, Tajkarimi et al. 2010 Weerakkody et al. 2009
Taurine	*Klebsiella* spp.		Chien 2008
Tea saponins		Various bacteria and fungi	Li et al. 2009
The root of *G. glabra* (licorice-root)		*Arthinium sacchari, Bacillus subtilis, Candida albicans, Chaetomium funicola* and *Staphylococcus aureus*	Sato et al. 2000

4.7.5 Ethanol

Ethanol is present in ciders, alcopops and BMBs at concentrations up to 8.5% (abv). At these concentrations ethanol alone is not able to prevent spoilage by fermentative yeasts or acid-tolerant bacteria. The most ethanol-tolerant yeast species include *D. anomala*, *D. bruxellensis*, *I. orientalis*, *P. anomala*, *P. galeiformis*, *S. cerevisiae*, *Sc. ludwigii* and *Z. bailii* (Stratford 2006). BMBs with the highest ethanol level (6%, abv) were less susceptible to yeast spoilage than products with lower levels of ethanol (Hutzler 2008). The concentration at which fermentation activity appeared to be suppressed was between 4.8% and 6%.

LAB can tolerate up to 20% (abv) (Suzuki et al. 2008a). Some strains of *Lb. plantarum* and *Lb. suebicus* were able to grow in fruit mashes in the presence of 12% and 14% ethanol at pH 2.5 (Hammes and Hertel 2006). Other ethanol-tolerant lactobacilli include *Lb. brevis*, *Lb. fructivorans* and *Lb. hilgardii* (Back 2005). *Lb. collinoides* resists the ethanol and acidity levels encountered in cider making (Laplace et al. 1999). *P. parvulus* and *P. inopinatus* have been reported to grow in 12–14% (v/v) ethanol concentrations (Edward and Jensen, 1992). New *Pediococcus* species from distilleries tolerated 6.5–10% ethanol at pH 3.5 (Zhang et al., 2005, Zhang and Dong 2006). Ethanol stimulates exopolysaccharide synthesis by *P. pentosaceus*, indicating that these polymers may protect cells against ethanol (Manca de Nadra and Strasser de Saad, 1995). *Z. mobilis* also tolerates ethanol well, the maximum concentration lying between 6.5% and 10% (v/v) (Sahm et al. 2006). A cider-spoiling *Z. mobilis* strain was able to grow with 8% (v/v) ethanol in a synthetic medium (Coton et al. 2006). Pathogenic *E. coli* 0157:H7 and *Salmonella* were able to grow in 4% (v/v) ethanol concentration in sweet wort (pH 5.5). *L. monocytogenes* was inhibited by 3% ethanol, but it survived more than 5 days in 5% ethanol concentration (Mentz et al. 2010).

Relatively rapid inactivation of protozoans at the ethanol levels of mild alcoholic beverages or higher has been reported (Dawson et al. 2004, Erickson and Ortega 2006). Reports were not found concerning the survival of viruses in alcohol-enriched environments.

4.7.6 Water activity

Water activity alone does not restrict the growth of spoilage microbes in ready-to-drink beverages (Sperber 2009), but it is an important preservative factor in

juice concentrates, syrups and similar ingredients used in beverage production in breweries. These ingredients are generally protected from spoilage due to their high sugar concentration (Lawlor et al. 2009). However, spoilage by osmotolerant yeasts such as *Z. bailii* and *Z. rouxii* may result during storage and transport when condensate water leads to increase in water activity (Stratford, 2003). Sugar-tolerant bacteria can also grow in these dilute microenvironments (Stenius et al. 1991, Lawlor et al. 2009). The rate of bacterial pathogen inactivation increased in cranberry juice concentrates with increasing ° Brix levels (Enache and Chen 2007).

4.7.7 Storage temperature

The majority of beverages currently produced in breweries are stored at ambient temperatures. Nutritious non-carbonated beverages, such as fruit juices, and low acid products require cold storage for microbiological stability unless they have been processed for commercial sterility (Lawlor et al. 2009, Rystad and Johnstone 2009). Refrigeration prevents juice spoilage by *Alicyclobacillus* spp., as these bacteria do not grow below 20–25 °C (Smit et al. 2011). Fruit juices and concentrates used as beverage ingredients may be transported at reduced temperatures. Cold storage (0 – -23 °C) can improve the safety of acidic juice ingredients by reducing the number of pathogens (Oyarzabal et al. 2003, Nogueira et al. 2003, Enache and Chen 2007, Enache et al. 2009. The rate of pathogen inactivation depends on the juice type, °Brix level, the specific temperature, and the physiological state of the pathogen. Time-temperature combinations recommended for 5-log reduction of bacterial pathogens have been proposed for specific juices in the literature (FDA 2000, Nogueira et al. 2003, Enache and Chen 2007). Pathogens (bacteria, protozoa) appear to survive better at refrigeration temperatures than at freezing or ambient temperatures (Vojdani et al. 2008, Estrada et al. 2010).

4.7.8 Microbial adaptation and stress resistance

Many conditions that microbes encounter in beverage production are potential stress-factors to cells. It is well documented that a sub-lethal stress can induce an adaptive stress tolerance response, which may provide protection against subsequent lethal stresses of the same or different kind (cross-protection) (Chung et al. 2006, van de Guchte et al. 2002, Smits and Brul 2005). Microbial

populations generally respond non-homogeneously to stress. Spoilage and path-pathogenic microbes may adapt to biochemical stresses such as weak organic acids, carvacrol, nisin and hop compounds, and to physico-chemical stresses such as heat and high pressure (Brul et al. 2003). Adapted cells may also show increased virulence. Many typical spoilage phenomena in soft drinks and alcoholic beverages, such as ropiness (induced by ethanol), production of volatile phenols (induced by phenolic acids), and arginine metabolism are induced by stress. Stress adaptation may also lead to morphological changes that may have implications in food spoilage (Asano et al. 2007).

In adverse conditions, many bacteria may also enter into a viable but non-culturable state. These bacteria cannot be detected on normal culture media and their detection requires either special cultivation conditions or molecular biological tools. Viable but non-culturable form has been detected in over 60 species to date (Skovgaard 2007), including beer-spoilage LAB (Suzuki et al. 2006). Media have been developed to improve the recovery of these refractory forms from beer (Suzuki et al. 2008b). Studying the gene expression of spoilage and pathogenic microbes under various conditions may allow identification of biomarkers for stress resistance development.

4.8 Prevention of contaminations

4.8.1 Primary production

It is well documented that some pathogenic bacteria, viruses and parasites are able to remain infective in fresh produce (berries, fruits) and juices made from them and can cause illness after consumption (Parish 2009). Good agricultural practices during pre-harvest, harvest and processing of produce are the first steps in preventing contamination of fresh beverage ingredients (Murphy et al. 2006, Tribst et al. 2009). In fruit juice production, compliance with good manufacturing practices (GMP), the application of HACCP system and proper processing are important for reducing the risk of contamination by pathogenic microbes (Tribst et al. 2009, Newell et al. 2010). Prevention is particularly important for controlling food-borne viruses, since there is insufficient data about their inactivation in food preservation (Baert et al. 2009). For example, adequate selection and storage of raw materials is critical. The practice of washing raw fruits and vegetables with sanitizers often has limited efficacy against harmful microbes (Burnett & Beuchat 2000; Erickson & Ortega 2006;

Tribst et al., 2009). In the USA, fruit juices must be processed in a manner that results in a 5 log reduction in pathogenic microbes (FDA 2001). When designing inactivation treatments for fruit juices, *Salmonella* is a good target for orange juice, *E. coli* and *Cryptosporiudium* for apple juices and *L. monocytogenes* for various juices that have never been involved in outbreaks (Tribst et al. 2009).

