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ORIGINAL PAPER

Biofilm growth of individual and dual strains of *Klebsiella oxytoca* from the dairy industry on ultrafiltration membranes

Xuemei Tang · Steve H. Flint · Rod J. Bennett · John D. Brooks · R. Hugh Morton

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Abstract Formation of biofilms in dairy membrane plants causes membrane pore blocking, product contamination and subsequent economic loss. To investigate the biofilm growth, two *Klebsiella oxytoca* strains, *K.* B006 and TR002, previously isolated from New Zealand dairy membrane plants, were grown both individually and combined on three types of ultrafiltration (UF) membranes in different concentrations of whey medium in biofilm reactors (CBR 90, BioSurface Technologies, Bozeman, USA). Biofilms of both the individual and combined strains grew on the membrane surfaces to levels of 4.9–7.99 log colony-forming units (CFU) cm^{-2} measured by standard plate counting after removing the cells by sonication. More biofilm grew on used polyethersulfone (PES) membranes than on new PES and polyvinylidene fluoride (PVDF) membranes. Both strains formed good biofilms, although *K.* B006 formed a denser biofilm than TR002. This corresponded to our previous study on the attachment of these organisms, where *K.* B006 attached in greater numbers than *K.* TR002. The dual strains produced a higher biofilm density than single strains on the new membranes. Biofilm density tended to increase with increased whey concentration. The saturated biofilm was approximately 10^8 CFU cm^{-2}. PES membranes appeared to support biofilm growth less readily than did PVDF membranes and therefore may be the preferred material for UF membranes to reduce problems with microbial colonisation. Used membranes were more readily colonised with biofilm than were new membranes. Therefore, selecting a membrane type and monitoring membrane age will help manage biofilm development during UF.

Keywords Biofilm · Ultrafiltration · Membrane · *Klebsiella* · Dairy

X. Tang (✉) · S. H. Flint · R. J. Bennett · R. H. Morton
Institute of Food, Nutrition and Human Health,
Massey University, Palmerston North, New Zealand
e-mail: X.Tang@massey.ac.nz

J. D. Brooks
School of Applied Sciences,
Auckland University of Technology,
Auckland, New Zealand

Introduction

Ultrafiltration (UF) membranes are widely used in the dairy industry [20]. The limitation on membrane usage is the frequent cleaning required due to fouling involving solute adhesion and microbial fouling [5, 11, 15, 16, 18]. Biofilms on membrane surfaces have been studied in water-processing environments [10, 17] but not in dairy manufacturing. In the dairy industry, UF is used most frequently to treat whey [14], with membrane plants processing cheese whey having a greater potential for microbial fouling due to the high pH (6.0–6.5). Biofilm growth is influenced by the substrate concentration [12]. Therefore, by choosing whey as the medium and using different concentrations of whey proteins, the relationship between biofilm growth and whey protein concentration can be investigated.

Some in vitro continuous systems that can be used to investigate biofilm growth in a laboratory include rotating disc reactors, the modified Robbins device, the annular reactor, and specifically designed commercial laboratory reactor systems such as the CBR 90 (BioSurface Technologies, Bozeman, USA) [9]. The CBR 90 reactor system is a biofilm reactor containing 24 removable polycarbonate coupons that allows controllable shear rate, continuous flow and temperature control. This system has been used for

monitoring biofilm formation and characterising biofilm structure [7] and statistical assessment for growing biofilms [9]. In our study, the CBR 90 reactor was modified to enable membranes to be fixed into the coupon holders.

Our objective in this study was to investigate how the biofilm growth of single and dual *Klebsiella* strains is affected by the membrane type [polyethersulfone (PES) and polyvinylidene fluoride (PVDF)] and whey medium concentration.

Materials and methods

Sources of strains

Two *Klebsiella oxytoca* strains were previously isolated from the dairy membrane plants, and both of them had high ability to attach to surfaces [19]. *Klebsiella* B006 was isolated from a liquid sample taken from a UF membrane plant processing whey at dairy manufacturing plant A, and *Klebsiella* TR002 was isolated from a biofilm sample scraped from a UF membrane plant processing whey at dairy manufacturing plant B.

