Microbiologically sensitive beverages – a risk assessment system

ASSESSMENT SYSTEM | Microbiological stability of non-alcoholic, low-alcohol and mixed beer beverages is frequently inferior to that of comparable full beers. Protective factors inhibiting microbial growth in beer are fewer or "diluted" or not present at all. However, new protective factors may also come to the fore. As reliable data for objectively assessing microbiological risks associated with these beverages is, in most instances, not available, a system for assessing such risks has been developed.

THE GERMAN BEER MARKET is in the process of change. For years, market share of beer has been declining in Germany whereas low-alcohol, non-alcoholic and mixed beer beverages are becoming ever more popular [1,2]. However, these beverages are considerably more sensitive in terms of microbiology compared to classical straight full beer. In low-alcohol or non-alcoholic beers and in mixed beer beverages, protective barriers that render beer an unsuitable nutrient medium for most microorganisms, i.e. ethanol content, hop bitter acid content, low pH, lack of nutrient and growth substances as well as the anaerobic environment, are present to a lesser extent or completely absent. These beverages can thus be classed as microbiologically sensitive beverages [3]. As reliable data on the microbial risk that these beverages are subjected to is oftentimes not available, a microbiological analysis and assessment system has been developed that makes it possible to assess this risk potential.

Microbiologically sensitive beers

Mixed beer beverages and sensitive beer types such as low-alcohol and non-alcoholic beers as well as hop-reduced beers may be more susceptible to microbiological spoilage than "standard" beer types. In this study, sensitive beer types and mixed beer beverages were inoculated with a selected set of pre-adapted microorganisms in order

Authors: Dipl.-Ing. Robert Riedl (photo), Dipl.-Ing. Jennifer Koob (photo) and Dr.-Ing. Mathias Hutzler, Weihenstephan Research Centre for Brewing and Food Quality (BLQ), TU München, Freising, Germany; Dipl.-Ing. Christian Hackl, Brau Union Österreich AG, Linz, Austria; and Dr.-Ing. Fritz Jacob, Weihenstephan Research Centre for Brewing and Food Quality (BLQ), TU München, Freising, Germany

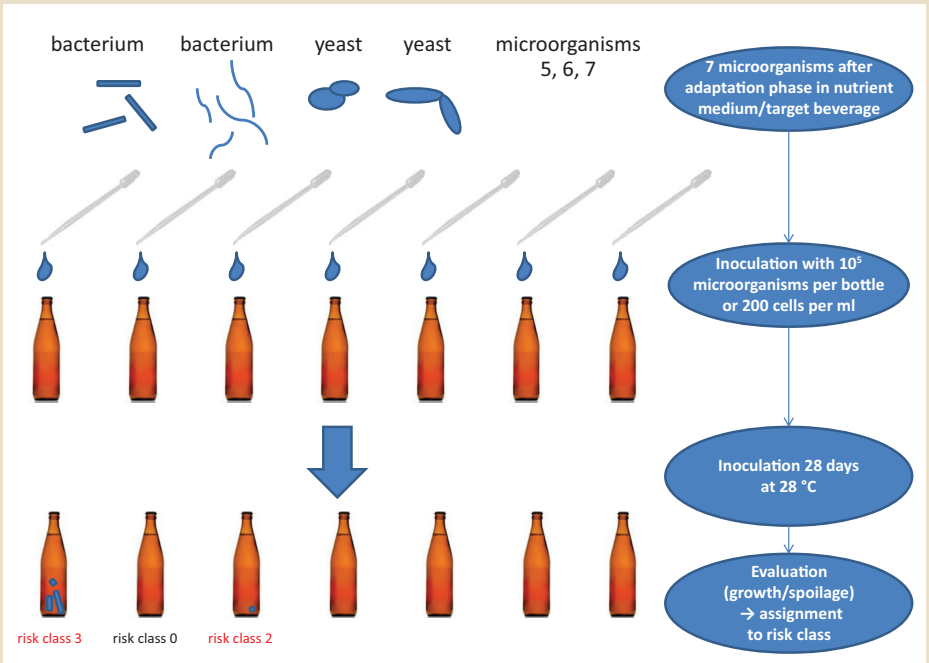


Fig. 1 Schematic representation of risk analysis (7 different microorganism strains serve as an example)