4.8.2 Beverage production

High quality ingredients, their proper handling and storage and the maintenance of good production hygiene are important for the production of microbiologically stable and safe beverages (Ashurst and Hargitt 2009). Most products produced in breweries are not pasteurized in pack and harmful microbial metabolites already formed, such as mycotoxins, may not be destroyed during processing. High quality of ingredients is inversely proportional to the need for chemical preservatives and physical processing. Ingredients should ideally be purchased on specification from reliable and audited suppliers (Ashurst and Hargitt 2009). Remote plants in developing countries are usually most in need of auditing (Ashurst and Hargitt 2009). Food imported into the European Community must comply with the general requirements laid down in EU regulations (EC 178/2002) or satisfy equivalent rules.

It is also important to consider the effects of transport conditions and the intrinsic properties of ingredients (pH, a_w) on microbiological risks (Lawlor et al. 2009). Low-acid juices (pH > 4.6) and unfermented wort can be a safety risk, and should be handled and stored accordingly (Parish 2009, Tribst et al. 2009, Mentz et al. 2010). Microbiological stability and the shipping efficiency of liquid ingredients can be both improved by removal of water (Lawlor et al. 2009). Many botanical extracts can currently be purchased as powders. Condensation of water inside tanks and pipelines should be prevented in order to inhibit significant growth of sugar-tolerant yeasts in syrups and juice concentrates (Stenius et al. 1991, Lawlor et al. 2009). The real challenge is to control temperature fluctuations and condensation water formation during long transport. Regular in-house control of ingredients with high spoilage or safety risk is advisable to ensure their microbiological quality.

In the production of fermented beverage bases, active fermentation should be started as soon as possible in order to control unwanted activity of contaminants (Jarvis 2003, Malfeito-Ferreira et al. 2009).

GMPs, cleaning and disinfection and hygienic equipment design in every step of manufacturing help to minimise contaminations from factory environment (Lawlor et al. 2009). General principles for hygienic food production have been laid down in the code of practice from Codex Alimentarius (CAC/RCP 1-1969), European Food Hygiene regulations (EC 852/2004). Regular cleaning and disinfection of the manufacturing area equipment prevent biofilm formation. Biofilms increase the resistance of bacteria to sanitizers and make sanitation increasingly difficult (Bower and Daeschel 1999, Storgårds et al. 1999, Friedrich et al. 2009). Breweries normally use cleaning-in-place for closed processing lines, whereas open surfaces in the filling hall are cleaned using low-pressure foam systems or thin film cleaning with subsequent disinfection of high risk equipment (Storgårds & Priha 2009). The requirements for hygienic equipment design are well documented (EHEDG 2011). Maintenance of good hygiene in factories requires competent and well-trained personnel, especially today when beverage production is becoming technically more and more complex (Stratford 2006).

GMPs and hygiene alone are not sufficient for ensuring the quality of modern industrial production (Zeuthen et al. 2003). Monitoring of the quality of raw materials and manufacturing steps is also needed. HACCP System is a legal requirement for EU-based companies (EC 852/2004). HACCP is a systematic approach consisting of identification and assessment of all hazards associated with the final product, identification of the steps within production at which the hazards may be controlled or reduced (CCPs), and the implementation of monitoring procedures at these CCPs (Zeuthen et al. 2003).

The importance of factory hygiene is increased in the production of functional beverages that tend to be more vulnerable to spoilage than simple synthetic drinks. Preservative-free products will require stringent hygiene to prevent microbial contaminations (Stratford and James 2003). The established and emerging means to prevent biofilm formation in breweries were recently reviewed by Storgårds and Priha (2009). Potential novel tools for controlling brewery process hygiene could be application of functional materials that hinder microbial attachment to process surfaces, and interference with microbial cell-to-cell signalling (quorum sensing), which is needed for biofilm formation.

4.9 Preservation of beverages

Despite all preventive actions, contaminations are always possible. The intrinsic product properties are only rarely sufficient to ensure acceptable keeping quality (Lücke 2003). Hence, some additional control measures are often required. There are basically two ways to control microbial growth and activity in the products, i.e. **chemical** (chemical preservatives) and **physical** (heat, filtration, aseptic packaging) (Sperber 2009). The choice of the preservation method depends on the acidity, carbonation level and ingredients of the beverage, and to some extent also on the marketing needs (e.g. preservative-free products). The pH and nutritional status are the most decisive factors in choosing the preservative method. In general, preservative systems are only effective when the initial contamination level is low. Most microbiological problems arise because of poor quality raw materials and poor process hygiene, which lead to overcoming of the preservation system applied during manufacture by the spoilage organisms.

4.9.1 Chemical preservation with weak organic acids

Shelf-life of beverages can be extended by the addition of chemical preservatives. Regulations in Finland (KTM 752/755) permit a maximum of 150 mg/l benzoic acid and 300 mg/l sorbic acid in soft drinks. When a mixture is used, the maximum level of sorbic acid is 250 mg/l. Ciders can be preserved with 200 mg/l sorbic acid and 200 mg/l SO_2. In other mild alcoholic beverages, 200 mg/l of sorbic and benzoic acid is permitted alone or in combination. Dimethyldicarbonate (Velcorin) can be used as a preservative in soft drinks and alcoholic beverages at a level of 250 mg/l. Simple high acid soft drinks and alcoholic beverages can be preserved only chemically. Since chemical preservatives in BMBs are only permitted in the non-beer portion, the levels are not usually sufficient for microbial control (Schwarzenberger 2002).

Sorbates and benzoates are weak acids which are mainly effective in their undissociated form. The pH value at which 50% is undissociated is 4.76 and 4.19 for sorbic and benzoic acids, respectively (Lücke 2003). In general, sorbates are useful in preventing mould and yeast growth as well as some bacterial spoilage Benzoic acid is highly effective against yeast growth and it is also useful against bacterial growth, but only moderately active against moulds

(Sperber 2009). A mixture of preservatives is usually more effective than a sin-single preservative due to their synergistic action (Wind and Restaino 1995).

The most problematic spoilage organisms of soft drinks are notoriously resistant to preservatives. Chemical preservatives in their legal doses do not prevent spoilage by preservative-resistant yeasts. Particularly *Zygosaccharomyces* spp. usually tolerate both sorbic and benzoic acids (James and Stratford 2003). The inhibition of *A. acidocaldarius* and *P. cyclohexanicum* in juices with sorbates and benzoates required higher concentration than permitted in several countries (Walker and Phillips 2008, Bevilacqua et al. 2008). Many moulds such as *Penicillium roqueforti, P. paneum, P. carneum, Monascus ruber* and *Paecilomyces variotii* were able to grow at high concentrations of preservatives and organic acids (Samson et al. 2004). Therefore, in most cases preservatives are not the primary solution to protect foods and beverages from mould contamination. The probable effect of decreasing preservative concentration is that a higher proportion of products will be subject to yeast spoilage.