Preparation of medium

Whey medium was prepared by mixing 1%, 5% and 20% of whey protein concentrate powder (WPC 80 containing 80% whey proteins, Fonterra Co-operative Group Ltd, Auckland, New Zealand) with sterilised lactose (Fonterra Co-operative Group Ltd, Auckland, New Zealand) and artificial whey permeate, which was prepared by mixing the following minerals in deionised water to make 1 l (pH 6.0–6.1) [52.7 ml 2 mol l^{-1} KOH (BDH, Poole, England), 24.29 g Na_3citrate·$2H_2O$ (Merck KGaA, Darmstadt, Germany), 4.99 g K_3citrate·$2H_2O$ (UNIVAR, Auckland, New Zealand), 3.67 g $CaCl_2 \cdot 2H_2O$ (Biolab, Clayton, Australia), 5.85 g $MgCl_2 \cdot 6H_2O$ (J.T. Baker, Phillipsburg, Mexico), 23.36 g KH_2PO_4 (Merck KGaA, Darmstadt, Germany) and 17.1 ml 3 mol l^{-1} H_2SO_4 (Biolab, Clayton, VIC, Australia)]. To mimic the composition of lactose and minerals in three different stages of UF, lactose and minerals were added in appropriate concentrations to make the final approximate composition. At the beginning of UF, the product composition was 1% whey proteins, 6% lactose and 6% minerals. The middle stage contained 5% whey proteins, 6.1% lactose and 6.1% minerals, and the final concentrated stage contained 20% whey proteins, 2.4% lactose and 2.4% minerals.

Preparation of inocula

Pure cultures of *K. oxytoca* were grown on skim milk agar (SMA) at 30°C for 24 h, and then a large loopful of colony was inoculated into 10 ml whey and incubated for 24 h. This was diluted in whey to reach a density of 10^6–10^7 colony-forming units (CFU) ml^{-1}, confirmed by agar plate counting.

Description of the CBR and the target membrane surface

The CBR 90 reactor was described by Goeres et al. [9]. For our study, the polycarbonate disk coupons were covered with UF membrane by clipping a 1.3-mm × 1.3-mm membrane square into a hole on the rod with a disk coupon. New coupons were made 1-mm diameter smaller than the original coupons so that the actual surface exposed and available for biofilm growth surface was still a 1.27-cm^2 circle surface. Three types of the UF membranes were used, including new PES membranes (10,000 MWCO) (Synder Filtration, Vacaville, CA, USA), new PVDF membranes (800,000 MWCO) (Synder Filtration) and used PES membranes from a New Zealand dairy manufacturing membrane plant processing cheese whey. All these membranes were inserted into the rods and cleaned in the CBR 90 reactor according to the procedures provided by the membrane manufacturer and the dairy manufacturing plant.

Experiment design

A full factorial design was used for testing three factors (Table 1). This involved completing $3^3 = 27$ experiments plus some extra experiments as controls. The results were based on four randomly sampled coupons in each experiment.

Biofilm experiments

The CBR 90 was used to grow biofilms in order to determine the biofilm growth rate and biofilm densities of the two strains grown individually and in combination for three concentrations of whey medium and for three different types of membrane. Membrane pieces were clipped onto the rods of the CBR 90. Clean-in-place (CIP) for new and used membranes, provided by the supplier and dairy manufacturing plant respectively, was completed before

Table 1 Factors in the experiment design

Factors	Levels		
	L1	L2	L3
Strains	*K.* B006	*K.* TR002	Mixture (1:1 ratio)
Whey protein concentration (%)	1	5	20
Membrane type	New PES	New PVDF	Used PES

K. Klebsiella, *PES* polyethersulfone, *PVDF* polyvinylidene fluoride

Table 2 Biofilm log density of two strains and their combination in whey on ultrafiltration (UF) membranes