SELECTION OF MICROORGANISMS USED AND THEIR CHARACTERISATION IN TERMS OF BREWING BIOLOGY

Classification microorganisms	Microorganism set A: non-alcoholic, low-alcohol beer, beer, standard beer	Microorganism set B: mixed beer beverages, standard mixed beer beverage	Characterisation in terms of brewing microbiology
Beer-spoilage bacteria	1. *L. brevis* FZ BLQ 4	1. *L. brevis* FZ BLQ 4	**Slime former *Lactobacillus brevis***. Strain isolated from beer with 4.5 vol %, 18 BU. Infection established in filler surroundings and also in wet cardboard packaging. This microorganism occurs in biofilms in formation **steps 2 and 3** i.e. in the **facultative to strictly anaerobic phase**. As a result of slime formation, evaluation is also possible in hazy beers.
	2. *Pectinatus portalensis* FZ BLQ HBS1	–	***Pectinatus* is a typical secondary contaminant**. It occurs in biofilms in the **3rd formation step** i.e. in the strictly anaerobic phase. It is not hop-sensitive i.e. high bitterness units have no influence on its growth. Depending on place of isolation, some *Pectinatus* strains can be sensitive to elevated alcohol contents (> 3 vol %) and reduced pH values (pH < 4.4). However, under laboratory conditions, *Pectinatus* strains have gradually been trained to survive in up to 12.5 vol % and pH = 3.5. *Pectinatus* very frequently occurs in the filler surroundings as so-called spreading infections. This microorganism is increasing in importance. In 2010 and 2011, *Pectinatus* infections were on the increase.
***Saccharomyces* brewer's culture yeasts**	3. *Saccharomyces cerevisiae* FZ BLQ H TUM 68	3. *Saccharomyces cerevisiae* FZ BLQ H TUM 68	**Most common top-fermenting wheat beer yeast**. Top-fermenting brewer's yeast is usually not found in biofilms. Carry-over into the filling section (e.g. biofilms) from the primary production section is possible. Top-fermenting culture yeast can readily exist in biofilms in the 2nd and 3rd formation steps. However, *Saccharomyces cerevisiae* wild yeasts are more frequently encountered as secondary contaminants in the filling section.
	4. *Saccharomyces pastorianus ssp. carlsbergensis* FZ BLQ H TUM 34/70	4. *Saccharomyces pastorianus ssp. carlsbergensis* FZ BLQ H TUM 34/70	**Most common bottom-fermenting brewer's yeast**. Carry-over into the filling section (e.g. biofilms) from the primary production section is possible. Bottom-fermenting culture yeast can exist in biofilms of the 2nd formation step. Under ambient conditions, it is more sensitive than top-fermenting culture yeast. As, in general, it grows more slowly than wild yeasts at higher temperatures, it cannot compete with the latter in biofilms in the long term.
***Saccharomyces* foreign yeast**	5. *Sacch. cerevisiae* var. *diastaticus* FZ BLQ TUM SY 1	5. *Sacch. cerevisiae* var. *diastaticus* FZ BLQ TUM SY 1	**Overfermenting *Saccharomyces* spoilage yeast** that, as a result of glucoamylases, can ferment long-chain dextrins that cannot be fermented by standard brewer's yeasts. *Sacch. cerevisiae* var. *diastaticus* is a typical secondary contaminant. Due to its overfermenting properties, it is very much dreaded for causing beverage containers to burst. This yeast typically occurs in biofilms of step 2 and 3.
Non-*Saccharomyces* foreign yeasts	6. *Dekkera anomala* FZ BLQ 2-C-1	6. *Dekkera anomala* FZ BLQ 2-C-1	***Dekkera anomala*** is a typical spoilage yeast for beer and non-alcoholic beverages. Some of these yeasts can grow in beverages with relatively few nutrients, **similar to *Saccharomyces cerevisiae* var**. diastaticus. This yeast is increasingly present in the context of mixed beer beverages and carbonated sugar-containing non-alcoholic beverages.
	7. *Wickerhamomyces anomalus* FZ BLQ 17-C-3	–	***Wickerhamomyces anomalus* is one of the most frequent yeast types** in breweries. It can arise as soon as biofilms start to form and is the dominant yeast species in biofilms in breweries. This yeast can normally not grow in beers with standard alcoholic contents, low residual extract and a very low residual oxygen concentration i.e. it is present latently. When several of these factors are absent, growth can generally occur.
	–	8. *Rhodotorula mucilaginosa* FZ BLQ17-L-2	***Rhodotorula mucilaginosa*** is a yeast that forms red colonies. It is a **typical aerobic yeast** and can grow in products only in the presence of high residual oxygen contents. It can **occur in the aerobic phase of biofilms** (mostly **steps 1-2**). It frequently presents itself as **spoilage yeast in non-carbonated non-alcoholic beverages**.
	–	9. *Kazachstania exigua* FZ BLQ H 2-G-7	***Kazachstania exigua* is a potential spoilage yeast** i.e. this yeast type can occur when selective properties are reduced and, among other things, when residual extract is elevated. Little is found about the presence of this yeast in biofilms. It should be well equipped to exist in **steps 2 and 3. This yeast was formerly called *Saccharomyces exiguus*.**