Weak acids are fungistatic rather than fungisidic. It is commonly thought that weak organic acids inhibit growth by acidifying the cytoplasm, but it is now clear that different acids may not all operate in the same way. It has been suggested that intracellular accumulation of anions is a general reason for the inhibitory effect (Carpenter and Broadment 2009). The resistance mechanisms of microbes can be divided into three strategies: 1) preservatives are destroyed or their entry is prevented, 2) they are removed from the cell, or 3) they are metabolically altered. The mechanisms of weak acid resistance have been much studied in *Z. bailii* and *S. cerevisiae* (for a review see Mollapur and Piper 2008). The studies indicate that *Z. bailii* either metabolizes acidic preservatives or prevents their entry into cytoplasm. It has been proposed that *Z. bailii* has an active “sorbate pump”, which extrudes the preservatives from the cytoplasm (James and Stratford 2003). Demidchik et al. (2005) proposed that the resistance to weak organic acids in *Z. bailii* is related to its high conductance pathway for low-affinity K+ uptake.

Dimethyldicarbonate is occasionally used for cold sterilization of soft drinks. It dissolves in water to form ethanol and CO_2. It should not be detectable in the final products. The use of dimethyldicarbonate has been limited because of its possible carcinogenity (Sperber 2009).

Sulphites are permitted in ciders (total 200 mg/l of total SO_2) and fruit cordials in Finland (KTM 752/755). SO_2 is a weak acid forming in solution a

pH-dependent equilibrium with molecular SO_2 at low pH and with bisulphite and sulphite ions at higher pH. Antimicrobial efficacy is mainly based on the molecular form that freely diffuses through cell membranes and decreases intracellular pH. Sulphites are also highly reactive, inactivating various macromolecules (Ought and Were 2005). Bacteria are more sensitive to SO_2 than fungi. Furthermore, Gram-negative bacteria appear to be more sensitive than Gram-positive ones. In ciders, free sulphite levels of 30–50 mg/l helps to prevent CO_2 production by fermentative yeasts (Jarvis 2003). However, it does not inhibitt sulphite-resistant yeast strains that can tolerate 6–12 mM sulphite (pH 4.0) (Stratford 2006). The resistant strains often belong to the species *Sc. ludwigii*, *S. cerevisiae*, *S. bayanus*, *Z. bailii* and *Z. lentus*. They can even grow in the presence of 1000–1500 mg/l sulphite (Jarvis 2003). Molecular SO_2 inhibited *Lb. plantarum* up to 1.5 mg/l levels, higher concentrations causing cell death. For the inhibition of *Acetobacter* spp. in wine, 0.8–1.2 mg/l molecular SO_2 was needed (Bartowsky and Henschke 2008). The maximum permitted level of SO_2 did not prevent the growth of cider-spoiling *Z. mobilis* in a growth medium (pH not specified) (Coton et al. 2006). Greater than 5-log reduction of *E. coli* O157:H7 was achieved with 50 ppm SO_2 after 6 h. Dimethyldicarbonate at 250 mg/l was also effective (Basaran-Akgul et al. 2009).

Sulphites are reactive compounds and their association with certain juice components and microbial metabolites (especially carbonyls) reduces their antimicrobial efficacy. Therefore, sulphite additions to cider should be made after the juice pressing (Jarvis 2003). SO_2 also rapidly inactivates patulin (Ought and Were 2005). It is noteworthy that some cultured and wild yeasts are able to produce sulphite from sulphates in significant amounts (Ought and Were 2005). Sulphites are possible allergens.

Product spills in the production area and the use of preservatives in ingredients may allow contaminants to adapt to chemical preservatives, which can increase their preservative-resistance. For example, *E. coli* may adapt to sodium benzoate and potassium sorbate under sub-lethal conditions (Santiesteban-Lopez 2009). Yeasts have also been shown to adapt to even higher proportions of preservatives in sub-inhibitory levels (James and Stratford 2003). Hence, the best way to prevent fungal spoilage would be to use preservatives and preservative techniques to which fungi have not yet become adapted, and to use a compilation of different preservative techniques (Smits and Brul 2005).

4.9.2 Biological preservation with natural antimicrobials

New natural antimicrobials are constantly being sought from safe microbes, animals and botanicals in order to reduce the need for chemical preservatives and heat processing in the food and beverage industry. They have been extensively studied for inactivation of spoilage and pathogenic microbes, with many promising results.

Several potential antimicrobials have been identified from microbes. **Bacteriocins** are low-molecular-weight antimicrobial peptides produced by bacteria. Nisin is the best-known and most studied example. It is produced by strains of *Lactococcus lactis* subsp. *lactis*. It is the only approved bacteriocin for specific food applications (Labbé and Nolan 2009), but not yet for beverage preservation. Nisin (E234) is able to form pores in cell membranes and is mainly active against Gram-positive bacteria (Labbé and Nolan 2009). At 5–10 IU/ml it has also been shown to be effective against spore-forming *A. acidoterrestris* (Walker and Phillips 2007). Enterococcal bacteriocin AS-48 (enterocin) has a broad-spectrum activity against Gram-positive bacteria, including spore-formers, LAB and many food pathogens (Abriouel et al. 2009, Martinez-Viedma et al. 2008 and 2009). AS-48 (2.5 µg/ml) killed vegetative cells and spores of *A. acidoterrestris* in a commercial fruit juice stored for 14 days at 37 ºC (Khan et al. 2010). In commercial energy drinks adjusted to pH 5.0, AS-48 was able to inhibit the growth of *L. monocytogenes, Staphylococcus aureus, Bacillus cereus* and *B. licheniformis* (Viedma et al. 2010). The general limitation of bacteriocins is their rather narrow antimicrobial spectrum, which is why they could best be used in combination with other preservation technologies. When adding bacteriocins as preservatives in beverages, milder heat treatments could be used (Khan et al. 2010). For a review of enterocins in food preservation see Khan et al. (2010).

Lysozyme (muramidase, EC 3.2.1.17, E1105) is an antimicrobial cell wall lytic enzyme that can be applied to control microbial growth in food and beverages (Silvetti et al. 2010). Silvetti et al. (2010) reported a new application of lysozyme for prolonging the shelf-life of unpasteurized beer. It had a strong inhibitory effect against LAB. Sensory analysis revealed that lysozyme had no negative impacts on beer flavour. Although the use of lysozyme in beverages is not permitted in beverages in Europe, this substance is considered safe by GRAS and an upper limit of 500 mg/kg for perries and ciders is specified (Codex stan

192-1995). The use of this enzyme in beverages is not currently permitted in Finland.

Berries, herbs, spices and their derived essential oils and extracts are a rich source of natural antimicrobials (NAs) (Puupponen-Pimiä et al. 2005, Tajkarimi et al. 2010). Some of these substances contribute to the self-defence of plants against microbial infections, oxygen radicals and radiation. More than 1340 plants have been identified as potential sources of such compounds. The antimicrobial compounds in plants are commonly contained in the essential oil fractions. These fractions contain components from many chemical classes, but phenolics are chiefly responsible for the antimicrobial activity (Tajkarimi et al. 2010). Plant phenolics can be divided into simple phenols and phenolic acids (e.g. vanillic, gallic), hydroxycinnamic acid derivatives (e.g. p-coumaric, caffeic, ferulic), flavonoids (e.g. catechins, proanthocyanins, flavonols) and polymeric tannins (Lopez-Malo et al. 2005).