Strains	Whey protein concentration	1%			5%			20%		
	Membrane type	New PES	New PVDF	Used PES	New PES	New PVDF	Used PES	New PES	New PVDF	Used PES
B006	Mean density (log CFU cm^{-2})	5.17	5.92	6.88	6.28	6.30	7.64	6.87	6.81	7.55
	Four-coupon repeatability SD	0.41	0.36	0.10	0.18	0.21	0.22	0.08	0.09	0.30
TR002	Mean density (log CFU cm^{-2})	5.15	5.62	6.17	4.90	5.59	7.82	5.92	6.03	7.99
	Four-coupon repeatability SD	0.37	0.25	0.05	0.18	0.10	0.06	0.12	0.31	0.30
Mixture	Mean density (log CFU cm^{-2})	6.91	7.07	6.23	6.75	6.66	7.64	7.18	7.07	7.98
	Four-coupon repeatability SD	0.24	0.22	0.20	0.07	0.30	0.27	0.21	0.19	0.24

CFU colony-forming units, *SD* standard deviation, *PES* polyethersulfone, *PVDF* polyvinylidene fluoride

pumping 330 ml medium into the reactor from a supply stored at 4°C. The medium in the reactor was then heated to 25°C.

Experiments with no inoculum injected were run as negative controls. In order to grow biofilms of individual strains, 1 ml of inoculum was injected into the reactor using a sterile syringe; 1 ml of each inoculum was injected for growing biofilms of dual strains. To allow the microorganisms to attach to the membrane surface, the reactor was run for 1 h at 25°C with a rotating speed of 180 rpm (Reynold's number was approximately 1,850, [4]) before continuously pumping medium through at 5.5 ± 0.5 ml min^{-1}. The flow rate was set, based on calculated planktonic growth data obtained from the batch experiments, to ensure that the hydraulic detention time was less than the shorter cell doubling time of the two strains [13] (B006 1.15 h, TR002 1.23 h). Membrane samples were taken after 24 h incubation and rinsed in a sterile glass bottle containing 15 ml sterilised reverse-osmosis (RO) water for 1 min. Then they were transferred into 10 ml sterilised peptone water (Merck KGaA, Darmstadt, Germany) with glass balls and treated for 2 min in a sonicator water bath (Soniclean Pty Ltd, Thebarton, SA, Australia) to remove biofilm from the membrane surface and disrupt biofilm clumps. The peptone containing biofilms was then diluted in peptone in serial ten-fold dilutions and surface plated (0.1 ml) onto standard plate count agar (SPCA) (Merck KGaA, Darmstadt, Germany).

Scanning electron microscopy (SEM)

Membrane samples were cut to 4 mm × 4 mm using sterile blades. Then they were fixed with 3% glutaraldehyde and 2% formaldehyde in 0·1 mol l^{-1} phosphate buffer pH 7.2 for a day at room temperature. The fixed samples were washed through buffer three times, dehydrated through a graded series of ethanol solutions (25–100%) and critical point dried using liquid CO_2. Dried samples were mounted onto aluminium specimen support stubs using conductive silver paint and then sputter coated with gold.

Statistical analysis

All statistical calculations were performed on the log density values. Each mean and standard deviation of log density came from four identical tested membrane samples. The analysis of variance (ANOVA) in Minitab software (Release 15; Minitab Inc., State College, PA, USA) was used to analyse the variance of factors affecting biofilm development. These included whey concentrations, membrane types and strains. To assess significance, P values were provided for all effects.

Results

Biofilm growth

Samples of new and used membranes, prepared for this study by cleaning using standard procedures, without any inoculum, showed no biofilm growth over 24 h in CBR 90. The biofilm growth on membranes following inoculation under various experimental conditions is summarised in Table 2.

Impact of whey concentration, membrane type and strains

The impact of whey protein concentration, membrane type and strain type on biofilm growth were analysed using Minitab statistical software. All three factors by themselves (whey concentration $P = 0.004$, membrane type $P < 0.001$ and strains $P = 0.004$) showed a significant effect on biofilm growth (Fig. 1). The biofilm density increased with increasing whey protein concentration. The used PES membrane supported more biofilm than the new membranes. Biofilm grew slightly better on new PVDF membranes than on new PES membranes. *K. oxytoca* B006 grew denser biofilm than TR002. The mixture of the two strains showed more stable biofilm growth with higher density than either single strain (Fig. 1).