Table 1

to observe the product-specific spectrum of harmful microorganisms. All or almost all microorganisms selected exhibit growth in a very sensitive beverage. This boils down to the fact that a wider range of microorganisms can grow in a sensitive beverage compared to a standard beer. The objective of this study was to determine the microbiological risk status or, in other words, the microbiological stability of beers and mixed beer beverages analysed. Figure 1 is a schematic of the analysis steps i.e. adaption, inoculation, incubation and evaluation.

Schematic of analysis

Seven different microorganism strains were inoculated into seven bottles of product (one strain per bottle). All microorganisms had been adapted to the target product beforehand. This adaptation took the form of inoculating the microorganisms into a mixture of 75 per cent target product and 25 per cent double concentrated nutrient broth (MRS or MIB for bacteria, YM for yeasts). In respect of all beverages tested, the strains tested were able to start growing in the target product/nutrient broth mixture. The adapted microorganisms were inoculated into the target product with a concentration of 200 cells/ml. In the case of non-alcoholic beers (NAB) and low-alcoholic beers (LAB), malt beers and standard beers, microorganism set A listed in table 1 was inoculated. Table 1 describes the microorganisms selected from a beverage technology and biology aspect. Microorganism set B listed in table 1 was inoculated into mixed beer beverages. The bottles were closed and incubated for 28 days at 28 °C. After 7, 17 and 28 days, the samples were visually assessed for haze, gas formation, biofilm formation and agglomeration. Spoilt samples were subdivided into "+/-" showing slight growth and "+" for growth. The bottles were opened after 28 days and the cell concentration was determined either microscopically using a Thoma chamber or by using the method "decadic dilution series/agar culture" (depending on degree of growth). Each result of the growth analysis of a microorganism was assigned to risk categories listed in table 3. Risk categories range from 0 (no growth) to 3 (strong growth). The risk categories associated with the individual microorganisms were added up and averaged for each beverage. A microorganism set of seven microorganisms can add up to a maximum sum of risk

SPECIFICATIONS OF BEER TYPES TESTED

NAB1	Non-alcoholic beer			bottom-fermented	
	vol %	**BU**	**pH**	**CO_2 w/v**	**F.S. g/100 ml**
	0.42	25	4.26	0.52	3.14
NAB2	Non-alcoholic beer			top-fermented	
	vol %	**BU**	**pH**	**CO_2 w/v**	**F.S. g/100 ml**
	0.4	11.7	4.26	0.58	3.46
ARB1	alcohol-reduced beer			bottom-fermented	
	vol %	**BU**	**pH**	**CO_2 w/v**	**F.S. g/100 ml**
	3.04	24.5	4.34	0.51	1.6
ARB2	alcohol-reduced beer			top-fermented	
	vol %	**BU**	**pH**	**CO_2 w/v**	**F.S. g/100 ml**
	4.2	10.5	4.32	0.6	1.3
B1	dark beer with elevated residual extract			bottom-fermented	
	vol %	**BU**	**pH**	**CO_2 w/v**	**F.S. g/100 ml**
	4.39	19.5	4.39	0.49	1.9
Standard beer	Helles vollbier			bottom-fermented	
	vol %	**BU**	**pH**	**CO_2 w/v**	**F.S. g/100 ml**
	5.1	22	4.4	0.53	0.2
MBB1	MBB			bottom-fermented	
	vol %	**BU**	**pH**	**CO_2 w/v**	**F.S. g/100 ml**
	1.9	8	3.5	0.5	5.3
MBB2	MBB			bottom-fermented	
	vol %	**BU**	**pH**	**CO_2 w/v**	**F.S. g/100 ml**
	2.5	9.8	3.65	0.5	0.06
					aspartame
Standard MBB BF	Standard mixed beer beverage (beer and lemonade)			bottom-fermented	
	vol %	**BU**	**pH**	**CO_2 w/v**	**F.S. g/100 ml**
	2.39	14.3	3.77	0.53	4.15