Antimicrobial properties of phenolic constituents from berries, spices and herbs have been extensively studied and reviewed (Burt 2004, Puupponen-Pimiä et al. 2005, Lopez-Malo et al. 2005, Tiwari et al. 2009, Tajkarimi et al. 2010). Most studies have looked at the effects of purified substances or extracts in laboratory media or model foods. Phenolic compounds from plants have also shown potential to inhibit spoilage microbes and bacterial pathogens associated with beverages. Several mechanisms are involved in the growth inhibition (Puupponen-Pimiä et al. 2005, Lacombe et al. 2010). In general, Gram-positive bacteria appear to be more sensitive to plant phenolics than Gram-negative species. Phenolic compounds from spices and especially from thyme, cinnamon and clove have shown a wide antimicrobial spectrum (Lopez-Malo et al. 2005). They are often effective in the range of 0.05–0.1% (Tajkarimi et al. 2010). Tea extracts have also exerted activity against various Gram-positive and negative bacteria (Lopez-Malo et al. 2005). Among different berries and berry phenolics, cranberry, cloudberry, raspberry, strawberry and especially bilberry have shown clear antimicrobial effects against e.g. *Salmonella* (Puupponen-Pimiä et al. 2005). Vanillin is a phenolic compound present in vanilla pods. It has inhibited various food-related bacteria such as *E. coli, Lb. plantarum* and *Listeria innocua* (Fitzgerald et al. 2004). Hydroxycinnamic acids have shown some activity against a variety of spoilage yeasts, especially against those strains less tolerant to potassium sorbate (Stead 1995). Only limited data is currently available about the efficacy of natural antimicrobials from plants in real foods or beverages (Tiwari et al. 2009). Vanillin has been found to have potential for preventing the

growth of spoilage yeasts in peach-flavoured soft drink (Fitzgerald et al. 2003). Synergism between different essential oil components and between essential oils and mild preservation methods has also been observed (Burt 2004, Belletti 2007, Belletti et al. 2010). Belletti et al. (2010) showed that beverages with 500 ppm of citron essential oil needed only 3 min treatment at 55 °C to prevent the growth of *S. cerevisiae*.

Extracts from herbs, spices and berries are widely used in the beverage sector for their antioxidant and flavouring properties and their use could also be optimised whith regard to antimicrobial activity. Their efficacy is affected by many factors related to the food matrix, target microbes and the extract composition, and should be studied case by case. The use of plant extracts in effective concentration may be limited by their strong aroma and flavour, and they may also give rise to off-flavours in case of spoilage (Hammond et al. 1999; Harris et al. 2008). Purified plant antimicrobials require novel food acceptance if they are considered to be novel food ingredients. Although plant-derived antimicrobials are not likely to perform miracles, their combined use with other mild preservation technologies may produce a synergistic effect allowing preservation without chemical additives and enhancement of nutritional and sensorial qualities of beverages. Antimicrobial effects of natural herb, spice, and fruit substances in soft drink environments should be further studied to evaluate their usefulness as a part of the preservative systems.

European Commision maintains a list about permitted novel foods and food ingredients (EC 2011). Various antimicrobials can also be incorporated into packaging materials. This topic has recently been thoroughly reviewed by Vartiainen (2009).

4.9.3 Biological acidification with well characterized bacteria and fungi

Fermentation is an ancient technique for improving the microbiological safety and quality of cereal-based beverages (Blandino et al. 2003). Several types of cereal-based fermented drinks, both alcoholic and non-alcoholic sour beverages, are produced around the world. Extracts made of malted cereals (worts) contain natural sugars and provide an excellent source of health-promoting compounds, such as antioxidants and vitamins. Thus, they form an excellent base for novel types of functional drinks. However, untreated wort as such is highly susceptible to spoilage organisms. Moreover, wort contains undesirable flavour compounds,

such as carbonyls, which restrict the usage of wort in beverage bases. Bioacidifi- Bioacidification of wort with selected microbes showing good antimicrobial properties is a potential tool to increase the shelf life of cereal extracts and malt-based beverages. Thus, the stability and nutritional properties of unfermented cereal worts in soft drink formulations may be improved by controlled fermentation.

LAB are perhaps the most widely applied microbial group in food and feed fermentations. The success of LAB is due to their ability to improve safety, flavour, nutritional value and structure of the products (Salminen and von Wright 2004). Several investigations have also been conducted to examine the antimicrobial properties of LAB isolates from barley and malt and their potential against microbial contaminants in malting and brewing (Hartnett et al. 2002, Laitila et al. 2002, O'Mahony et al. 2000, Niku-Paavola et al. 1999, Vaughan et al. 2001, 2003, 2004). The microbistatic and/or microbicidic action of LAB is based on both the competition for nutrients and production of various antimicrobial compounds such as organic acids, hydrogen peroxide, bacteriocins and low-molecular weight antimicrobials (Ouwehand & Vesterlund 2004). Lowe and Arendt (2004) reviewed the potential of LAB in malting and brewing applications.

In addition to their antimicrobial potential, the use of LAB in malting and in wort bioacidification has led to improvements in malt and wort properties. Malt-derived thermophilic LAB such as *Lactobacillus delbrueckii* or *Lb. amylovorus* strains have traditionally been used in the production of biologically acidified malt, mash or wort (Back 1988, Englmann & Reichert 1991, Lewis 1998, Narziss and Heiden 1971). Biological acidification has been practised for centuries in brewing applications in which Reinheitsgebot i.e. German Purity Law is strictly enforced. The ultimate goal is to establish a defined pH level in the mash or wort without using additional acids for pH adjustment. In addition to improved microbiological stability, biological acidification has contributed to the technological and organoleptic properties of malt, wort and beer (Lewis 1998, Pittner & Back 1995, Lowe et al. 2004, Lowe et al. 2005). Several LAB have been screened for the production of new functional drinks (Tenge and Geiger 2001, Sibakov et al. 2008). LAB selected for production of functional drinks in the brewery environment should not be hop tolerant in order to avoid possible beer spoilage.

Novel types of beverages can also be produced with mixed cultures consisting of acetic acid bacteria, lactic acid bacteria and weak fermenting yeasts. Bader et

al. (2007) utilised acetic acid bacteria (*Gluconobacter)*, LAB (*Lactobacillus)* and *Kluyveromyces* yeast strains for the production of new wort-based beverage. A fruity- tasting beverage containing CO_2 contained health-beneficial acids such as gluconic acid and L(+) lactic acid. The ethanol concentration was below 0.5% and the beverage can be classified as an "alcohol-free" product. The oral intake of gluconic acid has an impact on butyrate formation in the colon, which inhibits cancer formation and may activate apoptosis in cancer cells (Tsukahara et al. 2002).

Non-alcoholic refreshment drinks produced on an organic basis right from the beginning are becoming more and more popular in the European market. Examples of such a products are Bionade and Malt Plus X, which are already on the market. Innovative beverages based on natural cereal and berry products have gained consumer attention and may open new opportunities for the breweries.

4.9.4 Traditional physical preservation techniques

Physical preservation techniques either aim at reducing the number or preventing the entry of unwanted microbes into the products. The traditional physical techniques used in the beverage industry include thermal processing and filtration.