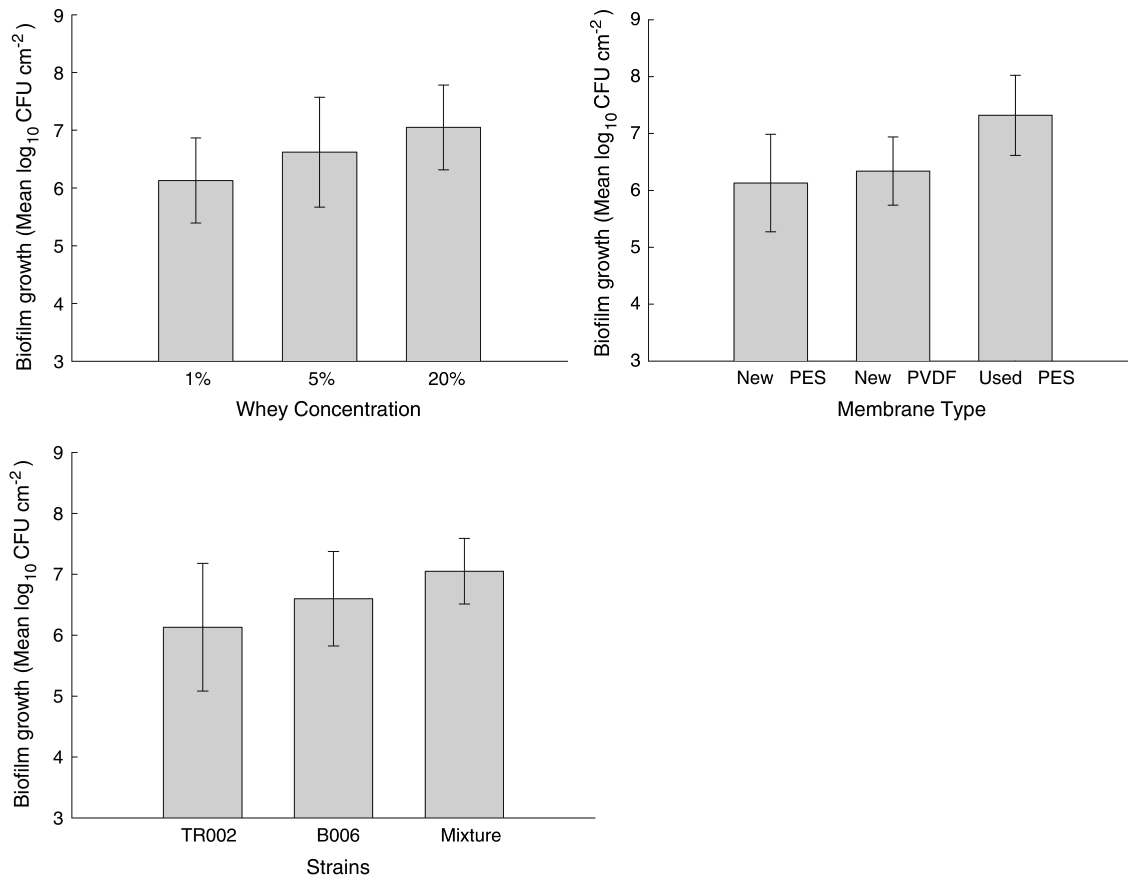

Fig. 1 Main effects of single factors on biofilm growth (data are the mean of nine mean log densities for each factor)

The membrane type and strain interaction had a significant effect on biofilm growth at the 6% level ($P = 0.059$). The biofilm log densities on the used membranes were generally higher than those on the new membranes, no matter what strains were used. However, on the new membranes, the dual strains showed much higher biofilm density than the single strain, and *K.* B006 produced more biofilm than *K.* TR002 (Fig. 2).

The dual strains produced higher biofilm density than did single strains on both types of new membranes (Fig. 3a, b). However, on used PES membranes, there was little difference between single and dual strains (Fig. 3c). This indicated that biofilms on the used membranes were saturated with an approximate density of 10^8 CFU cm^{-2}.

Scanning electron microscopy

Plate counts showed that the used membranes tended to have high biofilm densities. Images from SEM also confirmed that there were areas of used PES membrane that were highly colonised with microorganisms (Fig. 4).