vol % = per cent by volume alcohol *BU = bitterness units*

CO_2 w/v = carbon dioxide concentration in weight/volume per cent

F.S. g/100 ml = fermentable sugars in gram per millilitre

Table 2

RISK CATEGORIES CLASSIFIED BY GROWTH

Risk class	**Categorisation**
	(indication based on parameters)
0	**no growth**
	(no haze after 28 days)
1	**very slight/little growth**
	(after 28 days ± or >0.005 million cells/ml and ≤0.05 million cells/ml)
2	**weak growth**
	(± or + after 28 days and > 0.05 million cells/ml and ≤ 1 million cells/ml)
3	**strong growth**
	(+ after 7 days or 14 days or > 1 million cells/ml after 28 days)

Table 3

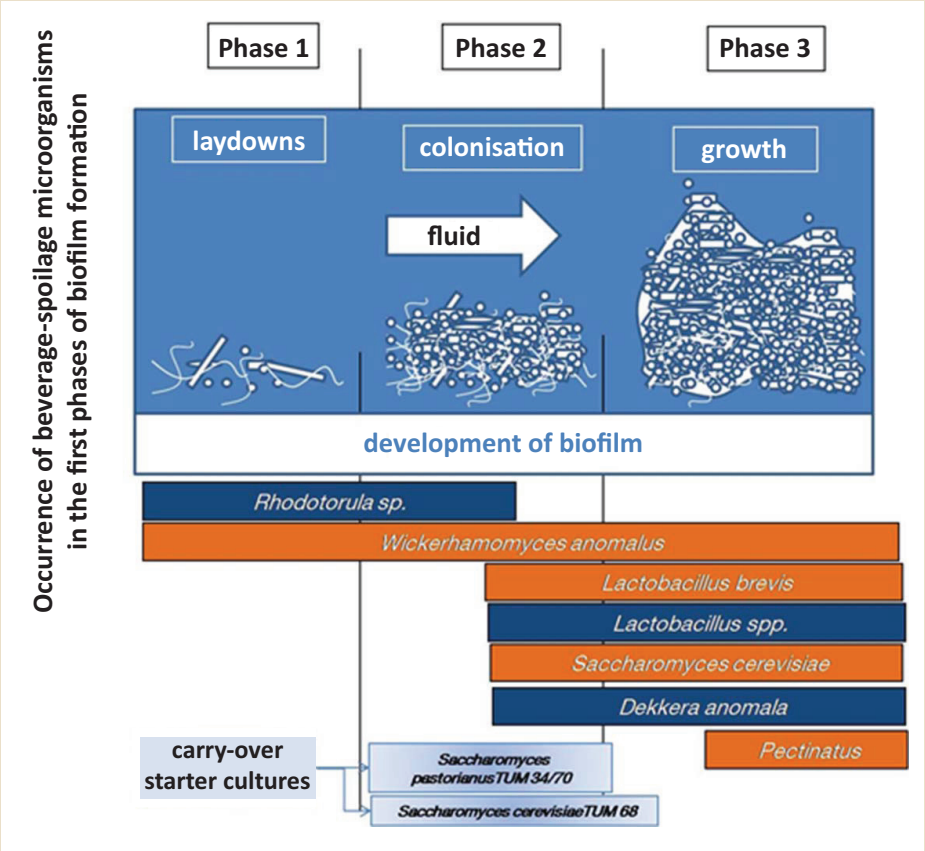


Fig. 2 Schematic of selection of microorganisms based on contribution to biofilm formation

EVALUATION OF ALCOHOL-REDUCED WHEAT BEER ARB2 USED AS AN EXAMPLE ...