Thermal processing is one of the most classical and effective preservation techniques. It may be used not only to preserve beverages but also to inhibit unwanted enzymatic activities (Back 2005). There are regulations about minimal thermal processing for low-acid products, since there is a significant risk for pathogen growth in these products. The regulations are set to inactivate spores of pathogenic *C. botulinum* or to prevent their outgrowth (Sperber 2009). In the beverage sector, tooth-friendly beverages and vegetable juices fall into the category of low-acid foods. Other soft drinks and alcoholic beverages are usually acid products ($pH < 4.6$) with limited food safety concerns and do not require heating by law (Sperber 2009). However, disease outbreaks from unpasteurized acid juices have led to the establishment of criteria for pathogen inactivation (FDA 2000). Acid beverages may be given a mild heat treatment (pasteurization) to eliminate vegetative cells (Ashurst and Hargitt 2009). When extra nutrients, such as fruit juice, are added as ingredients, pasteurization is usually necessary. Preservative-free soft drinks require harsher thermal processing than chemically preserved products in order to ensure

microbiological stability. The production of yeast-fermented beverages such as ciders and BMBs usually requires pasteurization to inactivate possible starter yeast cells that could otherwise cause product spoilage (Schwarzenberger 2002).

Pasteurization can be achieved by flash pasteurization by bulk liquid passage through a heat plate exchanger or in-pack pasteurization in a tunnel (Table 10). Pasteurized product can then be cold-filled or hot-filled (Stratford 2006). Flash pasteurization is typically applied to simple carbonated soft drinks that are rather resistant to spoilage (Back 2005). In-pack pasteurization is often preferred for acid products with high risk of spoilage. Flash pasteurization can also be applied to sensitive products when the filling is aseptic (Ashurst and Hargitt 2009). Hot filling is another approach for high risks products and is often used for ambient fruit juice drinks (Eckert and Riker 2007). The product is heated to the required temperature for sterilizing and filled hot in packages. Then, the filled containers are closed and inverted on line to ensure that the hot product is in contact with the closure (Ashurst and Hargitt 2009). Low-acid beverages (pH > 4.6) require harsher preservative methods and are usually processed at ultra-high temperature and aseptically filled (Eckert and Riker 2007).

Table 10. Examples of typical thermal processes in the beverage industry.

Thermal processing	Temp.	Time	Inactivation of microbes
Flash pasteurization, pH < 4.6	75–85 °C 90–96 °C	1–4 min 30–90 s	Heat-resistant moulds, alicyclobacilli may survive
Hot filling, pH < 4.6	83–88 °C 92–95 °C	0.5–1.5 min 10–15 s	Heat-resistant moulds, alicyclobacilli may survive
Tunnel pasteurization, pH < 4.6	72–80 °C	5–20 min	Heat-resistant moulds, alicyclobacilli may survive
High temperature short time treatment (pH < 4.6)	105–115 °C	0.5–4.2 min	Sterile
Ultra high temperature treatment (pH > 4.6	130–150 °C	1–9 s	Sterile, ambient storage in hermetic package possible

References: Back 2005, Ashurst and Hargitt 2009.

Yeasts and vegetative cells of bacteria are typically very sensitive to heat (Stratford 2006). Yeast D-values rarely exceed 1 min at 55 °C. Vegetative cells of beverage-spoiling microbes are usually more thermotolerant than bacterial pathogens or protozoans (Tribst et al. 2009, Lawlor et al. 2009). Shearer et al. (2002) compared the heat-resistance of several beverage-spoiling bacteria and

fungi. *S. cerevisiae* was the most thermotolerant microbe, showing D values of 13 s at 71.1 °C in apple juice. A heat resistant *Pediococcus* sp. required 16 s heating at 71.1 °C for 5-log reduction in a simulated apple cider (Piyasena et al. 2003). For 5-log-reduction of *E. coli* H157:07 and *C. parvum* in apple juice, 3 s and 6 s treatment at 71.1 °C is recommended (FDA 2000). Yeast ascospores can have 30–350 x higher thermotolerance compared to vegetative cells. For example, D60 °C values of 7–22 min have been reported for asci of *S. cerevisiae* (Tribst et al. 2009). Pasteurization treatments used in the beverage industry are usually sufficient to inactivate yeast ascospores. Vegetative cells of *P. cyclohexanicum* have been reported to survive 10 min at 95 °C in orange juice (Walker and Phillips 2007). Therefore, if initially present in raw materials this microbe might survive pasteurization treatments. Heat-resistance of food-borne viruses appears to vary greatly. The available data from surrogates suggests that some food-borne viruses may be extremely tolerant to heat (Baert et al. 2009). In milk products, heating at 71 °C resulted in 4–4.4 log reduction in infective particles of hepatitis A virus. Spore-forming *A. acidoterrestris* and certain ascomycetous moulds are the most heat-resistant microbes in the beverage industry (Silva et al. 1999, Tribst et al. 2009). The ascospores of several heat-resistant fungi such as *Byssochlamys* need an inactivation temperature of 90–100 °C (Scholte et al. 2004). For alicyclobacilli, D values from 1 min to 9.98 min at 95 °C have been reported (Sim et al. 2011).

Thermotolerance of microbes greatly depends on the beverage composition (Tchango Tchango et al. 1997) and physiological state of the cells. For example, previous adaptation to low pH may increase the thermal resistance of harmful beverage- associated bacteria such as *E. coli* 0157:H7 (Chung et al. 2006, Walker and Phillips 2007, Fernández et al. 2009). Juice fortification with calcium has been found to markedly elevate the heat resistance of *S. cerevisiae* (Shearer et al. 2002). Furthermore, fibres and other solids such as high sugar concentration may increase the heat resistance and survival of harmful organisms (Dens et al. 2003, Stratford 2006, Tribst et al. 2009). Therefore, the efficacy of thermal processing should be verified for new products. Back (2005) proposed suitable test organisms for evaluation of thermal processes in the beverage industry. Thermal death characteristics of beverage- and food-associated microbes have been collected in Lemgo Database (Schwarzer et al. 2010).

Harsh thermal processing adversely affects the sensory and nutritional qualities of beverages (Salomao et al. 2007). Hence, the control of heat-resistant

moulds in fruit processing is best established when adequately washed, high quality fruits and good manufacturing practices are used (Salomao et al. 2007). This also holds true for the control of other heat-resistant microbes.

The entry of microbes into beverages can be minimised or prevented by **factory design**, **filtration**, and **aseptic filling** techniques. It is important to separate the different production steps properly in order to prevent cross-contaminations. For example, unwashed return bottles should not be handled in the production and filling areas. In the production of BMBs and ciders, an efficient filtration step is necessary to minimise the migration of yeast cells from fermentation into the final products (Schwarzenberger 2002). It is now known that beer-adaptation of LAB may reduce cell size and increase the penetration rate of cells through membrane filters, which can be a threat to the microbiological stability of unpasteurized products (Asano et al. 2007). Filling lines in the beverage industry can be classified into three basic categories based on the magnitude of antimicrobial barriers. For a more detailed description of aseptic filling techniques see Lawlor et al. (2009) and Rysstad and Johnstone (2009).

4.9.5 Emerging non-thermal preservation techniques

Thermal processing at high temperatures easily destroys many bioactive and aroma compounds and causes changes in flavour, taste and nutritional value. Increased demand by consumers for "fresh-like" foods has led to development of technologies that do not employ heat to eliminate the spoilage microbes. One of the major limitations to their application in the beverage sector is that their use may require acceptance by the novel foods commission. Moreover, they often require highly expensive specialized equipment. Currently the leading non-thermal technology is high pressure processing.