Comparing Fig. 4 (PES) and Fig. 5 (PVDF), it was found that the two membrane surface structures appeared to

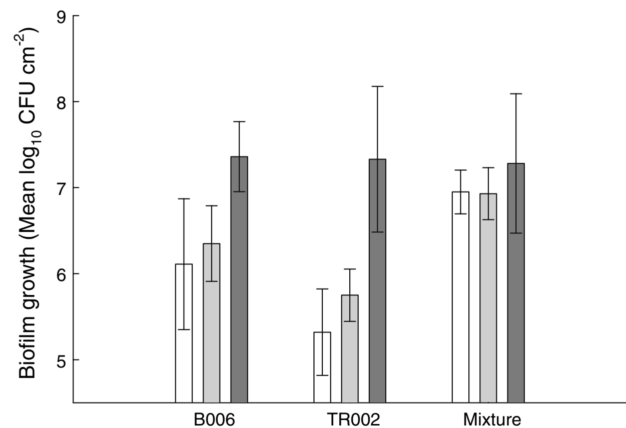

Fig. 2 Effect of the interaction of membrane type and strains on biofilm growth. Membrane types: (*open square*) new polyethersulfone (PES), (*light shaded square*) new polyvinylidene fluoride (PVDF) and (*dark shaded square*) used PES (data are the mean of nine replicates for each factor)

be different. The PVDF structure looked more open. Nevertheless, the biofilms on new PVDF membranes were generally less dense compared with the used PES membrane, as indicated in Table 2. Interestingly, on new PVDF membranes, the foulant (probably protein, encircled in Fig. 5)