... including classification into risk classes and risk assessment ∑and Ø

Parameter, time:	Haze/ visual damage			Cell concentration (million/ml)**	Risk assessment
	Day 7	Day 14	Day 28	Day 28	
Microorganisms					
1. *L. brevis*	+	+	+	50.4	3
2. *Pectinatus portalensis*	*	*	*	0	0
3. *Saccharomyces cerevisiae*	+/–	+/–	+/–	0.04	1
4. *Saccharomyces pastorianus ssp. carlsbergensis*	*	+/–	+	0.89	2
5. *Sacch. cerevisiae var. diastaticus*	+	+	+	4.0	3
6. *Dekkera anomala*	+	+	+	1.9	3
7. *Wickerhamomyces anomalus*	+/–	+	+	0.64	3
(+) strong growth, haze (+/–) slight growth, haze (–) no growth, haze					∑
**haze caused by microorganisms visually not evaluable: hazy product*					15
***if microorganism concentration is below the detection threshold of microorganism counting chambers, cell concentration is set at 0 to facilitate graphic representation*					Ø
					2.14

Table 4

points of 7 x 3 = 21 risk points and an average risk rating of (7 x 3) / 3 = risk group 3. This means that a sample belonging to risk group 3 is at a high risk of being spoiled by a microorganism. A beverage is generally very sensitive to microbiological spoilage when all microorganisms inoculated exhibit strong growth and are, accordingly, classed in risk group 3 so that the average maximum risk group 3 is obtained. The sum as well as the average risk category was determined for each beer sample and mixed beer beverage tested. The sum and average values for beers and mixed beer beverages can be compared with the standard beer and standard mixed beer beverage. Beer types tested and their chemical-physical properties are shown in detail in table 2.

Targeted selection of microorganisms

Table 1 and figure 2 "describe the characteristics of the microorganisms used for brewing microbiology and their role in biofilms as well as the phases of biofilm formation in which the microorganisms used typically grow". Figure 1 shows a simplified model of biofilm development phases up to the growth phase (different models with different determinations of phase description are presented in the literature). Species/strains were selected from the microorganism sets, these have different risk potential. Typically, they can arise as secondary contamination, in particular in biofilms [4-9]. Due to production errors, live culture yeasts can be carried over into the secondary section, usually downstream of filtration. For that reason, two typical representatives of culture yeasts were also tested.

Assessment using an example

Table 4 lists the assessment of the alcohol-reduced ARB2 wheat beer by way of example. Based on visually monitored growth, a risk class is assigned reflecting growth vigour and averaged over the organism set.

Lactobacillus brevis, *Saccharomyces cerevisiae* var. *diastaticus*, *Dekkera anomala* and *Wickerhamomyces anomalus* show strong growth in this beer type. This results in a sum of 15 risk class points and an average of 15/7 = 2.14.

Pectinatus did not grow in this beer type, beer culture yeasts showed only weak growth. It is interesting that microbial growth and product damage by the *Lactobacillus brevis* FZ-BLQ4 strain due to its characteristic of being able to form slime was observed, despite the natural haze. Thus, this *Lactobacillus brevis* strain is particularly suitable for inoculation into naturally hazy beers. Accordingly, the microbiological risk of this beverage should be regarded as high. The risk groups for the individual microor-

ganisms tested are summarised in table 5 for microorganism set A and in table 6 for microorganism set B. Figure 3 is a graphic representation of the sum of risk category points and their average.

Sensitive non-alcoholic and alcohol-reduced beers

According to this scheme, the two non-alcoholic beers are classed as being microbiologically very sensitive because all microorganism strains tested grow – with the exception of *Lactobacillus brevis* in NAB1. When looking both at the sum and the average of the risk category points, values are thus clearly higher, also compared to the other beer types tested. It was found in follow-on studies that the *Lactobacillus brevis* strain tested can survive up to a bitter acid content of, on average, 22 BU. This goes to explain the absence of growth in NAB1 having a higher value i.e. 25 BU. When using a hop-tolerant strain, the associated growth of *Lactobacillus brevis* would further increase the average risk category points. Strong growth of culture and wild yeasts can be attributed to the relatively high content of fermentable sugars.