High pressure processing (HPP) can be used to reduce microbes in liquid foods such as beverages. The pressure, ranging from 50 to 1000 MPa for periods ranging from seconds to minutes, disrupts non-covalent bonds in microbes without affecting the organoleptic properties of the product (Sperber 2009). In general, vegetative cells of bacteria and fungi are regarded as sensitive to HPP, whereas endospores are not inactivated. Inactivation of fungal spores is dependent on the solute concentration and pH. Spores may survive in the extracts with low water activity and grow rapidly when the juices are diluted. Chapman et al. (2007) showed that the age (maturity) of ascospores influences

the resistance, old spores typically showing increased resistance. HPP would need to be combined with other hurdles in order to ensure product stability. The combination of preservation methods is recommended for reducing the load of highly barotolerant strains in food (Chapman et al. 2007, Bevilacqua et al. 2008). HPP is still an expensive and laborious technique, and its use is mainly limited to premium products.

Hydrodynamic cavitation is defined as the formation of gas bubbles in a fluid due to induced pressure fluctuation (Milly et al. 2007). Hydrodynamic cavitation has been shown to be promising for inactivating *S. cerevisiae* in apple juice. Effective inhibition was achieved in reduced processing temperatures (Milly et al. 2008). Common beverage spoilers such as LAB and *Z. bailii* were also eliminated when hydrodynamic cavitation was used with mild temperature treatments (Milly et al. 2007). Hydrodynamic cavitation could be used to obtain minimally processed pasteurized low acid foods and commercially sterilized high acid fluid products.

Ionizing irradiation is electromagnetic radiation which ionizes the molecules it contacts. The doses needed to inactivate bacterial spores are much higher compared to vegetative cells (Sperber 2009). In addition, high doses are required to inactivate small targets such as viruses. The use of ionizing irradiation is strictly regulated, still belonging to the group of accepted preservation techniques.

Other emerging physical preservation techniques for beverages are **pulsed high-voltage electric field (PEF)** and ultraviolet (UV) irradiation treatments**.** PEF disrupts the microbial cell membrane, killing the microbes by lysis (Sperber 2009). PEF has not been effective against heat-resistant moulds (Tribst et al. 2009). Some papers have reported good pathogen inactivation in fruit juices when PEF was combined with antimicrobials such as citric acid, cinnamon and bark oil (Mosqueda-Melgar et al. 2008). *Lb. brevis* and *Z. bailii* were more sensitive to PEF in alcoholic beverages than in alcohol-free media (Beveridge et al. 2004). Different results have been obtained concerning the efficacy of PEF against bacterial spores (Rajkovic et al. 2010).

UV inactivates cells by cross-linking adjacent thymine molecules on DNA strands (Sperber 2009). UV irradiation is a promising alternative to thermal treatments, and may have the added benefits of retention of product quality, simplicity and lower operation costs (Koutchma 2009). FDA has approved UV treatment as an alternative to heat pasteurization for fresh juice products (FDA 2000). A novel thin film apparatus for UV light was also shown to be efficient

against *Lb. brevis* and *E. coli* in beer (4–5 log reduction) but not against *S. cerecerevisiae* (Lu et al. 2010). It shows promise for treatment of beverages, although its use is still limited due to the low UV transmittance of liquid foods. However, UV irradiation may cause undesirable effects such as damage to vitamins and proteins and formation of off-flavours and aromas. UV irradiation has also been used for increasing process surface hygiene (Tapani et al. 2009).

4.9.6 Hurdle technology and multitarget preservation

The concept of hurdle technology is widely applied in food preservation. It aims at improving total quality of foods by applying gentle preservative factors (hurdles) with synergistic effect (Leistner 2000). The hurdles are intentionally combined to improve the microbiological stability, sensory and nutritional properties of the products (Leistner 2000, Labbé and Nolan 2009). The most important hurdles used in preservation systems of beverages and their ingredients are acidity (pH), temperature (high and low), low water activity (a_w), low redox potential (E_h), carbonation, preservatives (sorbates, sulphites), and competitive microbes (such as LAB). Hurdle technology allows minimal processing, and hence preserves sensory and nutritional quality without compromising microbiological stability or safety. Appropriate application of hurdle technology requires understanding of the interaction of hurdles and physiological responses of harmful microbes. This so called multitarget preservation aims at applying hurdles that act on multiple cellular targets to further minimise processing (Leistner and Gorris 1995). This means that the hurdles included in food should impact on undesirable microbes in several different ways.

Microbiological stability and safety of beverages has traditionally been based on interactions of several antimicrobial hurdles. With increasing understanding of the mode of action of different preservative agents and techniques, more rational application of this approach is becoming possible. Modelling helps in studying interactions of multiple factors by reducing the number of required tests to a practical level.

Hurdle technology has been successfully applied to prevent germination of *A. acidoterrestris* spore,s as reviewed by Bevilacqua et al. (2008). For example, the combination of thermal treatment at 90ºC for 8 min with the use of 80 ppm of cinnamaldehyde was effective in reducing sporulation. Predictive models have been developed to assess the probability of yeast spoilage as a function of

acidity, sugar content and binary preservative combinations (Battey et al. 2001), and as a function of thermal treatment time, added aroma compounds and initial contamination level (Belletti et al. 2007 and 2010). Dai et al. (2010) studied the effects of sorbate and benzoate alone and with lauric arginate and cinnamic acid on the growth of spoilage yeasts. A model was developed to identify triplet antimicrobial combinations with equal efficacy to that of yeast growth inhibitors. The three antimicrobials acted synergistically at pH 3, 3.5 and 4. Battey et al. (2001) noticed that a combination of low pH and higher preservative concentrations caused inhibition of the spoilage moulds *A. niger* and *P. spinulosum.* Contamination by *C. parvum* could be controlled by hydrogen peroxide alone or in combination with inactivation methods such as UV, freezing, or ozone treatments (Kniel et al. 2003).

4.10 Experimental assessment of beverage safety and stability

Whenever new formulations are developed it is important to go through every change made in the recipe in order to consider the microbial risks. The conventional way to ensure microbiological safety and stability of new formulations is to run **challenge tests** on them (Sperber 2009). In microbial challenge tests the tested food is inoculated with relevant spoilage microbes. The growth is monitored and the alterations to recipes can be made according to knowledge obtained from monitoring. Challenge tests should be performed with stressed cells, because they usually tolerate harsher environmental conditions than unstressed ones (Rowan 1999). Moreover, it is advisable to use recently isolated cultures of spoilage organisms that have retained their spoilage properties better than “laboratory adapted” cultures.

The use of **mathematical models** to describe and predict microbial responses (growth, survival, and inactivation) in relation to controlling factors in food environment has been popular for the last 20 years (Panagou et al. 2010). **Predictive microbiology** translates the primary patterns of microbial behaviour to mathematic models that can be used to predict microbial behaviour under various environmental conditions. Predictive microbiology has typically focused on the creation of pathogen models, and these models are nowadays widely used as risk management tools in the food industry (for a review see Tamplin 2009). Predictions of the spoilage sensitivities of certain foods are made with spoilage models. Prediction of spoilage is more difficult than prediction of pathogen

growth (Sutherland 2003), because spoilage of food is usually estimated from organoleptic changes. Spoilage models could be useful, for example, to predict microbial stability in new product development and to evaluate the stability of products treated with combined preservation techniques. Predictive models potentially reduce the time and cost involved in microbial challenge testing. The need to generate predictive data for fruit juice microbiology is clear (Tribst et al 2009). Predictive modelling for understanding mycotoxin production in fruit juices would be highly important. It is often said that "all models are wrong: the question is how wrong they have to be not to be useful?" (Sutherland 2003). In the future, the data generated from system biological studies (proteomics, genomics, metabolomics) combined with process- and product-specific factors may facilitate the building of mechanistic models.