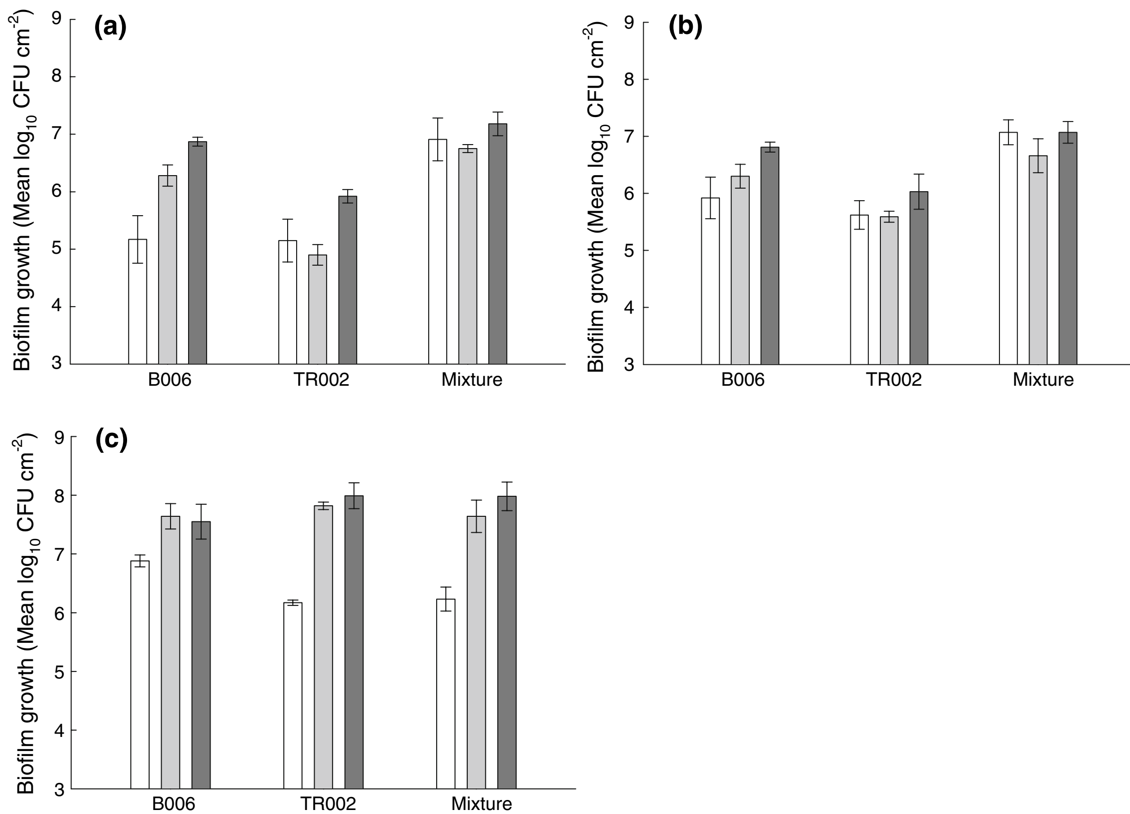

Fig. 3 Biofilm growth of single and dual strains on membranes in whey. Whey protein concentration: (*open square*) 1%, (*light shaded square*) 5% and (*dark shaded square*) 20%. **a** Biofilm growth on new polyethersulfone (PES) membranes, **b** biofilm growth on new polyvinylidene fluoride (PVDF) membranes and **c** biofilm growth on used PES membranes

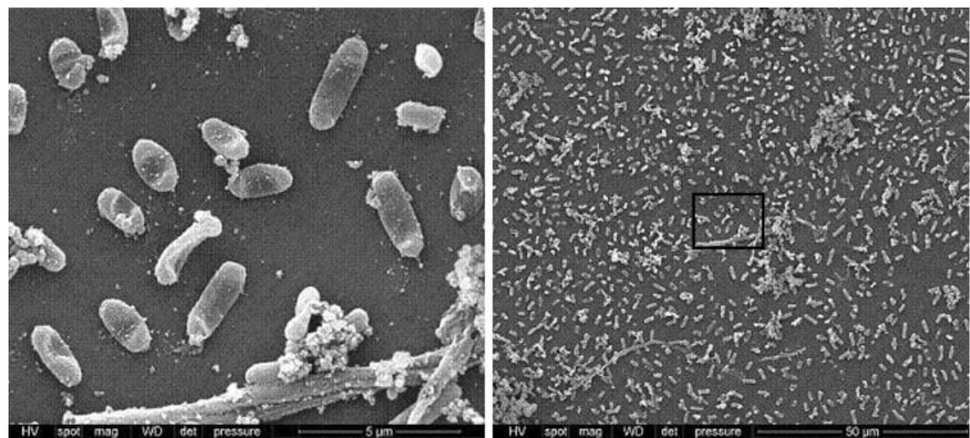

Fig. 4 Scanning electron (SE) micrographs of biofilm of *Klebsiella oxytoca* B006 on used polyethersulfone (PES) membranes after 24 h incubation with 5% whey. (The *left* is the *zoomed* in image of the *rectangular area* in the *right* image)

was much higher than on the used PES membrane (Fig. 4) but apparently did not facilitate bacterial attachment and biofilm formation.

Discussion

This study aimed to elucidate which factors influence biofilm growth on UF membrane surfaces using a laboratory scale system. The CBR 90 system was confirmed to be effective for growing biofilms on membrane samples. As far as we are aware, this is the first time that the CBR 90 system has been used for studies on membrane systems, although the system has been used to study biofilm growth on a variety of surfaces [9].

The two *Klebsiella* strains were isolated from two different UF membrane plants processing whey. Therefore, whey was chosen as the nutrient medium for these trials. To mimic conditions in the manufacturing plants, the whey medium was made artificially by mixing minerals, lactose

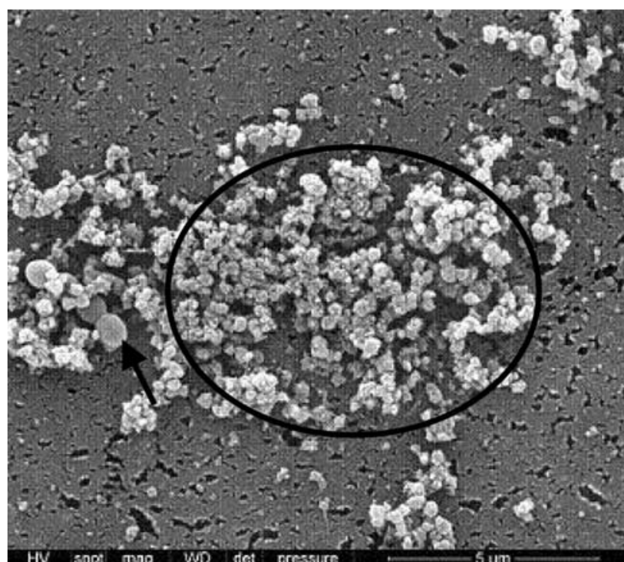

Fig. 5 Scanning electron (SE) micrograph of biofilm of *Klebsiella oxytoca* TR002 on a new polyvinylidene fluoride (PVDF) membrane after 24 h incubation with 5% whey.(*Arrow* indicates microorganisms and *circle* shows the foulant, which is probably whey proteins)