The two alcohol-reduced beers show growth of wild yeasts comparable to that in the non-alcoholic beers. Culture yeasts start vigorous growth also in ARB1 whereas growth was weak in ARB2. *Lactobacillus brevis* grows only in ARB2, this is attributable to the bitter acid content as was observed in non-alcoholic beers. Build-up of tolerances during adaptation to a new medium was observed for various species [10]. The reverse would be also conceivable, i.e. that the same tolerances can drop back during strain maintenance and storage. This would explain why the *Pectinatus* strain used grew only in non-alcoholic beers. During storage, the alcoholic content could have diminished on non-selective media. When using more hop or alcohol resistant bacterial strains, risk category points would be even higher. Thus, the microbiological risk should be classed as being high also in alcohol-reduced beers.

RISK ASSESSMENT BASED ON MICROORGANISMS AND BEER TYPES (MICROORGANISM SET A)

	NAB		ARB		Beer	
Beverage	**NAB1 BF**	**NAB2 TF**	**ARB1 BF**	**ARB2 TF**	**B1 BF**	**Standard beer**
Microorganisms						
1. *L. brevis*	0	3	0	3	3	3
2. *Pectinatus portalensis*	3	3	0	0	0	0
3. *Saccharomyces cerevisiae*	3	3	3	1	1	0
4. *Saccharomyces pastorianus ssp. carlsbergensis*	3	2	3	2	2	0
5. *Sacch. cerevisiae* var. *diastaticus*	3	3	3	3	3	3
6. *Dekkera anomala*	3	3	3	3	3	3
7. *Wickerhamomyces anomalus*	3	3	2	3	2	0

NAB = non-alcoholic beer *ARB = alcohol-reduced beer*
TF = top-fermented *BF = bottom fermented*

Table 5

Vollbier (beer with 11 - 14 % original wort) relatively stable

Beer B1 has a similar high microbiological risk as the alcohol-reduced beer ARB2. Only the *Wickerhamomyces anomalus* yeast starts to grow slower in B1 than in ARB2. The extraordinarily vigorous yeast growth in a vollbier can be attributed to the relatively high content of fermentable sugars. This beer type has an average risk category rating of more than 2.0 and is thus considerably more sensitive than the standard beer tested.

As had been expected, the standard beer can be classed as microbiologically stable. The dextrin-splitting *Saccharomyces cerevisiae* var. *diastaticus* yeasts as well as the overfermenting *Dekkera anomalus* started to grow. Growth of *Lactobacillus brevis* is attributed to the fact that the bitter acid content of 22 BU of the standard beer is at the very limit of the strain used.

Mixed beer beverages stable beverage type (compared to control)

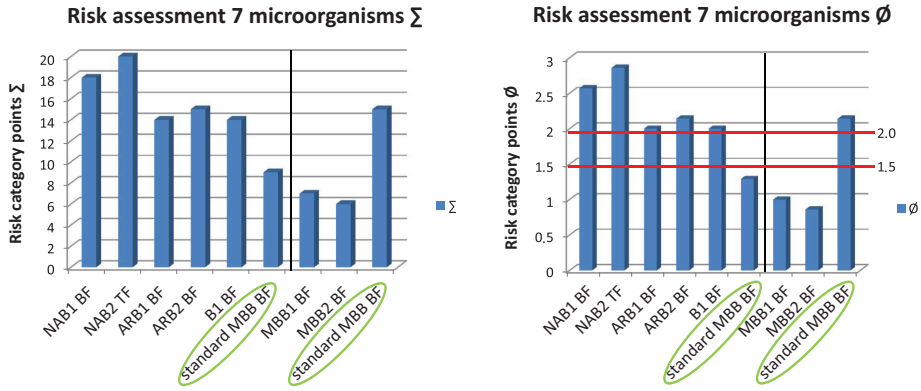
Saccharomyces cerevisiae var. *diastaticus* grows in very low quantities in MBB1 and beer culture yeasts do not grow at all. The two non-*Saccharomyces* yeasts *Kazachstania exigua* and *Dekkera anomala* exhibited

RISK ASSESSMENT BASED ON MICROORGANISMS AND MBB (MICROORGANISM SET B)