5. Conclusions

Microbiology of functional and specialty beverages as well as novel beverage ingredients has been poorly studied. In this review, potential microbiological risks related to their production have been evaluated mainly based on their intrinsic and extrinsic properties and on the microbiology of similar products.

Many modern beverages produced have **less antimicrobial hurdles** compared to traditional carbonated soft drinks due to higher level of nutrients supporting microbial growth, lower acidity and/or milder carbonation level. There is also growing pressure to reduce thermal processing and the use of chemical preservatives in order to produce "natural" products. The reduction of the antimicrobial hurdles in beverage production can be expected to increase the product spoilage rate unless the gap is filled with an increase in process hygiene or with new hurdles.

The major **spoilage microbe types** in modern beverages will probably remain the same as in the traditional soft drinks, but the range of spoilage species is expected to increase. Spoilage cases by previously harmless yeast and LAB that are common in the brewery environment may be expected to become increasingly common. As a harmless organism in one product can spoil the other one, the filling practices in breweries producing non-beer beverages should be reviewed.

Bacteria are expected to gain increasing importance especially in the spoilage of mildly acidic (pH > 3.5–4.0) and carbonated products. The growth of enterobacteria and spore-forming bacteria may become possible in mildly acidic conditions. Still and mildly carbonated products filled in PET bottles may allow the growth of aerobic spoilage bacteria. In juice-rich formulations, a variety of juice spoilage bacteria such as **aerobic acid-tolerant bacteria** (*Alicyclobacillus*, acetic acid bacteria) and **aerotolerant propionibacteria** may emerge as spoilage organisms. *Propionibacterium cyclohexanicum* is a new fruit juice contaminant

that survives pasteurization and tolerates well both weak organic acids and low pH. New functional and exotic ingredients and the unconventional applications of established ingredients may also provide new growth substrates or bring new harmful microbes and their metabolites into the products. For example, acetic acid bacteria of the genus *Asaia* have emerged as spoilage organisms in flavoured mineral waters.

Increasing the **ingredient import** and the increasing trend of using **low-acid juice ingredients (pH > 4.6)** may give rise to new microbial safety risks. Pathogenic bacteria may not only survive but can also grow in the low-acid fruit and vegetable juices and sweet wort. Juices and cereal-based ingredients, especially apple juices, may also contain elevated levels of mycotoxins. Many modern beverages could also allow better survival of pathogens compared to the traditional soft drinks. Some products resemble more and more natural fruit juices with known associations with food-borne illnesses or contain ingredients that could protect microbial cells. *Escherichia coli* 0157:H7 is the most likely known microbial threat in juice-rich beverages due to extreme acid-tolerance and the low infective dose of this organism. Low-acid products are able to support the growth of various food pathogens which needs to be taken into consideration in the product development and preservation. More information is needed about the behaviour of pathogens in the modern beverages to evaluate the real risks.

Changes in **climate conditions** may result in the appearance of “emerging” or “new” pathogens. Irrigation of crops is expected to increase, possibly using poor-quality waters, which is then likely to increase the occurrence of microbial contaminants in raw materials used in food and beverage production. Examples already exist. Irrigation of raspberries with contaminated water has transmitted noroviruses. This could be hazardous especially in the smoothie type beverages.

Mycotoxins are considered as a worldwide problem and their occurrence in food and feed chains is expected to increase due to climate change. Therefore, there is a need 1) to control toxigenic fungi in the production of raw materials, 2) to develop means to remove preformed toxins in the contaminated materials and 3) to develop early-warning tools for mycotoxin production and detection of mycotoxins. Production of fungal hydrophobins is also linked to variable environmental conditions and attachment of fungi on plant surfaces. Changes in weather conditions may lead to increased fungal contamination and production of fungal hydrophobins in cereals and fruits (such as apples and grapes), thus increasing the gushing risk of beverages.

It is generally accepted that the microbiological quality of beverage and food production should primarily be ensured by a **preventative approach**. Special attention should be paid to microbial quality of the risk ingredients. Whenever new formulations are developed it is important to go through every change made in the recipe in order to consider the microbial risks. For instance, some functional components, such as dietary fibres, may protect microbes from thermal inactivation. It must also be taken into account that low-acid beverages (pH > 4.6) will require pasteurization and subsequent cold-storage or sterilization for microbiological safety. **Predictive microbiology** helps in describing and predicting the behaviour of harmful microbes and optimising the preservative systems, reducing the costs and time involved in the challenge testing.

Microbial adaptation to stressful conditions should be taken into account when designing the preservation and quality control of beverages. Traditional culture methods may underestimate the magnitude of real contamination due to the presence of viable but non-culturable microbes. Research is needed for optimization of the detection of stressed cells and to develop early warning systems for the quality control of beverages. **Molecular techniques** are the only feasible option for the detection of viruses and parasites in the beverage industry.

A future challenge in the beverage and food sector is to produce **safe and acceptably stable products with minimal processing**. Current preservatives and natural antimicrobials have somewhat limited antimicrobial activity. It is unlikely that new food preservatives will be accepted in the near future. Therefore, exploitation of the synergistic effects of the existing natural antimicrobials with the existing GRAS substances and mild physical preservation methods is the most potential approach for controlling harmful microbes in beverages in the future. Botanical extracts and aroma compounds already used in the beverage formulations have potential as natural antimicrobials, and could be exploited for the purpose of preservation. Biological acidification of the beverage bases is another approach with great potential for enhancing stability, safety and nutritional and sensory quality of the beverages. The future of beverage preservation techniques will be a skilled combination of antimicrobial hurdles to maintain microbiological stability and safety while maintaining maximum sensory and nutritional quality.

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Appendix A: Properties of common beverage-spoiling microbes

Spoilage microbes	min pH	Growth (ºC)	Oxygen	Energy sources	Nitrogen sources	Vitamin requirement	Carbonation tolerance	Resistance to sorbates and benzoates	Special properties
LACTIC ACID BACTERIA:	2.9-3.5	3-55	Micro-aerophilic	Sugars, organic acids	Amino acids	Variable	High		
Leuconostoc mesenteroides	4.8	10-<45	Micro-aerophilic	Various sugars	Amino acids (glutamate, valine)	B-group, folic acid not	High		Dextran from sucrose
Lactobacillus paracasei		10-40	Micro-aerophilic	Sugars, organic acids	Amino acids	Panthotenic nicotinic acid	High		
ACETIC ACID BACTERIA:	3.0-4.5	5-40	Aerobic	Sugars, alcohols, organic acids	Amino acids, NH_4		<1.5 vol	Possible	Heat-sensitive
Gluconobacter oxydans	≤3.6	≤42	Aerobic	Simple sugars	NH_4			Possible	Acetic acid from ethanol
Acetobacter aceti	3.2	≤48	Aerobic	Sugars, alcohols, organic acids	NH_4			Possible	CO_2 and H_20 from acetic acid
ALICYCLO-BACILLI	2-2.5	25-60	Aerobic	Sugars, polyols	Amino acids		<1.5 vol		Extremely heat-resistant spores.
MOULDS:	0.5-3.5	6-47	Aerobic	Many organic substrates	Amino acids, NO_3, NH_4	Some need	<1.5 vol	Possible	Degradation of preservatives
Aspergillus niger			Aerobic	-II-	-II-		<1.5 vol	High	
Penicillium spinolosum			Aerobic	-II-	-II-		<1.5 vol	High	
YEASTS:	1.5-3.5	0-48	Aerobe/ Facultative	Simple sugars	Amino acids, NH_4, (NO_3, NO_2)	B-group by some species	High	Possible	
Dekkera spp.	1.8-2.3	>5	Facultative	Simple sugars	Amino acids, NH_4, NO_3	Thiamine and biotine	5-6 vol	Moderate sorbate tolerance	Acetate from glucose, hydrolytic enzymes
Saccharomyces cerevisiae	1.8	4-13	Facultative	Sugars	Amino acids, NH_4	Variable	5	Some strains. Possibly SO2 tolerant.	Ascospores D60°C 5.1-17.5 min
Zygosaccha-romyces bailii	2.2-2.5	6.5-13	Facultative	Sugars. NO sucrose fermented.	Amino acids, NH_4	B-group	5	High. Also tolerant to SO_2 and acetate.	Osmotolerant, ascospores D60°C 8-14 min