and WPC 80 in the proportions to reflect concentrations expected during whey processing. Thus, the three whey protein concentrations used were representative of three stages (start, middle and end) in the UF membrane whey processing plant. This method of preparing the whey medium ensured consistency throughout the study and avoided variation that would be expected with different batches of whey.

All three factors (whey protein concentration, strain and membrane type) were shown to be significant factors by themselves, but only one-two-factor interaction (strain and membrane type) had a significant effect on biofilm growth. The mixture always reached high biofilm density on any membrane surface, but single strains, especially *K.* TR002, reached much higher biofilm density on used membranes than on new membranes (Fig. 2). Whey protein concentration played an important role in biofilm development of the two strains. The higher the whey protein concentration, the more biofilm was formed. This indicates that *K. oxytoca* growth was limited by the medium concentration. Some other studies also found that *K. oxytoca* reached higher biofilm density in higher substrate concentration [12].

The two *Klebsiella* strains used differed in their ability to form biofilms. *K.* TR002, which was isolated from PES UF membrane surfaces, showed less ability to grow as a biofilm than did *K.* B006, which was isolated from the liquid in a UF membrane plant. This corresponds to our previous study in which B006 had a greater ability to attach to surfaces than did TR002 [19].

On new membranes, the mixture of strains showed higher biofilm density than the single strains for each whey concentration. Therefore, there might be some interactions between these two strains that facilitated their growth in the biofilm. However, on the used membranes, the biofilm density of single strains and the mixture were not significantly different. The maximum biofilm density in our experiments was about 10^8 CFU cm^{-2} (Table 2; Fig. 2). This might indicate that the biofilm growth reached a steady state [6] due to the nutrient concentration, shearing forces or other limiting conditions [15].

The biofilms formed on used membranes were significantly denser (mean = 7.32 log CFU cm^{-2}) than those on new membranes (mean = 6.23 log CFU cm^{-2}). This might be because the surfaces of used membranes had a rougher surface than the new membranes. It might also be because used membranes had more open surfaces, which could trap more bacteria, having been used and cleaned with chemicals at high temperatures (e.g. 50°C). However, we speculate that the most likely cause is that repeated cleaning of the used membranes modified the physicochemical properties of the surface.

Our observations may be explained by the presence of organic material, especially protein, on the membrane surface. Proteins may either enhance or block microbial attachment [1], and these influences may depend upon whether the proteins are in a denatured or native form. In the first case, in our trials with used PES membranes, we presumed that the organic material that was not completely removed by standard cleaning had been denatured during the CIP process and might have acted as a conditioning film, easily binding or trapping bacteria on the surface. Such conditioning layers composed of denatured proteins may have enhanced bacterial adhesion. In the other case, the probable fresh whey proteins, as observed on the SEM (Fig. 5), were seen to foul the new PVDF membrane surface but not the used PES membrane surface (Fig. 4). The lower microbial biofilm density observed on the new PVDF membrane (Fig. 5) might be explained by the attachment of fresh whey proteins to the surface of new PVDF membranes more readily than to PES membranes and by these fresh, native proteins blocking the attachment of bacteria. This protein blocking of attachment has been seen by others [2]. It has also been suggested that fresh proteins present in the liquid inhibit bacterial adhesion [3, 8]. It is clear that the behaviour of biofilms on new and used membranes merits further investigation.

With the continuous flowing CBR system, we aimed to create a similar environment to that experienced in a whey UF processing plant, with the exception that the product was not actually passing through the membrane. The whey composition, temperature and turbulence were all representative of what could be expected in a whey processing plant. The results of our study suggested that the whey protein concentration, the membrane type and the interactions

between different microorganisms are all important factors for biofilm development on UF membrane surfaces. Further studies will investigate cleaning strategies using this model system.

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