	MBB		
Beverages	**MBB1 BF**	**MBB2 BF**	**Standard MBB**
Microorganisms			
1. *L. brevis*	0	0	0
2. *Saccharomyces cerevisiae*	0	0	3
3. *Saccharomyces pastorianus ssp. carlsbergensis*	0	0	3
4. *Sacch. cerevisiae* var. *diastaticus*	1	3	3
5. *Dekkera anomala*	3	3	3
6. *Rhodotorula mucilaginosa*	0	0	0
7. *Kazachstania exigua*	3	0	3

MBB = mixed beer beverage

Table 6



Risk assessment					
	∑	Ø		∑	Ø
NAB1 BF	18	2.57	MBB1 BF	7	1
NAB2 TF	20	2.86	MBB2 B	6	0.86
ARB1 BF	14	2	standard MBB BF	15	2.14
ARB2 BF	15	2.14			
B1 BF	14	2			
standard beer BF	9	1.29			

Fig. 3 Overview assessment of beer types tested ∑ and Ø

strong growth. Poor growth of the *Saccharomyces* yeasts indicates that the natural non-beer portion of the naturally hazy MBB1 contains inhibitor substances that inhibit growth of *Saccharomyces* yeasts. As a result of the low pH value, the *Lactobacillus brevis* strain used cannot grow. The biological monitoring system in beverage production operations should thus focus on non-*Saccharomyces* wild yeasts. *Saccharomyces cerevisiae* var. *diastaticus* and *Dekkera anomala* show vigorous growth in MBB2. Growth of other microorganisms was not observed. MBB2 is a sweetener-based mixed beer drink, the amount of fermentable sugars in this beverage is thus very low, limiting growth of most yeasts. *Saccharomyces cerevisiae* var. *diastaticus* and *Dekkera* yeasts are able to split dextrins and thus grow in media that cannot be utilised by other yeasts. In hygiene monitoring, attention should be given to the so-called overfermenting yeasts. In total, both mixed beer beverages can be regarded as being as microbiologically stable as the control mixed beer beverage (control standard MBB BF = mixed beer beverage bottom fermenting) (fig. 3).

Summary

The microbiological analysis and assessment system presented is very suitable for testing novel sensitive products in terms of their microbiological sensitivity and assessing their microbiological risk. The two non-alcoholic and alcohol-reduced beers as well as beer type B1 have to be classed as being microbiologically less stable than the standard beer tested. In filling these non-alcoholic and alcohol-reduced beers, stringent filler hygiene is required and stringent checks have to be carried out. It is also recommended that additional monitoring systems be installed. The mixed beer beverages tested are more stable than the standard mixed beer beverage.

Lactobacillus brevis and, thus, most other *Lactobacillus spp.* possibly present in the filler surroundings can grow in the top-fermenting beers as well as in beer type B1 tested. *Pectinatus* started to grow in both non-alcoholic samples. Beer culture yeast and *Wickerhamomyces anomalus* can grow in all

beer samples. The non-alcoholic beers can be regarded as excellent nutrient media for biofilms and beer-spoilage microorganisms from the filler section. Beer residues should be minimised and removed as often as possible. In addition to tunnel pasteurisation, a monitoring system that detects biofilm-forming bacteria (acetic acid bacteria), lactobacilli, biofilm-forming *Wickerhamomyces anomalus* and *Saccharomyces* yeasts should be installed. For these beer types, biofilm phase I should not be exceeded.

The mixed beer beverages tested can be classed as microbiologically stable. When maintaining impeccable and continuously monitored filler hygiene, filling of the two beverages MBB1 and MBB2, after flash pasteurisation but without subsequent tunnel pasteurisation, should be reliably assured without any critical risk of contamination. Installation of dedicated monitoring systems is recommended or necessary. In as far as MBB1 and MBB2 are concerned, overfermenting yeasts such as *Saccharomyces cerevisiae* var. *diastaticus* and *Dekkera* yeasts as well as non-*Saccharomyces* yeasts should be monitored. For both products, there should be additional monitoring growth of *Wickerhamomyces* yeasts. This may be an instrument for detection of biofilms containing yeasts before highly product-spoiling foreign yeasts can establish themselves. ■

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