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Appendix B: Some of the regulations on mycotoxins in foods and beverages (Table 1), and on *Fusarium* mycotoxins in foods and beverages (Table 2)

Table 1. Some of the regulations on mycotoxins in foods and beverages (EC Regulation 1881/2006).

Mycotoxin	Foodstuffs	Maximum levels (μg/kg)
Aflatoxin	2.1.5 Dried fruit and processed products thereof, intended for direct human consumption or use as an ingreadient in foodstuffs	B_1: 2 Sum of B1,B_2, G_1 and G_2 :4
	2.1.6. All cereals and all cereal products derived from cereals, including processed cereal products, with the exception of foodstuffs listed in 2.1.7 (maize), 2.1.10 (baby-foods and young children), 2.1.12 (dietary foods for medical purposes)	B_1: 2 Sum of B1,B_2, G_1 and G_2 :4
	2.1.10 Processed cereal-based food and baby-foods for infants and yound children	0.10
	2.1.12 Dietary foods for special medical purposes, intended specifically for infants	0.10
Ochratoxin A	2.2.1 Unprocessed cereals	5.0
	2.2.2 All products derived from unprocessed cereals, including processed cereal products abd cereals intended for direct human consumption with the exception of foodstuffs listed in 2.2.9 and 2.2.10	3.0
	2.2.3 Dried vine fruits (currants, raisins and sultanas)	10
	2.2.4 Roasted coffee beans and ground roasted coffee, excluding soluble coffee	5.0
	2.2.5 Soluble coffee (instant coffee)	10.0
	2.2.6. Wine (including sparkling wine, excluding liquer wine and wine with an alcoholic strength of not less than 15% vol and fruit wine	2.0
	2.2.7 Aromatised wine, aromatised wine-based drinks and aromatised wine product coctails	2.0
	2.2.8 Grape juice, concentrated grape juice as reconstituted, grape nectar, grape must and concentrated grape must as reconstituted, intended for direct human consumption	2.0
	2.2.9. Processed cereal-based foods and baby foods for infants and young children	0.50
	2.2.10 Dietary foods for special medical purposes intended specifically for infants	0.50
Patulin	2.3.1 Fruit juices, concentrated fruit juices as reconstitute and fruit nectars	50
	2.3.2 Spirit drinks, cider and other fermented drinks derived from apples or containing apple juice	50
	2.3.3 Solid apple products, including apple compote, apple puree intended for direct consumption	25
	2.3.4 Apple juice and solid apple products, including apple compote and apple puree, for infants and young children and labelled and sold as such	10.0
	2.3.5 Baby foods and other processed cereal-based foods for infants and young children	10.0

Table 2. Some of the regulations on *Fusarium* mycotoxins in foods and beverages (EC Regulation 1881/2006).

Mycotoxin	Foodstuffs	Maximum levels (µg/kg)
Deoxynivalenol	2.4.1 Unprocessed cereals other than durum wheat, oats and maize	1250
	2.4.2 Unprocessed durum wheat and oats	1750
	2.4.3 Unprocessed maize with the exception of unprocessed maize intended to be processed by wet milling	1750
	2.4.4 Cereals intended for human consumption, cereal flour, bran and germ as end product marketed for direct human consumption, with the exception of foodstuffs listed in 2.4.7, 2.4.8 and 2.4.9	750
	2.4.7 Processed cereal-based food and baby-foods for infants and young children	200
Zearalenone	2.5.1 Unprocessed cereals other than maize	100
	2.5.2 Unprocessed maize with the exception of unprocessed maize intended to be processed by wet milling	350
	2.5.3 Cereals intended for human consumption, cereal flour, bran and germ as end product marketed for direct human consumption, with the exceptions listed in 2.5.6, 2.5.7, 2.5.8, 2.5.9 and 2.5.20	75
	2.5.7 Processed cereal-based foods (excluding processed maize-based foods) and baby foods for infants and young children	20
Fumonisins	2.6.1 Unprocessed maize with the exception of unprocessed maize intended to be processed by wet milling	4000
	2.6.2 Maize intended for direct human consumption, maize-based foods for direct human consumption, with the exceptions of foodstuffs listed in 2.6.3 and 2.6.4	1000
	2.6.3 Maize-based breakfast cereals and maize-based snacks	800
	2.6.4 Processed maize-based foods and baby-foods for infants and young children	200
T-2 and HT-2	2.7.1 Unprocessed cereals and cereal products	sum of T-2 and HT-2 toxin onhold

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Microbiological spoilage and safety risks in non-beer beverages

Abstract

During the past ten years major changes have occurred in the global beverage market. Functional beverages and bottled waters constitute the fastest growing sectors. Energy drinks and non-alcoholic malt beverages are also gaining popularity. This literature review aims to provide state-of-the-art knowledge on microbial spoilage and safety risks in non-beer beverages with emphasis on functional and specialty products.

Many modern beverages have higher level of nutrients for microbial growth, lower acidity and / or milder carbonation level compared to traditional soft drinks. Thermal and chemical preservation have also been reduced. These changes in the beverage production are expected to increase the spoilage rate unless the gap is filled with new antimicrobial hurdles. Yeasts and lactic acid bacteria will probably remain the major spoilage microbe types also in the modern products, but the range of species is expected to increase. Bacteria are likely to gain increasing importance in the spoilage. Emerging spoilers include e.g. alicyclobacilli, *Asaia*, clostridia, entero- and propionibacteria.

Possible new microbial health risks may arise from the use of low-acid juice ingredients and increasing ingredient import. Pathogenic bacteria may not only survive but can also grow in the low-acid fruit and vegetable juices. Moreover, novel ingredients may improve the survival of pathogens in the beverages, or bring new harmful microbes or their metabolites into products. More research is needed about the occurrence and faith of pathogens and emerging spoilers in the modern non-beer beverages.

Whenever new products are developed, it is important to go through every change in the recipe, packaging and processing in order to consider microbial risks. The future challenge is to produce microbiologically safe and stable beverages while maintaining maximum sensory and nutritional quality. Exploiting the synergistic effect of natural antimicrobials together with GRAS substances and mild physical preservation is a potential approach for controlling harmful microbes in beverages. Predictive microbiology can help in optimising the preservative systems and describing the behaviour of contaminants in complex non-beer beverages.

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